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Use of Viability-Based Methods for Improved Detection of Recent Fecal Contamination in a Microbial Source Tracking Study Near Tijuana, Mexico

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Abstract Current microbial source tracking methods heavily rely on the use of quantitative PCR (qPCR) assays to differentiate human and non-human sources of fecal contamination. However, traditional qPCR measures DNA from viable, viable but not culturable (VBNC), and dead cells, which may confound the use of this technique for detecting recent fecal contamination from waters receiving treated sewage effluent. In this study, fecal indicator bacteria (FIB), six host-associated markers, and two viability-based methods for rapid detection and assessment of fecal contamination were used in a microbial source tracking study to identify sources impairing water quality and sediments within the San Antonio de los Buenos watershed in Tijuana, Mexico. Horse- and gull-associated markers were detected in 4 and 8% of samples tested, respectively. The human- and dog-associated markers were positive in 74 and 63% of watershed samples and 92 and 75% of storm drain samples, respectively. Propidium monoazide (PMA) successfully inhibited amplification of DNA from dead cells in environmental creek waters that receive large volumes of treated wastewater effluent. Accordingly, PMA-qPCR measurements were more comparable to measurements made by culture-based methods (IDEXX). The covalently linked immunomagnetic separation/adenosine triphosphate (Cov-IMS/ATP) method showed a strong linear relationship to culture methods when compared to measurements made by the qPCR Entero1a assay. Both the PMA-qPCR and the Cov-IMS/ATP methods show

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promise for improved assessment of water quality and recent fecal contamination in sewage impacted waters, including areas receiving discharge from wastewater treatment plants, where measurement by qPCR does not effectively differentiate between DNA from live and dead cells. This study serves as an important positive control for non-point source pollution studies.

Keywords Propidium monoazide · Fecal indicator bacteria · qPCR · Microbial source tracking · Cov-IMS/ATP

1 Introduction

In Tijuana, Mexico, inadequate infrastructure results in untreated wastewater that has potential to contaminate groundwater and surface water (BECC 2009b). Due to limited recreational water quality monitoring and inadequate source tracking studies in this region, the impact and extent of sewage discharges to the coastal environment are not well understood. Although significant progress has been made using quantitative PCR for monitoring DNA sequences associated with different host organisms, these methods do not effectively differentiate between DNA from live and dead target organisms making it difficult to determine between effluent that has been properly disinfected and discharge of raw sewage (He and Jiang 2005; Varma et al. 2009). Viability-based methods that can rapidly detect recent fecal pollution events and differentiate between wastewater treatment effluent and fecal pollution events are needed in order to better protect recreational beach visitors and assess public health risk, particularly at beaches that lack effective infrastructure and/or are affected by a known point source (wastewater discharges), such as the beaches in Tijuana and near Rosarito, Mexico.

Although not necessarily pathogenic in nature, fecal indicator bacteria (FIB) such as *Escherichia coli* and enterococci are used as proxies for fecal contamination (Gerba 2000). Epidemiological studies have demonstrated an increased risk of contracting swimming related illnesses at wastewater impacted beaches (Cabelli 1982; Kay 1994; Fleisher 1996; Wade et al. 2006; Wade et al. 2010). Yet, the use of FIB alone to assess water quality presents certain limitations. FIB can originate from both human and animal sources, and some strains can be largely associated with plants and soil (Ferguson et al. 2013). Therefore, presence of FIB does not confirm human contamination and a lack of correlation between FIB and human contamination has been noted in some cases (Lemarchand et al. 2003; Mika at el. 2014; Riedel et al. 2015). Further, current techniques are primarily culture-based, requiring a minimum of 18–24 h for analysis.

The development of rapid detection assays, allowing enumeration of microbial contaminants as quickly as 1 h, has progressed significantly in recent years. One such method, the covalently linked immunomagnetic separation/ATP quantification (Cov-IMS/ATP) method, allows for rapid enumeration of *E. coli* and enterococci from fresh and marine waters (Lee et al. 2010). The Cov-IMS/ATP assay for enterococci has been found advantageous because it is specific to a range of *Enterococcus* species, found comparable to other USEPA standard methods but allows for rapid detection of FIB in less than one hour processing time, and is a viability-based field portable method (Zimmer-Faust et al. 2014).

In recent years, microbial source tracking (MST) techniques have also advanced and become more widely used to help detect fecal inputs that are associated with specific sources of contamination (Boehm et al. 2013). Specifically, library-independent molecular-based methods that distinguish between sources of contamination for fecal indicators such as *Bacteroidales* are being widely applied (Harwood et al. 2014; Griffith et al. 2003; Seurinck et al. 2005; Kildare et al. 2007). Detection methods using qPCR for host-associated *Bacteroidales* markers are highly repeatable, specific, and sensitive methods for tracking human pollution back to its source (Ebdon et al. 2007; Gawler et al. 2007; Layton et al. 2013; Ebentier et al. 2013; Boehm et al. 2013).

Despite the increasingly widespread use of host-associated markers for identification of fecal contamination in impaired watersheds, current molecular assays using quantitative PCR are limited in that they do not discriminate between viable and non-viable cells as these methods amplify DNA of both live and dead cells as well as extracellular DNA (Bae and Wuertz 2009a, b). Extracellular DNA has been found to persist in soil for greater than four weeks, (Nielsen et al. 2007) and DNA of the *Bacteroidales* human marker (HF183Taqman) has been found to persist greater than two weeks in seawater (Nielsen et al. 2007); therefore, detection of human-associated markers by qPCR alone may not necessarily indicate recent contamination events. Further, presence of human-associated markers is expected in wastewater effluent (Varma et al. 2009), even when

wastewater has been effectively treated. The high human marker concentration in treated sewage makes it difficult to detect additional human sources in watersheds subject to wastewater discharge; therefore, these markers alone are not always appropriate for assessing coastal water quality near wastewater outfalls.

