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Real-Time Quantitative PCR Measurements of Fecal Indicator Bacteria and Human-Associated Source Tracking Markers in a Texas River following Hurricane Harvey

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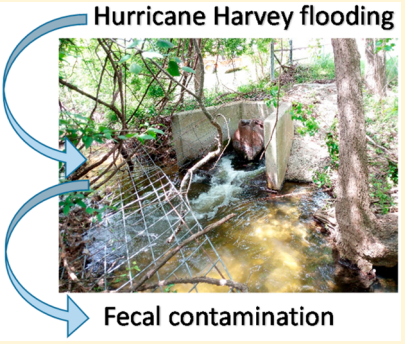
Supporting Information

ABSTRACT: Hurricane Harvey has caused unprecedented devastation to huge parts of southeastern Texas, particularly damaging the wastewater infrastructure resulting in release of sewage contamination into environmental waters. The purpose of this study was to conduct a preliminary assessment of fecal indicator bacteria (*Escherichia coli* and enterococci) and human-associated fecal genetic markers (human-associated Bacteroidales), measured using qPCR assays, across a Texas river impacted by Hurricane Harvey. Water samples were collected along the Guadalupe River during September−December 2017. The most heavily flooded sites showed the highest abundance of fecal indicator bacteria and human-associated Bacteroidales markers, indicating that a large number of sewage overflows and stormwater runoff occurred during Harvey flooding. These findings suggest that high levels of human fecal contamination were introduced into waterways draining into the Gulf of Mexico and impaired surface water quality. The human-associated Bacteroidales markers exhibited a low to slightly strong correlation with conventional fecal indicators, suggesting the variable occurrence of different markers and uncertainty of enterococci and *E. coli* for detection of human fecal pollution. In general, results of this initial microbiological contaminant assessment will serve as baseline information for follow-on studies to monitor existing and emerging public health risks to residents of Texas and potential long-term environmental impacts on the water resources in the impacted regions.



INTRODUCTION

Hurricane Harvey originated as a tropical storm over the Atlantic Ocean, becoming a Category 4 hurricane on August 25, 2017, as it approached the coast of Texas.[1] After a week of storms and heavy flooding, Hurricane Harvey devastated huge parts of southeastern Texas, particularly damaging the wastewater infrastructure resulting in release of sewage contamination into environmental waters.[2,3] Immediately following Harvey, both the Texas Commission on Environmental Quality (TCEQ) and the U.S. Environmental Protection Agency (EPA) reported the release of wastewater from sanitary sewers due to the historic flooding, and more than 800 wastewater treatment plants reported spills.[4,5] The widespread flooding resulted in sewage overflows and contamination of floodwaters that eventually made their way into surface waters, before finally discharging into the Gulf of Mexico. These sewage overflows contain high levels of fecal bacteria and potentially pathogenic organisms posing a serious risk to human and environmental health via waterborne disease outbreaks, deterioration of recreational and drinking water quality, and degradation of aquatic ecology.[6] Initial water samples collected from floodwaters in affected areas showed *Escherichia coli* levels greater than 100 times the EPA criterion for recreation water quality,[7] strongly suggesting a mixture of sewage in water.[2,8] Water quality impacts have been reported for previous hurricanes but have primarily been limited to chemical contaminants, and transient increases in nutrient loading.[9−12] Moreover, these studies have focused on contamination of localized water bodies such as the contamination of Lake Pontchartrain (Louisiana) during Hurricane Katrina,[12,13] and limited data about the distribution of microbial contaminants in impacted rivers and streams are available.

