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Detection of human fecal pollution in environmental waters using human mitochondrial DNA and correlation with general and human-associated fecal genetic markers

A. B. M. Tanvir Pasha, Jessica Hinojosa, Duc Phan, Adrianne Lopez and Vikram Kapoor

ABSTRACT

Human mitochondrial DNA (mtDNA) genetic markers are abundant in sewage and highly human-specific, suggesting a great potential for the environmental application as human fecal pollution indicators. Limited data are available on the occurrence and co-occurrence of human mtDNA with fecal bacterial markers in surface waters, and how the abundance of these markers is influenced by rain events. A 1-year sampling study was conducted in a suburban watershed impacted by human sewage contamination to evaluate the performance of a human mtDNA-based marker along with the bacterial genetic markers for human-associated Bacteroidales (BacHum and HF183) and *Escherichia coli*. Additionally, the human mtDNA-based assay was correlated with rain events and other markers. The mtDNA marker was detected in 92% of samples ($n = 140$) with a mean concentration of 2.96 $\log_{10}$ copies/100 ml throughout the study period. Human mtDNA was detected with greater abundance than human-associated Bacteroidales that could be attributed to differences in the decay of these markers in the environment. The abundance of all markers was positively correlated with rain events, and human mtDNA abundance was significantly correlated with various bacterial markers. In general, these results should support future risk assessment for impacted watersheds, particularly those affected by human fecal pollution, by evaluating the performance of these markers during rain events.

Key words | fecal source tracking, human mitochondrial DNA, human-associated *Bacteroidales*, quantitative PCR, rainfall, water quality

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INTRODUCTION

Identifying the sources of fecal pollution in environmental surface waters used for human recreation and/or fish breeding is the first step for reducing the potential for human contact with enteric pathogens (Simpson *et al.* 2002; Jeong *et al.* 2019). Human waste can be introduced into environmental waters in a variety of ways, including leaking septic tanks, untreated sewage, sewer malfunctions, contaminated storm drains, urban runoff, and other sources (Marsalek & Rochfort 2004; Bakir *et al.* 2006; Badgley *et al.* 2010). Hence, the accurate detection of human fecal pollution is imperative for mitigating human and environmental health risks, reducing economic losses, and maintaining water quality. For over a century, microbiological water quality has been assessed by measuring the densities of two fecal indicator bacteria (FIB), *Escherichia coli* and *Enterococcus* spp. As monitoring tools, these fecal bacterial indicators often provide a useful baseline for establishing public health risks. However, many studies have recognized the limitations of conventional bacteria-based fecal source tracking, including the facts that most of these microorganisms are able to inhabit the intestines of several hosts, including humans, livestock, and wildlife, and are able to grow and survive

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naturally in the environment (Gordon 2001; Anderson *et al.* 2005; Ishii *et al.* 2006). Recently, new microbial source tracking assays, such as the human-associated Bacteroidales genetic markers, have been developed using the quantitative polymerase chain reaction (qPCR) and aim to detect bacteria derived specifically from human feces (Seurinck *et al.* 2005; Kildare *et al.* 2007; Sauer *et al.* 2011; Layton *et al.* 2013; Kapoor *et al.* 2015). Nonetheless, an alternative to these human fecal microbial targets is the use of approaches that detect the presence of human DNA itself, derived from human fecal waste, specifically in the form of mitochondrial DNA (mtDNA) (Caldwell *et al.* 2007; Vuong *et al.* 2013). In geographic regions where Bacteroidales markers are not abundant in human hosts due to differences in diet, culture, or other environmental influences, mtDNA-based markers may be particularly useful in determining human fecal pollution (Yatsunenko *et al.* 2012; Nshimyimana *et al.* 2017). In addition, few studies have demonstrated the cross-reactivity of human-associated Bacteroidales assays with samples from other host feces (Johnston *et al.* 2013; Layton *et al.* 2013). A direct marker in the form of human mtDNA could add a layer of confirmation to current source tracking studies guiding mitigation efforts, which require strong stakeholder support and are often very expensive to implement.

The detection of fecal bacterial indicators may be influenced by rainfall events (Santiago-Rodriguez *et al.* 2012; Stachler *et al.* 2018). It has been previously reported that rainfall may lead to an increase of fecal indicators and pathogens in environmental surface waters and, thus, may represent a heightened risk to human health (Shehane *et al.* 2005; Santiago-Rodriguez *et al.* 2012). Specifically, rainfall events may result in stormwater runoff, resuspension of sediments, and sewage overflows that can contribute to an increase of indicators in surface waters (Parker *et al.* 2010; Sidhu *et al.* 2013). Nevertheless, the contribution of fecal pollution due to rainfall is often not considered when assessing water quality, and limited information is available on the correlation between rainfall and source tracking markers, including human mtDNA. Studies have examined the occurrence of human mtDNA in environmental waters; however, there is still little observational data on the occurrence and co-occurrence of human mtDNA with human-associated Bacteroidales and general *E. coli* markers in surface waters over extended temporal and spatial scales (Martellini *et al.* 2005; Kapoor *et al.* 2013, 2014). In this study, qPCR assays targeting human mtDNA, human-associated Bacteroidales markers (HF183 and BacHum), and *E. coli* were applied to identify and quantify human fecal contamination in environmental surface waters. The Cibolo Creek in the Upper Cibolo Creek (UCC) watershed (Texas, USA) was used for sampling due to a history of significant human fecal contamination (Bass & Burger 2013). Surface waters of the Cibolo Creek receive discharge from two wastewater treatment systems and are subject to occasional stormwater runoff from nearby agricultural fields during rainfall events.

The objective of this study was to identify the sources of human fecal pollution in a suburban watershed by using general and human-associated fecal genetic markers, as well as human mtDNA as a direct and robust human fecal marker. The study aimed to understand indicator prevalence in environmental surface waters and what that might suggest about the sources of these indicators. The influence of rainfall events on the abundance of these indicators was also evaluated. In addition, the correlation between human mtDNA, human-associated Bacteroidales markers, and *E. coli* was studied to better understand the efficacy of each marker for the detection of human fecal pollution. The results of this study provide valuable information on the sources of fecal pollution in the UCC watershed and important insights on the distribution of traditional (*E. coli*) and more novel indicators (human mtDNA and human-associated Bacteroidales).

