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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

Vikram Kapoor,[a,b] Tarja Pitkänen,[c,d] Hodon Ryu,[a] Michael Elk,[a] David Wendell,[b] Jorge W. Santo Domingo[d]

Pegasus Technical Services, Inc., Cincinnati, Ohio, USA[a]; Department of Biomedical, Chemical, and Environmental Engineering, University of Cincinnati, Cincinnati, Ohio, USA[b]; National Institute for Health and Welfare, Water and Health Unit, Kuopio, Finland[c]; Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, Ohio, USA[d]

The identification of fecal pollution sources is commonly carried out using DNA-based methods. However, there is evidence that DNA can be associated with dead cells or present as "naked DNA" in the environment. Furthermore, it has been shown that rRNA-targeted reverse transcription-quantitative PCR (RT-qPCR) assays can be more sensitive than rRNA gene-based qPCR assays since metabolically active cells usually contain higher numbers of ribosomes than quiescent cells. To this end, we compared the detection frequency of host-specific markers and fecal bacteria using RNA-based RT-qPCR and DNA-based qPCR methods for water samples collected in sites impacted by combined sewer overflows. As a group, fecal bacteria were more frequently detected in most sites using RNA-based methods. Specifically, 8, 87, and 85% of the samples positive for general enterococci, *Enterococcus faecalis*, and *Enterococcus faecium* markers, respectively, were detected using RT-qPCR, but not with the qPCR assay counterpart. On average, two human-specific *Bacteroidales* markers were not detected when using DNA in 12% of the samples, while they were positive for all samples when using RNA (cDNA) as the template. Moreover, signal intensity was up to three orders of magnitude higher in RT-qPCR assays than in qPCR assays. The human-specific *Bacteroidales* markers exhibited moderate correlation with conventional fecal indicators using RT-qPCR results, suggesting the persistence of nonhuman sources of fecal pollution or the presence of false-positive signals. In general, the results from this study suggest that RNA-based assays can increase the detection sensitivity of fecal bacteria in urban watersheds impacted with human fecal sources.

Sewage overflows and stormwater runoff introduce high levels of fecal bacteria into surface waters and are considered the primary cause of water quality impairments in urban watersheds, particularly those affected by combined sewer overflows (CSOs) (1, 2). Sewage contamination of surface waters poses a serious risk to human and environmental health via waterborne disease outbreaks (3–5), deterioration of recreational and drinking water quality (6, 7), and degradation of aquatic ecology (8, 9). Hence, identifying the primary source(s) of fecal contamination is imperative to enable best management practices for mitigating pollution and public health risks.

Microbial source tracking (MST) methods targeting fecal bacteria have recently been used to identify the sources of fecal contamination impacting water systems (10, 11). Many of these MST methods are based on quantitative PCR (qPCR) assays targeting the bacterial rRNA genes present within water DNA extracts (12, 13). However, the value of DNA-based monitoring in microbial ecology studies is limited by the possibility of DNA being associated with dead cells or the extent to which "naked DNA" may survive in the environment once bacteria are lysed (14–16). These facts pose a significant challenge to the environmental fate and transport of fecal bacteria which are often assessed via quantification of targets in spatial and temporal studies. Consequently, source tracking data based on amplification of genetic markers by DNA-targeted qPCR methods may not distinguish between recent and past contamination events since DNA of some bacteria can persist after cell death for a prolonged period of time (16). The use of intercalating DNA-binding chemicals, such as propidium monoazide, have been previously suggested to discriminate between viable and dead cells via inhibition of PCR amplification of DNA derived from dead cells (15). Interferences due to particulate matter present in environmental samples limit the use of these chemicals for MST assays. To circumvent the aforementioned limitations, rRNA has been proposed as an alternate target in the detection of fecal bacteria, primarily because of its poor stability outside the cell (17) and its correlation with cell activity (18). However, one disadvantage of RNA based detection is that it may be difficult to equate rRNA copy numbers to actual cell densities due to the strong correlation of rRNA expression with physiological state of the cell. RNA-based reverse transcription-qPCR (RT-qPCR) assays have been utilized in a number of microbiological studies, such as the detection of bacterial contamination in blood (19), subdominant bacteria in human intestines (20), and assessment of nitrification in wastewater treatment (21).

To unambiguously identify fecal pollution associated with recent contamination events (22, 23), it is necessary to distinguish

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Address correspondence to Jorge W. Santo Domingo, santodomingo.jorge@epa.gov, or David Wendell, wendeldw@uc.edu.
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TABLE 1 Description of the Duck Creek watershed sampling sites

Site	Location	Presumed primary fecal contamination source	Surroundings
1	5076 Wooster Road (Duck Creek)	CSO, urban runoff	Industrial
2	Red Bank Road and Columbia Parkway overpass (Duck Creek)	CSO, urban runoff	Industrial
3	3601 Old Red Bank (Duck Creek)	CSO	Residential
4	Germania and Bancroft (Little Duck Creek)	Urban runoff	Residential, vegetation
5	Settle Street (Little Duck Creek)	Urban runoff, septic tanks	Residential, vegetation
6	Camargo Road (Little Duck Creek)	Urban runoff, septic tanks	Residential, vegetation
7	Red Dog Hotel (confluence of Duck Creek and Deerfield Creek)	CSO, domestic pets	Pet care facility, vegetation
8	John Parker School (Deerfield Creek)	CSO, domestic pets	School, grasslands
9	BMW store (Deerfield Creek)	CSO, urban runoff	Interstate, car showroom
10	3715 Madison Road (Duck Creek)	CSO, urban runoff	Concrete channels
11	Indian Hills (Little Duck Creek)	Urban runoff	Residential, railroad

active microbial cells from dead cells. Fecal bacterial groups such as *Bacteroidales* are obligate anaerobes presumed to survive only for short periods of time after released into water (24, 25). Hence, it would be preferable to detect host-specific markers associated with metabolically active *Bacteroidales* populations. We theorize that in order to better assess the impact of fecal pollution it is important to target active cells via rRNA-based detection of gene markers in fecal source tracking studies. Since metabolically active cells usually contain higher numbers of ribosomes than quiescent cells (26), it is not a surprise that recent studies have shown that rRNA-targeted RT-qPCR assays can be more sensitive than rRNA gene-based qPCR assays (27, 28). In addition, the rRNA/rRNA gene ratio from each target bacterium is an important indicator of its metabolic status within bacterial communities and has been used to provide an estimate of *in situ* bacterial growth rates (29, 30). Thus, it is reasonable to propose that rRNA transcripts should better reflect the diversity of the predominantly metabolically active members of the microbial community.