Hurricanes and large storms play a significant role in the transport of water contaminants across environmental waters.[10,11] Among these contaminants, the mobilization and distribution of fecal indicator bacteria and human-associated fecal markers due to sewage overflows and stormwater runoff resulting from flooding are poorly understood. There is a critical need to understand the distribution and transport of fecal bacteria in contaminated surface waters due to ecological as well as human health concerns. Emerging real-time quantitative PCR (qPCR) methods designed to estimate the concentration of fecal pollution by targeting genomic DNA from fecal indicator bacteria such as *E. coli*, *Enterococcus* spp., and Bacteroidales are now available and can generate test results just a few hours after sampling, which is critical during extreme events such as hurricanes. These genetic markers may also detect viable but nonculturable (VBNC) cells that are not detected by conventional cultivation approaches but may still

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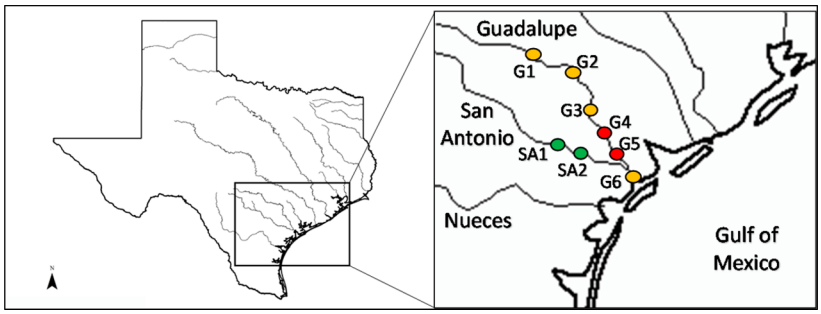

Figure 1. Locations of sampling sites. Sites G1−G6 were located on the Guadalupe River, and sites SA1 and SA2 were located on the San Antonio River. Sites G1−G3 and G6 were classified as nonflooded regions of the Guadalupe River (orange circles), while sites G4 and G5 were located in the flooded catchment of the Guadalupe River (red circles). Sites SA1 and SA2 were located in the nonflooded segments of the San Antonio River (green circles). This map was created using ESRI ArcGIS and shows the state of Texas boundary and major rivers of Texas.

pose a public health risk. In this study, we have documented the levels of fecal indicator bacteria (*E. coli* and enterococci), including human-associated fecal genetic markers (human-associated Bacteroidales), measured using qPCR assays, across a Texas river impacted by Hurricane Harvey. In addition, we studied the correlation between human-associated Bacteroidales markers and traditional fecal indicators to better evaluate the efficacy of using these markers for the detection of human fecal pollution.

The primary objective of this study was to investigate the extent to which the flooded waterways in Texas have been contaminated with microbial contaminants following Hurricane Harvey. To accomplish this, we have evaluated the presence and abundance of fecal indicator bacteria and selected human-associated markers in flood-impacted environmental waters. Surface water samples were collected along the Guadalupe River, ranging from sites in Victoria, TX [which recorded devastating flooding (see Figure S1)],[14] to its outfall in the Texas coast. Samples were also collected from the San Antonio River as a control site because it is in the proximity of the Guadalupe River and was not severely impacted by flooding.[14] We applied a suite of qPCR assays targeting *E. coli*, enterococci, and human-associated Bacteroidales markers (HF183 and BacHum) to identify and quantify the fecal contamination in water. The results presented here provide the much needed and critical data on the effects of human fecal contamination sources on the surface water quality following Hurricane Harvey.

MATERIALS AND METHODS

Sampling Sites and Sample Collection. The Guadalupe River runs from Kerr County, Texas, to the San Antonio Bay in the Gulf of Mexico. The excessive rainfall from Hurricane Harvey resulted in major flooding over the Guadalupe River at Victoria, TX, during which the river crested at ∼32 feet [i.e., around 10 feet above flood stage (see Figure S1)]. The water quality of the river is a major concern as it is an important drinking water source for several cities and some sections are used in recreational activities. Thus, fecal contamination of the river is a significant public health concern and has a negative economic impact.

