QUANTIFYING SOURCES OF FECAL CONTAMINATION IN A COASTAL SYSTEM WITH COMPLEX STORMWATER DYNAMICS

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ABSTRACT

Justin Daniel Hart: Quantifying sources of fecal contamination in a coastal system with complex stormwater dynamics.
(Under the direction of Rachel T. Noble)

Coastal North Carolina (NC) exhibits complex meteorological and hydrological dynamics that facilitate the delivery of fecal contaminants to downstream receiving waters via stormwater runoff. A quantitative microbial assessment of stormwater in Beaufort, NC was conducted to identify trends and potential sources of fecal contamination. During wet weather, the increase in microbial contaminants in receiving waters was substantial. Short-term rainfall (i.e. less than 12 hours) was predictive of *E. coli*, *Enterococcus* spp., and human-specific marker concentrations in receiving water, and strong correlation between 12-hr antecedent rainfall and *Enterococcus* spp. ($r = 0.57$, $p < 0.001$, $n=92$) suggests there is potential for a predictive model to be developed that would improve management of water quality impairment. These data will be used to inform ongoing stormwater mitigation projects in this region and serve as a conceptual model for the interaction between complex stormwater dynamics and water quality impairment in coastal NC.

To my mentors and teachers who have inspired me to marvel at our unseen companions.

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LIST OF ABBREVIATIONS

ACTB	β-actin gene
CFU	Colony-Forming Units
C_T	Cycle Threshold
ddPCR	Droplet Digital Polymerase Chain Reaction
DMF	North Carolina Division of Marine Fisheries
EC	*Escherichia coli* concentration
ENT	*Enterococcus* spp. concentration
GS	Gordon Street Stormwater Outfall
I/I	Infiltration and Inflow
LoB	Limit of Blank
LoD	Limit of Detection
LoQ	Limit of Quantification
mEI Agar	mebrane-Enterococcus Indoxyl-p-D-Glucoside Agar
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
MPN	Most Probable Number
MST	Microbial Source Tracking
NC	North Carolina
NCDEQ	North Carolina Department of Environmental Quality
NEC	Negative Extraction Control

NTC	No Template Control
OS	Orange Street Stormwater Outfall
PC	Polycarbonate
PCR	Polymerase Chain Reaction
RCR	Rachel Carson Reserve
qPCR	Quantitative Polymerase Chain Reaction
SCM	Stormwater Control Measure
SPC	Specimen Processing Control
UNC-IMS	University of North Carolina at Chapel Hill Institute of Marine Sciences
USEPA	United States Environmental Protection Agency

CHAPTER 1: INTRODUCTION

The expansion of urban development in coastal areas combined with variable rainfall patterns generates runoff that often impairs the quality of receiving water bodies in coastal North Carolina (NC), endangering ecosystems and human health (Sanger, et al., 2013). Two groups of fecal indicator bacteria (FIB), *Escherichia coli* (EC) and *Enterococcus* spp. (ENT), are measured to manage the risks posed by microbial fecal contaminants in water. FIB serve as a proxy for the presence of bacterial and viral pathogens associated with feces. Exposure to water with high concentrations of FIB can lead to gastrointestinal and other illnesses (Colford et al., 2007; Haile et al., 1999; Prüss, 1998; Soller et al., 2017). The North Carolina Department of Environmental Quality (NCDEQ) Division of Marine Fisheries (DMF) recreational water quality section measures *Enterococcus* spp. in water used for recreation based on regulatory limits suggested by the United States Environmental Protection Agency (USEPA; USEPA, 1986). Additional guidance was issued in 2012 and 2014 by USEPA but has not yet been adopted by NC (USEPA, 2014, 2012).

Typically, the recreational water quality along the coast of NC is excellent. In a 2014 comparison of national water quality, NC ranked 5th out of 30 coastal states in terms of number of exceedances of USEPA-recommended FIB thresholds (Dorfman and Haren, 2014). Maintaining a reputation for safe water quality is particularly important for the NC economy. North Carolina is the 6th most-visited state in the country, and in 2016 alone, there were 10.7 million person-trips to coastal NC, resulting in $337 million in spending in Carteret County alone (Visit North Carolina, 2017)

Even though beach and estuarine water quality is excellent a majority of the time, there are several hydrological mechanisms by which fecal contamination may reach recreational water in coastal NC, including stormwater runoff (Cahoon et al., 2016). In response, there is a need for applied microbiological assessment to inform mitigation strategies and resource allocation. Simply put, there are not engineering solutions for these coastal systems to mitigate all stormwater-related contamination events due to unpredictability, the lack of space and other resources, and other factors. Stormwater runoff is known to be the main causative agent adversely impacting water quality in coastal NC (Converse et al., 2011; Coulliette and Noble, 2008; Kirby-Smith and White, 2006; Parker et al., 2010; Stumpf et al., 2010). In Dare County, NC, stormwater was found to load between $\log_{10}$ 4–7 MPN of both ENT and EC to receiving waters over the duration of a storm (Converse et al., 2011). Loading estimates from other studies conducted in coastal NC have generated even higher estimates of loading for fecal indicator bacteria (Stumpf et al., 2010).

In coastal NC, there are several hydrological and meteorological factors that create unique challenges to stormwater management. For one, storm conditions are highly variable on a local scale. For instance, in 2016, the town of Beaufort, NC and Morehead City, NC received 59.1 and 70.4 inches of rainfall respectively, despite being adjacent and the weather stations being less than three miles from one another (Weather Underground Station ID: KMRH, MoreheadCityWeather.com). Rainfall amounts are typically highest in the late summer and early fall, coinciding with the end of the tourist and tropical storm seasons, but spring patterns of rainfall can bring long slow, steady storm events. Generally, storms occurring in the relatively drier winter and spring months are longer and have a lower rate of precipitation relative to summer and fall storms, which can be short in duration (hours to day) and intense (more than 30

inches of rain in September 2015; Weather Underground Station ID: KMRH). Typical summer storm events can surpass the capacity of engineered stormwater control measures (SCMs), leading to flooding and hazardous standing water (Flood and Cahoon, 2011).

The challenges posed by this variability are compounded by the terrain; the area is low-lying and the tidally-influenced surficial groundwater aquifers are shallow. As a result, there is limited space for SCM, such as stormwater retention ponds, or subterranean stormwater and wastewater conveyance. There is also little gradient to propel stormwater through a conveyance system using gravity. Even within the existing engineered conveyance systems there is evidence of tide- and storm-dependent interaction between groundwater and the stormwater and wastewater infrastructure in coastal NC (Flood and Cahoon, 2011). The volume of stormwater runoff is in part determined by an area's soil saturation and the ability of rainfall to infiltrate to surficial aquifers (Göbel et al., 2004; Line and White, 2007). As the amount of impervious surface upstream of tidal creeks continues to expand, the volume of stormwater runoff generated during storms and stormwater contamination will also increase the likelihood of major flood events (Kopp et al., 2015). Corroded wastewater pipes have been demonstrated to exfiltrate under dry weather conditions in California, indicating a likely mechanism for the delivery of human fecal contamination to stormwater discharge receiving waters (Sercu et al., 2011). The corrosion of intertidal stormwater and wastewater pipes may lead to greater exfiltration of fecal contaminants (Flood and Cahoon, 2011).

While cultured FIB are useful for predicting the magnitude of potential fecal contamination, they are not able to indicate the sources, such as leaking sewage (Field and Samadpour, 2007; Hagedorn et al., 2011; Tran et al., 2015). Several library-independent quantitative polymerase chain reaction (qPCR) assays have been developed to enumerate

molecular microbial source tracking (MST) markers that associate fecal contamination to particular species of warm-blooded animals. Among these, HF183 TaqMan is consistently one of the best performing human-specific MST markers (Boehm et al., 2013; Layton et al., 2013), with high specificity (81%, Staley et al., 2012) and sensitivity (95%, Ahmed et al., 2012; Shanks et al., 2010) to human feces. Sensitivity is the likelihood of not obtaining a false negative result with an assay, while specificity is the likelihood of not obtaining a false positive result. Other human-specific MST markers are powerful when used in tandem with HF183 TaqMan by increasing the certainty of sewage contamination (Ballesté et al., 2010; Griffith et al., 2016; Sidhu et al., 2013). In addition to HF183 TaqMan, BacHum-UCD and Fecal *Bacteroides* have demonstrated high sensitivity and specificity to human sewage, respectively (Ahmed et al., 2016; Converse et al., 2009). All three of these assays target different conserved sections of the 16S rRNA gene in human-specific bacteria of the genus *Bacteroides* genus or order *Bacteroidales* (Harwood et al., 2014; Kildare et al., 2007). Additionally, these particular human-specific assays have been incorporated to epidemiologic studies to predict the human health risk of recreational waters (Griffith et al., 2016). Gull feces are another common source of coastal fecal contaminants, and have been found to be the most prevalent source of fecal contamination in some cases in coastal NC (Lauer, 2015). In samples of water contaminated with gull feces, the Gull2 TaqMan marker had a 85% sensitivity and 90% specificity to the contamination, targeting the species *Catellicoccus marimammalium* (Ryu et al., 2012).

Distinguishing between human and non-human sources of fecal contamination is important to risk management and disease prevention as sewage inherently presents a high probability of causing illness due to the human enteric pathogens it contains (Hagedorn et al., 2011; Lim et al., 2017; Soller et al., 2014). For example, it has been suggested that the human

viral pathogen norovirus is the predominant causative agent of waterborne disease in the United States (Soller et al., 2014). Different non-human sources range in their contributions to the overall "fecal contamination portfolio" and the probability of causing illness depending on the setting. Given this, there is hope of standardizing human-specific assays into a regulatory instrument (Boehm et al., 2015; Fewtrell and Kay, 2015). It is necessary to sample across a range of conditions to comprehensively characterize trends in MST marker and FIB concentrations as various dynamics can determine the fate and transport of indicators of fecal contamination (Mattioli et al., 2017; Riedel et al., 2015; Wanjugi et al., 2016; Yau et al., 2014).

This study sought to accomplish three objectives. The primary objective was to determine the magnitude of fecal contamination in the stormwater discharge to highly-used receiving waters of a coastal town in coastal NC. This was done by measuring FIB during both dry and storm conditions over a ten-month period, including a wide range of meteorological events. The location was selected for study because of the complex intersection of coastal development, hydrology, unpredictable stormwater dynamics that often result in standing water and flooding, and the proximity to the Rachel Carson Reserve (RCR), a jewel of the NC State Reserve system and of the National Estuarine Research Reserve Program. The second objective was to discern the sources of fecal contamination, and to determine whether human sources could be responsible for observed FIB concentrations. A combination of human-specific MST markers along with a marker specific for bird fecal contamination was quantified in all samples using vetted, peer-reviewed, and published qPCR approaches. The third objective was to identify the potential for simple predictive models to be developed that may assist in the ability to adequately manage such a high-profile estuarine resource. This was accomplished by analyzing the statistical relationships between FIB and MST marker concentrations to a wide range of

environmental and meteorological parameters. Ultimately, this study sought to create evidence-based tools to assist in stormwater mitigation in the Town of Beaufort, NC. The characterization of these stormwater receiving waters will inform ongoing investigation into the effects of stormwater runoff from Beaufort in the RCR.

