Examining coastal dynamics and recreational water quality by quantifying multiple sewage specific markers in a North Carolina estuary

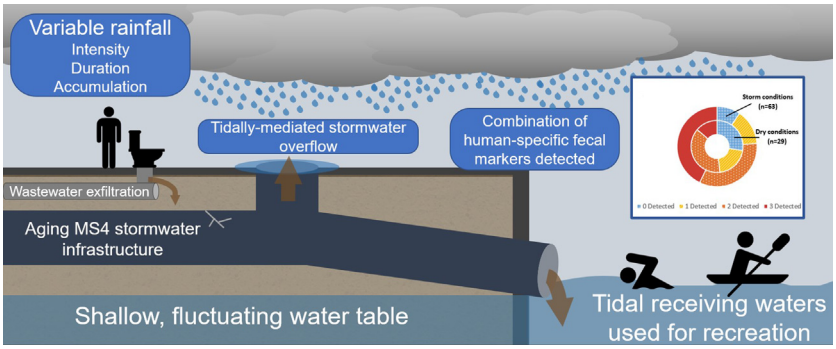
Justin D. Hart [a,b], A. Denene Blackwood [a], Rachel T. Noble [a,b,*]

[a] University of North Carolina Institute of Marine Sciences, Morehead City, NC, United States of America
[b] Department of Environmental Sciences and Engineering, University of North Carolina Gillings School of Global Public Health, Chapel Hill, NC, United States of America

HIGHLIGHTS

- FIB and 3 sewage-specific qPCR markers quantified concurrently in Beaufort, NC
- Short-term rainfall volume predicted elevated fecal indicator concentration
- Establishes a baseline for these markers before Hurricane Matthew in 2018
- Nuisance flooding conveyed sewage-related fecal contamination to street surface

GRAPHICAL ABSTRACT



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ABSTRACT

Fecal contamination is observed downstream of municipal separate storm sewer systems in coastal North Carolina. While it is well accepted that wet weather contributes to this phenomenon, less is understood about the contribution of the complex hydrology in this low-lying coastal plain. A quantitative microbial assessment was conducted in Beaufort, North Carolina to identify trends and potential sources of fecal contamination in stormwater receiving waters. Fecal indicator concentrations were significantly higher in receiving water downstream of a tidally submerged outfall compared to an outfall that was permanently submerged ($p < 0.001$), though tidal height was not predictive of human-specific microbial source tracking (MST) marker concentrations at the tidally submerged site. Short-term rainfall (i.e. <12 h) was predictive of *E. coli*, *Enterococcus* spp., and human-specific MST marker concentrations (Fecal *Bacteroides*, BacHum, and HF183) in receiving waters. The strong correlation between 12-hr antecedent rainfall and *Enterococcus* spp. ($r = 0.57$, $p < 0.001$, $n = 92$) suggests a predictive model could be developed based on rainfall to communicate risk for bathers. Additional molecular marker data indicates that the delivery of fecal sources is complex and highly variable, likely due to the influence of tidal influx (saltwater intrusion from the estuary) into the low-lying stormwater pipes. In particular, elevated MST marker concentrations (up to 2.56×10^4 gene copies HF183/mL) were observed in standing water near surcharging street storm drain. These data are being used to establish a baseline for stormwater dynamics prior to dramatic rainfall in 2018 and to characterize the interaction between complex stormwater dynamics and water quality impairment in coastal NC.

1. Introduction

In coastal North Carolina (NC) variable rainfall patterns generate irregular stormwater runoff that often impairs the quality of receiving

* Corresponding author at: 3431 Arendell Street, Morehead City, NC 28557, United States of America.
E-mail address: rtnoble@email.unc.edu (R.T. Noble).

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water bodies, endangering ecosystems and human health (Sanger et al., 2013). *Enterococcus* spp. (ENT) concentrations are monitored in marine recreational water to approximate the human health risk posed by microbial fecal contaminants. *Escherichia coli* (EC) are likewise used as fecal indicator bacteria (FIB) in freshwater systems. 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). The North Carolina Department of Environmental Quality Division of Marine Fisheries (NCDMF) recreational water quality section monitors ENT concentrations in coastal water used for recreation based on regulatory limits suggested by the United States Environmental Protection Agency for marine waters (USEPA; USEPA, 1986). Additional guidance was issued in 2012 and 2014 by USEPA but has not yet been adopted by NCDMF (USEPA, 2012, 2014).

Typically, 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 lowest 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 USA, and there were 11.8 million person-trips to coastal NC in 2018 alone, resulting in $377 million in spending in Carteret County (Visit North Carolina, 2019).

Even though beach and estuarine water quality is excellent the majority of the time, there are several hydrological mechanisms, including stormwater runoff, that transport fecal contamination to recreational water in coastal NC (Cahoon et al., 2016) Furthermore, stormwater dynamics in coastal NC vary widely from year to year, season to season and month to month. For example, in 2018, Carteret County NC recorded 101.7 in. of rainfall, including 30 in. of rainfall from Hurricane Florence alone (recorded by the National Weather Service, Newport, NC, https://www.weather.gov/mhx/Florence2018) causing devastating flooding and water quality impairments. There is a need for applied microbiological contaminant assessments to inform and evaluate stormwater management strategies. In many cases, there are not engineering solutions for NC coastal systems to mitigate the sheer volume of stormwater-related discharge due to the lack of in-ground space, unpredictability, lack of gradient in elevation, and soil type and quality. Stormwater runoff is known to be the main causative agent adversely impacting water quality in coastal NC (Converse et al., 2011; Parker et al., 2010; Stumpf et al., 2010). In Dare County, NC, the mean loading estimate for fecal indicator bacteria EC and ENT 10^4–10^7 MPN/s of each EC and ENT contributed to receiving water over the duration of a typical storm (Converse et al., 2011). Loading estimates from other studies conducted in coastal NC have generated similar rates of FIB loading, up to 10^{12} total EC and ENT cells (MPN) of over the course of a storm event (Stumpf et al., 2010).

In coastal NC, there are several hydrological and meteorological factors that create unique challenges to stormwater management. For one, regional weather patterns are highly variable on a local scale. For instance, in 2016, weather stations three miles apart in the town of Beaufort, NC and Morehead City, NC recorded 59.1 and 70.4 in. of annual rainfall, respectively (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, while spring rainfall patterns can bring long, steady storm events. Generally, storm events 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 in. in September 2015; Weather Underground Station ID: KMRH). Typical summer storm events can quickly surpass the capacity of engineered stormwater control measures (SCM), leading to flooding and hazardous standing water (Flood and Cahoon, 2011). A recent study on extreme tropical events predicts that they will increase for coastal NC with the onset of climate change driven meteorology (Paerl et al., 2019)

The challenges posed by this variability are compounded by the terrain; the area is low-lying, almost entirely devoid of slope, and tidally-influenced surficial groundwater aquifers are shallow, often within 2–3 ft of the surface of the land in Carteret County, NC when close to the land-water interface. This means there is limited space for SCMs to retain or divert stormwater. There is also little gradient to propel stormwater to another location without pumping. Even within the existing engineered conveyance systems there is evidence of tide- and storm-dependent infiltration and inflow (I/I) between groundwater and the stormwater and wastewater infrastructure in coastal NC (Flood and Cahoon, 2011). The volume of stormwater runoff is partly 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 (Kopp et al., 2015). Corroded wastewater pipes exfiltrate sewage under dry weather conditions, indicating a likely mechanism for the delivery of human fecal contamination to stormwater discharge receiving waters (Sercu et al., 2011). Corrosion of intertidal stormwater and wastewater pipes may therefore lead to greater exfiltration of fecal contaminants (Cahoon and Hanke, 2019).

