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Discrimination of Viable and Dead Fecal *Bacteroidales* Bacteria by Quantitative PCR with Propidium Monoazide[▽]

Sungwoo Bae and Stefan Wuertz*

Department of Civil and Environmental Engineering, 2001 EU III, University of California, Davis, One Shields Avenue, Davis, California 95616

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Propidium monoazide (PMA) was optimized to discriminate between viable and dead *Bacteroides fragilis* cells and extracellular DNA at different concentrations of solids using quantitative PCR. Conditions of 100 μM PMA and a 10-min light exposure also excluded DNA from heat-treated cells of nonculturable *Bacteroidales* in human feces and wastewater influent and effluent.

The aim of microbial source tracking (MST) methods is to identify, and in some cases quantify, the dominant sources of fecal contamination in surface waters and groundwater (2, 16). One of the most promising library- and cultivation-independent approaches utilizes fecal *Bacteroidales* bacteria and quantitative PCR (qPCR) assays to measure gene copies of host-specific genetic markers for 16S rRNA (4, 5, 10, 14). Currently, molecular assays do not directly discriminate between viable and nonviable cells since DNA of both live and dead cells and extracellular DNA can be amplified. Consequently, source tracking data based on detection of genetic markers by PCR cannot distinguish between recent and past contamination events since DNA of selected pathogens can persist after cell death for more than 3 weeks (6). Hence, it would be preferable to detect host-specific markers in viable cells of *Bacteroidales* bacteria, which are strictly anaerobic microorganisms and unlikely to survive in water.

Previous studies have suggested the use of intercalating DNA-binding chemicals combined with PCR to inhibit PCR amplification of DNA derived from dead cells (8, 9, 11, 15). For example, ethidium monoazide (EMA) has been investigated as a means of reducing the PCR signal from DNA originating from dead bacterial cells (7, 15, 19). However, the use of EMA prior to DNA extraction has been found to result in a significant loss of the genomic DNA of viable cells in the case of *Escherichia coli* 0157:H7, *Campylobacter jejuni*, and *Listeria monocytogenes* (3, 7). Recently propidium monoazide (PMA) has been proposed as a more selective agent, penetrating only dead bacterial cells but not viable cells with intact membranes (8). EMA/PMA in combination with PCR or qPCR has been applied to identify viable food-borne pathogens in a simple matrix (3, 7, 8, 11), and possible restrictions in the use of PMA in environmental samples were reported (9, 19). Yet the feasibility of applying PMA in environmental samples or MST studies using fecal *Bacteroidales* bacteria has not been systematically studied. Any meaningful application of EMA or PMA in stool or natural water samples must consider potential interferences due to particulate matter present in the environmental matrix. Similarly, procedures for the concentration of large volumes of water samples to simultaneously monitor pathogens and MST identifiers can lower the limit of detection (4, 12), but they concentrate solids or other inhibitors of quantitative PCR (qPCR) as well, which might interfere in the covalent binding of PMA to DNA.

The objectives of this study were, therefore, the following: (i) to evaluate the applicability of PMA-qPCR methods to detect culturable *Bacteroides fragilis*, (ii) to determine the feasibility of PMA-qPCR analysis for environmental samples containing different concentrations of solids, and (iii) to validate the utility of the PMA-qPCR method for the detection of fecal *Bacteroidales* bacteria in defined live and heat-treated mixtures of human feces and in wastewater treatment plant influent and effluent.

Pure cultures of *Bacteroides fragilis* (ATCC 25285) were grown in thioglycolate broth (Anaerobe System, Morgan Hill, CA) under anaerobic conditions in GasPak anaerobic jars (Becton Dickinson Microbiology Systems, Cockeysville, MD). The solids were obtained by hollow-fiber ultrafiltration as described previously (12, 13). Ultrasonification and heat sterilization in an autoclave were used for removing attached bacteria or DNA from solids and inactivating residual DNA. Finally, the solids were resuspended with 1× phosphate-buffered saline (PBS) solution to 100 mg liter^{-1} or 1,000 mg liter^{-1} of suspended solids. The concentration of total suspended solids (TSS) was measured using method 2450 C (1). Next, 1 ml of broth medium containing 2×10^9 viable or 2×10^8 heat-treated *B. fragilis* cells, which had been exposed at 80°C for 20 min, was spiked into 1× PBS buffer solutions containing 0 mg liter^{-1}, 100 mg liter^{-1}, or 1,000 mg liter^{-1} of TSS. Before the cells were spiked, 1 ml of *Bacteroides fragilis* cell suspension was enumerated with the Live/Dead BacLight bacterial viability kit (Molecular Probes Inc., Eugene, OR) using a hemacytometer and an Axioskop 2 Plus epifluorescence microscope (Zeiss, Thornwood, NY) equipped with two filter sets (fluorescein isothiocyanate and Texas Red). The inoculated samples were incubated under anaerobic conditions in GasPak anaerobic jars (Becton Dickinson Microbiology Systems, Cockeysville, MD) for 4 h at 20°C to allow sufficient time for the cells to sorb to solids.

