Integrating Culture and Molecular Quantification of Microbial Contaminants into a Predictive Modeling Framework in a Low-Lying, Tidally-Influenced Coastal Watershed

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ABSTRACT

Examinations of stormwater delivery in the context of tidal inundation are lacking. Along the coastal plains of the southeast, tidal inundation is increasing in frequency and severity, often with dramatic adverse impacts on stormwater discharge and "sunny day flooding". Therefore, a comprehensive study was conducted to examine tidally-influenced stormwater outfalls discharging to Taylor's Creek, an estuary off the coast of Beaufort, NC used regularly for recreation and tourism. Over a wide range of meteorological conditions, water samples were collected and analyzed for fecal indicator bacteria (FIB, used for regulatory decision-making) and published quantitative microbial source tracking (qMST) markers. Nineteen sampling events were conducted from July 2017 – June 2018 with samples classified as inundated, receding or transition depending on collection during tidal stage. A first-of-its-kind multiple linear regression model was developed to predict concentrations of *Enterococcus* sp. by tidal cycle, salinity and antecedent rainfall. We demonstrated that the majority of variability associated with the concentration of *Enterococcus* sp. could be predicted by *E. coli* concentration and tidal phase. FIB concentrations were significantly (<0.05) influenced by tide with higher concentrations observed in samples collected during receding (low) tides (EC: log 3.12 MPN/100 mL; ENT: 2.67 MPN/100 mL) compared to those collected during inundated (high) (EC: log 2.62 MPN/100 mL; ENT: 2.11 MPN/100 mL) or transition (EC: log 2.74 MPN/100 mL; ENT: 2.53 MPN/100 mL) tidal periods. Salinity, was also found to significantly (<0.05) correlate with *Enterococcus* sp. concentrations during inundated (high) tidal conditions (sal: 17 ppt; ENT: 2.04 MPN/100 mL). Tide, not precipitation, was shown to be a significant driver in explaining the variability in *Enterococcus* sp. concentrations. Precipitation has previously been shown to be a driver of

Enterococcus sp. concentrations, but our project demonstrates the need for tidal parameters to be included in the future development of water quality monitoring programs.

1. INTRODUCTION

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

The United States (US) Environmental Protection Agency (US EPA) has recommended the use of enterococci (ENT) and *Escherichia* coli (EC) as fecal indicator bacteria (FIB) to monitor both marine and fresh surface waters (US EPA, 2012). FIB serve as a proxy for the presence of microbial pathogens associated with feces. Ingesting water with high concentrations of FIB through recreation can lead to gastrointestinal and other illnesses (Colford et al., 2007; Haile et al., 1999; Soller et al., 2017). Additionally, FIB have been selected due to their low pathogenic potential and high concentrations in sewage and feces (Ahmed et al., 2008; Ahmed et al., 2019; Harwood et al., 2014; Sidhu et al., 2012). As such, FIB have been widely used by states as a mitigation tool to meet US EPA water quality requirements. States have the discretion, however, to implement either or both FIB in monitoring programs. The State of North Carolina (NC) utilizes enterococci solely to monitor recreational surface waters (NC DEQ, 2020). While studied significantly across coastal waters, one major drawback towards the use of FIB, however, is their lack of source-specificity (Ex. human vs. non-human) regarding fecal contamination. As such, quantitative microbial source tracking tools (qMST) have been proposed.

Quantitative microbial source tracking methods aim to discriminate between human and non-human fecal sources in contaminated waterbodies (Lee et al., 2020; Nguyen et al., 2018;

Shanks et al., 2015). The performance of human-specific (Ex. HF183) markers are of particular interest to mitigate public health risks, given their utility and strong relationships to observed risk in sewage-impacted waters (Badgley et al., 2019; Haugland et al., 2010; Jothikumar et al., 2005). Additionally, US EPA has published recommendations for concentrations for *Enterococcus* sp. quantified via a qPCR-based approach in fresh and marine surface waters (Haugland et al., 2005; US EPA Method 1609 &1611, 2012). Previous epidemiological studies have indicated a stronger link between swimming-associated gastrointestinal illnesses and molecular approaches for Entero1-qPCR compared to traditional culture-based methods (Arnold et al., 2016; Colford et al., 2012; Wade et al., 2008). Greater understanding of the concentrations of specific fecal qMST source markers relative to culture-based FIB enumeration used in routine water quality monitoring is necessary, especially within the context of coastal systems.

