A Bacterial Source Tracking Project to Identify Sources of Fecal Pollution at Cole and Ropes Parks: May 2017-May 2018

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Summary

Enterococci is the federal standard for assessing marine recreational water quality. In response to a history of elevated enterococci concentrations, two beaches at Cole and Ropes Parks were designated as impaired and placed on the Texas 303(d) list. This source tracking project was initiated with the support of the Cole and Ropes Parks Coordination Committee to identify possible sources of bacterial pollution. To that end, a 9-month study was conducted to 1) quantify the abundance of enterococci bacteria 2) survey the antimicrobial susceptibility of the enterococci population, 3) quantify the abundance of human-, canine-, and gull-associated molecular markers, and 4) characterize the taxonomic composition of the bacterial community at large. Findings showed that enterococci concentrations frequently exceeded the EPA recreational water quality criterion of 104 MPN 100 mL^{-1}. The most elevated enterococci concentrations followed storm events and were correlated with physical changes typical of storm events and stormwater runoff (e.g., decreased salinity and pH). Storm events also promoted shifts in the taxonomic structure of the bacterial community at large, resulting in a decrease in bacterial diversity. By contrast, storm events were not associated with the abundance of the human-, canine-, and gull-associated molecular markers. These markers were abundant throughout the study with gull being the most abundant, followed by human, and canine. The detection of these markers followed distinct trends in that the human marker was only correlated with decreased water transparency while the canine-associated and gull-associated markers were correlated with multiple parameters (e.g., increased water and air temperature, decreased dissolved oxygen, increased wind speed, and decreased barometric pressure). That the canine marker was also correlated with enterococci concentrations suggests that canine fecal pollution may have contributed to elevated enterococci levels. However, the absence of correlation between enterococci and the human- and gull-associated markers suggests that enterococci levels were not representative of human or gull fecal pollution. Findings indicate that stormwater runoff resulted in elevated enterococci and decreased bacterial diversity. Findings also indicate that gulls, humans, and canines contributed to bacterial loading. Future efforts to assess and remediate bacterial pollution would benefit from the inclusion of data that quantifies the abundance of specific host-associated markers.

Acknowledgements

We thank the Texas Commission on Environmental Quality and the Coastal Bend Bays and Estuaries Program for funding this project. We also thank the Cole and Roles Parks Coordination Committee for supporting this project, and we thank the Corpus Christi Nueces County Public Health District for their analysis of water samples. This project would not have been possible without the assistance and expertise of graduate and undergraduate students at Texas A&M University-Corpus Christi: Nicole C. Elledge, Hailey Wallgren, Sandra Marbach, Christina Remmes, and Lee J. Pinnell.

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Introduction

Recreational water quality is commonly assessed by quantifying the abundance of fecal indicator bacteria (FIB) (Gronewold et al. 2015). Enterococci are a ubiquitous group of Gram-positive FIB normally abundant in the feces of humans and other animals (Boehm and Sassoubre et al. 2014). The correlation between enterococci abundance and human health outcomes has prompted their widespread use as a measure of recreational water quality (Byappanahalli et al. 2012). In 2004, the Environmental Protection Agency (EPA) adopted enterococci as the federal standard for marine water quality (USEPA, 2004). The EPA recreational water quality criterion for enterococci is not more than 35 colony forming units (CFU) 100 mL^{-1} (geometric-mean standard) and 104 CFU 100 mL^{-1} (single-sample standard) (USEPA, 2011). In the state of Texas, the Texas Beach Watch Program, under the administration of the Texas General Land Office (TGLO), routinely monitors enterococci concentrations to assess water quality at marine recreational beaches.

Two recreational beaches at Cole and Ropes Parks in Corpus Christi Bay (Beach IDs TX259473 and TX821303) have exhibited a history of elevated enterococci levels. The water quality at both parks was designated as impaired by the Texas Commission of Environmental Quality (TCEQ) and both beaches were added to the Texas 303(d) list. In response to these impairments in water quality, the Cole and Ropes Parks Coordination Committee drafted an implementation plan for one total maximum daily load (TMDL) for bacteria (TCEQ, in review). The TMDL implementation plan identified bacterial source tracking as the first priority for implementation. Therefore, this bacterial source tracking project was initiated in fulfillment of the implementation plan, with the ultimate goal of providing data to strategize best management practices to remediate water quality.