New viability-based tools which provide rapid assessment of water quality are critical in areas with malfunctioning treatment systems. Propidium monoazide (PMA), a selective agent that penetrates and inhibits amplification of DNA from dead cells, has been coupled with qPCR to successfully discriminate between live and dead cells of pure cultures (*Bacteroides thetaiotaomicron*) and *Bacteroidales* in human feces and wastewater (Varma et al. 2009; Bae and Wuertz 2009a; Nocker et al. 2006). Yet the ability to measure fecal *Bacteroidales* with PMA in environmental samples and in microbial source tracking studies has not been well documented (Bae and Wuertz 2009a). Previous work with PMA has been predominately lab based.

A novel MST approach combining two viability-based methods (PMA-qPCR and Cov-IMS/ATP), qPCR source-specific assays, and traditional culture-based methods for FIB was tested in an urban sub-drainage basin in Baja California, Mexico that is receiving wastewater effluent. Specifically, we tested the following hypotheses: (1) wastewater effluent discharging into the San Antonio de los Buenos Creek is a source of FIB and human markers to coastal waters; (2) freshwater inputs from storm drains negatively impacts water quality in the SAB watershed; (3) enterococci levels measured by the viability-based technique, Cov-IMS/ATP, correlate with enterococci levels measured by standard culture-based IDEXX method and qPCR; and (4) the addition of a PMA treatment step to an existing qPCR assay improves detection of recent sewage contamination in environmental fresh and marine waters. To our knowledge, this is the first study to utilize two viability-based methods, PMA-qPCR and the Cov-IMS/ATP assays, and to incorporate PMA coupled with qPCR in an innovative microbial source tracking study.

2 Material and Methods

2.1 Field Site

The San Antonio de los Buenos (SAB) sub-watershed is located in Tijuana, Baja California, Mexico approximately 20 km south of the US-Mexico border (Table 1, Figure S1). The regional climate is Mediterranean, with most rainfall (approximately 261 mm annually) in winter months (Nov–Apr) and drier summer months (May–Oct). Currently, approximately 25 million gallons per day (MGD) of sewage from Tijuana is treated at the San Antonio de los Buenos (SAB) wastewater treatment plant. Treated sewage is mixed with five MGD raw sewage, chlorinated, and discharged at the shoreline near Punta Bandera, Tijuana (BECC 2009b). Tijuana Comisión Estatal de Servicios Públicos de Tijuana (CESPT), the local sanitation company, provides wastewater collection services for 87% of the region, and treats only 81% of wastewater generated in Tijuana (BECC 2009a). Further, many of the existing major sewer collection lines within the City are in a deteriorated condition. The inadequate infrastructure results in untreated wastewater and/or raw sewage that has the potential to contaminate groundwater and surface water (BECC 2009b).

Potential sources of fecal pollution within this region can include raw sewage from inadequate or lacking wastewater treatment, and feces from dogs, horses, dairy farm runoff, gulls, and other wildlife. In addition, feral dogs reside in the area and beaches are animal-friendly, thus increasing the amount of possible fecal pollution from canine sources.

2.2 Sample Collection

Samples were collected from eight sites within the SAB sub drainage basin between October 2011 and July 2013. Four marine samples were collected from residential beaches at Baja Malibu, San Antonio del Mar (SADM

Table 1 Coordinates and description of sampling site locations

	Coordinates		
Sample site	Water type	Latitude	Longitude
Baja Malibu Beach	Marine	32.41603	−117.096
Isla Storm drain	Fresh	32.4273	−117.0987
SADM Beach	Marine	32.43119	−117.1002
SADM Storm Drain	Fresh	32.43123	−117.0998
Real del Mar Creek	Fresh	32.4432	−117.1054
SAB Creek	Fresh	32.44655	−117.1075
SAB Beach	Marine	32.44633	−117.1075
Real del Mar Creek	Fresh	32.4432	−117.1054
Punta Bandera Beach	Marine	32.46311	−117.117

Beach), San Antonio de los Buenos (SAB Beach) and Punta Bandera along a 6 km long-shore transect (Figure S1). In addition, samples were collected from four freshwater inputs that discharged directly into the ocean: Real del Mar Creek (RDM Creek), San Antonio de los Buenos Creek (SAB Creek) and two storm drains, (SADB drain and Isla drain). SAB wastewater treatment plant discharges treated sewage into the SAB Creek, making up the majority of the creeks' flow. Water for bacterial and nutrient analyses were collected in two liter sterile polypropylene bottles at ankle-depth, stored on ice and processed within six hours of collection. Processing was done on site at a mobile laboratory in Tijuana, Mexico.

2.3 FIB Enumeration

Samples were analyzed using US EPA marine recreational water quality single sample limit for Total coliforms (TC), *Escherichia coli* (EC) and enterococci (ENT) bacteria. Sample values were also compared to two Mexican single sample water quality limits: the ENT water quality health standard of 200 MPN/100 mL and the TC daily limit for treated wastewater discharging to the ocean of 2000 MPN/100 mL (BECC 2009b).

Samples were processed according to standard methods for enumeration of presumptive ENT (Enterolert, IDEXX), EC, and TC (Colilert-18, IDEXX). Tenfold and 100-fold dilutions of water samples were used as recommended by the manufacturer. Higher dilutions (1000-fold and/or 10,000-fold) were periodically used for highly contaminated sediments and water samples. Subsets of samples were analyzed in parallel for enterococci by membrane filtration (Method 1600, USEPA 2010).

Sediment samples ($n = 28$) were collected from selected sites on five separate days. Samples were processed according to Mika et al. (2009) with the modification of adding greater sediment to buffer ratio for each wash. Specifically, approximately 45 g of sediment was resuspended in 60 mL of PBS (pH 7.0 + 0.2) and shaken by hand for 2 min. Supernatant was decanted after settling for 1 min into a fresh, sterile bottle (Lee et al. 2006). This process was repeated for a total of two washes to obtain a final volume of 120 mL resuspension, which was then used for all FIB enumeration of sediment samples according to the methods above.