MATERIALS AND METHODS

Study area and sampling sites

The Cibolo Preserve in the UCC watershed covers approximately 40 hectares of land in the southern part of Kendall County, Texas, USA, and was chosen for sampling due to a history of significant human fecal contamination (Bass & Burger 2013). The primary surface water body of the UCC is the Cibolo Creek, which is formed from the contributions of Brown and Champee springs in southern Kendall County and flows in a northwest to southeast direction for 161 km through five other counties before its confluence

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with the San Antonio River. The creek drainage area covers 200 km^2 and is characterized as rural/suburban with light ranch areas. Although it has a history of bacterial contamination often exceeding the Texas water quality standards for safe contact recreation, the creek is commonly used for recreational activities (Bass & Burger 2013). A previous study reported bacterial impairments in the Cibolo Creek as well as a high concentration of orthophosphorus and other nutrients; however, since 2013, no studies have been conducted to examine fecal contamination in the Cibolo Creek (Bass & Burger 2013). The geology of the area is fractured karst limestone, resulting in significant groundwater recharge through the streambed, and decreases in the measured flow of the lower 30 km of the creek during the dry season. As an effort to maintain the flow, the South Texas Wastewater Treatment Plant (STWT) and Wastewater Treatment and Recycling Center (WWTRC) in Boerne, TX, began discharging their treated effluent into the creek. Wastewater effluent has been suggested as a potential source of fecal pollution of surface waters (Servais *et al.* 2007; Naidoo & Olaniran 2014). Thus, the occurrence of human fecal contamination in the Cibolo Preserve may likely be linked to the effluent discharges from the two wastewater treatment plants (WWTPs).

A total of nine sampling sites were selected within the study area based on proximity to the WWTPs, discharge to the Cibolo Creek and in an attempt to cover the extent of the preserve area (Figure 1). Site 1 is located on the Currey Creek downstream of the STWT; since no flow upstream of the STWT was observed over the study period, no sampling site was selected in the upstream region of the Currey Creek. Site 2 is located approximately 1 km downstream of site 1 and is the only recreational site in the study area. Sites 7 and 8 are located before and after the contribution from the WWTRC, respectively, and site 3 is located just after the confluence of the Cibolo Creek and the Menger Creek. The WWTRC discharges effluent water into the Menger Creek, which converges with the Cibolo Creek at the preserve boundary. Sites 4 and 5 are located before and after the converging point of the Browns Creek with the Cibolo Creek. Site 6 is located at the eastern edge of the preserve area and was selected to observe the quality of the water before leaving the preserve area. Site 9 is located on the Browns Creek, which converges with the Cibolo Creek inside of the preserve area.

Sample collection and DNA extraction

Surface water samples ($n = 140$) were collected over a period of 1 year (March 2017 to March 2018) from nine different sites within the study area. All samples were collected 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 2–4 h of collection. The water samples were then filtered (300–500 ml) onto 0.45-μm pore size, 47-mm diameter mixed cellulose ester membranes (Millipore, Billerica, MA), after which the membranes were immediately frozen at −80 °C. Extraction controls with autoclaved distilled water were used during filtration to monitor for potential extraneous DNA contamination. The manufacturer's protocol was followed to extract DNA from the sample membranes using the DNeasy PowerLyzer PowerSoil Kit (Qiagen, Germantown, MD). The purity and concentration of DNA were determined using the NanoDrop One^C Spectrophotometer (Thermo Scientific, Wilmington, DE). DNA extracts were stored at −20 °C for subsequent qPCR analyses.

qPCR analyses

TaqMan qPCR assays were used to measure both the presence and relative abundance of the four genetic markers in environmental water samples (Table 1). The human mtDNA genetic marker using primer sets targeting the human mitochondrial gene NADH dehydrogenase subunit 5 (ND5) was used to detect human mtDNA (Caldwell *et al.* 2007). The remaining three markers used qPCR assays targeting the fecal bacterial groups: *E. coli* (EC23S857 assay) (Chern *et al.* 2011) and the two human-associated Bacteroidales (HF183 and BacHum assays) (Kildare *et al.* 2007; Haugland *et al.* 2010).

The methodology for the qPCR assays performed is described in detail in a previous study (Kapoor *et al.* 2018). Briefly, reaction mixtures (25 μl) contained 1× SsoAdvanced Universal Probes Supermix (Bio-Rad, Hercules, CA), 0.2 μM final concentration of forward and reverse primers, a 6-FAM (6-carboxyfluorescein)-labeled hydrolysis probe, and 2 μl of the extracted DNA template from the water samples. All reaction mixtures were prepared in duplicate, and qPCR assays were performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The qPCR

