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Persistence of nucleic acid markers of health-relevant organisms in seawater microcosms: Implications for their use in assessing risk in recreational waters

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ABSTRACT

In the last decade, the use of culture-independent methods for detecting indicator organisms and pathogens in recreational waters has increased and has led to heightened interest in their use for routine water quality monitoring. However, a thorough understanding of the persistence of genetic markers in environmental waters is lacking. In the present study, we evaluate the persistence of enterococci, enterovirus, and human-specific *Bacteroidales* in seawater microcosms. Two microcosms consisted of seawater seeded with human sewage. Two additional seawater microcosms were seeded with naked *Enterococcus faecium* DNA and poliovirus RNA. One of each replicate microcosm was exposed to natural sunlight; the other was kept in complete darkness. In the sewage microcosms, concentrations of enterococci and enterovirus were measured using standard culture-dependent methods as well as QPCR and RT-QPCR respectively. Concentrations of human-specific *Bacteroidales* were determined with QPCR. In the naked-genome microcosms, enterococci and enterovirus markers were enumerated using QPCR and RT-QPCR, respectively. In the sewage microcosm exposed to sunlight, concentrations of culturable enterococci fell below the detection limit within 5 days, but the QPCR signal persisted until the end of the experiment (day 28). Culturable enterococci did not persist as long as infectious enteroviruses. The ability to culture enteroviruses and enterococci was lost before detection of the genetic markers was lost, but the human-specific *Bacteroidales* QPCR signal persisted for a similar duration as infectious enteroviruses in the sewage microcosm exposed to sunlight. In the naked-genome microcosms, DNA and RNA from enterococci and enterovirus, respectively, persisted for over 10 d and did not vary between the light and dark treatments. These results indicate differential persistence of genetic markers and culturable organisms of public health relevance in an environmental matrix and have important management implications.

1. Introduction

Monitoring fecal indicator bacteria (FIB), including *Escherichia coli* and enterococci (ENT) in waters used for recreation and shellfish harvesting aids in mitigating health risks associated with human exposure to water polluted with feces. These indicators were chosen for health risk monitoring because their concentrations are quantitatively

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linked to adverse health outcomes in swimmers exposed to waters polluted by urban runoff and treated wastewater effluent (Cabelli et al., 1983; Haile et al., 1999; Prüss, 1998). Currently, cultivation of enterococci (ENT) is the standard method used to monitor marine waters for fecal pollution (USEPA, 2004).

Despite their widespread use, the suitability of FIB for assessing risk has been challenged for multiple reasons. FIB occurrence often does not correlate with the presence of fecal pathogens (Baggi et al., 2001; Boehm et al., 2003; Bonadonna et al., 2002; Geldenhuys and Pretorius, 1989; Horman et al., 2004; Lemarchand and Lebaron, 2003; Noble and Fuhrman, 2001; Pusch et al., 2005; Wade et al., 2003), so there is concern about whether they really indicate a health risk. FIB can grow and survive in extra-intestinal environments including beach sand, soil and sediments (Anderson et al., 2005; Fujioka et al., 1999; Hardina and Fujioka, 1991; Whitman et al., 2003; Yamahara et al., 2009) and therefore the presence of FIB in a water sample may not indicate the presence of feces. Finally, standard enumeration of FIB is accomplished using culture-based methods that require 24 h incubations (USEPA, 2004). This has several drawbacks. First, because of the lengthy incubation, exposure to polluted waters can occur before beach advisories are posted (Hou et al., 2006; Kim and Grant, 2004). Second, the risk of exposure to polluted waters may no longer exist by the time results are available, making beach advisories and closures costly and futile. Third, culture-based methods can miss starved and damaged cells or cells that are in a viable but non-culturable (VBNC) state (Menon et al., 2003; Pommepuy et al., 1996; Rahman et al., 1996), thereby underestimating the true number of FIB and the risk to human health.

To address the problems with using traditional FIB to assess risk of recreational waterborne illness, newly proposed methods of diagnosing fecal pollution have been developed. The majority of these are culture-independent methods that detect a genetic marker using polymerase chain reaction (PCR) and quantitative PCR (QPCR) (Bae and Wuertz, 2009: Bernhard and Field, 2000b; Haugland et al., 2005; McQuaig et al., 2006; Seurinck et al., 2005; Shanks et al., 2006). Since nucleic acids can remain in the environment and be detected by PCR after an organism is no longer alive (Espinosa et al., 2008) it is important to understand how long the genetic markers persist in environmental waters as entities separate from intact cells or viruses.

The present study evaluated culturability and genome persistence of enteroviruses and ENT, and persistence of the human-specific fecal *Bacteroidales* marker in seawater microcosms. We chose to focus on ENT because it is the preferred health indicator for monitoring marine waters (Wade et al., 2003) and used a human virus as a model pathogen since viruses are believed to be the main etiology of recreational waterborne illness (Jiang and Chu, 2004; Rusin et al., 1999). The human-specific marker in *Bacteroidales* was chosen because it shows promise as a human-specific marker of fecal pollution (Bernhard and Field, 2000a; Kildare et al., 2007; Seurinck et al., 2005). Our goal was to measure persistence and decay rates of genetic markers and naked genomes to understand indicator and pathogen fate and to evaluate the ability of ENT and human-specific *Bacteroidales* genetic markers to predict the presence of human viruses in marine waters.

2. Methods

Four microcosms were used; two were exposed to natural sunlight and two were kept in complete darkness. Sunlight exposure and darkness were the only treatments. From this point forward the treatments are referred to as ''light'' and ''dark'' microcosms. One light and one dark microcosm were seeded with sewage influent. Subsequently, we followed the persistence of sewage-derived culturable ENT, infectious enterovirus, and sewage-derived ENT, enterovirus, and human-specific *Bacteroidales* genetic markers. The remaining light and dark microcosms were seeded with naked *Enterococcus faecium* and Poliovirus 3 (PV3) genomes. Hereafter, the sewage-seeded microcosms are referred to as ''sewage microcosms'', and the microcosms seeded with genomes are referred to as ''naked-genome microcosms''.