* Corresponding author. Mailing address: Department of Civil and Environmental Engineering, 2001 EU III, University of California, Davis, One Shields Avenue, Davis, CA 95616. Phone: (530) 754-6407. Fax: (530) 752-7872. E-mail: swuertz@ucdavis.edu.

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A fresh human fecal specimen was obtained from a healthy adult. Two grams of feces was suspended in 25 ml 1× PBS. The fecal suspension was diluted 1:10 and 1:100 in a 1× PBS solution, and aliquots were subjected to heat treatment at 80°C for 20 min. The heat-treated fecal portions were mixed with fresh diluted samples (1:10 and 1:100 dilutions) in defined ratios, with fresh feces representing 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% of the total, respectively. Effluent and influent water samples were collected in sterile 2-liter bottles from the University of California, Davis, wastewater treatment plant. The effluent samples were concentrated to approximately 200 ml by hollow-fiber ultrafiltration (12).

PMA (Biotium Inc., Hayward, CA) was prepared, stored, and used as described in previous studies (8, 9), but PMA concentrations and light exposure time were varied to determine the optimal condition of PMA-qPCR; the PMA concentrations were 2 μM, 6 μM, 20 μM, and 100 μM. Light exposure times were 1 min, 5 min, 10 min, and 20 min. Genomic DNA was extracted using the FastDNA spin kit for soil (Biomedicals, Solon, OH). Cell lysis was achieved by bead beating using a bead mill Minibread beater (Biospec Products Inc., Bartlesville, OK) at 2,400 rpm for 20 s. Otherwise, DNA extraction was performed according to the manufacturer's instructions. TaqMan probe and primer assays targeting the rRNA genes of all fecal *Bacteroidales* bacteria (BacUni-UCD) and mixed human-specific *Bacteroidales* bacteria (BacHum-UCD), developed by Kildare et al. (4), were used to detect and quantify fecal *Bacteroidales* bacteria present in fecal and (waste)water samples.

We explored the ability of PMA-qPCR to discriminate between viable and heat-killed cells at different solids concentrations using *Bacteroides fragilis* cultures (Fig. 1). PMA did not influence the PCR amplification of DNA derived from viable cells when no solids were present (TSS = 0 mg liter^{-1}) (Fig. 1A). The level of PMA concentration slightly affected the mean cycle threshold differences (ΔC_T) of viable cells at higher solids concentrations (TSS = 100 and 1,000 mg liter^{-1}) (Fig. 1C and E). The signal reductions in the amplification of heat-killed cells were a function of both the PMA concentration and exposure time (Fig. 1B, D, and F). Lower solids concentrations did not inhibit the efficacy of discrimination from heat-killed cells. However, solids at 1,000 mg liter^{-1} affected the amplification of DNA derived from heat-killed cells. Higher solids concentrations affected the suppression of PCR amplification from heat-treated cells by interfering with the cross-linking of PMA. In agreement with previous reports, the number of viable *Bacteroides fragilis* cells was underestimated in our study when EMA-treated and untreated samples containing only viable cells were compared because mean ΔC_T values were as high as 10 (data not shown). In contrast to EMA, PMA seems to not penetrate live cells, since higher selectivity of PMA is most probably associated with the higher charge of the molecule (8).