CHAPTER 2: MATERIALS AND METHODS

Sampling Location

The study sites for this study are located in the Town of Beaufort, a coastal community in Carteret County, NC. Beaufort had a permanent population of 4,153 residents as of 2015, although Carteret County's population grows by as much as 150,000 residents seasonally (Carteret Economic Development). Beaufort is bordered by the Newport River to the west, Taylor's Creek and Back Sound to the south, and unincorporated Carteret County to the east and north (Figure 1). Taylor's Creek separates the town from the RCR, which includes a group of undeveloped barrier islands. During the tourist season, there is a high level of secondary contact with the water of Taylor's Creek through boating, kayaking, and upright paddle boarding. There is also considerable primary contact with the water at the beaches of RCR as well as near private

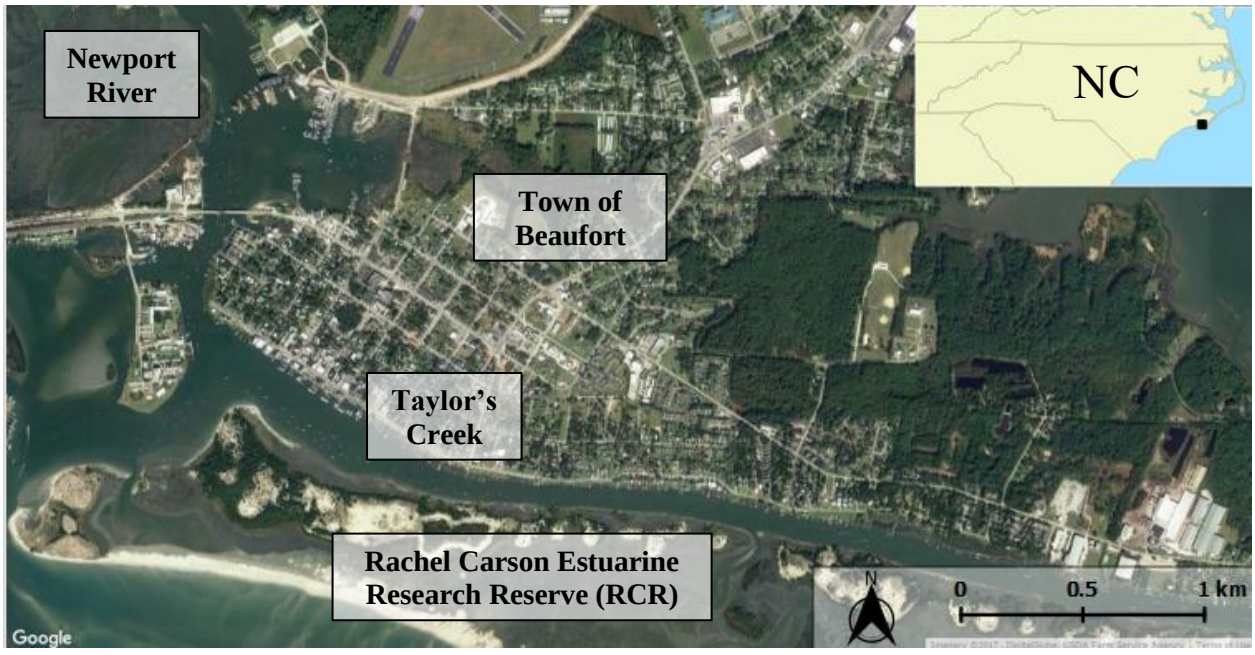

Figure 1. Map of aerial view of Beaufort and location within NC

and public docks on the Beaufort waterfront. The wastewater sewer and stormwater system are separate in Beaufort, although in some locations the systems were built in close proximity due to the shallow groundwater in the area.

For the purposes of this study, dry conditions were those which had zero in. of five d antecedent precipitation. Storm sampling conditions were triggered by the anticipation of at least 0.25 in. of rain. Sampling efforts were conducted within 90 min. of low tide, using the projections of a nearby tide sensor (NOAA Tides and Currents Station ID: 8656483). A total of 22 storm condition events and five dry condition events were sampled between August 17, 2016 and June 14, 2017 (Table 1).

Sampling efforts focused on receiving waters downstream of two stormwater conveyance outfalls that discharge to Taylor's Creek near the intersections of Front Street and Orange Street (OS), and Front Street and Gordon Street (GS). While there are several other stormwater outfalls along the Taylor's Creek waterfront, these two were selected because of their accessibility, their size, and their proximity to recreational areas in Taylor's Creek. The stormwater conveyance systems that discharge at these two outfalls drain primarily residential sections of Beaufort. Samples were collected in stormwater discharge receiving waters. At low tide, OS is exposed and discharges to the surface of Taylor's Creek. There is a weak but present flow even during dry conditions. GS discharges submerged beneath a public dock. Each outfall is the terminus of a 24-in. diameter reinforced concrete pipe.

Samples were occasionally gathered in the stormwater system upstream from discharge locations with cooperation from the Town of Beaufort Division of Public Works (Figure 2). Puddles throughout Beaufort were also sampled based on observation of flooding and familiarity with areas that tend to flood during storm conditions. At storm drains throughout Beaufort, there

was frequently evidence in the form of sediment and leaf litter that the stormwater system had recently overflowed to the street. On two occasions, samples were collected by boat outward from both OS and GS during dry conditions in an attempt to capture any discharge plume.

Sample Collection

The following environmental parameters were recorded *in situ* using a multi-parameter sonde (6920 V2, YSI, Yellow Springs, OH): water temperature (°C), conductivity (ms/cm^2), salinity (PSU), turbidity (NTU), and dissolved oxygen (percent saturation). Weather information, including antecedent precipitation (inches) and air temperature (°C) was mined from the weather station hosted at the Michael J Smith Airport on Weather Underground (ID: KMRH).

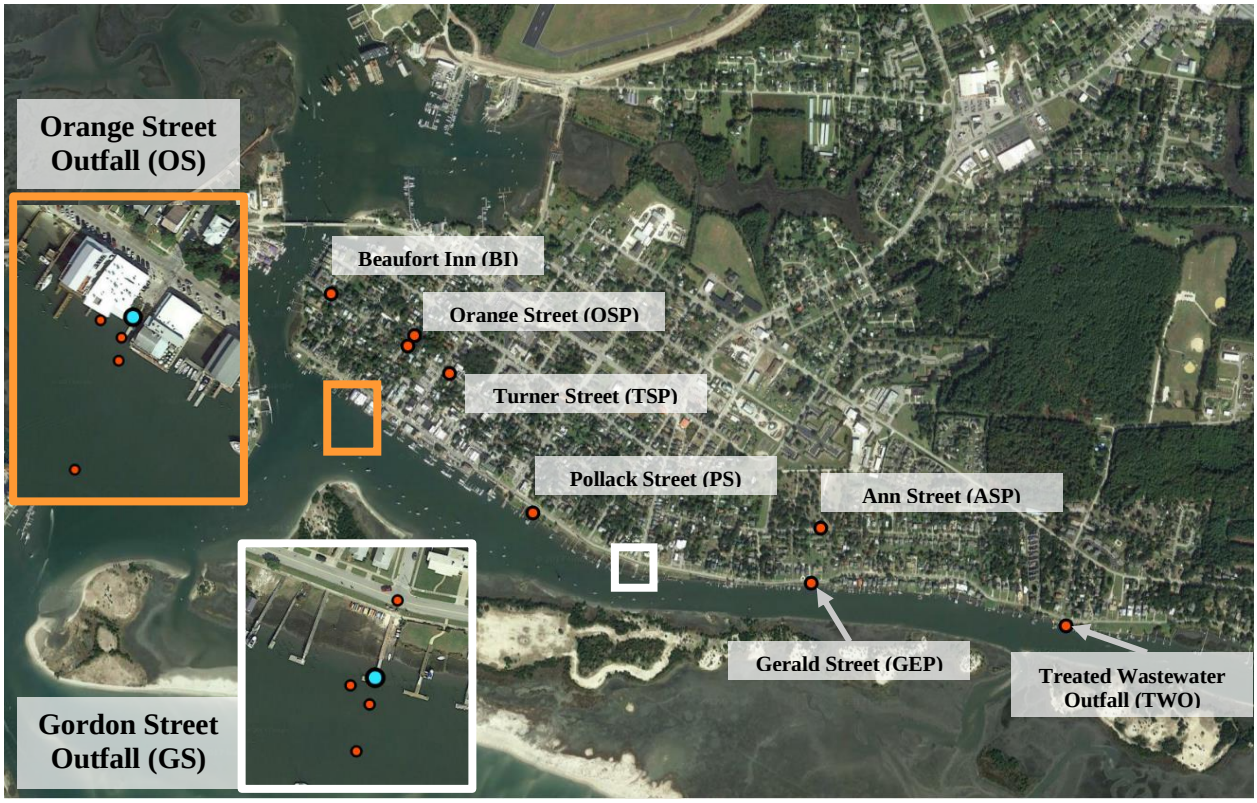

Figure 2. Map of the outfalls of interest (blue circles) and other sampling locations (orange circles) in Beaufort, NC.

Grab samples were collected in sterile, pre-rinsed 1 L acid-washed polypropylene (Nalgene^{TM}) bottles downstream from the sampling location. Samples were transported to the

University of North Carolina at Chapel Hill Institute of Marine Science (UNC-IMS) on ice and processed upon return within 3 hours of collection.

Sample Preparation

E. coli and *Enterococcus* spp. concentrations were quantified for each sample using USEPA-approved Defined Substrate Technology™ Enterolert™ and Colilert-18© kits combined with high most probable number (MPN) Quantitray/2000© trays (IDEXX Laboratories, Westbrook, ME) following manufacturer instructions. Samples were diluted 1:10 or 1:100 in deionized water to dilute competing bacterial species as recommended by the manufacturer and measured in duplicate.

The NC Department of Marine Fisheries (DMF) Division of Shellfish Sanitation and Recreational Water Quality has used both Enterolert™ and a membrane filtration technique described in USEPA Method 1600 to quantify *Enterococcus* spp. in marine water samples (Potts, pers. comm, (USEPA, 2006). USEPA Method 1600 has also been demonstrated to outperform Enterolert™ as an indicator of illness risk in other settings (Griffith et al., 2016). Given this, samples for this study were also membrane filtered onto gridded 0.4m, 47mm diameter Mixed Cellulose Ester filters (EMD Millipore Corporation, Billerica, MA) according to USEPA Method 1600 in order to compare the concentrations to those determined using Enterolert™. These membranes were plated onto membrane-Enterococcus-Indoxyl-p-D-Glucoside (mEI) agar (Becton Dickinson, Franklin Lakes, NJ) and incubated for 24 hours at 41°C. Colonies that were > 0.5 mm with a blue halo were counted (USEPA, 2006).