While cultured FIB are useful for predicting the magnitude of potential fecal contamination stemming from stormwater, they are not able to indicate the fecal sources, such as leaking sewage (Dila et al., 2018; Hagedorn et al., 2011; Olds et al., 2018). Assays that rely on qPCR for quantification of source-specific genes, viruses, or bacteria are now well-accepted in the field of microbial source tracking (MST). Among these, HF183 is consistently one of the best performing human-specific MST markers (Bernhard and Field, 2000; Boehm et al., 2013), with high specificity (Staley et al., 2012) and sensitivity (Ahmed et al., 2012; Green et al., 2014; Shanks et al., 2010) to human feces. Other human-specific MST markers are powerful when used in tandem with HF183 by increasing the certainty of human fecal contamination (Ballesté et al., 2010; Griffith et al., 2016; Sidhu et al., 2013). In addition to HF183, BacHum 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* or order *Bacteroidales* (Harwood et al., 2014; Kildare et al., 2007). Additionally, these particular human-specific assays have been incorporated to epidemiological studies to predict the human health risk of recreational waters (Griffith et al., 2016). Furthermore, recent research on the HF183 marker has pursued an understanding of the linkage between HF183 and calculated microbial risk through an assessment of wastewater-based HF183 concentrations, along with basic assumptions about pathogen:FIB relationships (Boehm et al., 2015). Distinguishing between human and non-human sources of fecal contamination is important to on-the-ground infrastructure remediation as well as 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). Given this, there is hope of standardizing human-specific assays as a regulatory instrument (Boehm et al., 2015; McLellan et al., 2018; Shanks et al., 2016). Various local dynamics can determine the fate and transport of these indicators; thus, it is necessary to sample across a range of conditions to comprehensively characterize trends in MST marker and FIB concentrations (Mattioli et al., 2017; Riedel et al., 2015; Wanjugi et al., 2016).

The primary objective of this study was to quantify the dynamics and magnitude of fecal contamination in the stormwater discharge to highly-used receiving waters of a coastal town in NC. This was accomplished by measuring FIB and molecular markers of sources of fecal contamination, as well as detailed analysis of environmental and physical,

and chemical parameters during a wide array of dry and storm conditions over a ten-month period. The location was selected for study because of the complex intersection of coastal development, hydrology, unpredictable stormwater dynamics, shellfish harvesting and recreational water usage that often result in standing water and flooding. Furthermore, the Rachel Carson Estuarine Research Reserve (RCR) is within hundreds of meters and is highly-recreated within the NC Coastal and National Estuarine Research Reserve Systems. The RCR attracts both recreators and researchers and is ideal for this assessment precisely because of the other data that are collected close by. An objective of this study was to use quantitative approaches 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 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 a foundation of knowledge to assist in stormwater mitigation in the Town of Beaufort, NC through an ongoing collaborative stakeholder engagement process. The characterization of these stormwater receiving waters will inform ongoing investigation into the effects of stormwater runoff from Beaufort to the RCR.

2. Method

The sample sites for this study are located in the Town of Beaufort, a coastal community in Carteret County, NC (Fig. 1). Beaufort has a municipal separate storm sewer system (MS4), though the shallow surficial aquifer has meant the space to construct storm and sanitary sewer systems is constrained and the two are often close together. Taylor's Creek separates the town from the RCR, which includes a group of undeveloped barrier islands. Several of the Beaufort storm sewer outfalls discharge into Taylor's Creek. 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 and public docks on the Beaufort waterfront, with hundreds of bathers each day during the summer tourist season.

Samples were collected during both dry ($n = 29$) and storm ($n = 63$) weather conditions to distinguish the effect of stormwater input from ambient water quality conditions. For the purposes of this study, dry conditions were those which had zero mm of 120-hr (5 days) antecedent precipitation. Storm conditions were classified as periods when at least 6 mm of rain were forecast in a 12-hr period near the sampling locations. 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.

Sampling efforts focused on receiving waters downstream of two stormwater conveyance outfalls that discharge to Taylor's Creek. While there are several other stormwater outfalls along the Taylor's Creek waterfront, these two were selected because of their accessibility, size, and proximity to recreational areas in Taylor's Creek. The stormwater conveyance systems that discharge at these two outfalls drain primarily residential sections of Beaufort. To the west, the Intertidal Outfall at Orange Street (Outfall I) sits at an intertidal elevation. At low tide, Outfall I is exposed and discharges to the surface of Taylor's Creek. A weak but persistent dry weather flow spills into Taylor's Creek