A factorial three-way analysis of variance including the PMA concentration, exposure time, and TSS concentration was performed to determine the interferences of solids and the optimal PMA-qPCR condition in the differentiation of viable cells from dead cells (Table 1). The mean ΔC_T of viable cells in the PMA experiments was slightly influenced by the PMA concentration ($P = 0.05$) in the absence of solids (TSS = 0 mg liter^{-1}), but the effect was biologically insignificant (mean $\Delta C_T = 0.004$). The PMA concentration had a significant effect on ΔC_T values for both viable and dead cells in the presence of higher solids concentrations (TSS = 100 and 1,000 mg liter^{-1}), as shown in Table 1. However, the effect of exposure time in PMA treatment was insignificant at a TSS concentration of 1,000 mg liter^{-1} ($P > 0.4$). The solids concentration caused significantly different ΔC_T values for viable and dead cells in the PMA treatments ($P < 0.001$) as determined by factorial three-way analysis. The greatest differences in the mean ΔC_T values between viable and dead cells were seen at 100 μM of PMA and with a 10-min exposure time, as determined by Tukey's comparison test, for TSS concentrations of 100 mg liter^{-1} and 1,000 mg liter^{-1}. Ideally, shorter light exposure and a lower concentration of dye can minimize the penetration of live cells. However, these conditions were not compatible with sufficient inhibition of amplification of DNA from dead cells for PMA treatment.

The factorial design study revealed that the mean ΔC_T of *B. fragilis* cells was a function of both the concentration and the exposure time. An optimal set of conditions consisted of applying PMA at 100 μM for a 10-min exposure time. By comparison, in the case of *E. coli* 0157:H7, a PMA concentration of 50 μM was sufficient for avoiding a potential DNA loss from viable cells, but a longer incubation time (15 min) for the PMA cross-linking step and a higher PMA concentration (240 μM) resulted in a moderate DNA loss (8). Yet a factorial design was not employed in that study.

PMA-qPCR was applied to defined mixtures of viable and heat-treated cells prepared from fresh human stool samples. PMA-qPCR resulted in selective exclusion of DNA from heat-treated stool, and there was no effect on PCR amplification from fresh feces. Gene copy numbers for human-specific *Bacteroidales* detected by BacHum-UCD were directly related to the percentage of fresh feces present in 1:10 (higher TSS content) and 1:100 (lower TSS content) dilutions of fecal material, with R^2 values of 0.98 and 0.88, respectively (Fig. 2A and B). PMA also suppressed the signals from heat-treated feces, with a reduction in the number of gene copies detected of 2.5 logs in 1:10 dilutions of fecal samples and 3.2 logs in 1:100 dilutions of fecal samples, respectively. The greater variability in the data at the lower feces concentration and hence lower target numbers for PMA-qPCR would suggest that there may be some penetration of PMA into undamaged cells, an effect that was not noticeable when there were many cells present. A close look at Fig. 2B reveals that the relationship is not perfectly represented by a linear fit, hence the lower R^2 value. However, the standard deviation of C_T values for different percentages of fresh fecal material ranged from 0.52 to 1.17, an acceptable value which would not significantly affect the interpretation of the linear relationship.

Influent and effluent water samples from the University of California, Davis, wastewater treatment plant were analyzed with BacUni-UCD and BacHum-UCD *Bacteroidales* molecular markers (4) to evaluate the PMA-qPCR method in environmental samples. In the influent samples, the concentration of viable and dead *Bacteroidales* cells was 7.6×10^6 gene copies/ml, compared to 2.3×10^6 gene copies/ml for viable *Bacteroidales* bacteria alone, as determined by PMA-qPCR