Impairment of water quality is thought to stem from unknown point and nonpoint sources of fecal pollution. In 2010, a previous bacterial monitoring and source tracking study at Cole and Ropes Parks (CBBEP Project Number 1010) reported the detection of a human-associated *Enterococcus faecium* gene (*esp*) following storm events, suggesting that humans comprise at least one source of bacterial pollution (Mott et al. 2010). However, the *esp* gene was detected only three times, and the authors cited detection limits and polymerase chain reaction (PCR) inhibition as limiting factors. Regardless, the authors concluded that the detection of host-associated molecular markers is a promising methodological development and recommended that future studies utilize similar markers to identify sources of fecal pollution.

More recently, the exhaustive Source Identification Protocol Project (SIPP) analyzed 41 bacterial source tracking methods and confirmed that detection of host-associated molecular markers (e.g., human, gull, cow, horse, and canine) is the preferred method to identify sources of fecal pollution (Boehm et al. 2013; Harwood et al. 2013; Steward et al. 2013). Additionally, the use of 16S rRNA gene sequencing, to monitor population-level changes in the bacterial community, is an emergent methodological development (Tan et al. 2015; Vierheilig et al. 2015). The first approach results in the quantification of bacterial strains associated with specific hosts while the second approach results in the high-resolution census of the bacterial community at large. Collectively, these two approaches represent the most advanced source tracking methodologies.

The purpose of this project was the identification of fecal pollution sources at Cole and Ropes Parks in Corpus Christi Bay. For this purpose, this project utilized traditional culture-dependent and molecular culture-independent methods to monitor and identify sources of fecal pollution at Cole and Ropes Parks. The objectives of this project were to 1) quantify the abundance of enterococci bacteria 2) survey the antimicrobial susceptibility of the enterococci population, 3) quantify the abundance of molecular markers associated with specific hosts (i.e., human, gull, and canine), and 4) characterize the bacterial community at large. We hypothesized that storm events and stormwater runoff would be correlated with the abundance of enterococci, host-associated makers, as well as larger shifts in the bacterial community.

Methods

Water Sampling. Surface water samples (2 L) were collected in duplicate at six Texas Beach Watch Stations (https://cgis.glo.texas.gov/Beachwatch/) distributed between Cole Park (N = 4 stations) and Ropes Park (N = 2 stations) (Figure 1). The sampling stations include Cole Park #1 (Latitude 27.77545, Longitude -97.39112), Cole Park #2 (Latitude 27.77199, Longitude -97.38829), Cole Park #3 (Latitude 27.76991, Longitude -97.38717), Cole Park #4 (Latitude 27.76762, Longitude -97.3845), Ropes Park #1 (Latitude 27.75477, Longitude -97.37623), and Ropes Park #2 (Latitude 27.75279, Longitude -97.37587). Samples were collected on a near bimonthly schedule starting May 9, 2017 and ending January 23, 2018. Additional samples were collected immediately after storm events. All samples were collected using sterile bottles per the guidelines established by the TCEQ Surface Water Quality Monitoring Procedures Manual (TCEQ, 2012). All samples were stored immediately on ice at 4ºC and held at that temperature for no longer than four hours.