Investigations examining the simultaneous occurrence and prevalence of both rRNA and rRNA gene targets of fecal indicators in surface waters are limited. To our knowledge, there has been minimal data on the evaluation of rRNA-targeted qPCR assays (i.e., rRNA RT-qPCR) for the detection and quantification of fecal bacteria in water samples affected by CSOs. In this study, we applied RT-qPCR and qPCR assays targeting human-specific *Bacteroidales* markers (HF183 and BacHum), *Escherichia coli*, *Enterococcus* spp., *Enterococcus faecalis*, and *Enterococcus faecium* to identify and quantify fecal contamination in water. The templates used in this study were RNA and DNA extracted from surface water samples collected from Duck Creek Watershed (Cincinnati, OH), since CSOs and sanitary sewer overflows (SSOs) are assumed to be the primary sources of fecal bacteria in the watershed during wet weather events (31, 32).

The objectives of this study were to compare the detection frequency of the different rRNA gene-targeting assays using both RNA and DNA as the templates and to evaluate the distribution of targeted fecal bacteria, including source-specific markers, across an urban watershed impacted by sewage pollution. In addition, we studied the correlation between human-specific *Bacteroidales* markers and conventional fecal indicators using rRNA based RT-qPCR to better evaluate the efficacy of using these markers for the detection of human fecal pollution.

MATERIALS AND METHODS

Study sites and sampling. Water samples were collected from 11 different sampling sites within the Duck Creek Watershed (Table 1) over a period of 10 weeks from October 2012 to December 2012. Streams in the watershed do not meet Ohio Water Quality Standards (WQS; OAC 3745-1) for bacteria during dry or wet weather (32). The Duck Creek, Little Duck Creek, and Deerfield Creek are the primary tributaries in this watershed, with their confluence located within the combined sewer service area in the city of Cincinnati. These three streams discharge into Little Miami River which eventually drains into the Ohio River. CSOs and SSOs are considered the primary sources of human fecal pollution in the watershed (31), and the sampling sites were chosen based on CSO locations and watershed runoff. All sites have been previously monitored for the presence of human fecal pollution through human-specific molecular markers (33).

Water samples were collected using sterilized 1-liter bottles (Nalgene, Rochester, NY) and transported on ice to the laboratory at the University of Cincinnati (Clifton, OH) within 2 h of collection. The water samples (200 to 1,000 ml) were filtered onto 0.45-μm-pore-size, 47-mm-diameter mixed cellulose ester membranes (Millipore, Billerica, MA) at the University of Cincinnati and frozen immediately at −80°C. Extraction controls with autoclaved distilled water were used during filtration to monitor for potential extraneous DNA contamination. The membranes were transported on ice coolers prior to the laboratory in the U.S. Environmental Protection Agency (Cincinnati, OH), where the nucleic acid extractions were conducted.

RNA and DNA extraction. RNA and DNA were extracted from the membranes as described elsewhere (27). Briefly, frozen membranes were subjected to bead-beating using DNase and RNase free glass beads (Mo Bio Laboratories, Inc., Carlsbad, CA) in the presence of lysis buffer (Buffer RLT Plus; Qiagen GmbH, Hilden, Germany) containing β-mercaptoethanol (Sigma-Aldrich Co., St. Louis, MO). The cell lysate was centrifuged, and the supernatant was used for simultaneous extraction of RNA and DNA with the AllPrep DNA/RNA minikit, following the manufacturer's instructions (Qiagen GmbH). RNA was further purified using an Ambion Turbo DNA-free DNase kit prior to the reverse transcription step, following the manufacturer's instructions (Life Technologies, Grand Island, NY). The concentration and purity of RNA and DNA was determined by using Qubit RNA and dsDNA HS assay kits and a Qubit 2.0 Fluorometer (Life Technologies). cDNA was synthesized on the extraction day from the purified RNA extracts using random hexamer primed Superscript III system for RT-PCR as described previously (27). Both cDNA and DNA were stored at −20°C for subsequent analyses.

qPCR analyses. The occurrence and relative abundance of six different fecal bacterial markers in environmental water samples was measured using TaqMan qPCR assays (Table 2) and cDNA and DNA extracts as the templates. The targeted fecal bacterial groups were *E. coli* (EC23S857 assay) (34), *Enterococcus* spp. (Entero1 assay) (35), *Ent. faecalis* (Faecalis

TABLE 2 Primers and probes used in this study

Assay	Primer or probe		Reference
	Name or type[a]	Sequence (5′–3′)[b]	
Human-specific *Bacteroidales* (HF183)	HF183-1	ATCATGAGTTCACATGTCCG	38
	BthetR1	CGTAGGAGTTTGGACCGTGT	38
	BthetP1	6FAM-CTGAGAGGAAGGTCCCCCACATTGGA-TAMRA	38
Human-specific *Bacteroidales* (BacHum)	BacHum-160f	TGAGTTCACATGTCCGCATGA	39
	BacHum-241r	CGTTACCCCGCCTACTATCTAATG	39
	BacHum-193p	6FAM-TCCGGTAGACGATGGGGATGCGTT-TAMRA	39
General *Enterococcus* (Entero1)	ECST748F	AGAAATTCCAAACGAACTTG	35
	ENC854R	CAGTGCTCTACCTCCATCATT	35
	GPL813TQ	6FAM-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA	35
Enterococcus faecalis (Faecalis)	FaecalF	CGCTTCTTTCCTCCCGAGT	36
	FaecalR	GCCATGCGGCATAAACTG	36
	FaecalP	6FAM-CAATTGGAAA GAGGAGTGGCGGACG-TAMRA	36
Enterococcus faecium (Faecium1)	CiumF	TTCTTTTTCCACCGGAGCTT	37
	CiumR	AACCATGCGGTTTYGATTG	37
	CiumP	6FAM-AGTAACACGTGGGTAACCTGCCCATCAGA-TAMRA	37
Escherichia coli (EC23S857)	F	GGTAGAGCACTGTTTtGGCA	34
	R	TGTCTCCCGTGATAACtTTCTC	34
	P	6FAM-TCATCCCGACTTACCAACCCG-TAMRA	34
Total bacteria (BacT)	BACT1369F	CGGTGAATACGTTCYCGG	40
	PROK1541R	AAGGAGGTGATCCRGCCGCA	40
	TM1389F	6FAM-CTTGTACACACCGCCCGTC-TAMRA	40

[a] F, forward; R, reverse; P, probe.
[b] 6FAM, 6-carboxyfluorescein, fluorescence reporter dye; TAMRA, 6-carboxytetramethylrhodamine, fluorescence quencher dye. Lowercase letters denote deliberately mismatched bases.

assay) (36), *Ent. faecium* (Faecium1 assay) (37), and human-specific *Bacteroidales* (HF183 and BacHum assays) (38, 39).

The qPCR assays were performed as previously described by Ryu et al. (37). Standard curves were generated by using plasmids containing the sequences for each of the targeted genes. Controls containing no template were used to check for cross contamination. In addition, PCR inhibition was tested in cDNA and DNA extracts by using undiluted and 10-fold dilutions of each extract as qPCR template. The absence of PCR inhibition in samples was also confirmed by amplifying with general bacterial 16S rRNA gene primers (BacT assay) (40). No-reverse-transcription controls (undiluted and 10-fold diluted RNA samples) were used to confirm the absence of DNA in RNA extracts.