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

Coastal NC has over 5900 km^2 of land below 1-m elevation (Figure 1), making it the third largest low-lying region in the US (Poulter et al., 2009; Titus & Richman, 2001). Additionally, much of the coastal zone in NC has a low topographic slope increasing at less than 0.09 m elevation for every horizontal mile (Corbett et al., 2008). As such, coastal NC remains susceptible to the effects of global climate change, including sea level rise, intensifying extreme storm events and increasing tidal ranges and sunny-day flooding (Hino et al., 2019). Sea level off the NC coast has increased 0.28 m as compared to 1950. The rate of rise accelerating over the last decade to now increasing by over 0.03 m every 2 years (NOAA, 2020; NC Coastal Resources Commission, 2015). This coupled with increased nuisance flooding frequency events suggest coastal surface waters along the coast of NC are at risk for continual impairment (King Tides Project, 2020; Sweet et al., 2014).

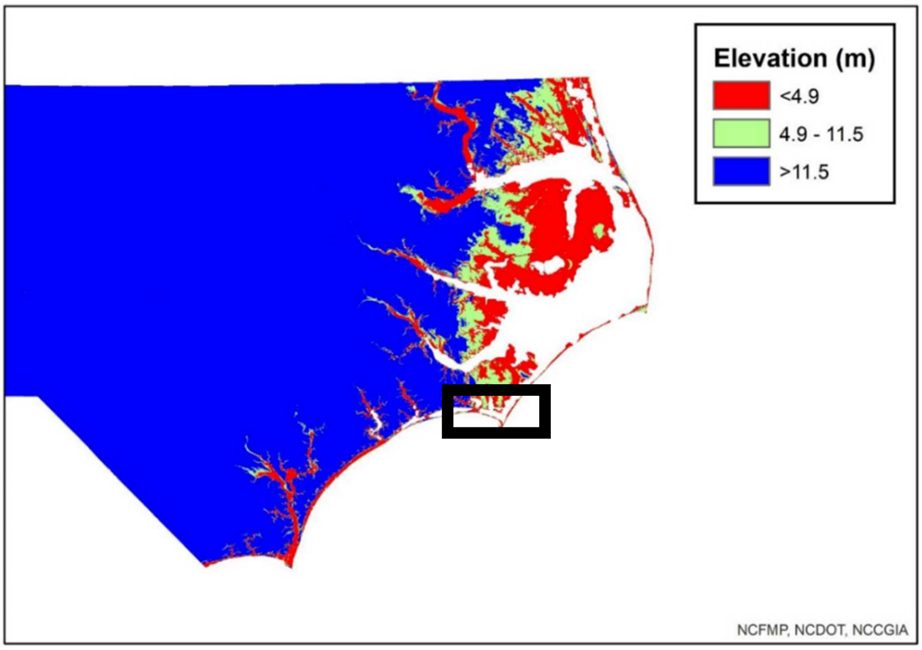

Figure 1: Digital Elevation Model (DEM) depicting elevation in coastal, eastern NC and sampling area.

The study site for this research is located in Beaufort, NC, a coastal community situated in the coastal plain region of southeastern NC with a relatively small permanent population (4,391) that experiences seasonal growth given its proximity to coastal waters and productive tourism industry (US Census Bureau, 2020). The town sits proximal to the Rachel Carson Reserve (RCR), an important draw of tourists and part of the NC National Estuarine Research Reserve System (NERRS). The stormwater outfalls that were studied as part of this project discharge into Taylors Creek, the body of water that sits in between Beaufort and the RCR (Figure 2).

To our knowledge, this is the first study that has evaluated the success of multiple linear regression (MLR) models over a wide range of climatic conditions to examine the importance of tidal phase on stormwater contaminant delivery. Several studies have utilized MLR as a predictive tool in determining bacterial concentrations within estuarine systems (Gonzalez et al., 2012; Molina et al., 2014; Zimmer-Faust et al., 2018), however the novelty of this study is that this is the first-time tide has been incorporated into the predictive framework of water quality monitoring and has subsequently been coupled with quantitative metrices of human fecal contamination. The primary objectives of this research were to 1) determine the concentrations and sources of fecal contaminants in discharge conveyed to receiving waters using multi-sample, time-paced sampling during both storm events and dry weather conditions at various times throughout the tidal cycle, 2) relate FIB and qMST marker concentrations to parameters such as tidal height, 24-h rainfall, salinity and total suspended solids (TSS), and 3) use a multiple linear regression tool to predict concentrations of *Enterococcus* sp. in the context of tidal height, cycle and phase. This research advances the understanding of patterns of delivery of microbial contaminants in low-lying coastal systems.

