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Differential decay of human faecal *Bacteroides* in marine and freshwater

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Summary

Genetic markers from *Bacteroides* and other faecal bacteria are being tested for inclusion in regulations to quantify aquatic faecal contamination and estimate public health risk. For the method to be used quantitatively across environments, persistence and decay of markers must be understood. We measured concentrations of contaminant molecular markers targeting *Enterococcus* and *Bacteroides* spp. in marine and freshwater microcosms spiked with human sewage and exposed to either sunlight or dark treatments. We used Bayesian statistics with a delayed Chick–Watson model to estimate kinetic parameters for target decay. DNA- and RNA-based targets decayed at approximately the same rate. Molecular markers persisted (could be detected) longer in marine water. Sunlight increased the decay rates of cultured indicators more than those of molecular markers; sunlight also limited persistence of molecular markers. Within each treatment, *Bacteroides* markers had similar decay profiles, but some *Bacteroides* markers significantly differed in decay rates. The role of extracellular DNA in persistence appeared unimportant in the microcosms. Because conditions were controlled, microcosms allowed the effects of specific environmental variables on marker persistence and decay to be measured. While marker decay profiles in more complex environments would be expected to vary from those observed here, the differences we measured suggest that water matrix is an important factor affecting quantitative source tracking and microbial risk assessment applications.

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Introduction

Water-borne human faecal contaminants harbour many pathogens, pose serious health risks to humans (Haile *et al*., 1999), cause economic losses and may disrupt aquatic ecosystems (van der Putten *et al*., 2007; Stewart *et al*., 2008). In recent years, researchers have developed specific methods of faecal contaminant detection and identification using *Bacteroides* targeted polymerase chain reaction (PCR), and sensitive and quantitative methods using quantitative real-time PCR (qPCR) (Dick and Field, 2004; Layton *et al*., 2006; Kildare *et al*., 2007; Shanks *et al*., 2008; 2009; Converse *et al*., 2009). Compared with culturing methods, qPCR offers advantages for estimating bacterial and viral concentrations, both because of its speed (same day results) and because it can detect difficult-to-cultivate organisms. Application of these methods could therefore reduce uncertainty in faecal source identification and associated risk assessment. Nevertheless, in order to interpret quantitative molecular data for risk assessment, it is necessary to understand marker decay in environmental matrices (Wade *et al*., 2006; Field and Samadpour, 2007; Santo Domingo *et al*., 2007).

Decay of culturable faecal indicator bacteria (FIB) in natural water sources has been studied and reviewed extensively over the last 50 years (Gainey and Lord, 1952; Chamberlin and Mitchell, 1978; McCambridge and McMeekin, 1981; Sinton *et al*., 1999; Noble *et al*., 2004; Boehm *et al*., 2009). Far less is known about how genetic markers from indicators and pathogens behave in the environment (Leach *et al*., 2007), both within, and when released from the cell, although the fate of qPCR targets under environmental conditions is receiving increased attention. Temperature, particulate concentration, particulate size, predation, salinity and sunlight all affect marker decay (Kreader, 1998; Okabe and Shimazu, 2007; Bell *et al*., 2009; Walters and Field, 2009; Walters *et al*., 2009; Bae and Wuertz, 2009a; Dick *et al*., 2010; Klein *et al*., 2011; Schulz and Childers, 2011). Although sunlight contributes most to the deactivation of culturable bacteria (Davies-Colley *et al*., 1994), observations on the effects of

sunlight on the decay of molecular markers have been mixed (Walters and Field, 2009; Walters *et al*., 2009; Bae and Wuertz, 2009a). Studies that have compared indicator and pathogen decay provide insights into indicator/pathogen relationships (Walters *et al*., 2009; Klein *et al*., 2011). An indicator that correlates highly with an infectious pathogen or group of infectious pathogens through the environment is a more accurate predictor of human health risk (USEPA, 2005).

Because DNA can persist in metabolically inactive or dead cells, and in the environment after cell lysis, its detection does not directly indicate viability of environmental bacteria (Masters *et al*., 1994; Deere *et al*., 1996; Keer and Birch, 2003). Two methods have been used to estimate the extent of detection of extracellular DNA. The first, propidium monoazide treatment before PCR, causes only membrane enclosed DNA to be detected (Nocker *et al*., 2007; Bae and Wuertz, 2009b). When this method was used in decay studies, authors reported that extracellular DNA accounted for much of the signal in the environment (Bae and Wuertz, 2009a). Alternatively, significant presence of ribosomal RNA (rRNA) suggests viable or dead cells with intact cell membranes, because rRNA is actively degraded by cellular mechanisms under conditions of starvation or cold shock (Chen and Deutscher, 2005) and deteriorates faster than DNA when liberated from the cell (Novitsky, 1986). Furthermore, cellular ribosome content is correlated with growth and metabolic rate (Kemp *et al*., 1993; Kerkhof and Ward, 1993; Poulsen *et al*., 1993; Wawer *et al*., 1997) and is used as a proxy for cell activity in microbial ecology studies using fluorescent *in situ* hybridization and community sequence analysis (Mills *et al*., 2004; 2005; Gentile *et al*., 2006; Akob *et al*., 2007; Gaidos *et al*., 2011). Quantification of rRNA with reverse transcriptase quantitative PCR (RT-qPCR) has previously been used to suggest presence of membrane enclosed cells in human faecal *Bacteroides* decay studies (Walters and Field, 2009).

Microcosms are often used to study environmental processes, because they allow the effects of isolated environmental variables to be studied under highly controlled conditions (e.g. see Kreader, 1998; Okabe and Shimazu, 2007; Bell *et al*., 2009; Walters and Field, 2009; Walters *et al*., 2009; Bae and Wuertz, 2009a; Dick *et al*., 2010; Klein *et al*., 2011; Schulz and Childers, 2011). We investigated the decay of culturable enterococci and molecular markers from *Bacteroides* and *Enterococcus* spp., in marine and freshwater microcosms in sunlight and dark treatments. To address the correlation of molecular and culturable indicators with pathogens, we monitored decay of *Campylobacter* molecular markers with qPCR using a published assay (Lund *et al*., 2004). We extracted nucleic acids from microcosms over a period of 21 days. A delayed Chick–Watson (DCW) model, previously used for pathogen decay (Sivaganesan *et al*., 2003) and *Nitrosomonas europaea* disinfection (Wahman *et al*., 2009), was used to estimate lag times (Z) and decay rates (k) of both rRNA genes (rDNA) and rRNA. We compared marker decay using Z and k, and also compared marker persistence, the length of time that markers remained above the limit of quantification (LOQ). Independent of DCW model analysis, we also calculated human-specific to general *Bacteroidales* ratios, as these ratios have been suggested as a means to estimate contributions from human sources.

