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Detection limits and cost comparisons of human- and gull-associated conventional and quantitative PCR assays in artificial and environmental waters

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ABSTRACT

Some molecular methods for tracking fecal pollution in environmental waters have both PCR and quantitative PCR (qPCR) assays available for use. To assist managers in deciding whether to implement newer qPCR techniques in routine monitoring programs, we compared detection limits (LODs) and costs of PCR and qPCR assays with identical targets that are relevant to beach water quality assessment. For human-associated assays targeting *Bacteroidales* HF183 genetic marker, qPCR LODs were 70 times lower and there was no effect of target matrix (artificial freshwater, environmental creek water, and environmental marine water) on PCR or qPCR LODs. The PCR startup and annual costs were the lowest, while the per reaction cost was 62% lower than the Taqman based qPCR and 180% higher than the SYBR based qPCR. For gull-associated assays, there was no significant difference between PCR and qPCR LODs, target matrix did not effect PCR or qPCR LODs, and PCR startup, annual, and per reaction costs were lower. Upgrading to qPCR involves greater startup and annual costs, but this increase may be justified in the case of the human-associated assays with lower detection limits and reduced cost per sample.

1. Introduction

Recreational water quality monitoring often involves measuring fecal indicator bacteria (FIB) via culture-based enumeration methods by either membrane filtration (U.S. Environmental Protection Agency, 2006, 2010) or defined substrate technology (IDEXX, Westbrook, ME) (American Public Health Association et al., 2005). Molecular-based water quality monitoring methods for detection and quantification of host-associated fecal bacterial DNA can address some of the inherent drawbacks associated with culture-based methods (Boehm, 2007; Byappanahalli and Ishii, 2011; Ferguson and Signoretto, 2011; Kim and Grant, 2004) by not requiring overnight incubations and using host-associated genomic sequences to identify the original host of fecal pollution in environmental water samples.

For some fecal host sequences there exists both conventional polymerase chain reaction (PCR) and a more complex quantitative polymerase chain reaction (qPCR) detection assay option. Numerous performance comparisons of PCR and qPCR have been conducted previously in clinical research (Balamurugan et al., 2009; Carson et al., 2010; Dagher et al., 2004; Flori et al., 2004; Mygind et al., 2002) that indicates that the sensitivity improvement (if any) of qPCR over PCR is assay specific. The mechanisms for why qPCR can be more sensitive than PCR have been well described (Ginzinger, 2002; Smith and Osborn, 2009). While thermal cyclers

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for PCR are relatively inexpensive and readily available in many public health laboratories besides academic and research institutions, qPCR platforms are more expensive and less widely adopted.

The goal of this study is to present a comparison of environmentally relevant PCR and qPCR assay detection capabilities and costs to help inform decisions regarding whether an environmental laboratory should invest in new qPCR instrumentation. We focused this study on a human-associated PCR (HF183 Endpoint) and two human-associated qPCRs (HF183 Taqman, HF183 SYBR) all sharing the same target sequence. A gull-associated PCR (Gull2 Endpoint) and qPCR (Gull2 Taqman) sharing the same target sequence were also evaluated (Table 1). These five assays were chosen for their relevance in beach management decisions, since these sources can be abundant in coastal areas and (especially sewage/wastewater) can expose swimmers to potential pathogens in contaminated waters. All five assays in the current study have previously been evaluated for their specificity and sensitivity through a highly rigorous method evaluation study using artificial freshwater as the medium (Boehm et al., 2013). We expanded on this research by testing assay detection limits over a broader range of target concentrations and with targets diluted in creek and marine environmental water matrices. Additionally, we detail the startup, annual and per reaction cost differences between the assays.

2. Materials and methods

2.1. Method background

For this study, we used the standardized protocols (reagents and standards) that were tested in a large method evaluation study (Boehm et al., 2013). The standardized protocols (SOPs) used from the Boehm et al. (2013) study were HF183 Taqman, HF183 SYBR, HF183 Endpoint, Gull2 Taqman, Gull2 Endpoint, DNA extraction SOP, and a gel visualization SOP. Brief SOP details follow and full descriptions are provided in Supplementary materials.

2.2. Dilutions

2.2.1. Diluent water types

Either sewage or gull feces were spiked into three water types – artificial freshwater (AW, distilled water with 0.3 mM $MgCl_2$, 0.6 mM $CaCl_2$, and 1.4 mM $NaHCO_3$), environmental freshwater collected from a creek (CW), or environmental marine water (MW) collected from a coastal site (Table 2). Samples of environmental waters were collected within and at the discharge of a 47 km^2 watershed drained by Topanga Creek in the Santa Monica Mountains in Southern California. Over 70% of the watershed is undeveloped public, open space (GeoPentech, 2006). Approximately 3 L grab samples of CW and MW were collected on two occasions in the morning in polypropylene plastic bottles (prewashed with 10% HCl and rinsed three times with source water before collection) and immediately stored on ice for transport to the laboratory. Creek water was collected from actively flowing sections of the lower reach of this creek in Topanga State Park that did not indicate a significant presence of human fecal contamination during snapshot monitoring in 2011–2012 (data not shown). CW was collected at a site named Shale Falls for the sewage dilutions and at a site named Lower Brookside Drive (Dagit et al., 2013) for the gull feces dilutions. Marine water was collected on incoming waves in knee deep surf at Topanga State Beach approximately 100 m up-current from the outlet of Topanga Creek at a location that was free from a significant presence of human or gull fecal contamination during spot monitoring in 2011–2012 (data not shown). Physical and chemical parameters of CW and MW collected as the source water for the gull spiked samples were measured *in situ* using a Hydrolab Quanta Multiparameter Sonde (Hach Hydromet, Loveland CO) (Table 2).

2.2.2. Sewage dilutions

Primary influent sewage was obtained from the Orange County Sanitation District (Fountain Valley, CA) and transported on ice to the laboratory on the same morning that environmental water samples were collected. Three 1:20 dilutions of the sewage were generated using AW, CW, or MW as the water matrix. These dilutions were made by first spiking 20 ml of each matrix with 20 ml of sewage and then transferring 5 ml from these tubes into another 50 ml Falcon centrifuge tube preloaded with 45 ml of matching matrix type. The 1:20 dilutions in each matrix were equilibrated on a rotating platform for one hour at 24 °C. After equilibration, *Enterococcus* spp. (ENT) concentrations were measured for each dilution (Table 3) using the Enterolert with Quanti-Tray/2000 method (IDEXX, Westbrook ME). Ten ml volumes of each 1:20 dilution were then filtered onto Isopore 0.4 μm HTTP membrane polycarbonate filters (EMD Millipore, Billerica MA) depositing a final volume of 0.5 ml sewage onto each filter. Each filter was placed into a separate 2 ml polypropylene screw cap tube (Sarstedt Inc., Newton NC) preloaded with 0.3 g 212–300 μm (50–70 U.S. sieve) acid-washed glass beads (Sigma–Aldrich, St. Louis MO). Tubes containing filters were immediately archived at −80 °C until DNA extraction. These filters will be referred to as 0.5S-AW, 0.5S-CW, and 0.5S-MW (Table 3). For the purposes of negative controls, filters were created by filtering 10 ml of AW, CW or MW without prior spiking with sewage. These controls will be referred to as AW_NS, CW_NS and MW_NS.

2.2.3. Gull feces dilutions

Moist gull feces was collected at Mariners Point in Mission Bay San Diego, CA (32° 45′ 50″ latitude, −117° 14′ 47″ longitude) in a

Table 1
PCR and qPCR assays used in this study.

Name	Type	Target	Forward primer/reverse primer	Probe/dye	Amplicon size (bp)	Reference
HF183 endpoint	PCR	*Bacteroides* 16S	ATCATGAGTTCA CATGTCCG/CAATCGGAG TTCTTCGTG	N/A	525	Bernhard and Field, 2000
HF183 Taqman	qPCR	*Bacteroides* 16S	ATCATGAGTTCACATGTCCG/CGTAGGAGTT TGGACCGTGT	FAM-CTGAGAGGAA GGTCCCCCACA TTGGA-TAMRA	167	Haugland et al., 2010
HF183 SYBR	qPCR	*Bacteroides* 16S	ATCATGAGTTCACATGTCCG/TACCCCGCCT ACTATCTAATG	SYBR Green	82	Seurinck et al., 2005
Gull2 Endpoint	PCR	*Catellicoccus marimammalium*	TGCATCGACCTAAAGTTTTGAG/GTCAAAG AGCGAGCAGTTACTA	N/A	412	Lu et al., 2008
Gull2 Taqman	qPCR	*Catellicoccus marimammalium*	TGCATCGACCTAAAGTTTTGAG/GTCAAAG AGCGAGCAGTTACTA	FAM-CTGAGAGGGTGA TCGGCCACATTGGGACT-BHQ1	412	Shibata et al., 2010

Table 2
Water types. Environmental water was collected during dry weather for same day use in the sewage spiking event and again at the same sites two weeks later for the gull feces spiking event. Chemical and physical CW and MW values were measured *in situ* during the 7/17/2012 collection event.

Water	Spike	Location lat./long.	Date, Time	ENT MPN/100 ml	Temp. °C	Sp. cond. ms/cm	DO mg/L	pH	Salinity PSU	ORP mV	Turb. NTU
AW			7/1/12	<10	24	1.4	7.33	8.15	0.69	159	2.1
CW	Sewage	34° 3′ 26″/−118° 35′ 1″	7/2/12, 9:25a	41	N/D	N/D	N/D	N/D	N/D	N/D	N/D
MW	Sewage	34° 2′ 16″/−118° 35′ 5″	7/2/12, 10:10a	10	N/D	N/D	N/D	N/D	N/D	N/D	N/D
CW	Feces	34° 3′ 26″/−118° 35′ 1″	7/17/12, 6:20a	10	17.7	1.5	9.06	8.08	0.75	158	4.6
MW	Feces	34° 2′ 16″/−118° 35′ 5″	7/17/12, 6:50a	<10	19.56	52	7.28	7.66	33.9	160	N/D

N/D = not determined.

Table 3
Characteristics of extractions. Sewage and Gull feces refer to the amount that was filtered. Water Type indicates whether artificial (AW), creek (CW), or marine (MW) water was used as the matrix. *Enterococcus* spp. (ENT) values reported are the concentration prior to filtration. The DNA value is the concentration (as determined via absorbance at 260 nm) of the extracted eluent.

Filter	Sewage (ml/10 ml)	Gull feces (mg/10 ml)	Water type	ENT (MPN/100 ml)	DNA (ng/μl)
0.5S-AW	0.5		AW	15500	4
0.5S-CW	0.5		CW	29100	7
0.5S-MW	0.5		MW	21400	8
0.67G-AW		0.67	AW	199000	1
0.67G-CW		0.67	CW	242000	2
0.67G-MW		0.67	MW	242000	3

location that is known for a large congregation of avian species representing mostly gulls. Twenty five to 30 individual droppings were collected from beach sand with a sterile spatula into a single sterile 50 ml centrifuge tube, transported on ice to the laboratory, stored overnight at 4 °C, and processed the following day. AW was added to 16.7 g of feces to make up the final volume of the AW-feces mixture to 50 ml. Ten ml of this slurry was transferred to 40 ml of AW, CW, or MW for a 1:5 dilution. These 1:5 dilutions were further diluted 1:10 three times by adding 5 ml–45 ml of matrix in a 50 ml falcon tube for final dilutions of 1:5000. The 1:5000 dilutions were equilibrated on a rotating platform for one hour at 24 °C. After equilibration, ENT concentrations were measured for each dilution (Table 3) using Enterolert with Quanti-Tray/2000. Ten ml volumes of each 1:5000 dilution were then filtered onto Isopore 0.4 μm HTTP membrane polycarbonate filters (EMD Millipore, Billerica MA) depositing a final mass of 0.67 mg onto the filters. Each filter was placed into a separate 2 ml polypropylene screw cap tube (Sarstedt Inc., Newton NC) preloaded with 0.3 g 212–300 μm (50–70 U.S. sieve) acid washed glass beads (Sigma–Aldrich, St. Louis MO). Tubes containing filters were immediately archived at −80 °C until DNA extraction. These filters will be referred to as 0.67G-AW, 0.67G-CW, and 0.67G-MW (Table 3).

2.3. DNA extraction

DNA extraction of the 0.5S-AW, 0.5S-CW, 0.5S-MW, AW_NS, CW_NS, and MW_NS filters (Section 2.2.2) and the 0.67G-AW, 0.67G-CW, and 0.67G-MW filters (Section 2.2.3) were completed with the DNA-EZ ST1 Extraction Kit (GeneRite, North Brunswick NJ) (see Section S1 for SOP details). The extracted DNA was eluted into 100 μl of elution buffer and DNA concentration (Table 3) was determined using UV absorption with a Nanodrop 2000C (Thermo Scientific, Waltham MA).

2.4. PCRs and qPCRs

Working dilutions of the 0.5S-AW, 0.5S-CW, 0.5S-MW, 0.67G-AW, 0.67G-CW, and 0.67G-MW extractions (Section 2.3, Table 3) spanning up to 6 orders of magnitude were made using molecular biology grade water (MO BIO Laboratories, Inc., Carlsbad CA). HF183 Endpoint (Bernhard and Field, 2000) (Table S1, S2), HF183 Taqman (Haugland et al., 2010) (Table S3, S4), and HF183 SYBR (Seurinck et al., 2005) (Table S5, S6) SOPs were followed as written in Sections S2 and inoculated with 2 μl of template from the extractions and their working dilutions. Gull2 Endpoint (Lu et al., 2008) (Table S7, S8), and Gull2 Taqman (Shibata et al., 2010) (Table S9, S10) SOPs were followed as written in Section S3 and inoculated with 2 μl of template from the 0.67G-AW, 0.67G-CW, or 0.67G-MW extractions and working dilutions of these extractions.

qPCR reactions were amplified for 50 cycles in an Applied Biosystems StepOnePlus with instrument performance verified with a TaqMan RNase P Fast 96-Well Instrument Verification Plate (Life Technologies, Carlsbad, CA). PCR reactions were cycled 35 rounds in an Applied Biosystems GeneAmp PCR System 9700. Two μl of each PCR products were visualized and scored by eye (see Section S4 for SOP details) utilizing a FlashGel DNA System (Lonza, Allendale NJ). It should be noted that a less subjective digital analysis of the gel visualization was attempted with ImageJ computer software (Schneider et al., 2012), but the detection algorithm did not detect bands that were visually apparent (data not shown). The failure of the detection algorithm may be an artifact of the resolution (640 by 480 8-bit pixels) of the images taken by the camera in the FlashGel DNA System.

2.5. Statistical analysis

Statistical analyses were done using Stata version 12.1 (StataCorp, 2011). For human-associated assays, an initial linear regression was run to analyze performance differences of SYBR versus Taqman. To adjust for performance differences between water type, water was included in the model as a factor variable and an interaction term was included between feces/sewage concentration and water type to allow for differences in slope.

Separate analyses were also completed for SYBR and Taqman for comparison between assay performances between the three water types (AW, CW, and MW) for human and gull associated assays. Water type and feces/sewage concentrations were regressed on C_q values. Firth's logistic regression analyses (Firth, 1993) were performed to quantify the relationship between probability of detects and analyte (feces or sewage concentration). Firth's logistic regression analyses use a penalized likelihood estimation method to deal successfully with problems of separation (Heinze and Schemper, 2002). Likelihood ratio tests were completed comparing a full model, which allowed the slope to vary by water type, to a condensed model that allowed for a common slope across water types for each assay. Predicted probabilities of detection were computed for varying levels of sewage and feces concentration and were applied as in Burns and Valdivia (2007) to determine generalized detection limits (LODs) for each assay type.

2.6. LOD determinations

Many assumptions typically applied to the structure of the data distributions used for LOD analysis (Currie, 1995, 1968) fail for qPCR data (Burns and Valdivia, 2007). Using the methods described in Burns and Valdivia (2007), we constructed logistic models to determine LODs at a Currie detection decision, i.e. detection frequency, of 95%. Concentrations above the LOD are predicted by the logistic model to have a false negative rate lower than 5%. Confidence intervals (95%) are reported for each LOD. For PCR assays, samples were scored as detected when a reaction was visualized by eye as a band on an electrophoresis gel (Section 2.4) after 35 cycles. For qPCR, samples were scored as detected when an amplification signal greater than a fluorescence threshold of 0.03 (ΔRn) was detected within 40 thermal cycles. A PCR maximum cycle number of 35 and qPCR of 40 were determined as optimal by the SIPP study (Boehm et al., 2013). Any SYBR qPCR reactions with a melting temperature deviating greater than 0.8 °C from the expected value known for plasmid control products were scored as non-detects.

For this study, six replicate qPCR reactions of each dilution were analyzed, with the exception of HF183 SYBR MW which had an instrument failure that resulted in one less replicate at each dilution. For PCR assays, three replicate reactions of each dilution were analyzed, with the exception of HF183 Endpoint CW which was analyzed with 6 replicate reactions.

2.7. Cost analysis

HF183 Taqman qPCR, HF183 SYBR and HF183 Endpoint conventional PCR assays were used to model cost differences between PCR and qPCR. The PCR and qPCR thermal cycler prices used in the analysis are both quoted from Applied Biosystems to provide some level of consistency; however, prices from various manufacturers vary considerably. Annual maintenance costs are based on the instrument manufacturer's recommended procedures and frequencies. Consumables used in the cost analysis are, when possible, matched to the exact catalog numbers of those used in this study. The cost analysis does not include service contracts, many materials that are consistent between PCR and qPCR (such as pipettes and microcentrifuge tubes), technician training, or infrastructure requirements (such as dedicated rooms, refrigeration equipment, and hoods). No educational or other discounts were factored into the prices used for the cost analysis and all prices are in 2012 United States dollars and taken directly from the manufacturer.