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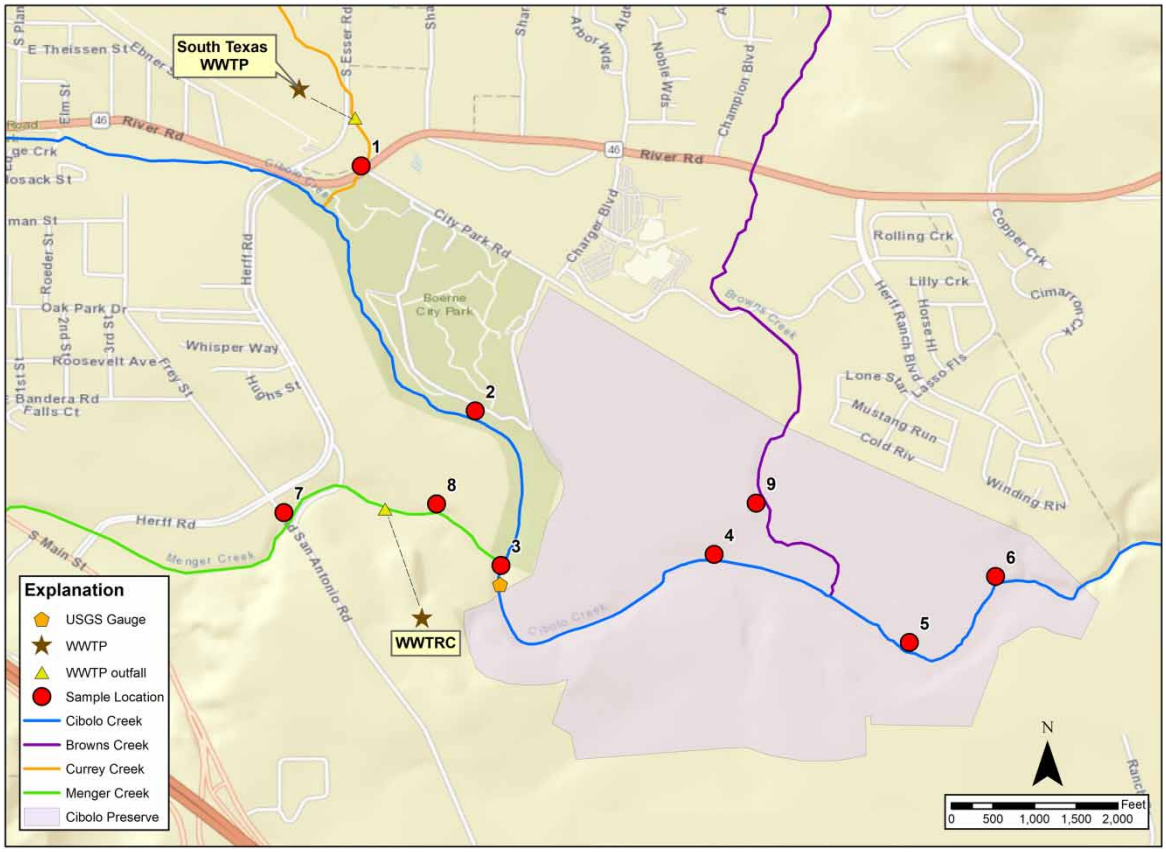

Figure 1 | Study sampling locations in Boerne, Texas. Sites 2–6 were located on the Cibolo Creek, site 1 on the Currey Creek, sites 7 and 8 on the Menger Creek, and site 9 on the Browns Creek. This map was created using the ESRI ArcGIS.

Table 1 | Primers and probes used in this study

Assay	Primer and probe sequences (5′–3′)	Reference
Human mtDNA	Forward: CAGCAGCCATTCAAGCAATGC Reverse: GGTGGAGACCTAATTGGGCTGATTAG Probe: TATCGGCGATATCGGTTTCATCCTCG	Caldwell *et al.* (2007)
Human-associated Bacteroidales (HF183)	HF183-1: ATCATGAGTTCACATGTCCG BthetR1: CGTAGGAGTTTGGACCGTGT BthetP1: 6-FAM-CTGAGAGGAAGGTCCCCCACATTGGA-TAMRA	Haugland *et al.* (2010)
Human-associated Bacteroidales (BacHum)	BacHum-160f: TGAGTTCACATGTCCGCATGA BacHum-241r: CGTTACCCCGCCTACTATCTAATG BacHum-193p: 6-FAM-TCCGGTAGACGATGGGGATGCGTT-TAMRA	Kildare *et al.* (2007)
E. coli (EC23S857)	Forward: GGTAGAGCACTGTTTtGGCA[a] Reverse: TGTCTCCCGTGATAACtTTCTC[a] Probe: 6-FAM-TCATCCCGACTTACCAACCCG-TAMRA	Chern *et al.* (2011)

6-FAM, 6-carboxyfluorescein, fluorescence reporter dye; TAMRA, 6-carboxytetramethylrhodamine, fluorescence quencher dye.
[a]Lower case 't' denotes deliberately a mismatched base.

data were analyzed using the Bio-Rad's CFX Manager Software (version 3.1). Standard curves were generated by using plasmids containing the sequences for each of the targeted genes. No template controls were used to check for

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cross-contamination for each qPCR run. In addition, undiluted and 10-fold dilutions for each DNA extract were used as the DNA template in qPCRs to test for PCR inhibition.

Data analyses

All data for each genetic marker were calculated as the copy number per 100 ml of water for every sample analyzed using qPCR with a cycle threshold (C_T) value above background. Before performing statistical analyses, all data were transformed to $\log_{10}$ copies per 100 ml of the water sample. The difference in the detection frequency for human mtDNA, *E. coli*, and human-associated Bacteroidales markers was determined using cross-tabulation by means of Venn diagrams. Differences in marker concentrations among the study sites were examined using a nonparametric Kruskal–Wallis one-way analysis of variance. Spearman's rank correlation coefficients (r) between qPCR indicators were calculated using the means of $\log_{10}$ transformed marker concentrations in GraphPad Prism 8 (La Jolla, CA) using two-tailed 95% confidence intervals. For comparison purposes, Spearman's coefficients are ranked according to the following scale: 0.1–0.39 (weak correlation), 0.4–0.59 (moderate correlation), 0.6–0.79 (strong correlation), and 0.8–1 (very strong correlation). All statistical analysis results were regarded as significant at $p < 0.05$. Precipitation data reported 24 h before sample collection was obtained from the US Geological Survey (USGS) station number 08183890, located on the Cibolo Creek within the Cibolo Preserve.

RESULTS AND DISCUSSION

Performance of qPCR assays

The linear range and amplification efficiencies of the qPCR assays were determined by plotting the standard curves generated using serial dilutions of known copy numbers of each marker. The linear range of quantification for all the qPCR assays was between 10 and 10^6 copies per reaction. The qPCR amplification efficiencies for all the assays ranged from 86 to 105%, with r^2 values above 0.9. To determine PCR inhibition, reactions were performed with 10-fold dilutions of each DNA extract as described in a previous study (Kapoor *et al.* 2015). In these tests, PCR inhibition did not interfere with the amplification efficiency, since a C_T value proportional to a 10-fold dilution relative to the undiluted DNA templates was observed for the reactions. DNA extraction controls and no template controls indicated the absence of contamination in the qPCR assays.