2.4 Sample Filtration and DNA Extraction

Each sample was filtered through 47-mm, 0.4-μm pore size HTTP polycarbonate filters (Isopore Millipore, Billerica MA) in triplicate of varying volume (due to turbidity) from 15 to 500 mL on a standard platform manifold with sterile disposable filtration devices (Thermo Scientific, Logan, UT). Filters were stored in 2-mL polypropylene screw cap tubes containing acid-washed glass beads (Sigma-Aldrich, St. Louis MO) at −80 °C until DNA extraction. DNA was extracted using commercial kits. DNA was recovered from samples collected before 2012 according to manufacturer's guidelines with a slight modification of the Mo Bio UltraClean fecal DNA extraction kit (Mo Bio Laboratories Inc., Carlsbad, CA). Sample filters were placed on the Biospec 8-Mini Bead Beater for 1.5 min in lieu of vortexing samples for ten minutes (Mika et al., 2014). For samples collected in 2012 to 2013, the DNA was recovered using the DNA-EZ ST1 extraction kit (GeneRite, North Brunswick NJ). To assess possible differences due to a change in extraction kits during the study, a subset of samples extracted with the DNA-EZ ST1 kit was run for qPCR analyses in parallel with samples extracted with the Mo Bio UltraClean fecal DNA extraction kit for comparison. No significant difference was shown in qPCR amplification results between the two kits ($R^2 = 0.91$; data not shown).

2.5 Host-Associated Markers

Samples collected from 2010 to 2012 were analyzed for human-associated *Bacteroidales*. The HF183 SYBR assay was applied with DNA primers HF183F and HF183R (Bernhard and Field 2000a, b; Seurinck et al. 2005; Kildare et al. 2007). Each sample was measured in duplicate and a subset of samples on each plate spiked with 1 μl (~10^4 copies positive control) *Bacteroidales* standard to evaluate recovery and any possible inhibition by contaminants in DNA extracts (Mika et al. 2014; Bustin et al. 2009). In the case of interference, samples were diluted twofold and reprocessed (Noble et al. 2006; Cao et al. 2012). qPCR assays were conducted on 25-μl reaction mixtures with a StrataGene MX3000P thermocycler.

Samples taken in 2012 and 2013 were processed for a suite of host-associated markers (Table 2) to identify human and non-human sources within the watershed. Samples collected from 18 March 2012 to July 11 2013 were tested for the following assays: HF183 Taqman

Table 2 Endpoint and qPCR assays used in study

Name	Source	Type	Target	Forward primer/reverse Primer	Probe/dye	Reference
HF183 Taqman	Human	qPCR	*Bacteroides* 16S	ATCATGAGTTCACATGTCCG/OGTAGGAGT TTGGACOGTGT	FAM-CTGAGAGG AAGGTOCCOCACATTGA-TAMRA	Haugland et al. 2010
HF183 SYBR	Human	qPCR	*Bacteroides* 16S	ATCATGAGTTCACATGTCCG /TACCOCGOCTACTATCTAATG	SYBR Green	Seurinck et al. 2005
BacHum	Human	qPCR	*Bacteroides* 16S	TGAGTTCACATGTOOGCATGA/CGTTACCC OGOCTACTATCTAATG	FAM-CTGAGAGG AGGTOCCOCACATTGGA-TAMRA	Kildare et al. 2007
Gu112 Taqman	Gull	qPCR	*Catellicoccus marimammalium*	TGCATCGACCTAAAGTTTTGAG/ GTCAAAGAGCGAGCAGTTACTA	FAM-CTGAGAGG GTGATOGGOCACATTGGGACT-BHQ1	Shibata et al. 2010, Sinigalliano et al. 2013
DogBact	Dog	qPCR	*Bacteriodales spp.*	COCTTGTATGTACOGGTACGCAATCGGA GTTCGTG	FAM-ATTOGTGGTGTAGOGGTGAAAT GCTTAG-BHQ1	Sassourbre et al. 2012a, b
HoF597	Horse	Endpoint	*Bacteriodales spp.*	CCAGOCGTAAAATAGTCGGCAATCG GAGTTCTTCGTG	N/A	Dick et al. 2005
Enterococcus	General	qPCR	*Bacteriodales spp.*	AGAAATTCCAAACGAACTTGCAGTGC TCTACCTCCATCATT	6FAM-TGGTTCTCTCCGAAATAGCT TTAGGGCTA-TAMRA	Haugland et al. 2010, USEPA Method A

Samples collected within the first phase of the study period (2009–2011) were analyzed for HF183 SYBR only. Samples collected from the second phase (2012–2013) were analyzed for all markers, excluding HF183 SYBR

(Haugland et al. 2010), BacHum (Kildare et al. 2007), DogBact (Shibata et al. 2010), Gull2Taqman (Shibata et al. 2010), and Entero1a (US EPA Method A). qPCR analyses were carried out according to previously published and standardized protocols detailed in Boehm et al. 2013. Host-associated qPCR assays were conducted on 25-μl reaction mixtures in triplicate using an Applied Biosystems StepOnePlus thermocycler. Cell concentrations were calculated according to pooled standard curve analysis based on the relative quantity of target DNA compared to that of a known quantity of target DNA (either genomic or plasmid) (Table S1). DNA concentration and quality were determined using UV absorption with a Nanodrop 2000C (Thermo Scientific, Waltham MA).