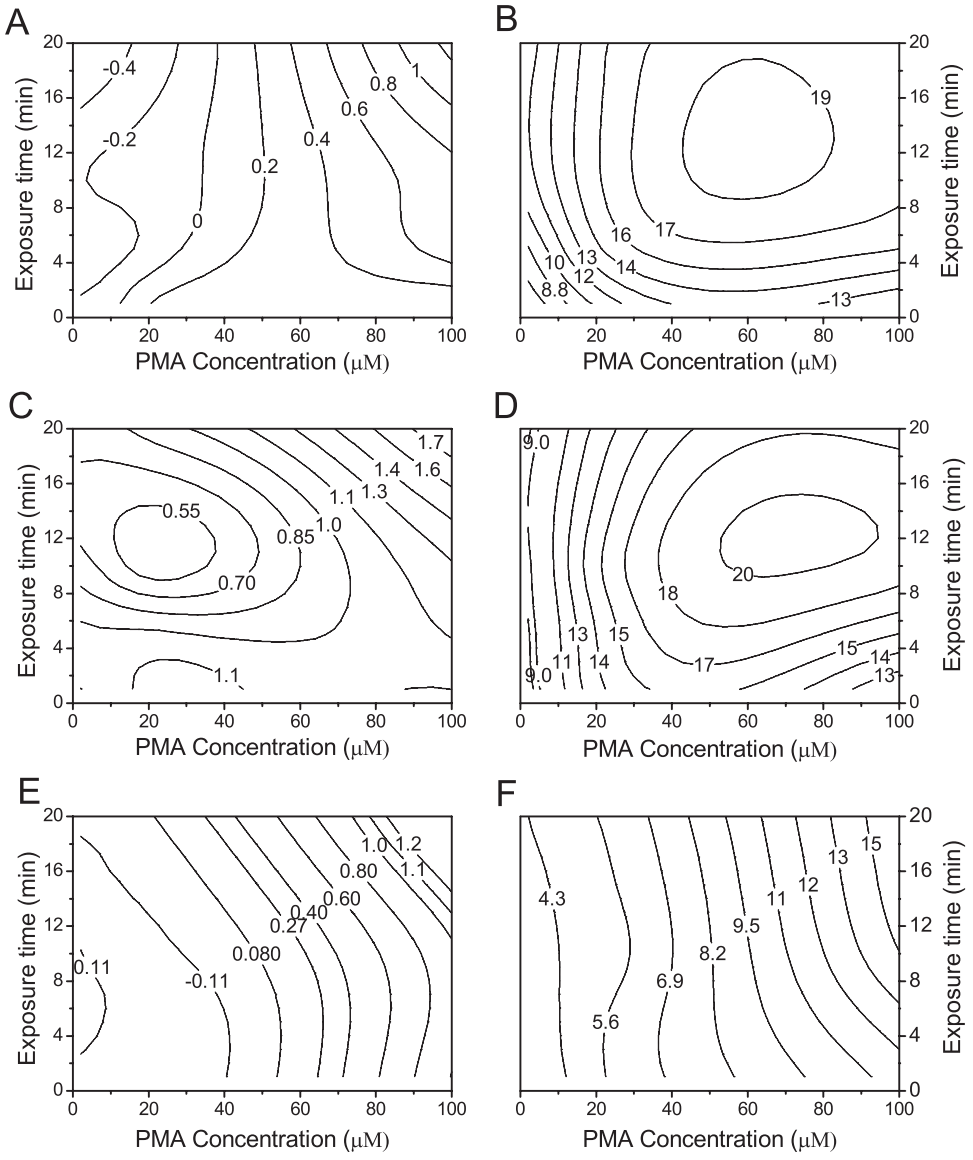

FIG. 1. Effect of PMA on amplification of BacUni-UCD universal marker in viable and dead *Bacteroides fragilis* cells with different concentrations of solids. The contour lines represented ΔC_T values and were generated by the Origin Pro 8 software program. The mean cycle threshold differences (ΔC_T) were calculated by subtracting C_T values obtained without PMA treatment from C_T values obtained with PMA treatment. (A and B) ΔC_T for viable cells (A) or dead cells (B) in the absence of added solids. (C and D) ΔC_T for viable cells (C) or dead cells (D) at a solids concentration of 100 mg liter^{-1}. (E and F) ΔC_T for viable cells (E) or dead cells (F) at a solids concentration of 1,000 mg liter^{-1}.