The PCR TaKaRa Ex Taq DNA Polymerase RR001B Kit (Clontech, Mountain View CA) contains 200 μl of 1000 U TaKaRa Ex Taq DNA Polymerase, 4000 μl of 10X Ex Taq Buffer, 4000 μl of 25 mM $MgCl_2$ solution and 3200 μl of dNTP mixture (2.5 mM each dNTP). For each PCR reaction, 0.1 μl, 2.5 μl, 1.5 μl and 2 μl of Ex Taq Polymerase, buffer, $MgCl_2$ and dNTP mixture were required, respectively. The total number of reactions possible with one kit was calculated to be 1600 (Table S11 and S14).

qPCR primers, probes and plasmids (used for standard curves and positive controls) were purchased from Integrated DNA technologies (Coralville, IA). A 5-point plasmid standard curve, measured in triplicate, was used for each 96-well reaction plate in the qPCR assay in addition to three no-template negative controls. For PCR it was assumed that a single concentration standard would be run in triplicate along with three no-template negative controls. This limited the wells available for samples to 78 reactions per qPCR plate and 90 reactions per PCR plate. As such, the "Units/Sample" for the optical adhesive films and 96-well reaction plates are listed as 1/78 for qPCR (Tables S12, S13, S15) and 1/90 for PCR (Tables S11, S14). A plasmid containing sequences of the marker is used as a positive control for making standards. This plasmid is estimated to cost $240 per 4 μg. The cost of the plasmid per sample amounted to such a small cost that it is reported as $0.00 due to the very small amount of plasmid (3×10^{-7} ng) required to generate a 5-point dilution series that begins at 1×10^5 target copies (Tables S12, S13, S15).

3. Results

3.1. Human-associated assays

3.1.1. HF183 endpoint PCR

Extractions in each water type (Section 2.3) were serially diluted and the HF183 Endpoint PCR assay was tested for its ability to detect sewage at concentrations spanning 0.5 ml sewage/10 ml water to the equivalent of 1.4×10^{-7} ml sewage/10 ml water. In AW, CW and MW, a <100% detection rate was observed at and below a 5.0×10^{-3} ml sewage/10 ml, 2.5×10^{-2} ml sewage/10 ml and 5.0×10^{-3} ml sewage/10 ml concentration, respectively (Fig. 1A–C). Two replicates of the AW_NS, CW_NS, and MW_NS (Section 2.2.2) non-spiked negative control extractions were analyzed and none showed visible bands via gel visualization. One AW, three CW and two MW no-template-controls (NTC) were also run with none showing visible bands.

To predict a 95% detection frequency LOD and associated confidence intervals (CI) a Firth's logistic model was used. The model was run on combined data from all water types. We feel this is justified based on the results from the Taqman and SYBR data which showed no statistically significant (Taqman $P = 0.4092$, SYBR $P = 0.441$) influence of water type on assay LOD (Section 3.1.2, 3.1.3). The logistic model for HF183 Endpoint is plotted against sewage concentrations (Fig. 1A–C, black lines) and predicts an LOD of 4.5×10^{-2} ml sewage/10 ml water (95% CI = $1.8 \times 10^{-2} - 1.6 \times 10^{-1}$, Wald $Chi^2 = 143.7$, $P = 0.000$).

3.1.2. HF183 Taqman qPCR

Extractions in each water type (Section 2.3) were serially diluted and the HF183 Taqman qPCR assay was tested for its ability to detect sewage at concentrations spanning 0.5 ml sewage/10 ml water to the equivalent of 1.4×10^{-7} sewage/10 ml water. To determine LODs, the C_q results (Fig. 1G–I, grey points) were analyzed in a binary fashion. In AW, CW, and MW, a <100% detection rate was observed at and below a 5.0×10^{-5}, 5.0×10^{-4}, 2.5×10^{-4} ml sewage/10 ml concentration, respectively (Fig. 1D–F, grey points). Six replicates of the AW_NS, CW_NS, and MW_NS (Section 2.2.2) non-spiked negative control extractions did not amplify by 40 cycles. Six CW and MW no-template-controls (NTCs) were also run with none amplifying by 40 cycles, while 1 AW NTC had a C_q of 38.6 and the other 5 did not amplify by 40 cycles.

Neither the main effects term for water type ($F_{2,\ 73} = 0.92$, $P = 0.4092$) nor the interaction term between water type and sewage ($F_{2,73} = 0.01$, $P = 0.99$) were significant, indicating no significant performance difference between the three water types. A Firth logistic model was created with the combined HF183 Taqman AW, CW, and MW binary data. This combined model (Fig. 1D–F, grey lines, note: the model shown on each panel is identical and replicated for comparison purposes) predicts the HF183 Taqman LOD at a concentration of 6.4×10^{-4} ml sewage/10 ml water (CI 3.6×10^{-4}–1.4×10^{-3}, Wald $Chi^2 = 17.11$, $P = 0.000$).

3.1.3. HF183 SYBR qPCR

The HF183 SYBR qPCR assay was tested with the same dilutions used for the Taqman assay (Section 3.1.2). Results for HF183 SYBR assay were inconsistent in this study. Out of eight experimental runs, utilizing multiple batches of dye and enzymes, only three runs, one in each matrix type, were considered acceptable for data analysis. The five failed runs all showed poor amplification of positive controls. We were unable to determine the source of failure

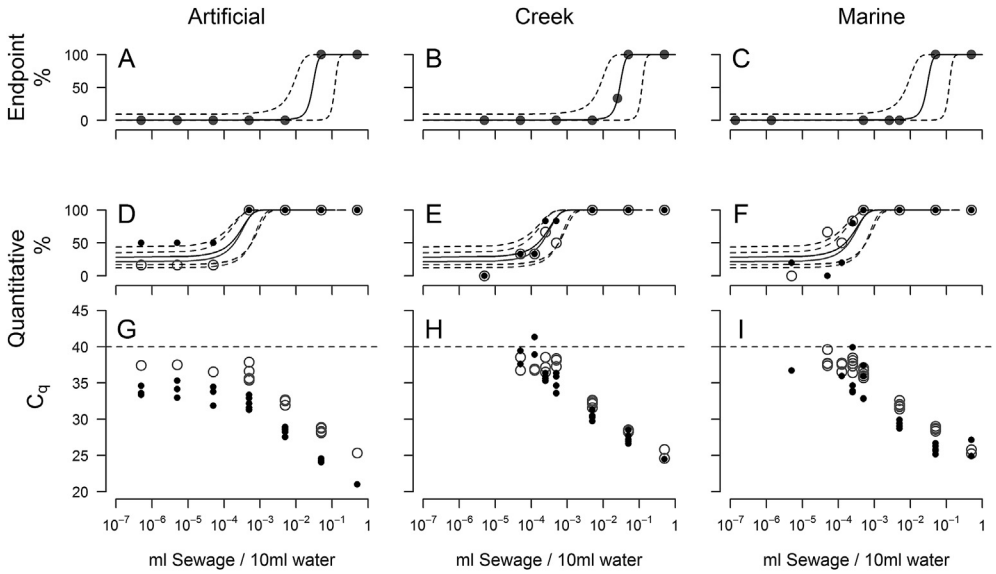

Fig. 1. Detection of the HF183 gene target in dilutions of extracted artificial, creek, or marine water spiked with sewage. Target was detected with either the PCR Endpoint assay, the Taqman qPCR assay, or the SYBR qPCR assay. A, B, C) The percentage of replicate reactions detected via gel visualization are indicated for Endpoint (grey filled circles). A logistic model of combined Endpoint data is shown (black line) on all three panels with corresponding CIs (dashed lines). D, E, F) The percentage of replicate reactions amplifying by 40 cycles are indicated for Taqman (open gray circles) and SYBR (black circles). Logistic models of Taqman (grey line) and SYBR (black line) are shown with corresponding CIs (dashed lines). G, H, I) Quantification cycle (C_q) is shown for Taqman (open grey circles) and SYBR (black circles).

and, moreover, every SYBR run had a complementing Taqman run on the same working dilutions that successfully amplified. To determine LODs, the C_q results of the successful runs (Fig. 1G–I, black points) were analyzed in a binary fashion. The MW SYBR qPCR run experienced an instrument malfunction (drifting baseline) in one column of samples resulting in only five replicates. In AW, CW, and MW, a <100% detection rate was observed at and below a 5.0×10^{-5}, 5.0×10^{-4}, 2.5×10^{-4} ml sewage/10 ml concentration, respectively (Fig. 1D–F, black lines). Three replicates of the AW_NS, CW_NS, and MW_NS (Section 2.2.2) non-spiked negative control extractions were analyzed. AW_NS had a C_q of 31.7, 32.3, and 32.2 with the last replicate melting temperature 5.6° off from expected. CW_NS had C_qs of 39.3, 38.2, and 38.7. MW_NS had a C_q of 37.7, ND, and ND. AW NTCs had C_qs of 32.5, ND, and 34.9. CW and MW NTCs did not amplify by 40 cycles.

For the HF183 SYBR assay, there was no significant difference on the slope of C_q on sewage between water types ($F_{2,71} = 0.83$, $P = 0.441$). However, the main effect was significant indicating a difference in average C_q value, after adjusting for sewage concentration, for the SYBR AW reactions when compared to the SYBR CW reactions ($\beta = 2.47$, $t = 2.58$, $p = 0.012$). A Firth logistic model was created with the combined HF183 SYBR AW, CW, and MW binary data. This combined model (Fig. 1D–F, black lines, note: the model shown on each panel is identical and replicated for comparison purposes) predicts an LOD of 6.5×10^{-4} ml sewage/10 ml (CI 3.5×10^{-4}–1.7×10^{-3}, Wald $Chi^2 = 13.47$, $P = 0.0002$).

The lower two concentrations (5×10^{-7} and 5×10^{-6} ml sewage/10 ml water) of the AW data and the 5×10^{-6} ml sewage/10 ml water MW sample have a detection frequency greater than zero, in spite of concentrations lower than the LOD. For AW, these detections along with those in non-spiked and NTC reactions suggest that the HF183 SYBR AW assay run had a low level of sewage contamination throughout the reactions. To explore the impact of these possible false positives on the model output, the HF183 SYBR combined water model was also run with these putative false positives changed to a 0% detection rate. This new (false positive removed) model predicts an LOD of 5.3×10^{-4} ml sewage/10 ml water (CI 3.0×10^{-4}–1.1×10^{-3}, Wald $Chi^2 = 19.0$, $P = 0.0$).

3.2. Gull-associated assays

3.2.1. Gull2 endpoint PCR

Extractions in each water type (Section 2.3) were serially diluted and the Gull2 Endpoint PCR assay was tested for its ability to detect feces at concentrations spanning 0.67 mg feces/10 ml water to the equivalent of 6.7×10^{-10} g feces/10 ml water. In AW, CW and MW, a <100% detection rate was observed at and below a 3.3×10^{-6}, 6.7×10^{-6}, and 6.7×10^{-6} mg feces/10 ml water, respectively (Fig. 2A–C). One reaction each of the AW_NS, CW_NS, and MW_NS (Section 2.2.2) non-spiked negative control extractions did not show visible bands via gel visualization. Two AW, CW, and MW no-template-controls (NTC) were also run with none showing visible bands.

A Firth logistic model (Fig. 2A–C, black lines) of the combined endpoint data was used to predict an LOD for Gull2 Endpoint at 9.8×10^{-6} mg feces/10 ml water (CI 4.3×10^{-6}–3.1×10^{-5}, Wald $Chi^2 = 143.7$, $P = 0.000$).

3.2.2. Gull2 Taqman qPCR

The Gull2 Taqman qPCR assay was tested with the same dilutions used for the Endpoint assay (Section 3.2.1). When the C_q results (Fig. 2G–I) are analyzed in a binary fashion AW, CW, and MW have a <100% detection rate at and below a 3.4×10^{-6}, 6.7×10^{-6}, 3.4×10^{-6} mg feces/10 ml water, respectively (Fig. 2D–F). Three replicates of the AW_NS, CW_NS, and MW_NS (Section 2.2.2) non-spiked negative control extractions did not amplify by 40 cycles. Three AW, CW, and MW no-template-controls (NTC) were also run with none amplifying by 40 cycles.

When water type and gull feces concentrations were regressed on C_q values neither the interaction of water type and feces ($F_{2,66} = 0.20$, $P = 0.82$), nor the water type main effects terms ($F_{2,66} = 0.68$, $P = 0.50$) were significant for feces concentration versus C_q value, indicating no significant performance difference between the three water types. To determine the LOD, a Firth logistic model was created with the combined Gull2 Taqman AW, CW, and MW binary data. This combined model (Fig. 2D–F, grey lines, note: the model shown on each panel is identical and replicated for comparison purposes) predicts an

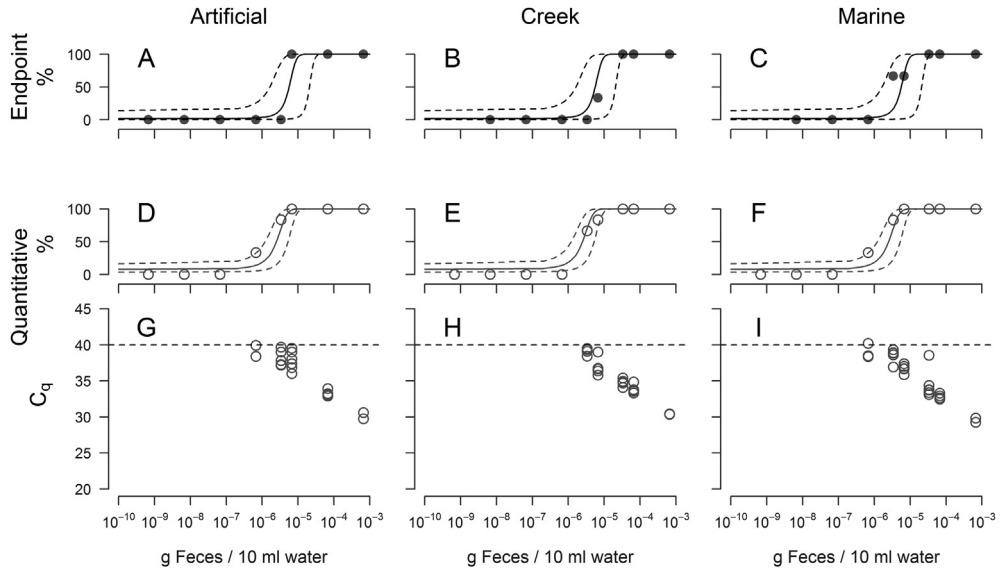

Fig. 2. Detection of the Gull2 gene target in dilutions of extracted artificial, creek, or marine water spiked with gull feces. Target was detected with either a PCR Endpoint assay or a Taqman qPCR assay. A, B, C) The percentage of replicate reactions detected via gel visualization are indicated for Endpoint (grey filled circles). A logistic model of combined Endpoint data is shown (black line) on all three panels with corresponding CIs (dashed lines). D, E, F) The percentage of replicate reactions amplifying by 40 cycles are indicated for Taqman (open circles). A logistic model of combined Taqman data is shown (grey line) on all three panels with corresponding CIs (dashed lines). G, H, I) Quantification cycle (C_q) is shown for Taqman (open grey circles).

LOD for Gull2 Taqman at 5.5×10^{-6} mg feces/10 ml water (CI 3.4×10^{-6}–9.9×10^{-6}, Wald $Chi^2 = 28.84$, $P = 0.000$).

3.3. Assays cost analyses

To assist managers in deciding which assays are best for their needs, the costs associated with setting up and running a qPCR versus a conventional PCR system were estimated (Table 4). The instrument costs, annual maintenance costs, and assay consumable costs were compiled for HF183 Endpoint PCR (Table S11), HF183 SYBR qPCR (Table S12), HF183 Taqman qPCR (Table S13), Gull2 Endpoint PCR (Table S14), and Gull2 Taqman qPCR (Table S15). The PCR instrument cost (Table S11 and S14) is about 51% of the cost of a qPCR instrument (Table S12, S13, S15). PCR instrumentation requires a temperature validation every two years requiring a $6097 temperature verification kit which is included in the instrument cost. Alternatively, temperature validation may be included with a service contract if one is purchased (service contract costs are not included in this study). Excluding a Temperature Verification Kit reduces the PCR startup cost to $9037 or 30% of the qPCR startup cost.

qPCR instrumentation requires a calibration and performance verification test every 18 months resulting in a qPCR annual maintenance cost of $716 (Table S12, S13, S15). PCR has no associated annual costs (Table S11 and S14).

For both HF183 and Gull2 assays the Endpoint PCR cost per reaction is approximately 63% lower than the Taqman qPCR cost per reaction (Table 4). While the Endpoint reactions have a $0.43 per reaction cost associated with FlashGel visualization that qPCR assays do not have (Table S11 and S14), the higher costs of the Taqman polymerase, primers, and probes (Table S13 and S15) more than offsets the visualization costs leading to Taqman assays being the most expensive option. The SYBR assay cost per reaction is 56% lower than the Endpoint PCR assay (Table 4) due to the primers and polymerase being approximately the same cost for both assays and the SYBR assay not requiring a FlashGel visualization step (Table S11 and S12).

Table 4
Cost comparison between PCR and qPCR assays.

Assay name	PCR or qPCR	Cost/reaction (US$)	Annual cost (US$)	Startup cost (US$)
HF183	Endpoint	0.93	0	15,100
	SYBR	0.52	716	29,900
	Taqman	1.51	716	29,900
Gull2	Endpoint	1.08	0	15,100
	Taqman	1.70	716	29,900

4. Discussion

4.1. Detection discussion

LODs in this study were determined at the 95% detection frequency threshold using a logistic modeling approach (Burns and Valdivia, 2007). When an LOD model was rerun with possible false positive samples adjusted to non-detects (Section 3.1.3), the new model did not predict a significantly different LOD than the unmodified model, given the CIs of the LODs. This suggests that using a Firth logistic model approach to predicting LODs is a robust technique not overly sensitive to low levels of false positives. We found that the HF183 qPCRs have a lower detection limit than their respective PCR. The Boehm et al. (2013) study, which tested the protocols used in this study across several labs, found varying sensitivity (between 80% and 100%) for HF183 Endpoint, most labs reporting 100% sensitivity for HF183 SYBR, and all labs reporting 100% sensitivity for HF183 Taqman. This is in strong agreement with the findings of this study that the qPCR version of the HF183 assay are better at detecting low levels of target DNA. We did not find a lower detection limit for Gull2 qPCR versus Gull2 PCR. Again, the Boehm et al. (2013) study found similar results with both Gull2 assays sensitivities reported from ~60% to 100%. These results show that the improvement of qPCR detection limits over PCR are not to be assumed and are assay specific. This is similar to previous medically-related comparisons of PCR versus qPCR which also found a range of detection improvement from zero to two orders of

magnitude (Balamurugan et al., 2009; Carson et al., 2010; Dagher et al., 2004; Flori et al., 2004; Mygind et al., 2002). All five assays considered in this study performed similarly in artificial, creek, and marine waters. Staley et al. (2012) tested HF183 Taqman against sewage spikes in 5 different water types (tannic, river, lake, estuarine, and marine) on two dates and reported an average process LOD dilution of 10^{-5} with a standard deviation of 1 order of magnitude. This level of variation is greater than that reported in this study and suggests that assay analytical variation needs to be monitored per location. The results from Staley et al. (2012) and this study both indicate that the HF183 PCR and qPCR environmental assays are not greatly affected by a diverse range water matrix types.