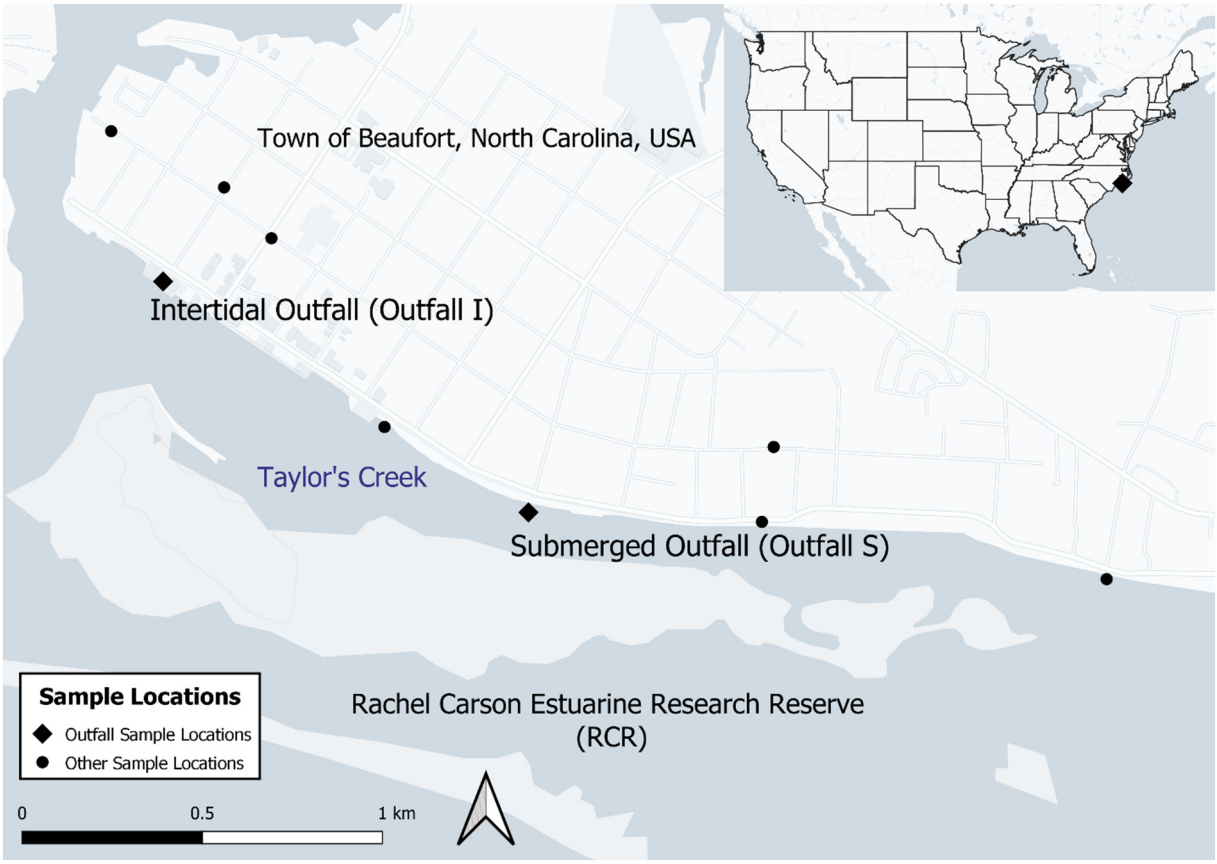

Fig. 1. Sample locations in the Town of Beaufort, NC.

at low tide at Outfall I. Further east, the Submerged Outfall at Gordon Street Dock (Outfall S) discharges submerged beneath a public dock. Each outfall is the terminus of a 0.61 m diameter reinforced concrete pipe. Samples were occasionally gathered in-pipe from the stormwater sewer upstream from discharge locations with cooperation from the Town of Beaufort Division of Public Works. Standing water throughout Beaufort was also sampled based on observation of ponding to determine the water quality of these nuisance floodwaters (Fig. 1).

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). Sterile, pre-rinsed 1 L acid-washed polypropylene (Nalgene™) bottles were used to collect 1 L samples <1 m downstream from the end of the pipe at each sampling location at depths of 0.5–1 m below the surface. Samples were transported to the University of North Carolina at Chapel Hill Institute of Marine Sciences (UNC-IMS) in a cooler on ice and processed upon return within 3 h of collection.

2.1. Sample preparation

Taylor's Creek is a tidal creek in which brackish water from the Newport River Estuary (see Gonzalez et al., 2012, and Coulliette et al., 2009) and marine saltwater from the Atlantic Ocean mix. ENT and EC 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's 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. 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° for 1–7 months until 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 their use in qPCR analysis.

2.2. qPCR calibration standards

Plasmid standards were used for fecal *Bacteroides*, BacHum and HF183 qPCR assays. Standards were synthesized by GenScript (Piscataway, NJ). Gene sequences relating to the target sequences were synthesized and inserted into a linearized pUC57 vector which was cloned into DH5α competent cells. Plasmids containing the insert were extracted using Wizard® Plus SV Minipreps DNA Purification System (Promega Corp., Madison, WI). Plasmids were linearized using Eco R1 digestion and verified via a 1% agarose gel in Tris-Acetate-EDTA buffer. The weight of purified plasmids was then determined spectrophotometrically (Nanodrop 2000c, Thermo Scientific, Waltham, MA). Nanograms of purified plasmids were converted 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. The quantity of each standard was verified via droplet digital PCR (ddPCR) using a QX200™ Droplet Digital™ PCR System (Bio-Rad Laboratories, Inc., Hercules, CA). The same primers and probes for each target were used for both ddPCR and qPCR. Standard concentrations ranged from 8.91×10^7 gene copies/100 mL for Fecal *Bacteroides* to 1.56×10^8 gene copies/100 mL for HF183 (Table 1).

For these reactions, 5 μL of each standard was transferred to 500 μL of buffer AE (QIAGEN), bead beaten for 2 min in a 48-place Mini-Bead Beater™ (BioSpec Products, Inc. Bartlesville, OK), then centrifuged at 10,000*g* for 1 min. 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 min, 40 cycles of 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 30 s, 98 °C for 10 min 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 Bio-Rad QuantiSoft software (Bio-Rad) (Table 1).

Table 1
Concentrations of plasmid standards and SPC.

Target assay	Standard concentration (copies/100 mL water)	
Fecal *Bacteroides*	8.91×10^7	(95% CI: $8.56–9.26 \times 10^7$)
BacHum	1.16×10^8	(95% CI: $1.09–1.23 \times 10^8$)
HF183	1.56×10^8	(95% CI: $1.32–1.80 \times 10^8$)
ACTB (SPC)	5.40×10^7	

A specimen processing control (SPC) was added to all unknowns, standards, and negative controls to 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 extraction tubes 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 the absence of cross-contamination. In no case was cross-contamination observed as a result of 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 as a positive control for the *ACTB* SPC assay. None of the samples in this study were determined to be inhibited relative to the NEC. Following qPCR analysis for the *ACTB* marker, an unknown sample was considered inhibited if its cycle threshold (C_T) exhibited greater than a 2.32 C_T delay (equivalent to a half-log difference in concentration) relative to the C_T of the NEC (Gonzalez and Noble, 2014). None of the samples in this study exhibited inhibition according to this metric. However, 32 samples (out of total n = 92) were diluted 1:2 to increase the volume available to perform the assays.

2.3. qPCR analyses

The concentrations of fecal-associated molecular markers in water samples were determined through previously published real-time qPCR assays (Table 2) 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.

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

Table 2
Primer and probe sequences of target assays.

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	BacHum-160f	TGAGTTCACATGTCCGCATGA	1000 nM	Kildare et al. (2007)
	BacHum-241r	CGTTACCCCGCCTACTATCTAATG	1000 nM	
	BacHum-193p	6-FAM-TCCGGTAGACGATGGGGATGCGTT-BHQ-1	100 nM	
HF183	HF183	ATCATGAGTTCACATGTCCG	1000 nM	Haugland et al. (2010)
	BFDRev	CGTAGGAGTTTGGACCGTGT	1000 nM	
	BFD TM FAM	6-FAM-CTGAGAGGAAGGTCCCCCACATTGGA-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		

et al., 2005). For unknown reasons, the *ACTB* SPC demonstrated higher concentrations in samples than in controls and was therefore not used to calibrate 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.

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 3). The quality control characteristics of the master curves for each marker appear in Table 3.

NTC and NEC were not positive for any of the MST marker assays. The limit of blank (LoB) for each assay was calculated using the corresponding master curve assuming a C_T value of 40 (Table 4). 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 master curve. The limit of quantification (LoQ) was assumed to be identical to the LoD.

2.4. Data analyses

Colilert-18® and Enterolert ™ values were averaged in Microsoft Excel using MPN equations from Hurley and Roscoe (1983). Samples exceeding the detection limit for IDEXX Quantitray/2000© were assigned the highest value within the averaged limits of detection (24,560 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. For samples where an MST marker was not detected, the marker was assigned a value of 1.0 copy/100 mL to simplify the dataset for log-adjustment. For samples with discordant duplicate detection—where the marker was detected in one but not both duplicate wells– the copy number was calculated as half of the detected value.