2.1. Microcosm design

Seawater (pH = 7.85, salinity = 34.2) from the Upper Elkhorn Slough (Monterey, CA, USA) was collected in 20 l, acid washed, collapsible polyethylene containers. Containers were rinsed three times with source water before collection. Sewage influent from the Regional Water Quality Control Plant (Palo Alto, CA, USA) was collected in sterile 1 l polyethylene bottles. Seawater and sewage influent were transported back to the lab and kept at 4 °C, in the dark, until use.

Microcosms consisted of four, five gallon, high density polyethylene buckets that were weighted and placed in a 162 qt marine cooler (2 buckets per cooler). The sewage microcosms received 14 l of unfiltered seawater and the naked-genome microcosms received 15 l. The coolers were filled with tap water to 5 cm below the lip of the buckets and served as an insulated water bath for the microcosms. Microcosm temperature was maintained near the *in situ* temperature of the Elkhorn Slough (~17 °C) by chilling water to 10 °C using a NESLAB RTE-7 Digital One Refrigerated Bath (Thermo Fisher Scientific, Waltham, MA) and continually circulating the chilled water through ¾ ″ copper tubing piped through the water bath, surrounding the buckets. The water bath was protected from solar radiation and ambient heat by placing white, 5 cm foam board insulation on top of the coolers with openings for the buckets to allow exposure to sunlight.

Sewage microcosms received 1 l of sewage influent to make the final volume 15 l. Naked-genome microcosms were seeded with naked, purified genomes from *E. faecium* (ATCC #19434) and Poliovirus 3 (ATCC VR-300) (see extraction and purification method below). Light microcosms remained uncovered, and dark microcosms were kept from sunlight by an opaque black cover. To mimic mixing of the water column, an airstone was placed in each microcosm and air was pumped continuously for the duration of the experiment. Evaporation occurred over the course of the experiment in light microcosms. The volume of water lost due to evaporation was noted on each sampling day. Concentrations of targets in the light microcosms were corrected for evaporation.

2.2. Enumeration of culturable ENT (cENT)

Culturable ENT (cENT) were enumerated, in triplicate, according to EPA method 1600 (USEPA, 2002). The microcosm exposed to sunlight was sampled on days 0, 1, 5, and 6; the dark microcosm was sampled on days 0, 1, 4, 5, 6, 8, and 11. Plates were incubated at 41 ± 0.5 °C for 24 h on days 0–4. On days 5–11 an extra 24 h of incubation was provided for recovery of cells that may have entered a viable but non-culturable (VBNC) state, or slowed their metabolism to accommodate a reduced nutrient environment (Shrestha et al., 2007). The detection limit was 0.01 CFU/ml.

2.3. Detection of infectious enteroviruses

Samples from sewage microcosms were assayed for infectious enteroviruses using plaque assays on days 0, 1, 4, 6, 8, 11, 14, 18, 22, and 28. On each sampling day, triplicate 30 ml sample volumes were concentrated to 200 μl using Amicon® Ultra-15 centrifugal filtration devices with a 10,000 NMWL cutoff (Millipore, Billerica, MA) by concentrating 15 ml volumes twice. The concentration efficiency of using Amicon Ultra-15 filtration devices for recovery of infectious enteroviruses was nearly 100% (data not shown). Plaque assays were carried out according to methods developed by Vincent Racaniello's Laboratory (personal communication, 2007). Briefly, 2×10^6 HeLa cells/plate were grown for 24 h (or until 85–90% confluency) on 60 mm Costar® cell culture plates (Thermo Fisher Scientific Inc.) in Dulbecco's Modified Essential Medium (DMEM; Hyclone, Thermo Fisher Scientific Inc.) supplemented with 10% fetal bovine serum and 1 × Penicillin/Streptomycin (Invitrogen, Carlsbad, CA). Growth medium was removed and cells were rinsed once with phosphate buffered saline (PBS, pH = 7.4). Dilutions, 50% and 10%, of each concentrated sample, were prepared in viral buffered saline (VBS; 1× PBS (pH = 7.4) and 0.2% fetal bovine serum (Invitrogen)). Cells were infected using 100 μl of each dilution; duplicate infections were performed for the 50% and 10% dilutions. Infections were allowed to proceed for 45 min then cells were overlaid with 0.9% agar + 1× DMEM supplemented with 5% fetal bovine serum, 1× Penicillin/Streptomycin, and 0.024 units/ml Nystatin (Sigma–Aldrich, St. Louis, MO) warmed to 45 °C. After solidifying, plates were incubated at 37 °C in 5% CO_2. After 48 h, 2 ml of 10% trichloroacetic acid was applied to each plate and allowed to soak into the agar for 10 min. The agar was removed and discarded. Cells were stained with crystal violet for 10 min. A positive result for infectious enterovirus was determined by the presence of visible plaques. The limit of detection was determined to be 1 pfu/30 ml.

2.4. Nucleic acid preparation

Samples from sewage microcosms were collected on days 0, 1, 4, 6, 8, 11, 14, 18, 22, and 28 for all molecular marker analyses. Samples were collected in sterile high density polyethylene bottles, transported back to the laboratory on ice and processed immediately. One hundred milliliters of each sample were filtered onto 47 mm, 0.45 μm HAGW membranes (Thermo Fisher Scientific Inc.), under 15 mm Hg vacuum. Filters were rolled and placed in sterile, 15 ml polypropylene tubes containing 500 μl of guanidine isothiocyanate (GITC) buffer (5 M guanidine thiocyanate, 100 mM EDTA, 0.5% N-lauroyl sarcosine; Sigma–Aldrich). Tubes were vortexed for 30 s to saturate the filter with GITC buffer and were stored at −80 °C until extraction.