2. MATERIALS AND METHODS

2.1 Study Sites and Sample Collection

Water samples were collected at three sampling locations throughout Beaufort (Figure 2): two at stormwater outfall locations (Orange St. and Marsh/Pollock) proximal to downstream receiving waters (Taylor's Creek) and a third site (Ann St.) one block inland. The two downstream locations were selected to underline the performance of the stormwater conveyance system, while the inland site was selected to characterize upstream watershed conditions. Following a land survey campaign, the Orange St. and Marsh/Pollock outfall sites were found to be the only two with an above-ground end-of-pipe access point and, as such, were selected as sampling locations. Nineteen sampling events were conducted seasonally over the course of 11 months from July 2017 – June 2018, with samples collected during both storm and ambient conditions. Storm sampling was initiated after a sustained period of moderate to heavy rainfall which produced accumulation of at least ~0.25 in until ~1 h after the storm ended. Dry weather samples were collected following three days without rainfall accumulation.

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

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

2.2 Environmental Parameters

Water temperature, total suspended solids (TSS) and salinity were measured in situ using a YSI probe (YSI 6600 multiparameter probe, USA). Additionally, meteorological observations (Ex. 24-h antecedent rainfall, tidal height and air temperature) were collected from publicly available data provided by NOAA: Station (ID: 8656483). We were able to determine the relative meteorological conditions by rounding sample collection time to the nearest NOAA sampling point (6-minute increments).

2.3 Tidal Characterization

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

Table 1: Latitude, longitude, elevation and pipe size for OS, MP and AS sampling locations

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

2.4 Sample Preparation

FIB *E. coli* and enterococci were enumerated using Colilert-18® and Enterolert™ at a 1:10 dilution (sample: DI water) per manufacturer instructions (IDEXX Laboratories, Westbrook, ME). For downstream molecular analysis, triplicate 100-150 mL samples were vacuum filtered through 0.45 µm pore size, 47 mm polycarbonate (PC) filters (HTTP, Millipore, Bedford, MA) using a six-place filtration manifold and vacuum pump assembly. The filters were placed into sterile, DNase/RNase-free microcentrifuge tubes and stored at -80 °C. DNA extractions were performed using the NUCLISENS® MINIMAG® extraction kit per manufacturer instructions, with extracts then stored at -20 °C. Assays were performed in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories., Hercules, CA) with the following cycling conditions: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Extracted samples were processed using TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, Waltham, Massachusetts). Primers (100 µM) and probes (10 µM) were synthesized by LGC Biosearch Technologies (Petaluma, CA). Each reaction had a total volume of 25 µL, 20 µL including nuclease-free water, TaqMan® Environmental Master Mix 2.0, as well as appropriate primers and probes, and 5 µL of unknown sample, standard, or control. No template controls (NTCs) were processed with every plate.

2.5 Assessment of qPCR Specimen Processing Control and Inhibition Control

Performance of the qPCR assays through evaluation of recovery efficiency and qPCR inhibition was measured using β actin (*ACTB*) cDNA as a specimen processing control (SPC) as previously conducted by Conn et al. (2012). 5 µL of *ACTB* solution (4000 copies/µL) was pipetted into each of the samples, calibrators, and negative controls prior to processing. Following this, samples were extracted. Inhibition was determined by calculating the difference between the cycle threshold (Ct) of the SPC in samples with (experimental) and without (control, only SPC) target DNA. Extracts were analyzed without dilution with samples having more than 0.5 log units (2.32 Ct) difference from control samples deemed inhibited (Lambertini et al., 2008). Since the total number of inhibited samples (11 out of 167 samples) constituted only 6.6%

of total samples inhibited, no adjustment for inhibition was made. For all qPCR runs, appropriate controls were employed and showed no contamination: no template control (omission of DNA template from the qPCR reaction), and negative extractions control (inclusion of filter blank during DNA extraction). Plasmid standards were used for HF183 and Entero1-qPCR assays. Standards were synthesized by GenScript (Piscataway, NJ). Gene sequences were synthesized and inserted into a linearized pUC57 vector which was cloned into DH5α competent cells. Plasmids were extracted using Wizard® Plus SV 10 Minipreps DNA Purification System (Promega Corp., Madison, WI) and linearized using Eco R1 digestion. They were then confirmed via a 1% agarose gel in Tris-Acetate-EDTA buffer. The weight of purified plasmids was then calculated spectrophotometrically (Nanodrop 2000c, Thermo Scientific, Waltham, MA). Nanograms of plasmids were transformed to copy number by using a copy number calculator (SciencePrimer.com). Linearized plasmids were diluted and stored at a concentration of 1×10^8 copies per µL at -20°C.