Detection of human mtDNA and fecal bacterial markers

The concentrations of human mtDNA, human-associated Bacteroidales, and *E. coli* were measured for the water samples using TaqMan qPCR assays. The human mtDNA marker was detected in 92% of water samples (Table 2), which indicates that human fecal pollution was predominant in the study area. Both human-associated Bacteroidales (HF183 and BacHum) markers were present in the majority of the water samples, while *E. coli* showed the maximum detection frequency (99%) for samples in this study. The mean marker abundance for the human mtDNA marker was measured the highest among all the markers, followed by *E. coli*.

The detection of human mtDNA was compared with the bacterial markers (Table 3). Specifically, 92% (129 out of 140) of the samples detected both *E. coli* and human mtDNA markers. For human-associated Bacteroidales, 50% (70 out of 140) of the samples were positive for both HF183 and BacHum markers, while 7 and 18% were positive for only HF183 and BacHum, respectively. In comparison, human mtDNA was detected in 96% (91 out of 95) of water samples positive for BacHum, and 95% (76 out of 80) of water samples positive for HF183. However, when comparing total samples, both human mtDNA and BacHum were detected concurrently in 65% (91 out of 140) of water

Table 2 | Distribution of molecular markers ($\log_{10}$ copies per 100 ml) used in this study detected via qPCR assays ($n = 140$)

Assay	Mean	Range	+(%)
Human mtDNA	2.96	0.00–6.69	92
Human-associated Bacteroidales (HF183)	1.57	0.00–4.89	57
Human-associated Bacteroidales (BacHum)	1.80	0.00–5.17	68
E. coli (EC23S857)	2.90	0.00–4.96	99

+(%) = percentage of samples detected positive for the marker.

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Table 3 | Comparison of the detection of the human mtDNA marker with bacterial markers from water samples ($n = 140$) using qPCR assays

Detection	Human mtDNA[1] vs. BacHum[2]	Human mtDNA[1] vs. HF183[2]	Human mtDNA[1] vs. *E. coli*[2]
Only marker 1	38	53	0
Only marker 2	4	4	10
Both 1 and 2	91	76	129
None	7	7	1
Total	140	140	140

samples, and both human mtDNA and HF183 were detected concurrently in 54% (76 out of 140) of water samples.

Spatial distribution of markers

The spatial distribution of the levels of markers across the study sites is represented in Figure 2. The two human-associated Bacteroidales markers, BacHum and HF183, displayed a similar spatial distribution pattern across the sampling sites, although the mean marker level of the BacHum marker was higher than HF183. The levels of the human-associated Bacteroidales markers were statistically different ($p < 0.05$, Kruskal–Wallis one-way analysis of variance) from each other among the study sites using qPCR assays. Site 1, located just downstream of the STWT, had the highest mean copy number for the human-associated Bacteroidales markers, while site 9 had the lowest copy numbers. Site 2, located downstream of site 1, after the convergence of the Currey Creek with the Cibolo Creek, showed a decrease in copy numbers for both of the human-associated Bacteroidales markers. A gradually decreasing trend in the concentration was observed for both BacHum and HF183 in a downstream direction, from site 2 to site 6 on the Cibolo Creek, suggesting that the human fecal contamination is coming from upstream sources, including the outfall of WWTPs. Previous studies have reported a high abundance of fecal indicators in treated waters at the outlet of WWTPs (Servais *et al.* 2007). Moreover, it has been reported that the persistence of human-associated Bacteroidales in the environment begins to decrease after 4 days of separation from its host and is completely decayed after 7 days, although the decay rate depends on multiple variables such as temperature, sunlight, and nutrients (Walters & Field 2009). Surprisingly, there were no notable differences in human mtDNA marker levels among the study sites, except for site

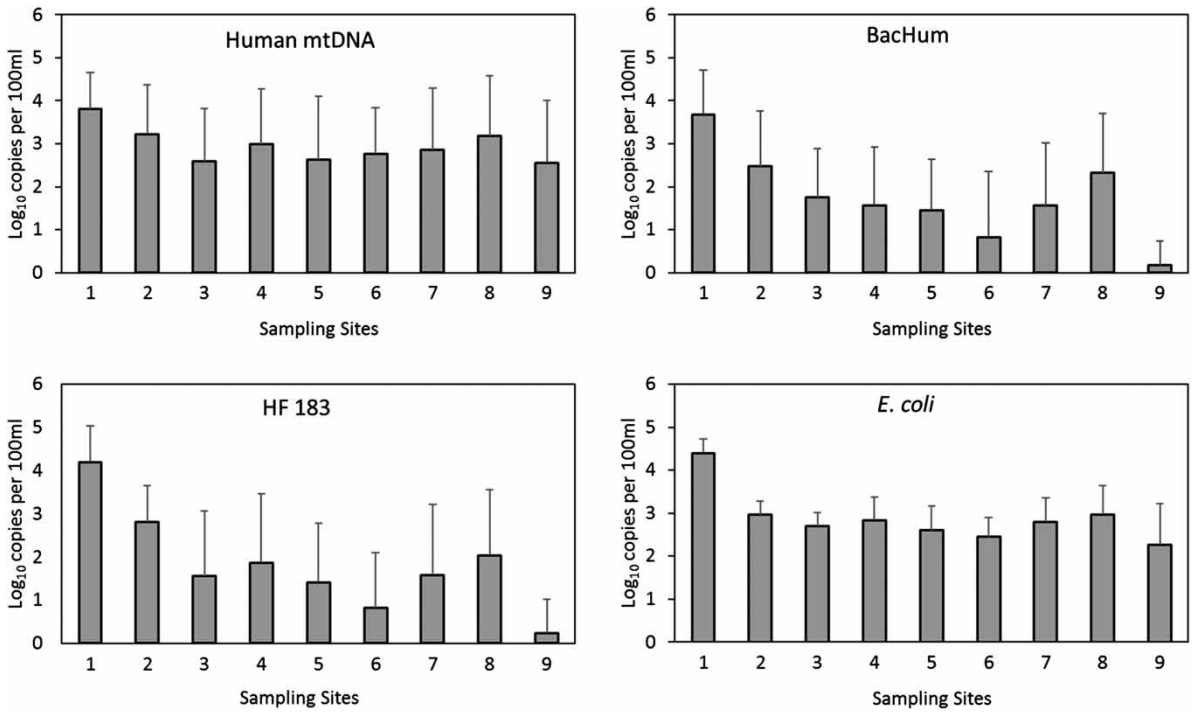

Figure 2 | Spatial variation in levels of markers across study sites as determined using qPCR assays. Bars represent averages, and error bars represent standard deviations ($n = 15$).