2.6 PMA-qPCR for HF183 SYBR

Propidium monoazide (PMA) treatment was applied to a set of samples for analysis of viability of human-associated marker detected using the HF183 SYBR assay. Each sample was filtered through a 47-mm, 0.4-μm pore size HTTP polycarbonate filters (Isopore Millipore, Billerica MA) in varying volume from 15 to 500 mL on a standard platform manifold with sterile disposable filtration devices (Thermo Scientific, Logan, UT). The filter was placed in 5 mL of PBS, and vortexed for one minute. One mL of resuspension was aliquoted for analysis of PMA treatment. PMA was applied to samples as described in Nocker et al. (2007) and Bae and Wuertz (2009a) with slight modifications. Briefly, a final concentration of 100 μM PMA (stock originally dissolved in 20% dimethylsulfoxide) was added to one mL of sample resuspension and incubated in the dark for five minutes. The sample tubes were then placed in a container with ice and agitated on an orbital mixer while exposed to a 700-W halogen lamp for ten minutes. Tubes were manually rotated approximately every minute to prevent excessive heating. Samples were then centrifuged at 10,000×*g* for 5 min at room temperature. The pellet was resuspended in one mL of AE buffer and filtered as described above through a 47-mm, 0.4-μm pore size HTTP polycarbonate filter (Isopore Millipore, Billerica MA). The filter was stored in a 2 mL polypropylene screw cap tub containing acid-washed glass beads (Sigma-Aldrich, St. Louis MO) at −80 °C until DNA extraction.

2.7 Cov-IMS/ATP for ENT

The covalently linked immunomagnetic separation/ATP quantification method (Cov-IMS/ATP) was applied for analysis of viable enterococci in water samples. Cov-IMS/ATP, the near-real time assay for *Enterococcus spp*. and *E. coli* based on immunomagnetic separation IMS/ATP quantification (Lee and Deininger 2004; Bushon et al., 2009), has been developed for use in freshwater and marine water sources within impaired watersheds (Lee et al. 2010; Mika et al. 2014). Samples in this study were processed in three main steps: (1) isolation of target organisms, during which samples were incubated on a rotating mixer with antibody-magnetic bead biosorbents; (2) lysing of target organisms, exposing specific ATP into solution; and (3) the addition of the luciferin/luciferase enzyme, which catalyzes a light-emitting reaction while consuming ATP. This emission was quantified using a luminometer (GloMax Microplate Multimode Reader; Promega; New Horizon Diagnostics, model 3560), and light intensity was measured in relative light units (RLU) and correlated to cell concentration.

Samples in this study were processed according to methods detailed in Zimmer-Faust et al. (2014). Briefly, a solution of antibody-magnetic bead complexes was generated: 200 μl of Dynabead particles (Invitrogen, Carlsbad, CA) washed in borate buffer in phosphate-buffered saline (PBS) (10% [wt/vol] borate buffer in PBS, pH 9.5) were incubated with 40 μl *Enterococcus spp*. antibodies (cat #B65173R, Meridian Life Sciences) on a rotating mixer at 37 °C for 18–24 h. Following incubation, the anti-ent biosorbent mixture was washed with and stored in bovine serum albumin (0.1% BSA *w*/*v* in PBS) buffer at continuous rotation at 4 °C for up to 2 weeks. Approximately 200–500 mL per sample was filter-concentrated and analyzed using the anti-ent biosorbent mixture. Samples were filtered through a 0.45-μm filter (SA1J792H5; Millipore) to capture bacteria. Filters were then submerged in 10 mL PBS and bacteria were resuspended after 1 min of vortexing. One milliliter of resuspension was added to the anti-ent biosorbent and incubated on a Dynal Rotary Mixer (Life Technologies, Grand Island, NY) for 40 min. After incubation, the bound target enterococci was separated from the biosorbent with use of a magnetic separator. After two washes with 1 mL of Tween 20 in PBS (1% wt/vol, Tween in PBS), bound complexes were washed with 200 μl of somatic-cell releasing agent and lysed with 200 μl of bacterial cell-releasing agent. Supernatant and 100 μl of Bactiter-Glo reagent (Promega, Madison, WI)

was transferred to each well of a white, 96-well plate. A luminometer quantified luminesce in RLU for all sample wells.

2.8 Ancillary Parameters

Water samples were analyzed in situ for dissolved oxygen, salinity, specific conductivity, pH. and water temperature with a Hach Quanta Hydrolab multi-parameter probe (Hach, Loveland, Colorado) deployed in knee-depth water. A subset of water samples collected were also analyzed for nitrate and ammonia (TNT 835 and TNT 813; Hach, Loveland, Colorado) using the Hach Spectrophotometer (model DR 280).

2.9 Statistical Analyses

Linear regression models were applied to estimate relationship between culture-based, IMS/ATP and qPCR measurements and were computed using $\log_{10}$-transformed data. Pearson's correlation coefficients were calculated to further examine the linear relationship between methods. Regression analyses were done in STATA 12.1 (STATA Corp LP, College Station, Texas). Box plots were generated using the default settings of R Statistical Software version 12.1 (Team 2011). Horizontal lines in the boxes represent the second quartile (median). The upper and lower edges of the boxes (hinges) extend to the 1st and 3rd quartiles. The whiskers (upper/lower adjacent) extend to the most extreme data point which is no more than 1.5 times the length of the box beyond the box. Data points beyond the whiskers are declared outliers and represented with open circles.

3 Results

3.1 Spatial FIB Results

FIB exceeded US EPA Recreation-1 water quality single-sample standards within the SAB sub-watershed in 42, 45, and 44% of samples collected for TC, EC, and ENT and 44% of samples for both EC and ENT, respectively (Table S2). A comparable fraction of samples (42%) also exceeded the Mexican water quality threshold for ENT. ENT and EC concentrations were well correlated ($R^2 = 0.91$).