Additionally, four 100 mL subsample replicates were vacuum filtered through 0.4 µm, 47 mm diameter polycarbonate (PC) filters (GE Osmonics, Minnetonka, MN) and stored in DNase/RNase-free microcentrifuge tubes at -80° until later extraction and analysis. All samples,

positive, and negative controls were extracted and purified using the PowerSoil kit (QIAGEN, Valencia, California) according to manufacturer instructions and eluted at a volume of 100 μL. Extracts were stored at -20°C until use.

Table 1. Sample collection dates and weather conditions

Sampling Date	Weather Conditions	Number of Samples
August 17, 2016	Dry	8
September 2, 2016	Storm	2
September 3, 2016	Storm	2
September 12, 2016	Storm	3
September 15, 2016	Storm	3
September 29, 2016	Storm	7
September 30, 2016	Storm	4
November 2, 2016	Dry	3
November 4, 2016	Storm	5
November 14, 2016	Storm	2
November 30, 2016	Storm	4
December 6, 2016	Storm	2
December 14, 2016	Storm	2
January 23, 2017	Storm	2
February 8, 2017	Storm	5
March 12, 2017	Storm	2
March 14, 2017	Storm	2
March 18, 2017	Storm	2
March 28, 2017	Dry	9
April 6, 2017	Storm	2
April 17, 2017	Sewage	1
April 24, 2017	Storm	2
April 25, 2017	Storm	4
May 5, 2017	Storm	3
Mary 24-25, 2017	Storm	4
May 30, 2017	Storm	2
June 5, 2017	Dry	3
June 13, 2017	Dry	3
June 14, 2017	Dry	3

Sewage Collection

Raw wastewater influent was collected from the Town of Beaufort Wastewater Treatment Plant to determine the copy numbers of human-specific markers in local sewage and to verify that the bird-specific Gull2 TaqMan marker did not cross-react with bacteria found in human sewage. The sample was transported on ice to UNC-IMS and processed within 3 hours of collection. Two 25 mL and two 50 mL subsamples were vacuum filtered through 0.4 μm, 47 mm diameter PC filters (GE Osmonics), extracted using the PowerSoil kit and eluted in 100 μL of elution buffer. These reduced filtration volumes were selected to avoid concentrating inhibitory substances. The concentration of each marker was converted to copies/100 mL.

qPCR Calibration Standards

Plasmid standards for fecal *Bacteroides*, BacHum-UCD, HF183 TaqMan, and Gull2 TaqMan qPCR assays were linearized and diluted according to Lauer (2015). The quantity of each standard was verified using droplet digital PCR (ddPCR) using a QX200™ Droplet Digital™ PCR System (Bio-Rad Laboratories, Inc., Hercules, CA). For these reactions, 5 μL of each standard was transferred to 500 μL of buffer AE (QIAGEN), bead beaten for 2 minutes in a 48-place Mini-Bead Beater™ (BioSpec Products, Inc. Bartlesville, OK), then centrifuged at 10,000 g for 1 minute. Both the crudely extracted standard and the standards extracted with the PowerSoil kit were diluted 1:10 and 1:100 in nuclease-free water so that the final copy number would fall in the dynamic range of ddPCR. To generate droplets, a 20 μL solution containing the extracted standard dilutions, nuclease-free water, 250 nM probes, 2.5 μM primers, and ddPCR Supermix for Probes (no dUTP) (Bio-Rad, Catalog #1863024) was added to a DG8 cartridge (Bio-Rad) with 70 μL Droplet Generation Oil for Probes (Bio-Rad) and run on a QX200 Droplet

Generator (Bio-Rad). Once the cycle was completed, 40 μL of the droplets containing the reaction mixture were transferred to a 96-well plate. The plate was placed in a C1000 Thermocycler (Bio-Rad) and cycled according to the following conditions: 95°C for 10 minutes, 40 cycles of 94°C for 30 seconds, 58°C for 1 minute, and 72°C for 30 seconds, 98°C for 10 minutes and then cooled to room temperature. Once the cycle was completed, the plate was read using the QX200 Droplet Reader (Bio-Rad). The values were calculated using QuantiSoft software (Bio-Rad) and are shown in Table 2.

Specimen Processing Control

A uniform quantity of specimen processing control (SPC) was added to all unknowns, standards, and negative controls to determine recovery and identify inhibition in samples. Mouse β-actin (*ACTB)* cDNA which had been previously reverse transcribed and the copy number determined by ddPCR was used as the SPC. *ACTB* cDNA was spiked into PowerSoil Solution C1 at an intended concentration of 4×10^6 copies per extraction, resulting in a qPCR amplification at a cycle threshold (C_T) of 27-29 assuming loss from extraction.

Negative extraction controls (NECs) were used to verify cross-contamination. In no case was cross-contamination evidenced to have occurred during sample extraction. Blank PC filters were added to each NEC extraction tube, spiked with SPC, and extracted alongside all unknowns and/or standards. The extracted NEC acted as a negative control for MST marker assays and a positive control for the *ACTB* SPC assay. Following qPCR analysis for the *ACTB* marker, an unknown sample was considered inhibited if its cycle threshold (C_T) differed by greater than a 2.32 C_T delay (equivalent to a half-log difference in concentration) relative to the C_T of the NEC. None of the samples in this study were determined to be inhibited according to this metric.

However, 32 samples (34%) were diluted 1:2 to increase the volume available to perform the assays. This dilution was accounted for in the final calculations.

Table 2. MST marker standard concentrations

MST Marker or SPC	Standard Concentration (copies/100 mL water)
Fecal *Bacteroides* (95% CI)	8.91×10^7 ($8.56 – 9.26 \times 10^7$)
BacHum-UCD (95% CI)	1.16×10^8 ($1.09 – 1.23 \times 10^8$)
HF183 TaqMan (95% CI)	1.56×10^8 ($1.32 – 1.80 \times 10^8$)
Gull2 TaqMan (95% CI)	6.21×10^7 ($4.67 – 6.97 \times 10^7$)
ACTB	5.40×10^7

qPCR Analyses

The concentrations of fecal-associated molecular markers in water samples were determined through previously published real-time qPCR assays following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). All assays were performed on a CFX96™ Real-Time System (Bio-Rad) using TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, Waltham, Massachusetts). Primers and probes were synthesized by LGC Biosearch Technologies (Petaluma, CA). Each reaction had a total volume of 25 μL, including nuclease-free water, TaqMan® Environmental Master Mix 2.0, 100 nM probes, 1000 nM primers, and 2.5 μL of unknown sample, standard, or control. Information about the assays can be found in Table 3.

The quantity of each MST marker was determined using a modification of the Pfaffl method for the relative quantification of qPCR products that accounts for the amplification efficiency of the reaction (Haugland et al., 2005). For unknown reasons, the *ACTB* SPC demonstrated higher concentrations in samples than in controls and was therefore not used to

correct sample concentrations. All samples and controls were run in duplicate while standards were run in triplicate to create a dilution curve for each plate that was run.

Table 3. MST marker assay information

Assay	Oligo ID	Sequence	Concentration	Reference
Fecal *Bacteroides*	BFDFor	CGTTCCATTAGGCAGTTGGT	1000 nM	Converse et al. (2009)
	BFDRev	CGTAGGAGTTTGGACCGTGT	1000 nM	
	BFD TM FAM	6-FAM-CTGAGAGGAAGGTCCCCCACATTGGA-BHQ-1	100 nM	
BacHum-UCD	BacHum-160f	TGAGTTCACATGTCCGCATGA	1000 nM	Kildare et al. (2007)
	BacHum-241r	CGTTACCCCGCCTACTATCTAATG	1000 nM	
	BacHum-193p	6-FAM-TCCGGTAGACGATGGGGATGCGTT-BHQ-1	100 nM	
HF183 TaqMan	HF183	ATCATGAGTTCACATGTCCG	1000 nM	Staley et al., 2012
	SSHBacR	TACCCCGCCTACTATCTAATG	1000 nM	
	SSHBac-PRB	6-FAM-TTAAAGGTATTTTCCGGTAGACGATGG-BHQ-1	100 nM	
Gull2 TaqMan	Gull For	TGCATCGACCTAAAGTTTTGAG	1000 nM	Sinigalliano et al. (2010)
	Gull Rev	GTCAAAGAGCGAGCAGTTACTA	1000 nM	
	Gull TM FAM BHQ	6-FAM-CTGAGAGGGTGATCGGCCACATTGGGACT-BHQ-1	100 nM	
ACTB cDNA (SPC)	Mouse *ACTB*	20× concentration of primer and probe stock labeled with FAM and TAMRA Proprietary. Refer to ThermoFisher Scientific Catalog Number: 4352933E		

Standard dilution curves were aggregated to form a single master curve for each of the MST markers and the *ACTB* reference gene. The C_T values for each reaction were calculated by

the CFX96 ™ Real-Time System. The number of MST marker copies was determined by extrapolating from the respective master curve (Table 4).

Table 4. Master curve information

Targets	# of Individual Standard Curves, (Total # of Data Points Included)	Master Curve	R^2	Efficiency
ACTB	5 (75)	-3.50x + 42.9	0.960	93.07%
Fecal *Bacteroides*	4 (66)	-3.55x + 42.1	0.987	91.35%
BacHum	4 (61)	-3.55x + 43.2	0.986	91.16%
HF183	5 (92)	-3.53x + 41.8	0.983	91.94%
Gull2 TaqMan	4 (33)	-3.42x + 40.2	0.961	95.93%

A total of zero NTC and NEC were positive for any of the MST marker assays. The limit of blank (LoB) for each assay was calculated using the corresponding standard curve assuming a C_T value of 40 (Table 5). The limit of detection (LoD) was set as the average C_T of the lowest dilution with detected values. Each LoD was extrapolated from the respective linear model. The limit of quantification (LoQ) was assumed to be identical to the LoD.

Table 5. Limits of blank and detection for each MST marker

MST Marker	Limit of Blank (copies/reaction)	Limit of Detection (copies/reaction)
Fecal *Bacteroides*	6.52	54.3
BacHum-UCD	8.20	32.0
HF183 TaqMan	3.21	7.03
Gull2 TaqMan	2.00	2.52

Data Analyses

Coliert-18© and Enterolert ™ values were averaged in Microsoft Excel 2016 (Redmond, WA) using MPN equations from Hurley and Roscoe, (1983). Samples that exceeded the detection limit for IDEXX Quantitray/2000© were assigned the highest value within the averaged limits of detection (24560 MPN/100 mL); values below the limit of detection were assigned value of 5.0 MPN/100 mL, the lowest value within the averaged limits

of detection. All values were corrected to the unit of MPN/100 mL based on dilution. ENT values determined by EPA Method 1600 were averaged and converted to colony-forming units (CFU)/100 mL. For samples where an MST marker was not detected, the marker was assigned a value of 1.0 copy/100 mL.