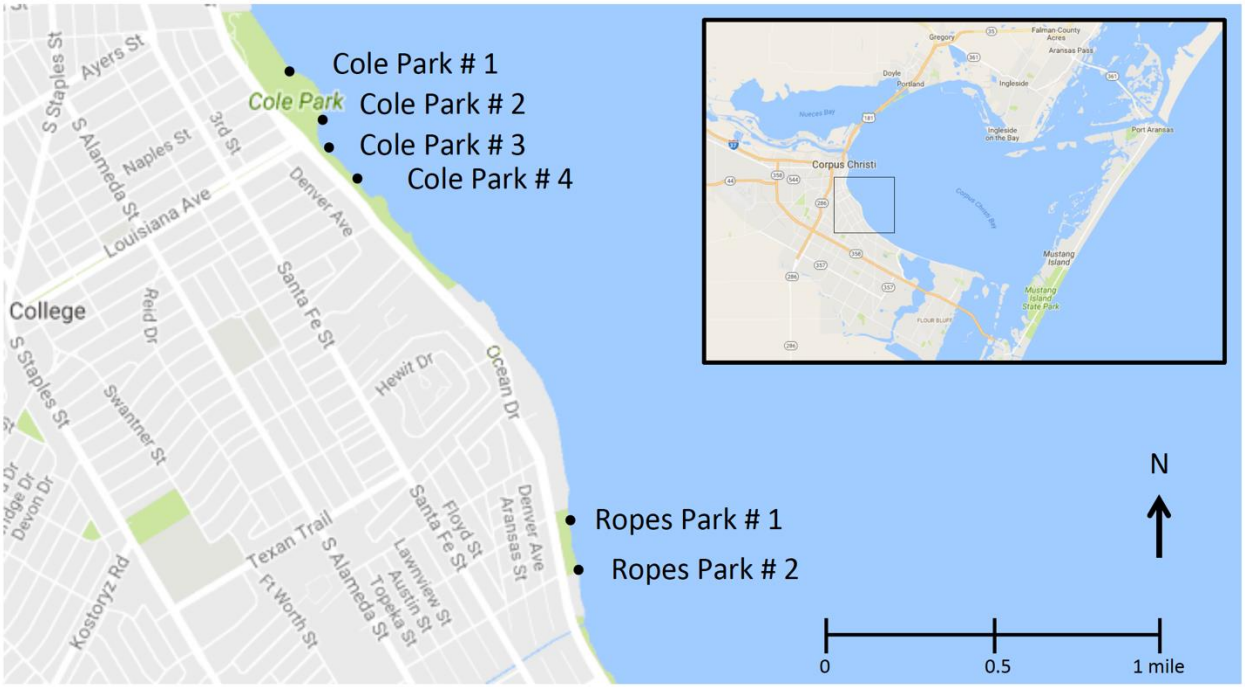

Figure 1. Map of the six sampling stations at Cole Ropes Parks in Corpus Christi Bay, Texas. The insert shows the location of the parks relative to the bay and Mustang Island.

Physical Parameters. Physical parameters [water temperature (ºC), dissolved oxygen (mg mL^{-1}), specific conductance (µS cm^{-1}), barometric pressure (mmHg), salinity, and pH] were measured with a YSI 556 Multi Probe System (YSI Incorporated, Yellow Springs, OH). Weather conditions [e.g., air temperature (ºC) and and days since precipitation] were obtained from https://www.wunderground.com/. Water transparency (m) was measured with a 120 cm transparency tube (Ben Meadows, Janesville, WI, USA). Wind speed (mph) was measure with a Kestral wind meter (Kestrel Instruments, Boothwyn, PA, USA).

Enterococci Concentrations. Duplicate 100 mL water samples were transported to the NELAP-accredited Corpus Christi Nueces County Public Health District Laboratory (CCNCPHDL) for

Enterolert testing (IDEXX Laboratories, Westbrook, Maine, USA). The CCNCPHDL followed procedures established for enterococci sampling as performed by the Texas Beach Watch Program (https://cgis.glo.texas.gov/Beachwatch/docs/QAPP2016-2017.pdf). Relationships between enterococci concentrations and physical parameters were determined by the cenken test which computes the Kendall's tau correlation coefficient. Relationships between enterococci concentrations and storms events were determined by comparing wet- and dry-loading concentrations using a Cendiff test.

Antimicrobial Susceptibility. The antibiotic susceptibility of enterococci isolates was determined by disk-diffusion assay. *Enterococcus* were cultured by the EPA 1600 method (USEPA, 2006). DNA was isolated from these cultures using the heat lysis method (Englen and Kelley, 2000). A traditional PCR assay targeting the *sodA* gene was used to identify *E. faecalis* and *E. faecium* (Jackson et al. 2004). See Table 1 (below) for primer sequences. *E. faecalis* and *E. faecium* isolates were then tested for susceptibility to two antibiotics (ampicillin and vancomycin) as described previously (Traub et al. 1998). For this purpose, bacterial were cultured on Mueller-Hinton agar (Becton, Dickinson and Company, Sparks, MD) and susceptibility was quantified with Sensi-Disc antimicrobial susceptibility test discs per the manufacturer's instructions (Becton, Dickinson and Company, Sparks, MD).