3.2 FIB Results Near Freshwater Inputs

FIB results in freshwater showed elevated levels at RDM Creek and in runoff samples from SADM and Isla storm drains. All three sites exceeded standards in 100% of samples tested (Table S2). FIB concentrations measured from creek and storm drain sites were two to four orders of magnitude higher than those from other sites (Figure S2). FIB levels for beach sites receiving direct inputs were generally higher than for other marine sites (Baja Malibu and Punta Bandera). FIB levels ranged from two to 35 times higher at SADM Beach and twelve to 270 times higher at SAB Beach in comparison to beaches free from inputs. Both SADM and SAB beaches had a greater frequency of exceedance for FIB than other beaches tested. Storm drains adjacent to these marine sites consistently showed elevated levels of FIB and likely contributed to exceedances.

Variable levels of FIB were observed for SAB Creek and Beach throughout the study. These sites were in exceedance of USEPA guidelines for recreational coastal waters 25–27% of the time. Elevated FIB (>241960) concentrations for all three indicators were measured from SAB Creek on three different sampling days during the study period. During those events, TC concentrations were above the Mexican allowable daily limit for treated wastewater discharging to the ocean and were in exceedance of US EPA single sample limits. On these dates, concentrations of FIB at upcoast and downcoast sites remained two to three orders of magnitude lower, despite elevated levels at the SAB discharge point.

3.3 FIB Results in Sand Samples

FIB levels in sediment were higher in sand collected near creek sites and at SAB Beach, and followed a similar trend to FIB levels measured in water. FIB enumerated from sand collected near creek sites ranged from <1–1468 MPN/g dry weight for ENT, and sand samples from SAB beach reached concentrations of 651 MPN/g dry weight for ENT (Fig. 1). Highest levels were measured from wet sand collected adjacent to SAB Creek, with values greater than 163,904 MPN/g dry weight for all three indicator bacteria on 19 February 2010. Low levels or non-detectable levels of FIB were measured in sand from all other sites. Median concentrations of FIB in sand for the study were 10.12, 11.41, and <1 MPN/g dry weight for TC, EC and ENT, respectively.

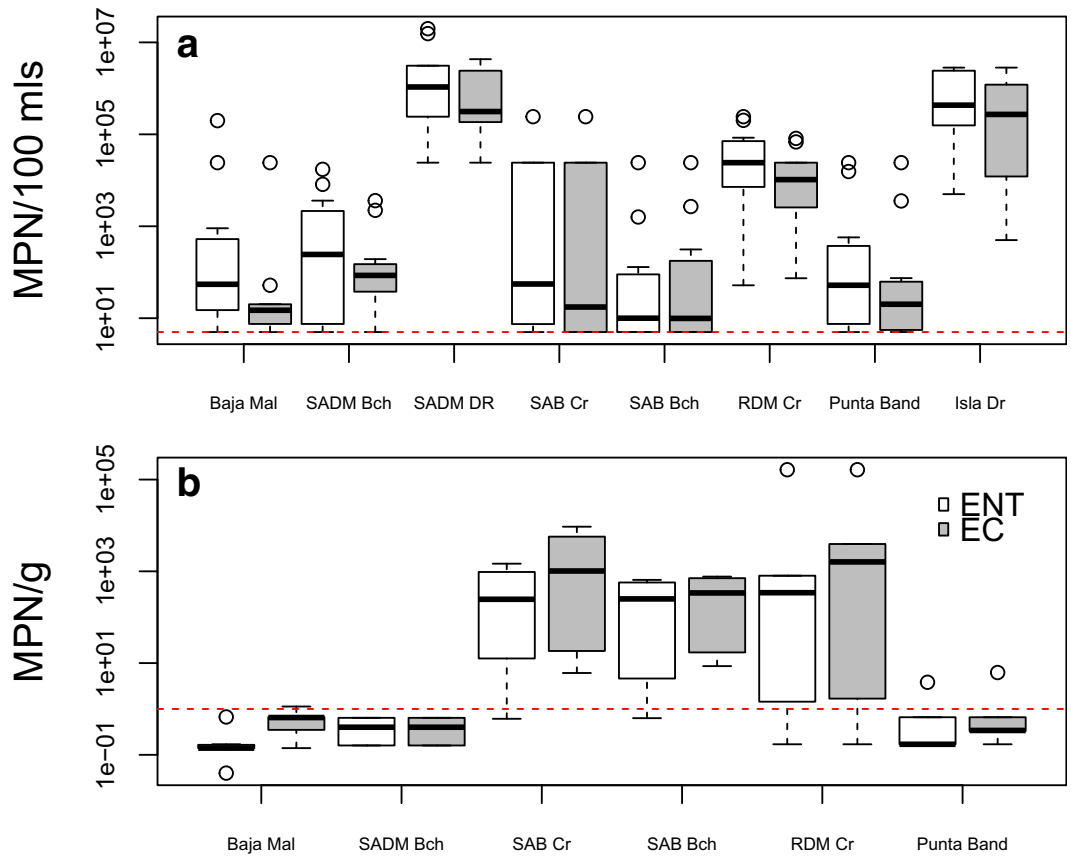

Fig. 1 *Box* and *whisker* plot of *E. coli* and enterococci concentrations for the study period. Plots are shown for FIB results in water (*top panel*) and in sand (*bottom panel*) at each sampling site. FIB levels measured from water are reported in units of MPN per 100 ml while FIB levels in sand are reported in units of MPN/g dry weight. The *dotted red line* depicts detection limit. The *box* signifies the 25th, 50th, and 75th percentiles and the *whiskers* depict the 10th and 90th percentiles

Table 3 Exceedance of FIB. Percent exceedance of FIB is listed for samples measured for total coliforms (TC), *E. coli* (EC), and enterococci (ENT) above the US EPA water quality standards and the Mexico water quality standards for enterococci

		% Exceedance of USEPA standard	% Exceedance of MX standard		
Sample site	*n*	TC	EC	ENT	ENT
A	12	0	8	0	0
B	12	25	33	33	17
C	12	25	25	25	25
D	11	18	27	27	27
E	12	100	100	100	100
F	12	8	8	8	0
Bd	9	100	100	100	89
I	6	100	100	100	100
All sites	86	42	45	44	42