Results

Decay curves fit a DCW model

Assay performance characteristics obtained from standard curves are reported in Table 1. Regression lines obtained from measured versus predicted values using each model suggested that the data set as a whole fit a DCW model, with a lag phase followed by pseudo-first-order decay, better than standard Chick–Watson (CW) ($R^2_{DCW} = 0.92$, $R^2_{CW} = 0.76$). Therefore all comparisons between molecular data sets were made using DCW unless otherwise stated.

Molecular targets persisted longer in marine water than in freshwater

We monitored the DNA decay of seven *Bacteroides* markers, one enterococci marker and one *Campylobacter* spp. marker (Table 1). Despite decay curve variations dependent on assay or light treatment, there was a highly significant difference in the length of the lag phase (Z) between water types: on average it was 3.1 days ($p < 0.005$) longer in marine water than in freshwater. However, post-lag decay (k) was faster in marine water ($p < 0.05$). The lack of post-lag data points limited model estimate comparisons with the light marine treatment. On average, DNA markers persisted above the method LOQ 2.5 days longer in marine water compared with freshwater (Table 2, $p < 0.01$).

Sunlight had a small effect on the decay of rDNA and rRNA markers

Differences in marker decay attributable to light were less pronounced (Fig. 1). ANOVA of rDNA decay estimates of Z and k resulted in no significant differences between light treatments in either matrix, but in freshwater, GenBac3 and BuniF2 markers exhibited a significantly higher decay of rDNA markers in light compared with dark treatments when comparing estimate credible intervals (Table 3 and Table S1). Light had a similar effect on decay rates and

Table 1. Assay performance characteristics.

Assay	Target	Reagent	Fluor. threshold	Cal. equation	Cal. method	Amplification efficiency (%)	Range of quantification	%CV across range of quant.	Reference
BsteriF1	Human *Bacteroides* 16S	FastMix	0.02	$y = 38.08 - 3.30x$	M	100.4	10^1–10^5	1.13	Haugland *et al.* (2010)
BuniF2	Human *Bacteroides* 16S	FastMix	0.02	$y = 38.07 - 3.42x$	M	97.9	10^1–10^5	1.08	
GenBac3	*Bacteroidales* 16S	Universal	0.02	$y = 39.26 - 3.41x$	M	97.1	10^1–10^5	1.30	Seifring *et al.* (2008)
HF183 Taq	Human *Bacteroides* 16S	FastMix	0.02	$y = 37.48 - 3.39x$	M	98.6	10^1–10^5	1.11	Seurinck *et al.* (2005)
HF134/303R	Human *Bacteroides* 16S	SYBR	0.8	$y = 38.00 - 3.26x$	M	101.3	10^2–10^5	1.24	Bernhard and Field (2000); this study
HF183/303R	Human *Bacteroides* 16S	SYBR	0.8	$y = 39.01 - 3.35x$	M	99.4	10^2–10^5	1.48	Bernhard and Field (2000); this study
HumM2	*Bacteroides*-like functional gene	Universal	0.02	$y = 39.26 - 3.42x$	M	98.1	10^1–10^5	1.14	Shanks *et al.* (2009)
Entero1 Multiplex	*Enterococci* and internal amplification control	Universal	0.02	$y = 37.98 - 3.38x$	M	98.8	10^1–10^5	1.13	Ludwig and Schleifer (2000)
Camp	*Campylobacter* spp.	FastMix	0.02	$y = 37.44 - 3.34x$	M	99.7	10^1–10^5	1.24	Lund *et al.* (2004)
groEL	*C. jejuni gro*EL gene	Universal	0.02	$y = 40.90 - 3.41x$	S	98.1	10^1–10^5	1.82	Love *et al.* (2006)
mapA	*C. jejuni map*A gene	SYBR	0.8	$y = 41.66 - 3.97x$	S	89.3	10^1–10^5	1.73	Price *et al.* (2006)
pAW 109	pAW 109 RNA (ABI)	FastMix	0.02	$y = 38.59 - 3.45x$	S	97.6	10^1–10^5	0.80	Cook *et al.* (2004)
PAO	*Pseudomonas aeruginosa* strain PAO-T7	SYBR	0.8	$y = 36.75 - 3.57x$	M	95.3	10^2–10^5	1.54	This study; Hoang *et al.* (2000)

qPCR reactions used either TaqMan Fast Universal PCR Master Mix ('FastMix'), TaqMan Universal PCR Master Mix ('Universal') or SYBR Green® in-house Master Mix ('SYBR'). Calibration equations were obtained using either the master calibration curve method (M) or a single standard curve (S). Amplification efficiency is equal to $10^{(1/-\text{slope})}/2$. Range of quantification (ROQ) refers to the range in which the logarithm of copies per reaction maintains a linear relationship with Ct. Assay limits of quantification (LOQs) are defined as the lowest target concentration within the ROQ. Method LOQs are the lower limits of quantification per 100 ml of water sample and are defined as the assay LOQ times the processing dilution factor (50 for DNA and 213.3 for RNA). Per cent coefficient of variation (%CV) indicates the average precision in measuring standard concentrations across the ROQ.

Table 2. Persistence of BsteriF1, BuniF2 and GenBac3 rDNA and rRNA within the method LOQs.

	rDNA		rRNA	
	Fresh	Marine	Fresh	Marine
BsteriF1				
Light	5	7	5	7
Dark	6	7	6	9
BuniF2				
Light	5	7	5	7
Dark	6	7	6	7
GenBac3				
Light	7	11	7	9
Dark	9	20	11	13

Values represent the number of days post-seeding markers were detected at concentrations above the assay LOQ on all three filters for that day. Sampling did not occur on days 8, 10, 12 and 14–19.

Table 3. Comparison of decay rates (k_{DCW}) and their lower (LCI) and upper (UCI) 95% credible intervals between BsteriF1, BuniF2 and GenBac3 rDNA and rRNA in dark fresh (DF) and light fresh (LF) treatments.