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1, which had the highest concentration. *E. coli* also exhibited the highest concentrations at site 1, which is located downstream of the STWT. In summary, all the markers exhibited the highest concentrations at site 1, which suggest that the STWT effluent discharge significantly contributed to the fecal pollution in the Cibolo Preserve.

E. coli was present in 99% of the samples using the EC23S857 assay with mean marker abundance $>10^2$ copies per 100 ml of water. Unlike human-associated Bacteroidales, there was no decrease in the *E. coli* concentrations downstream of site 2. Previously, many studies have confirmed non-point sources as the major contributor of *E. coli* in the environment (Anderson *et al.* 2005; Ishii *et al.* 2006; Tran *et al.* 2015). Based on the United States Department of Agriculture (USDA) estimates for the animal populations of Kendall County, there are more than 27,000 animals in the area, including cattle, goats, sheep, horses, feral hogs, and deer, with a manure deposition of approximately 10.4 kg/hectare/day. Surface runoff from upstream sources with manure deposition may have contributed to the variable concentration of *E. coli* in the creek waters, as also suggested in a previous study (Bass & Burger 2013).

Sites 7 and 8 were chosen to observe the difference in contamination between the upstream and downstream waters of the Menger Creek due to the effluent water discharged from the WWTRC. All markers showed a slight increase in median concentrations downstream of the treatment plant. As discussed previously, many studies have reported fecal bacteria in the effluent water of WWTPs, mainly attributable to the poor design of the plants (Glassmeyer *et al.* 2005; Daneshvar *et al.* 2012). Additionally, a thick layer of the algal bloom was present during the dry season at the downstream location at site 8. A similar phenomenon was observed in another study (Carey & Migliaccio 2009), and they suggested that excessive nutrients like nitrogen and phosphorus from the effluent water may be responsible for the high algae growth. These data suggest effluent from the WWTRC as a point source of contamination.

Correlation of markers with rainfall events

To discern the effect of rainfall events, the precipitation data reported 24 h before sample collection was obtained for each sampling event. The average concentrations for all markers at sites 2, 3, and 4 were used for the analysis due to their proximity to the USGS station. In total, there were five rainfall events with rainfall ranging from 0.51 to 41.91 mm of rain in a 24 h period. Results for marker copy number per 100 ml volume are shown in Figure 3 along with plots of the rainfall throughout the study. One-way analysis of variance (ANOVA) was performed on all fecal markers to assess if the concentrations of the markers were significantly affected by rainfall events. The abundance of all markers was positively correlated with rain events, indicating that the indicator marker concentration increased after a rainfall event. Furthermore, the abundance of all markers was found to be considerably different ($p < 0.05$) between dry and wet weather conditions. The results of this study suggest that the loading of markers into surface waters may be influenced by precipitation events. As suggested in previous studies, it is possible that sediments and runoff could contribute to the input of bacterial markers into surface waters. Results presented here are consistent with previous studies in which higher numbers of bacterial markers were detected after rainfall events (Santiago-Rodriguez *et al.* 2012; Stachler *et al.* 2018).

Correlation of human mtDNA and fecal bacterial markers

Spearman's rank correlation coefficients were calculated between each pair of human mtDNA and fecal bacterial markers as measured by the qPCR (Table 4). All Spearman's correlation coefficients were positive and showed statistical significance based on the *p*-value of 0.05. Human mtDNA exhibited a moderate correlation with fecal bacterial markers (*E. coli*, HF183, and BacHum). When compared with human-associated Bacteroidales, human mtDNA was detected with higher abundance (approximately 1–2 orders of magnitude) than HF183 and BacHum. This could be attributed to differences in the decay of human-associated Bacteroidales and human mtDNA markers in the environment. As discussed above, human-associated Bacteroidales can be completely decayed in 7 days after release from a host, whereas human mtDNA can be detected for up to 15 days in the environment (Martellini *et al.* 2005). There was a strong correlation between both the human-associated Bacteroidales markers, HF183 and BacHum. The human-associated Bacteroidales and *E. coli* marker showed the moderate-to-strong

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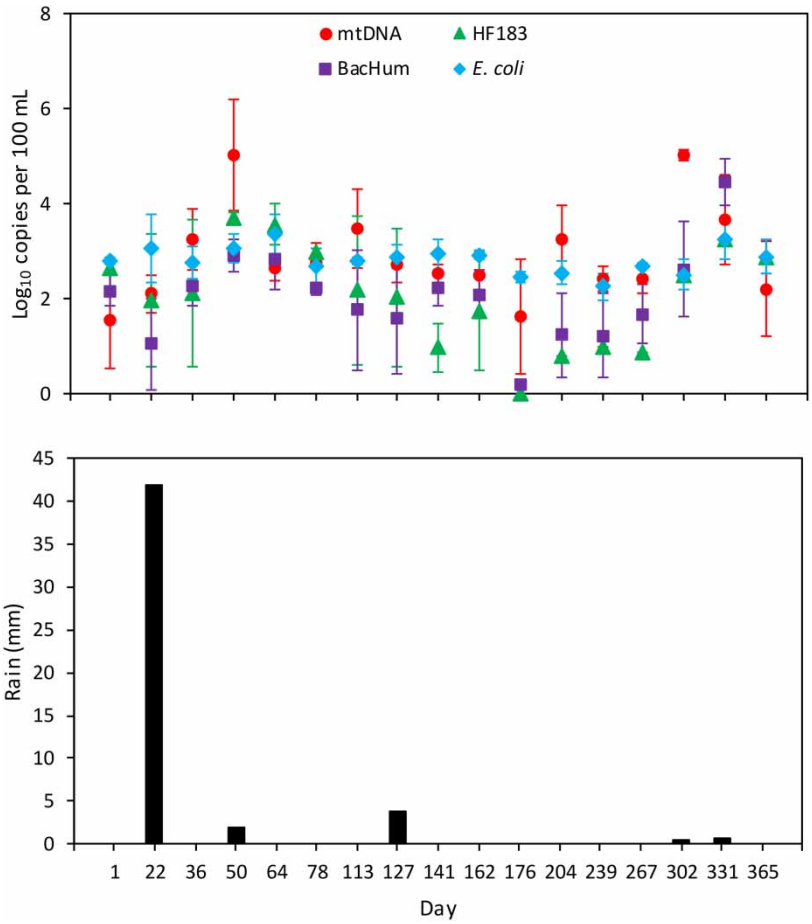