3.4 Non-human Marker Results

Combined data for presence and range of quantification of source-associated molecular markers is shown in Table 3a–f and Fig. 2. Three animal host-associated markers were used to assess if dog, gull and horse fecal waste were polluting the SAB sub-drainage basin. The dog-associated marker (DogBact) was detected in 66% of all samples analyzed from field sites. One hundred percent of samples tested at the SAB Creek and SAB Beach samples were positive for DogBact. Frequent detection of Dogbact (67–83%) was also observed in storm drain samples. The SAB Creek and storm drains (SADM and Isla) were the most impacted sites with the highest concentrations of dog marker ranging from 10^4–10^6 copies/100 mL (Table 3e). Gull marker was not frequently detected in the SAB sub-drainage basin, with only 8.5% (4/47)

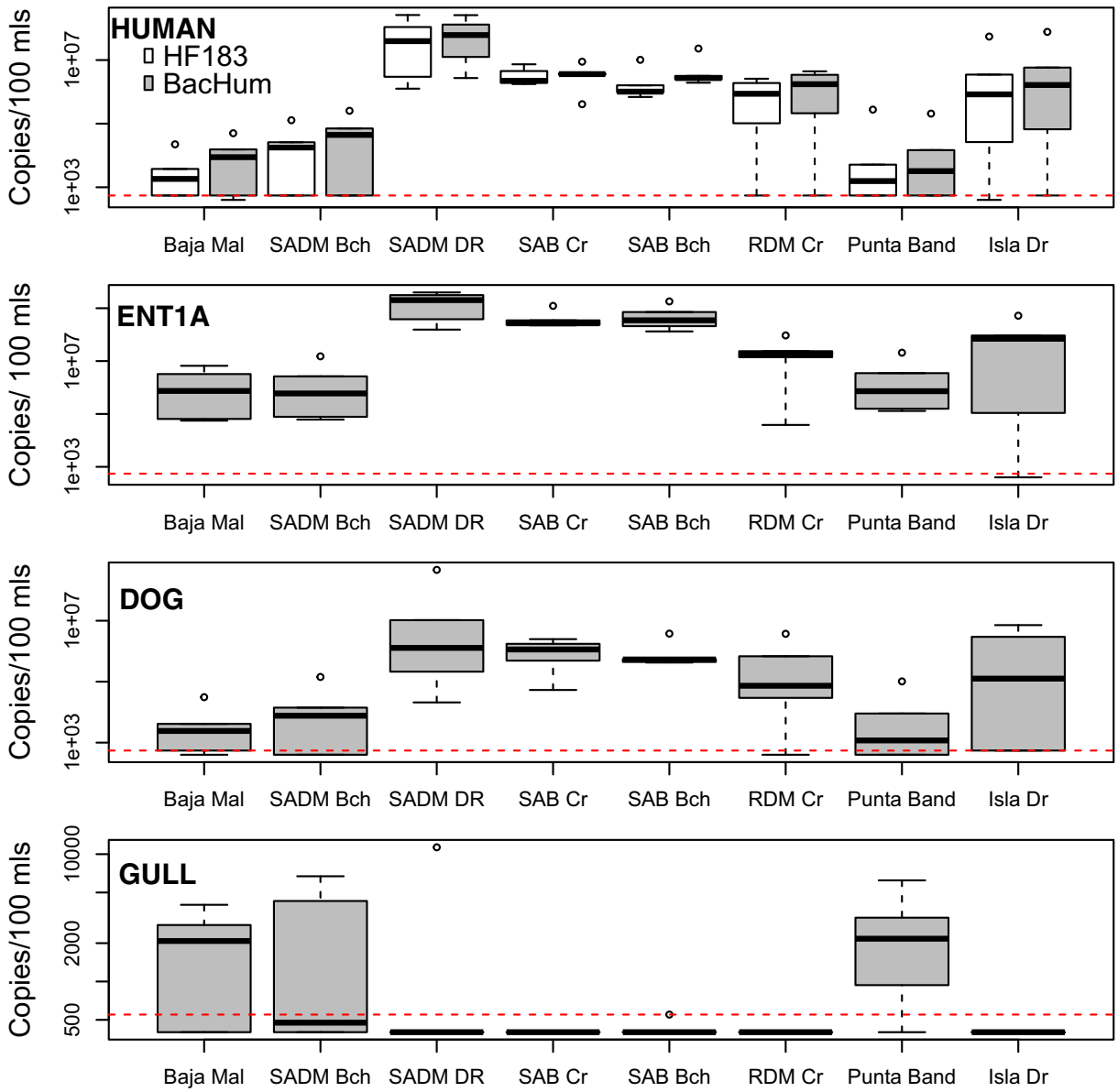

Fig. 2 *Box* and *whisker* plot of MST markers for the study period, reported in copies/100 mL. The *dotted red line* slows the lower limit of quantification for each marker. The *box* signifies the 25th, 50th, and 75th percentiles and the *whiskers* depict the 10th and 90th percentiles

of all samples positive for the gull-associated marker. Gull marker was only detected at Baja Malibu, SADM Beach and Punta Bandera beach sites and concentrations ranged between 2565–2787 copies/100 mL (Table 3f).

The horse-associated (HoF597) marker was also tested in fresh and marine waters. The HoF597 marker was positive for two of 47 samples tested and was not found to be a significant source at this field site.