	DF			LF		
	k_{DCW}	LCI	UCI	k_{DCW}	LCI	UCI
BsteriF1						
rDNA	−0.89	−1.00	−0.78	−1.03	−1.15	−0.95
rRNA	−0.78	−0.93	−0.63	−1.04	−1.36	−0.80
BuniF2						
rDNA	−1.07	−1.26	−0.88	−1.35	−1.45	−1.26
rRNA	−0.97	−1.18	−0.75	−1.35	−1.67	−1.11
GenBac3						
rDNA	−0.72	−0.79	−0.64	−0.88	−0.94	−0.82
rRNA	−0.49	−0.57	−0.43	−0.79	−0.90	−0.66

Data sets that fit DCW with R^2 values > 0.90 are shown. Estimates with credible intervals that overlap are not significantly different at the 95% significance level. Data are extracted from the complete data set (Table S1).

persistence of rRNA markers (Fig. 1). BsteriF1, BuniF2, Entero1 and GenBac3 all showed significantly higher decay rates of rRNA in light versus dark freshwater treatments (Table 3 and Table S1). Both rDNA and rRNA markers displayed biphasic decay in dark treatments (Fig. 1) and had shorter persistence times in light (Table 2 and Table S1, paired *t*-test $p < 0.05$).

In freshwater microcosms we also estimated decay rates of culturable enterococci and compared them with rDNA and rRNA decay rates, using a CW decay model. In this case, the CW model fit the data better than DCW (Table S1), providing a more accurate comparison of decay rates. In dark treatments, analysis of covariance indicated that decay rates for rRNA and culturable cells were not significantly different from that of DNA ($p > 0.2$,

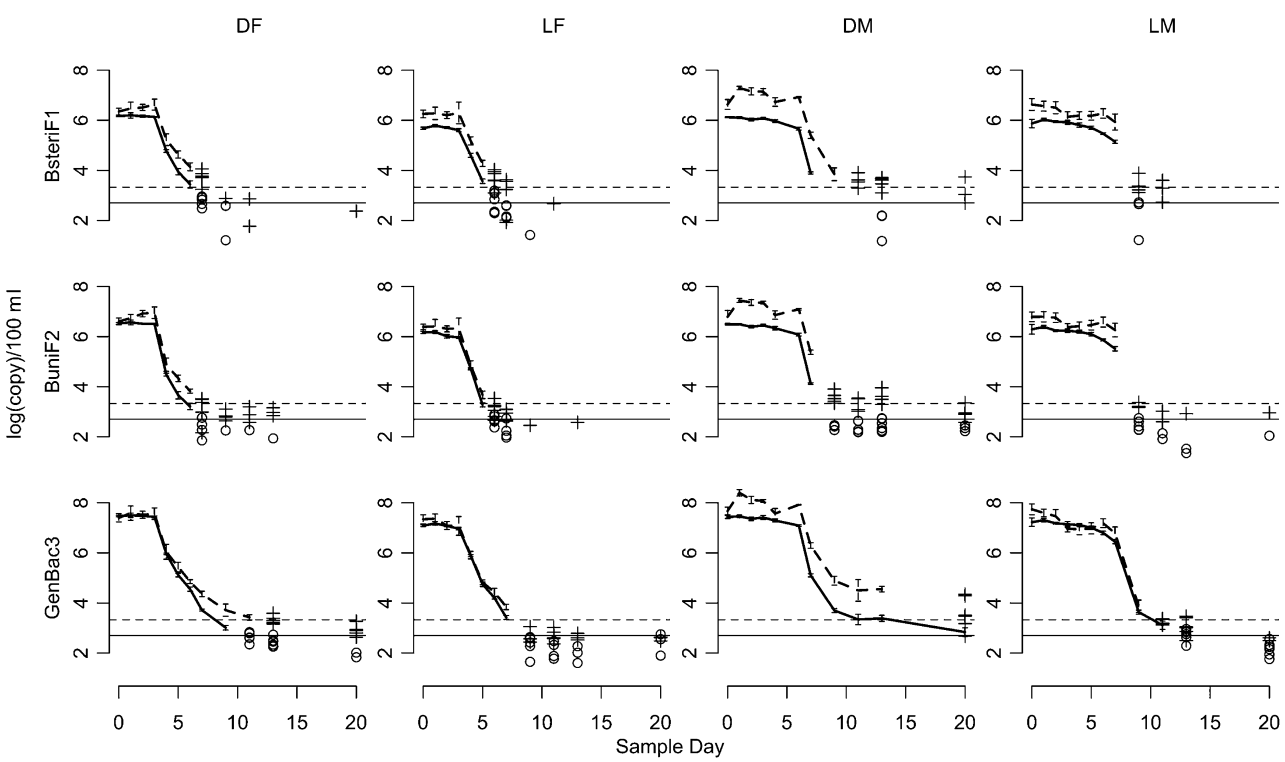

Fig. 1. Decay profiles of BsteriF1, BuniF2 and GenBac3 rDNA and rRNA. Thick solid and dashed lines represent rDNA and rRNA marker concentrations within the method LOQ respectively. Circles and crosses represent rDNA and rRNA marker detects below the method LOQ respectively. Horizontal solid and dashed lines represent method LOQs for rDNA and rRNA analysis respectively. Error bars represent the standard deviation of six Ct values. DF, dark fresh water. LF, light fresh water. DM, dark marine water. LM, light marine water.

Figure S1), suggesting that a similar set of factors determined their decay rates. Under light conditions, DNA decay rates were lower than that of culturable enterococci ($P < 0.05$), but were not significantly different than RNA decay rates ($P = 0.053$) (Table 4) when using a standard cut-off for significance.

Decay rate dependence on molecular target

Weighted one-way ANOVA was used to analyse the difference in lag phase *Z* and decay rate *k* between targeted clades, independent of treatment. The Entero1 marker experienced a shorter lag phase than BsteriF1, BuniF2, GenBac and HF 183 Taq ($p < 0.05$) and slower decay than Buni and HF 183 Taq ($p < 0.05$) (Table 5).

Despite the close genetic relatedness of groups targeted by *Bacteroides* assays, we observed differences in post-lag decay in some treatments, particularly in freshwater (Table 3 and Table S1). These differences led to changes in the ratios between markers. Independent of model estimates, initial and final human-specific : GenBac3 ratios were significantly different in most cases (Table 6). Results of parametric and non-parametric analyses agreed: the human specific : GenBac3 ratios increased in those human-specific assays with lower decay rates (BsteriF1 and HumM2), while BuniF2 had a higher decay rate and the BuniF2:GenBac3 ratio declined.

Camp marker increased in concentration in marine water

We used a published qPCR assay (Camp) to monitor *Campylobacter* decay (Lund *et al*., 2004). While Camp markers remained below quantification limits in freshwater treatments throughout the study, concentrations of both DNA and RNA Camp markers increased in marine water, reaching maximums at about day 6, followed by rapid decay, and showing another period of increase before day 20 (Fig. S2). We retested microcosm DNAs that tested positive for Camp with *Campylobacter jejuni*-specific assays mapA (Price *et al*., 2006) and groEL (Love *et al*., 2006), and both were below assay limits of detection. A search for database sequences matching Camp primers and probe found no exact matches outside the genus *Campylobacter* (NCBI nr/nt). To identify the cells that grew in the marine microcosms and tested positive with the Camp assay, we cloned ~ 1300 bp fragments produced when DNA or cDNA from day 6 dark marine treatment was amplified with the Camp reverse primer paired with a

Table 4. Results of ANCOVA analysis on decay rates of rDNA, rRNA and cultured cells of enterococci [most probable number (MPN)] in freshwater.