DNA Isolation. Duplicate 100 mL water samples were vacuum filtered onto two 0.45 µm mixed cellulose esters (MCE) filters (47 mm in diameter) (Millipore Sigma, Bedford, MA, USA). Filters were stored at -80 ºC for no longer than 14 days. DNA was isolated from the filters using a DNeasy Powersoil Kit (Qiagen, Valencia, CA, USA). The isolated DNA was quantified and assessed for quality (260/280 nm) using a BioSpectrometer (Eppendorf, Hamburg, Germany) and stored at -20ºC. The DNA isolated from the first filter was utilized for the quantitation of host-associated markers while the DNA isolated from the second filter was utilized for the bacterial community composition analysis.

Host-Associated Markers. The abundance of host-associated molecular markers was quantified using droplet digital PCR (ddPCR) as described previously (Cao et al. 2015). Primers targeting three host-associated bacterial strains (see Table 1 for primer sequences) were recommended by the Source Identification Protocol Project (Boehm *et al.,* 2013; Harwood *et al.,* 2013; Stewart *et al.,* 2013). The targets included the human-associated *Bacteriodales*, the gull-associated *Catellicoccus*, and the canine-associated *Bacteriodales*. Each ddPCR reaction was run in triplicate, including no template controls (NTCs) and positive controls for each run. The positive controls were designed in the form of synthetic gBlock gene fragments (Integrated DNA Technologies, Skokie, Illinois, USA). Each PCR reaction had a total volume of 20 µL and was composed of 10 µL EvaGreen Supermix, 1 µL forward primer, 1 µL reverse primer, and 3 µL of sample DNA. Droplets were generated with the QX200 Droplet Generator (BioRad Laboratories, Hercules, California) according to manufacturer's instructions and transferred to a thermal cycler with the conditions listed in Table 2. The optimum annealing temperature (59ºC) was determined by a running temperature gradient experiment for all three sets of primers. After thermal cycling, the droplets were read with the QX200 Droplet Reader (BioRad Laboratories, Hercules, California) and analyzed with the QuantaSoft software. The threshold for positive droplets was set manually according to the NTC peaks present in each run. The QuantaSoft software reported the concentration of each marker in copies 20 μL^{-1} PCR reaction. These values were converted to

gene copies 100 mL^{-1} water sample with the following equation: (copies μL^{-1})(20/3)(50) = copies 100 mL^{-1} water sample. The original concentration was multiplied by (20/3) as each PCR reaction had a total volume of 20 µL, 3 µL of which was sample/template DNA. This number was then multiplied by 50, as this was the final volume of DNA obtained from the DNA extractions. The Pearson's product moment correlation was used to test relationships between the abundance of host-associated markers, physical parameters, and enterococci concentrations. ANOVA and Tukey post-hoc tests were conducted to test if the abundance of host-associated markers was related to storm events (i.e., periods of wet-loading, dry-loading, or neither).

Table 1. Sequences and probes used for the detection of host-associated molecular markers.

Target	Primer and probe sequences	Reference
Enterococcus faecalis (*sodA* gene)	Forward: 5'-ACTTATGTGACTAACTTAACC-3' Reverse: 5'-TAATGGTGAATCTTGGTTTGG-3'	Jackson et al. 2004
Enterococcus faecium (*sodA* gene)	Forward: 5'-GAAAAAAACAATAGAAGAATTAT-3' Reverse: 5'-TGCTTTTTTGAATTCTTCTTTA-3'	Jackson et al. 2004
Human-associated *Bacteroidales* HF183	Forward primer: 5′-ATCATGAGTTCACATGTCCG-3′ Reverse primer: 5′-TACCCCGCCTACTATCTAATG-3′	Seurinck et al. 2005
Gull-associated *Catellicoccus* LeeSeaGull	Forward primer: 5′-TGCATCGACCTAAAGTTTTGAG-3′ Reverse primer: 5′-GTCAAAGAGCGAGCAGTTACTA-3′	Lee et al. 2013
Canine-associated *Bacteroidales* DogBac	Forward primer: 5'-CGCTTGTATGTACCGGTACG-3' Reverse primer: 5'-CAATCGGAGTTCTTCGTG-3'	Sinigalliano et al. 2010
16S rRNA	Forward: 515fMod 5'-GTGYCAGCMGCCGCGGTAA-3' Reverse: 806rMod 5'-GGACTACNVGGGTWTCTAAT-3'	Walters et al. 2016