Given the seasonally variable intensity of recreational use of Taylor's Creek, the NCDEQ Tier 1 standard of 104 ENT MPN/100 mL was applied to place the results of this study into the context of recreational water quality management. The NC Coastal Recreational Water Monitoring program also includes a threshold of 35 ENT MPN/100 mL for the geometric mean of five samples collected over a 30-day period. Samples were not collected frequently enough during each 30-day period to reach the five samples required to calculate the geometric mean because the samples were collected using an adaptive monitoring framework based on precipitation (USEPA, 2014). 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 recommended by the EPA (USEPA, 2014). USEPA and NCDEQ guidance include thresholds of 100 EC MPN/100 mL and 35 ENT MPN/100 mL, respectively, for the geometric means of five samples collected over a 30-day period. These thresholds were not referenced in the analyses due to the adaptive monitoring framework based on precipitation. Samples were not collected frequently enough during each 30-day period to reach the five samples required to calculate the geometric mean for each 30-day period during the study.

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 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. A power test was performed to avoid Type I error in these correlations by confirming $\beta > 0.8$ for the sample size. The variabilities of FIB 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. ENT and 12-hour cumulative antecedent rainfall were plotted against HF183 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).

Table 4
Limits of blank and detection for qPCR assays.

MST marker	Limit of blank (copies/reaction)	Limit of detection (copies/reaction)
Fecal *Bacteroides*	6.52	54.3
BacHum	8.2	32
HF183	3.21	7.03

Table 3
qPCR master curves.

Targets	# of individual standard curves (total # of data points included)	Master curve	R^2	Efficiency
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)	−3.53 + 41.8	0.983	91.94%
ACTB	5 (75)	−3.50 + 42.0	0.960	93.07%

3. Results

EC concentrations ranged from no detection to 5.88×10^4 MPN/100 mL. ENT concentrations ranged from no detection to 1.70×10^4 MPN/100 mL. The mean concentrations of both EC and ENT were significantly greater ($p < 0.001$) in receiving waters during storm conditions (EC mean = 158 MPN/100 mL, ENT mean = 214 MPN/100 mL) than during dry conditions (EC mean = 25.7 MPN/100 mL, ENT mean = 15.8 MPN/100 mL), (Fig. 2). Of the samples collected from receiving waters, 19 of 53 (35.8%) exceeded the NC ENT threshold of 104 MPN/100 mL (Fig. 3) and 8 samples (15.1%) exceeded the USEPA EC threshold of 320 MPN/100 mL. All exceedances occurred during storm conditions.

When considering samples collected from storm (n = 63) and dry (n = 29) weather conditions over the duration of the entire study period, FIB concentrations were significantly higher ($p < 0.01$) at Outfall I (EC mean = 95 MPN/100 mL, ENT mean = 151 MPN/100 mL) relative to Outfall S (EC mean = 45.7 MPN/100 mL, ENT mean = 29.5 MPN/100 mL) (Fig. 3). Of the regulatory exceedances measured, 14 exceedances (n = 19, 73.7%) of the NCDEQ ENT threshold and seven exceedances (n = 8, 87.5%) of the USEPA EC threshold occurred at Outfall I. Six of the ENT exceedances at Outfall I were an order of magnitude greater than the threshold (Fig. 3).

Samples collected in-pipe or from standing water upstream of the outfalls (hereafter "land-based sites") had significantly higher concentrations of both ENT (mean = 3.72×10^3 MPN/100 mL) and EC (mean = 2.09×10^3 MPN/100 mL) compared to either outfall. Of the 16 samples taken from land-based sites, 5 (31.5%) exceeded the ENT threshold by two orders of magnitude and 3 (18.8%) exceeded the EC threshold by two orders of magnitude.

The human-specific marker HF183 was detected in 65 of the samples (n = 92, 70.6%), BacHum in 59 (n = 92, 64.1%), and fecal *Bacteroides* in 48 (n = 92, 52.1%; Table 5). All three human-specific markers were detected together in 31 of the samples (n = 92, 33.7%; Table 5). Of the 92 samples, 42 (45.7%) were below the limit of detection for fecal *Bacteroides*, 14 (15.2%) for BacHum, and 5 (5.43%) for HF183. These concentrations 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, a practice described in e.g. Cao et al. (2013). None of the negative controls used for these assays had detectable gene copies, suggesting the observed gene copy quantities in samples were not due to cross-contamination during field or laboratory processing.

Of the 18 storm events sampled for this study, HF183 was detected in Taylor's Creek during all 18 storms, BacHum was detected during 17 storms, and Fecal *Bacteroides* was detected during 16 storms. Nine of the 21 samples collected from Outfall I during storm events were positive for all three human-specific markers. All three human-specific markers were detected at both Outfall I and Outfall S even during dry conditions (Table 5). There was no significant difference in the distributions of HF183 and BacHum between Outfall I and Outfall S. However, there was a significant difference between the distribution of fecal *Bacteroides* concentrations at the two sites ($p < 0.01$). For human-specific MST marker concentrations at Outfall I and Outfall S, there was a significant difference (all $p < 0.05$) in concentrations between dry (HF183: dry mean = 12.9, BacHum: dry mean = 22.4 copies/100 mL fecal *Bacteroides:* dry mean = 6.89 copies/100 mL) and storm (HF183: storm mean = 97.7 copies/100 mL, BacHum: storm mean = 109 copies/100 mL, fecal *Bacteroides*: storm mean = 20.9 copies/100 mL) conditions.

At least one human-specific marker was detected in a majority of samples during all weather conditions (n = 74/92, Table 5). Of the three human-specific markers, the highest concentrations of each human-specific MST marker were detected in the in-pipe and standing water samples, with maximum concentrations of 2.57×10^4 copies/

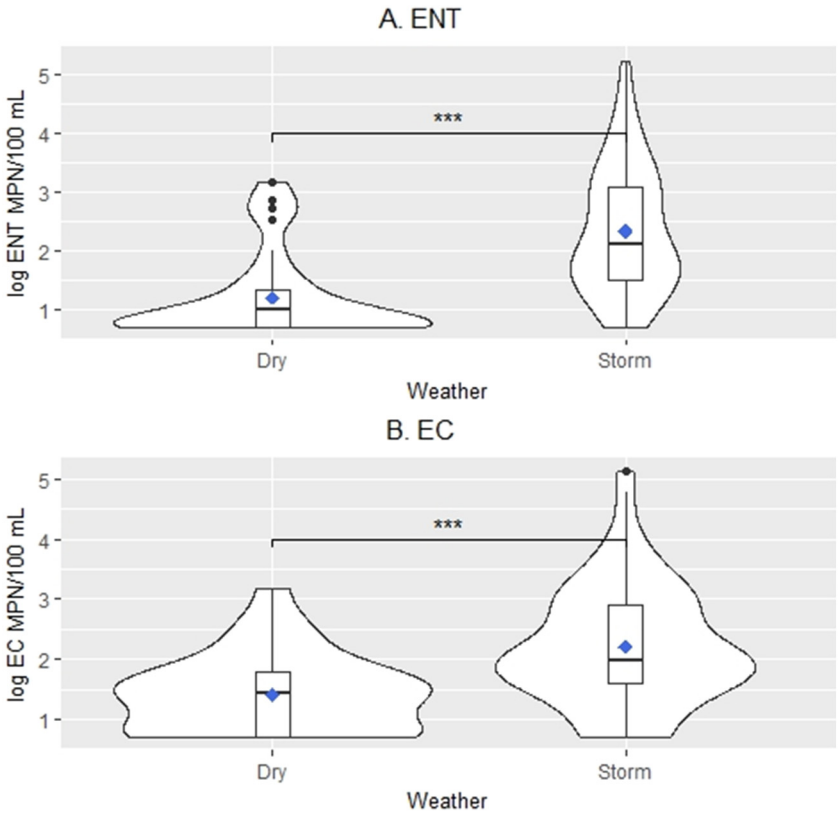

Fig. 2. Violin plots of ENT and EC concentrations during dry and storm weather conditions. The blue diamond represents the mean concentration.