Figure 3 | Temporal variation in levels of markers along with rainfall during the sampling period. Rainfall is presented as the amount of rain that fell within the 24-h period before that day's sampling. Data points represent averages, and error bars represent standard deviations ($n = 9$).

Table 4 | Spearman's rank correlation coefficient matrix for human mtDNA, HF183, BacHum, and *E. coli* markers as measured by qPCR

	Human mtDNA	HF183	BacHum	*E. coli*
Human mtDNA	1			
HF183	0.571	1		
BacHum	0.468	0.664	1	
E. coli	0.561	0.610	0.548	1

correlation, indicating that human fecal pollution was predominant in the study area. A similar correlation has been previously reported between *E. coli* concentration and the concentrations of the human-associated Bacteroidales markers in environmental water samples (Kapoor *et al.* 2013).

The mitochondrial marker and the fecal bacterial markers targeting human were tested on more than 100 water samples. These samples were collected over a period of 1 year from nine different sites in a fast-growing suburban watershed, representing natural areas with minimal human impact (Cibolo Preserve) and small urban areas close to the natural environment or surrounded by agricultural activities. The preserve area was overrepresented to observe the potential impact of discharging treated wastewater effluent into the creeks in the preserve. High incidences of the human mtDNA and human-associated Bacteroidales markers were observed, especially for human mtDNA with a 92% occurrence in the water samples. Among the 140 samples tested with the human markers, human mtDNA marker and BacHum were concurrently positive in 91 samples and negative in seven samples, representing a 70% convergence for these markers. The remaining samples were detected by either human mtDNA (27.1%) or BacHum

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(2.8%). Given that the BacHum marker has a bacterial origin and the human mtDNA marker is derived from human cells themselves, we expected this variability due to the differential persistence of mitochondria and/or mtDNA vs. Bacteroidales in surface water.

Human sewage contamination of environmental surface waters is a serious issue for populations living in fast-growing semi-urban settings and for the surrounding natural environment. Our study clearly showed a high occurrence of human markers in the water samples, even in areas with relatively low human activities (preserve area). This could be attributed to the discharge of municipal WWTP effluents containing considerable levels of fecal waste (Servais *et al.* 2007; Naidoo & Olaniran 2014) as well as leaking septic tanks. Other sources of fecal pollution include municipal waste from household sewage treatment systems, sanitary sewer overflows, combined sewer overflows, and stormwater and urban runoff (Bakir *et al.* 2006; Badgley *et al.* 2010). In the present study, we quantified the level of human fecal contamination in a suburban watershed by an integrated analysis of human-specific markers and FIB. The evaluation of study sites for human fecal markers demonstrates that sewage sources of fecal pollution are major contributors to water quality degradation within the Cibolo Preserve. Thus, the primary source of human mtDNA and human-associated Bacteroidales markers can be attributed to the waste influx from nearby WWTPs. Since creeks in the watershed provide critical recharge for both the Trinity and Edwards aquifers, these sources appear to represent a chronic and relatively constant source of human fecal pollution.

This study demonstrated the correlation of human mtDNA with commonly used fecal pollution indicators. The bacterial markers (*E. coli*, BacHum, and HF183 assays) showed a moderate correlation with the human mtDNA marker. This is expected based on the difference in survival and the persistence of human and bacterial cells in environmental waters. For instance, most Bacteroidales are obligate anaerobes and die quickly in oxygenated surface water and, therefore, are expected to survive for shorter periods in the environment after release from their hosts (Kapoor *et al.* 2013; Layton *et al.* 2013). Conversely, *E. coli* can grow and persist in environmental waters and sediments, can come from other nonhuman sources such as domesticated animals, and thus, are not inherently correlated with recent human fecal contamination. Based on the results of this study, human mtDNA-based markers could be an indicator of the presence of human fecal pollution in environmental waters (Yang *et al.* 2015; Kapoor *et al.* 2017). By comparing a direct marker of human feces (human mtDNA) with bacterial fecal indicators, we were able to overcome the limitations of conventional fecal indicators such as *E. coli* that yield false positives from bacteria already living in the environment. As our study was limited to samples collected in a localized geographic location, further research is necessary to determine if the relationships among the fecal markers noted here are significantly different when the number of geographically different samples is increased.

The detection of mitochondrial genes via PCR-based assays can be used to identify animal waste directly through its own discharged eukaryotic cells. In addition to our study, the application of mtDNA for fecal source tracking has also been demonstrated in a few recent studies. For example, qPCR assays targeting mitochondrial genes have been used to detect human-, bovine-, and swine-specific contamination (Caldwell *et al.* 2007; Villemur *et al.* 2015). A mitochondrial-based microarray (mitoArray) has been developed for rapid identification of the presence of a large number of animals potentially implicated in fecal pollution in mixed activity watersheds (Vuong *et al.* 2013). It should be noted that although mtDNA genes provide a reliable PCR target, it is still an indirect indicator of the presence of bacterial pathogens and the risk estimation of human health.