Data analysis. The range of quantification and the limit of detection for all of the qPCR assays were established in a previous study (27). The marker copy number per 100 ml of water was calculated for all samples subjected to qPCR with a cycle threshold (C_T) value above background, and all data were $\log_{10}$ transformed before statistical analysis. Cross-tabulation by means of Venn diagrams was used to detect the difference in the detection frequency of RT-qPCR and qPCR assays for *E. coli*, *Enterococcus* spp., and human-specific *Bacteroidales*. Differences in marker concentrations were analyzed a using nonparametric Kruskal-Wallis one-way analysis of variance. The correlation between human-specific *Bacteroidales* and conventional fecal indicators using RT-qPCR results was analyzed using the logistic regression analysis. Pearson correlation coefficients were also determined for the data sets. All analyses were performed using Microsoft Excel (2011), and correlation strength was interpreted according to an accepted scale for biological statistics (41). All statistical test outcomes were regarded as significant at a P of <0.05.

RESULTS

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 of the assays ranged from 80.1 to 96.0%, with r^2 values between 0.937 and 0.998. The linear range of quantification for the qPCR assay of human-specific *Bacteroidales* (HF183 and BacHum) and *E. coli* markers were between 10 and 10^5 copies, while the linear range for qPCR assay of Entero1 was 50 to 5×10^5 copies, and those for Faecalis and Faecium1 were between 10^2 and 10^5 copies. PCR inhibition tests were performed with 10-fold dilutions of each DNA extract as described in Pitkänen et al. (27). In these tests, a C_T 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 indicated the absence of contamination in the qPCR experiments.

Detection of markers with RNA and DNA extracts. When the results from the RNA- and DNA-based assays were combined, all of the markers were detected in >90% of water samples ($n = 66$) using both extracts. However, many of the water samples tested here were positive for some of the markers only when RNA was used as the template (Fig. 1). Specifically, 8% (5 out of 66), 87% (45 out of 52), and 85% (46 out of 54) of the samples positive for Entero1, Faecalis, and Faecium1 markers, respectively, were detected using RT-qPCR, but not with the qPCR assay counterpart. For human-specific *Bacte-*

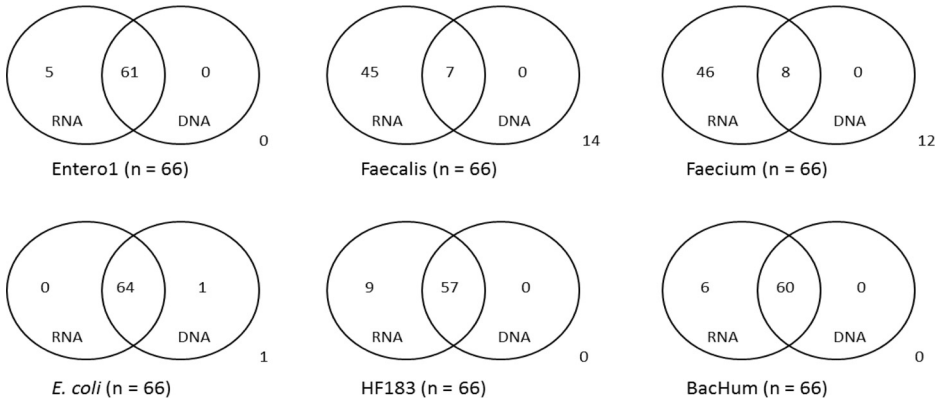

FIG 1 Occurrence of *Enterococcus* spp., *E. coli*, and human-specific *Bacteroidales* presented as Venn diagrams from CSO water samples using RT-qPCR and qPCR. Numbers outside the circles represent numbers of samples that tested negative with both the methods.

roidales, all samples were positive for both markers using RNA as the template, while only 86 and 91% samples were positive for HF183 and BacHum, respectively, using DNA as the template. In contrast, the EC23S857 marker was detected in all water samples using both RNA and DNA as the templates, except in one sample where it was only detected using DNA.

Distribution of host-specific and fecal bacteria marker levels. The concentration of human-specific *Bacteroidales* and fecal bacterial markers were measured for the water samples using both RT-qPCR and qPCR assays. Overall, there was a good correlation ($r = 0.85$) between RNA- and DNA-based qPCR results for all of the markers used in the present study. More interestingly, the mean marker abundance using RNA was significantly higher ($P < 0.01$) for all of the markers (Table 3).

The spatial distribution of the levels of markers across the study sites is represented in Fig. 2. The two human-specific *Bacteroidales* markers, HF183 and BacHum, exhibited a similar spatial distribution pattern across the sampling sites, although the level of BacHum marker was 1 order of magnitude higher for all of the samples. Both of the markers tested positive at all of the sites using RNA and DNA as the templates, with the exception of sites 6 and 11, where HF183 was not detected in most of the samples when DNA was used as the template. The levels of the human-specific *Bacteroidales* markers were statistically different ($P < 0.001$, Kruskal-Wallis one-way analysis of variance) from each other among the study sites using both RT-qPCR and qPCR assays. Site 9 had the highest mean copy number for the human-specific *Bacteroidales* markers, while site 6 had the lowest copy numbers, for both RNA and DNA based qPCR results.

TABLE 3 Distribution of molecular markers used in this study detected via TaqMan RT-qPCR and qPCR assays ($n = 66$)

Assay	Target	Distribution ($\log_{10}$ copies/100 ml)		% positive[a]
		Mean	Range	
Human-specific *Bacteroidales* (HF183)	RNA	4.56	0.81–5.96	100
	DNA	3.52	0.00–4.86	86
Human-specific *Bacteroidales* (BacHum)	RNA	5.68	1.98–7.18	100
	DNA	4.38	0.00–5.72	91
General *Enterococcus* (Entero1)	RNA	5.76	3.49–6.80	100
	DNA	3.73	0.00–4.78	92
Enterococcus faecalis (Faecalis)	RNA	3.39	0.00–4.82	79
	DNA	1.83	0.00–3.14	11
Enterococcus faecium (Faecium1)	RNA	2.74	0.00–3.61	82
	DNA	1.39	0.00–2.54	12
E. coli (EC23S857)	RNA	3.64	0.00–4.69	97
	DNA	2.53	0.00–3.41	98

[a] That is, the percentage of samples detected positive for the marker.