An *a priori* identification of representative sampling locations was not possible because of the considerable logistical complications involving access to the sites and safety concerns. An initial rapid assessment was performed on September 8, 2017, whereby multiple water samples were collected from the Guadalupe River in flooded regions in Victoria, TX, and potential sampling sites were identified. A more intensive sampling campaign was conducted 2 weeks later on September 22, 2017, followed by three more sampling events each in October, November, and December 2017. During the intensive sampling campaign, water samples were collected from six different sites along the Guadalupe River (G1−G6) and two sites on the San Antonio River (SA1 and SA2) over a period of 10 weeks from September to December 2017 (Figure 1). The sampling locations were chosen and categorized into flooded or nonflooded sites on the basis of the severity of impact due to rainfall and flooding (see Figure S2). The description and coordinates of the sites are listed in Table 1. An additional description of the sampling sites is given in the Supporting Information.

Table 1. Description of Sampling Sites

sampling site	descriptive location	flooded/ nonflooded	latitude, longitude
G1	Guadalupe River (GR) at I-10	nonflooded	29.56656, −98.02294
G2	GR near Independence Park	nonflooded	29.48377, −97.44888
G3	GR at US 59T Business	nonflooded	28.79334, −97.01316
G4	GR at US 59	flooded	28.75168, −97.0063
G5	GR before confluence of San Antonio River	flooded	28.6554, −96.95924
G6	GR after confluence of San Antonio River	nonflooded	28.47814, −96.86275
SA1	San Antonio River at US 239	nonflooded	28.73567, −97.64348
SA2	San Antonio River near Goliad State Park	nonflooded	28.64992, −97.38478

Water samples were collected in duplicate from each site using sterilized 1 L bottles (Nalgene, Rochester, NY) and transported on ice to the laboratory at the University of Texas at San Antonio (San Antonio, TX) within 6 h of collection. The water samples (200−1000 mL) were filtered onto 0.45 μm pore-size, 47 mm diameter polycarbonate membranes (Pall Life Sciences, Ann Arbor, MI) and stored at −20 °C until DNA could be extracted.

qPCR Analyses. The occurrence and relative abundance of four different fecal bacterial markers in environmental water samples were measured using TaqMan qPCR assays (Table S1) with DNA extracts as the templates. DNA was extracted from filter samples using the DNeasy PowerLyzer PowerSoil Kit

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(Qiagen, Germantown, MD) according to the manufacturer's protocol. The targeted fecal bacterial groups were *E. coli* (EC23S857 assay),[15] *Enterococcus* spp. (Entero1 assay),[16] and human-associated Bacteroidales (HF183 and BacHum assays).[17,18] The qPCR assays were performed as previously described.[19] Additional details regarding DNA extraction and qPCR assays are provided in the Supporting Information. The genetic markers used in our study for the detection of *E. coli* and enterococci have been correlated to public health risk,[20] and the Entero1 assay has been incorporated by the EPA into water quality standards in the United States.[7] The two human-associated Bacteroidales qPCR markers have been evaluated in a number of studies and shown to be highly specific to human feces, with little to no cross reactivity with other host feces.[21,22] In addition, all four qPCR markers have been validated and tested in several studies measuring human fecal contamination in environmental waters.[19,21−23]

Analyses of Data. The marker copy number per 100 mL of water was calculated for those samples with values above the limit of quantification for each assay, and all data were $\log_{10}$ transformed before statistical analysis. The qPCR results for initial grab samples were excluded from any subsequent analyses as these samples were obtained from locations slightly different from the sampling sites established in this study. Differences in marker concentrations were analyzed using nonparametric Kruskal−Wallis one-way analysis of variance. The correlation between human-associated Bacteroidales and conventional fecal indicators was analyzed using the logistic regression analysis. All analyses were performed using Microsoft Excel (2016), and correlation strength was interpreted according to an accepted scale for biological statistics.[24] All statistical test outcomes were regarded as significant at the $p < 0.001$ level.