	Dark			Light		
Method	Estimate	R^2	*p*-value	Estimate	R^2	*p*-value
rDNA	–0.306	0.918		–0.152	0.926	
rRNA	–0.234	0.943	0.241	–0.542	0.955	0.053
MPN	–0.275	0.873	0.606	–1.012	0.996	0.007

p-values represent the significance in decay rate differences when compared with DNA markers.

Table 5. Significance matrix of *p*-values from weighted ANOVA testing estimate differences between assays.

			Z		
	BsteriF1	BuniF2	Entero1	GenBac3	HF 183 Taq
BsteriF1		0.87	**0.02**	0.92	0.80
BuniF2	0.87		**0.02**	0.82	0.66
Entero1	**0.02**	**0.02**		**0.04**	**0.01**
GenBac3	0.92	0.82	**0.04**		0.93
HF 183 Taq	0.80	0.66	**0.01**	0.93	
			k		
	BsteriF1	BuniF2	Entero1	GenBac3	HF 183 Taq
BsteriF1		0.42	0.08	0.97	0.10
BuniF2	0.42		**0.02**	0.39	0.30
Entero1	0.08	**0.02**		0.08	**0.01**
GenBac3	0.97	0.39	0.08		0.09
HF 183 Taq	0.10	0.30	**0.01**	0.09	

Estimates from all treatments were used.

Table 6. Comparison of initial and final human-specific : GenBac3 marker ratios.

	Initial ratio	Final ratio	*p*-value
BsteriF1			
DF	0.05	0.08	0.068
LF	0.04	0.066	0.032
DM	0.049	0.063	0.011
LM	0.047	0.05	0.393
BuniF2			
DF	0.114	0.046	0.001
LF	0.122	0.034	0.002
DM	0.113	0.106	0.102
LM	0.125	0.119	0.427
HF 183 Taq			
DF	0.074	0.035	< 0.001
LF	0.05	0.027	0.038
DM	0.076	0.075	0.158
LM	0.069	0.082	0.546
HumM2			
DF	0.005	0.01	0.015
LF	0.008	0.01	0.032
DM	0.005	0.007	0.031
LM	0.006	0.008	0.136

Initial ratios are from day 0. Final ratios are from the last sample day. Concentrations of human-specific markers were quantifiable. Means of filter triplicates were used to calculate ratios. *p*-values were obtained assuming unequal variances in one-tailed *t*-tests and represent the significance level when comparing initial and final ratios with each marker within each treatment.

universal rRNA primer [27F (Weisburg *et al*., 1991)]. We screened the resulting 27F/CampR2 clones with the original Camp qPCR assay. Sequences from clones testing positive with the Camp assay revealed that almost all clones clustered near or within *Kordiimonadales*, *Sphingomonadales*, or elsewhere within the *Alphaproteobacteria* (Fig. S3). There were no *Campylobacter* sequences. Almost all of the sequence fragments contained mismatches corresponding to the 3′ end of CampF2 or CampP2 oligo sequences (Fig. S4). An unclassified clade of *Alphaproteobacteria* designated as microcosm clone group E, comprising nine DNA clones and 20 cDNA clones, represented a common actively growing group of bacteria unintentionally identified by the Camp assay.

Matrix effects on sample processing and qPCR

Matrix-specific compounds did not significantly affect DNA recovery or qPCR amplification efficiency. Variability in estimated DNA marker concentrations within the assay limits of quantification between triplicate microcosm water samples was very low (CV = 1.2%). Estimated DNA marker concentrations for all assays on day 0 did not significantly differ ($P > 0.3$) between marine and freshwater microcosm samples. Internal amplification control (IAC) (plasmid) and an engineered strain of *Pseudomonas aeruginosa* strain PAO-T7 (PAO) (genomic) controls, used to indicate the presence of carry-over compounds, did not have significantly different Ct values ($P > 0.5$) between marine and freshwater microcosm DNA extracts.

In contrast, estimates of recovery of the RNA processing control pAW 109 RNA (ABI) through DNase treatment and reverse transcription steps were significantly higher in marine water ($p < 0.005$) compared with freshwater RNA. Variability in RNA processing from marine water limited our ability to draw conclusions from these data sets.

Discussion

DCW model

The DCW model describes experimental data when a shoulder (lag phase) occurs before pseudo-first-order decay. In disinfection studies, the lag phase is usually interpreted as representing a survival period before the disinfectant has built up enough to cause cell death (e.g. see Wahman *et al*., 2009). Our decay curves suggest a period of survival of *Bacteroides* cells, followed by cell death and lysis.

Effects of water matrix on decay of molecular markers

The decay profiles in this study were similar to those of *Bacteroides fragilis* (Okabe and Shimazu, 2007). Although the exact conditions of that experiment were unclear, we showed that similar decay profiles extend to complex and genotypically diverse *Bacteroides* communities encountered during contamination events. Walters and Field inoculated human faeces into freshwater microcosms and observed a similar 4-day lag phase in *Bacteroides* decay (Walters and Field, 2009). Highly similar freshwater decay profiles in all three studies imply that biological and chemical differences between the freshwater sources did not greatly affect *Bacteroides* decay.

We suspect that the differences between lag-phase durations in marine and fresh microcosms are largely due to differences in predator populations. Microcosm conditions before addition of sewage likely reflect ‘bottom-up’ regulatory conditions that are the result of relatively low nutrient availability. In these conditions, bacterial populations remain relatively low and thus restrict growth of predator populations. However, with the influx of sewage ($\sim 10^{10}$ bacteria), predatory organisms are no longer limited by prey scarcity and their populations expand, resulting in a transition to a ‘top-down’ regulated, or predator-controlled, bacterial community. The end of lag phase and the beginning of post-lag decay in this study may mark rapid increase in predator abundance and bacterial mortality. Thus, the length of lag phase would correspond to the time required for predator population

growth to a level that results in rapid bacterial decay. In this framework, several factors could explain why the lag phase was shorter in freshwater microcosms. The freshwater used in microcosms could contain a more abundant and/or faster growing predator population, resulting in an earlier onset of rapid decay. Alternatively, increased salinity may delay predator growth in marine water (Okabe and Shimazu, 2007; Schulz and Childers, 2011).