2.6 Standard Curves

Standard curves for HF183 and Entero1-qPCR consisted of the calibration standard and five 10-fold serial dilutions that were run in triplicate. For each of the molecular markers, standard dilution curves were aggregated to form a singular curve. The theoretical limit of detection (LOD) was the lowest concentration where the standard could be detected reliably in at least 50% of qPCR replicates. The limit of quantification (LOQ) for qPCR assays was defined as the lowest concentration above the lowest point on the standard curve where amplification was observed in at least 50% of qPCR replicates.

2.7 Multiple Linear Regression Models

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

$$\text{Sin}(2 \text{ x } \pi \text{ x } (\frac{\textit{Minutes from high tide}}{\textit{Total minutes between high tides}}))$$

$$\text{Cos}(2 \text{ x } \pi \text{ x } (\frac{\textit{Minutes from high tide}}{\textit{Total minutes between high tides}}))$$

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

Using the regression model formula:

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

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

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

2.8 Statistical Analysis

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

3. RESULTS

3.1 Summary Statistics

In total, 137 samples were collected and analyzed using culture-based FIB enumeration, qPCR-based *Enterococcus* sp. enumeration and qMST marker enumeration using vetted, published qPCR-based approaches. Concentrations of EC (log 0.7 – 4.94 MPN/100 mL) and ENT (log 0.7 – 4.78 MPN/100 mL) were comparable to those of the molecular markers, HF183 (log 0.7 – 4.07 copies/100 mL) and *Enterococcus* sp. quantification via qPCR (log 0.7 – 5.03 copies/100 mL). Significant correlations were observed across combinations of FIB and qMST markers with significant positive correlations found between ENT and EC (r: 0.65; $p < 0.01$), Entero1-qPCR (r: 0.71; $p < 0.01$) and HF183 (r: 0.45; $p < 0.01$).

In an attempt to understand stormwater conveyance as it relates to tidal cycle, samples were collected over a wide range of precipitation and tidal conditions (Figure 3). On average, log EC and ENT concentrations in samples collected during storm events were 2.90 and 2.39 MPN/100 mL respectively, compared to average concentrations of 2.41 and 2.14 MPN/100 mL respectively during dry conditions. This was also true for qMST markers as HF183 and *Enterococcus* sp. quantified via qPCR were also found at mean higher concentrations in samples collected during storm conditions (HF183: log 2.08 copies/100 mL; Entero1-qPCR: log 3.36 copies/100 mL) compared to those collected under ambient conditions (HF183: log 2.03 copies/100 mL; Entero1-qPCR: log 2.70 copies/100 mL). When tested for significance, none of the differences in concentration between wet vs. dry conditions were found to be significantly different ($p<0.05$).