RESULTS AND DISCUSSION

Performance of qPCR Assays. Standard curves were generated using serial dilutions of known copy numbers to determine the amplification efficiencies and linear range of the qPCR assays. The qPCR amplification efficiencies for all the assays ranged from 92.5 to 105.6%, with r^2 values between 0.986 and 0.999. The linear range of quantification for the qPCR assay of human-associated Bacteroidales (HF183 and BacHum) and *E. coli* markers was between 10 and 10^6 copies, while the linear range for the qPCR assay of Entero1 was between 20 and 2×10^6 copies. PCR inhibition tests were performed with 10-fold dilutions of each DNA extract as described in a previous study.[23] In these tests, a Ct value proportional to a 10-fold dilution relative to the undiluted DNA templates resulted, suggesting that PCR inhibition did not interfere with the amplification efficiency. No template controls (two per PCR plate) and DNA extraction controls indicated the absence of contamination in the qPCR experiments.

Distribution of Fecal Indicator Bacteria and Human-Associated Marker Levels. Initial grab water samples ($n = 4$) were collected on September 8, 2017, near sites G4 and G5. The average concentrations of fecal bacterial indicators in these samples were 1.31×10^2 and 5.05×10^5 copies/100 mL of water for *E. coli* and enterococci, respectively. The average concentrations of human-associated Bacteroidales in these samples were 6.74×10 and 5.67×10 copies/100 mL of water for BacHum and HF183, respectively. This was followed by a more intensive sampling campaign from September to December 2017.

The targeted fecal bacterial groups were frequently detected in surface water samples ($n = 32$) (40−100%). *E. coli* and enterococci were present in all samples using the EC23S857 and Entero1 assays, respectively, while the human-associated Bacteroidales were detected less frequently. Enterococci exhibited the highest levels across all the sampling sites with a mean marker abundance of $>10^3$ copies/100 mL of water. The spatial distribution of the levels of markers across the study sites is represented in Figure 2. The water samples yielded

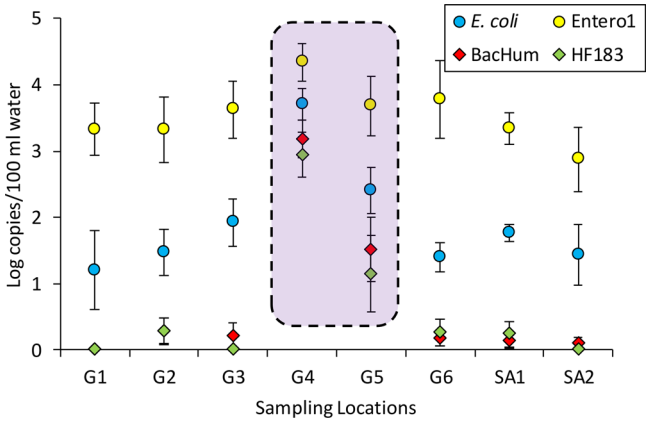

Figure 2. Spatial variation in levels of markers across study sites as determined using qPCR assays. Sites G4 and G5 exhibited the highest concentrations of fecal genetic markers (highlighted in purple) and were located in the flooded segment of the Guadalupe River. Error bars may be obscured by marker sizes in some cases.

significantly different amplification results across study sites with both the EC23S857 and Entero1 assays (Kruskal−Wallis test; $p < 0.001$). Site G4 had the highest concentrations of *E. coli* and enterococci among all the study sites. The two human-associated Bacteroidales markers, HF183 and BacHum, exhibited a similar spatial distribution pattern across the sampling sites, although the level of the BacHum marker was slightly higher for all samples. Both of the markers tested positive at most of the sites, with the exception of sites G1 and SA2, where HF183 was not detected in any of the samples. The levels of the human-associated Bacteroidales markers were statistically different (Kruskal−Wallis test; $p < 0.001$) from each other among the study sites. Site G4 had the highest mean copy number for the human-associated Bacteroidales markers, which is consistent with the excessive flooding at this site, and also because it was located right after a wastewater treatment plant (WWTP) and the discharge from the WWTP was fed into this region. Site G5 also had a relatively high concentration of human-associated Bacteroidales compared to those of other sites. However, for site G6, which is located right after the confluence of the Guadalupe River and San Antonio River, the concentrations of both human-associated Bacteroidales markers decreased, indicating that the San Antonio River was not severely impacted by human fecal contamination. This was also true for sites SA1 and SA2 (control sites on the San Antonio River) that had considerably lower levels of human-associated Bacteroidales.