Given the variable intensity of recreactional use of Taylor's Creek, the NCDEQ Tier 1 standard of 104 ENT MPN/100 mL ($\log_{10}$ 2.02 MPN/100 mL) was applied to place the results of this study into the context of recreational water quality management. Additionally, while NCDEQ does not monitor EC concentrations to manage water quality, EC results were compared to the statistical threshold value of 320 EC MPN/100 mL ($\log_{10}$ 2.51 MPN/100 mL) recommended by the EPA (USEPA, 2012).

The Shapiro-Wilks test was used to determine the normality of the distributions of each bacterial quantification method and environmental parameter. None were found to be normally distributed at $\alpha = 0.05$. FIB and MST marker concentrations were $\log_{10}$-transformed to partially resolve this skewness. All statistical tests were performed at a significance level of $\alpha = 0.05$. Non-parametric Spearman's Rank correlation coefficients (Spearman, 2010) were used to evaluate the correlation of microbial concentrations to the following environmental parameters: water temperature, air temperature, air pressure, conductivity, salinity, turbidity, dissolved oxygen, and antecedent precipitation. The variabilities of microbial indicator concentrations between sites and between weather conditions were evaluated using the non-parametric Mann-Whitney U Test since the samples were independent of one another. The variability between *Enterococcus spp.* concentrations determined by EPA Method 1600 and Enterolert™ was evaluated using the non-parametric Wilcoxon signed-ranks test since the samples were dependent. ENT and 12-hour cumulative antecedent rainfall were plotted against HF183 TaqMan

concentrations to assess their potential predictive capability. All statistical correlations were tested in R software (R Core Team, Vienna, Austria) using the Hmisc package (Harrell et al., 2016).

CHAPTER 3: RESULTS

Overview of FIB Quantification in Stormwater

Both ENT and EC values spanned a four-$\log_{10}$ range in all samples collected from Beaufort, NC over the 9-month sampling period. EC values ranged from no detection (corrected to $\log_{10}$ 0.69 MPN/100 mL) at both outfalls to $\log_{10}$ 4.77 MPN/100 mL at the Beaufort Inn (BI) sampling site (Figure 3). ENT values ranged from no detection (corrected to $\log_{10}$ 0.69 MPN/100 mL) at both outfalls to $\log_{10}$ 4.23 MPN/100 mL in floodwater on Gerald Street (GEP). The mean concentrations of EC and ENT were significantly greater during storm conditions (EC μ=2.20 $\log_{10}$ MPN/100 mL, ENT μ=2.33 $\log_{10}$ MPN/100 mL) than during dry conditions (EC μ=1.41 $\log_{10}$ MPN/100 mL, ENT μ=1.20 $\log_{10}$ MPN/100 mL, Figure 5). Based upon the ENT threshold of 104 ENT MPN/100 mL, 19 off the 53 samples collected from receiving waters (35.8%) exceeded the NC ENT threshold of 104 MPN/100 mL and 8 samples (15.1%) exceeded the USEPA EC threshold of 320 MPN/100 mL. All exceedances occurred during storm conditions.

During all weather conditions, FIB values were significantly higher at OS (EC μ=1.98 $\log_{10}$ MPN/100 mL, ENT μ=2.18 $\log_{10}$ MPN/100 mL) relative to GS (EC μ=1.66 $\log_{10}$ MPN/100 mL, ENT μ=1.47 $\log_{10}$ MPN/100 mL) (Figure 5). Of the regulatory exceedances measured, 14 exceedances (n=19, 73.7%) of the NCDEQ ENT threshold and 7 exceedances (n=8, 87.5%) of the USEPA EC threshold occurred at OS. Three of the EC exceedances and 6 of the ENT exceedances at OS were an order of magnitude greater than the threshold (Figure 3).

Samples collected in-pipe or from standing water upstream of the outfalls (hereafter "land-based sites") had higher concentrations of both ENT (μ=3.57 $\log_{10}$ MPN/100 mL) and EC

(μ=3.32 $\log_{10}$ MPN/100 mL) compared to either outfall (Figure 4). Of the 16 samples taken from land-based sites, 5 (31.5%) exceeded the ENT threshold by 2 $\log_{10}$ and 3 (18.8%) exceeded the EC threshold by 2 $\log_{10}$ (Figure 3).

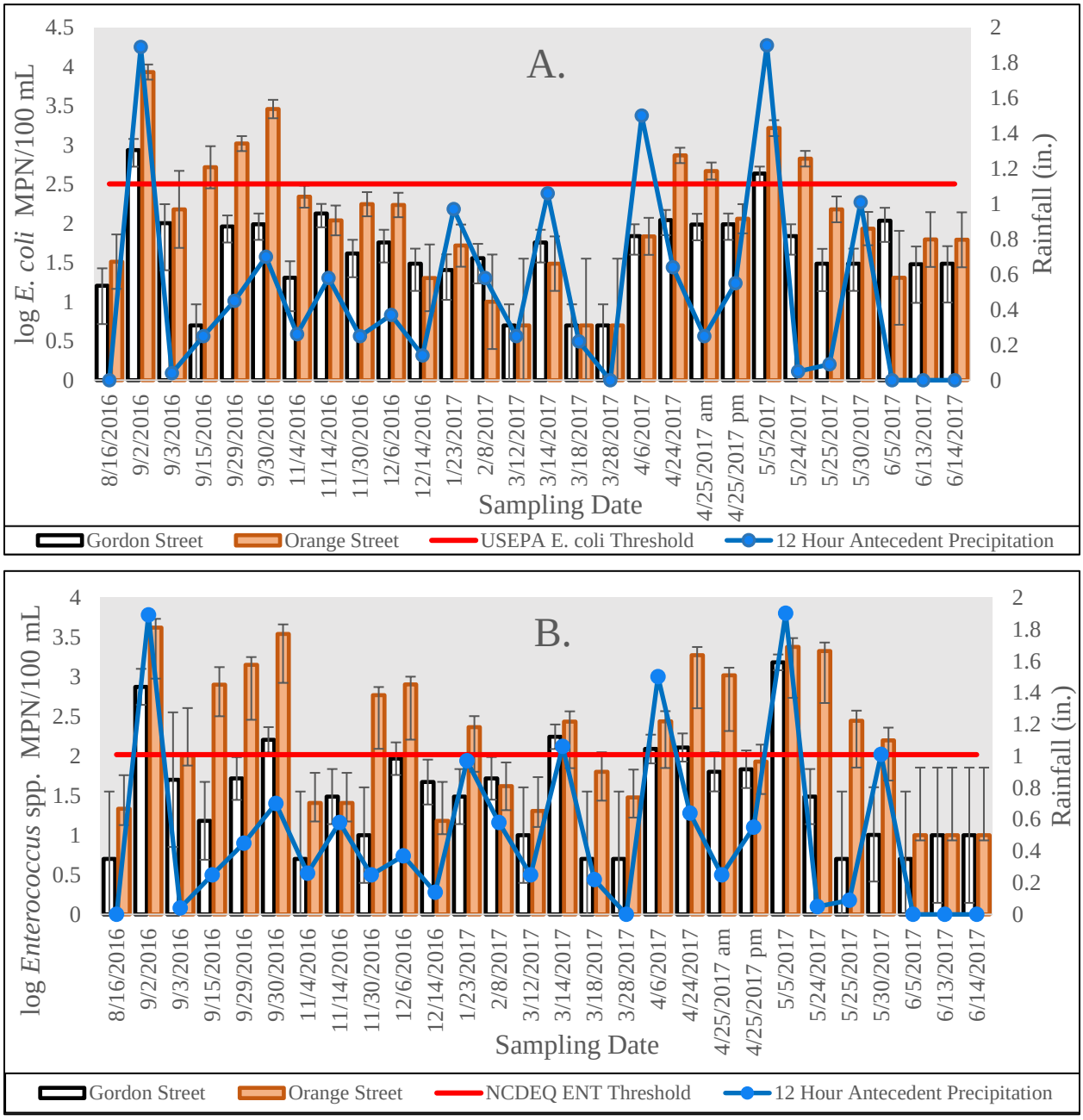

Figure 3. (A) EC measured with Colilert-18© and (B) ENT measured with Enterolert™ with 12-hour cumulative antecedent rainfall overlaid in blue. The blue line does not represent rainfall between sampling events. Error bars represent the standard error calculated using equations from Hurley and Roscoe (1983). The red horizontal lines indicate the USEPA-recommended freshwater recreational water quality standards for *E. coli* (used here for context, not used for water quality management in NC), and the NCDEQ ENT standard of 104 MPN/100 ml.

Overview of MST Marker Quantification in Stormwater

HF183 TaqMan was detected in 65 of the samples (n=92, 70.6%), BacHum-UCD in 59 (n=92, 64.1%), fecal *Bacteroides* in 48 (n=92, 52.1%), and Gull2 TaqMan in 21 (n=92, 22.1%) (Figure 4). All three human-specific markers were detected together in 31 of the samples (n=92, 33.7%; Figure 6). Of the 92 samples, 42 (45.7%) were below the limit of detection for fecal *Bacteroides*, 14 (15.2%) for BacHum-UCD, 5 (5.43%) for HF183 TaqMan, and 3 (3.26%) for Gull2 TaqMan. These values were not excluded from the following analyses and interpretation as they were useful for identifying MST marker trends according to the objectives of this study as previously described. None of the negative controls used for these assays yielded positive results, suggesting the concentrations observed were not due to cross-contamination.

All three human-specific MST markers were found at both GS and OS during storm conditions and at OS even during dry conditions (Figure 4). There was no significant difference in the distributions of HF183 TaqMan and BacHum-UCD between OS and GS. However, there was a significant difference between the distribution of fecal *Bacteroides* values at the two sites. For human-specific MST marker values at OS and GS, there was a significant difference in values between dry (HF183 TaqMan: μ=1.11 $\log_{10}$ copies/100 mL, BacHum-UCD: 1.35 $\log_{10}$ copies/100 mL fecal *Bacteroides:* μ=0.838 $\log_{10}$ copies/100 mL) and storm (HF183 TaqMan: μ=1.99 $\log_{10}$ copies/100 mL, BacHum-UCD: μ=2.04 $\log_{10}$ copies/100 mL, fecal *Bacteroides:* μ=1.32 $\log_{10}$ copies/100 mL) conditions.