Enterococci were present in all samples using the Entero1 assay with RNA as the template with mean marker abundance $>10^4$ copies per 100 ml of water, while they were less frequently detected when using the DNA-based qPCR assay. Using the RT-qPCR assay, Entero1 marker was positive in 5 of 66 water samples that were determined to be negative using qPCR assay. The detection frequencies and concentrations of both Faecalis and Faecium1 markers via RT-qPCR assay were greater than the qPCR assay ($P < 0.01$) (Table 3). Sites 4, 5, and 6 tested negative for both Faecalis and Faecium1 when using DNA as the template, while they were positive when using RNA as the template. *E. coli* was tested positive in most of the samples using the EC23S857 assay either by RT-qPCR (97%) or qPCR assay (98%). There were no significant differences in *E. coli* numbers among the study sites using both RNA and DNA as the templates.

rRNA/rRNA gene ratio used to estimate the activity status of cells. The copy number of rRNA and the rRNA gene for the different bacterial markers allowed us to estimate the ratio between rRNA and the rRNA gene. The difference was quantified by using RT-qPCR and qPCR to obtain the rRNA/rRNA gene ratio for different bacterial cells present in the water samples.

The relationship between rRNA and rRNA gene copies for each marker was examined (Fig. 3). There was a positive correlation between individual rRNA and rRNA gene concentrations ($0.2 < r^2 < 0.8$, $n = 396$), suggesting that the activity of a bacterial species (rRNA transcripts) frequently followed its relative abundance in the community (rRNA gene frequency). The samples showing rRNA/rRNA gene ratios of >1 were presumed to contain on average more active populations than those samples with ratios of <1 (i.e., they have relatively high number of ribosomes per bacterial cell). Most samples tested with the species- or source-specific markers had higher rRNA

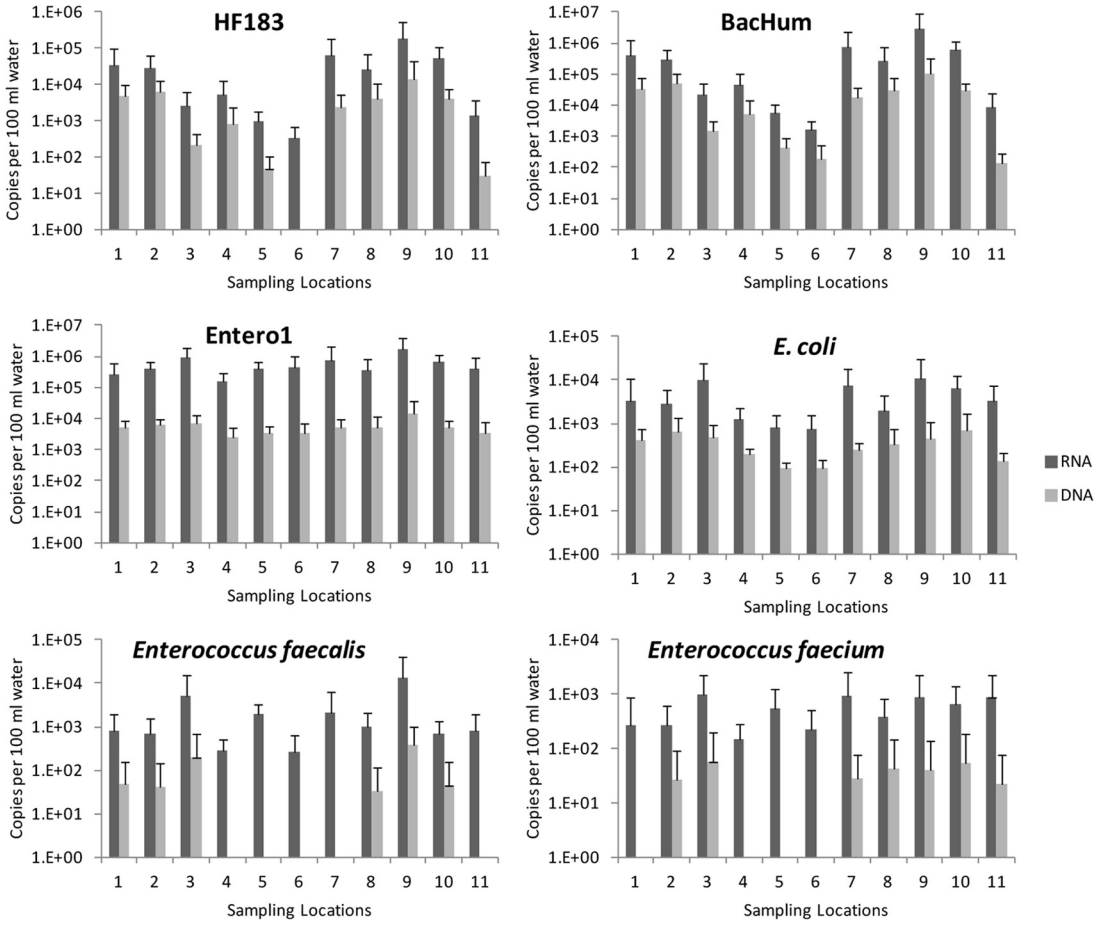

FIG 2 Spatial variation in levels of markers across study sites using RNA based RT-qPCR and DNA-based qPCR assays.

levels than rRNA gene. *Enterococcus* spp. exhibited the highest rRNA/rRNA gene ratios, suggesting that on average the targeted populations are indeed active.

Correlation between human-specific *Bacteroidales* and fecal indicator bacteria. The numbers of rRNA transcripts were used to study the correlation between human-specific *Bacteroidales* and conventional fecal indicators (enterococci and *E. coli*). The human-specific *Bacteroidales* markers showed moderate correlation with enterococci and *E. coli* RT-qPCR results, which are the most frequently used water quality indicators (Table 4). There were low to moderate correlations between human-specific *Bacteroidales* and fecal enterococci markers (Entero1, *E. faecalis*, and *E. faecium*) in the present study (Fig. 4). There was a weak correlation between the HF183 and Faecalis markers in the samples (correlation coefficient, $r^2 = 0.14$, $P < 0.001$), although Faecium1 exhibited slightly moderate correlation with the HF183 marker ($r^2 = 0.32$, $P < 0.001$). HumBac also showed similar correlation as HF183 with both Faecalis ($r^2 = 0.14$, $P < 0.001$) and Faecium1 ($r^2 = 0.30$, $P < 0.001$). Only few samples generated quantifiable qPCR results using Faecalis and Faecium1 assays, and therefore it was not possible to determine a relationship between these variables when using DNA as the template.

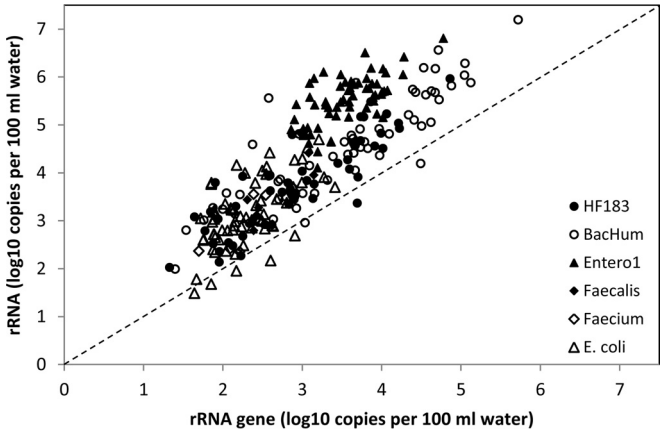

FIG 3 Relationship between rRNA and the rRNA gene for fecal markers as determined by RT-qPCR and qPCR. The dashed line represents the 1:1 rRNA/rRNA gene ratio to highlight the relative activity levels as established by Campbell et al. (30).