The 1-year-long source tracking study provided information regarding the seasonal variability of fecal indicators and their correlation with rainfall events. This study demonstrates the high abundance of the human mtDNA-based marker and their positive association with other indicators of human fecal pollution in the environment. Overall, levels of the human mtDNA marker were detected at similar concentrations to the *E. coli*-based assay and approximately 1–2 orders of magnitude greater than the HF183 and BacHum assays.

CONCLUSION

The study goal was to identify the sources of human fecal pollution in an urban watershed by using human mtDNA

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as a direct marker as well as conventional fecal genetic markers. The co-occurrence of human mtDNA with the presence of bacterial fecal indicators was evaluated in sewage-impacted environmental waters. Human mtDNA abundance significantly correlated with various bacterial markers. In addition, the concentration of all the markers increased after rainfall events. By applying this human DNA as a direct indicator of contamination, we were able to overcome the limitations of using microbial markers to more adequately reflect the fecal pollution input. Further investigations are necessary to establish the association of human mtDNA with human fecal pollution in more ambient waters along with water bodies impacted by other fecal pollution sources (e.g., wildlife and agriculture). While more studies should be performed in additional water systems to further elucidate the range of correlation between human mtDNA and fecal bacterial occurrence, the correlations observed in this study broaden the range of potential applications for human mtDNA-based assays. Although limited in the surface area, these results should assist with future risk assessments for semi-urban/rural watersheds, particularly those affected by human fecal pollution, by providing the improved fecal source tracking approach.

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REFERENCES

Anderson, K. L., Whitlock, J. E. & Harwood, V. J. 2005 Persistence and differential survival of fecal indicator bacteria in subtropical waters and sediments. *Applied and Environmental Microbiology* **71** (6), 3041–3048.

Badgley, B. D., Thomas, F. I. & Harwood, V. J. 2010 The effects of submerged aquatic vegetation on the persistence of environmental populations of *Enterococcus* spp. *Environmental Microbiology* **12** (5), 1271–1281.

Bakir, M. A., Sakamoto, M., Kitahara, M., Matsumoto, M. & Benno, Y. 2006 *Bacteroides dorei* sp. nov., isolated from human faeces. *International Journal of Systematic and Evolutionary Microbiology* **56** (7), 1639–1643.

Bass, R. & Burger, D. 2013 *Upper Cibolo Creek Watershed Protection Plan*. City of Boerne. Retrieved from: http://www.ci.boerne.tx.us/DocumentCenter/View/3690/.

Caldwell, J. M., Raley, M. E. & Levine, J. F. 2007 Mitochondrial multiplex real-time PCR as a source tracking method in fecal-contaminated effluents. *Environmental Science & Technology* **41** (9), 3277–3283.

Carey, R. O. & Migliaccio, K. W. 2009 Contribution of wastewater treatment plant effluents to nutrient dynamics in aquatic systems: a review. *Environmental Management* **44** (2), 205–217.

Chern, E. C., Siefring, S., Paar, J., Doolittle, M. & Haugland, R. A. 2011 Comparison of quantitative PCR assays for *Escherichia coli* targeting ribosomal RNA and single copy genes. *Letters in Applied Microbiology* **52** (3), 298–306.

Daneshvar, A., Aboulfadl, K., Viglino, L., Broséus, R., Sauvé, S., Madoux-Humery, A. S., Weyhenmeyer, G. A. & Prévost, M. 2012 Evaluating pharmaceuticals and caffeine as indicators of fecal contamination in drinking water sources of the Greater Montreal region. *Chemosphere* **88** (1), 131–139.

Glassmeyer, S. T., Furlong, E. T., Kolpin, D. W., Cahill, J. D., Zaugg, S. D., Werner, S. L., Meyer, M. T. & Kryak, D. D. 2005 Transport of chemical and microbial compounds from known wastewater discharges: potential for use as indicators of human fecal contamination. *Environmental Science & Technology* **39** (14), 5157–5169.

Gordon, D. M. 2001 Geographical structure and host specificity in bacteria and the implications for tracing the source of coliform contamination. *Microbiology* **147** (5), 1079–1085.

Haugland, R. A., Varma, M., Sivaganesan, M., Kelty, C., Peed, L. & Shanks, O. C. 2010 Evaluation of genetic markers from the 16S rRNA gene V2 region for use in quantitative detection of selected Bacteroidales species and human fecal waste by qPCR. *Systematic and Applied Microbiology* **33** (6), 348–357.

Ishii, S., Ksoll, W. B., Hicks, R. E. & Sadowsky, M. J. 2006 Presence and growth of naturalized *Escherichia coli* in temperate soils from Lake Superior watersheds. *Applied and Environmental Microbiology* **72** (1), 612–621.

Jeong, J., Wagner, K., Flores, J. J., Cawthon, T., Her, Y., Osorio, J. & Yen, H. 2019 Linking watershed modeling and bacterial source tracking to better assess *E. coli* sources. *Science of the Total Environment* **648**, 164–175.

Johnston, C., Byappanahalli, M. N., Gibson, J. M., Ufnar, J. A., Whitman, R. L. & Stewart, J. R. 2013 Probabilistic analysis showing that a combination of Bacteroides and Methanobrevibacter source tracking markers is effective for identifying waters contaminated by human fecal pollution. *Environmental Science & Technology* **47** (23), 13621–13628.

Kapoor, V., Smith, C., Santo Domingo, J. W., Lu, T. & Wendell, D. 2013 Correlative assessment of fecal indicators using human mitochondrial DNA as a direct marker. *Environmental Science & Technology* **47** (18), 10485–10493.

Kapoor, V., DeBry, R. W., Boccelli, D. L. & Wendell, D. 2014 Sequencing human mitochondrial hypervariable region II as a molecular fingerprint for environmental waters. *Environmental Science & Technology* **48** (18), 10648–10655.

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Kapoor, V., Pitkänen, T., Ryu, H., Elk, M., Wendell, D. & Santo Domingo, J. W. 2015 Distribution of human-specific bacteroidales and fecal indicator bacteria in an urban watershed impacted by sewage pollution, determined using RNA- and DNA-based quantitative PCR assays. *Applied and Environmental Microbiology* **81** (1), 91–99.