Table 2. ddPCR thermal cycler conditions used to quantify host-associated fecal markers.

Step	Temp (°C)	Time	Ramp rate	Number of cycles
Enzyme activation	95	5:00		1
Denaturation	95	0:30		40
Annealing/extension	59	1:00	2ºC s^{-1}	40
Signal stabilization	4	5:00		1
	90	5:00		1

Bacterial Community Composition. The composition of the bacterial community at large was determined by 16S rRNA gene sequencing. For this purpose, the sequencing of 72 wet-loading

and 72 dry-loading samples was contracted with Molecular Research LP (Shallowater, TX, USA). Briefly, the V4 region of the 16S rRNA gene was amplified by PCR as described previously [Walters *et al.,* 2016]. See table 1 for primer sequences. The 16S rRNA gene amplicons were then sequenced (Illumina HiSeq 250 PE) and the sequence reads were analyzed using the open-source Quantitative Insights Into Microbial Ecology (QIIME) software [Kuczynski *et al.,* 2012]. Alpha diversity values were computed in QIIME using Shannon's diversity index. Measurements of diversity were compared between dry-loading and wet-loading events using QIIME's Kruskal-Wallis pairwise H test [Kruskal and Wallis, 1952]. A principal coordinate analysis (PCoA) of beta diversity was computed in QIIME using unweighted UniFrac distance values.

Data Analysis. All tests were completed with R and R Studio. For all tests, significance was declared at $p < 0.05$.

Results and Discussion

Water Sampling. A total of 20 sampling events produced 120 water samples (N = 20 sampling events, N = 6 sampling sites, N = 120 total water samples). Six sampling events occurred immediately after a storm event (N = 36 samples). Strom events occurred May 22, June 5, June 26, September 27, October 4, and November 13, 2017. Stormwater runoff is a primary stressor in aquatic ecosystems, loading embayments with fecal waste, residual pharmaceuticals, and bacteria (Williamson et al. 2014).

Physical Parameters. Water temperature (ºC), salinity, dissolved oxygen (mg L^{-1}), and pH varied seasonally over the 9-month sampling period (May 2017 to January 2018) (Figure 2). Water temperature ranged from 10.7 to 32.5ºC. Salinity ranged from 28.5 to 38.3. Specific conductance ranged from 44,094 to 57,627 µS cm^{-1}. Dissolved oxygen ranged from 4.0 to 11.3 mg mL^{-1}. pH ranged from 7.3 to 8.3. Air temperature ranged from 9.8 to 37.1ºC. Water transparency ranged from 0.06 to 1.20 m. Wind speed ranged from 0.6 to 32.7 mph.