The greater performance difference between the human versus the gull assays could be due to the reduction in qPCR product size for the HF183 Taqman and HF183 SYBR assays (Bernhard and Field, 2000; Haugland et al., 2010; Seurinck et al., 2005). The Gull2 primers and amplicon sizes are equivalent (Lu et al., 2008; Shibata et al., 2010) which suggests that optimization is possible for the qPCR assay that may lead to a lower LOD. The SYBR based version of the Gull2 assay not tested in this study uses the same primers as Endpoint and Taqman and therefore may have a similar LOD.

4.2. Cost comparison discussion

The improvement of detection of HF183 qPCRs over PCR (Section 4.1) comes at an increased instrument and maintenance costs. HF183 Taqman reaction costs are more than Endpoint while, in contrast, HF183 SYBR costs less per reaction. This lower cost may be negated by the much higher failure rate of the SYBR reactions. In our experiments we experienced a failure rate as high as 60% with the HF183 SYBR assay. We do not feel that this failure level is typical in our lab, but we were unable to determine the cause. If this high failure rate were to persist, then 2.5 times the number of SYBR reactions would need to be run to analyze the same number of samples as Endpoint or Taqman. This would increase the equivalent per reaction cost for SYBR to $1.30. This equivalent per reaction cost is now greater than Endpoint but still lower than Taqman. If the failure rate of HF183 SYBR is not considered, then the higher startup and annual costs of the HF183 SYBR versus HF183 Endpoint will eventually be offset by the lower cost per reaction (Table 4). The time it will take for HF183 SYBR to become less expensive than HF183 Endpoint is dependent on the number of reactions run per year. For example, if a laboratory is analyzing 9600 reactions per year (100 96-well plates), then the two assays will reach an equivalent “break-even” cumulative cost by 5 years (Figure S1). There is no break-even point for the Gull2 assays tested in this study. Since the SYBR version of Gull2 uses the same primers as Gull2 Endpoint, it can be assumed to have a similar reduction in the per reaction costs as the HF183 assays. Further investigation into the performance of the possibly less expensive SYBR qPCR version of Gull2 is warranted.

4.3. Shortcomings of this study

Not all managers may find the HF183 and Gull2 assays relevant to their work. Larger studies have evaluated a broad range of assays that associate with other fecal host animals but these studies tend to focus more on specificity and sensitivity (Boehm et al., 2013; Shanks et al., 2012). We are not aware of a large scale LOD study using similar techniques as this study. Therefore, managers may be forced to either run LOD analyses on their assays of interest or extrapolate upon the results presented here. It should also be noted that the FlashGel DNA System used with the endpoint assays in this study uses a proprietary DNA stain reported to be more sensitive than ethidium bromide (Riley and White, 2008). Therefore, labs using ethidium bromide visualization may experience an even greater difference between PCR and qPCR LODs than this study determined. More replicates analyzed at each concentration would improve the LOD model confidence levels and possibly allow for distinguishing performance differences within water matrices or between Gull2 Endpoint and Gull2 Taqman. One obvious difference between PCR and qPCR not addressed in this study is the ability of qPCR to go beyond target detection to target quantification. While determining target levels is an important piece of information, the interpretation of this information is still in its infancy and important aspects such as marker decay rate in the environment are still being established. Due to this and other factors, many researchers believe that the most conservative interpretation of qPCR data is presence versus absence and that frequency of detection, not magnitude of detection, is the most appropriate metric for policy relevant situations (Cao et al., 2013). Finally, a significant limitation of this study is the continual lowering of costs associated with PCR and qPCR methods. Managers are advised to use the cost analysis in this study as a guide for their own cost analysis with current prices.

4.4. Other factors to consider if deciding to upgrade

In addition to differences in LOD and cost, there are several other factors that may influence the decision to invest in either an endpoint or qPCR technology. First, qPCR requires the preparation, storage, and handling of reference DNA standard materials. Previous studies have shown that these materials are critical for the successful estimation of DNA target concentrations and that improper use can lead to erroneous results (Shanks et al., 2012). Second, it is well documented that the amplification of DNA targets isolated from environmental samples can partially or completely inhibit PCR-based methods (Wilson, 1997). While strategies are available to detect inhibition in endpoint PCR methods (Shanks et al., 2006), these methods can only detect the presence or absence of complete amplification inhibition. In contrast, there are many strategies available to characterize the presence of amplification inhibition (even partial) with qPCR technologies (King et al., 2009). Because environmental water samples can harbor many types of substances that only partially inhibit amplification, the use of qPCR inhibition screening strategies may result in a lower incidence of false negatives and higher confidence in results. Third, substances that can co-extract with DNA target from environmental samples can not only lead to partial or complete inhibition, but may also interfere with DNA target isolation (Stoeckel et al., 2009). Just like inhibition, endpoint PCR strategies are available, but they can only identify the complete failure of the DNA isolation step (for example see Rossen et al., 1992), whereas qPCR methods offer more refined strategies that can detect more subtle changes in the efficiency of DNA recovery needed for accurate quantification (Fredricks et al., 2005). Fourth, DNA amplification-based technologies can be severely confounded by the presence of contaminating DNA molecules. It is important to consider that the lower the LOD is for a given method, the easier it is to potentially contaminate experiments with extremely low concentrations of extraneous DNA. Finally, it is important to consider the amount of training required for laboratory personnel. While both methods are very similar in terms of mixing reagents, qPCR methods require additional steps where a high level of proficiency is required for successful application. For qPCR, small deviations in protocols can lead to large differences in results (Shanks et al., 2012). As DNA amplification-based technology use becomes more widespread for water quality monitoring applications, it will be necessary for sample processing laboratories to carefully consider and weigh all these issues before selecting to invest in endpoint PCR or qPCR technology.

4.5. Conclusions

In conclusion, there was not a predictable difference in LOD between endpoint and qPCR assays evaluated in this study. For the human-associated HF183 assays, the Taqman and SYBR LODs are 70X lower than the Endpoint LOD (but SYBR performed erratically on a run by run basis). In contrast, for the gull-associated Gull2 assays, the Taqman LOD is not statistically distinguishable from Endpoint LOD. Results for HF183 and Gull2 were not affected by artificial, creek, or marine water matrices indicating the assays evaluated can be robust across waters tested in this study. Instrument and annual maintenance costs of qPCR methods are more expensive than endpoint PCR. SYBR based assays can be cheaper per reaction (if failure rate is low enough) than endpoint assays, while Taqman based assays are more expensive. If enough reactions are run per year the increased startup costs of SYBR qPCR can be offset by the reduced reaction costs. The significant LOD improvement of HF183 qPCR assays compared to Endpoint may help justify the greater costs for human-associated qPCR methods while the similar performance of the gull-associated assays does not justify adapting Taqman qPCR technology (a SYBR version may be preferred but was not tested in this study).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jenvman.2014.01.029.

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Performance and specificity of the Cov-IMS/ATP method for rapid detection and enumeration of enterococci in coastal environments

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Running Head: Cov-IMS/ATP for coastal water quality assessment

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Abstract

The performance and specificity of the Covalently-linked Immunomagnetic Separation/ Adenosine triphosphate (Cov-IMS/ATP) method for the detection and enumeration of enterococci was evaluated in recreational waters. Cov-IMS/ATP performance was compared with standard methods: defined substrate technology (Enterolert, IDEXX Laboratories), membrane filtration (EPA Method 1600), and an Enterococcus-specific qPCR assay (EPA Method A). We extend previous studies by 1) analyzing the stability of the relationship between the Cov-IMS/ATP method and culture-based methods at different field sites, 2) evaluating specificity of the assay for seven ATCC *Enterococcus* species, 3) identifying cross-reacting organisms binding the antibody-bead complexes with 16S rRNA gene sequencing and evaluating specificity of the assay to five non-enterococci species, and 4) conducting preliminary tests of preabsorption as a means of improving the assay. Cov-IMS/ATP was found to perform consistently and with strong agreement rates (based on exceedance/compliance with regulatory limits) of between 83% and 100% when compared to the culture-based Enterolert method at a variety of sites with complex inputs. The Cov-IMS/ATP method is specific to five of seven different *Enterococcus* spp. tested. However, there is potential for non-target bacteria to bind the antibody which may be reduced by purification of the IgG serum with preabsorption at problematic sites. The findings of this study help to validate the Cov-IMS/ATP method, suggesting a predictable relationship between the Cov-IMS/ATP method and traditional culture-based methods, which will allow for more widespread application of this rapid and field portable method for coastal water quality assessment.

Keywords: Adenosine triphosphate quantification, *Enterococcus*, fecal indicator bacteria, Immuno-magnetic separation, rapid method, recreational water quality

40 **Introduction**

41 Pollution from diverse and numerous sources, as well as extreme variability in
42 enterococci concentrations, make microbial source tracking (MST) challenging (1-3). The
43 development of rapid detection technologies has advanced significantly in recent years (4-6).
44 Such assays enumerate microbial contaminants in as quickly as one hour and may be more
45 protective of human health for monitoring as well as beneficial for MST when compared to
46 traditional methods (7). Traditional reporting has relied on culture-based methods (*e.g.* defined
47 substrate technology, membrane filtration), which can take between 24-48 hours to yield results,
48 making these methods less effective for assessment of short duration beach contamination events
49 and MST (5, 8). The method explored in this study, Covalently-linked Immunomagnetic
50 Separation/ Adenosine Triphosphate (Cov-IMS/ATP), is field portable and the quickest of the
51 current rapid methods being explored for coastal water quality assessment; environmental
52 enterococci concentrations can be enumerated in marine and fresh waters within one hour of
53 sample collection (9). Further, the Cov-IMS/ATP method measures ATP of viable bacteria only,
54 potentially allowing for better comparison with traditional culture-based technologies than
55 nucleic-acid based technologies. Cov-IMS/ATP also has reasonable startup costs and is user-
56 friendly, eliminating the need for highly experienced technicians.

57 Immunomagnetic separation (IMS) has been used in the past for isolation and
58 measurement of *Giardia* (10, 11) and *Cryptosporidium parvum* (12). IMS for isolation in
59 combination with DAPI for enumeration of *Giardia* and *C. parvum* in drinking water is U.S.
60 EPA approved. More recently, IMS/ATP has been used to analyze recreational water quality.
61 Lee and Deininger first applied the IMS/ATP assay to measure *E. coli* in recreational freshwater
62 in 2004 (13). The IMS/ATP assay was later optimized by Bushon to quantify *Enterococcus* in

63 recreational water (14) and *Enterococcus* and *E. coli* in wastewater (15). The selective magnetic
64 bead–antibody complex applied in these studies relied on hydrophobic interactions between the
65 antibody and the magnetic bead as the primary attachment mechanism for isolation of target
66 organisms from environmental samples. Lee et al. (2009) optimized the use of the IMS/ATP
67 assay to quantify *E. coli* and *Enteroccous* in marine waters with the development of the Cov-
68 IMS/ATP assay. The Cov-IMS/ATP assay relies on a more robust covalently-linked antibody-
69 bead complex that cannot be destabilized as easily as the original adsorption-based complex (9).
70 These earlier studies suggest that the *Enterococcus* IMS/ATP method can be useful for rapid
71 assessment of coastal water quality assessment in fresh and marine waters; however, Cov-
72 IMS/ATP has only been validated at a few sites, and its application potential as well as
73 limitations have not been expressly evaluated. Further, Bushon et al. (2009) found that the
74 IMS/ATP assay was site specific, requiring a different calibration curve when comparing the
75 assay to culture-based methods at different sites (15).

76 Differential specificity has been reported to lead to intrinsic differences in enumeration
77 by mEI (Difco, Becton, Dickinson and Company, San Jose, CA) and Enterolert (IDEXX) media
78 (16) and potentially may influence site specific performance of the Cov-IMS/ATP assay. A
79 similarly constructed IgG *Enterococcus* antibody showed potential for cross-reactivity (17), and
80 the specificity of the polyclonal *Enterococcus* antibody used in the Cov-IMS/ATP assay has not
81 been examined (18).

82 In this study, performance of the Cov-IMS/ATP assay is evaluated and compared to
83 traditional culture-based methods and an *Enterococcus*-specific qPCR assay at several complex,
84 diverse environmental sites through development of calibration curves with both ambient
85 concentrations of enterococci and wastewater-spiked ambient water. Further, specificity of the

Cov-IMS/ATP assay is tested against different strains of *Enterococcus*. In addition, potential cross-reacting organisms are identified with 16S rRNA gene sequencing, and the ability of the assay to detect non-enterococci species is evaluated. In the final stage of the study, an additional preabsorption step is evaluated for potential to decrease non-specific binding of the antibody-bead complex. These results help validate the Cov-IMS/ATP method for successful application of the assay to coastal water quality assessment and for identification of hot spots of fecal contamination, through rapid in-field enumeration of enterococci.

Materials and Methods

Cov-IMS/ATP Methodology

Enterococcus spp. antibodies (cat #B65173R, Meridian Life Sciences) and Dynabead particles (Invitrogen, Carlsbad, CA) were used to generate antibody-bead complexes. Dynabead particles (M-280) are uniform, superparamagnetic, polystyrene beads functionalized with sulfonyl ester groups permitting covalent binding to immunoglobins. *Enterococcus* spp. antibodies (polyclonal IgG) were applied for isolation of target organisms.

Samples were processed according to the Cov-IMS/ATP method developed by Lee et al. (2009) with several modifications (9). Briefly, 200 µL of Dynabeads were washed in borate buffer in phosphate-buffered saline (PBS) (10% borate buffer w/v in PBS, pH 9.5) and separated for one minute from solution using a magnet. After two washes, the clean Dynabeads were added to 40 µL of IgG solution creating an anti-ent biosorbent. This antibody-bead complex was incubated at 37°C for 18-24 hours. Following incubation, the anti-ent biosorbent mixture was stored in bovine serum albumin (0.1% BSA w/v in PBS) buffer at continuous rotation at 4°C for up to two weeks.

For sample analysis, one mL of sample was incubated with 200 µL of the antibody-bead complex for 40 minutes on a Dynal Rotary Mixer (Life Technologies, Grand Island, NY) in 1.7 mL low binding microcentrifuge tubes. A magnetic separator was used to separate the biosorbent with bound target enterococci from remaining solution. Bound complexes were washed twice with one mL of tween 20 in PBS (1% Tween w/v in PBS). Following, bound complexes were washed once with 200 µL somatic cell-releasing agent and lysed with the addition of 200 µL bacterial releasing agent. Supernatant was plated in duplicate on a white, 96 well plate and 100 µL of Bactiter-Glo reagent (Promega, Madison, WI) was added to each well. A luminometer (model GloMax® Microplate Multimode Reader, Promega) quantified luminescence in relative light units (RLU) for all analyses except sewage-spiked calibration curves. A handheld lumoinometer (model 3550, New Horizons Diagnostics, Baltimore, MD) was used to quantify luminescence for sewage-spiked calibration curves.

Culture-based methods

Measurements made by Cov-IMS/ATP were compared with counts of *Enterococcus* determined by two standard methods, USEPA Method 1600 and defined substrate technology (Enterolert, IDEXX). For membrane filtration (MF) (USEPA Method 1600), water samples were filtered (1-5 mL for ambient and 0.1-1 mL for spiked samples, depending on sample concentration) on a GN-6 mixed cellulose-gridded membrane filter with a standard platform manifold (in triplicate) and incubated on mEI agar (BD, Franklin Lakes, NJ, USA) for 24 hours at 41°C (19). Presumptive enterococci colonies were then enumerated as colony forming units (CFU) per 100 mL. Detection of *Enterococcus* with Enterolert Quanti-Tray 2000 was performed according to the manufacturer's instructions (IDEXX Laboratories,Westbrook, ME). Environmental creek and marine samples were diluted to1:10 and spiked samples were diluted to

no more than 1:1000. Positive identification of presumptive enterococci was determined by samples presenting fluorescence under UV light (365 nm) and quantified in units of most probable number (MPN) per 100 mL.

Quantitative PCR

Cell densities of *Enterococcus* were determined by quantitative PCR (qPCR) using USEPA Method A (20). For the measurement of *Enterococcus* gene copies per 100 mL, sample water was filtered through 47 mm, 0.4 µm pore size, HTTP polycarbonate filters (EMD Millipore, Billerica, MA) in duplicate. Each filter was placed in an individual two mL polypropylene screw cap tube, containing 0.3 g, 212 – 300 µm (50 – 70 U.S. sieve) acid washed glass beads (Sigma-Aldrich, St. Louis, MO) and stored at -80°C until DNA extraction. Filter blanks, consisting of 50 mL of PBS passed through the polycarbonate filter, were also generated with each set of processed samples.

Briefly, the PCR reaction mixture consisted of 2 µL of DNA template added to 12.5 µL 1x ABI Universal Master Mix, 2.5 µL of 2 mg mL^{-1} BSA, 3.5 µL of primer/probe working solution and 4.5 µL molecular grade RNase free water for a final reaction volume of 25 µL. The reaction was cycled at 50^{o}C for two min, 95^{o}C for 10 min, 40 cycles of 95^{o}C for 15 sec, and 60^{o}C for two min. Primer and probe sequences are detailed in Table 1. All qPCR samples were run in triplicate. Standard curves covering five orders of magnitude were constructed with serial dilutions of known amounts of genomic DNA extracted from *E. faecalis* ATCC 29212. Quantification thresholds (Cq) were converted into units of gene copies using a pooled master standard calibration curve (efficiencies 90-105%, R^2>0.99). Environmental samples were spiked with salmon testes DNA to assess for possible inhibition (21). Samples were not found to be inhibited in the study, with sample values deviating <1 Cq from internal controls.