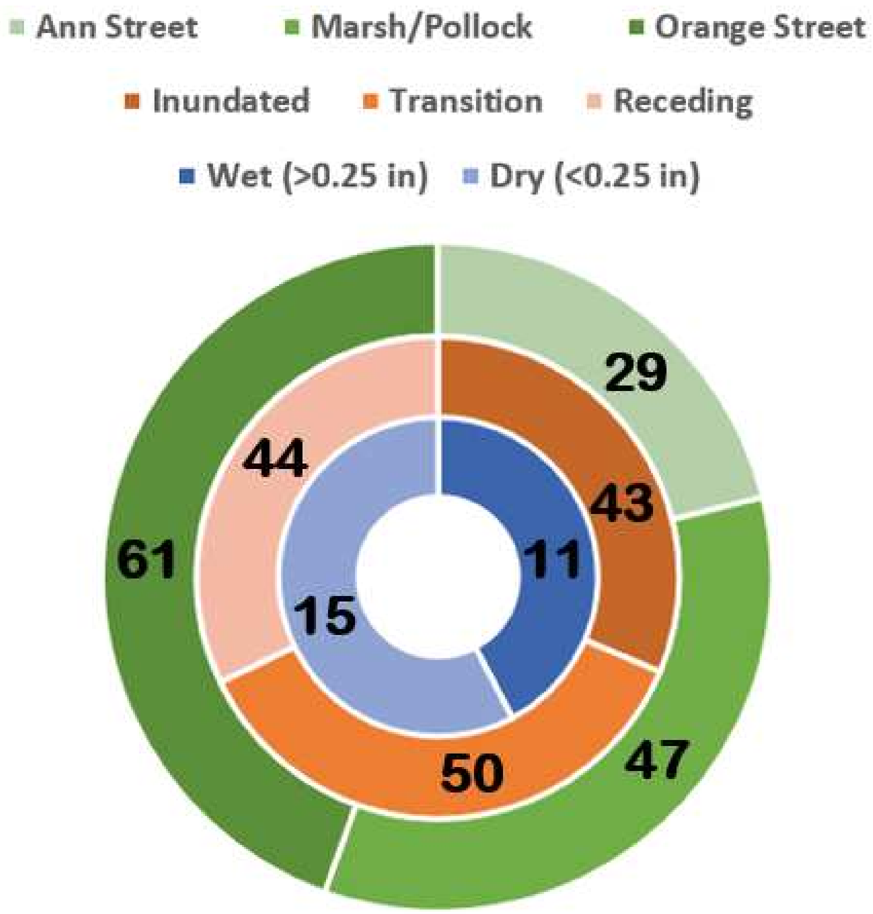

Figure 3: Number of samples collected at sampling sites: AS (n = 29), MP (n = 47) and OS (n = 61), during tidal phases: inundated (n = 43), transition (n = 50) and receding (n = 44) and wet (n = 11) vs. dry (n = 15) conditions.

Samples were collected across a diverse range of environmental conditions (Table 2), with salinity measurements indicating an array of samples were collected across both storm and tidal variations, as these values ranged from 0-35 parts per thousand (ppt). This suggests periods of both fresh, stormwater inundation and marine, creek water inundation were included in overall analysis. Additionally, a wide range of water temperatures that ranged from 9.0°C during the winter months, to 28°C during the summer months, indicate seasonality was also considered in sample collection.

Table 2: Summarized data for environmental parameters: salinity (ppt), TSS (mg/L), water temp. (°C), 24h antecedent rainfall (in), tidal height (m) and air temp. (°C) across the three sampling sites (OS, MP and AS).

Parameter	N	Average	Min	Max
Salinity (ppt)	58	15	0	35
TSS (mg/L)	70	18.5	0.71	64.4
Water Temp (°C)	84	19	9	28
24h Ant. Rainfall (in)	137	0.50	0	3.06
Tidal Height (m)	137	0.062	-0.634	0.692
Air Temp (°C)	118	20	4	28

3.2 Inter-Site Variability

On average, mean FIB and qMST marker concentrations where consistently higher at AS compared to those at the OS and MP locations (Table 3). Concentrations of EC, ENT and Entero1-qPCR concentrations at the upstream, inland AS location averaged 3.62 MPN/100 mL, 3.10 MPN/100 mL and 3.96 copies/100 mL respectively, compared to average values of 2.15 MPN/100 mL, 1.76 MPN/100 mL and 2.19 copies/100 mL at OS and 2.69 MPN/100 mL, 2.39 MPN/100 mL and 3.08 copies/100 mL at MP. The distributions of qMST marker and FIB marker concentrations measured across the sample sites were skewed, with relatively low average EC and ENT concentrations observed for the two downstream locations (OS and MP), and high concentrations at the inland location. As such, we wanted to assess FIB and qMST marker concentrations in samples that would exceed US EPA recommended criteria based on either molecular (Entero1-qPCR: 1280 copies/100 mL (log 3.11)) or culture (EC: 320 MPN/100 mL (log 2.51); ENT: 104 MPN/100 mL) (log 2.04)) criteria defined in 2012 by US EPA and the NC Department of Environmental Quality (NC DEQ, 2020; US EPA, 2012). Previous reports in the literature have cross-linked the risk associated with *Enterococcus* sp. in sewage to measured concentrations of the qMST marker-HF183 (equivalent to 4200 copies/100 mL (log 3.62) (Boehm et al., 2015). Table 5 below summarizes the samples as they relate to recommended exceedance thresholds for each individual group of FIB and qMST markers.

Table 3: Summarized data for EC, ENT, HF183 and Entero1-qPCR concentrations at sampling sites (Orange St., Marsh/Pollock and Ann St.) including the distribution and prevalence of samples that exceeded recreational contact standards.