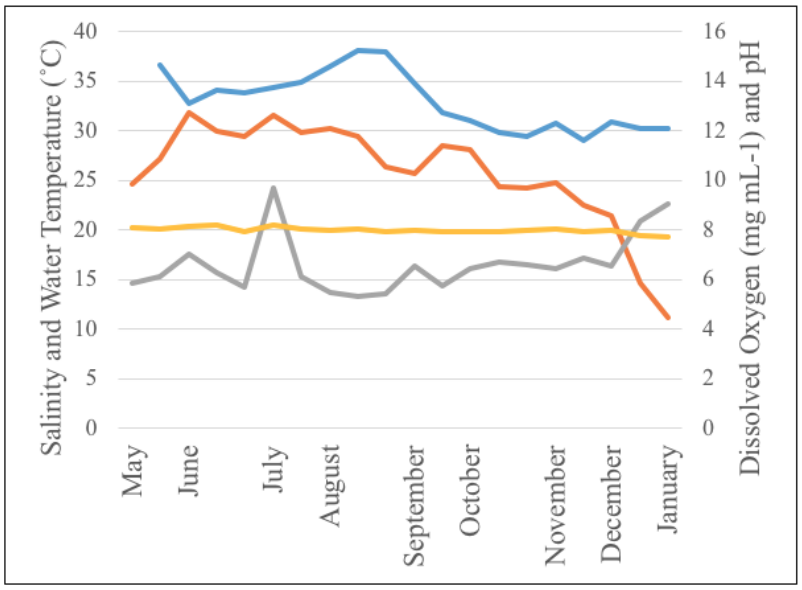

Figure 2. Seasonal variation in averaged water temperature (red), salinity (blue), dissolved oxygen (gray), and pH (yellow) over the 9-month sampling period (May 2017 to January 2018).

Enterococci Concentrations. Enterococci concentrations ranged from fewer than 10 to 22,029 MPN 100 mL^{-1}, but concentrations were not correlated with seasonal variation in temperature (Figure 3). Concentrations were significantly correlated with salinity (-0.138, $p < 0.05$), specific conductance (-0.143, $p < 0.05$), pH (-0.146, $p < 0.05$), days since precipitation (-0.441, $p < 0.001$), and water transparency (-0.325, $p < 0.001$). The strong correlation between days since precipitation and water transparency indicates that increased enterococci concentrations were related to storm events. This relationship between enterococci and storm events was clearly illustrated in Figure 3 by the vertical red lines that mark the occurrence of storm events. Previously, Mott et al. 2010 correlated storm events and the influx of stormwater runoff with increased enterococci concentrations at Cole and Ropes Parks (Mott et al. 2010).

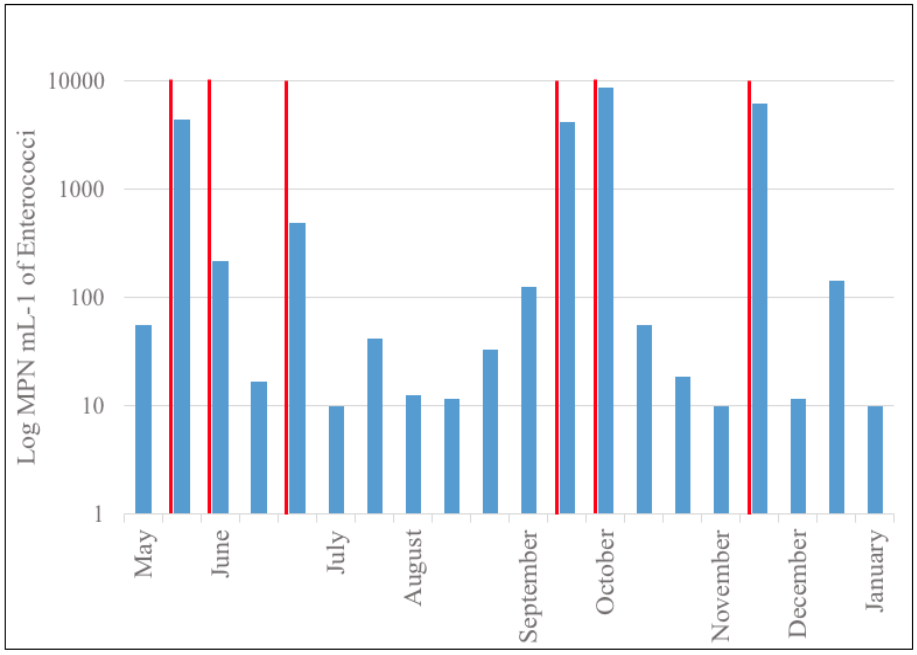

Figure 3. Variation in averaged enterococci concentrations (Log MPN 100 mL^{-1}) in surface water samples collected in Cole and Ropes Parks. Vertical red lines mark storm events.

Antimicrobial Susceptibility. The antimicrobial susceptibility testing was proposed in an amendment to the Quality Assurance Project Plan (QAPP), but antimicrobial susceptibility testing was not completed before the QAPP expired. Results will be included as an amendment to the final report.