The temporal distribution of the average levels of fecal indicator bacteria and human-associated markers is presented in Figure 3. Sites G4 and G5 were classified as flooded regions of the Guadalupe River, while sites G1−G3 and G6 were located in the nonflooded areas. Sites SA1 and SA2 were located in the

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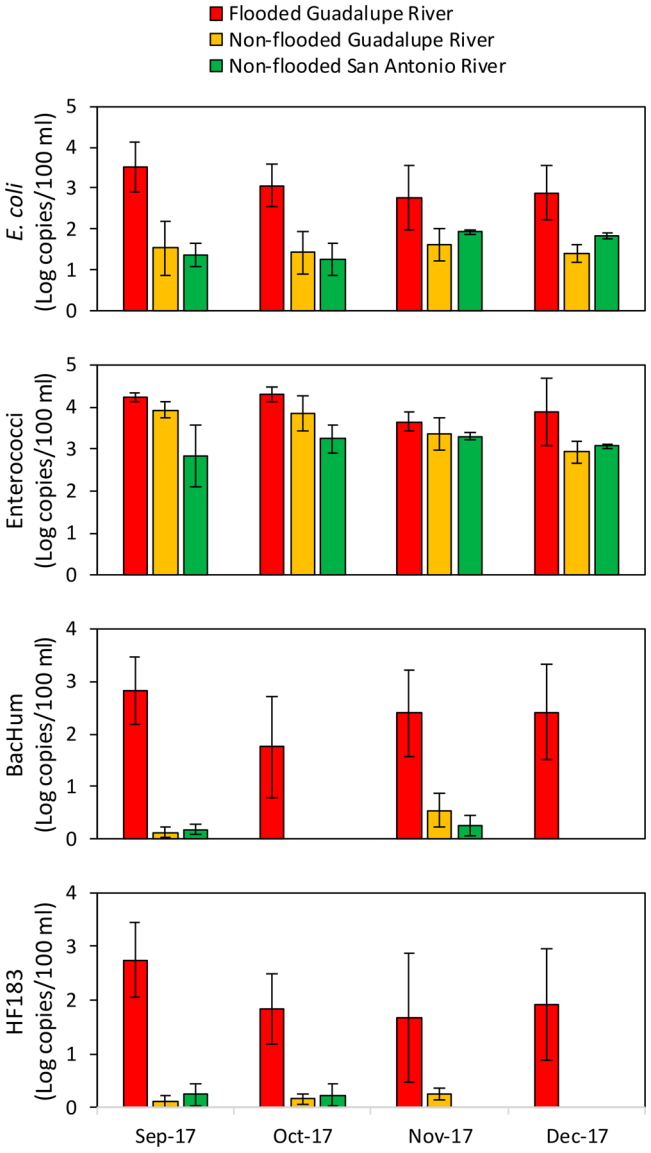

Figure 3. Temporal variation in levels of *E. coli*, enterococci, and human-associated Bacteroidales in water samples collected in this study. Sites G1−G3 and G6 were classified as nonflooded regions of the Guadalupe River (orange bars), while sites G4 and G5 were located in the flooded catchment of the Guadalupe River (red bars). Sites SA1 and SA2 were located in the nonflooded segments of the San Antonio River (green bars).

nonflooded segments of the San Antonio River. On average, all markers had the highest concentrations for the flooded regions of the Guadalupe River. Although the concentrations of human-associated Bacteroidales were significantly higher for the flooded sites than the nonflooded sites throughout the collection period, the observed levels of human-associated fecal markers were considered relatively low for an impacted site.[25] However, the concentrations for human-associated Bacteroidales remained elevated for flooded sites even 2−3 months after Hurricane Harvey, suggesting that human fecal pollution may be a chronic and relatively constant source of fecal contamination for the flood-impacted sites, both before and after the hurricane. Samples collected on September 22, 2017, ∼3 weeks after flooding, demonstrated the highest levels for *E. coli*, BacHum, and HF183, in the flooded and nonflooded segments; however, no significant variation occurred over time (Kruskal−Wallis test; $p > 0.5$). Initial grab samples collected on September 8, 2017 (during the period of recovery from flooding), from the flooded segment of the Guadalupe River (near sites G4 and G5) had concentrations of the Entero1 marker higher than those of the samples collected on September 22, 2017 (3 weeks after flooding); however, the concentrations of other markers were slightly lower. This may be due to the differences in the locations of the initial grab samples and the actual sampling sites, which were not accessible earlier because of safety concerns.