The naked-genome microcosms were sampled on days 0–8, 11, 14, 18 and 22. On each sampling day triplicate 30 ml volumes were concentrated to 200 μl using Amicon® Ultra-15 Centrifugal Filter Devices with a 10,000 NMWL cutoff (Millipore). For storage purposes each 200 μl concentrated sample received 500 μl of Qiagen buffer RLT, containing 1% (v/v) β-mercaptoethanol (Sigma–Aldrich) and was stored at −80 °C until extraction.

Nucleic acids (DNA and RNA) were extracted and purified from all samples using the AllPrep™ DNA/RNA Micro Kit (Qiagen, Valencia, CA) with modifications. For sewage microcosm samples 500 μl of GITC buffer was used to preserve the sample for freezing. After thawing, 350 μl of Buffer RLT Plus, containing 1% (v/v) β-mercaptoethanol (Sigma–Aldrich) and 20 ng of carrier RNA (supplied by manufacturer), was added to each filter containing tube. Since samples from the naked-genome microcosms received 350 μl Buffer RLT Plus prior to freezing, these samples were thawed and 20 ng carrier RNA was added. Sample tubes were vortexed for 1 min. Next, lysates were added to the AllPrep DNA spin column and were processed from this point forward following the manufacturer's instructions. Filtration blanks and extraction blanks were included with all nucleic acid extractions. All DNA preparations were eluted in 50 μl Buffer EB and RNA preparations were eluted in 14 μl RNase free water. DNA and RNA samples were stored at −20 and −80 °C respectively, until used.

2.5. *Human-specific* Bacteroidales PCR

Using conventional PCR we followed persistence of the human-specific *Bacteroidales* fecal marker (Bernhard and Field, 2000a,b) in DNA extracts from the sewage microcosms. PCRs and cycling parameters were performed using primers HF183F and Bac708R (Table 1) as previously described (Walters et al., 2007). Two microliters of DNA extract were used as template in each reaction. PCR products were visualized on 1% agarose gels stained with ethidium bromide (Sigma–Aldrich), using a BioRad Gel Doc XR system (BioRad, Hercules, CA). No template controls were included with each PCR.

2.6. QPCR and RT-QPCR standards

RNA standards were synthesized in vitro for quantification of enterovirus genomes in reverse transcriptase-QPCR (RT-QPCR). Primer PV1279R (Table 1) was used to create Poliovirus 3 (PV3) cDNA during RT-PCR using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), according to manufacturer's instructions. Ten microliters of cDNA from the RT-PCR were used as template in PCR for cloning. PCR mixtures consisted of 1× PCR SuperMix which contained 220 μM dNTPs and 1 U recombinant Taq DNA polymerase (Invitrogen), 2.65 mM $MgCl_2$, 500 μM of each primer, PV228F and PV1191R (Table 1). Thermal cycling was performed using a GeneAmp 9700 thermal cycler (Applied Biosystems) with an initial denaturation of 95 °C for 5 min,

Table 1 – Primer and probe sequences used in PCR, QPCR, and RT-QPCR.

Name	Sequence 5′ → 3′	Target	Reference
HF183F	ATCATGAGTTCACATGTCCG	Human-specific *Bacteroidales*	Bernhard and Field (2000a)
708R	CAATCGGAGTTCTTCGTG	*Bacteroidales* bacteria	Bernhard and Field (2000b)
PV1279R	GCACCGTGTAGCCTGATCTCC	PV3 genome	This study
PV228F	CGCTCAGCACTCTACCCCG		
PV1191R	CCACCACCCCTTGGATTCC		
EVupstream	CCTCCGGCCCCTGAATG	Pan-enterovirus 5′-UTR	DeLeon et al. (1990)
EVdownstream	ACCGGATGGCCAATCCAA		
EV probe	FAM-ACGGACACCCAAAGTAGTCGGTTC-BHQ-1	Pan-enterovirus 5′-UTR	Gregory et al. (2006)
ECST748F	AGAAATTCCAAACGAACTTG	ENT 23S rRNA gene	Haugland et al. (2005), Ludwig and Schleifer (2000)
ENC854R	CAGTGCTCTACCTCCATCATT		
GPL813TQ	6FAM-GGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA		Ludwig and Schleifer (2000)
BacHum-160f	TGAGTTCACATGTCCGCATGA	Human-specific *Bacteroidales*	Kildare et al. (2007)
BacHum-241r	CGTTACCCCGCCTACTATCTAATG		
BacHum-193p	6FAM-TCCGGTAGACGATGGGGATGCGTT-TAMRA		

followed by 35 cycles of 95 °C for 45 s, 57 °C for 45 s, and 72 °C for 60 s and a final extension step of 72 °C for 7 min. PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide (Sigma–Aldrich). The 963 bp fragment was excised and gel purified using the QIAquick Gel Extraction Kit (Qiagen) and cloned into the pCR 2.1-TOPO TA cloning vector (Invitrogen). Quantification standards were created following the procedure outlined by Gregory et al. from this point forward (Gregory et al., 2006).

Whole genomic DNA from *E. faecium* and plasmid DNA from an *E. coli* clone harboring the *Bacteroidales* human-specific rRNA gene were used to generate standard curves for the ENT and BacHum QPCR assays respectively.

DNA and RNA standards were quantified using an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). ENT cell equivalents (CE) were calculated using 6 rrn operons per genome of *E. faecium* with a genome size of 2.6 Mb (Oano et al., 2002; Sechi et al., 1994), and were serially diluted to a range of 1.76×10^2 to 1.76×10^6 copies/μl. Enterovirus RNA standards were serially diluted to a range of 3.75×10^0 to 3.75×10^6 copies/μl, calculated based on the size of the in vitro transcribed RNA fragment. The number of rRNA gene copies for the *Bacteroidales* human-specific DNA standard was calculated using 4425 bp (the size of the insert, 525 bases, plus the size of the vector, 3.9 kb), and were serially diluted to 2.04×10^0 to 2.04×10^5 copies/μl. Human-specific *Bacteroidales* cell equivalents were calculated using 6 rrn operons/genome (Walters and Field, 2009). All standards fall within the range of quantification of each QPCR assay. Standard dilutions were included in triplicate with each QPCR or RT-QPCR and were used to determine the C_T and efficiency of each run. Copy number estimates of targets in unknown samples were extrapolated from the linear fit of the standard copy number versus threshold cycle.