DNA Isolation. The DNeasy Powersoil method of DNA isolation yielded approximately 10 ng ul^{-1} of DNA per 100 mL water sample. The quality of the DNA, as determined by the 260/280 nm ratio, was approximately 1.9.

Host-Associated Markers. The three host-associated markers (i.e., human, gull, and canine) were readily detectable in all water samples (Figure 4). Table 3 shows the minimum, maximum, and average abundance of each marker. On average, the most abundance marker was gull followed by human and canine, although each was abundant. The Pearson's correlation values between the host-associated markers and physical parameters indicated that the human-associated marker was only correlated with water transparency (see Table 4). However, relationships between the abundance of the human-associated marker and physical parameters (e.g., water and air temperature) were obscured by an unexplained spike in abundance in January 2018 (see Figure 4). By contrast, the canine-associated and gull-associated markers were correlated with multiple parameters (e.g., water and air temperature, dissolved oxygen, wind speed, and barometric pressure) (Table 4). Importantly, the canine-associated marker was the only marker correlated with enterococci concentrations.

Table 3. Minimum, maximum, and average abundance (gene copies 100 mL^{-1}) of the three host-associated markers.

Marker	Minimum	Maximum	Average
Human	66.7	3680.0	642.6
Canine	17.8	3681.1	560.1
Gull	75.6	3133.3	689.3

Table 4. Pearson's product moment correlation between the fecal markers (i.e., human, gull, and canine) and the measured physical parameters. *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001. Non-significant values are indicated by a dash.

Parameter	Human	Gull	Canine
Water temperature (°C)	-	0.329*	0.304*
Barometric pressure (mmHg)	-	-0.524***	-0.514***
DO (mg/L)	-	-0.365*	-0.314**
Specific conductance (µS/cm)	-	0.474***	-
Salinity	-	0.475***	-
Air temperature (°C)	-	0.464***	0.36**
Wind speed (mph)	-	0.395**	0.293*
Transparency (m)	0.398**	-	-
Enterococci (MPN/100 mL)	-	-	0.352**

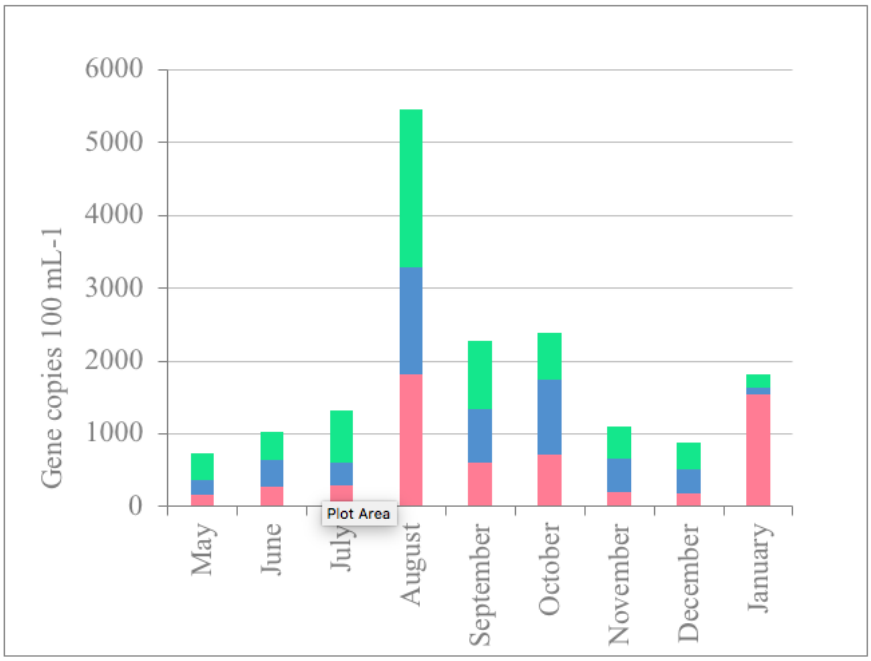

Figure 4. Averaged abundance (gene copies 100 mL^{-1}) of human (pink), canine (blue), and gull (green) markers in surface water samples collected in Cole and Ropes Parks.