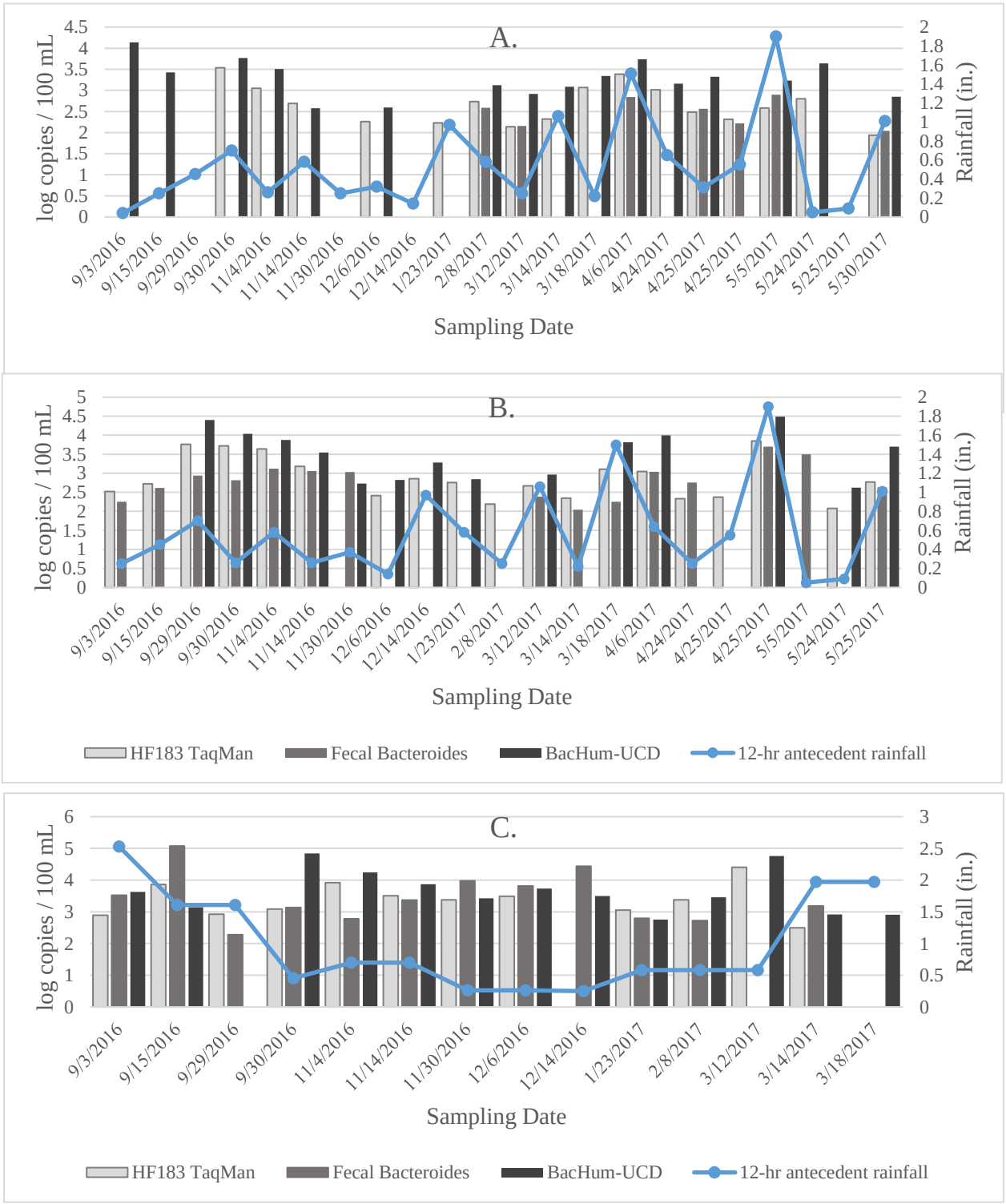

Figure 4. Concentrations of Human-specific MST Markers during storm conditions at (A) GS, (B) OS, and (C) Land-based sampling sites over the sampling period. 12-hour antecedent rainfall is denoted by blue points. The blue line does not reflect the precipitation between events.

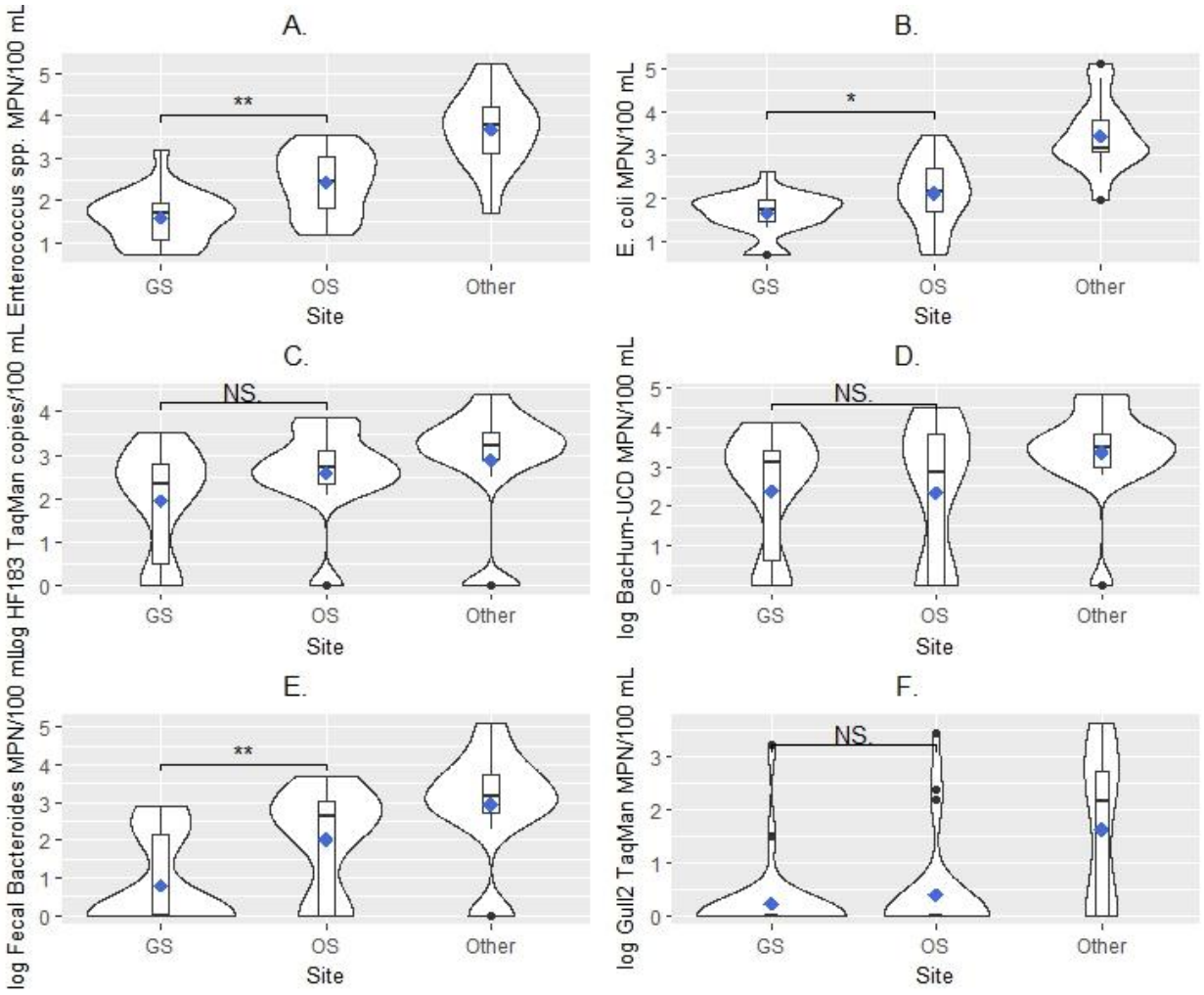

Figure 5. Violin plots demonstrating the concentration of (A) EC, (B) ENT, (C) HF183 TaqMan, (D) BacHum-UCD, (E) Fecal *Bacteroides*, and (F) Gull2 TaqMan concentrations during both dry and storm conditions at GS, OS, and all other sampling sites. These violin plots are modified box and whisker plots. The thick black line of the box plot represents the median value while the top and bottom of the box represent the 75th and 25th percentile, respectively. The whisker lines extend to the maximum and minimum values of the distribution. Outliers of the distribution are denoted by black dots. The mean of the distribution is denoted by a blue diamond. The width of the curves surrounding each box and whisker plot represents the frequency of each concentration. The p-value for the Mann Whitney U test comparing the distributions for GS and OS is denoted for each FIB and MST marker (* = < 0.05, ** = < 0.01).

At least one human-specific marker was detected in a majority of all samples (Figure 6). Of the three human-specific markers, the highest concentrations of each human-specific MST marker were detected in the land-based in-pipe and standing water samples, with 4.41 $\log_{10}$ copies/100 mL, 5.08 $\log_{10}$ copies/100 mL, and 4.84 $\log_{10}$ copies/100 mL for HF183 TaqMan,

fecal *Bacteroides*, and BacHum-UCD, respectively. Each of the land-based samples was taken from a stormwater manhole or overflowing stormwater intake, with the exception of Ann Street (ASP), which formed on the side of the road that lacked a stormwater drain. A low concentration of BacHum-UCD (2.91 $\log_{10}$ copies/100 mL) was detected at ASP, but the other two human-specific markers were not detected.

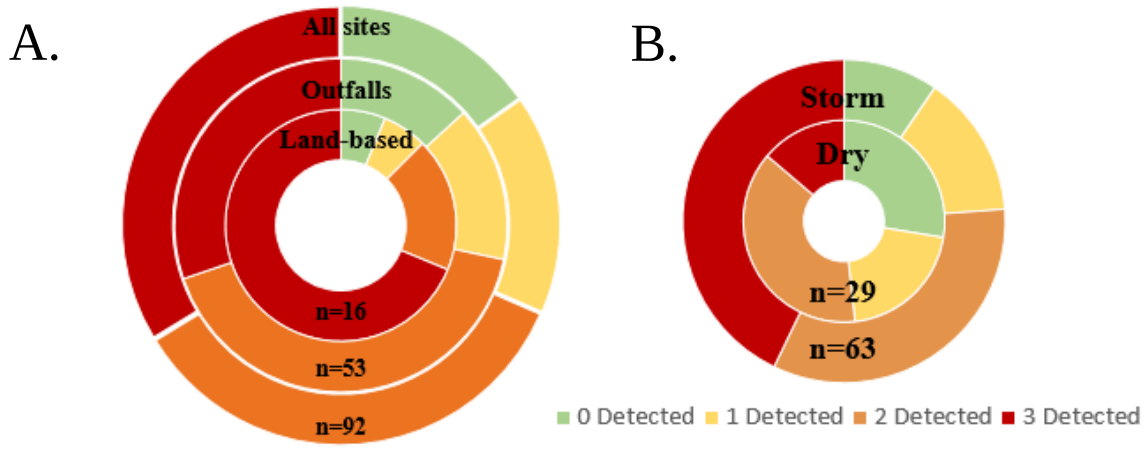

Figure 6. Donut charts demonstrating the proportion of samples collected with detection of 0, one, two, or three human-specific MST markers by (A) site, and (B) weather conditions. The number of samples within each subset is displayed.