The presence of high concentrations of bacterial indicators in surface water samples collected from flooded regions of the river was indicative of the fecal contamination in the environmental waters of the impacted areas. The most heavily flooded sites showed the highest abundance of fecal indicator bacteria and human-associated Bacteroidales, indicating that the large number of sewage overflows and stormwater runoff that occurred during Hurricane Harvey flooding introduced high levels of fecal bacteria into waterways draining into the Gulf of Mexico thereby impairing surface and coastal water quality. The concentrations of fecal indicator bacteria, such as *Enterococcus* spp., in recreational waters impacted by sewage have been linked to adverse health outcomes in swimmers through epidemiology studies.[20] The EPA recently published revised recreational water quality criteria for enterococci that aim to keep the risk of gastrointestinal illness (GI) in swimmers below approximately 30 illnesses per 1000 primary contact recreators.[7] All initial water samples collected from the flooded regions of the Guadalupe River had *E. coli* and enterococci concentrations above the regulatory level for contact recreational waters,[7] although it should be noted that our results were measured by qPCR methods while the Recreational Water Quality Criteria are based on culturable *E. coli* or enterococci. A recent study used quantitative microbial risk assessment (QMRA) to simulate the risk of GI illness associated with swimming in waters containing different concentrations of the HF183 qPCR marker and estimated there was a linear relationship between HF183 median concentrations (in units of copies per 100 mL) and simulated GI illness rates [$\log_{10}$(GI risk) = −4.93 + 0.94 × $\log_{10}$ HF183; RSQ = 0.99].[25] On the basis of this regression relationship, the simulated GI illness rate per 1000 swimmers varied between 5.2×10^{-4} and 0.005 for the flooded sites in the Guadalupe River. This level of predicted human health risk based on the HF183 marker is relatively low compared to the EPA-adopted health target of ∼30 GI illnesses per 1000 swimmers. Although the QMRA model used here relies on a scenario in which raw sewage contaminates recreational waters, there are many other potential sources of human fecal pollution such as leaking septic systems, open defecation, and treated wastewater. Future studies to explore the relationships between human health and human-associated qPCR marker levels in recreational waters impacted by sewage, treated effluents, and human feces are needed.

Stormwater runoff resulting from flooding can cause an influx of indicator bacteria to receiving waters. Previous studies have demonstrated increased levels of indicator bacteria in coastal waters influenced by stormwater runoff.[26−28] However, there are limited studies relating the impact of flooding caused by hurricanes to the sources of fecal pollution in inland waterways. Fecal bacteria may be introduced into surface waters during flooding through numerous sources such as municipal waste from household sewage treatment systems, combined sewer overflows (CSOs), sanitary sewer overflows (SSOs), damaged

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WWTPs, leaky septic tanks, and stormwater and urban runoff.[6,19] In this study, we quantified the extent of human fecal contamination in environmental waters by an integrated analysis of host-specific markers and fecal bacteria. The assessment of study sites for human-associated Bacteroidales demonstrates that sewage sources of fecal pollution are major contributors to water quality deterioration within our study area. Because the Guadalupe River does not encompass agriculture or farming runoff, and there are no CSOs in the sampling region, the primary source of human-associated Bacteroidales can be attributed to the influx of waste from damaged wastewater infrastructure and stormwater runoff. As the river flows into the Gulf of Mexico, these sources appear to represent a chronic and relatively constant source of human contamination for inland and near shore communities.