(Fig. 3). There was a significant difference between results with PMA treatment and those with no treatment for both gene copies/ml and the C_T number ($P < 0.01$), yet this result nonetheless indicates that many *Bacteroidales* cells detected in the influent were viable. In general, the residence time in a sewer network is less than 24 h, and even though *Bacteroidales* bacteria are anaerobic organisms, they appear to be somewhat protected in the wastewater collection system, perhaps due to the formation of oxygen gradients in solids. A 2.5-log reduction of human-specific *Bacteroidales* DNA from influent samples to effluent samples was observed, but human-specific *Bacteroidales* DNA was still present at 10^4 gene copies ml^{-1} in effluent samples after UV treatment when no PMA treatment was applied (Fig. 3). Similarly, the concentration of the universal *Bacteroidales* gene marker BacUni-UCD was 10^4 gene copies ml^{-1} in effluent after a 3-log reduction during wastewater treatment (data not shown). As determined by PMA-qPCR, 30% of *Bacteroidales* cells containing the human-specific molecular marker BacHum-UCD were still viable in influent samples, whereas only human-specific *Bacteroidales* DNA but no viable cells were detected in effluent samples (Fig. 3). This result can be explained by the highly oxygenated environment in the aeration tank of the wastewater treatment plant and a typical cell residence time in the activated sludge process of 3 to 15 days (18), followed by UV treatment. The total coliform count in the effluent was less than 2.2 most probable number/100 ml. Consequently, the absence of viable *Bacteroidales* cells in the effluent would be expected.

TABLE 1. Statistical analysis for differences (ΔC_T) between nontreatment and PMA treatment for experiments where *Bacteroides fragilis* was spiked[a]

TSS concn (mg liter^{-1})	Factor	Effect of factor with PMA treatment									
		Viable *Bacteroides fragilis*					Dead *Bacteroides fragilis*				
		Mean ΔC_T	SD	*df*[b]	*F*[c]	*P* value[d]	Mean ΔC_T	SD	*df*[b]	*F*[c]	*P* value[d]
0	Conc (μM)	0.003	0.792	3	2.77	0.050	12.29	3.78	3	44.04	0.001
	Time (min)	0.003	0.926	3	0.06	0.980	12.29	3.21	3	23.79	0.001
	Interaction			9	1.92	0.087			9	1.49	0.209
100	Conc (μM)	0.91	0.935	3	11.44	0.001	11.92	4.76	3	15.05	0.001
	Time (min)	0.91	0.961	3	10.09	0.001	11.92	5.96	3	1.36	0.274
	Interaction			9	1.80	0.111			9	0.97	0.484
1,000	Conc (μM)	0.22	0.702	3	12.10	0.001	6.49	3.05	3	48.90	0.001
	Time (min)	0.22	0.963	3	0.86	0.472	6.49	6.49	3	0.88	0.464
	Interaction			9	0.60	0.784			9	1.13	0.373

[a] A general linear model, which is the foundation for the *t* test, analysis of variance, regression analysis, and multivariate methods including factor analysis, was used to analyze the effects of the PMA concentration, exposure time, and interaction at different concentrations of solids.
[b] Degrees of freedom.
[c] The statistic used to test the hypothesis that the variance of a factor is equal to zero.
[d] The *P* value is the smallest level of significance that would lead to rejection of the null hypothesis with the given data. We chose the common α-level of 0.05 to determine an acceptable level of significance.

A combination of large-volume water filtration and qPCR assays to simultaneously detect pathogens and MST molecular markers in water has been successful in lowering sample limits of detection and in improving detection of target pathogens present at low concentrations (4, 12, 16). However, the viability of target bacteria must be addressed to ensure broad application of nucleic-acid-based methods to environmental monitoring. A recent study reported that a limitation regarding PMA treatment was observed in samples with higher solid contents such as sediments and some environmental samples during denaturing gradient gel electrophoresis analysis of viable cells (9). Wagner et al. (19) suggested that the particles of diluted fermentor sludge could inhibit the cross-linking step when the chemicals should be light activated, since the radiation probably cannot penetrate the liquid. Similarly, the presence of eukaryotic DNA in stool samples and that of various inhibitors in matrices with a high solid content, like storm water, can hamper sensitivity in distinguishing viable cells in the application of PMA-qPCR. In our hands, PMA-qPCR was successful at relatively high solids concentrations (TSS = 1,000 mg liter^{-1}) only after optimization.