Gull2 TaqMan was detected in just 21 samples (n=92, 22.8%), of which 9 were land-based samples. Water impaired with gull feces in coastal NC can demonstrate concentrations toward 6 $\log_{10}$ copies/100 mL, and gull feces themselves were found to have a concentration of 11.8 $\log_{10}$ copies/100 mL (Lauer, 2015). The highest Gull2 TaqMan concentration detected in this study was 2.5 $\log_{10}$ lower than the level in impaired water. There was also no significant difference in Gull2 TaqMan concentration between dry and storm conditions.

Samples were taken upstream and downstream of GS and OS during dry weather to determine whether fecal contaminants upstream of the outfalls were being discharged to receiving waters (Figure 7). During one sampling event, all three human-specific MST markers as well as Gull2 TaqMan were detected both upstream and downstream of OS. On the same day,

no MST markers were detected either upstream or downstream of GS. On another day with dry conditions, BacHum-UCD and HF183 TaqMan were detected in samples collected from boat-based sampling further downstream of OS. Fecal *Bacteroides* was detected at one of two sites sampled by boat downstream of GS, but the other human-specific MST markers were not detected (Figure 7).

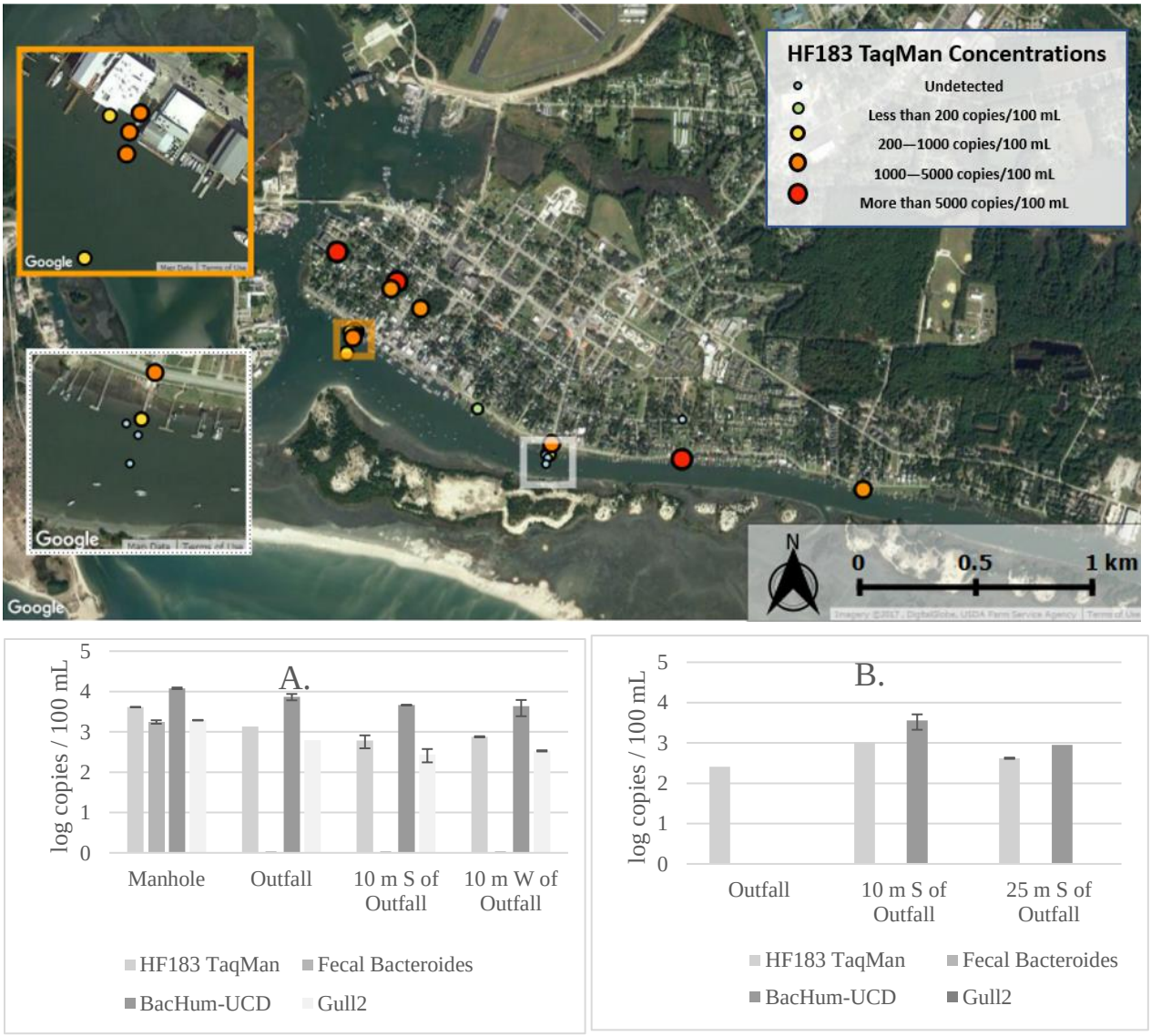

Figure 7. Map of HF183 TaqMan concentrations observed throughout Beaufort, NC, as well as bar graphs of (A) concentrations of MST markers upstream, at the OS outfall, and downstream during dry weather conditions, and (B) concentrations of MST markers at and downstream of the OS outfall during a separate dry condition sampling event.

Quantification of MST Markers in Beaufort Sewage

All three human-specific markers were detected in raw sewage influent collected from the Town of Beaufort on the order of 8 $\log_{10}$ copies/100 mL (Table 6). Gull2 TaqMan was not detected in the sewage influent, confirming that the assay does not cross-react with this particular human source.

Table 6. Average concentrations (n=4) of MST markers in raw sewage in Beaufort, NC as determined by qPCR

MST Marker	Average Concentration
Fecal *Bacteroides* (95% CI)	1.4×10^8 copies/100 mL raw sewage influent ($1.24–1.56 \times 10^8$)
BacHum-UCD (95% CI)	4.88×10^8 copies/100 mL raw sewage influent ($3.83–5.93 \times 10^8$)
HF183 TaqMan (95% CI)	5.49×10^8 copies/100 mL raw sewage influent ($5.06–5.92 \times 10^8$)
Gull2 TaqMan	not detected

FIB and MST Marker Correlation with Environmental Parameters

Across all sites and weather conditions, EC and ENT strongly correlated with one another (r=0.781), indicating similar factors are responsible for the increase of FIB concentrations (Figure 8). During all weather conditions, all three human-specific MST markers significantly correlated with all FIB and each other. At GS and OS, all FIB and human-specific MST markers significantly correlated with short-term (6-hr or 12-hr) cumulative rainfall, whereas Gull2 TaqMan concentrations significantly correlated with long-term (14 and 30 d) cumulative rainfall (Figure 8). These same relationships were not significant and were weaker when applied to all sampling locations. There was no significant difference between values obtained using Enterolert™ and EPA Method 1600and a significant correlation between the two (r=0.897) during all weather conditions. This verifies that both methods are appropriate for measuring the concentration of *Enterococcus* spp. in this setting.

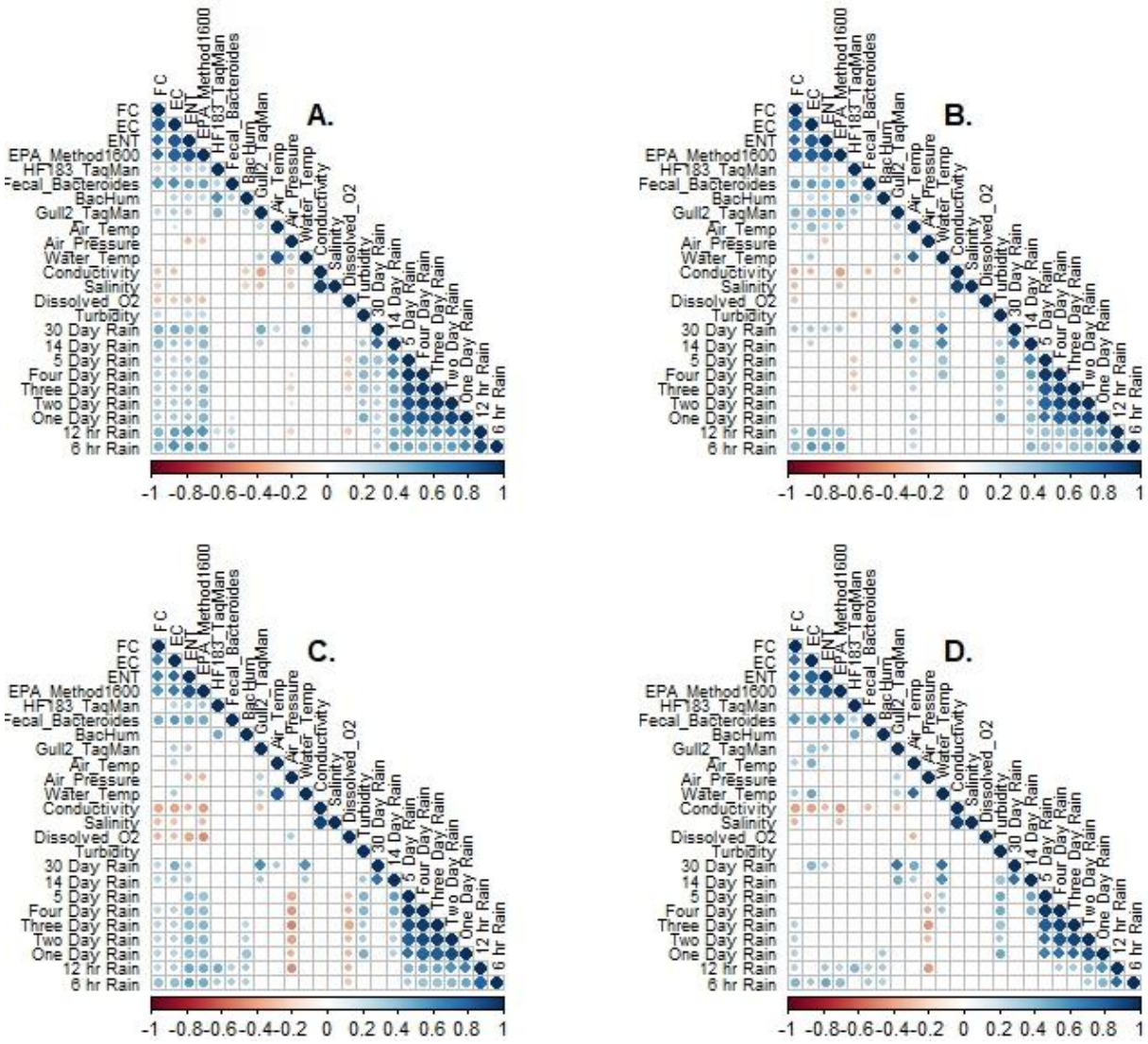

Figure 8. Correlograms of FIB concentrations, MST marker concentrations, and environmental and weather parameters under the following conditions: A. all sampling sites during all weather conditions, B. all sampling sites under storm conditions, C. GS and OS samples only during all weather conditions, and D. GS and OS samples during storm conditions only. The correlations are shaded so that strongly positive correlations are blue and strongly negative correlations are red. Blank boxes indicate the correlation was not significant at a p-value of 0.05.