155 All four methods were run in parallel in order to calibrate Cov-IMS/ATP measurements
156 of relative light units (RLU) to standard units of CFUs, MPNs, and gene copies per 100 mL.

157 ***DNA Extraction***

158 DNA was recovered according to manufacturer's guidelines of the DNA-EZ ST1
159 Extraction Kit (GeneRite, North Brunswick NJ). Extracted DNA was eluted into 100 µl of
160 elution buffer and aliquots were stored at -20^{o}C until analysis with qPCR. DNA concentration
161 was determined using UV absorption with a Nanodrop 2000C (Thermo Scientific, Waltham
162 MA). A preloaded tube (containing 0.3 g of glass beads) was extracted in the same manner as a
163 sample and used to assess any possible contamination during the extraction process (extraction
164 blank). Additionally, extraction efficiency was evaluated by spiking a preloaded tube with 1µL
165 of sewage extracted DNA, known to amplify at a specific cycle threshold value (Cq).

166 Bacteria attached to the bound portion of the bead-antibody complex were also identified
167 by sequencing. DNA was extracted according to Shanks et al. 2012 (22). Universal primers were
168 used to amplify partial 16S rRNA genes by PCR (Table 1). The UltraClean PCR Clean-Up kit
169 (MoBio Laboratories, Carlsbad, CA) was used according to manufacturer's guidelines. Further
170 processing and sequencing of the 16S gene was performed at UCLA Genotyping and Sequencing
171 Core (GenoSeq, Los Angeles, CA). Sequences were realigned with CLUSTALW (SDSC
172 WorkBench 3.2) and blasted against the NCBI nucleotide database (NCBI-BLAST).

173 ***Sample Collection and Processing- Environmental Calibration Curves***

174 Grab samples were collected by immersing a two L polypropylene bottle at the water
175 surface for creek and lagoon samples and at ankle depth (approximately 40 cm) for marine
176 samples. Approximately 400 - 500 mL of sample was filter concentrated and analyzed using the
177 Cov-IMS⁄ATP assay. Samples were filtered through a 0.45 µm filter (SA1J792H5; Millipore),

and bacteria were resuspended by vortexing the filter for 1 min in 10 mL of PBS (1x pH 7.2). One mL of the resuspended solution was added to the anti-ent biosorbent and processed according to methods described previously. The remaining sample was analyzed with Enterolert, MF, and qPCR for enterococci at each site, respectively.

Water samples were collected on multiple field days between August 2012 – June 2013 from Topanga State Beach, California; Doheny State Beach, California; and Tijuana, Mexico (for full site descriptions and sampling details see Supplemental Information Table S1). At Topanga State Beach, coastal and lagoon samples were collected. Potential sources of fecal indicator bacteria (FIB) include significant bird populations, local septic systems, dogs, lagoon input, algae and kelp. At Doheny State Beach five sites were sampled – three ocean sites and two at the discharge points of Harbor Creek and San Juan Creek. Potential sources of FIB include transient populations as well as faulty infrastructure and significant bird populations.

Samples were also collected from eight sites in Tijuana, Mexico, approximately 17 miles south of the US-Mexico border. Samples were collected from ocean sites as well as four freshwater inputs that discharge directly into the ocean including Real del Mar Creek, San Antonio de los Buenos (SADB) Creek and two storm drains (San Antonio del Mar drain and Isla drain). SADB wastewater treatment plant discharges approximately 25 MGD of secondary treated and chlorinated sewage into the SADB Creek, making up the majority of the creek flow (23). Sources of fecal pollution to ocean water include raw sewage from inadequate or lack of treatment, dogs, and gulls.

Sample Collection and Processing- Sewage Spiked Calibration Curves

Grab samples were collected, as stated above, for sewage-spiked calibration curves at Topanga State Beach (34º2’19.67”, -118º34’56.21”), Topanga Lagoon (34º2’19.85”,

-18º34'58.42"), Topanga Creek (34º3'50.48", -118º35'13.95"), Doheny State Beach (33º27'40.40", -117º40'54.45") and Santa Monica Beach (34º0'12.41", -118º29'29.57"). Primary influent collected same day from the Orange County Sanitation District (Fountain Valley, CA) was serially diluted in ambient waters and analyzed with the Cov-IMS/ATP and Enterolert methods.

Cov-IMS/ATP Specificity: Enterococcus spp.

Specificity of the Cov-IMS/ATP assay was tested with seven ATCC strains representative of *Enterococcus* species found in environmental waters: *Enterococcus hirae* (ATCC 8043), *Enterococcus mundtii* (ATCC 43186), *Enterococcus durans* (ATCC 6056), *Enterococcus faecium* (ATCC 35667), *E. faecalis* (ATCC 29212), *Enterococcus casselflavus* (ATCC 12755), and *Enterococcus gallinarum* (ATCC 70025) (Difco, Becton, Dickinson and Company, San Jose, CA). Each ATCC strain described was grown to semi-logarithmic phase and adjusted to a concentration of 10^8 cells mL^{-1} PBS (1x, pH 7.4) using optical density (OD 595) before being serially diluted ten-fold in phosphate-buffered saline (1x, pH 7.4) to concentrations ranging between approximately 10 cells mL^{-1} and 1000 cells mL^{-1}. Standard curve measurements were taken in parallel by the MF, Enterolert, qPCR, and the Cov-IMS/ATP assays as described above.

Cov-IMS/ATP Specificity: Non-Target Bacteria

Samples from each field site were evaluated for potential binding to non-enterococcal bacteria. After the bead-antibody complex had been incubated with environmental sample for 40 minutes, a subset of the bound antibody-bead complex was plated on nutrient agar (pH 7.2, Becton Dickinson and Company) and/or tryptic soy agar (pH 7.2, Becton Dickinson and Company) for 24 hours at 37°C. Following incubation, approximately 50 isolates were randomly

selected and purified. A subset of these isolates was then further purified and sequenced using 16S rRNA primers described above.

The IgG serum was further tested for cross-reactivity with five non-target bacterial species, four of which were previously identified by our laboratory in high prevalence from Doheny and Topanga beach water by 16S rRNA sequencing. *E. faecalis* and potential cross reactors were grown to semi-logarithmic phase and adjusted to a concentration of 10^8 cells mL^{-1} PBS (1x, pH 7.4) using optical density (OD 595) before being serially diluted to a starting concentration of 100 cells mL^{-1} PBS (1x pH 7.4). A standard curve generated with *E. faecalis* was compared to a standard curve generated with *E. faecalis* spiked with an equivalent volume of *Staphylococcus gallolyticus* (no ATCC number provided), *Bacillus cereus* (ATCC 14579), *Acinetobacter calcoaceticus* (ATCC 23055), *Enterobacter cloacae* (ATCC 13047), or *S. saprophyticus* (ATCC 15305), to test for possible cross-reactivity of the non-target bacteria with the IgG serum.

Preabsorption process for purified antibody-bead complexes

The antiserum was purified by preabsorption with mixtures of *S. saprophyticus*, *E. cloacae,* and *B. cereu*s, bacterial species found in marine waters and isolated with high prevalence from the bound-bead population. Cell cultures used for preabsorption were initially grown for 24 hours at 37 °C. Bacterial densities were adjusted to 10^8 cells mL^{-1} using optical density (595 nm). One mL of each bacterial strain was centrifuged at 12,000 x g at room temperature for ten min. The pellet was then washed and re-suspended three times in one mL of 0.1% Tween in PBS (1x pH 7.4). Fifty µL of the cell pellet was then used to inoculate and preabsorb the antibodies (500 µL) according to Saraswat (1994) (24). Following a 40 min incubation period, the cell-antibody solution was centrifuged at 12,000 x g for 10 min at room

247 temperature. Supernatant was then retrieved and the concentration of the purified antibody serum
248 was measured using the ELISA IgG Rabbit assay (Immunology Consultants Laboratory,
249 Portland, OR). The Cov-IMS/ATP assay was run in parallel using purified antibody serum and
250 non-preabsorbed antibodies to compare potential performance improvements.

251 ***Statistical Analyses***

252 Statistical analyses were performed in STATA 12.1 (STATA Corp LP, College Station,
253 Texas). Linear regression models were applied to estimate MPN per 100 mL as a function of
254 RLU per 100 mL and were computed using $\log_{10}$-transformed data. Pearson's correlation
255 coefficients were calculated to further examine the linear relationship between methods. Cohen's
256 kappa coefficient was used to assess agreement of the Cov-IMS/ATP and Enterolert methods in
257 indicating samples exceeding or in compliance of the 104 MPN per 100 mL threshold. The
258 kappa coefficient was characterized according to Fleiss (1981) guidelines: kappa values over
259 0.75 were defined as in excellent agreement, between 0.40-0.75 defined as in fair to good
260 agreement, and kappa values below 0.40 being characterized as in poor agreement (25, 26).

261 **Results**

262 Water samples (sewage-spiked and environmental) were measured using the Cov-
263 IMS/ATP, Enterolert, and USEPA Method A qPCR assays. Luminescence, reported by the Cov-
264 IMS/ATP method as RLU per 100 mL, was plotted against MPN per 100 mL, per the Enterolert
265 method. Linear regression was used to model the association between the Enterolert and Cov-
266 IMS/ATP methods based on the USEPA enterococci single standard of 104 MPN per 100 mL as
267 well as an equivalent single sample threshold for the Cov-IMS/ATP method. Table 2 presents the
268 results of linear regression models applied for sewage-spiked and environmental data.
269 Confidence intervals (CIs) were applied to the exceedance threshold predicted for the Cov-

IMS/ATP measurements by the linear regression equation. Since there is not an established threshold for Cov-IMS/ATP measurements, the threshold applied in this context represents the best estimate from the data equivalence point as opposed to a hard threshold which has been established for Enterolert measurements. Frequency of observations falling within the 95% CI were calculated and reported as percentage of observations within uncertainty region (Table 3, Column Unc.). Agreement rates were calculated based on the percentage of data points where the Cov-IMS/ATP and Enterolert methods agreed in indicating samples exceeding or in compliance of the 104 MPN per 100 mL threshold. Values falling within the 95% CI were excluded from Cohen's kappa calculations and associated inter-rater agreement rates.

Sewage-Spiked Calibration Curves

Raw influent samples from the Orange County Sanitation District were diluted and spiked into ambient waters and analyzed with the Cov-IMS/ATP and Enterolert methods at Doheny State Beach, Dockweiler Beach, Santa Monica Beach, Topanga Creek, Topanga State Beach, and Topanga Lagoon. Average *Enterococcus* concentrations, as measured by Enterolert and Cov-IMS/ATP, correlated well among the six sites ($R>0.90$, $P<0.05$) and between the six sites when data were combined ($R=0.88$, $P<0.05$) (Table 2). When data were combined between all sites, between all marine sites, and between all sites within the Topanga watershed, Pearson's correlation coefficients remained high ($R>0.80$, $P<0.05$) as did the inter-rater agreement (AR> 83%, $\kappa>0.75$) when results were examined in comparison to the USEPA recreational water enterococci single standard of 104 MPN per 100 mL (Figure 1).

Due to the low number of points per site, a mixed-effects model that allowed for a random slope and random intercept for each site was applied to further quantify potential site-specific effects on the relationship between Enterolert and Cov-IMS/ATP measurements. There

was no evidence of a site effect. The variance associated with site effects on slope and intercept were negligible when compared to the variance due to randomness in the model: σ^2 =0.00 and 0.02 versus 0.12, respectively (see supplementary information Table S2).

Environmental Calibration Curves

For Doheny State Beach, Topanga State Beach, and Tijuana, Mexico samples, calibration curves were created using ambient source water over an approximately six month period. Luminescence, reported by the IMS/ATP method as RLU per 100 mL, was plotted against MPN per 100 mL, as reported for the Enterolert method (Figure 2). Significant correlations and linear relationships between the Cov-IMS/ATP and Enterolert results ($R > 0.75$, $P < 0.05$) were observed at each site.

Due to differences in the relationship between MPN and RLU measurements between sites for environmental calibration curves, an additional linear regression model was run to allow for differences between the three sites (see supplementary information Table S3). There was not a significant difference between Mexico and Doheny Beach ($F_{1,63}$= 0.66, P=0.42) or between Doheny Beach and Topanga State Beach profiles (t=-0.61, p=0.542), only between the Topanga and Mexico profiles (t=0.22, p=0.030).

The linear relationship between Enterolert and Cov-IMS/ATP results differed among the three sites, with the slope coefficient varying between 0.25 at Topanga State Beach and 0.58 at Tijuana, Mexico (Table 2). However, site-specific calibration curves improved predictability of Cov-IMS/ATP measurements for the Topanga site only and did not offer an improvement over the combined calibration curve for either the Doheny or Tijuana sites. Here, the improvement was small, the AR was increased from 83% to 89%, when applying the site-specific calibration curve to Topanga site measurements. The measurements falling within the region of uncertainty

316 did increase from 5% to 32%, signaling a larger region of uncertainty for certain measurements
317 when applying the more generalized combined calibration curve to environmental data for the
318 Topanga site. Site specific calibration curves may still be utilized for best performance at certain
319 sites, as indicated by the improved performance at Topanga State Beach. However, overall
320 performance of the combined calibration curve was strong (R =0.83, AR=90%, κ=0.79) and
321 comparable to the performances of individual site-specific calibration curves when they were
322 applied to each site (Table 3).

323 Luminescence, reported by the IMS/ATP method as RLU per 100 mL, was also plotted
324 against copies per 100 mL, as reported for the USEPA Method A qPCR assay. The linear
325 relationship between Enterolert and Cov-IMS/ATP results differed among the three sites, with
326 the slope coefficient varying between 0.31 at Tijuana, 0.40 at Mexico, and 1.06 at Doheny State
327 Beach (Table 4). For Topanga State Beach samples, there was a linear relationship between
328 qPCR and Cov-IMS/ATP measurements (R=0.68). Similarly, at Doheny State Beach, qPCR and
329 Cov-IMS/ATP measurements were correlated (R=0.71). Measurements at the Tijuana site, both
330 when comparing Cov-IMS/ATP and qPCR (R=0.37) measurements and when comparing the
331 Enterolert and qPCR assay (R=0.30) measurements, were not as predictable.

332 ***Model Variability***

333 Model variance was analyzed on smoothed data for the combined environmental curves
334 and sewage-spiked calibration curves. Sum of squared error (SSE) was calculated for binned
335 data, such as between 0.5 to 1 MPN. Estimated sum of squared error was plotted against cell
336 concentration (MPN per 100 mL) (see supplemental information Figure S1). For the sewage-
337 spiked samples, the range of SSE was small overall, ranging between 0.5 and 2.8. For the
338 environmental model, predictions made were the most robust around the enterococci exceedance

339 threshold of 104 MPN per 100 mL. At higher concentrations (over 1000 MPN per 100 mL) there
340 was a trend of consistent, increased error.

341 ***Limit of Detection***

342 Blank samples were processed by analyzing PBS in a similar manner to that of the
343 environmental samples. The limit of detection for the Cov-IMS/ATP method was as per Bushon
344 et al. (2009) (14). The limit of detection was 6190 RLU per 100 mL based on 15 blank samples.

345 ***Speciation of Enterococci at study sites***

346 Site water was further characterized at Doheny State Beach, Topanga State Beach, and
347 Imperial Beach through examination of enterococci species assemblages. Enterococci isolates
348 were found to include the following six species: *E. faecalis, E. faecium, E. gallinarum, E.*
349 *casseliflavus, E. mundtii*, and *E. hirae,* as well as additional unidentified enterococci and
350 unidentified non-enterococci individuals. Enterococci isolates examined in this study from
351 Doheny State Beach and Imperial Beach were previously collected and identified as part of a
352 comprehensive comparison of *Enterococcus* species diversity but have not been analyzed
353 previously by site (12). Out of 65 isolates examined at Doheny Beach, *E. faecalis* and *E. faecium*
354 were the most prevalent species obtained, representing 42% and 22% of the 65 isolates
355 examined, respectively. The remaining isolates were characterized as follows: *E. casseliflavus*
356 (8%), *E. gallinarum* (11%), unidentified enterococci (6%), and non-enterococci (8%).

357 At Imperial Beach, isolates were examined from a mixture of the Tijuana River mouth
358 and Imperial Beach waters. Water samples were collected approximately ten miles from the
359 Tijuana site sampled in this study, due to difficulty associated with transporting bacterial isolates
360 across an international border. Out of 60 isolates, 43% were identified as non-*Enterococcus*. *E.*
361 *faecium* and *E. casseliflavus* were the most pre-dominant enterococci species, representing 18%

362 and 17% of isolates examined, respectively. The remaining isolates were characterized as: *E.*
363 *hirae* (7%), *E. faecalis* (8%), *E. mundtii* (2%), and unidentified enterococci (5%). In addition, 20
364 isolates were isolated from mEI agar at Topanga State Beach and Topanga Lagoon, and 100% of
365 these isolates were identified as *E. faecalis*. Isolates were identified according to Ferguson et al.
366 (2013) (16).

367 ***Cov-IMS/ATP Assay Specificity***

368 Specificity of the Cov-IMS/ATP assay was verified by testing seven common enterococci
369 species found in marine waters. Measurements made by the Enterolert and Cov-IMS/ATP
370 methods correlated well for five out of the seven species enterococci species tested (Table 5). *E.*
371 *gallinarum* and *E. hirae* measurements made by Cov-IMS/ATP correlated poorly with
372 measurements made by the Enterolert assay. Measurements for six species were well correlated
373 for the Cov-IMS/ATP and MF assays (Table 5). Cov-IMS/ATP exhibited differential sensitivity
374 to the seven enterococci species when compared to traditional methods (MF and Enterolert)
375 (Figure 3). The Cov-IMS/ATP method may potentially be the most sensitive to *E. faecalis* and *E.*
376 *faecium* and the least sensitive to *E. gallinarum*, *E. casseliflavus*, and *E. mundtii.* Average
377 *Enterococcus* concentrations as measured by Enterolert, MF and qPCR methods correlated well
378 (R> 0.80) for all seven *Enterococcus* spp. (Table 5).