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

Samples collected at the AS location consistently exceeded recommended concentrations for both culture- and qPCR-based quantification of FIB concentration. For ENT, 79% of samples collected during all environmental conditions exceeded the NC Department of Environmental Quality (DEQ) state threshold of 104 MPN/100 mL. This was also true when samples were analyzed for concentration of Entero1-qPCR, which exceeded US EPA recommended criteria in approximately 83% of samples. When we compare these exceedances to the two downstream locations, which are influenced more greatly by tidal inundation, exceedance of FIB concentrations decreases. FIB exceedances were lowest at the OS outfall with approximately 32% and 27% of samples exceeding recommended EC and ENT concentrations respectively. This compares to an exceedance rate of 14% for samples analyzed for ENT concentrations via qPCR. HF183 concentrations, which are specifically associated with human fecal sources, only exceeded suggested thresholds (4200 copies per 100 mL, (Boehm et al., 2015)) in approximately one-third of samples at AS and MP with fewer samples (15%) exceeding suggested thresholds at OS.

3.3 Tidal Characterization

Descriptive statistics were calculated across sample sites as characterized by collection time within the tidal cycle (Table 4). Across the three tidal categories (inundated, transition and receding), FIB and qMST marker concentrations were consistently higher at the AS location when compared to the two downstream sites: OS and MP. FIB and qMST marker concentrations were compared across tidal classifications using one-way ANOVA calculations with only EC concentrations significantly ($p<0.05$) differing between inundation and receding tidal periods. The same analyses were performed between FIB characterized by sites across the different tidal phases. At OS, significant ($p<0.05$) differences were found between ENT and HF183 concentrations between inundated (high) and receding (low) tides, while EC and *Enterococcus* sp. determined via qPCR concentrations were found to be significantly different at MP. No significant differences in FIB concentrations were found at the AS location across the tidal classifications, which corroborates the inland location of this site.

Table 4: Descriptive statistics of FIB characterized by tidal cycle (inundated, receding or transition) sampling location.

	Inundated (N = 43)			Receding (N = 44)			Transition (N = 50)		
Mean Value	**OS**	**MP**	**AS**	**OS**	**MP**	**AS**	**OS**	**MP**	**AS**
Tidal Height (m)	0.42	0.40	0.46	-0.33	-0.32	-0.33	0.06	0.12	0.14
EC (MPN/100 mL)	1.98	2.31	3.58	2.50	3.19	3.67	1.99	2.68	3.56
ENT (MPN/100 mL)	1.37	1.93	3.04	2.06	2.85	3.09	1.77	2.52	3.30
Entero1-qPCR (copies/100 mL)	2.05	2.48	3.48	2.59	3.94	4.34	1.84	2.93	4.26
HF183 (copies/100 mL)	0.7	2.24	1.96	1.72	2.55	2.94	1.99	2.03	2.99

A representative number of samples were collected across the tidal cycle in order to better represent FIB and qMST marker concentrations in the context of storm events and ambient (dry) conditions. Across the three tidal classifications, correlation coefficients were determined between ENT concentrations and EC, *Enterococcus* sp. concentrations determined via qPCR and HF183. A similar analysis was conducted with environmental parameters such as water temperature, salinity and TSS. Regardless of tidal cycle, ENT concentrations were found to significantly ($p<0.05$) correlate with other FIB concentration and qMST marker concentration, regardless of enumeration approach (culture vs. molecular). Only salinity measurements (r = -0.448, p-value = 0.042) revealed a significant relationship, with regards to the environmental parameters measured, indicating negative correlation with ENT concentrations only during periods of tidal inundation.

3.4 Multiple Linear Regression Models

Three models in total were created to predict concentrations of ENT in a tidally-influenced estuarine system. The models were created using data from all sampling locations, however only the two downstream location (OS and MP) were significant ($p<0.05$) in their prediction of variation in ENT concentrations; therefore, the models are appropriate for locations regularly influenced by tidal inundation. For all three models, a combination of biological (EC concentrations) and environmental parameters (24-h antecedent rainfall, tidal height, tidal cycle and tidal phase) were found to maximize the ability to predict the observed variation in ENT concentrations explained. FIB and qMST markers, such as HF183 and *Enterococcus* sp. determined via qPCR, as well as environmental parameters, such as water temperature, salinity, TSS, 24h antecedent rainfall and water temperature, were considered when making a data training set. However, the five variables used in our models that consistently performed the best across the three sites, when compared to other data training sets. Models were evaluated by comparing the p-value and adjusted R^2 values. Table 5 summarizes the model performances for the pooled data from the three sites. The OS model demonstrated that 55% of its variation could be explained by five variables, with EC concentration and tidal phase and cycle exhibiting significant influences on ENT concentrations. Similar results were observed for the MP model with 63% of the variation in *Enterococcus* sp. concentration explained by the same variables. In this model, however, only EC concentration and tidal cycle were found to significantly contribute to ENT concentrations. Interestingly enough, 24-h antecedent rainfall was not a significant contributor to the variation observed in *Enterococcus* sp. concentrations for any of the models.