3.5 Human-Associated Marker Results

Human-associated markers were detected in 72% (HF183Taq) and 81% (BacHum) of samples collected from field sites. Creek sites (SAB Creek and RDM Creek) both had extremely high and consistent levels of BacHum and HF183Taq, ranging between 10^4 and 10^6 copies/100 mL. Storm drains were also impacted by human fecal contamination. HF183 and BacHum were

detected in 83–100% of samples tested from storm drains (Table 3b, c). The highest concentration and the highest median values (10^7–10^8 copies/100 mL) of human-associated markers were measured in runoff from the SADM storm drain. HF183 and BacHum markers were detected in 50–67% of samples collected from marine sites (Baja Malibu, SADM Beach and Punta Bandera beaches). Human-associated marker concentrations at marine sites were typically measured at one to two orders of magnitude lower than concentrations at creek sites, and up to five orders of magnitude less than storm drain sites.

The HF183 SYBR marker was detected in 71% (n = 41) of samples tested from field sites, with a median concentration (in ROQ) of 4.45×10^4 copies/100 mL (Table 3a). SAB Creek, SAB Beach and RDM Creek were impaired for the human-associated marker, with 100% detection of HF183 SYBR at creek sites and 83% detection at SAB Beach. HF183 SYBR was also detected in a 33–50% of samples analyzed for other sites (Baja Malibu, SADM Beach and Punta Bandera), with lowest frequency of detection at the furthest upcoast (Punta Bandera) and downcoast (Baja Malibu) sites. Concentrations in SAB Creek water ranged on from 2.87×10^3–9.15×10^4 copies/100 mL; SAB Beach had comparable levels (2.28×10^2–1.93×10^5 copies/100 mL). Median values for RDM were measured at 7.24×10^4 copies/100 mL for HF183 SYBR. All four samples collected from SADM and Isla storm drains were positive for the HF183 marker with concentrations reaching as high as 7.30×10^6 copies/100 mL in runoff samples.

3.6 PMA-qPCR Results

A subset of water samples were analyzed for the human-associated HF183 SYBR marker with and without PMA treatment. PMA-qPCR (n = 20) and qPCR samples are plotted against FIB enumerated with culture-based methods (Figure S2). PMA-qPCR samples showed a stronger relationship with FIB than did qPCR. PMA treated samples and measurements made by IDEXX for both EC and ENT had an increased R^2 ($R^2 = 0.43$ and $R^2 = 0.55$) when compared against samples processed without the PMA step ($R^2 = 0.33$ and $R^2 = 0.36$). Samples falling below ROQ are estimated and non-detects were set to a value of 200 copies/100 mL. In addition, several samples exceeded the detection limit for FIB; therefore, actual correlations may be stronger for PMA-qPCR and FIB than captured here (Figure S2).

3.7 Viable vs Non-viable Method Performance

Data were analyzed with and without WWTP outflow points and increased performance was observed when SAB Creek and mixing zone discharge points were removed from analysis. When data from all sites were included, viable methods had stronger linear relationship to FIB (Tables 4 and 5). Viability-based measurements agreed well, whereas viability-based measurements compared to qPCR showed a week relationship. Cov-IMS/ATP results correlated well with IDEXX Enterolert measurements for measurement of ENT ($R^2 = 0.71$). However, the correlation was weak between Cov-IMS/ATP measurements and ENT 1A qPCR measurements ($R^2 = 0.33$). A poor linear relationship was seen when comparing qPCR and IDEXX EC and ENT measurements. When data points from SAB Creek and Beach were removed, R^2 increased from 0.33 and 0.36 to 0.73 and 0.77, for EC and ENT, respectively, illustrating a significant linear relationship between IDEXX and qPCR measurements at the other sites.

4 Discussion

Elevated concentrations of FIB and exceedances in water quality standards, along with detection of several host-associated markers, indicate widespread fecal contamination in the San Antonio de los Buenos sub-watershed. Dog and human fecal contamination were both important sources and frequently detected. High levels of fecal contamination were associated with freshwater storm drain inputs and in creek sites. Previous studies have also reported high FIB values associated with the wastewater discharge from the SAB outfall (Orozco-Borbon et al. 2006). These studies did not evaluate potential inputs from storm drains. This study identified that degraded water quality at SADM Beach may be the combined effect of SAB Creek discharge (outlet of the SAB WWTP) as well as urban runoff from storm drains. Further, creek sand samples had FIB levels consistent with sewage impacted sand (Mika et al. 2009).

To investigate the extent of contamination associated with the SAB outfall, samples were collected at sites upcoast and downcoast of the discharge point. During this study, human-associated markers and high levels of FIB were detected at the SAB outfall and on occasion at Punta Bandera, located 2.05 km upcoast from the SAB

Table 4 Results of host-associated molecular markers. Summary of molecular markers tested in study for (A) HF183 SYBR, (B) HF183Taqman, (C) BacHum, (D) ENT, (E) DogBact, and (F) Gull2Taqman