Viral lysis causes the decline of abundant bacterial hosts that are susceptible to viral infection. Seeded *Bacteroidales*, at concentrations between 10^7 and 10^8 per litre, present abundant targets for viruses, qualifying as 'winners' according to the 'kill the winner' phenomenon (Thingstad and Lignell, 1997; Thingstad, 2000). However, in each treatment, post lag decay began simultaneously for genetically distant *Bacteroides*, *Enterococci*, and the range of *Alphaproteobacteria* detected by the Camp assay, suggesting a mechanism of decay less discriminatory than viral lysis. This also suggests that, at least under these conditions, factors causing decay did not greatly differ according to bacterial growth rate (growing *Alphaproteobacteria* versus stationary *Bacteroides*).

Several lines of evidence demonstrated that matrix-specific compounds did not drastically affect DNA recovery or qPCR amplification efficiency. A matrix effect would be expected to increase the variability between extraction and/or qPCR replicates, yet the coefficient of variation was very low between triplicate microcosm water samples. Had DNA extraction efficiency or qPCR amplification kinetics been dependent on matrix, we would expect to see a difference in the starting concentrations on day 0 between marine and freshwater microcosms; such a difference was not seen. In addition, we used both plasmid (IAC) and cellular (PAO) spiked process controls, and neither demonstrated matrix effects.

Since RNA processing includes the additional steps of DNase treatment and reverse transcription, we included the RNA processing control pAW 109 RNA after extraction, but before the DNase step, to estimate potential target loss or matrix effects from this point forward. We made use of a published qPCR assay, with primer and probe sequences, complementary to sequences on pAW 109 RNA, that was originally used to estimate recovery of RNA extracted from human serum samples (Cook *et al*., 2004). Higher estimates of RNA recovery in marine water compared with freshwater suggested that matrix effects altered the processing efficiency of DNase treatment and/or reverse transcription.

Effects of light on cultured and molecular indicators

The detrimental effects of UV and visible light on the culturability of indicator bacteria are well documented (Davies and Evison, 1991; Davies-Colley *et al*., 1994; Sinton *et al*., 1999; 2002; Boehm, 2007), but observations on the effects of light on molecular markers targeting *Bacteroides* and enterococci vary (Walters and Field, 2009; Walters *et al*., 2009; Bae and Wuertz, 2009a). Here, sunlight decreased the length of time *Bacteroides* markers persisted, presumably by killing cells and terminating DNA maintenance mechanisms, or by damaging DNA templates directly or via photosensitized intermediates (reviewed in Ravanat *et al*., 2001). Similar decay profiles of enterococci by culture and molecular markers suggest that a similar set of factors cause decay of enterococci genomic DNA, rRNA and culturable cells under dark conditions in freshwater. Furthermore, the tight linear correlation (Pearson's $r = 0.933$, $p = 0.002$) between culturable enterococci and enterococci DNA markers in dark fresh treatments suggests that the detectable Entero1 markers were not only enclosed by a cell membrane, but also contained within culturable cells. However, under light conditions culturable cells decayed much faster than both ribosomal DNA and RNA, strengthening previous assertions that light has a much greater impact on the culturability of cells than on the persistence of DNA and RNA targets.

In dark treatments, we observed a biphasic decay pattern, not only with general *Bacteroidales* as previously observed (Dick *et al*., 2010), but also with BsteriF1, BuniF2, HF 183 Taq and Entero1 markers. Biphasic decay may have also occurred to some degree in light treatments, as suggested by some detection below assay LOQs. The onset of decay of these markers was simultaneous and independent of marker concentration. Biphasic decay, or tailing, is typical of heterogeneous populations, owing to genetic variability among organisms targeted by these assays, or to differences in growth phase of contaminant bacteria upon introduction into water (Hellweger *et al*., 2009).

Previous studies have reached opposing conclusions about the effects of sunlight on molecular markers. In our microcosms and in the environment, UVA radiation is the predominant form of UV light. UVA damages cellular components mostly via photosensitized intermediates versus direct DNA absorption (Sinton *et al*., 2002). UVA therefore has a greater effect on culturability than on direct modification of nucleic acid and deterioration of the primer/probe target region. It is possible that exposure to a higher level of UVB light could result in a higher decay by direct DNA damage than estimated in this study, for both culturable indicators and molecular markers.

Role of extracellular DNA detection

It has been suggested that extracellular DNA often contributes to the signal in environmental qPCR methods (Bae and Wuertz, 2009b). However, the persistence of

RNA outside of the cell is limited to very short time periods (Novitsky, 1986). Therefore, similar decay rates between DNA and RNA in this study suggests that we detected mostly DNA and RNA targets enclosed within a cell membrane; if we had detected large amounts of extracellular DNA, we would expect to see RNA concentration fall below DNA. This is a desirable result for the purpose of estimation of risk, as survival of infectious pathogens is likely to be better correlated with indicator cells than with extracellular DNA. The difference between our results and previous studies may be due to sample concentration methods; our filtration methods may be less likely to capture extracellular DNA. However, extracellular DNA has been detected for up to 18 days using similar filtration methods (Walters *et al*., 2009). Abiotic features of samples, such as increased concentrations of particles that associate with DNA, could facilitate capture of extracellular DNA. Furthermore, bactericidal mechanisms that attack the cell membrane specifically (e.g. viral lysis and membrane oxidation) may be more likely to produce detectable extracellular DNA.

Decay profiles differ by bacterial target group

Bacteria targeted by Entero1 experienced an earlier onset of decay, but slower decay, than targeted *Bacteroides*. Protozoan predators have been shown to prefer prey based on prey outer membrane characteristics (Gonzalez *et al*., 1990; Tarao *et al*., 2009), size (Simek and Chrzanowski, 1992), morphology (Justice *et al*., 2008) and perhaps growth rate (Pernthaler, 2005). Gram-positive *Actinobacteria* are notably resistant to grazing (Pernthaler *et al*., 2001) due to surface layer characteristics (Tarao *et al*., 2009). Furthermore, *Enterococcus faecalis* mutants lacking genes involved in capsular polysaccharide biosynthesis displayed enhanced susceptibility to phagocytosis, suggesting a defensive role for capsule formation in some *Enterococcus* spp. (Hancock and Gilmore, 2002). Alternatively, higher susceptibility to abiotic factors such as reactive oxygen could also explain the earlier onset of decay of bacteria targeted by Entero1. Indicators and pathogens with cellular similarities, such as cell wall composition, morphology and resistance to the effects of reactive oxygen, may show higher correlation in environmental waters. While we have not determined the exact causes, the observed differences in decay between Entero1 and *Bacteroides* markers support separate interpretations of data obtained using these tools.