DISCUSSION

We demonstrated that the use of rRNA-based RT-qPCR assays increased the detection frequency of some fecal bacteria and host-specific markers in surface water samples from the Duck Creek Watershed, which is mainly impacted by CSOs and SSOs. The increased sensitivity with the rRNA based approach can be attributed to the high numbers of rRNA molecules present within the

TABLE 4 Pearson's correlation coefficients between human-specific *Bacteroidales* and fecal bacteria using RT-qPCR results ($n = 66$)

	Pearson's correlation coefficient (r)					
Bacteroidales and fecal bacteria	Human-specific *Bacteroidales* (HF183)	Human-specific *Bacteroidales* (BacHum)	General *Enterococcus* (Entero1)	*E. faecalis* (Faecalis)	*E. faecium* (Faecium1)	*E. coli* (EC23S857)
Human-specific *Bacteroidales* (HF183)	1	0.991	0.587	0.379	0571	0.597
Human-specific *Bacteroidales* (BacHum)		1	0.588	0.377	0.551	0.605
General *Enterococcus* (Entero1)			1	0.718	0.772	0.680
E. faecalis (Faecalis)				1	0.669	0.534
E. faecium (Faecium1)					1	0.693
E. coli (EC23S857)						1

bacterial cells. Since the abundance of rRNA molecules is the product of cell growth and its physiological state (18, 30), the rRNA-based approach should also provide information on the general metabolic status of the targeted bacterial populations. From a public health standpoint, this information may be important for a better understanding of the potential risks associated with the presence of fecal bacteria in water, assuming that the presence of active waterborne pathogens is likely to correlate better with the presence of active fecal bacterial indicators than with dead cells (which may be detected via extracellular DNA).

Since DNA can persist in metabolically inactive cells or in dead cells, and in the environment as extracellular or "naked" DNA (16), it has been suggested that this DNA fraction often contributes to the signal in qPCR-based MST methods (15). The frequency and significant contribution of "naked" DNA and DNA from dead cells is not determined in most environmental studies. However, it has been reported that the presence of nonreplicating DNA in marine water samples may range between 75 to 90% of total extractable DNA, some of which could be considered detrital in nature (i.e., associated with dead cells or absorbed to particulate matter) (42). More recently, Collins and Deming (43) reported that the amount of extracellular DNA exceeded the concentration of DNA within bacteria present in seawater. Although the amount of nonreplicating DNA in freshwater systems has been studied in less detail, dissolved DNA has been detected in river water samples (44). In addition, the occurrence of small bacteria (i.e., <1.0 μm) in river samples, some of which may be associated with nonactive bacteria, has been reported to be ca. 40% of the total bacterial community (45). These studies suggest that a significant fraction of bacterial signals detected via PCR and qPCR methods may be the result of organisms that are ecologically irrelevant to ecosystem function, although the role of this DNA pool in genetic exchange (i.e., via natural transformation) may be significant in some cases. The public health relevance, however, could be significant via the introduction of artifacts (false positives) that may not correlate with the levels of recent contamination events, the identification of predominant pollution sources and the fate and transport of fecal bacterial groups. In contrast, rRNA is actively degraded by cellular mechanisms under certain stress conditions (e.g., starvation) and deteriorates in the environment much faster than DNA (17). However, due to the dependence of rRNA levels on the physiological state of the cells, it is difficult to correlate rRNA numbers with actual bacterial cell densities. Therefore, the applicability of DNA-based methods to measure cell numbers is still relevant. From a regulatory perspective, we need additional studies to further evaluate the use of rRNA based methods for setting regulatory thresholds and developing risk assessment models.

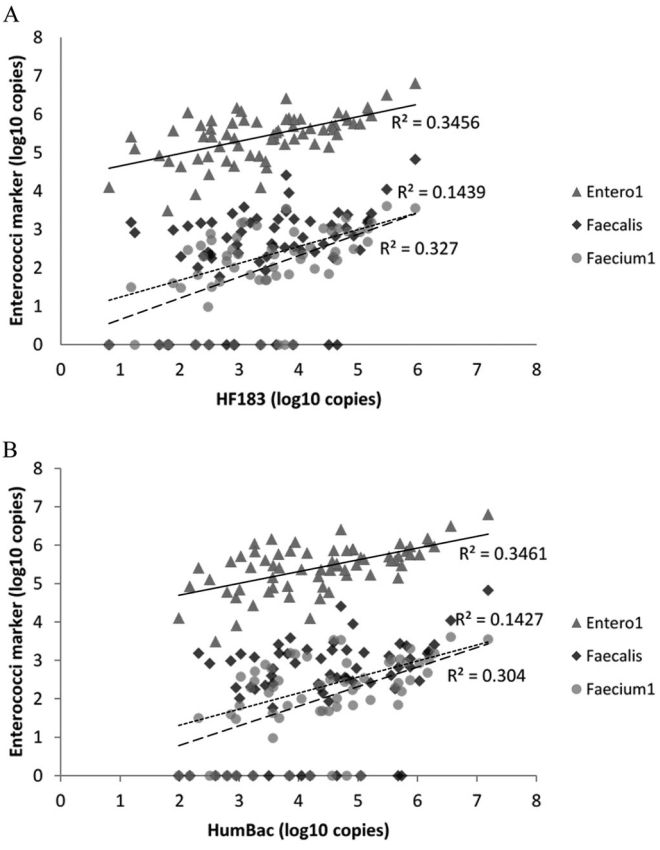

FIG 4 Correlation between human-specific *Bacteroidales* and fecal indicators using RT-qPCR. (A and B) HF183 versus enterococcus markers (A) and HumBac versus Enterococci markers (B) ($n = 66$). The linear regression lines between human-specific *Bacteroidales* and enterococci are represented as a solid line for Entero1, a dotted line for Faecalis, and dashed line for Faecium1.