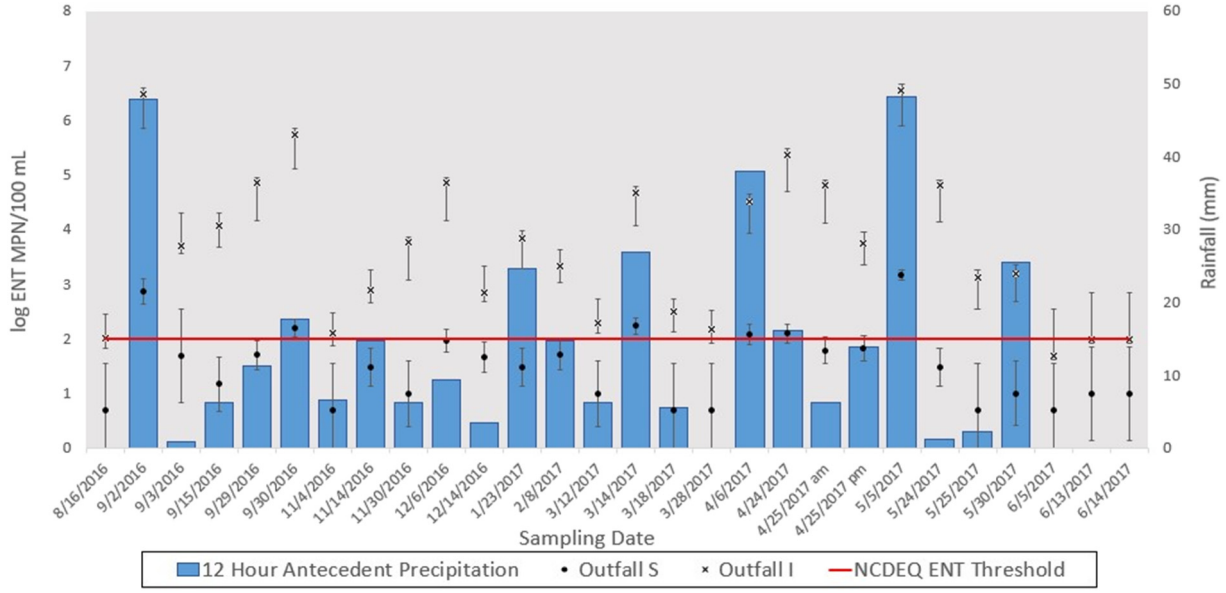

Fig. 3. ENT concentrations and 12-hr antecedent precipitation relative to the NCDEQ ENT threshold of 135 MPN/100 mL.

100 mL, 1.20×10^5 copies/100 mL, and 6.92×10^4 copies/100 mL for HF183, fecal *Bacteroides*, and BacHum, respectively. In Beaufort wastewater treatment plant sewage influent, the human-specific MST marker concentrations used in this study ranged from $1–5 \times 10^8$ copies/100 mL. Each of the land-based samples was taken from a stormwater manhole or overflowing stormwater intake, often during active precipitation.

Samples were collected up-watershed of both outfall sites during dry weather to determine the path and concentration of human fecal contamination receiving waters (Fig. 4). During one dry weather sampling event, all three human-specific MST markers were detected near Outfall I. On the same day, no MST markers were detected near Outfall S. On a separate day with dry conditions, BacHum and HF183 were detected in samples collected from boat-based sampling >100 m downstream of Outfall I. Fecal *Bacteroides* was detected at one of two sites sampled by boat 50 m downstream of Outfall S, but the other human-specific MST markers were not detected.

3.1. FIB and MST marker correlations with environmental parameters

Across all weather conditions, EC and ENT strongly correlated with one another ($r = 0.772$) in receiving water samples, indicating similar factors drive FIB concentration trends in Taylor's Creek (Fig. 5). During all weather conditions, all three human-specific MST markers significantly correlated with all FIB and each other. At both outfall sites, all FIB and human-specific MST markers significantly correlated with short-term (6 h or 12 h) cumulative rainfall (Fig. 5). These same relationships were not significant and were weaker when on-land sampling locations were included. 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.

Table 5
Detection of multiple markers in samples according to weather condition and sample location.

Detected marker(s)	Dry (n = 28)	Wet (n = 64)	Outfall (n = 75)	Land-based (n = 17)	Total (n = 92)
None	25%	11%	15%	18%	15%
Only HF183	0%	11%	9%	0%	8%
Only BacHum	4%	6%	1%	24%	5%
Only Fecal Bacteroides	4%	3%	4%	0%	3%
HF183 + BacHum	4%	13%	12%	0%	10%
HF183 + Fecal Bacteroides	29%	16%	19%	24%	20%
BacHum + Fecal Bacteroides	7%	5%	7%	0%	5%
All three	29%	36%	33%	35%	34%

3.2. Site-based associations with antecedent rainfall

To further examine the role that the type of sampling location plays on these associations, linear models were plotted to compare HF183, EC, ENT, and 12-hr rainfall (Fig. 5). 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 at Outfall I, the relationship was negative at land-based sites. Similarly, HF183 demonstrated a positive association with rainfall at Outfall I, but a negative association at land-based sites. No significant relationship was observed between HF183, FIB, and antecedent rainfall at Outfall S.