Silkie and Nelson have suggested using the ratio between host-specific (e.g. BsteriF1, BuniF2) and general *Bacteroidales* markers (e.g. GenBac3) as a means to estimate the proportion of contamination from host sources (Silkie and Nelson, 2009). Similar decay of host-specific and general *Bacteroidales* markers supports the utility of this method and others (Wang *et al*., 2010), because decay rates would remain out of the equation. However, using the DCW model, we found that host-specific and general *Bacteroidales* markers can have different decay rates. Accordingly, we found that host-specific and general *Bacteroidales* marker ratios changed over time. The ability to reveal different decay rates between diverse lineages of *Bacteroides* may have been aided by the analytical precision offered by qPCR and an appropriate decay model, in contrast to previous studies that used clone library analysis (Schulz and Childers, 2011). In addition, background levels of general *Bacteroidales* markers due to chronic contamination and/or extended persistence in sediments (Dick *et al*., 2010) could lead to underestimates of source contributions using a ratio approach. Another untested assumption inherent in such approaches is that general *Bacteroidales* markers from different sources decay at the same rate. Information may be gained from ratio approaches, but differential decay should be considered, and targeting markers that decay similarly to general *Bacteroidales* (e.g. BsteriF1) may be more accurate in such approaches.

Both study conditions and results are relevant to Pacific Northwest estuaries that experience chronic contamination from terrestrial sources. Microcosm temperature (12.8°C) reflects that of Tillamook Bay, OR (11.6 $\pm$ 2.0°C) and one of its major tributaries, Wilson River (13.5 $\pm$ 3.7°C), during the summer months (NOAA, 2011; USGS, 2011) when aquatic faecal concentrations are highest (Shanks *et al*., 2006). In a molecular source tracking study in this area, researchers observed that the probability of detecting *Bacteroides* human-specific markers, HF183 and HF134, in the saline bay was double the probability of detecting the same markers in rivers (Shanks *et al*., 2006), despite the rivers being the source of contaminants to the bay. Our results suggest that the higher occurrence of markers in bay samples could have been due to an accumulation of slowly decaying *Bacteroides* cells.

Camp assay non-specificity

The *Campylobacter* assay we used is reported to target pathogenic and non-pathogenic *Campylobacter* species, and was previously tested by others for specificity using 63 *Campylobacter* strains and 14 non-*Campylobacter* species (Lund *et al*., 2004). Cloning and sequencing of partial 16S genes from microcosm organisms detected by the Camp assay revealed that it detected mostly *Alphaproteobacteria*, whose assay target regions only partially match Camp primer and probe sequences, in this study. The change from the original protocol (Lund *et al*., 2004) to an ABI platform using the Fast Universal PCR Master Mix may have caused a decrease in specificity

and allowed the assay to detect a diverse range of bacteria in a separate lineage of *Proteobacteria*. Alternatively, previous testing may have been insufficient to reveal the assay's non-specificity in genotypically complex environmental samples. In future environmental studies using modified qPCR protocols, specificity should be confirmed independently.

Limitations of the study

One important caveat to any microcosm study results is provided by previous experiments with nutrient enriched microcosms, which have resulted in rapidly changing community structures (Schäfer *et al*., 2001; Allers *et al*., 2007). These changes may reflect a response to confinement, and thus may not necessarily predict the types and rates of community change in the native setting.

We expect that the decay profiles observed in this study may not perfectly predict those found in the environment, due to microcosm set-up or to variables not tested in this study, such as sediments, turbidity, salinity, temperature and bactivore concentration. For example, we would expect higher decay rates had we incubated microcosm at higher temperatures. Results from microcosm studies are sometimes criticized because their controlled conditions do not correspond to complex natural ecosystems (Downing *et al*., 2008). However, because of their lack of complexity, microcosms allow critical factors influencing persistence to be identified (Downing *et al*., 2008). Here we showed that both light and water type influenced genetic marker persistence and rate of decay.

Another limitation of this study is its lack of replication. However, although microcosms were not replicated, microcosms of the same water type (e.g. the two freshwater microcosms versus the two marine water microcosms), or light type, displayed similar decay, increasing the confidence in the observed decay profiles. It will be important in future studies to measure the coefficients of variance among microcosm replicates.

Concluding remarks

Molecular methods, such as qPCR, have potential to surpass culture-based methods in terms of specificity and sample-to-answer turn around time. However, basic questions concerning viability of cells and extracellular persistence of targets under environmental conditions hamper development of standards for their application and data interpretation.

In the presence of sunlight, our study showed that markers may be bound within cells that are non-culturable, but enclosed in a cell membrane. We found that at least in some conditions, nearly all Entero1 molecular markers were contained within culturable enterococci cells, removing the persistence of extracellular DNA from the equation and simplifying interpretation.

Despite their phylogenetic relationship, not all *Bacteroides* markers decayed at the same rate. It is unclear whether this variability of survival traits at the level of species or phylotype will affect the utility of these tools in the environment. Small differences observed in this study may be absent or amplified under other conditions not tested. Currently, differential decay of molecular markers under varying environmental conditions is not considered when choosing appropriate molecular monitoring tools or interpreting the data. Divergent decay profiles of *Bacteroides* markers between marine and freshwater, however, suggest that separate sets of standards may be appropriate for *Bacteroides* qPCR when applied to these sample types.

Characterizing the effects of environmental variables on molecular markers of faecal contamination is the biggest challenge to molecular source tracking and risk assessment. The increasing number of environmental variables that can dramatically change quantitative interpretations of environmental molecular marker data warrants further investigation. Additional pathogen and illness correlation studies are needed to determine the predictive power of faecal molecular markers across all aquatic environments.

Experimental procedures

Sewage and water samples

Raw sewage influent was obtained from the Corvallis sewage treatment plant. Marine water was collected from just under the surface three miles off the central Oregon coast. Freshwater was collected from Canyon Creek, about 30 miles east of Sweet Home, OR. The land use for Canyon Creek catchment is exclusively timber. Water samples had no visible turbidity or sediment.

Microcosms

Two marine water and two freshwater 15 l microcosms, consisting of plastic buckets, were inoculated with 150 ml of raw sewage influent and partially submerged in constant 12.8°C outdoor water baths at the Salmon Disease Lab (Oregon State University, Corvallis, OR), as described previously (Walters and Field, 2009). Continuous airflow was supplied to the bottom of each microcosm with sterile 4 mm tubing and a fish-tank pump to prevent stratification. A four-way valve was used to ensure equal airflow among tanks.