Table 5: Multiple regression model for the association of log_{10} Enterococci with biological and environmental characteristics by sampling location (Orange St., Marsh/Pollock and Ann St.). The regression model looks to better characterize the effect of tidal cycle on bacterial concentrations delivered with the system.

Factor	Coefficient	Std. Error	t-value	Prob>|t|
Orange St.				
R^2 = 0.55, p = 3.12 e-05				
Intercept	0.743	0.920	0.807	0.424
EC	0.680	0.123	5.513	1.46e-06***
24h Rainfall	0.102	0.108	0.946	0.349
Tidal Height	2.900	2.432	1.192	0.239
[a]Inundated	0.000	0.000	0.000	0.000
Receding	1.227	1.212	1.012	0.317
Transition	2.199	0.758	2.900	0.006**
Sin(TidalCycle)	-2.478	0.931	-2.662	0.011*
Cos(TidalCycle)	1.083	0.534	2.029	0.048*
Marsh/Pollock				
R^2 = 0.63, p = 2.01 e-04				
Intercept	1.408	1.072	1.314	0.198
EC	0.772	0.181	4.273	1.61e-04***
24h Rainfall	-0.035	0.168	-0.211	0.835
Tidal Height	5.167	2.590	1.995	0.055
[b]Inundated	0.000	0.000	0.000	0.000
Receding	0.675	1.333	0.506	0.616
Transition	1.731	0.962	1.800	0.081
Sin(TidalCycle)	-2.426	0.995	-2.439	0.020*
Cos(TidalCycle)	0.178	0.799	0.222	0.826
Ann St.				
R^2 = 0.62, p = 0.058				
Intercept	2.843	2.127	1.337	0.200
EC	0.325	0.235	1.385	0.185
24h Rainfall	-0.872	0.562	-1.553	0.140
Tidal Height	9.816	7.466	1.315	0.207
[c]Inundated	0.000	0.000	0.000	0.000

Receding	1.582	3.687	0.429	0.674
Transition	1.484	3.080	0.482	0.636
Sin(TidalCycle)	-2.702	2.702	-1.000	0.332
Cos(TidalCycle)	-1.121	2.393	-0.469	0.646

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

4. DISCUSSION

Historically, rainfall has long been associated with elevated FIB concentrations in receiving waters (Coulliette & Noble, 2008; Hart et al., 2020; Silva et al., 2014). However, the influence of tide on contaminant delivery during storms is poorly understood, particularly in low-lying coastal plain systems. This study evaluated the relationships of both culture- and qPCR-based FIB and qMST markers in the context of tidal cycle in an estuarine system exposed to stormwater delivery across a wide range of weather conditions. To further evaluate relationships observed for ENT, EC and qMST marker concentrations according to tide, we developed a MLR tool to better understand stormwater contamination dynamics in a complex, tidally-influenced estuarine system. MLR has been recommended as part of the US EPA 2012 "Update to the Recreational Water Quality Criteria," but to our knowledge there are no published models that incorporate tide. Predictive modeling tools have previously shown their utility in NC estuaries such as the one we studied (Gonzalez et al., 2012; Gonzalez & Noble, 2014) and therefore may be used to better serve coastal water quality managers by better explaining microbial contaminants in the context of tide and other environmental parameters. We hope to provide a framework for stormwater researchers needing to incorporate a tidal parameter in their monitoring regimes for the future, while also highlighting some of the major limitations associated with using such an approach.

4.1 Summary Statistics

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

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

4.2 Multiple Linear Regression Models

To our knowledge, this was the first application of a MLR that incorporated both qualitative and quantitative tidal variables to examine drivers of microbial contaminant concentrations. This approach, when compared to other statistical methods, may serve as more appropriate tool to routinely evaluate stormwater-impacted water quality. As evidenced by the recent USEPA buy-in, predictive modeling tools (Cyterski et al., 2013) can offer an opportunity to identify drivers of contamination, especially as related to stormwater inputs, and environmental parameters. In the end, these tools conserve valuable resources by allowing predictions rather than routine sample collection and monitoring to manage recreational exposure and risk. Previous modeling done by Gonzalez et al., (2014) was conducted in a neighboring system and demonstrated successful application of MLR. In this study, however, no tidal variable was incorporated to explain variation in either EC or ENT concentrations. Furthermore, rainfall was found to be a significant driver of FIB concentrations. The utility of our study is the incorporation of both well-established biological parameters (Hamilton et al., 2017; Jin et al., 2004; Parker et al., 2010) with less-understood environmental influences, such as tidal condition.