2.7. QPCR

QPCR was used to enumerate ENT 23S rRNA genes (Haugland et al., 2005; Ludwig and Schleifer, 2000) and human-specific *Bacteroidales* 16S rRNA genes (Kildare et al., 2007) in each of the microcosms over time. All samples were analyzed by QPCR in triplicate. Two microliters of DNA were used as template in each reaction. For ENT QPCR each 25 μl reaction included 1× TaqMan™ Universal PCR Master Mix (Applied Biosystems), 1000 nmol primer ECST748F and ENC854R and 200 nmol probe GPL813TQ (Table 1). For human-specific *Bacteroidales* QPCR each 25 μl reaction consisted of 1× TaqMan™ Universal PCR Master Mix, 400 nM of each primer, BacHum-160f and BacHum-241r, and 80 nM BacHum-193p probe (Table 1). QPCR for both assays was carried out using an ABI StepOnePlus thermocycler (Applied Biosystems) with the following cycling parameters: an initial 2 min incubation at 50 °C to activate uracil-*n*-glycosylase (UNG) activity, a 95 °C denaturation step of 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. No template controls, extraction blanks and filtration blanks were included with each QPCR run. Two additional human-specific *Bacteroidales* QPCRs for each sample were spiked with a known quantity of DNA target prior to account for inhibition. The spiked reactions consisted of 1 μl sample DNA and 1 μl of ~2000 copies of target DNA. The copy number of the spike was confirmed during each QPCR run. The limit of detection was 4 gene copies for both ENT and human-specific *Bacteroidales* QPCR assays. Limits of detection were determined by QPCR on serial dilutions of standard DNA, and were defined as the lowest concentration at which all replicates were reliably detected. Our limits of detection were 0.17 CE/ml water sample for enterococci and human-specific *Bacteroidales* markers in the sewage microcosms, and 0.55 enterococci CE/ml water sample in the naked-genome microcosms.

2.8. *Enterovirus* RT-QPCR

We used RT-QPCR to enumerate the number of enterovirus genomes in each microcosm over time. RT-QPCR was carried out using an ABI StepOnePlus thermocycler (Applied Biosystems). The TaqMan® RNA-to-C_T™ *1-Step* Kit (Applied Biosystems) was used in all enterovirus RT-QPCRs. Each 25 μl reaction mixture consisted of 2 μl RNA template, 1× TaqMan® RT-PCR Mix, 1× TaqMan® RT Enzyme Mix, 400 nM each pan-enterovirus primer (EVupstream and EVdownstream) and 200 nM EV probe (Table 1). The amplification conditions included a 30 min reverse transcription step at 48 °C, followed by a 10 min denaturation step of 95 °C and 40 cycles of 60 °C for 60 s and 95 °C for 15 s. All samples were analyzed by RT-

QPCR in triplicate. No template controls, extraction blanks and filtration blanks were included with each RT-QPCR run. The limit of detection for the enterovirus RT-QPCR assay was 1 copy of the enterovirus target which corresponds to 0.07 copies/ml water sample in the sewage microcosms and 0.23 copies/ml in the naked-genome microcosms. This was estimated from RT-QPCR on serial dilutions of RNA standards and was defined as the lowest concentration at which all replicates were reliably detected.

2.9. Decay rate estimations and statistical comparisons

Replicates tended to be log-normally distributed, thus we chose to describe the central tendency of the replicates from each time point using the geometric mean and 95% confidence intervals about the geometric mean. Data were normalized by dividing each sample mean by the mean of the sample on the day of microcosm set up (C/C_0). Normalized data were transformed with the natural logarithm ($\ln(C/C_0)$). First-order decay rates (k) and their 95% confidence intervals were estimated as the slope of the regression line: $\ln(C/C_0) = kt$, where t is time (days) and k has units of d^{-1}. This model corresponds to the Chick–Watson model. Only data points above the assay limit of detection (shown in Fig. 1) were used in the regression. R^2 values are reported for all curve fits. We compared decay rates using the model: $Y_i = (\beta_0 + \beta_2) + (\beta_1 + \beta_3)t$ (Neter et al., 1990). This model tests the null hypothesis of no difference between regression slopes (decay rates).

3. Results

3.1. Bacterial persistence in sewage microcosms

There was a marked difference in the culturability of enterococci (ENT) in the light and dark sewage microcosms (Fig. 1A). Concentrations of culturable ENT (cENT) were equal between the light and dark microcosms at the start of the experiment. The concentration of cENT decreased rapidly in the light microcosm; cENT decreased nearly 5-logs and fell below the detection limit of 0.01 CFU/ml by day five. In the dark microcosm the numbers of cENT remained above the detection limit until day 12. The presence of sunlight produced a statistically significant increase in the decay rate of cENT ($k_{light} = -2.21\ d^{-1}$, $k_{dark} = -0.907\ d^{-1}$, $p = 0.029$; Table 2). cENT decayed an order of magnitude in concentration two and a half times as fast in the light compared to the dark microcosms ($T_{90(light)} = 1.04$ d, $T_{90(dark)} = 2.54$ d).