379 Fourteen samples used in the specificity experiments were run in duplicate to quantify the
380 variability of the Cov- IMS/ATP assay. On average, duplicates differed by 10040 RLU or 13%.

381 Non-enterococcal bacteria that were bound to the antibody-bead complex were identified
382 by sequencing (Table 6). A rarefaction curve indicated that even if considerably more isolates
383 were sequenced, similar species richness would still be observed (see Supplemental Information
384 Figure S2). *Bacillus spp*. (29%), *Exiguobacterium spp*. (19%), and *Enterobacter spp*. (15%) were

385 the most common non-enterococcal bacteria isolated followed by *Staphylococcus spp*. (10%) and
386 *Aeromonas spp*. (8%). For a full species list of isolates sequenced see supplemental information
387 Table S4.

388 Specificity of the antibody-bead complex was tested against four ATCC species of cross
389 reactors identified in high frequency from 16S rRNA sequencing results: *B. cereus*, *A.*
390 *calcoaceticus*, *E. cloacae*, and *S. saprophyticus*, as well as against *S. gallolyticus*. Sensitivity of
391 the Cov-IMS/ATP assay was decreased when several of these species were present, individually
392 or in combination, in samples spiked with different concentrations of *E. faecalis*. *S. gallolyticu*s
393 (P=0.07), *E. clocae* (P=0.03), and *S. saprophyticus* (P=0.10) affected measurements made by the
394 Cov-IMS/ATP method. Further, combinations of various cross reactors, including a mixture of
395 *E. clocae* and *B. cereus* (P=0.00) and a mixture of *E. clocae, B. cereus,* and *S. saprophyticus*
396 (P=0.01), were also found to significantly affect Cov-IMS/ATP measurements (Table 7).

397 ***Preabsorption***

398 To improve the specificity of the assay, *Enterococcus* IgG serum was preabsorbed with
399 bacterial strains that affected Cov-IMS/ATP measurements including *S. aureus*, *E. cloacae,* and
400 *B. cereus.* An ELISA assay (Immunology Consultants Laboratory, Portland, OR) was used to
401 quantify the portion of antibody remaining in suspension. On average, concentration was
402 decreased from approximately 1 mg mL^{-1} to 0.85 mg mL^{-1} signaling a 15 % loss of antibodies to
403 preabsorption. The ratio of antibody to magnetic bead was adjusted for this loss.

404 Preabsorbed antibodies were applied in laboratory experiments to increase specificity of
405 the assay (subset of trial results included in supplemental information Figure S3). Preabsorbed
406 antibody-bead complexes bound less of the interfering species. Measurements were on average

reduced by 60% for *S. aureus* and 40% for *B. subtilis*. Preabsorption did not appear to improve potential cross reactivity from *E. cloacae*.

Discussion

Standard enterococci detection methods for coastal water quality are culture-based and require a lengthy incubation period. A rapid, portable method can be an important facet of a multi-tiered approach to source tracking that has previously relied on culture- and nucleic-acid based methods (27, 28). Molecular methods based on qPCR can be highly sensitive, but require experienced technicians, expensive startup costs, and measure a genetic endpoint that does not distinguish between viable and non-viable organisms. In comparison, the Cov-IMS/ATP method requires minimal equipment, is affordable and simple to perform. The Cov-IMS/ATP method also has the ability to filter higher volumes through the HAWG filter when compared to the HTTP filter required for qPCR, which is advantageous for analyzing turbid water samples including runoff. Moreover, Cov-IMS/ATP quantifies viable bacteria only making it more comparable to culture-based methods. Therefore, it is important to have a comprehensive understanding of the empirical relationship between culture-based and Cov-IMS/ATP measurements.

In this study, a strong, positive association was observed between the measurements produced by the Enterolert and Cov-IMS/ATP assays in different water samples and with different environmental source inputs of enterococci. Less variability was noted in the Cov-IMS/ATP estimates among samples with mid-range concentrations, between 100 and 300 MPN per 100 mL. When data were combined between sites with a common input (raw influent), a site-specific calibration curve did not appear necessary or provide a particular advantage. Although

there was evidence of some site heterogeneity in proportion of Enterolert to Cov-IMS/ATP measurements between sites when measurements were compared across ambient waters, a combined environmental calibration curve was applied effectively and demonstrated excellent inter-rater agreement with Enterolert for the enterococci single sample limit and a strong correlation between the two methods. The three environmental sites reported represent various challenging inputs, both freshwater and marine, thus the combined calibration curve is robust to a variety of complex inputs. Best performance at certain sites may require site-specific calibration curves, however a combined calibration curve was applied effectively and with strong predictability for both ambient and sewage-spiked waters.

Site heterogeneity and method variability may result in a region of uncertainty for the Cov-IMS/ATP assay. Therefore, values falling in this region may require verification from another method. Even so, these results suggest that the relationship between Enterolert and Cov-IMS/ATP measurements may reasonably predict and help differentiate sites with high and low *Enterococcus* levels from sites that may need further verification.

Quantitative PCR measurements were also compared with both Enterolert and Cov-IMS/ATP measurements. The weaker relationship at certain sites could be due to environmental factors, such as different point-sources of FIB. Sources of fecal pollution have been found to be an important part of describing the relationship between culture and qPCR results and can result in decreased correlation between the two methods at certain sites (29). At the discharge point of the SADB wastewater treatment plant at the Tijuana site, high levels of ENT were measured by qPCR, yet low to non-detectable levels were observed with culture-based methods. The elevated signal of ENT at this site may be the result of qPCR amplifying DNA from both live and dead cells of *Enterococcus*, contributing to the decoupling of qPCR and culture-based methods.

453 However, Cov-IMS/ATP measurements still correlated well with Enterolert measurements at the
454 Tijuana sites, which suggest that Cov-IMS/ATP may be useful for detecting complex and recent
455 inputs of FIB.

456 To explain discrepancies between sites, differential sensitivity to various *Enterococcus*
457 species and potential cross-reactive binding of the antibody-bead complex was evaluated using
458 the seven most common *Enterococcus* species in marine water (30, 31). To our knowledge, this
459 is the first report on the comparison of these culture-based methods to Cov-IMS/ATP using pure
460 cultures of varied *Enterococcus* species. The antibody used in the Cov-IMS/ATP is polyclonal in
461 nature and has not been absorbed on other *Enterococcus* species besides *E. faecium* (18). Caruso
462 et al. (2008) analyzed specificity of a similar *Enterococcus* IgG serum for application to a
463 fluorescent antibody technique and found the serum to be effective at labeling *E. faecium* species
464 only (17). However, the IgG serum as utilized in the Cov-IMS/ATP assay appears to have a more
465 robust specificity profile to *Enterococcus* spp. Variations in assay sensitivity were observed for
466 the different *Enterococcus* spp. and could contribute to differences between Cov-IMS/ATP and
467 the culture-based measurements at certain sites where *Enterococcus* species other than *E.*
468 *faecium* are dominant. Depending on the site and source input, enterococcal communities can
469 differ drastically (32, 33), as found between the three sites tested in this study.

470 Potential for cross reactivity of the antiserum to non-specific antigens was also assessed
471 using several common species isolated in high frequency from the antibody-bead complex. *E.*
472 *cloacae, S. saprophyticus,* and *S. gallolyticus* were found to significantly affect Cov-IMS/ATP
473 measurements. A serum purification procedure, based on preabsorption of the polyclonal
474 antibody with non-enterococcal bacteria, shows promise to increase specificity of the assay.

475 However, further research is needed to develop more target-specific antibodies and to better
476 optimize preabsorption of the antibody to reduce cross reactivity.

477 This study extends previous efforts by sampling a variety of location types, during both
478 the wet and dry seasons, and bacterial concentrations. Previous reports have documented poorly
479 correlated measurements between IMS/ATP and traditional methods in primary influent obtained
480 from the Orange County Sanitation District (OCSD) (15), as well as at Doheny State Beach (Jay
481 Lab, unpublished data). In this study, Cov-IMS/ATP and traditional methods correlated well in
482 OCSD sewage-spiked ambient waters, OCSD sewage spiked directly into PBS, and
483 environmental waters (Topanga State Beach, CA, Doheny State Beach, CA and Tijuana,
484 Mexico).

485 The Cov-IMS/ATP method rapidly measures viable enterococci, providing a useful field
486 tool for assessment of coastal water quality and for identification of hot spots of fecal
487 contamination. In this study, the Cov-IMS/ATP assay illustrated robust measurements and a
488 predictable relationship between enterococci measurements made by the Cov-IMS/ATP and
489 Enterolert methods. A reliable and consistent relationship between Cov-IMS/ATP and culture-
490 based methods would substantially increase ease and efficiency of application of the Cov-
491 IMS/ATP method for rapid assessment of water quality in coastal watersheds.

492

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601 **Figure 1**. Logarithmic MPN versus RLU for sewage-spiked calibration curves generated with
602 ambient water at A) marine sites ($F_{1, 17}$=59.69, P=0.00, RMSE=0.33), B) sites within Topanga
603 watershed ($F_{1,13}$=32.10, P=0.00, RMSE=0.41), C) data combined for all sites ($F_{1, 27}$=89.92,
604 P=0.00, RMSE=0.36). Solid line: linear mean trend between MPN and RLU; grey dashed line:
605 95% CI of exceedance threshold predicted by Cov-IMS/ATP; black dashed lines: exceedance
606 threshold at 104 MPN/100 mls for MPN and predicted exceedance threshold for RLU/100 mls.
607
608 **Figure 2.** Logarithmic MPN versus RLU for environmental calibration curves generated with
609 ambient water at A) Topanga State Beach sites ($F_{1, 17}$=22.21, P=0.00, RMSE=0.54), B) Doheny
610 State Beach sites ($F_{1, 16}$=22.71, P=0.00, RMSE=0.56), C) Tijuana, Mexico sites ($F_{1, 30}$=72.13,
611 P=0.00, RMSE=0.0.72), and D) all sites combined ($F_{1, 67}$=149.46, P=0.00, RMSE=0.61). Solid
612 line: linear mean trend between MPN and RLU; grey dashed line: 95% CI of exceedance
613 threshold predicted by Cov-IMS/ATP; black dashed lines: exceedance threshold at 104
614 MPN/100 mL for MPN and predicted exceedance threshold for RLU/100 mL.
615
616 F**igure 3**. Linear regression results for *Enterococcus* spp. specificity experiments. Logarithmic
617 cell concentration versus RLU for A) *E. faecalis*, B) *E. faecium*, C) *E. gallinarum*, D) *E. hirae*,
618 E) *E. durans*, F) *E. mundtii*, G) *E. casseliflavus*. Dashed line: relationship between RLU and cell
619 concentration for *E. faecium* for comparison; solid lines: linear mean trend between cell
620 concentration and RLU/100 mL. Hollow circles= cell concentration as measured by membrane
621 filtration (CFU/100 mL); black circles= cell concentration as measured by Enterolert (MPN/100
622 mL).

Table 1. Specific primers used in this study for 16s rRNA sequencing and Ent 1A qPCR assays.

PCR and QPCR primers/probes used in this study					
Assay			Primer/Probe Sequences		Reference
Name	Type	Target	For Primer/ Rev Primer	Probe/Dye	
EPA Method A (ENT 1A)	QPCR	Enterococcus	AGAAATTCCAAACGAACTTG/ CAGTGCTCTACCTCCATCATT	FAM-TGGTTCTCTCCGAAA TAGCTTTAGGGCTA-TAMRA	34
Sanger Seq. (16S r RNA)	PCR	Total Bacteria	AGAGTTTGATCCTGGCTCAG/ ACGGCTACCTTGTTACGACTT	N/A	35
Sketa	QPCR	Salmon DNA	GGTTTCCGCAGCTGGG/ CCGAGCCGTCCTGGTCTA	FAM-AGTCGCAGGCGGCC ACCGT-TAMRA	24

Table 2. Results of linear regression models for sewage-spiked and environmental calibration curves. Relationship between MPN and RLU described by slope (ß) of the model.

Enterolert vs. Cov-IMS-ATP Measurements					
Treatment	Site (n)	Slope (ß)		Model	
		ß	95% CI	R^2	R
Sewage-spiked	Dockweiler	0.80	[0.66, 0.94]	0.99	0.99
	Doheny	0.48	[0.23, 0.73]	0.93	0.96
	Santa Monica	0.40	[0.04, 0.76]	0.81	0.90
	Topanga Creek	0.59	[0.17, 1.01]	0.87	0.93
	Topanga Beach	0.26	[.05, 0.46]	0.92	0.96
	Topanga Lagoon	0.39	[0.15, 0.63]	0.90	0.95
	Marine (19)	0.40	[0.0.29, 0.51]	0.78	0.88
	Topanga (15)	0.44	[0.27, 0.60]	0.71	0.84
	All Sites (29)	0.45	[0.35, 0.55]	0.77	0.88
Ambient	Doheny (18)	0.51	[0.28, 0.74]	0.59	0.77
	Topanga (19)	0.25	[0.14, 0.36]	0.57	0.75
	Tijuana (32)	0.58	[0.44, 0.72]	0.71	0.84
	All Sites (69)	0.56	[0.47, 0.65]	0.69	0.83

Table 3. Statistical analysis of Enterolert versus Cov-IMS/ATP measurements for the detection of *Enterococcus* concentrations exceeding/in compliance with 104 MPN/100 mL threshold. Uncertainty (Unc.) calculated as percentage of measurements falling within 95% CI of predicted *Enterococcus* threshold for Cov-IMS/ATP method.

Agreement Rate (AR) Calculations Between Cov-IMS/ATP and Enterolert Measurements							
Treatment	Site (n)	Site-Specific			Combined		
		AR	Unc.	Cohen kappa (SE)	AR	Unc.	Cohen kappa (SE)
Sewage-spiked	Marine (19)	83%	37%	0.43 (0.24)	100%	42%	1.00 (0.30)
	Topanga (15)	92%	20%	0.75 (0.28)	92%	13%	0.75 (0.27)
	All Sites (29)				95%	28%	0.83 (0.22)
Ambient	Doheny (18)	83%	33%	0.63 (0.29)	86%	22%	0.70 (0.25)
	Topanga (19)	89%	5%	0.78 (0.24)	83%	32%	0.00*
	Tijuana (32)	90%	9%	0.79 (0.19)	93%	13%	0.86 (0.19)
	All Sites (69)	90%	14%	0.79 (0.13)	90%	13%	0.79 (0.13)

*Were not able to calculate statistically significant Cohen's kappa value, when combined curve applied no measurements were in exceedance

Table 4. Results of linear regression models for qPCR measurements of *Enterococcus* concentrations of ambient water at Topanga State Beach, Doheny State Beach, and Tijuana, Mexico sites. qCPR measurements regressed against Cov-IMS/ATP and Enterolert measurements.

Environmental Calibration Curves QPCR Results								
Site	QPCR and Cov-IMS/ATP				QPCR and Enterolert			
	Slope (ß)		Model		Slope (ß)		Model	
	ß	95% CI	R^2	R	ß	95% CI	R^2	R
Topanga	0.4	[0.14, 0.66]	0.46	0.68	0.37	[0.06, 0.68]	0.37	0.61
Doheny	1.06	[0.42, 1.70]	0.50	0.71	0.33	[0.14, 0.53]	0.56	0.75
Tijuana	0.31	[0.02, 0.60]	0.14	0.37	0.25	[-0.04, 0.54]	0.09	0.30

Table 5. Results of linear regressions models for *Enterococcus* spp. specificity experiments. *Enterococcus* spp. concentrations measured with MF (membrane filtration), Enterolert, Cov-IMS/ATP, and the Ent 1A qPCR assay. Separate regressions run comparing rapid methods to culture-based as well as culture vs culture-based methods.