In a recent watershed study, MST data using qualitative (presence/absence) markers of bovine-specific (CF128) and human-specific (HF183) *Bacteroidales* genotypes were more reliable on high-flow samples with higher concentrations of culturable fecal indicators and could not discriminate precisely between livestock- and human-derived feces in the larger land use pattern (17). The reason for this outcome may have been the use of nonquantitative MST data and/or the presence of free DNA or extracellular DNA, which can persist in marine water, freshwater, and sediment for up to 55 days, 21 days, or 40 days, respectively (6). Significant concentrations of dissolved DNA have been found in marine water, freshwater, and sediments at concentrations ranging from 1 μg to 80 μg liter^{-1} (6). It is also possible that a case of positive detection of a *Bacteroidales* genetic marker in a 2.5-μl creek sample using direct PCR without DNA extraction (5) could have been caused by the presence of free DNA and not by a recent fecal

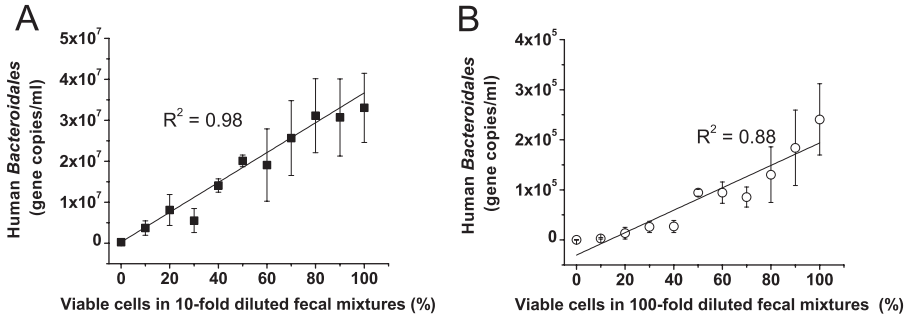

FIG. 2. Effect of PMA treatments at 100 μM and a 10-min light exposure on PCR amplification in human fecal samples containing defined ratios of fresh and heat-treated feces. The black squares (■) denote a 1:10 dilution of fecal material, and the white circles (○) denote a 1:100 dilution of fecal material. The error bars represent standard deviations for three samples. (A) Least-squares linear regression between the concentration of BacHum-UCD marker and defined ratios of 10-fold-diluted fresh and heat-treated feces. (B) Least-squares linear regression between the concentration of the BacHum-UCD marker and defined ratios of 100-fold-diluted fresh and heat-treated feces.

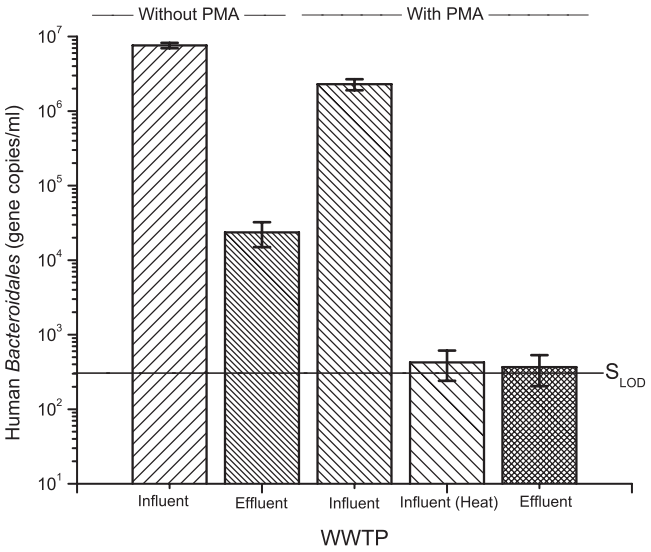

FIG. 3. Comparison of *Bacteroidales* gene copies determined using the BacHum-UCD assay in the presence and absence of PMA. Wastewater treatment influent, heat-treated influent, and effluent after UV disinfection were analyzed by quantitative PCR. The effluent was concentrated from 2 liters to 200 ml by hollow-fiber ultrafiltration (12), and DNA was extracted from the concentrated effluent and the influent samples. S_{LOD}, sample limit of detection.

contamination event. PMA combined with qPCR assays for host-specific *Bacteroidales* genetic markers may be used in the future to simultaneously identify the sources of different fecal loadings and estimate recent and past fecal contamination by both measuring molecular markers in viable cells and separately quantifying their gene copies in dead cells and in extracellular DNA. This rapid and simple method should greatly advance the utility of *Bacteroidales* assays in microbial source tracking. Moreover, it could be an extremely useful method to determine survival of host-specific *Bacteroidales* cells or waterborne pathogens and their DNA, to estimate recent fecal contamination in water, and to inform remedial action plans.

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