4. Discussion

The concentrations of FIB and human-specific MST markers in Beaufort stormwater and in the receiving waters of stormwater discharge are seriously concerning. Both EC and ENT concentrations in standing water and receiving waters increased significantly during storm conditions as compared to dry weather conditions (both $p < 0.001$). During storm conditions, concentrations of EC and ENT strongly and significantly correlated with one another ($r = 0.833$, $p < 0.05$). Antecedent rainfall correlated significantly for all cumulative rainfall periods analyzed for this study with both ENT and EC concentrations (all $p < 0.01$), supporting the prediction that observed fecal contamination results in part from cumulative stormwater input. The strongest correlations were at 30-day antecedent rainfall (EC: $r = 0.473$, $p < 0.001$; ENT: $r = 0.415$, $p < 0.001$), 12-hr antecedent rainfall (EC: $r = 0.545$, $p < 0.001$; ENT: $r = 0.570$, $p < 0.001$), and 6-hr antecedent rainfall (EC: $r = 0.586$, $p < 0.001$; ENT: $r = 0.564$, $p < 0.001$). For samples taken during storm conditions, only 6-hr and 12-hr antecedent rainfall correlated with EC and ENT ($p < 0.001$). In some samples, the concentrations of EC and ENT exceeded regulatory thresholds recommended by NCDEQ

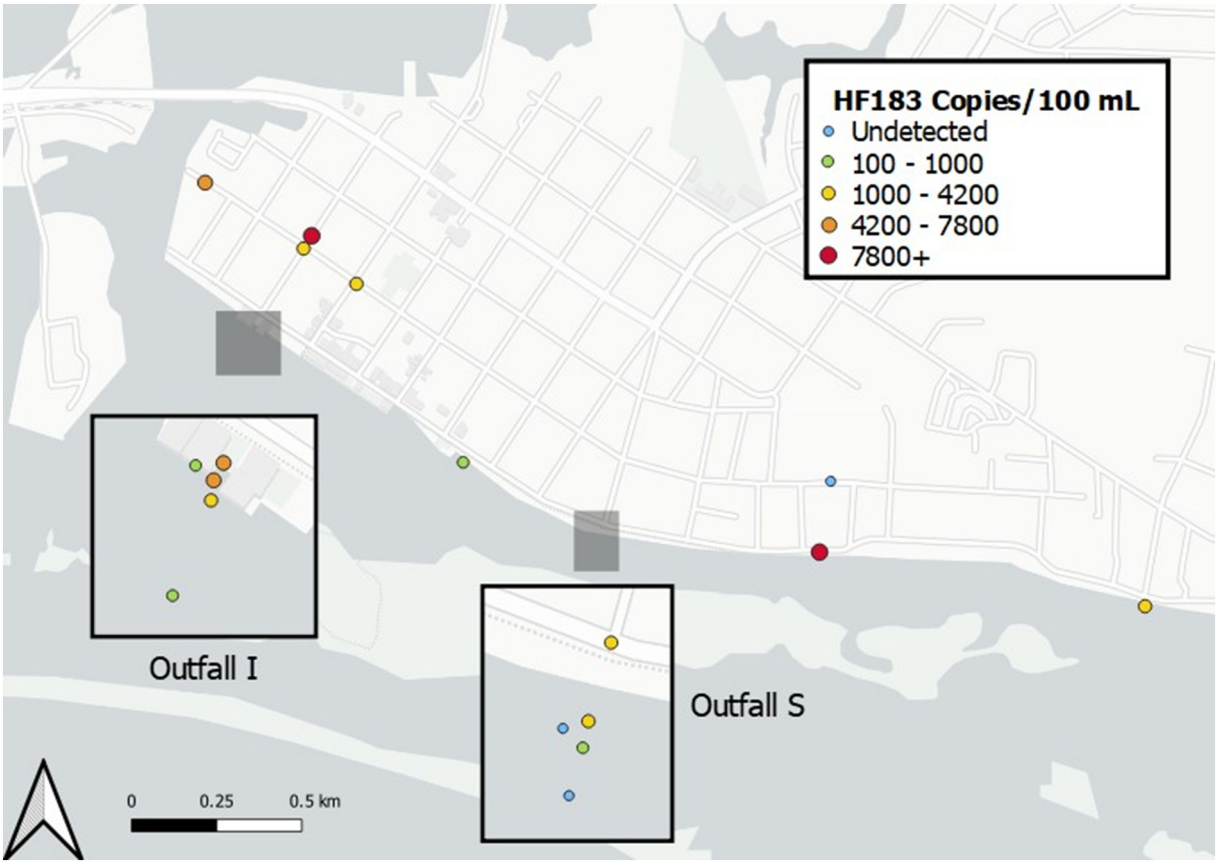

Fig. 4. Map of relative HF183 concentrations at sampling sites throughout Beaufort. (Inset: HF183 concentrations of transects downstream from Outfall I and Outfall S).

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 warrant further modeling of rainfall-based recreational advisories for this section of Taylor's Creek because of its heavy recreational use and visibility and prominence to local tourism.

At least one of the three human-specific markers was found at each land-based in-pipe or standing water 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 fecal contamination at specific nodes of the stormwater conveyance system, and that this contamination contributes to the elevation in observed FIB concentration during storm events. Human fecal markers have previously been detected and quantified in MS4 communities, negating the assumption that separation prevents sanitary sewage from contaminating stormwater (Sercu et al., 2011; Steele et al., 2018; Olds et al., 2018). The human-specific markers detected in stormwater discharge to Taylor's Creek not only indicate the possible presence of human pathogens, but also of organic pollutants, nutrients, and pharmaceuticals typically found in human sewage (Dila et al., 2018; Templar et al., 2016).

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 animals (e.g. dogs, cats, deer) in other locations (Harwood et al., 2014; Layton et al., 2013). The use of multiple source-specific markers seeks to overcome these variations, providing greater certainty of human contamination (Harwood et al., 2014). Other studies have also quantified human fecal contamination using HF183 in combination with other human-specific markers to compare performance of the markers and improve certainty of human fecal contamination (Lenaker et al., 2018; Li et al., 2019). The repeated quantification of all three markers in this study, often at concentrations that are representative of significant human fecal sources, indicates a strong likelihood of human contamination originating from sewage infrastructure in this circumstance (Lenaker et al., 2018). For instance, six samples were negative for HF183 but positive for at least one of the other human-specific markers. The presence of these markers in standing water also suggests that during overflow conditions in the stormwater conveyance system (e.g. during a storm at high tide), diluted sewage is reaching the surface and streets. Saltwater from the estuary that has infiltrated the storm sewer may also be present in these puddles, complicating the relationship between precipitation volume and FIB concentrations. These puddles may be a hazard to human health as the high concentrations of human-specific MST markers indicates that human pathogens may also be present. While these short-lived puddles are not regulated as recreational waters, further investigation of the patterns and quantities of source-specific markers in storm-related standing water may provide important clues regarding the condition of the sewer system, (e.g. a contaminated puddle may appear near a compromised sanitary sewer pipe), and in particular will offer clues to the impact of estuarine tidal influx to the system.

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. The storm-related increase in the concentration of human-specific markers suggests they are more concentrated in stormwater than in the receiving waters (Templar et al., 2016). These concentrations offer insight to the water quality in Taylor's Creek itself and a conservative approximation of the human fecal pollution 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 sites in RCR that are used for recreation.