On the day the microcosm experiments were started the concentration of ENT cell equivalents (CE) obtained by QPCR was approximately twice as high as the concentration of cENT (Fig. 1A). While cENT were readily inactivated in the light microcosm, it took eight times as long for ENT cell equivalents to decline by the same order of magnitude ($T_{90(cENT\_light)} = 1.04$ d, $T_{90(ENT\_CE\_light)} = 8.28$ d). In the dark microcosm there was also a difference in decay rates between the two enumeration methods (membrane filtration and ENT 23S QPCR). cENT decreased an order of magnitude approximately four times faster than the ENT genetic markers ($k_{cENT\_(dark)} = -0.907\ d^{-1}$, $k_{ENT\_CE\_(dark)} = -0.241\ d^{-1}$, $p < 0.001$; $T_{90(cENT\_dark)} = 2.54$ d, $T_{90(ENT\_CE\_dark)} = 9.55$ d). We did not observe a significant difference between decay rates of ENT cell equivalents between the light and dark treatments in sewage microcosms over the course of the experiment ($k_{light} = -0.278\ d^{-1}$, $k_{dark} = -0.241\ d^{-1}$, $p = 0.595$; Table 2). In both treatments the concentration of ENT cell equivalents reached the QPCR limit of detection by day 28 (Fig. 1A). Though there was not a statistically supported difference in the decay rates of ENT cell equivalents between the light and dark sewage microcosms, ENT cell equivalents concentrations were consistently higher in the dark microcosm. Initial concentrations were equal between the light and dark microcosms but in the first 24 h of the experiment a substantial post-inoculation increase in the concentration of ENT was observed in the dark microcosm.

We followed persistence of the *Bacteroidales* human-specific fecal marker in the sewage microcosms using conventional PCR and QPCR. A statistically significant difference in decay rate of the human-specific *Bacteroidales* genetic marker was observed between the light and dark microcosms ($k_{light} = -1.30\ d^{-1}$, $k_{dark} = -0.264\ d^{-1}$, $p = 0.015$; Table 2). The marker concentration decreased nearly 5 times faster in the light microcosm than the dark ($T_{90\_(light)} = 1.77$ d, $T_{90\_(dark)} = 8.72$ d). The human-specific *Bacteroidales* marker was not detected in the light microcosm after 8 d. In the dark microcosm concentrations decreased to 0.1 CE/ml by day 18 and fell below the limit of detection by day 28 (Fig. 1B). The human-specific *Bacteroidales* QPCR assay was completely inhibited in light microcosm samples on days 22 and 28, the last two days of the experiment. This observation is based on the fact that when environmental DNA was spiked with ~2000 copies of the target these samples did not amplify. This finding does not alter the interpretation of our data about the persistence of the human-specific *Bacteroidales* genetic marker because the marker was not detected after day 8 in these samples. Inhibition was not detected until day 22 and was not observed in QPCR performed on DNA from any samples from the dark microcosm. The inhibition could have been caused by a phytoplankton bloom in the light microcosm near the end of the experiment. We did not observe the same level of inhibition in the ENT QPCR assay based on the fact that we were always able to amplify our target. This difference in QPCR assay susceptibility to inhibition has been previously observed between the human fecal *Bacteroidales* and ENT QCR assays (data not shown) and is not surprising given the various mechanisms of PCR inhibition (Wilson, 1997).

Samples from the sewage microcosms were also assayed for the presence of the human-specific *Bacteroidales* marker using conventional PCR. The human-specific *Bacteroidales* genetic marker was detected through day 4 in the sewage microcosm exposed to sunlight and until day 11 when kept in the dark, this is approximately half the number of days we detected the human-specific *Bacteroidales* marker with QPCR in both treatments.

3.2. Persistence of infectious enterovirus and enterovirus genomes in sewage microcosms

Enteroviruses in samples from sewage microcosms remained infectious for 8 days in the light microcosm and 14 days in the dark. The enterovirus genome was detected in the same samples through day 28 regardless of sunlight exposure.

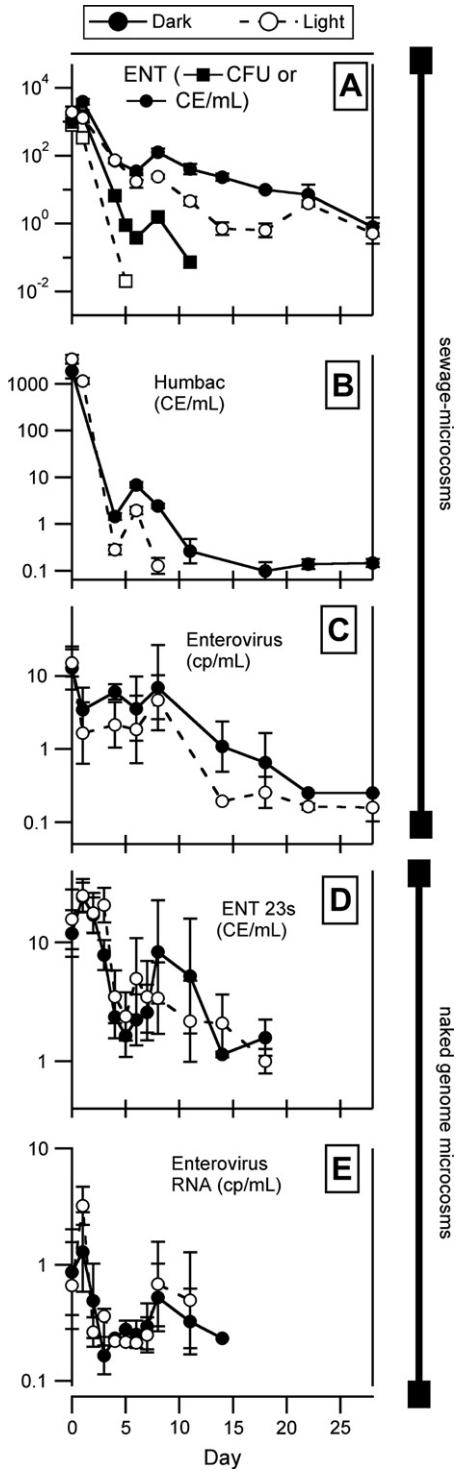

Fig. 1 – (A)–(C) Concentrations obtained from the *sewage-seeded* microcosms. (D)–(E) Concentrations obtained from the *naked-genome* microcosms. Concentrations of (A) cultured enterococci (cENT) cells (CFU/ml), ENT (cell equivalents/ml),(B) human-specific *Bacteroidales* (cell equivalents/ml),(C) enterovirus (copies/ml),(D) naked ENT genomes (cell equivalents/ml),(E) naked enterovirus genomes (copies/ml) in light and dark microcosms over time (days). Note: data from days where the sample quantities dropped below the limits of detection for the assay are omitted from the figure.