Bacterial Community Composition. The PCoA analysis of beta diversity indicated that storm events promoted profound shifts in bacterial community composition (Figure 5). Similarly, the Kruskal-Wallis pairwise H test comparing the diversity of wet- and dry-loading events showed

that dry-loading samples exhibited the highest community diversity ($p < 0.05$). Previous studies have concluded that storm events and stormwater runoff are a primary stressor/disturbance in coastal systems (Williamson et al. 2014). These results support that conclusion and indicate that stormwater-associated pulses of freshwater were a disturbance event that reduced biodiversity.

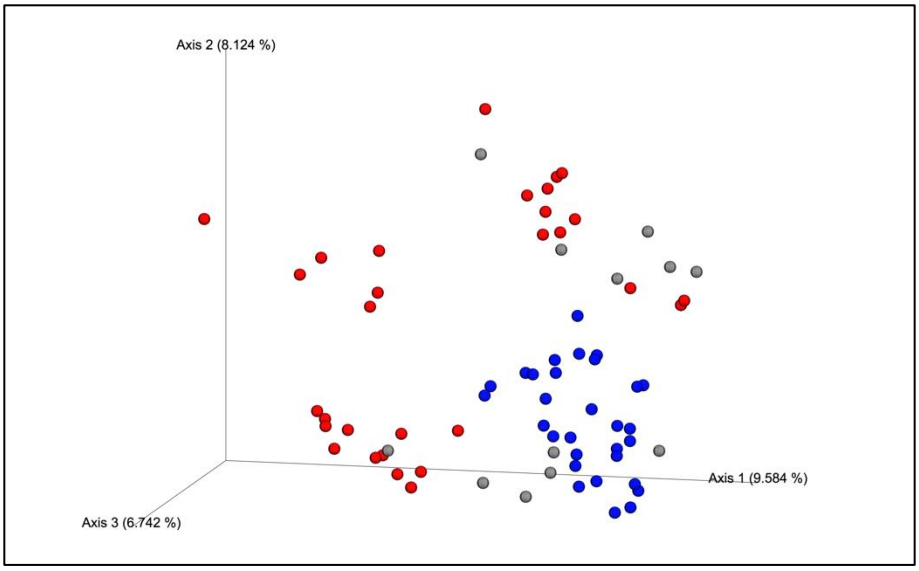

Figure 5. Principal coordinate analysis (PCoA) of beta diversity computed using unweighted UniFrac distance values. The wet-loading samples are shown in blue and the dry-loading samples are shown in red. Gray dots represent samples that did not cluster with their respective sampling type (i.e., wet- or dry-loading).

Figure 6. Results of the Kruskal-Wallis pairwise H test showing the diversity of wet- versus dry-loading samples. Dry-loading samples are significantly more diverse than wet-loading samples ($p < 0.05$).

Conclusions

This project utilized traditional culture-dependent and molecular culture-independent methods to monitor enterococci concentrations and identify sources of fecal pollution at Cole and Ropes Parks, Texas. Enterococci concentrations frequently exceeded the EPA's water quality criterion of 104 CFU 100 mL^{-1}, and were strongly correlated with precipitation, with the highest concentrations occurring immediately following storm events. Indeed, data analysis revealed that elevated enterococci concentrations were correlated with physical changes typical of storm events: decreases in salinity, specific conductance, pH, and water transparency. The taxonomic composition of the larger bacterial community, as evidenced by 16S rRNA gene sequence analysis, followed a similar trend. By contrast, the abundance of host-associated markers (i.e., human, canine, and gull) was not associated with precipitation. Rather, the human marker was only correlated with water transparency while the canine and gull markers were correlated with multiple parameters (e.g., water and air temperature, dissolved oxygen and wind speed). In general, the abundance of these host-associated markers followed a temperature-driven seasonal trend, with the highest abundances detected during the warmest month (i.e., August), but that trend was obfuscated by an unexplained increase in the abundance of the human marker during the coldest month (i.e., January). The correlation between the abundance of the canine marker and enterococci concentrations suggests that canine fecal pollution may have contributed to elevated enterococci levels. However, enterococci concentrations were not predictive of human-associated *Bacteroidales* and therefore, we recommend that future studies incorporate the detection of host-associated markers.

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