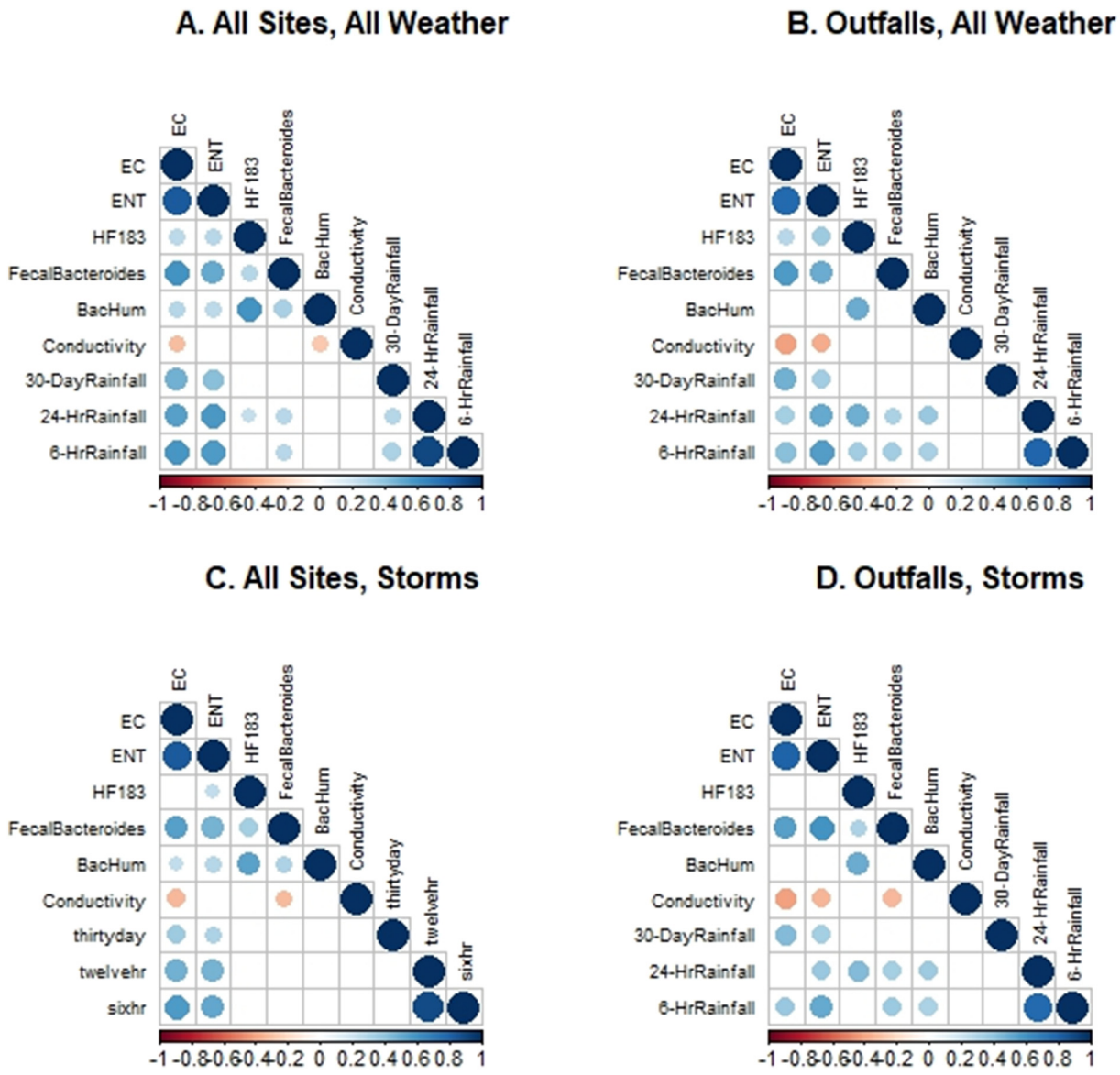

Fig. 5. Correlation plots comparing the distributions of rainfall and water quality parameters with FIB and human-specific marker concentrations for A) all sites and weather conditions, B) samples collected from receiving water during all weather conditions, C) all sites during storm conditions only, and D) samples collected from receiving water during storms only. Blue circles indicate positive correlation, orange circles indicate significant negative correlation, the absence of a circle indicates the correlation was not significant, and the size of the circle indicates the strength of the correlation, with larger being stronger.

The detection of human-specific markers up watershed of Outfall I suggests that sewage enters the stormwater system upstream of Outfall I even during dry conditions. There is a visible, consistent flow at low-tide at Outfall I, which may result from wastewater exfiltration or simply dry weather runoff. In MS4 communities, exfiltration occurs when the wastewater sewer is above the water table, which in Beaufort would likely correspond to low tide (Sercu et al., 2011).

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; Jardé et al., 2018; Wanjugi et al., 2016). These factors may help explain the return to excellent water quality conditions and the lack of MST markers detected at Outfall S during dry conditions. This also suggests the relatively high concentrations of MST markers detected at Outfall I during dry conditions originate from a fresh fecal source.

Different relationships were observed between rainfall and MST markers in land-based and receiving water samples. In receiving water samples, cumulative rainfall was predictive of MST marker concentrations. The correlations were significant for 6-hr antecedent rainfall (fecal *Bacteroides*: $r = 0.340$, $p < 0.05$; BacHum: $r = 0.330$, $p < 0.05$; HF183: $r = 0.344$, $p < 0.05$) and 12-hr antecedent rainfall (fecal *Bacteroides*: $r = 0.310$, $p < 0.05$; BacHum: $r = 0.377$, $p < 0.05$; HF183: $r = 0.488$, $p < 0.01$) (Fig. 5). However, for land-based samples there is an inverse relationship between 12-hr antecedent rainfall and the concentration of HF183 (Fig. 6). This suggests that increases in overland stormwater runoff volume do not contribute an increase in MST markers to the stormwater system. Rather, this indicates the bulk of the human-associated contamination originates within the sanitary sewer system.

5. Limitations and future directions

Since they are present in the feces of warm-blooded animals, FIB concentrations are not influenced solely by human fecal sources. A gull-specific qPCR assay to detect *Catellicoccus* spp. was used, however it was only found in 22.8% of samples and concentrations were generally