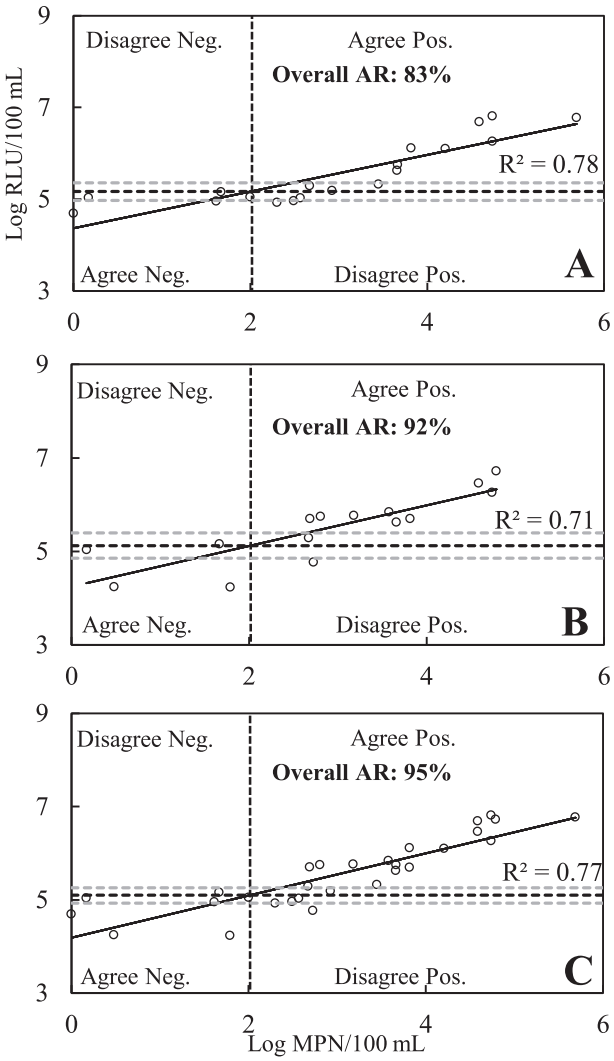
		Culture vs. Rapid Method (IMS-ATP/ QPCR) Measurements								Culture vs. Culture Measurements			
Treatment		IDEXX and Rapid Method				MF and Rapid Method				MF and IDEXX			
	Rapid	Slope (ß)		Model		Slope (ß)		Model		Slope (ß)		Model	
Ent sp.	Method	ß	95% CI	R^2	R	ß	95% CI	R^2	R	ß	95% CI	R^2	R
E. faecalis	IMS	0.84	[0.14, 1.54]	0.83	0.91	1.17	[0.20, 2.14]	0.74	0.86	0.99	[-0.29, 2.27]	0.67	0.82
E. faecium	IMS	1.31	[0.86, 1.75]	0.97	0.98	1.30	[-0.32, 2.92]	0.99	0.99	0.96	[-0.35, 2.27]	0.98	0.99
E. gallinarum	IMS	-0.21	[-6.19, 4.12]	0.03	0.17	0.37	[-2.65, 2.99]	0.31	0.56	0.59	[-0.67, 1.85]	0.04	0.20
E. hirae	IMS	0.60	[-1.21, 2.42]	0.27	0.52	0.62	[0.42, 0.82]	0.97	0.98	0.37	[-0.20, 0.95]	0.42	0.65
E. durans	IMS	0.90	[-0.88, 2.49]	0.74	0.86	0.69	[-0.08, 0.85]	0.89	0.94	0.81	[-0.44, 2.05]	0.76	0.87
E. mundtii	IMS	0.31	[0.00, 0.62]	0.66	0.81	0.61	[0.04, 1.17]	0.69	0.83	1.68	[0.45, 2.92]	0.85	0.92
E. casselfavis	IMS	0.22	[-0.86, 1.29]	0.87	0.93	0.26	[-0.13, 0.65]	0.99	0.99	0.22	[-0.86, 1.29]	0.94	0.97
E. faecalis	QPCR	0.79	[-0.33, 1.90]	0.63	0.79	1.14	[0.84, 1.44]	0.97	0.98				
E. faecium	QPCR	0.81	[0.06, 1.99]	0.35	0.59	1.26	[-0.07, 2.14]	0.59	0.77				
E. gallinarum	QPCR	1.13	[-0.45, 2.70]	0.99	0.99	0.68	[-0.19, 1.59]	0.92	0.96				
E. hirae	QPCR	0.76	[-1.01, 2.53]	0.63	0.79	0.37	[-0.20, 0.95]	0.80	0.89				
E. durans	QPCR	0.55	[-0.26, 1.25]	0.61	0.78	0.26	[0.31, 0.51]	0.45	0.67				
E. mundtii	QPCR	0.40	[-0.02, 0.81]	0.64	0.80	0.89	[0.45, 1.32]	0.89	0.94				
E. casselfavis	QPCR	0.45	[-0.72, 1.61]	0.96	0.98	0.50	[0.20, 0.81]	0.99	0.99				

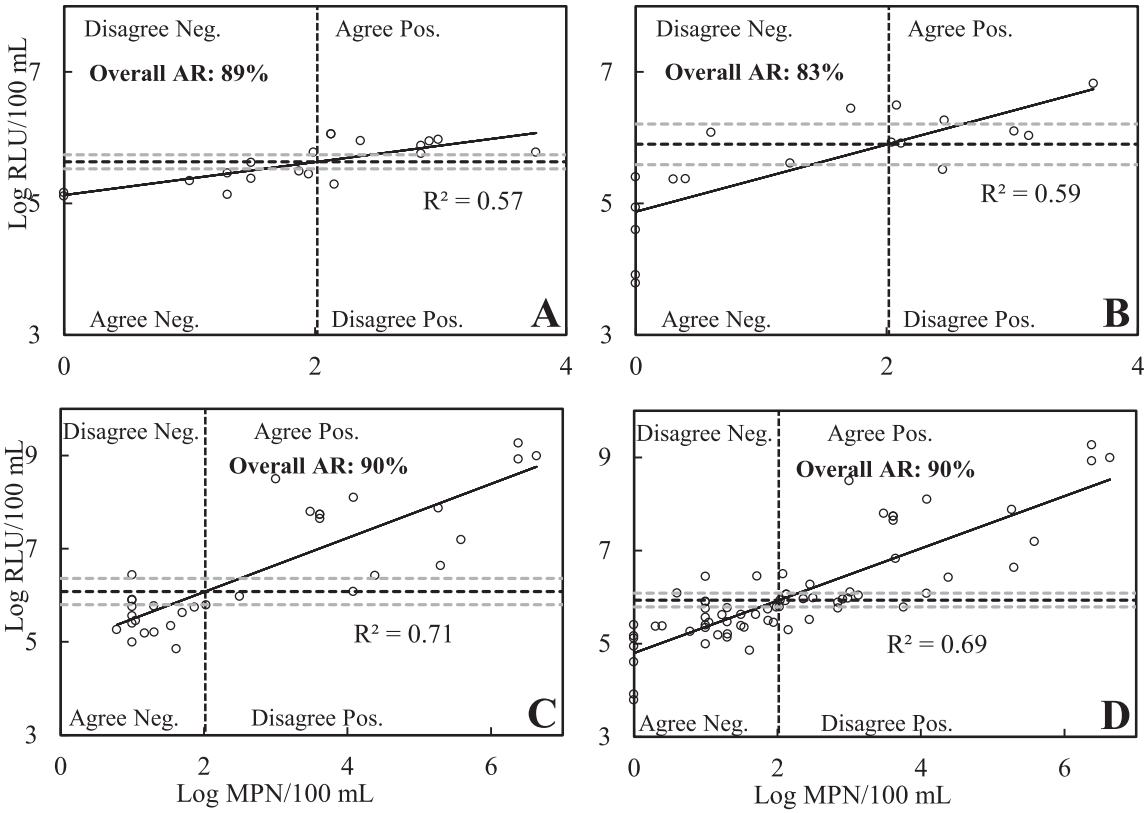
Table 6. Bacteria isolated from bound antibody-bead complexes at either Topanga or Doheny State Beaches and sequenced with 16s rRNA sequencing.

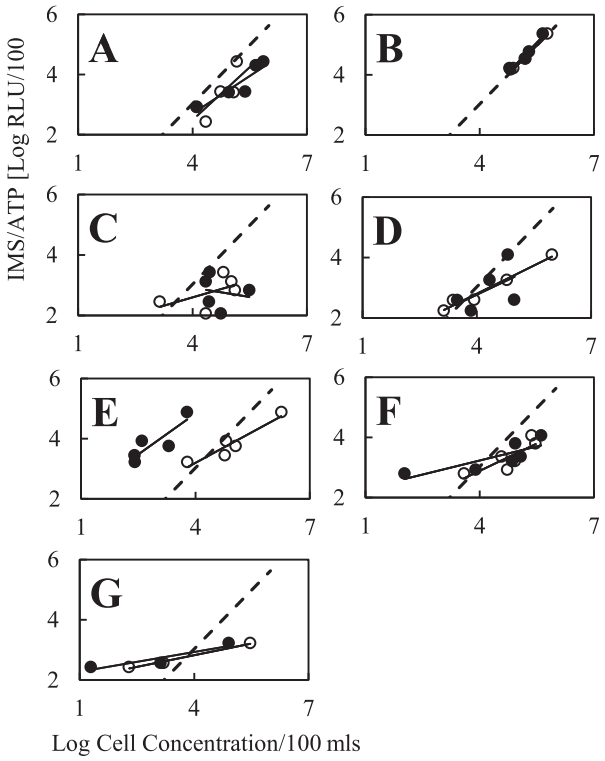
	Site no. isolates from % (n):		%
Species	Doheny	Topanga	Total
Acinetobacter spp.	-	16% (8)	8%
Aeromonas spp.	4% (2)	12% (6)	8%
Bacillus spp.	49% (22)	12% (6)	29%
Delftia spp.	-	2% (1)	1%
Enterobacter spp.	24% (11)	6% (3)	15%
Exiguobacterium sp	13% (6)	24% (12)	19%
Pantoea spp.	-	2% (1)	1%
Pseudomonas spp.	2% (1)	-	1%
Sphingopyxis spp.	4% (2)	-	2%
Sporosarcina spp.	-	4% (2)	2%
Staphylococcus spp	2% (1)	18% (9)	10%
Vogesella spp.	-	6% (3)	3%
Total (n)	45	51	96

Table 7. Linear regression results testing potential effect of non-target bacteria on Cov-IMS/ATP measurements. ENT= *E. faecalis* spiked standard curve; ENT + CR = *E. faecalis* spiked with potential non-target bacterium: EC= *E. cloacae*, BC= *B. cereus*, AC= *A. calcoaceticus*, SS= *S. saprophyticus*. Presence of cross reactor included as indicator variable; significance of change in slope (Δ Slope) due to cross reactor included below.

Cov-IMS/ATP and IDEXX With Interference				
Treatment	Slope (ß)		Δ Slope	
Cross reactor (CR):	ENT	ENT + CR	t score	P-value
S. gallolyticus	0.72	0.06	2.84	0.07
B. cereus	0.66	0.78	1.87	0.158
A. calcoaceticus	0.72	0.64	-0.6	0.59
E. cloacae	1.42	0.59	3.91	0.03
S. saprophyticus	0.59	-0.03	2.09	0.13
EC + BC	1.21	0.44	9.38	0.00
EC +BC + AC	0.95	0.64	1.76	0.18
EC +BC +SS	0.80	0.17	5.25	0.01







1 Application of Inverse-Capture Immunomagnetic Separation/ATP quantification (Inv IMS-ATP) in Topanga Creek Watershed

1.1 Introduction

Novel technologies for rapidly assessing water quality are needed. Often, watersheds may be impacted by multiple fecal pollution sources which are difficult to track using traditional culture based methods that can take between 18 – 24 hours to obtain results. Rapid, viability-based methods that can enumerate microbial contaminants in as quickly as one hour may be more protective of human health for coastal recreational water quality monitoring. One such technique is the immunomagnetic separation (IMS) method, which has proven effective in isolation and quantification of *Giardia*, *Cryptosporidium parvum* and *Escherichia coli*.

The Jay lab at UCLA tested the Covalently-linked (Cov-IMS/ATP) and Inverse-capture (Inv-IMS/ATP) immunomagnetic separation/ATP quantification methods, for rapid quantification and detection of viable enterococci (ENT) and *Bacteroides thetaiotaomicron*. IMS/ATP concentrations were compared to traditional fecal indicator bacteria (FIB) numbers and molecular marker concentrations to determine the feasibility of adopting this technology to track fecal pollution in the Topanga Creek watershed. This chapter details the results of the Inv-IMS/ATP method for detection of *B. thetaiotaomicron,* a bacterium thought to be more associated with sources of human fecal pollution than traditional water quality indicators. Results of the Cov-IMS/ATP method are detailed in the manuscript titled *"Performance and specificity of the Cov-IMS/ATP method for rapid detection and enumeration of enterococci in coastal environments"* and can be found in full in Appendix J.

1.2 Methods

Field Site

The Inv-IMS/ATP method was tested in the Topanga Creek Watershed, California, USA. Marine water samples were collected once from Topanga Beach on XX during early morning hours from the Beach Upcoast (BU 175m) site. Samples were taken during an incoming wave at ankle depth. Freshwater creek samples were collected once on XX date during early afternoon hours from the Scratchy Trail (ST 4800 m) site. Samples were characterized for FIB and conductivity on day of collection. BU and ST were chosen to represent marine and freshwaters as these site often exhibited low background levels of FIB and human-associated markers during the first year of the study period. Site map and details regarding sampling locations are shown in Table 5-1 and Figure 5-1.

Table 1-1. Sampling Locations from Topanga Creek and Beach. Sample name is listed, followed by distance (m) to or distance upstream of lagoon discharge point (BO). Note Coordinate System: UTM , Zone 11N.

Site Name	Easting (m)	Northing (m)	Elevation (ft)	Description
Beach Upcoast -175m (BU)	353726	3767515	0	Ocean
Beach Outlet- 0m (BO)	353896	3767506	0	Ocean
Scratchy Trail – 4800m (ST)	353518	3771500	500	Creek

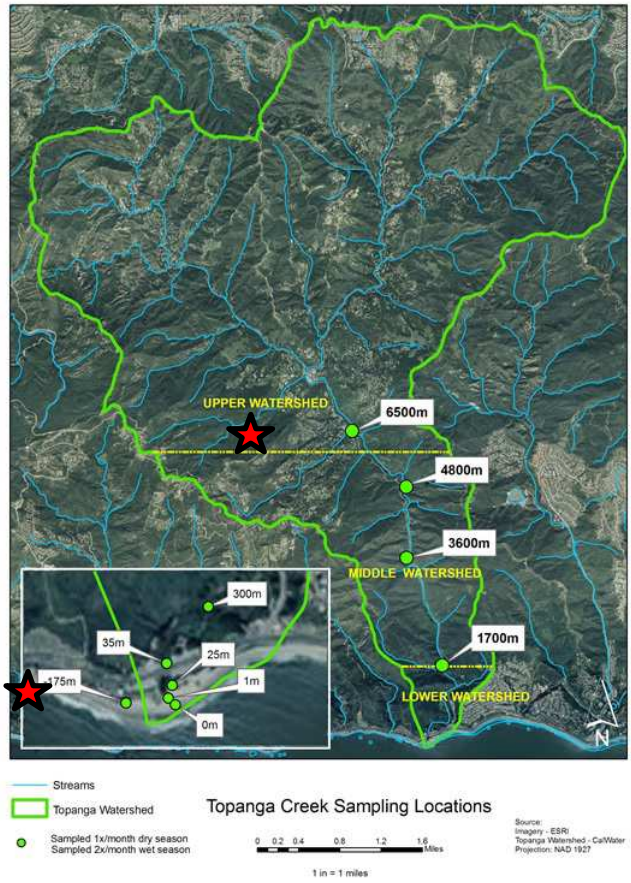


Figure 1-1 Map of the Topanga Creek Watershed and sampling locations, sites sampled specifically for the Inv-IMS/ATP method are indicated by red stars.

Inv-IMS/ATP Methodology

Ambient water sample processing

Ambient water samples were collected in sterile, acid washed (10% HCl) polypropelyene bottles, transported on ice and processed in the UCLA lab. Approximately 400 mL of ambient water was filtered through a 0.45 µm filter (SA1J792H5; Millipore) via vacuum filtration to concentrate the sample. The filter was placed in 5 mL of phosphate buffered saline (PBS, pH 6.8) and vortexed for one minute to resuspend the bacteria.

Inv-IMS/ATP Processing

One mL of the sample resuspension was added to a 2-mL microtube containing 20 ug of BT monoclonal antibody (IgM) (ab65441, AbCam, Cambridge, MA) and incubated on a bidirectional mixer for 30 minutes at room temperature. 330 uL of washed Dynabeads Rat anti-Mouse IgM (Invitrogen, cat no. 110-39D) were added to the sample-IgM mixture for a 30 minute incubation period on a rotating and tilting rotator. Dynabeads used in this study are superparamagnetic polystyrene beads with a 4.5 um diameter, that are pre-coated with Rat anti-mouse IgG. A rare earth magnet was used to separate the bound target from the supernatant. The bound target was then washed twice in PBS (with 2 mM EDTA, 0.1% BSA) and separated magnetically. 100 uL of lysing agent (Bacterial Cell-Releasing Agent, New Horizons Diagnostics, Columbia, MD) was added to the bound biosorbent and vortexed for 30 seconds on medium-high setting and separated. The extract (now in liquid phase) was transferred to a clean 96 well microplate. Equal volume of BacTiter-Glo (#G8231, Promega Corporation, Madison, WI) was added to each of the sample wells and pipette-mixed gently three times. The microplate was then transferred to Multi+ GloMax luminometer (E8031) Promega Corporation, Madison, WI) for luminescence measurements in Relative Light Units (RLU).

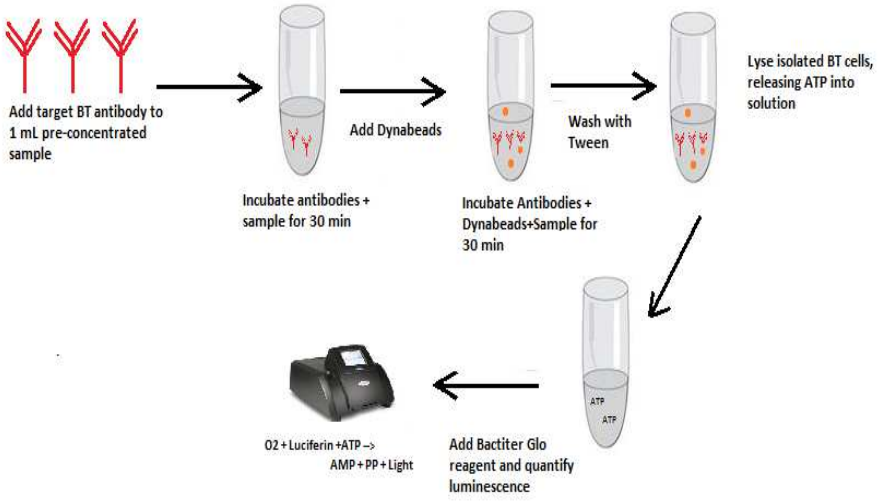


Figure 1-2 Flow diagram showing the Inverse Capture immunomagnetic separation/ATP quantification (Inv-IMS/ATP) method for rapid viable detection of Bacteroides thetaiotaomicron.

Culture-based processing

Measurements made by Inv-IMS/ATP were compared with counts of enterococcus determined by defined substrate technology (EnterolertTM, IDEXX). Enumeration of enterococcus with Enterolert^{TM} Quanti-Tray 2000 was performed according to the manufacturer's instructions (IDEXX Laboratories,Westbrook, ME). Environmental creek and marine samples were diluted to1:10 in sterile Milli-Q water. Spiked samples were diluted to no more than 1:1000. Positive identification of enterococci was determined by samples presenting fluorescence under UV light (365 nm) and quantified in units of most probable number (MPN) per 100 mL.