Correlation between Human-Associated Bacteroidales and Fecal Indicator Bacteria. The numbers of markers per 100 mL water sample were used to study the correlation between human-associated Bacteroidales and conventional fecal indicators (enterococci and *E. coli*). The human-associated Bacteroidales markers showed a weak to slightly strong correlation with enterococci and *E. coli* markers, which are the most frequently used water quality indicators (Figure S3). There was a weak correlation between the BacHum and Entero1 markers in the samples (correlation coefficient, $r^2 = 0.24$; $p < 0.001$), although *E. coli* exhibited a slightly stronger correlation with the BacHum marker ($r^2 = 0.72$; $p < 0.001$). HF183 also showed a correlation similar to that of BacHum with both Entero1 ($r^2 = 0.32$; $p < 0.001$) and *E. coli* ($r^2 = 0.75$; $p < 0.001$). The weak correlation of human-associated Bacteroidales with Entero1 could be due to overestimation of the Entero1 assay as reported in other studies.[29,30] These results are relevant to environmental monitoring, as the Entero1 assay has been proposed as an alternative method for the rapid detection of *Enterococcus* spp. in recreational waters.[31] As the overestimation of the Entero1 assay due to nontargeted bacteria could result in heightened human health risk assessment, additional studies are needed to determine more accurately the levels of false-positive signals in recreational settings.

The microbial source tracking study undertaken in the Guadalupe River after Hurricane Harvey substantiates that the large number of sewage overflows and stormwater runoff that occurred during Hurricane Harvey flooding introduced high levels of fecal bacteria into environmental waters. On the other hand, on the basis of the qPCR data, the low correlation between human-associated Bacteroidales and the conventional fecal indicator assays such as Entero1 highlights the ambiguity of enterococci as robust human fecal pollution surrogates.[32,33] In addition, relatively high levels of enterococci and *E. coli* were found in the samples with low to moderate levels of human-associated Bacteroidales markers, with the intensities of the Entero1 signals being consistently higher than those of the other markers. This may be due to the variable persistence of different markers after release from their hosts.[34,35] Because *E. coli* and enterococcus concentrations are not specific for human feces, it may be noted that counts of these indicator organisms may overstate the public health risks associated with stormwater runoff. This has been suggested in previous studies;[36,37] altogether, these data support the use of Bacteroidales markers as effective indicators of human fecal contamination. These findings also illustrate the extent to which *E. coli* and enterococci levels may be uncoupled from evidence of human sewage contamination in the environment.

Confronting water quality deterioration caused by fecal contamination remains a significant challenge for many countries in the world, especially during and after natural disasters, and can cause serious human health risks and have severe environmental and economic repercussions. For instance, surveys in the aftermath of Hurricane Katrina identified several cases of *Vibrio* infections and gastrointestinal illness.[38] An example of an economic impact is a case in which beaches are closed or posted with water quality advisories because concentrations of fecal indicator bacteria in coastal waters exceed standards. High levels of fecal pollution are the primary cause of river and stream impairment in the United States according to the National Water Quality Inventory,[39] with numerous often uncharacterized contamination sources.[40,41] Hence, accurate and reliable fecal source identification methods are essential for mitigating bacterial contributions to waterways and maintaining water quality. In this study, we have evaluated the presence and abundance of fecal indicator bacteria and human-associated markers in water samples collected along an impacted Texas waterway in the weeks following Hurricane Harvey. These data will provide the required information to produce a dynamic fecal source model for surface waters impacted by sewage overflows caused due to extreme flooding. The findings of this initial microbiological contaminant assessment will serve as the baseline information for follow-on studies to monitor existing and emerging public health risks to residents of Texas and potential long-term environmental impacts on the water resources in the impacted regions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.8b00237.

Description of sampling sites, DNA extraction, and qPCR assays; figures showing flooding of the Guadalupe River (Figure S1), a map showing the extent of flooding in Texas (Figure S2), and the correlation between human-associated Bacteroidales and fecal indicator bacteria (Figure S3); and a table listing the primers and probes used in this study (Table S1) (PDF)

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Notes

The authors declare no competing financial interest.

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