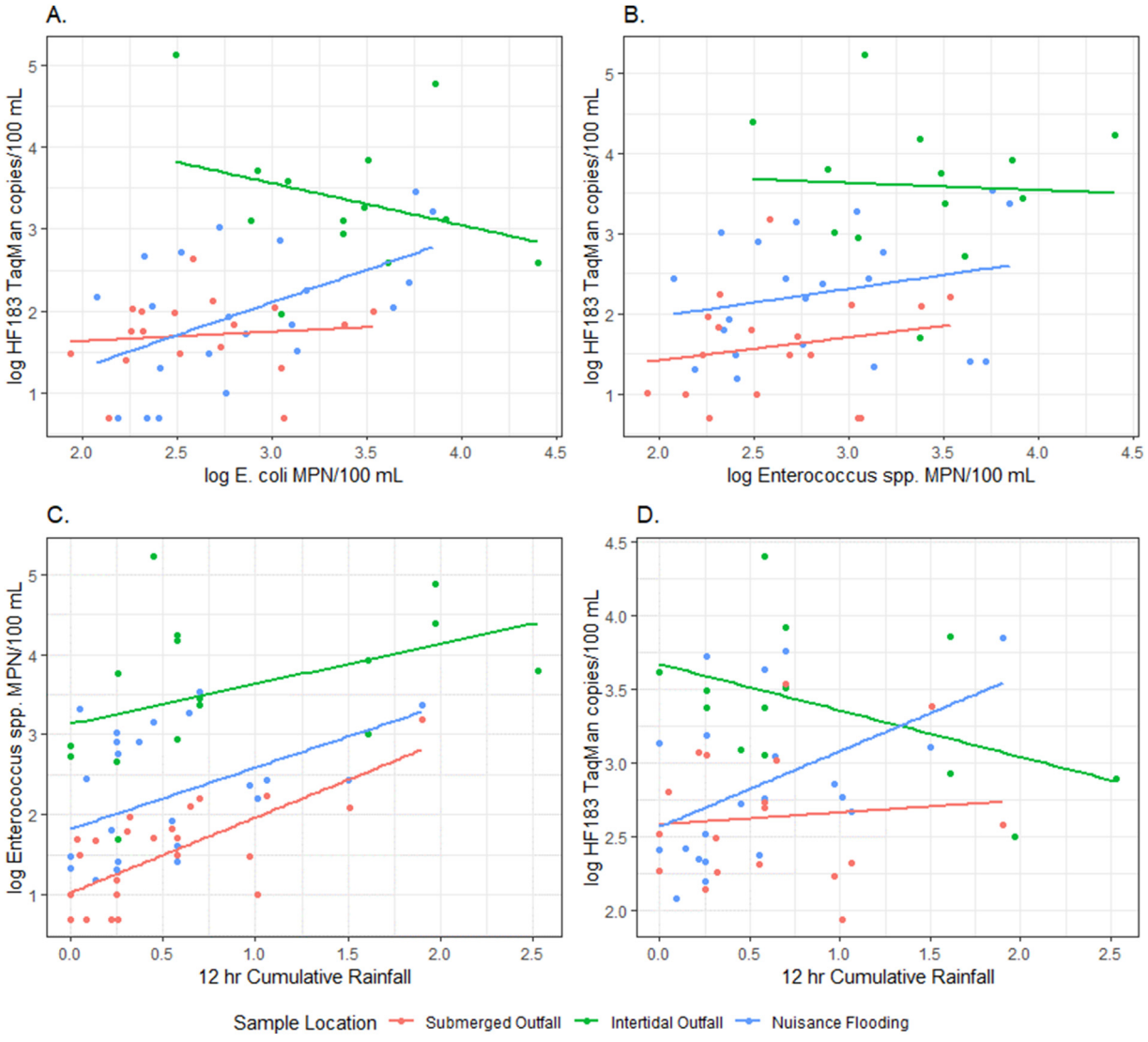

Fig. 6. Linear associations between a) EC and HF183, b) ENT and HF183, C) 12-hr antecedent rainfall and ENT, and d) 12-hr antecedent rainfall and HF183.

low, and not determined to be influenced by weather (data not shown). Due to the high concentrations of human-specific markers observed and their strong associations with FIB, it is reasonable to assume human sources contribute significant fecal contamination to the stormwater in this particular system.

The *ACTB* SPC was originally intended to be used to develop a correction factor for the qPCR analysis, but did not perform adequately or consistently. An accurate 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. Typically, 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). 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). While the *ACTB* SPC used for this study was able to approximate adequate recovery from the DNA extractions, it did not perform consistently enough to fully quantify inhibition of the qPCR reaction across a relevant linear range of concentrations. As a result, the concentrations of the MST markers were not calibrated according to recovery or inhibition and are conservative estimates of the true concentrations. The use of a ddPCR platform may have further reduced inhibition by partitioning inhibitory substances (Cao et al., 2015).

The *ACTB* SPC was intended to control for variation in specimen processing between extraction and thermocycling. The term specimen processing control can also refer to an SPC that is added prior to sample filtration and which may be used to determine recovery (Zhang and Ishii, 2018). This type of control was not selected for this study because the filtration process removes extracellular DNA present in the sample, thus providing a better approximation of the target cell concentration. A pre-filtration SPC would also control for this extracellular DNA, which was not the aim of this study.

Additionally, optimized HF183 primers have been developed that report high specificity and sensitivity to human sewage (Green et al., 2014). The use of more optimized primers in this study may have reduced the error of the HF183 results and improved the associations with other indicators.

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 for stormwater contamination. For instance, at high tide, seawater enters and occasionally fully submerges the outfall at Outfall I, causing significant dilution as brackish water enters the stormwater

system. Outfall elevation data were compared to tidal height collected from a nearby tidal gage at Duke Marine Lab (NOAA Station 8656483) to verify the inundation of the outfall at time of sample collection. There was no significant difference observed for any of the water quality indicators measured between samples collected when Outfall I was submerged (n = 43) versus when it was exposed (n = 49). However, this inundation may become increasingly challenging as Beaufort specifically and coastal NC generally experience more frequent high-tide flooding associated with sea level rise. In 2015, Beaufort experienced as many flood events as it had during the period of 1985–2000 (NOAA Station 8656483). By 2050, King Tides, current-driven high-tides that may cause coastal flooding regardless of weather, are anticipated to cause flooding in Beaufort between 25 and 100 days per year (NOAA, 2019). Coastal sewer infrastructure that is not protected against saltwater intrusion, as in Beaufort, may experience greater corrosion from these repeated inundations (Flood and Cahoon, 2011).

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 (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 surcharge conditions appear to bring fecal contaminants to the surface. While recreational bathing is an elective activity where exposure to fecal contaminants is voluntary, these nuisance floods bring the same fecal contaminants into communities where exposure may be involuntary, presenting a different risk paradigm relevant to stormwater management and coastal mitigation.

Simulated quantitative microbial risk assessment (QMRA) has been used in peer-reviewed literature to translate the illness rate benchmarks underlying USEPA guidelines to human specific markers. Boehm et al. (2015) benchmark of 4.2×10^3 4200 (3) HF183 gene copies/100 mL while McLellan et al. (2018) derived a benchmark of 7.8×10^3 7800 () Human *Bacteroides* gene copies/100 mL. In that study the "Human *Bacteroides*" primer and probe sequences are described in Sauer et al. (2011) and include the HF183 forward primer and BacHum reverse primer. Although the HF183 and Human *Bacteroides* markers are not a pathogen or causative agent of disease, they are indicative of human fecal contamination and the presence of viral and bacterial pathogens. While the reference material used in this study is different than that used to determine the benchmarks in these studies, it still serves as a gage for the relationship between the concentrations of human-specific markers observed in Beaufort stormwater and the potential relationship to human illness. For instance, a single sample taken during storm conditions from Outfall I receiving waters exhibited 5.37×10^3 HF183 gene copies/100 mL and 2.53×10^4 BacHum copies/100 mL. Concentrations in this range are concerning, particularly when corroborated by multiple human-specific indicators (Olds et al., 2018). BacHum 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 suggests an elevated and relevant risk to human health from contact with or ingestion of water from Taylor's Creek following storm events.

For instance, predictive models incorporating location-specific stormwater dynamics have been successfully developed to accurately predict FIB concentrations in the Great Lakes (Francy, 2009), Los Angeles (Thoe et al., 2014), the Gulf Coast (Zhang et al., 2012) and coastal NC (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. 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 human-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 a rainfall-based advisory.

CRediT authorship contribution statement

Justin D. Hart: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **A. Denene Blackwood:** Conceptualization, Methodology, Validation, Writing - original draft. **Rachel T. Noble:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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