The application of rRNA-based assays to detect active bacteria has proven useful in several studies (18–21). In a previous study, we demonstrated that the detection frequency of rRNA-based assays were in better agreement with the culture based detection of *E. coli* and enterococci in surface waters than that of rRNA gene-based assays, suggesting that rRNA signals were associated to active bacterial populations (27). Here, we applied rRNA based RT-qPCR assays for the identification and quantification of fecal bacteria and human-specific markers in an urban watershed impacted by fecal contamination. The greater sensitivity of rRNA-based assays over the rRNA gene-based detection obtained in our

results is also consistent with previous studies targeting RNA and DNA based on molecular markers for enumeration of bacteria in drinking water (46, 47), in coastal and environmental waters (30, 48), including waters impacted with fecal pollution (27), and in soil and sediments (26, 49, 50). The signals of human-specific *Bacteroidales*, *E. coli*, and *Enterococcus* spp. obtained by RT-qPCR analyses were compared to those determined using the conventional qPCR method (i.e., DNA-based signals), which is commonly utilized for determining the abundance of fecal bacteria in environmental waters. The RT-qPCR results provided higher quantitative values of fecal bacteria (up to three orders of magnitude; see Fig. 2) than the qPCR results obtained from the same sample. This is presumed to be based on the difference in metabolic activity of bacterial cells and their relative concentrations in the environment. These results demonstrate that targeting rRNA via RT-qPCR may provide up to 1,000-fold greater sensitivity over corresponding rRNA gene targets. Indeed, the higher sensitivity of RT-qPCR technique compared to qPCR have been reported for quantification of bacteria in natural and engineered systems such as human gut (28), marine sediments and grasslands (50, 51), environmental waters (27), and activated sludge processes (21, 52). Thus, in our study, the increased detection of fecal bacteria markers via rRNA-based signals suggests that RT-qPCR assays may be better suited for tracking and quantifying active fecal bacteria in environmental waters when the targeted groups are present at relatively low levels.

Human sewage contamination of surface waters is a serious concern for populations living in urban settings and for aquatic ecosystems in general. However, there are limited studies related to the identification of primary sources of fecal pollution in waterways using RNA as the target molecule. Fecal bacteria may be introduced into surface waters through numerous sources such as municipal waste from household sewage treatment systems, CSOs, SSOs, leaky septic tanks, and stormwater and urban runoff (2, 6, 7). In the present study, we quantified the extent of human fecal contamination in an urban watershed by an integrated analysis of host-specific markers and fecal bacteria. The assessment of study sites for human *Bacteroides* demonstrates that sewage sources of fecal pollution are major contributors to water quality deterioration within our study area. Because the Duck Creek Watershed does not encompass agriculture or farming runoff, and the creeks are too small for recreational use, the primary source of human-specific *Bacteroidales* can be attributed to the waste influx from nearby CSOs. Since tributaries in the Duck Creek Watershed flow into the Ohio River, these sources appear to represent a chronic and relatively constant source of human contamination. This has been suggested in a previous study (33); altogether, these data supports the use of *Bacteroidales* markers as effective indicators of human fecal contamination.

The microbial source tracking study undertaken in the Duck Creek Watershed substantiates that human fecal pollution is prevalent and is highly reflective of the architecture of the surrounding environment. On the other hand, based on the RT-qPCR data the low to moderate correlation between *Bacteroidales* and the fecal indicator assays highlights the ambiguity of enterococci and *E. coli* as robust fecal pollution surrogates (53, 54) (Table 4). For example, relatively high levels of *E. coli* were found in the samples with low to moderate levels of human-specific *Bacteroidales* markers. This may be due to variable persistence of different markers after release from their hosts (53–55). In addition, other sources of *E. coli*, besides human inputs, may be present in the watershed, which is consistent with other reports identifying nonhuman sources such as domesticated animals (56). These findings also illustrate the extent in which *E. coli* and enterococcus levels may be uncoupled to evidence of human sewage contamination in the urban environment. Furthermore, the moderate correlation of *E. faecalis* and *E. faecium* markers with the general enterococci (Entero1) suggests that other enterococci species may predominate in these waters. Since *E. faecalis* and *E. faecium* are considered the most abundant enterococcus species in human feces, these data highlight the need for further understanding the ecology of enterococci in natural settings.

Overall, we suggest that the results presented here should assist with future risk assessments for urban watersheds, particularly those affected by CSOs. However, future epidemiological studies must be conducted to determine whether RNA-based detection increases the correlation between the detection and activity levels of fecal indicators and illness associated with exposure to waterborne pathogens. Moreover, since MST methods may improve risk assessments of different pollution sources (57), it remains to be seen whether host-specific signals based on metabolically active cells add value to predictive risk models. In spite of these research gaps, our data suggest that RNA-based assays may be used in several applications, including in studies measuring the environmental fate and transport of fecal bacteria.

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This study has been subjected to the agency's administrative review and has been approved for external publication. Any opinions expressed in this study are those of the authors and do not necessarily reflect the views of the agency; therefore, no official endorsement should be inferred. Any mention of trade names or commercial products does not constitute endorsement or recommendation for use.

REFERENCES

1. **Santo Domingo JW, Bambic DG, Edge TA, Wuertz S.** 2007. Quo vadis source tracking? Towards a strategic framework for environmental monitoring of fecal pollution. Water Res **41:**3539–3552.
2. **Marsalek J, Rochfort Q.** 2004. Urban wet-weather flows: sources of fecal contamination impacting on recreational waters and threatening drinking-water sources. J Toxicol Environ Health Part A **67:**1765–1777. http://dx.doi.org/10.1080/15287390490492430.
3. **Samadpour M, Stewart J, Steingart K, Addy C, Louderback J, McGinn M, Ellington J, Newman T.** 2002. Laboratory investigation of an *Escherichia coli* O157: H7 outbreak associated with swimming in Battle Ground Lake, Vancouver, Washington. J Environ Health **64:**16–20.
4. **Mac Kenzie WR, Hoxie NJ, Proctor ME, Gradus MS, Blair KA, Peterson DE, Kazmierczak JJ, Addiss DG, Fox KR, Rose JB.** 1994. A massive outbreak in Milwaukee of Cryptosporidium infection transmitted through the public water supply. N Engl J Med **331:**161–167. http://dx.doi.org/10.1056/NEJM199407213310304.
5. **Gerba CP.** 2000. Assessment of enteric pathogen shedding by bathers during recreational activity and its impact on water quality. Quant Microbiol **2:**55–68. http://dx.doi.org/10.1023/A:1010000230103.
6. **Leenheer JA, Barber LB, Amy GL, Chapra SC.** 1995. Sewage contamination in the upper Mississippi River as measured by the fecal sterol,