Site-based Associations with Antecedent Rainfall

To further examine the role that the type of sampling location plays on these associations, simple linear models were performed to compare HF183 TaqMan, EC, ENT, and 12-hr rainfall (Figure 8). At all sites, there was a direct relationship between rainfall and ENT. However, there were discrepant relationships between HF183 and EC, ENT, and 12-hour cumulative rainfall by

site. While there was a positive association between EC and HF183 TaqMan at OS, the relationship was negative at land-based sites. Similarly, HF183 TaqMan demonstrated a positive association with rainfall at OS, but a negative association at land-based sites. No significant relationship was observed between HF183 TaqMan, FIB, and antecedent rainfall at GS.

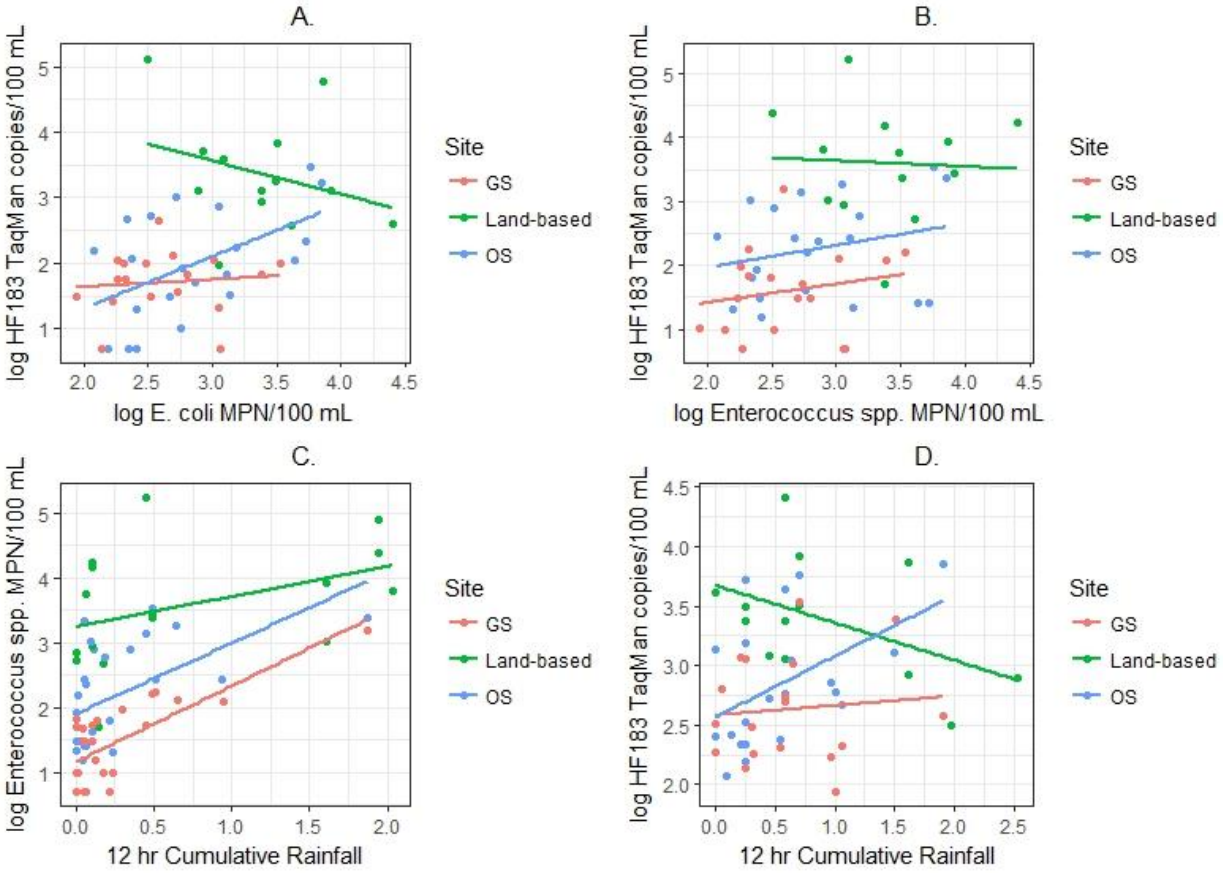

Figure 9. Scatterplots of (A). Colilert-18© vs. HF183 TaqMAn, (B). Enterolert™ vs. HF183 TaqMan, (C). 12-hr cumulative antecedent rainfall vs. Enterolert™, and (D). 12-hr cumulative antecedent rainfall vs. HF183 TaqMan. Lines of best fit have been fitted to each scatterplot according to the site from which the samples were collected.

Simple linear models were also created to interrogate seasonal patterns in FIB and MST marker concentrations. Certain FIB and MST markers also demonstrated seasonal patterns. EC concentrations were significantly lower in winter relative to other seasons, although this same relationship was not observed for ENT concentrations (Figure 3). No seasonal patterns were observed for Fecal *Bacteroides.* Both HF183 TaqMan and BacHum-UCD were significantly

lower in spring and summer compared to winter and fall. Gull2 TaqMan concentrations were significantly lower in spring and winter. Gull2 TaqMan was not detected in any samples in spring. There was no consistent, significant correlation between the human-specific MST markers and any of the other environmental parameters that were measured. The environmental parameter data were not collected at land-based sampling sites because the depth of the water at these sites was too shallow for the multiparameter sonde. However, measurements of air temperature and pressure were still made. While the environmental parameters were not significantly predictive of human-specific MST markers, there were several significant negative associations between FIB, conductivity, and dissolved oxygen at the receiving water sites.

CHAPTER 4: DISCUSSION

The concentrations of FIB and human-specific MST markers in Beaufort stormwater and in the receiving waters of this discharge are a cause for serious concern. Both EC and ENT concentrations in standing water and receiving waters increased significantly during storm conditions as compared to dry weather conditions. The concentrations of EC and ENT strongly and significantly correlated with one another (r=0.833), suggesting they originate from a common source. Antecedent rainfall correlated significantly for all cumulative rainfall periods analyzed for this study, with both ENT and EC concentrations supporting the prediction that observed fecal contamination results in part from stormwater input. The strongest correlations were at 30 d antecedent rainfall (EC: r=0.473; ENT: r=0.415), 12-hr antecedent rainfall (EC: r=0.545; ENT: r=0.570), and 6-hr antecedent rainfall (EC: r=0.586; ENT: r=0.564). For samples taken during storm conditions, only 6-hr and 12-hr antecedent rainfall correlated with EC and ENT. Occasionally, the concentrations of EC and ENT exceeded regulatory thresholds recommended by NCDEQ and USEPA by more than an order of magnitude. This suggests rainfall is predictive of microbial concentrations and severe water quality impairment can occur over short durations. There were strong enough correlations to potentially warrant further investigation of rainfall-based advisories for Taylor's Creek, a highly used area for recreation and boating, and an estuarine location proximal to the RCR.

The concentration of human-specific MST markers quantified in primary wastewater influent in Beaufort was similar to some of those previously reported in peer-reviewed literature (Table 6). In northern California, BacHum was quantified in the range of 8.0–9.8 $\log_{10}$

copies/100 mL in raw sewage (Silkie and Nelson, 2009). HF183 TaqMan has been quantified at 5–6 $\log_{10}$ copies/10 mL in composite wastewater in Victoria, Australia (Ahmed et al., 2012) and 8.6 $\log_{10}$ copies/100 mL in primary wastewater influent and 6.4–6.5 $\log_{10}$ copies/100 mL in treated wastewater in Vienna, Austria (Mayer et al., 2016). *Bacteroides thetaiotaomicron*, one of the target organisms of the fecal *Bacteroides* assay, was quantified at 7.25 $\log_{10}$ copies/100 mL in raw sewage in Michigan, although a different assay targeting the α-1-6-mannanase gene was used (Srinivasan et al., 2011). In Beaufort, mean values for the human specific markers from sewage influent collected specifically from the Town of Beaufort sewage treatment plant were 8.15 $\log_{10}$ fecal *Bacteroides* copies/100 mL, 8.69 $\log_{10}$ BacHum-UCD copies/100 mL and 8.74 $\log_{10}$ HF183 TaqMan copies/100 mL. While only one sample of sewage influent was analyzed for this study, the results confirm that the human-specific MST markers selected for this study were useful. However, additional analyses of sewage influent would yield important information about marker consistency and variability.

Simulated quantitative microbial risk assessment (QMRA) has been used in peer-reviewed literature to translate the illness rate benchmarks underlying USEPA guidelines to a benchmark of 4,200 (3.62 $\log_{10}$) HF183 TaqMan copies/100 mL (Boehm et al., 2015). Although the HF183 marker is not a pathogen or causative agent of disease, it is thought to be indicative of human fecal contamination and therefore has the potential to be used as a proxy for the presence of other important viral and bacterial pathogens. Although the reference material used in this study is different than that used to determine the benchmark of 4200 copies/100 mL, it still serves as a gauge for the relationship between the concentrations of HF183 TaqMan observed in Beaufort stormwater and the potential relationship to human illness. Over the course a storm on September 29-30, for example, samples taken from OS receiving waters reached 5,370

copies/100 mL (Figure 4). Values such as this could potentially be a cause for concern. Even though this concentration of HF183 TaqMan has corresponded to simulated elevation in risk to human health in peer-reviewed literature, an epidemiologic study has not been conducted specifically in Taylor's Creek to confirm the association with human illness (Boehm et al., 2015). Concentrations of BacHum-UCD reached in excess of 4.00 $\log_{10}$ copies/100 mL at both OS and GS during storm conditions. Fecal *Bacteroides* was detected less frequently than the other two human-specific markers, but reached a concentration of 5.08 $\log_{10}$ copies/100 mL in standing water at Gerald Street (GEP on Figure 2). BacHum-UCD and fecal *Bacteroides* have also been used in epidemiologic studies based on their presumed association with human health outcomes, although no similar threshold exists for these specific MST markers (Griffith et al., 2016). Together, the relevance to human health of these different markers suggest an elevated risk to human health from contact with or ingestion of water from Taylor's Creek following storm events. Yet these results are preliminary. Further study will be necessary to quantify such risk and consider the benefit of stormwater mitigation techniques toward the reduction of that risk.