Initial concentrations of enteroviruses were higher in the light microcosm than the dark microcosm but this relationship shifted the first day post-inoculation (Fig. 1C) after which the concentration of enterovirus genomes was consistently higher (1.5–2.5 times) in the dark microcosm. No difference in decay rates of the genomes was observed between the two treatments ($k_{light} = -0.147\ d^{-1}$, $k_{dark} = -0.140\ d^{-1}$, $p = 0.847$); ($T_{90\_(light)} = 15.7$ d, $T_{90\_(dark)} = 16.4$ d).

3.3. *Persistence of* E. faecium *and Poliovirus 3 genomes in naked-genome microcosms*

To provide additional understanding about the fate of genetic markers and their use as indicator species for fecal pollution and human health risks, we evaluated the effect of sunlight on persistence of naked genomes. We examined persistence of naked *E. faecium* genomes, because ENT are the current standard FIB for marine waters, and naked poliovirus genomes because of the labile nature of RNA.

Naked *E. faecium* genome was detected until day 18 in both treatments (Fig. 1D). There was no difference between decay of the ENT genome in sunlight or dark microcosms ($k_{light} = -0.167\ d^{-1}$, $k_{dark} = -0.126\ d^{-1}$, $p = 0.455$; Table 2). The concentration of ENT genomes remained constant over the first two days of the experiment at which time numbers began declining. Concentrations of detectable ENT genetic markers were reduced by 90% after approximately 14 days in the light and 18 days in the dark ($T_{90\_(light)} = 13.8$ d, $T_{90\_(dark)} = 18.3$ d), and persisted at low levels near the detection limit until the end of the experiment.

Naked PV3 genome fell to the detection limit by days 11 and 14 in the light and dark microcosms, respectively (Fig. 1E). Naked PV3 genome decay did not differ between light and dark microcosms ($k_{light} = -0.071\ d^{-1}$, $k_{dark} = -0.069\ d^{-1}$, $p = 0.989$; Table 2). Concentrations of poliovirus genomes decreased rapidly over the first 3 days of the experiment and hovered near the limit of detection for the RT-QPCR assay until days 11 (light) and 14 (dark). The time to 90% decay was 22.3 d and 32.4 d in the light and dark naked-genome microcosms, respectively.

4. Discussion

We sought to address two central issues in our study. First, because sunlight inactivation is an important factor influencing survival of enteric bacteria and persistence of infectious viruses (Boehm, 2007; Davies-Colley et al., 1994; Noble and Fuhrman, 1997; Sinton et al., 1999, 2002) we wanted to assess the impact of sunlight exposure on survival (measured by culturability) of sewage-derived ENT and enteroviruses, concomitant with persistence of the ENT, human-specific *Bacteroidales*, and enterovirus genetic markers. Second, because PCR-based methods detect nucleic acids from living and dead organisms, as well as exogenous nucleic acids, we wished to describe how exposure to sunlight influenced persistence of naked ENT and naked enterovirus genomes. To explore these issues we seeded unfiltered seawater microcosms with either (1) sewage influent, or (2) naked ENT DNA and poliovirus RNA, and exposed them to natural sunlight

Table 2 – Decay rates (k) and corresponding 95% confidence intervals for each indicator in 17 °C seawater microcosms exposed to sunlight (light) or darkness (dark). RSQ values for curve fits are provided in square brackets beneath k values. p-values represent comparison between decay rates in light and dark microcosm treatments. Units for decay rates are d^{-1}.

Indicator	Sewage-seeded microcosms					Naked-genome microcosms				
	Light		Dark		p-value	Light		Dark		p-value
	Rate (k) [RSQ]	95% C.I.	Rate (k) [RSQ]	95% C.I.		Rate (k) [RSQ]	95% C.I.	Rate (k) [RSQ]	95% C.I.	
cENT	−2.21 [0.987]	−5.48 to 1.06	−0.907 [0.836]	−1.32 to −0.493	0.029	–	–	–	–	–
Enterococci QPCR	−0.278 [0.745]	−0.410 to −0.145	−0.241 [0.836]	−0.344 to −0.138	0.595	−0.167 [0.732]	−0.239 to −0.096	−0.126 [0.452]	−0.206 to −0.046	0.455
Human *Bacteroidales* QPCR	−1.30 [0.830]	−2.37 to −0.219	−0.264 [0.606]	−0.462 to −0.066	0.015	–	–	–	–	–
Enterovirus RT-QPCR	−0.147 [0.755]	−0.222 to −0.072	−0.140 [0.885]	−0.196 to −0.084	0.847	−0.071 [0.080]	−0.265 to 0.124	−0.069 [0.229]	−0.182 to 0.044	0.989

with a diurnal light and dark cycle, or excluded sunlight completely. We followed survival and persistence of the indicator organisms and genetic markers in these microcosms using culture-based and culture-independent methods.

We assumed first-order decay to calculate inactivation rates. We found first-order decay was fairly good at describing the decay kinetics: 9 of the 12 curve fits have R^2 values greater than 0.6. One drawback to using this simple model is the inability to account for shouldering or tailing during decay. To account for these, more complex models can be used (e.g., Bae and Wuertz, 2009; Maier et al., 2000). Under some experimental conditions, biphasic decay, in which a resistant or protected sub-population persists, has been shown to occur (see Hellweger et al., 2009 and references therein). It is possible that biphasic decay may better describe the decline of the naked DNA and RNA genomes in the naked-genome microcosms given that some of the R^2 values are relatively low. However, given the large error bars in these measurements, we chose to retain the first-order decay model.