coprostanol. Water Res **29:**1427–1436. http://dx.doi.org/10.1016/0043-1354(94)00304-P.
7. **Sauer EP, VandeWalle JL, Bootsma MJ, McLellan SL.** 2011. Detection of the human specific *Bacteroides* genetic marker provides evidence of widespread sewage contamination of stormwater in the urban environment. Water Res **45:**4081–4091. http://dx.doi.org/10.1016/j.watres.2011.04.049.
8. **Fong T-T, Lipp EK.** 2005. Enteric viruses of humans and animals in aquatic environments: health risks, detection, and potential water quality assessment tools. Microbiol Mol Biol Rev **69:**357–371. http://dx.doi.org/10.1128/MMBR.69.2.357-371.2005.
9. **Walsh CJ.** 2000. Urban impacts on the ecology of receiving waters: a framework for assessment, conservation and restoration. Hydrobiologia **431:**107–114. http://dx.doi.org/10.1023/A:1004029715627.
10. **Scott TM, Rose JB, Jenkins TM, Farrah SR, Lukasik J.** 2002. Microbial source tracking: current methodology and future directions. Appl Environ Microbiol **68:**5796–5803. http://dx.doi.org/10.1128/AEM.68.12.5796-5803.2002.
11. **Simpson JM, Santo Domingo JW, Reasoner DJ.** 2002. Microbial source tracking: state of the science. Environ Sci Technol **36:**5279–5288. http://dx.doi.org/10.1021/es026000b.
12. **Layton A, McKay L, Williams D, Garrett V, Gentry R, Sayler G.** 2006. Development of *Bacteroides* 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. Appl Environ Microbiol **72:**4214–4224. http://dx.doi.org/10.1128/AEM.01036-05.
13. **Ebentier DL, Hanley KT, Cao Y, Badgley BD, Boehm AB, Ervin JS, Goodwin KD, Gourmelon M, Griffith JF, Holden PA.** 2013. Evaluation of the repeatability and reproducibility of a suite of qPCR-based microbial source tracking methods. Water Res **47:**6839–6848. http://dx.doi.org/10.1016/j.watres.2013.01.060.
14. **Keer J, Birch L.** 2003. Molecular methods for the assessment of bacterial viability. J Microbiol Methods **53:**175–183. http://dx.doi.org/10.1016/S0167-7012(03)00025-3.
15. **Bae S, Wuertz S.** 2009. Discrimination of viable and dead fecal *Bacteroidales* bacteria by quantitative PCR with propidium monoazide. Appl Environ Microbiol **75:**2940–2944. http://dx.doi.org/10.1128/AEM.01333-08.
16. **Nielsen KM, Johnsen PJ, Bensasson D, Daffonchio D.** 2007. Release and persistence of extracellular DNA in the environment. Environ Biosafety Res **6:**37–53. http://dx.doi.org/10.1051/ebr:2007031.
17. **Deutscher MP.** 2003. Degradation of stable RNA in bacteria. J Biol Chem **278:**45041–45044. http://dx.doi.org/10.1074/jbc.R300031200.
18. **Poulsen LK, Ballard G, Stahl DA.** 1993. Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. Appl Environ Microbiol **59:**1354–1360.
19. **Dreier J, Störmer M, Kleesiek K.** 2004. Two novel real-time reverse transcriptase PCR assays for rapid detection of bacterial contamination in platelet concentrates. J Clin Microbiol **42:**4759–4764. http://dx.doi.org/10.1128/JCM.42.10.4759-4764.2004.
20. **Matsuda K, Tsuji H, Asahara T, Matsumoto K, Takada T, Nomoto K.** 2009. Establishment of an analytical system for the human fecal microbiota, based on reverse transcription-quantitative PCR targeting of multicopy rRNA molecules. Appl Environ Microbiol **75:**1961–1969. http://dx.doi.org/10.1128/AEM.01843-08.
21. **Ahn JH, Kwan T, Chandran K.** 2011. Comparison of partial and full nitrification processes applied for treating high-strength nitrogen wastewaters: microbial ecology through nitrous oxide production. Environ Sci Technol **45:**2734–2740. http://dx.doi.org/10.1021/es103534g.
22. **Pintar K, Fazil A, Pollari F, Charron D, Waltner-Toews D, McEwen S.** 2010. A risk assessment model to evaluate the role of fecal contamination in recreational water on the incidence of cryptosporidiosis at the community level in Ontario. Risk Anal **30:**49–64. http://dx.doi.org/10.1111/j.1539-6924.2009.01321.x.
23. **Haas CN, Rose JB, Gerba CP.** 1999. Quantitative microbial risk assessment. John Wiley & Sons, Inc, New York, NY.
24. **Walters SP, Field KG.** 2009. Survival and persistence of human and ruminant-specific faecal *Bacteroidales* in freshwater microcosms. Environ Microbiol **11:**1410–1421. http://dx.doi.org/10.1111/j.1462-2920.2009.01868.x.
25. **Bae S, Wuertz S.** 2009. Rapid decay of host-specific fecal *Bacteroidales* cells in seawater as measured by quantitative PCR with propidium monoazide. Water Res **43:**4850–4859. http://dx.doi.org/10.1016/j.watres.2009.06.053.
26. **Nogales B, Moore ER, Llobet-Brossa E, Rossello-Mora R, Amann R, Timmis KN.** 2001. Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. Appl Environ Microbiol **67:**1874–1884. http://dx.doi.org/10.1128/AEM.67.4.1874-1884.2001.
27. **Pitkänen T, Ryu H, Elk M, Hokajärvi A-M, Siponen S, Vepsäläinen A, Räsänen P, Santo Domingo JW.** 2013. Detection of fecal bacteria and source tracking identifiers in environmental waters using rRNA-based RT-qPCR and rDNA-based qPCR assays. Environ Sci Technol **47:**13611–13620. http://dx.doi.org/10.1021/es403489b.
28. **Matsuda K, Tsuji H, Asahara T, Takahashi T, Kubota H, Nagata S, Yamashiro Y, Nomoto K.** 2012. Sensitive quantification of *Clostridium difficile* cells by reverse transcription-quantitative PCR targeting rRNA molecules. Appl Environ Microbiol **78:**5111–5118. http://dx.doi.org/10.1128/AEM.07990-11.
29. **Pérez-Osorio AC, Williamson KS, Franklin MJ.** 2010. Heterogeneous rpoS and rhlR mRNA levels and 16S rRNA/rDNA (rRNA gene) ratios within *Pseudomonas aeruginosa* biofilms, sampled by laser capture microdissection. J Bacteriol **192:**2991–3000. http://dx.doi.org/10.1128/JB.01598-09.
30. **Campbell BJ, Yu L, Heidelberg JF, Kirchman DL.** 2011. Activity of abundant and rare bacteria in a coastal ocean. Proc Natl Acad Sci U S A **108:**12776–12781. http://dx.doi.org/10.1073/pnas.1101405108.
31. **Metropolitan Sewer District of Greater Cincinnati.** 2013. Integrated sustainable watershed management manual. Metropolitan Sewer District of Greater Cincinnati, Cincinnati, OH.
32. **U.S. Environmental Protection Agency.** 2000. Biological and water quality study of the Little Miami River Basin. Ohio EPA Technical Report, USEPA, Washington, DC.
33. **Kapoor V, Smith C, Santo Domingo JW, Lu T, Wendell D.** 2013. Correlative assessment of fecal indicators using human mitochondrial DNA as a direct marker. Environ Sci Technol **47:**10485–10493. http://dx.doi.org/10.1021/es4020458.
34. **Chern E, Siefring S, Paar J, Doolittle M, Haugland R.** 2011. Comparison of quantitative PCR assays for *Escherichia coli* targeting rRNA and single copy genes. Lett Appl Microbiol **52:**298–306. http://dx.doi.org/10.1111/j.1472-765X.2010.03001.x.
35. **Ludwig W, Schleifer K-H.** 2000. How quantitative is quantitative PCR with respect to cell counts? Syst Appl Microbiol **23:**556–562. http://dx.doi.org/10.1016/S0723-2020(00)80030-2.
36. **Santo Domingo JW, Siefring SC, Haugland RA.** 2003. Real-time PCR method to detect *Enterococcus faecalis* in water. Biotechnol Lett **25:**261–265. http://dx.doi.org/10.1023/A:1022303118122.
37. **Ryu H, Henson M, Elk M, Toledo-Hernandez C, Griffith J, Blackwood D, Noble R, Gourmelon M, Glassmeyer S, Santo Domingo JW.** 2013. Development of quantitative PCR assays targeting the 16S rRNA genes of *Enterococcus* spp. and their application to the identification of *Enterococcus* species in environmental samples. Appl Environ Microbiol **79:**196–204. http://dx.doi.org/10.1128/AEM.02802-12.
38. **Haugland RA, Varma M, Sivaganesan M, Kelty C, Peed L, Shanks OC.** 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. Syst Appl Microbiol **33:**348–357. http://dx.doi.org/10.1016/j.syapm.2010.06.001.
39. **Kildare BJ, Leutenegger CM, McSwain BS, Bambic DG, Rajal VB, Wuertz S.** 2007. 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal *Bacteroidales*: a Bayesian approach. Water Res **41:**3701–3715. http://dx.doi.org/10.1016/j.watres.2007.06.037.
40. **Suzuki MT, Taylor LT, DeLong EF.** 2000. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5′-nuclease assays. Appl Environ Microbiol **66:**4605–4614. http://dx.doi.org/10.1128/AEM.66.11.4605-4614.2000.
41. **McDonald JH.** 2009. Handbook of biological statistics, vol 2. Sparky House Publishing, Baltimore, MD.
42. **Winn CD, Karl DM.** 1986. Diel Nucleic acid synthesis and particulate DNA concentrations: conflicts with division rate estimates by DNA calculation. Limnol Oceanogr **31:**637–645. http://dx.doi.org/10.4319/lo.1986.31.3.0637.
43. **Collins RE, Deming JW.** 2011. Abundant dissolved genetic material in Artic Sea ice. 1. Extracellular DNA. Polar Biol **34:**1819–1830. http://dx.doi.org/10.1007/s00300-011-1041-y.
44. **DeFlaun MF, Paul JH, Jeffrey WH.** 1987. Distribution and molecular weight of dissolved DNA in subtropical estuarine and oceanic environments. Mar Ecol Prog Ser **38:**65–73. http://dx.doi.org/10.3354/meps038065.
45. **Paul JH, Carlson DJ.** 1984. Genetic material in the marine environment:

implication for bacterial DNA. Limnol Oceanogr **29:**1091–1096. http://dx.doi.org/10.4319/lo.1984.29.5.1091.
46. **Revetta RP, Matlib RS, Santo Domingo JW.** 2011. 16S rRNA gene sequence analysis of drinking water using RNA and DNA extracts as targets for clone library development. Curr Microbiol **63:**50–59. http://dx.doi.org/10.1007/s00284-011-9938-9.
47. **Keinänen-Toivola MM, Revetta RP, Santo Domingo JW.** 2006. Identification of active bacterial communities in a model drinking water biofilm system using 16S rRNA-based clone libraries. FEMS Microbiol Lett **257:**182–188. http://dx.doi.org/10.1111/j.1574-6968.2006.00167.x.
48. **Miskin IP, Farrimond P, Head IM.** 1999. Identification of novel bacterial lineages as active members of microbial populations in a freshwater sediment using a rapid RNA extraction procedure and RT-PCR. Microbiology **145:**1977–1987. http://dx.doi.org/10.1099/13500872-145-8-1977.
49. **Martinez RJ, Mills HJ, Story S, Sobecky PA.** 2006. Prokaryotic diversity and metabolically active microbial populations in sediments from an active mud volcano in the Gulf of Mexico. Environ Microbiol **8:**1783–1796. http://dx.doi.org/10.1111/j.1462-2920.2006.01063.x.
50. **Luna GM, Dell'Anno A, Pietrangeli B, Danovaro R.** 2012. A new molecular approach based on qPCR for the quantification of fecal bacteria in contaminated marine sediments. J Biotechnol **157:**446–453. http://dx.doi.org/10.1016/j.jbiotec.2011.07.033.
51. **Griffiths RI, Whiteley AS, O'Donnell AG, Bailey MJ.** 2000. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA-and rRNA-based microbial community composition. Appl Environ Microbiol **66:**5488–5491. http://dx.doi.org/10.1128/AEM.66.12.5488-5491.2000.
52. **Park H, Rosenthal A, Ramalingam K, Fillos J, Chandran K.** 2010. Linking community profiles, gene expression and N-removal in anammox bioreactors treating municipal anaerobic digestion reject water. Environ Sci Technol **44:**6110–6116. http://dx.doi.org/10.1021/es1002956.
53. **Ishii S, Ksoll WB, Hicks RE, Sadowsky MJ.** 2006. Presence and growth of naturalized *Escherichia coli* in temperate soils from Lake Superior watersheds. Appl Environ Microbiol **72:**612–621. http://dx.doi.org/10.1128/AEM.72.1.612-621.2006.
54. **Whitman RL, Shively DA, Pawlik H, Nevers MB, Byappanahalli MN.** 2003. Occurrence of *Escherichia coli* and enterococci in Cladophora (Chlorophyta) in nearshore water and beach sand of Lake Michigan. Appl Environ Microbiol **69:**4714–4719. http://dx.doi.org/10.1128/AEM.69.8.4714-4719.2003.
55. **Badgley BD, Thomas FI, Harwood VJ.** 2010. The effects of submerged aquatic vegetation on the persistence of environmental populations of *Enterococcus* spp. Environ Microbiol **12:**1271–1281. http://dx.doi.org/10.1111/j.1462-2920.2010.02169.x.
56. **Ram JL, Thompson B, Turner C, Nechvatal JM, Sheehan H, Bobrin J.** 2007. Identification of pets and raccoons as sources of bacterial contamination of urban storm sewers using a sequence-based bacterial source tracking method. Water Res **41:**3605–3614. http://dx.doi.org/10.1016/j.watres.2007.04.013.
57. **Soller JA, Schoen ME, Bartrand T, Ravenscroft JE, Ashbolt NJ.** 2010. Estimated human health risks from exposure to recreational waters impacted by human and non-human sources of faecal contamination. Water Res **44:**4674–4691. http://dx.doi.org/10.1016/j.watres.2010.06.049.