ENT and EC have long shown co-occurrence within fecal waste natural environments, with some proposing that EC is a superior metric of fecal contamination given its specificity and relationship to human health (Cabelli et al., 1982; Soller et al., 2010). Therefore, the relevance of EC concentration within the model makes sense due to its known, previously published, positive correlation with ENT (Boehm & Sassoubre, 2014; Steele et al., 2018; Stumpf et al., 2010). Tidal cycle, however, which has been studied much less frequently, also appeared to exhibit great influence on ENT concentration variation. We believe this implies that contaminant transport is more dependent on the timing of storm events as they relate to the state of the tide, compared to simply the extent, intensity of the storm event itself. If this is true, downstream waters could be susceptible to impairment long after a storm event ceases and related to the release of the system as the tide retreats. Thus, contaminated waterways remain open during contamination events increasing the likelihood of deleterious public health effects (Leecaster & Weisberg, 2001; Noble, Blackwood, Griffith, McGee, & Weisberg, 2010). Furthermore, in this framework, antecedent rainfall patterns would carry increased weight and value to future predictive model development. This is because long periods of increased rainfall will begin to favor higher surficial groundwater levels, as well as decreased infiltration capacity, potentially driving a compounded issue of stormwater delivery hampered by localized increased tidal elevation due to increased localized runoff (Yau et al., 2014).

4.3 Application

In low-lying, rural systems, such as Beaufort, NC, it is not uncommon to find some degree of spatial autocorrelation in water quality studies (Partyka et al., 2017; Tu & Xia, 2008) suggesting that the qualities under investigation are determined somewhat by unmeasured, and possibly external factors. If these influences are not taken into consideration, bias can be introduced into microbial water quality monitoring programs and the subsequent management decisions. In this particular study, we considered tidal variation, which is surprisingly understudied. Coastal communities across the entire NC coast sit at elevations around or below those found in Beaufort (e.g. Currituck (7 ft), Hatteras (3 ft), and Ocracoke (3 ft)) and, as such, experience similar degrees of tidal inundation. By addressing this issue in more depth, stormwater researchers may have greater success in developing a more inclusive framework for stormwater management that may be applied in susceptible coastal communities throughout the US (Poulter et al., 2009; Pricope, Halls, & Rosul, 2019). We recognize the limitations of this study and the possible influence this may have on the reliability of model predictions. For instance, laboratory-based measures (e.g. salinity and TSS) not comprehensively conducted across all sample types throughout the study. Furthermore, it would have been of great interest to understand the elevation and pipe dimension and flow and discharge across the entire system, but these parameters were difficult to measure in practice and resulted in intermittent data collection. Additionally, sampling regimes varied between automatic and grab sampling, introducing bias related to sample collection frequency and type. Previous studies applying a tidal description in their sampling methods have primarily occurred during one tidal phase (Ex. low or high) which limits one's understanding of shifting FIB and qMST concentrations that change with the tide. Much of the previous literature shows geographic or socio-economic biases as many were conducted in the western US or in highly developed watersheds with lower tidal intrusion and greater financial resources to combat coastal flooding. With the greatest risks falling on low-lying, rural populations, accurate classifications of tidal inundation and its impact on microbial contaminant delivery in stormwater is necessary for future consideration.

We understand there is no “one-size-fits-all” model for the prediction of *Enterococcus* sp. concentration in discharge to coastal, surface waters. However, once baseline interactions between environmental parameters and microbial dynamics have been established through routine monitoring, data can then be interpreted in the context of tide. Without reliable spatial and temporal knowledge of tidal cycle, we cannot fully rely on the results of published models to answer today's questions of acceptable water quality.

5. CONCLUSIONS

- Concentrations of culture FIB (*E. coli* and enterococci), Entero1-qPCR and qMST (HF183) markers were significantly influenced by tide with higher concentrations found during receding (low) tides compared to those from inundated (high) or transition tidal periods.

- Environmental parameters, such as salinity, were found to significantly ($p<0.05$) correlate with ENT concentrations during periods of tidal inundation. Salinity is likely a valuable conservative marker for future dispersion studies.

- Study successfully showed the application of MLR using qualitative and quantitative tidal variables as driver of variation in both EC and ENT concentrations. However, 24-h antecedent rainfall was not determined to have a major influence on FIB concentration as has been previously reported.

- Monitoring programs in low-lying coastal communities with tidal inundation issues must incorporate a tidal parameter in order to evaluate the impact of tidal inundation on stormwater conveyance.

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