4.1. *Persistence of human-specific* Bacteroidales *in light and dark microcosms*

Genetic markers from the *Bacteroidales* group of fecal bacteria are promising alternative indicators of fecal pollution because they are host-specific and are unlikely to grow in extra-intestinal environments due to their anaerobic metabolism. Here, sunlight produced a difference in how long we detected the human-specific *Bacteroidales* genetic marker using traditional PCR and QPCR in sewage microcosms; the markers decayed more rapidly in the microcosm exposed to sunlight. This contrasts with the findings of Bae and Wuertz who saw no difference in decay rates of the human-specific *Bacteroidales* genetic marker due to sunlight exposure in seawater microcosms (Bae and Wuertz, 2009). Walters and Field also did not observe a difference in persistence of human-specific *Bacteroidales* markers due to sunlight exposure but reported a similar decay rate in freshwater microcosms ($-1.7\ d^{-1}$) as we observed in our seawater microcosms ($-1.3\ d^{-1}$) when exposed to sunlight (Walters and Field, 2009). The differences between these studies could be attributed to the physico-chemical differences between seawater and freshwater (pH, buffering capacity, ionic strength, dissolved nutrients, etc.), the type of inoculum (feces versus sewage), the presence of autochthonous organisms including grazers and bacteriophage, or the time of year the experiments were conducted. The use of propidium monoazide (PMA)-QPCR which tracks the concentration of cells with intact cell membranes may yield more insight into the persistence of the bacteria containing the human-specific marker (Bae and Wuertz, 2009).

We observed a difference in detection of the *Bacteroidales* human-specific fecal marker between conventional PCR (525 bp) and QPCR (81 bp). This difference was observed despite the fact that the same volume of DNA sample was used in both PCR assays. This observation could be explained by the higher number of cycles and sensitive fluorescent detection used in QPCR or differential persistence of different sized fragments, assuming the probability of UV damage increases with increasing fragment size as seen for RNA (Simonet and Gantzer, 2006).

4.2. Decay of sewage-derived enterococci in light and dark microcosms

Exposure to sunlight significantly impacts survival of ENT in culture-based studies (Barcina et al., 1997; Deller et al., 2006; Noble et al., 2004; Sinton et al., 2002; Walters and Field, 2009). The results of our microcosm experiments provide additional evidence that this is true. Where sunlight had a large influence on the ability to culture ENT, it did not have an effect on decay of the ENT genetic markers. ENT genetic markers were detected 23 days longer than cENT exposed to sunlight, and could have potentially been detected longer if the experiment had lasted longer. *Enterococcus* spp. can enter a viable but non-culturable (VBNC) state when subjected to adverse growth conditions in environmental waters (Hartke et al., 1998; Heim et al., 2002; Lleo et al., 2006; Signoretto et al., 2004, 2005), a phenomenon that could be responsible for the vast difference in detection of between the two methods. Alternately, QPCR could be detecting the genomes of dead enterococci that are protected from environmental degradation by the cell wall and envelope. To discern between these two, an approach such as PMA-QPCR may be useful.

4.3. Comparison of sewage-derived ENT and human-specific Bacteroidales genetic marker decay

The human-specific *Bacteroidales* markers and ENT genetic markers had different persistence profiles in the sewage microcosms with the human-specific *Bacteroidales* being more sensitive to sunlight. Since amplicon size for these two assays is similar (ENT is 106 bp, human-specific *Bacteroidales* is 81 bp), this cannot be explained by differential persistence of different size fragments. It is plausible that differential persistence is attributable to differences in cell wall composition between the two indicator organisms. ENT are gram positive cells with thick layers of peptidoglycan, whereas *Bacteroidales* bacteria are gram negative cells and have a thinner peptidoglycan layer. If gram negative cells are more prone to grazing by protists and cellular lysis than gram positive cells, this could explain the more rapid disappearance of the human-specific *Bacteroidales* makers. Another possible explanation is that since *Bacteroidales* are anaerobic, they are more sensitive to oxygen and reactive oxygen species generated from sunlight exposure.

4.4. Persistence of enterovirus in sewage microcosms

We examined the persistence of bacterial indicators relative to infectious enterovirus and enterovirus genomes measured using RT-QPCR in sewage-seeded microcosms. In the sewage microcosm exposed to sunlight infectious enteroviruses were detected 3 days longer than cENT (8 d versus 5 d). Persistence of infectious enterovirus was also not similar to the persistence of enterovirus and ENT genetic markers. These markers were detected 20 days longer than infectious enterovirus (28 d for markers versus 8 d for infectious virus) in microcosms exposed to sunlight. A close relationship between persistence of infectious enteroviruses and the human-specific *Bacteroidales* marker was seen in the sewage microcosm exposed to sunlight (both detected for 8 d) suggesting this marker could be useful in assessing health risks from exposure to waterborne enteric viruses. However, our comparison is one of relative persistence as we did not include a quantitative assay for infectious enteroviruses due to sampling volume constraints.

Multiple studies have explored the nature of viral infectivity and genome persistence over time in natural waters (Espinosa et al., 2008; Lipp et al., 2002; Noble and Fuhrman, 1997; Skraber et al., 2004; Tsai et al., 1995; Wetz et al., 2004). In our sewage microcosm exposed to sunlight we detected enterovirus genetic markers 3.5 times longer than infectious enteroviruses and twice as long in the dark microcosm. A similar observation was found for poliovirus when incubated in river water under dark conditions (Skraber et al., 2004). In unfiltered seawater (22 °C, in darkness), a 99% reduction in infectious poliovirus occurred after 4.5 days and detection of the genome by RT-PCR ceased after day 10 (Wetz et al., 2004). We detected infectious enteroviruses for 14 days and enterovirus genomes for the duration of our experiment (28 d) in the sewage microcosm kept in darkness. In fresh surface water incubated in the dark at 25 °C, poliovirus decayed at a rate of $-0.322\ d^{-1}$ as measured by RT-QPCR (Bae and Schwab, 2008). This rate is faster than we observed for seawater in the sewage microcosm ($-0.140\ d^{-1}$, in darkness). Loss of virus via culture or PCR-based detection is consistently higher in warmer waters (Bae and Schwab, 2008; John and Rose, 2005; Rzezutka and Cook, 2004; Wetz et al., 2004), due to increased grazing and enzyme activity (Sherr et al., 1988). This may explain our ability to detect infectious enteroviruses and genetic markers longer than previous studies – our water temperature was colder than previous studies. Our extended detection of enterovirus genetic markers over previous studies may also be due to the increased sensitivity of QPCR over conventional PCR.