Quantitative PCR processing

Measurements taken with the Inv-IMS/ATP method (RLU/100mL) were also compared against measurements by quantitative PCR (qPCR) using the human-associated *Bacteroidales* HF183Taqman (HF183) genetic marker. For the measurement of HF183 gene copies per 100 mL, sample water was filtered through 47 mm, 0.4 µm pore size, HTTP polycarbonate filters (EMD Millipore, Billerica, MA) in duplicate. Each filter was placed in an individual two mL polypropylene screw cap tube, containing 0.3 g, 212 – 300 µm (50 – 70 U.S. sieve) acid washed glass beads (Sigma-Aldrich, St. Louis, MO) and stored at -80°C until DNA extraction. Filter blanks, consisting of 50 mL of PBS passed through the polycarbonate filter, were also generated with each set of processed samples. DNA was recovered from water samples and calibration standards according to manufacturer's guidelines of the DNA-EZ ST1 Extraction Kit (GeneRite, North Brunswick NJ). Extracted DNA was eluted into 100 µl of elution buffer and aliquots were stored at -20^{o}C until analysis with qPCR. Calibration standards were prepared using *the OSTD1 plasmid* (IDT Technologies).

Briefly, the PCR reaction mixture consisted of 2 µL of DNA template added to 12.5 µL 1x ABI Universal Master Mix, 2.5 µL of 2 mg mL^{-1} BSA, 3.5 µL of primer/probe working solution and 4.5 µL molecular grade RNase free water for a final reaction volume of 25 µL. The reaction was cycled at 50^{o}C for two min, 95^{o}C for 10 min, 40 cycles of 95^{o}C for 15 sec, and 60^{o}C for two min. Primers and probe sequences are detailed in Table 3-1. Samples and calibration standards were run in triplicate. A five-point standard calibration curve was run alongside samples on each well plate. Standard curves had efficiencies between 90 - 110% and $R^2 > 0.99$. Quantification thresholds (Cq) were converted into units of gene copies using a pooled master standard calibration model. Negative controls and extraction blanks were included to ensure contamination of samples did not occur during either the filtration or extraction processes. This method was run in parallel in order to calibrate Inv-IMS/ATP measurements of relative light units (RLU) to standard units of MPNs and gene copies per 100 mL.

Calibration Curves

Calibration curves were created using both PBS (1X, pH 6.8) and ambient source water from Topanga to test how the Inv-IMS/ATP method performs at this field site. Calibration curves allow for measurements made by the Inv-IMS/ATP assay to be interpreted in the context of traditional FIB numbers. To construct curves, ambient waters (fresh or marine) were spiked with primary influent collected from Orange County Sanitation District (Fountain Valley, CA) on the same day. Sewage was serially diluted in ambient waters or PBS and analyzed with the Inv-IMS/ATP and Enterolert^{TM} (IDEXX, Westbrook Inc.) methods.

Decay of bacteria using Inv-IMS/ATP technology

Marine waters at Topanga Beach were also tested for decay of enterococci and the human-associated molecular marker. Approximately 12L of ocean water at BO was collected during early morning hours on 21 August 2013. Ocean water was spiked with primary influent collected from Orange County Sanitation District (Fountain Valley, CA) on the same day. Sewage was spiked into ambient waters to create an initial starting concentration of approximately 10^4 MPN/ 100 mL. Ocean water seeded with sewage mixture was divided into triplicate 2L beakers and placed in the light or dark in ambient conditions (rooftop of Boelter Hall, UCLA) and monitored over an 18 day period. Water samples were collected from beakers on eight various days and processed for enterococci, human-associated HF183 marker and with the Inv-IMS/ATP assay for rapid enumeration of BT. This laboratory decay experiment was conducted to test if the Inv-IMS/ATP method could track changes in FIB and human-associated molecular markers once an input of human fecal pollution was introduced to ambient source water.

1.3 Results

Calibration curves for Inv-IMS/ATP versus HF183 qPCR and Enterolert were generated effectively in PBS (phosphor-buffered saline), marine water (BO), and freshwater (ST) (Figure 1-3). Calibration curves are needed as the first step to demonstrating the Inv-IMS/ATP method can be applied at a field site. Strong linear relationships exist between Inv-IMS/ATP measurements and traditional measurements (with culture and qPCR) of sewage spiked fresh and marine (R^2 >0.92). Calibration curves show promise that the Inv-IMS/ATP assay can be successfully deployed in the Topanga Creek watershed to rapidly track fresh human fecal inputs. Such novel methods are required for quick and targeted remediation efforts of problem areas with chronically high FIB.

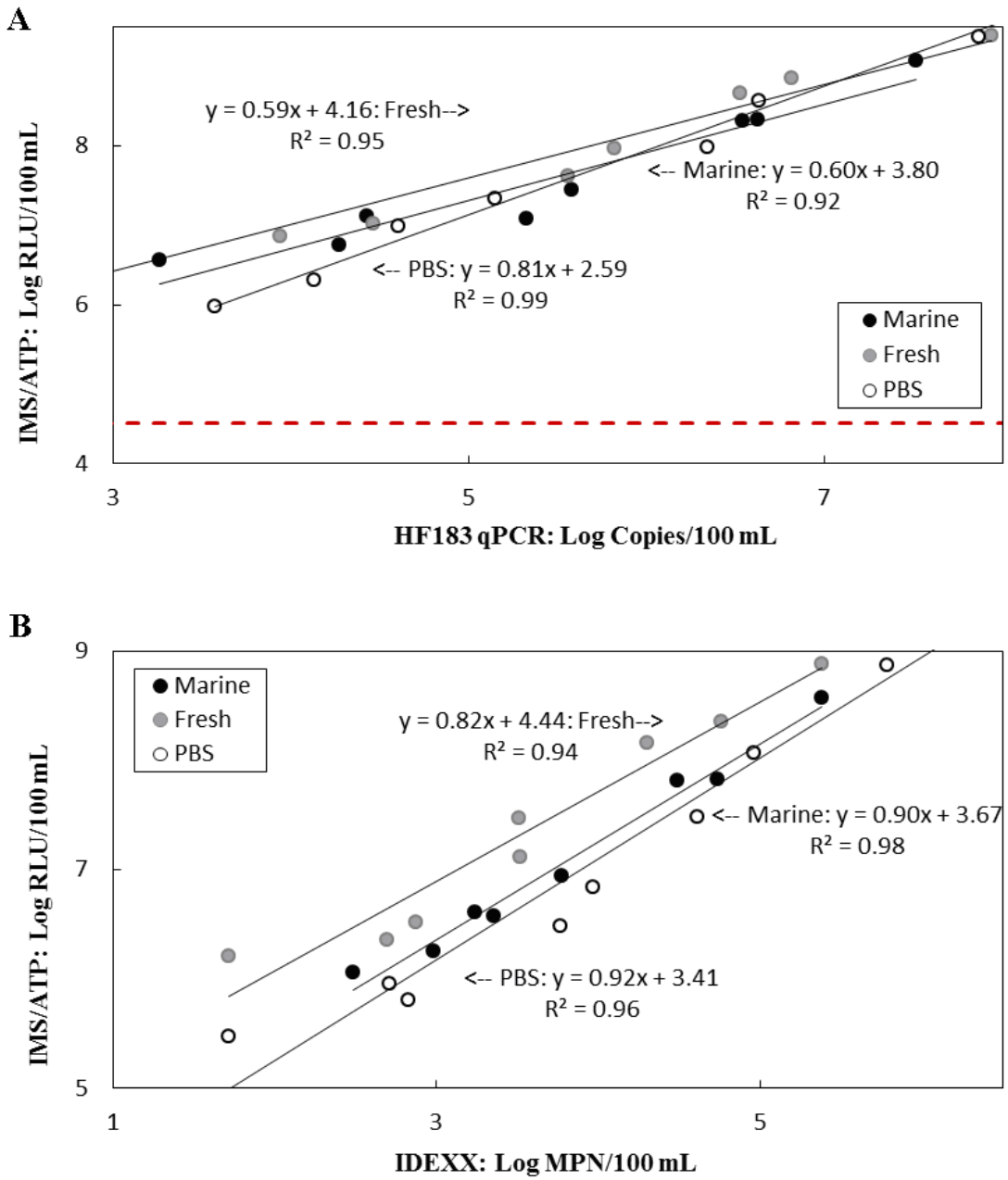


Figure 1-3 Calibration curves generated in sewage-spiked marine water, freshwater, and PBS (a lab-made saline solution). A. Inv-IMS/ATP (RLU/100mL) measurements versus HF183 qPCR (Copies/100mL) measurements. B. Inv-IMS/ATP (RLU/100mL) measurements versus Enterolert

Laboratory decay experiment was conducted to test if the Inv-IMS/ATP method could track changes in FIB and human-associated molecular markers once an input of human fecal pollution was introduced to ambient source water. The Inv-IMS/ATP assay effectively measured the decay of sewage-seeded seawater, when compared to measurements of the HF183 assay and Enterolert (Figure 1-4). Dark microcosms maintained higher concentrations of bacteria and markers throughout the experiment, however decay was similar regardless of light or dark conditions. Often microbial source

tracking studies will combine culture methods (IDEXX) with molecular methods (human-associated markers) to determine sources and hot spots of contamination. Measurements taken with the Inv-IMS/ATP method for rapid detection and quantification of BT were shown here to have more similar trends to enterococci than did molecular qPCR techniques. HF183 qPCR levels remained high, with levels staying constant until day 12. Yet, both BT and enterococci levels showed a sharp decline in signal after four days. Therefore, this experiment shows that fresh human inputs may be measured with these methods. In addition, Inv-IMS/ATP shows promise as the fasted way of the three methods tested to track human inputs, especially if the method is deployed in situ, though this was not tried at the site.

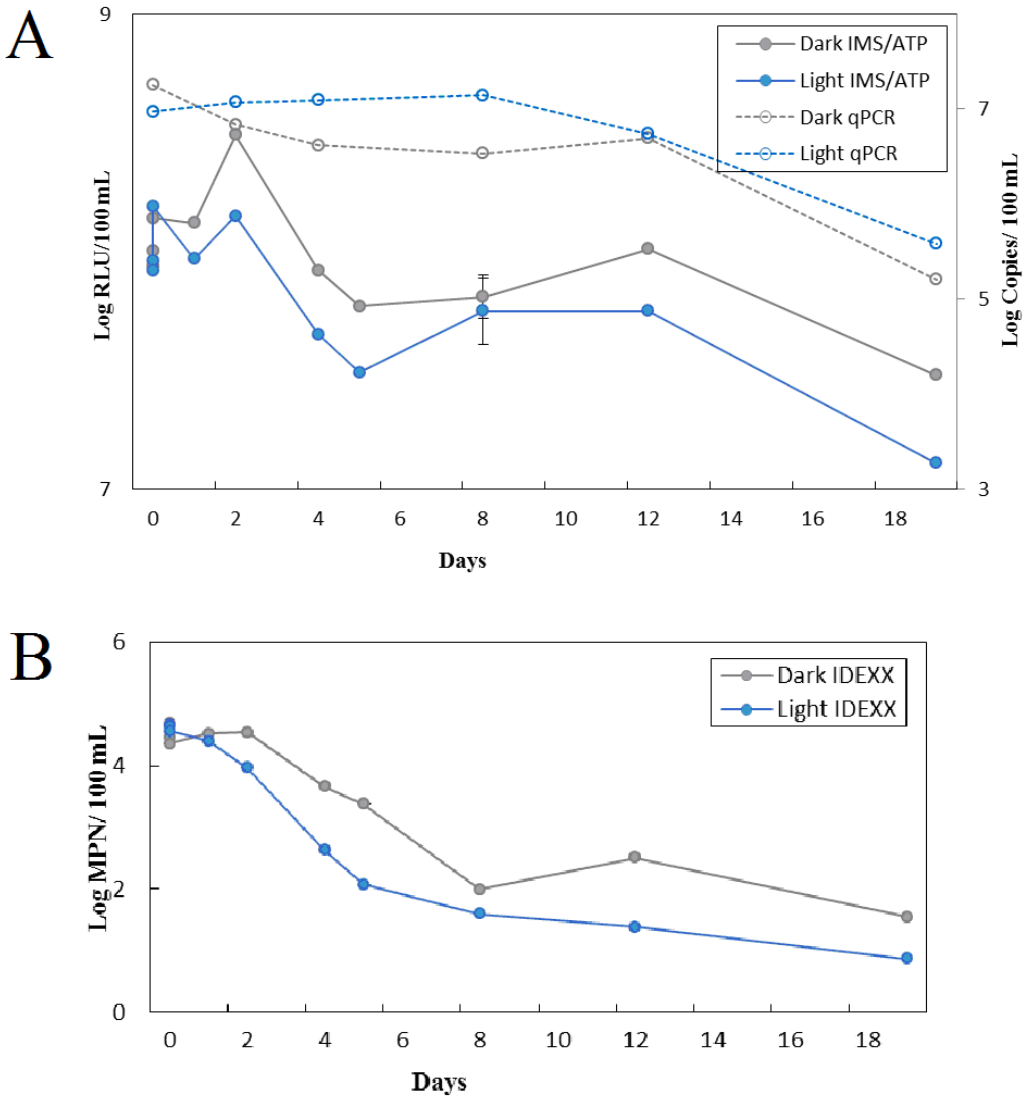


Figure 1-4 Laboratory decay experiment of sewage seeded ocean water in light (blue) and dark (grey) ambient conditions as measured by the A. INV-IMS/ATP method (RLU/100mL - solid line) and HF183 qPCR assay (copies/100mL - dashed line) and B. enterococcus measured

1.4 Discussion

There is a need for alternative methods for measuring human-associated fecal pollution in the environment. Traditional FIB cannot reliably be used in this capacity due to non-human and environmental sources and regrowth (Lee et al. 2006, Mika et al. 2009). Information regarding source (human-associated or not) allow for more effective source tracking and appropriate remediation strategies to be applied. Molecular techniques have advanced significantly in recent years and represent an opportunity to obtain source- or species-specific information about fecal contamination. However, qCPR is not viability-based, meaning that it is more protective than viability-based assays which do not measure dead cells. On the other hand, viability-based immuno-based techniques such as IMS/ATP (Lee et al. 2004; Bushon et al. 2009; Lee et al. 2010) have also shown much promise as a rapid, field-portable method for detecting and quantifying fecal pollution. Though this method has been limited to analyses of standard FIB, this work indicates that viable BT measurements by IMS/ATP were comparable to other sewage indicator measurements by other methods (culture-based techniques as well as qPCR.

Inv-IMS/ATP is, to our knowledge, the first IMS-based rapid method for human-associated fecal source-tracking using *B. thetaiotaomicron* and can provide results in one hour. The monoclonal nature of the antibody used to bind the target offers higher specificity than a polyclonal antibody (which are a broad-reacting antibodies suited for monitoring standard FIB, but may not be specific enough for source tracking.) Furthermore, the need for an IgG-coated magnetic bead eliminated the need to precoat the beads for specific organisms. Instead, the bead coupling protocol can be directly incorporated into the day-of analysis.

This study has demonstrated that IMS/ATP can yield measurements of *B. thetaiotaomicron* that are comparable to qPCR for the same organism in sewage and runoff-contaminated waters and during wet weather in Topanga site waters. Inv-IMS/ATP would provide an additional level of information regarding the persistence of the organism as only viable cells are responsive to the assay, potentially allowing for better comparison with traditional culture-based technologies than nucleic-acid based technologies. Finally Inv-IMS/ATP has reasonable startup costs and is user-friendly, eliminating the need for highly experienced technicians. Inv-IMS/ATP shows promise as a new tool for rapidly assessing water quality. This tool would allow managers tasked with monitoring water quality to have information regarding human contamination events in a number of hours, which is much more protective of human health. Further work is needed (larger in calibration curve of ocean waters) to test the method against frequency of exceedances at the beach, and to determine if this method can better track high levels of FIB consistently in the coastal environment.

1.5 Summary

- Calibration curves for Inv-IMS/ATP versus HF183 qPCR and Enterolert IDEXX were generated effectively in marine and freshwater from the Topanga watershed.
- Inv-IMS/ATP shows promise as a new tool for rapidly assessing water quality.

- Inv-IMS/ATP would allow managers tasked with monitoring water quality to have information regarding human contamination events in a number of hours.

1.6 References Cited

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Investigating decay of FIB and host-associated markers in sediment through laboratory microcosm experiments

Background

Microbial source tracking (MST) approaches utilizing real-time polymerase chain reaction (qPCR) quantification of source-associated DNA markers have greatly advanced. These methods, applied in the Topanga Source ID Study, enable more successful source tracking by allowing for same-day water quality monitoring results and information regarding the source of the fecal contamination. However, both FIB and DNA-based markers are poorly understood both in terms of how they decay in the environment and how their decay rates compare to each other. Molecular markers and FIB may decay differently under varied environmental conditions. FIB are measured using a culture-based technique, whereas DNA-based markers measure DNA from both culturable, viable but not culturable, and dead cells. Further, decay of fecal microorganisms is dependent on both the bacterium itself and on physical (e.g. organic matter content, temperature, salinity) and biotic factors (predation and competition) (e.g. Surbeck et al.2009, Korajic et al. 2013, Wanjugi et al. 2013), allowing for differences in environmental fate of different indicator organisms.

Decay in sediments is particularly understudied even though beach sands, sediments, and soils are a source of accumulation of FIB (Lee et al. 2006), may affect FIB deactivation (Mika et al. 2009), and have been shown to correlate with gastrointestinal illness (Heaney et al. 2009). Sediments can also promote persistence of fecal material by providing protection from ultraviolet radiation and by providing protection from predation by microorganisms in the overlying water column (Korajkic et al., 2013). Further, FIB have been found to survive and even regrow in sediments (e.g. Craig et al. 2004), which in turn complicates interpretation of FIB levels. Few MST studies have addressed how the presence of sediment and associated sediment characteristics (such as particle size distribution and organic matter content) may affect decay characteristics of DNA-based markers.