At least one of the three human-specific markers was found at each land-based sampling site, and frequently at concentrations that exceeded those measured in receiving waters. Taken together, this suite of human-specific markers offers powerful and compelling evidence of human-specific fecal contamination. The three human-specific assays used in this study vary in their specificity and sensitivity, and all three are known to cross-react with *Bacteroides* spp. present in the feces of other species of animal (e.g. dogs, cats, deer) in other locations (Harwood et al., 2014; Layton et al., 2013). However, the repeated patterns of all three markers indicate a strong likelihood of human contamination stemming from sewage infrastructure in this

circumstance. This suggests that during overflow conditions (e.g. during a storm at high tide), sewage is reaching the surface and streets. Evidence of this type of flooding was observed as sediment and debris deposition on road surfaces surrounding stormwater intakes at low tide following storm conditions. The high concentrations of human-specific MST markers present in these puddles suggest they may be a hazard to human health. While they are not regulated as recreational waters, further investigation of the patterns and quantities of source-specific markers in this type of standing water may provide important clues regarding contamination in the stormwater system.

The receiving waters of stormwater outfalls at OS and at GS were featured prominently for quantitative microbial assessment in this project (Figure 2). Because samples were taken in the receiving waters of Taylor's Creek and not directly from the end-of-pipe at each site, the concentrations are diluted relative to the conditions within the pipe. These values offer insight to the water quality in Taylor's Creek itself and a conservative approximation of the quality of the stormwater discharge. These two outfalls were focal points because they are major contributors of stormwater runoff to Taylor's Creek, are among the largest stormwater outfalls to Taylor's Creek, and are proximal to locations in RCR that are used for recreation. The sampling for this project was most intensive at these sites so that the differing patterns of microbial indicator concentrations at these major conveyances could be discerned. Different attributes of the discharges along the Beaufort waterfront inevitably mean relationships and inputs will not be identical. For instance, both ENT and EC concentrations, but not MST marker concentrations, were significantly greater at OS than at GS. This may result from site characteristics that allow for either more concentrated human fecal contamination at OS, or potentially greater dilution at GS. Importantly, the OS sampling site is shallower than at GS, and the entirety of the plume

from the submerged GS stormwater pipe may not necessarily reach near the surface where samples were taken. Importantly, in future efforts, it will be vital to assess the water quality of the entire expanse of the estuarine system, not just near major stormwater discharge pipes.

The detection of human-specific markers upstream and downstream of OS suggests sewage enters the stormwater system upstream of OS even during dry conditions. There is a visible, consistent flow at low-tide at OS, which may result from wastewater exfiltration. In separate stormwater and wastewater sewer systems, exfiltration occurs when the wastewater sewer is above the water table, which in Beaufort would likely correspond to low tide (Sercu et al., 2011). However, due to the cross-over design of the stormwater/wastewater sewer system in Beaufort, exfiltrate may leak from a wastewater pipe to a stormwater pipe and flow to the outfall and receiving waters. Additionally, a variety of biotic and abiotic factors not measured in this study (e.g. sunlight, predation) determine FIB and MST marker fate in the environment and would be expected to reduce their concentrations between rain events (Mattioli et al., 2017; Sassoubre et al., 2015; Wanjugi et al., 2016). These factors may help explain the return to excellent water quality conditions and the lack of MST markers detected at GS during dry conditions. This also suggests the relatively high concentrations of MST markers detected at OS during dry conditions originate from a fresh fecal source.

Site-Specific Associations with Antecedent Rainfall

Different relationships were observed between rainfall and MST markers at land-based and receiving water samples (Figure 9). In receiving water samples, cumulative rainfall was predictive of MST marker concentrations. The correlations were significant for 6-hr antecedent rainfall (fecal *Bacteroides*: r=0.340; BacHum-UCD: r=0.330; HF183 TaqMan: r=0.344) and 12-hr antecedent rainfall (fecal *Bacteroides*: r=0.310; BacHum-UCD: r=0.377; HF183 TaqMan:

r=0.488) (Figure 8). However, for land-based samples there is an inverse relationship between 12-hr antecedent rainfall and the concentration of HF183 TaqMan (Figure 9). This suggests that increases in overland stormwater runoff does not contribute an increase in MST markers to the stormwater system. Rather, this indicates that the bulk of the human-associated contamination originates within the system such that increases in stormwater volume deliver more human-specific contamination to receiving waters. Meanwhile, additional rainfall would only serve to dilute contaminated stormwater as it overflows to the surface at land-based sites. Opposing this trend, ENT concentrations were associated with antecedent rainfall at land-based sites, although stormwater runoff may not fully scour FIB present on the surface (McCarthy et al., 2011). Virtual Beach is a widely-used statistical modeling software developed by USEPA to develop models of the relationships between ambient environmental conditions and water quality indicators (Cyterski et al., 2013). Predictive models incorporating location-specific stormwater dynamics have been successfully developed to accurately predict FIB concentrations in the Great Lakes (Olyphant and Whitman, 2004; Francy, 2009; Telech et al., 2009; Francy et al., 2013), Los Angeles (Feng et al., 2015; Thoe et al., 2014), the Gulf Coast (Zhang et al., 2012) and coastal NC (Coulliette et al., 2009; Gonzalez et al., 2012). These models offer a rapid approximation of the concentration of FIB, saving regulators time and monitoring resources while facilitating timely risk communication to the public. Due to the relative ease of using Virtual Beach, as well as the observed relationships between water quality indicators and ambient environmental conditions in Taylor's Creek, it is possible a similar predictive model could be adapted for use in this setting.

Gull2 TaqMan was also detected both at land-based sites and in receiving waters. Gull2 TaqMan was also not detected in samples collected during the spring, and concentrations were

significantly lower in the winter compared to the fall and summer. These patterns generally reflect the migratory behavior of gulls and suggest gull feces were only a seasonal source. Because it was detected so infrequently and at relatively low concentrations, it does not appear gull feces were an important source of the observed fecal contamination.

Limitations and Future Directions

One limitation of this study is that it only considered two species as potential sources of fecal contamination. Based on anecdotal evidence, it is possible that dogs may also be a source of fecal contaminants and effective MST assays have been developed to quantify the presence of dog-specific contamination (Schriewer et al., 2013). Species-specific qPCR assays may also be used to distinguish fecal-specific *Enterococcus* spp. from non-fecal species, such as plant- or sediment-based species, although MST marker data indicate the fecal contamination in Beaufort largely comes from a fresh fecal source because of the predictive patterns observed (Bradshaw et al., 2016; Byappanahalli et al., 2012) Although other important source species of fecal contamination are not apparent, a library-based whole genome sequencing approach could allow for the discrimination of sediment and sewage-based indicator bacteria, as well as identify additional species with fecal contaminant contributions (Henry et al., 2016).

The use of an SPC assists in correcting the quantification of MST markers to account for inhibitory substances present in the sample matrix (Dorevitch et al., 2017; Haugland et al., 2005). While the SPC used for this study was able to approximate adequate recovery from the extraction, it was unable to perform consistently enough to fully quantify inhibition of the qPCR reaction across a relevant linear range of concentrations and as a result, the concentrations of the molecular markers for this study were not corrected according to recovery or inhibition. In the past, substantial inhibition has been detected in water samples collected from coastal NC and has

been alleviated by additional purification or dilution (Converse et al., 2011; Gonzalez and Noble, 2014). However, in our study, inhibition was not determined to have adverse impacts on the qPCR reactions. Therefore, the qPCR quantities determined for molecular markers employed in this study are conservative estimates of the contamination. Had the SPC that was employed for this study performed adequately and consistently over a range of concentrations, a correction factor may have improved observed associations between the concentrations of the MST markers and environmental parameters and could potentially improve the fidelity of a rainfall advisory.

Because samples were taken at low tide, they may not necessarily capture the effect of tidal inundation and dilution of the stormwater system. For that reason, low tide should be interpreted as a "worst case" scenario and results regarded accordingly. For instance, at high tide, seawater enters and occasionally fully submerges the outfall at OS, causing significant dilution and even allowing brackish water to enter the stormwater conveyance system. Front Street, the waterfront street in Beaufort, is prone to flooding during high water events, and smaller tides can cause overflow conditions in stormwater drains near the waterfront even during dry conditions. The tide-associated increase in groundwater infiltration was not monitored as a part of this study, but in previous years has been sizable (Flood and Cahoon, 2011). Traditionally, groundwater monitoring is required to fully assess wastewater exfiltration, although dye and smoke tests can be used to locate leaking wastewater pipes (Rutsch et al., 2006; Sauer et al., 2011). The presence of these human-associated markers in standing water near stormwater junctions, however, could also potentially point to areas in need of remediation as the tide appears to bring fecal contaminants to the surface.

Flow gauges and automated sampling units have been installed at OS and Pollock Street (PS) for future stormwater research. While flow and loading data collected in future work will

offer the ability to understand the delivery of microbial contaminants over the course of storm hydrographs, the data collected during this study were still valuable as an initial assessment of stormwater dynamics.

There has been historical interest in the development of rainfall-based advisories to inform the public of recreational water conditions in the sounds proximal to the Town of Beaufort. Taylor's Creek is a perfect example of a location that might benefit from such an advisory, as it is used extensively for recreation in the summer tourist months, and even is the location of a prominent summer-long children's sailing camp. Past predictive models developed for coastal NC have described associations between stormwater dynamics and molecular markers of fecal ENT, but have not been compared to source-specific molecular markers (Gonzalez and Noble, 2014). The data from this study suggest that FIB and MST marker information could be further explored to derive such a rainfall-based advisory with stakeholder input.

In coastal NC, stormwater managers must navigate complex hydrological and meteorological dynamics to effectively mitigate the risks to property and human health posed by stormwater. The challenges posed by contamination in stormwater runoff in coastal NC are only expected to increase in the future. The sea level is projected to continue rising throughout this century and intense storms are predicted to become more common in the Southeast, factors that will likely increase the amount of I/I in places like Beaufort (Kopp et al., 2015, National Climate Assessment, 2014). Urban area is expected to double in the southeastern United States by 2050 with implications for an increase in stormwater runoff due to an increase in the area of impervious surfaces (Terando et al., 2014). The combination of the increase in the volume of wastewater generated due to coastal development may lead to increases in costly pernicious damage to infrastructure caused by overflow and nuisance flooding (Flood and Cahoon, 2011;

Moftakhari et al., 2017). Efforts to periodically evaluate the infrastructure may mitigate these effects.

CHAPTER 5: CONCLUSIONS

- Persistent agreement across human-specific MST markers in stormwater discharge and land-based samples and the magnitude of measured concentrations of these markers indicate that human fecal contamination is significant in not only the stormwater discharge, and the receiving waters, but also during dry weather. For this reason, further study and honing in on the sources is warranted
- Gull feces were not an important source of fecal contamination during the study period, although ephemeral patterns of contamination were observed in standing water.
- Even though storm-based patterns of human-specific marker delivery were more pronounced that those during dry weather, the detection of human-specific MST markers both upstream and downstream of Orange Street (OS) outfall during dry weather indicates a potentially chronic source.
- Short-term rainfall (6-hr and 12-hr cumulative) was predictive of *E. coli* (EC), *Enterococcus* spp. (ENT), and human-specific microbial source tracking (MST) marker concentrations across all sites

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