4.5. Persistence of naked enterovirus RNA

We were surprised that naked PV3 RNA was detected for up to 14 days in the naked-genome microcosms (17 °C, in darkness). A previous study found that naked poliovirus RNA was not detected by RT-PCR after two days in unfiltered seawater incubated at room temperature in darkness (Tsai et al., 1995). The different results between our and Tsai et al.'s results may be due to differences in the starting concentrations of poliovirus RNA used in the experiments or the sensitivity of the two assays used for detection. No difference was observed in the persistence of naked poliovirus RNA between the treatments. This is somewhat consistent with the results of Simonet and Gantzer who showed that UV radiation-induced degradation of viral RNA followed first-order kinetics according to fragment size and that rates of degradation were low for fragments <200 bases (Simonet and Gantzer, 2006). The amplicon size for pan-enterovirus RT-QPCR is between 143 and 196 bases (PV3 genome yields a 143 bp amplicon). Perhaps if our RT-QPCR produced a larger product, we would have seen differences between light and dark treatments.

We did not observe a difference in decay of the *E. faecium* and PV3 genomes in the naked-genome microcosms ($p > 0.1$ for both light and dark treatments). This finding was unexpected because we hypothesized that the single stranded PV3 RNA genome would decay more rapidly than the ENT double

stranded DNA genome. One possible explanation for this result is that the pan-enterovirus primers target the 5′ untranslated region (UTR) of the RNA genome. This 5′-UTR contains secondary structure that could be responsible for the extended persistence of the enterovirus RNA marker. The comparison between first-order decay constants for these markers may be biased by the relatively poor fit of the first-order decay model used to estimate the RNA decay (see R^2 in Table 2). When comparing only the time until decay below the limit of detection, the PV3 RNA persisted until day 14 (dark) while the *E. faecium* genome was detected through day 18.

4.6. *Comparison of ENT and enterovirus marker decay in naked and sewage microcosms*

We compared decay rates of the ENT and enterovirus markers between the naked genome and sewage inoculated microcosms in order to elucidate the difference in persistence between encapsulated genomes and free genomes in our microcosms. In the naked-genome microcosms, enterovirus genomes did not decay at a rate that differed significantly from that of the enterovirus genomes in the sewage microcosms ($p > 0.1$ for both treatments). This was surprising because we hypothesized that the enterovirus capsid would provide protection to the genomic RNA and provide prolonged persistence relative to naked genomic RNA. We also found there was no difference in the rate of decay of the ENT markers between the sewage and naked-genome microcosms whether exposed to sunlight or kept in darkness ($p > 0.1$). This result was also unexpected because we anticipated that DNA genomes protected by cell membranes would decay less quickly than naked DNA. It is important to note that these comparisons may be biased by our assumption of first-order decay of targets, which based on the relatively low R^2 values for PV3 and *E. faecium* genome decay, may not be appropriate for naked nucleic acids (Table 2). If we compare the persistence of the targets in naked genome versus sewage microcosms, targets in the naked-genome microcosms fell below our detection limits more quickly than the targets in the sewage microcosms.

4.7. *Implications for water quality monitoring*

Caution should be exercised when extending results of microcosm studies to field settings. Nevertheless, the results reported here provide important information about the comparative persistence and decay of a human virus and health-relevant indicators in seawater. An ideal indicator of fecal pollution should be detected when fecal pathogens are present and persist in the environment in a way that is similar to fecal pathogens (Tamplin, 2003). Detection of cENT did not mimic detection of infectious enteroviruses; detection of cENT ceased three days before infectious enteroviruses were no longer detected. Therefore, enumeration of ENT using culture-dependent methods is probably not an adequate means of assessing risk of viral illness from exposure to seawater polluted with sewage. In contrast, both the ENT and enterovirus genetic markers were detected in the light and dark sewage microcosms until day 28, well past the last day infectious virus was detected. This suggests that detection of these genetic markers to assess risk may be overprotective. Assuming that the best indicator is one that persists the same amount of time as enteroviruses in seawater (Tamplin, 2003), our findings suggest that under the conditions of our study the best indicator for fecal pollution in marine waters may be the human-specific *Bacteroidales* genetic marker when detected using QPCR. This marker was detected through day eight in the sewage microcosm exposed to sunlight which was the same amount of time we detected infectious enterovirus in this microcosm.

5. Conclusions

- There is differential persistence among sewage-derived ENT, human-specific *Bacteroidales* and enterovirus genetic markers. Enterovirus and ENT genetic markers persisted longer than human-specific *Bacteroidales* genetic markers.
- The human-specific *Bacteroidales* QPCR marker was detected as long as infectious enteroviruses in the sewage-seeded seawater microcosm exposed to natural sunlight.
- Sunlight exposure exerted the greatest effect on decay of cENT and decay of the human-specific *Bacteroidales* genetic markers in the sewage microcosms.
- Sewage-derived ENT and enterovirus genetic markers disappeared at similar rates but were detected 2–3.5 times as long as infectious enteroviruses.
- Sunlight did not produce an effect on decay rates or persistence of the ENT and enterovirus genetic markers in the either the sewage or naked-genome microcosms.

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