Sampling of the Topanga watershed conducted between 2012-2014 illustrated that the Topanga Creek is a sink for inputs of nutrients and bacteria into the upper watershed. Levels of FIB that enter the upper watershed, near the developed portion and the town of Topanga, are subsequently reduced prior to reaching lower watershed sites at a rate exceeding dilution. Specifically, we observed a sharp decrease in FIB and nutrients in the Narrows portion of the creek, located between Owl Falls (6500 m) and Scratchy Trail (4800 m). It was hypothesized that different site specific characteristics within the Topanga watershed lead to differences in decay rate at the different sites. Microcosms were constructed in order to identify and evaluate if differences exist between analyte and site FIB and marker decay rates.

Hypotheses and Objectives

In this study, the effect of varied sediment characteristics (predation, organic content, nutrient concentrations, and particle size distribution) on decay rates of FIB and DNA-based markers in sediments and the overlying water column was analyzed. The hypotheses that FIB and markers would decay differently at different sites and that the Topanga upper watershed sites (ST and OF) would have faster decay than the lower watershed sites (BR and TL) were tested. There was a particular focus on analyzing 1. sediment decay rate differences between sites within the Topanga watershed for FIB/markers; 2. decay rate differences between ambient and oven-dried

sediment for FIB and markers and 3. Decay rate differences between different analytes (FIB-ENT and EC and marker-HF183 and ENT1A) in sediments.

Research Approach/Methods

1. Sampling Sites

Topanga field data indicated that FIB inputs from town are not transported downstream, therefore, two Topanga upper watershed and two Topanga lower watershed sites were selected. Further, in order to compare decay rates both within the Topanga watershed and within the context of the larger Santa Monica Bay watershed, sediment was collected and tested from a channelized section of Madea Creek within the Malibu watershed and from Ballona Creek freshwater marsh. Sediment that was used for the oven-drying treatment was collected on April 17th, 2014. Sediment used for ambient sediment treatment was collected on April 21st, 2014 and held at 4 °C in the dark prior to microcosm experiments.

Table 1. Description of sediment collection sites.

Site Name	Easting (m)	Northing (m)	Elevation (ft)	Description	Location
Scratchy Trail – 4800m (ST)	353518	3771500	500	Creek	Upper Topanga Watershed
Owl Falls (OF)-6500 m	352673	3772373	700	Creek	Upper Topanga Watershed
Brookside Drive (BR)	354075	3768713	0	Creek	Lower Topanga Watershed
Topanga Lagoon (TL)	353968	3767553	500	Lagoon (FW)	Lower Topanga Watershed
Ballona Creek (BC)	367685	3759839	0	Marsh (FW)	Ballona FW Marsh
Madea Creek (MC)	337802	3779288	800	Creek	Malibu Watershed

2. Microcosm Set Up

To better understand effect of sediment characteristics, sediment microcosm experiments were completed with samples from six sites located throughout the Santa Monica Bay Watershed of varied organic content, nutrients, particle size, and ambient microbiota. Microcosms were constructed in beakers with 2:1 sediment: water ratio by volume using homogenized sediment and artificial fresh water. Sediment was either oven dried at 176.6° C for 48 hours or ambient. Sediment was oven dried to reduce impact of ambient microbial community and predators on inoculum decay. Oven dried sediment microcosms were included for sites OF, ST, BR, and MC. Sediments were tested for effective disinfection by running an Enterolert and Coliert IDEXX on oven-dried sediment resuspension and by streaking sediment resuspension onto Tryptic Soy Agar. Oven dried sediment moisture content was adjusted prior to seeding of sewage inoculum by adding in appropriate volume of artificial freshwater, so that moisture content of oven dried and ambient sediment was equal at each site. Oven dried sediment was used in order to test for effect of competition and predation from ambient microbiota.

Microcosms were constructed with sewage (5% primary influent collected from the Orange County Sanitation District) seeded into sediment (1.5 L by volume) and allowed to incubate for two hours. After two hours, seeded sediment (400 mls) was distributed into each of three replicate, 2L Pyrex beakers. Two hundred mls of artificial freshwater was then added to each beaker. Microcosms were conducted in a Precision Environmental Chamber set at 20° C, average water temperature was 23.4° C, on a 12-hour light/dark cycle. Each beaker had an airstone to ensure mixed and oxygenated conditions.

3. Microcosm Sampling for FIB and Markers

Microcosms were sampled eight times over a 21 day period for decay of FIB and DNA-based markers. Prior to sampling the sediment at each time point, water was removed and replenished with 200 mL new artificial fresh water simulating semi-continuous flow conditions. Water column from one beaker of each set of three was processed for FIB and markers to account for resuspension of bacteria to water column. After water was removed, sediment was sampled using a 15 mL Falcon tube core. Three cores were taken from each beaker, composited, and processed for FIB and markers at each of the eight time points. Each sample (sediment and water column) was processed and analyzed for FIB (*E.coli*, enterococci) and qPCR host-specific markers for human (HF183) and enterococci (Entero1A). FIB was assayed by IDEXX and/or qPCR methods following EPA standard or published protocols.

From each sediment sample, particle size was determined using a hydrometer and organic content through loss on ignition. Benthic microalgal biomass was quantified by measuring chlorophyll a concentrations. Chlorpohyll a pigments were extracted with acetone and absorbance of the extract was determined using a Hach DR 2600 spectrophotometer (Armitage et al. 2004). Sediment was shipped to the Marine Science Institute, UCSB for carbon and nitrogen content analysis, determined using an automated organic elemental analyzer.
In each water sample (overlying water column), dissolved organic carbon was measured using a Shimadzu 5000 and nutrients (nitrate, ammonia and total phosphorus) were analyzed with spectrophotometric analysis (Hach DR 2600).

4. Decay Rate Calculation

A first-order decay equation was used to determine the decay rates of FIB and markers ($C=C_0e^{-kt}$). If markers or FIB levels reached a plateau, then subsequent time points were excluded from decay rate calculations. Following decay rate calculation, T_{90} values, the time in days to reach 90% reduction in abundance from starting concentration, was calculated as follows: $T_{90} = Ln10/k$.

Results

1. Sediment and Site Characteristics

Sediment characteristics differed between the different sites (Table 2). The Ballona Creek FW marsh site had the smallest particle size and highest carbon content of the sites tested. Although there was not a clear trend between particle size and decay rate; overall, sites with a larger particle size distribution had faster decay (ST, OF, and TL had faster decay of FIB and markers

that BC, MC and BR). There was no clear pattern detected between Chla levels and decay rates. BC and BR also had the highest % carbon and nitrogen content and the slowest decay for ENT and *E.coli*.

Table 2. Sediment characteristics of each site.

Site	Sediment Chla	Particle Size Distribution			CHN Analysis (Weight %)		Moisture Content
	(mg/g)	% Clay	% Sand	% Silt	Carbon	Nitrogen	H2O % By Mass
OF	1.19	5.50	93.20	1.30	0.08	0.01	13.85
TL	0.62	2.85	97.15	0.00	0.02	0.01	14.28
ST	0.00	5.51	93.19	1.30	0.13	0.02	14.30
BC	3.76	27.49	50.05	22.46	1.10	0.12	44.68
MC	8.31	9.31	89.50	1.19	0.17	0.02	16.35
BR	1.22	7.50	90.20	2.30	0.51	0.05	16.28

2. Decay Curves

The decay rates of ENT, *E.coli*, and the HF183 and ENT 1A marker in both ambient and oven dried sediments are illustrated in Table 3. To detect significant differences in decay rates between sites and anlaytes, two ANCOVA analyses were run in STATA V12 (STATACorp, College Station, Texas). For ENT decay, there was a significant difference between all ambient and oven dried sediments ($p<0.02$), which was less significant at ST ($p=0.09$). BR and MC oven dried sediment had significantly slower decay for ENT than OF and ST oven dried sediment ($p<0.02$). *E.coli* rates varied drastically between the oven dried and ambient sediments for all four sites tested ($p<0.02$), indicating potentially more of an effect of ambient population on controlling *E.coli* concentrations. There were no significant differences between ambient sediment *E.coli* decay rates for the six sites tested. For the human marker (HF183), there were also no significant differences between ambient sediment decay rates for the six sediments tested. For comparison of the HF183 decay rates between ambient and oven dried sediment at each site, there was only a significant difference at BR ($p=0.00$).

Overall, decay rates were less variable between sites for HF183 than for FIB, and HF183 decayed in both sterilized and ambient sediments, while FIB persisted or re-grew in most sterilized sediments (Table 3). Decay rates of both FIB and the human-associated HF183 molecular marker (HF183) were dependent on site with FIB and HF183 persisting more than twice as long in certain sediments (Table 4). Preliminary microcosm results reflect field data from a two year MST study where fecal contamination into the upper watershed sites (ST and OF) in Topanga Creek decayed more rapidly than at other sites for both the human marker (HF183) and FIB (*E.coli* and ENT). The Entero1A marker did not exhibit first order decay, which is consistent with Eichmiller et al. 2014.

Table 3. Decay rates (k day^{-1}), calculated using first order decay equation, of FIB and markers at each site.

Site	Ambient Sediment				Sterilized Sediment			
	E.coli	Ent	HF183	ENT1A	E.coli	Ent	HF183	ENT1A
OF	-0.90	-0.30	-0.97	-0.05	0.20	-0.22	-0.42	0.01
ST	-0.76	-0.29	-1.55	-0.07	0.00	-0.24	-1.03	-0.05
BR	-0.24	-0.10	-0.72	-0.03	0.25	0.11	-0.29	0.08
TL	-0.48	-0.25	-0.64	-0.18	–	–	–	–
BC	-0.19	-0.03	-0.58	-0.05	–	–	–	–
MC	-0.48	-0.21	-1.11	0.02	0.21	-0.03	-0.35	0.14

Table 4. Inactivation rate, T_{90}, (days) for molecular markers and FIB at each site.

Site	Ambient Sediment				Oven Dried Sediment			
	E.coli	Ent	HF183	ENT1A	E.coli	Ent	HF183	ENT1A
OF	-2.56	-7.67	-2.37	-46.00	*11.50*	-10.45	-5.48	*230*
ST	-3.03	-7.93	-1.48	-32.86	*2.E+04*	-9.58	-2.23	-46.00
BR	-9.58	-23.00	-3.19	-76.67	*9.20*	*20.91*	-7.93	*28.75*
TL	-4.79	-9.20	-3.59	-13	–	–	–	–
BC	-12.11	-76.67	-3.97	-46.00	–	–	–	–
MC	-4.79	-10.95	-2.07	115.00	10.95	-76.67	-6.57	16.43

*Values in italics indicate accumulation rates- time for increase in initial concentration by 90%.
*T_{90} refers to time for decrease in initial concentration by 90%.

Figure 1. Decay of FIB (A=enterococci, B= *E.coli*) over time at each of the 6 sites. Red lines indicate ambient sediment and black lines indicate oven dried sediment.

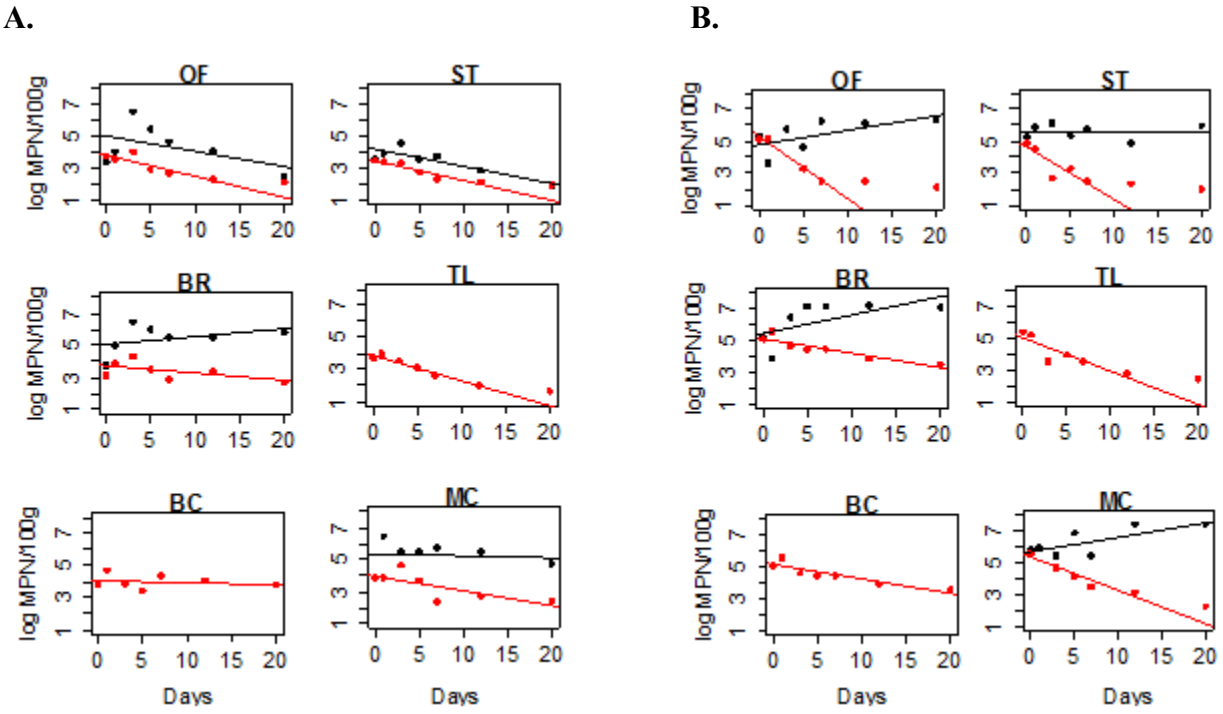
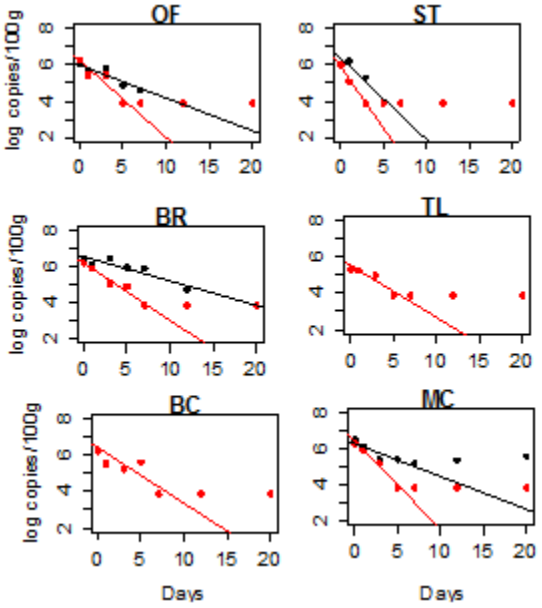
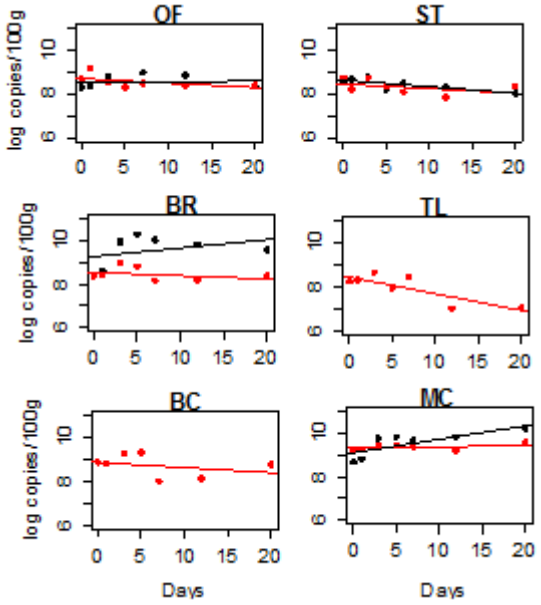


Figure 2. Decay of molecular markers (A=HF183, B= ENT1A) over time at each of the 6 sites. Red lines indicate ambient sediment and black lines indicate over dried sediment.

A.



B.



Discussion

As more pressure is put on local environmental resources, it becomes increasingly important to understand characteristics that effect fate and transport of fecal contamination. Recent studies have highlighted health risks associated with exposure to beach sand (e.g. Heaney et al., 2009). Despite this, little research has been conducted looking at the role of sediments in FIB, DNA-based marker, and pathogen dynamics. MST that utilizes DNA-based markers that consistently decay similarly to pathogens will be more protective of public health. This study showed both that site can affect decay rate of FIB and markers in sediment and that differences exist in decay rates between analytes.

1. Comparison of decay rates between the sites:

There was a trend for fastest decay for FIB (ENT and EC) and the human marker (HF183) at ST and OF, which is consistent with field observations. ST and OF had larger particle size and the lowest carbon and nitrogen content and exhibited the greatest decay, which is consistent with other studies which have found similar effects of sediment characteristics (e.g. Craig et al. 2004). There was a trend for the slowest decay of FIB (ENT and EC) and the human marker (HF183) at BC and BR; these sites had a higher percentage of silt and clay and had the highest nitrogen and carbon content of the sites tested.

2. Comparison of FIB and marker decay:

ENT and HF183 decay rates were correlated, although overall the HF183 marker decayed more rapidly than EC ($p<0.05$) and ENT ($p<0.05$) at all sites. The genetic marker for enterococci, Entero1, decayed much more slowly than did culturable *Enterococcus* spp., likely due to the presence of extracellular DNA, DNA from dead or dying cells, and the presence of viable but non-culturable cells (Bae and Wuertz, 2009a).

3. Comparison of decay between oven dried and ambient sediment (role of the ambient microbiota in decay):

E.coli regrew or persisted in oven dried sediment, but decayed in ambient sediment, which implicates the role of ambient microbiota on *E.coli* decay. There was less difference between oven dried sediment and ambient sediment for ENT and for the HF183 human marker.

Summary

- Inactivation of FIB and markers was influenced by sediment characeristics. Decay rates of FIB and markers varied between the different sites.
- FIB (ENT and EC) and the HF183 and Entero1A markers decayed at different rates, indicating differences in decay for the different analytes tested.
- There was a trend for faster decay rates of *E. coli*, ENT, and the HF 183 marker in the Topanga upper watershed site sediments (ST and OF). Decay may have been more rapid at these sites due to sediment characteristcs (larger particle size and lower carbon and nitrogen content).
- There was no regrowth of FIB or markers in any of the ambient sediments tested.

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