Light and dark treatments

To test the effects of ambient light on marker decay, one marine and one freshwater microcosm were individually

covered with an opaque lid and the water bath in which they were submerged was also covered with an opaque water bath lid. The water bath lid was left open for the marine and freshwater light treatment microcosms, and individual clear acrylic lids were used to prevent rain accumulation and evaporation. This allowed 92.5% of the natural light to penetrate the microcosms but prevented evaporation and dilution by rainwater (Walters and Field, 2009). Mean global horizontal solar radiation in August in this location is about 6.5 kWh/m^{-2}/day. (http://solardata.uoregon.edu).

Sampling and culturing

Five 50 ml samples were taken from each microcosm daily at 7:30 AM and stored at 4°C until processing. Microcosms were sampled daily for 1 week, every other day for the following week, and once the next week for a total of 12 sampling time points. From freshwater microcosms, two 50 ml samples were diluted with 50 ml distilled water and used for the quantification of enterococci with Enterolert® (Idexx Laboratories, Westbrook, Maine, USA), according to the manufacturer's instructions. Approximately, 30 min elapsed between sampling and culturing.

Filtration

Triplicate 50 ml samples were filtered simultaneously onto 47 mm 0.2 μM pore Supor-200® (Pall, Port Washington, NY, USA) filters using a filtration manifold and vacuum pump. Filters were placed in tubes containing 700 μl of GITC buffer (5 M GITC, 100 mM EDTA and 0.5% Sarkosyl) as previously described (Shanks *et al*., 2006). A maximum of 2.5 h elapsed between sampling and filtration. Tubes with filters were stored at –80°C for 2 days prior to nucleic acid extraction.

Nucleic acid extraction

DNA and RNA were extracted using the All Prep DNA/RNA Micro Kit® (Qiagen, Valencia, CA) according to the manufacturer's protocol. Total RNA was eluted with 14 μl of RNase-free water in low-retention 1.7 ml tubes, which resulted in a final elution volume of 12 μl (dead volume = 2 μl). DNA was eluted in 100 μl of elution buffer. RNA was stored at –80°C for no longer than 319 days before DNase treatment and reverse transcription.

Total RNA DNase treatment and reverse transcription

Each RNA sample was treated with DNase using the TURBO DNA-free kit® (ABI, Foster City, CA, USA). To control for target loss or potential matrix effects during DNase treatment and reverse transcription we spiked an equal amount of control pAW 109 RNA in each RNA extract. pAW 109 RNA is transcribed from a plasmid containing an array of target sequences and supplied at one million copies per microlitre (ABI). pAW 109 RNA was mixed with DNase buffer, DNase enzyme and molecular grade water before distribution to plate wells to equal one million copies per sample and incubated following the manufacturer's protocol. Five microlitres of each DNase-treated RNA sample was transferred directly to the reverse transcription reactions. Reverse transcription was performed in 25 μl of reactions with the High Capacity RNA-to-cDNA Master Mix®, according to the manufacturer's protocol (ABI). Fifty-five microlitres of buffer AE was added to each sample for a final volume of 80 μl and stored at –20°C until qPCR analysis. GenBac3 qPCR analysis on reverse transcriptase-negative samples indicated contaminant DNA concentrations below limits of detection for all samples.

qPCR

Assay chemistries and threshold settings are listed in Table 1. Twenty-five μl reactions were run on an ABI StepOne Plus® real-time thermalcycler. SYBR green® PCR reactions consisted of 3.5 mM $MgCl_2$, 1× PCR Buffer I (ABI), 2 mM each dNTP, 100 nM each primer, 1 μg of bovine serum albumin, 4% w/v acetamide, 4% v/v glycerol, 0.625 U of *Taq* polymerase (ABI, AmpliTaq), 50 μM ROX dye, 0.1× SYBR Green® nucleic acid stain and 2 μl of template. SYBR Green® reactions were cycled at 95°C for 2 min and 40 cycles of 95°C for 15 s and 60°C (64°C for mapA) for 32 s. Melt curve analysis with a resolution of 0.3°C was used after cycling to determine amplification specificity. TaqMan® reactions were performed as described previously (Shanks *et al*., 2009) using either Fast or Universal TaqMan Master Mix® (ABI). Reactions were cycled under the 'Fast' or 'Standard' default parameters stored by ABI StepOne Plus® software depending on the assay chemistry (Table 1). Primers, probes, BSA, acetamide and SYBR Green® dye were stored in single-use aliquots. Only DNA samples were analysed by SYBR Green® qPCR. Microcosm nucleic acid extracts were processed in batches to eliminate the impact of repeated freeze–thaw cycles. Microcosm DNA and cDNA samples were stored at 4°C between reaction set-ups (maximum storage time of 30 h). All microcosm samples were analysed in duplicate. Standard curves were run in triplicate.

qPCR standards, controls and quality criteria

Bulk standard and control DNA extracts were quantified with PicoGreen® (Molecular Probes, Eugene, OR), serially diluted and stored in single use aliquots in 0.65 ml low-retention tubes. At least five reaction wells on each plate contained positive control template for the appropriate assay. At least three wells on each plate were designated as no template controls (NTC). qPCR inhibition was monitored by two qPCR assays; a plasmid IAC multiplexed with the Entero1 assay and a SYBR Green® assay that targets genomes of an engineered strain of *P. aeruginosa* strain PAO-T7 (PAO) (Hoang *et al*., 2000). In each Entero1/IAC reaction, 50 copies of IAC linearized plasmid template were added prior to amplification of microcosm DNA. For the non-competitive inhibition control, 500 genomes of PAO were added to reactions with 2 μl of microcosm DNA and amplified using SYBR Green® chemistry PAO-T7 is a lab strain of *P. aeruginosa* originally designed for integration of single copy genes into the chromosome but used here as an inhibition control. Capitalizing on the integration of human generated sequence, we amplified the

region spanning the junction between PAO native and human derived sequences knowing that finding this strain in the environment is unlikely. PAO-F (5′-GAG TGG TTT AAG GCA ACG GT) and PAO-R (5′-ATG GAA ACA TCA ATG AAA ACA GCA) were used to prime amplification of the *attP*/*B* region (Hoang *et al*., 2000). As criteria for inhibition, we established bounds based on Ct values obtained from control amplification in molecular grade water at 2 standard deviations above the mean (Ct of 32.57 + 0.90 and 26.86 + 0.82 for IAC and PAO respectively). We concluded that nucleic acid extracts were free of inhibitors if mean Ct values for each extract fell below the bound for IAC and PAO assays. The mean IAC Ct for one of the 288 nucleic acid samples (144 DNA + 144 cDNA) fell just above the predefined bound and was omitted from data analysis (Fig. S5). In the final data set none of the 617 NTCs from all TaqMan runs showed amplification within the assay LOQ. Melt curve analysis indicated that of the 15 of 188 NTCs from SYBR runs that had Ct values within assay LOQs, none either contaminated with target DNA templates and positive amplification in these wells was assumed to be a product of primer-primer interactions. Melt curve analysis on microcosm DNA amplification reactions that were positive showed melt peaks corresponding to the proper melt peak for each assay.