Kapoor, V., Elk, M., Toledo-Hernandez, C. & Santo Domingo, J. W. 2017 Analysis of human mitochondrial DNA sequences from fecally polluted environmental waters as a tool to study population diversity. *AIMS Environmental Science* **4** (3), 443–455.

Kapoor, V., Gupta, I., Pasha, A. T. & Phan, D. 2018 Real-time quantitative PCR measurements of fecal indicator bacteria and human-associated source tracking markers in a Texas river following Hurricane Harvey. *Environmental Science & Technology Letters* **5** (6), 322–328.

Kildare, B. J., Leutenegger, C. M., McSwain, B. S., Bambic, D. G., Rajal, V. B. & Wuertz, S. 2007 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal Bacteroidales: a Bayesian approach. *Water Research* **41** (16), 3701–3715.

Layton, B. A., Cao, Y., Ebentier, D. L., Hanley, K., Ballesté, E., Brandão, J., Byappanahalli, M., Converse, R., Farnleitner, A. H., Gentry-Shields, J. & Gidley, M. L. 2013 Performance of human fecal anaerobe-associated PCR-based assays in a multi-laboratory method evaluation study. *Water Research* **47** (18), 6897–6908.

Marsalek, J. & Rochfort, Q. 2004 Urban wet-weather flows: sources of fecal contamination impacting on recreational waters and threatening drinking-water sources. *Journal of Toxicology and Environmental Health, Part A* **67** (20–22), 1765–1777.

Martellini, A., Payment, P. & Villemur, R. 2005 Use of eukaryotic mitochondrial DNA to differentiate human, bovine, porcine and ovine sources in fecally contaminated surface water. *Water Research* **39** (4), 541–548.

Naidoo, S. & Olaniran, A. 2014 Treated wastewater effluent as a source of microbial pollution of surface water resources. *International Journal of Environmental Research and Public Health* **11** (1), 249–270.

Nshimyimana, J. P., Cruz, M. C., Thompson, R. J. & Wuertz, S. 2017 Bacteroidales markers for microbial source tracking in Southeast Asia. *Water Research* **118**, 239–248.

Parker, J. K., McIntyre, D. & Noble, R. T. 2010 Characterizing fecal contamination in stormwater runoff in coastal North Carolina, USA. *Water Research* **44** (14), 4186–4194.

Santiago-Rodriguez, T. M., Tremblay, R. L., Toledo-Hernandez, C., Gonzalez-Nieves, J. E., Ryu, H., Santo Domingo, J. W. & Toranzos, G. A. 2012 Microbial quality of tropical inland waters and effects of rainfall events. *Applied and Environmental Microbiology* **78** (15), 5160–5169.

Sauer, E. P., VandeWalle, J. L., Bootsma, M. J. & McLellan, S. L. 2011 Detection of the human specific Bacteroides genetic marker provides evidence of widespread sewage contamination of stormwater in the urban environment. *Water Research* **45** (14), 4081–4091.

Servais, P., Garcia-Armisen, T., George, I. & Billen, G. 2007 Fecal bacteria in the rivers of the Seine drainage network (France): sources, fate and modelling. *Science of the Total Environment* **375** (1–3), 152–167.

Seurinck, S., Defoirdt, T., Verstraete, W. & Siciliano, S. D. 2005 Detection and quantification of the human-specific HF183 Bacteroides 16S rRNA genetic marker with real-time PCR for assessment of human faecal pollution in freshwater. *Environmental Microbiology* **7** (2), 249–259.

Shehane, S. D., Harwood, V. J., Whitlock, J. E. & Rose, J. B. 2005 The influence of rainfall on the incidence of microbial faecal indicators and the dominant sources of faecal pollution in a Florida river. *Journal of Applied Microbiology* **98** (5), 1127–1136.

Sidhu, J. P. S., Ahmed, W., Gernjak, W., Aryal, R., McCarthy, D., Palmer, A., Kolotelo, P. & Toze, S. 2013 Sewage pollution in urban stormwater runoff as evident from the widespread presence of multiple microbial and chemical source tracking markers. *Science of the Total Environment* **463**, 488–496.

Simpson, J. M., Santo Domingo, J. W. & Reasoner, D. J. 2002 Microbial source tracking: state of the science. *Environmental Science & Technology* **36** (24), 5279–5288.

Stachler, E., Akyon, B., de Carvalho, N. A., Ference, C. & Bibby, K. 2018 Correlation of crAssphage qPCR markers with culturable and molecular indicators of human fecal pollution in an impacted urban watershed. *Environmental Science & Technology* **52** (13), 7505–7512.

Tran, N. H., Gin, K. Y. H. & Ngo, H. H. 2015 Fecal pollution source tracking toolbox for identification, evaluation and characterization of fecal contamination in receiving urban surface waters and groundwater. *Science of the Total Environment* **538**, 38–57.

Villemur, R., Imbeau, M., Vuong, M. N., Masson, L. & Payment, P. 2015 An environmental survey of surface waters using mitochondrial DNA from human, bovine and porcine origin as fecal source tracking markers. *Water Research* **69**, 143–153.

Vuong, N. M., Villemur, R., Payment, P., Brousseau, R., Topp, E. & Masson, L. 2013 Fecal source tracking in water using a mitochondrial DNA microarray. *Water Research* **47** (1), 16–30.

Walters, S. P. & Field, K. G. 2009 Survival and persistence of human and ruminant-specific faecal Bacteroidales in freshwater microcosms. *Environmental Microbiology* **11** (6), 1410–1421.

Yang, Z., d'Auriac, M. A., Goggins, S., Kasprzyk-Hordern, B., Thomas, K. V., Frost, C. G. & Estrela, P. 2015 A novel DNA biosensor using a ferrocenyl intercalator applied to the potential detection of human population biomarkers in wastewater. *Environmental Science & Technology* **49** (9), 5609–5617.

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., Magris, M., Hidalgo, G., Baldassano, R. N., Anokhin, A. P. & Heath, A. C. 2012 Human gut microbiome viewed across age and geography. *Nature* **486** (7402), 222.

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