Camp target sequence analysis

We identified targets amplified by the Camp qPCR assay through cloning and sequence analysis. Amplicon libraries were constructed from both DNA and cDNA extracts from day 6 of the dark marine treatment using the primers 27F (5′-AGR GTT TGA TYM TGG CTC AG) and CampR2 (5′-GGC TTC ATG CTC TCG AGT T) in 30 cycles of PCR. Products from three identical parallel PCR amplifications were pooled and incorporated into the pCR4-TOPO plasmid vector as directed by the manufacturer (Invitrogen; Carlsbad, CA). Ninety-four per cent of clones containing inserts tested positive with the Camp assay. Sequencing of both strands of inserts was performed on an ABI PRISM 3730XL DNA Analyser (ABI). High-quality sequences were paired and queried against the NCBI-nr/nt database using BLAST (Altschul *et al*., 1990). Phylogenetic analysis was performed with Bosque (Ramírez-Flandes and Ulloa, 2008). These sequence data have been submitted to the GenBank databases under Accession No. HQ216233:HQ216358.

Copy number calculation

The master calibration curve method (Sivaganesan *et al*., 2008) was used to estimate the copy numbers in unknown samples for all assays except groEL, mapA and pAW 109. For these assays, a single standard curve was used to estimate copy numbers. Assay limits of quantification are defined as the lowest target concentration within the range of quantification (Table 1).

DCW model

Quantitative real-time PCR data collected for sample days that were above the method LOQ (all six Ct values > assay LOQ) were used for model fitting. Model fitting was performed on 32 DNA (8 assays × 4 treatments) and 20 RNA (5 assays × 4 treatments) data subsets. Twenty-six DNA and eight RNA data subsets had R^2 values greater than 0.90. Estimates from data subsets that fell below the 0.90 threshold were excluded from further statistical analysis.

The scatter plot of $\log_{10}(N_t/N_0)$ versus day showed a clear delayed phase before any post-shoulder decay (an example in Fig. S6), where N_t and N_0 are respectively the estimated copy numbers on day t and day 0. A DCW model was used to estimate the lag time Z (in days), and the post-shoulder decay rate constant k [$\log_{10}$(copies/100 ml)/day]. The Bayesian regression model for a given data set with n data points, is given by:

$$Y_i = \log_{10}(N_t/N_0)_i = \mu_i + \varepsilon_i, \quad i = 1, \ldots, n$$

where

$$\mu_i = 0 \quad \text{if } (day)_i \le Z$$

$$\mu_i = -k[(day)_I - Z] \quad \text{if } (day)_i > Z$$

Normal distribution with mean 0 and variance 10^4 was considered as the non-informative priors for k (> 0). As Z could be anywhere in the range of the number of days, a uniform prior was assumed for Z between 0 and the maximum number of days. In the equation above, ε_i values are independent and identically distributed normal random variables with mean 0 and variance σ^2. A diffuse Inverse-Gamma (0.0001, 0.0001) prior was used for σ^2. Thus Y_i values were all independent normal random variables with mean μ_i and variance σ^2. According to Bayes' theorem, the posterior distribution of the model parameters k, Z and σ^2 given the data $y_1, \ldots, y_n$ is proportional to the product of the normal densities (or likelihood) of all Y_i values evaluated at $y_1, \ldots, y_n$ (given μ_i, σ^2) and prior distributions of these parameters. This posterior distribution was used to estimate the rate constant k, Z and σ^2. Estimates of k and Z from data sets with R^2 values < 0.90 were excluded from further statistical analysis. Weighted one-way ANOVA (weight = 1/standard error of estimate) was used to compare estimates between conditions or between assays.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Enterococci decay. Decay of Entero1 rDNA and rRNA markers and enterococci culturable cells in freshwater microcosms. Culturable cell (MPN) and marker copy concentration share the *y*-axis.
Fig. S2. Decay/growth profiles of Camp rDNA and rRNA in marine treatments. DM, dark marine; LM, light marine. Solid and dash-dot lines represent rDNA and rRNA marker concentrations within the method LOQ respectively. Circles and crosses represent rDNA detects below the method LOQ. rRNA detects below the method LOQ were omitted for clarity. Horizontal dotted and dashed lines represent method LOQs for rDNA and rRNA analysis respectively. Error bars represent the standard deviation of six Ct values.
Fig. S3. Phylogenetic tree of sequences testing positive with the Camp assay from the dark marine microcosm day 6. Clones were created using amplification products from both DNA and cDNA. Representatives were selected from groups with 97% similarity and clustered using a maximum likelihood approach.
Fig. S4. A and B. Alignments of the Camp forward primer and probe with clone sequences indicated that almost all sequences testing positive for the Camp assay had mismatches with both the forward primer and probe (A). The majority of the mismatches occurred on the 3′ end of the oligonucleotide (B).

Fig. S5. Two controls were used to test for inhibition; PAO and an internal amplification control (IAC) multiplexed with Entero1. The bounds for inhibition were placed at 2 standard deviations from mean Ct values obtained from using 2 μl of laboratory grade substituted for microcosm sample DNA. One sample, D1DM17cDNA, may have displayed slight effects of inhibition as indicated by the IAC and was removed from the data set prior to analysis.

Fig. S6. Chick–Watson (CW) and delayed Chick–Watson (DCW) model comparison. HF183/303R decay under dark fresh. Decay rates (k) are calculated in both models. The change point (Z) indicates the beginning of rapid decay and is estimated through DCW only. The shaded area represents the 95% credible region estimated by DCW.

Table S1. Decay rate estimates and persistence of DNA and RNA molecular markers for all treatments. Estimates shown are change point (Z), decay rate constants (k_{DCW}), lower (2.5% LCI) and upper (97.5% UCI) credible intervals for each estimate, and r^2 values obtained using the DCW model. Two estimates for which credible intervals do not overlap are considered statistically different at the 95% level. For persistence, shown are the number of Ct values out of the 6 possible within the assay's range of quantification (3 filters $\times$ 2 qPCR replicates per filter) per sample day per assay are shown.

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