A	*n*	HF183 SYBR	%Frequency detected	%ROQ	Median (ROQ) copies/100 mL	(ROQ) copies/100 mL	
Site Description		%ND				lower	upper
Baja Malibu	6	67	33	17	9.02E + 02	–	–
SADM Beach	6	50	50	17	4.99E + 03	–	–
SAB Creek	6	0	100	83	2.72E + 04	2.87E + 03	9.15E + 04
SAB Beach	6	17	83	83	1.11E + 04	2.28E + 02	1.93E + 05
RDM Creek	7	0	100	86	7.24E + 04	1.19E + 04	1.26E + 06
Punta Bandera	6	67	33	0	–	–	–
All sites	37	29	71	54	4.46E + 04	2.28E + 02	7.30E + 06
B	n (#)	HF183 Taqman	%Frequency of detection	% ROQ	Median (ROQ) copies/100 mL	(ROQ) copies/100 mL	
Site Description		% ND				lower	upper
Baja Malibu	6	50	50	50	3.63E + 03	2.99E + 03	2.19E + 04
SADM Beach	6	33	67	67	2.27E + 04	1.48E + 04	1.27E + 05
SAB Creek	6	0	100	100	2.11E + 06	2.14E + 05	7.39E + 06
SAB Beach	5	0	100	100	1.00E + 06	6.86E + 05	1.02E + 07
RDM Creek	6	17	83	67	1.62E + 06	2.48E + 04	2.59E + 06
Punta Bandera	6	50	50	33	4.98E + 03	2.48E + 03	2.75E + 05
SADM Drain	6	0	100	100	4.00E + 07	1.25E + 06	2.69E + 08
Isla Drain	6	17	83	83	9.69E + 05	2.56E + 04	5.62E + 07
C	n (#)	BacHum	%Frequency of detection	% ROQ	Median (ROQ) copies/100 mL	(ROQ) copies/100 mL	
Site Description		% ND				lower	upper
Baja Malibu	6	33	67	67	1.46E + 04	2.44E + 03	4.85E + 04
SADM Beach	6	33	67	67	5.72E + 04	4.06E + 04	2.52E + 05
SAB Creek	6	0	100	100	3.48E + 06	3.74E + 05	9.00E + 06
SAB Beach	5	0	100	100	2.70E + 06	1.92E + 06	2.36E + 07
RDM Creek	6	17	83	83	2.98E + 06	2.04E + 05	4.48E + 06
Punta Bandera	6	50	50	50	1.41E + 04	5.50E + 03	2.05E + 05
SADM Drain	6	0	100	100	6.36E + 07	2.71E + 06	2.74E + 08
Isla Drain	6	17	83	83	1.82E + 06	6.38E + 04	8.11E + 07
D	*n* (#)	ENT	%Frequency of detection	% ROQ	Median (ROQ) copies/100 mL	(ROQ) copies/100 mL	
Site Description		% ND				lower	upper
Baja Malibu	6	2	67	67	1.21E + 04	1.22E + 03	3.65E + 04
SADM Beach	6	1	83	83	3.29E + 03	3.90E + 02	8.49E + 04
SAB Creek	6	0	100	100	1.53E + 06	1.34E + 05	7.18E + 06
SAB Beach	5	0	100	100	2.03E + 06	7.41E + 05	1.07E + 07
RDM Creek	6	1	83	83	1.03E + 05	7.46E + 04	5.29E + 05
Punta Bandera	6	0	100	100	3.82E + 03	6.58E + 02	1.17E + 05
SADM Drain	6	0	100	100	1.20E + 07	8.69E + 05	2.37E + 07
Isla Drain	6	1	83	83	5.04E + 05	8.91E + 04	3.06E + 06
E	n (#)	DogBact	%Frequency of detection	% ROQ	Median (ROQ) copies/100 mL	(ROQ) copies/100 mL	
Site Description		% ND				lower	upper
Baja Malibu	6	83	17	17	3.12E + 04	–	–
SADM Beach	6	50	50	50	1.41E + 04	1.14E + 04	1.45E + 05
SAB Creek	6	17	100	83	1.11E + 06	5.32E + 04	2.53E + 06
SAB Beach	5	0	100	100	5.40E + 05	4.28E + 05	3.85E + 06

Table 4 (continued)

RDM Creek	6	33	67	67	4.05E + 05	2.99E + 04	3.82E + 06
Punta Bandera	6	50	50	50	9.02E + 03	8.44E + 03	1.04E + 05
SADM Drain	6	17	83	83	1.36E + 06	2.14E + 05	4.89E + 08
Isla Drain	6	33	67	67	1.60E + 06	8.77E + 04	7.39E + 06
F	n (#)	Gull2Taq	%Frequency of detection	% ROQ	Median (ROQ) copies/100 mL	(ROQ) copies/100 mL	
Site Description		% ND				lower	upper
Baja Malibu	6	83	17	0	–	–	–
SADM Beach	6	67	33	17	2.79E + 03	–	–
SAB Creek	6	100	0	0	–	–	–
SAB Beach	5	100	0	0	–	–	–
RDM Creek	6	100	0	0	–	–	–
Punta Bandera	6	83	17	17	2.57E + 03	–	–
SADM Drain	6	100	0	0	–	–	–
Isla Drain	6	100	0	0	–	–	–

Number of samples included in analysis, percentages of samples detected for host markers and percentages for samples falling in the range of quantification (ROQ) and non-detect (ND) are provided. Median values and ranges of values for samples falling within ROQ are also shown. Samples with only one number falling in ROQ are reported as median value, but no range is given

discharge point. In addition, currents modeled with the Southern California Coastal Ocean Observing System (SCCOOS) were confirmed as flowing northward (upcoast) during the time of sampling on certain dates (data not shown). In other work, the SAB discharge plume was estimated to travel northward approximately 56 times a year, even contributing to pollution sources at San Diego beaches on occasion (Terrill et al. 2009; Sung Yung et al. 2009). In addition, the SAB outfall may also contribute to downcoast contamination. Sassourbre et al. (2012a, b) measured human enteric viruses, adenovirus and enterovirus at the SAB Creek discharge point and in marine waters 3.6 km downstream of the discharge point at Baja Malibu. In this study, frequent detection of markers downcoast of SAB Beach supports earlier work that currents can transport contamination as far as Baja Malibu.

Due to the widespread contamination associated with the SAB plume, it is critical to utilize effective monitoring methods for assessing water quality in the region. FIB and qPCR are routinely used in microbial source tracking studies to assess fecal contamination. In this study, HF183 SYBR qPCR measurements did not correlate with FIB measurements on days when water quality in creek and surf zone samples were impaired with treated sewage. Other studies have previously reported a lack of correlation between FIB and human-associated source markers (Santoro and Boehm 2007; Litton et al. 2010; Flood et al. 2011). Litton et al. (2010) found that HF183 marker and enterococci measurements did not covary in a stream impacted with treated wastewater effluent. Similarly, in this study a poor relationship was seen between HF183 SYBR qPCR and FIB measurements at the SAB Creek and Beach (R^2 = 0.13). For the overall sub-watershed, an improved relationship was seen ($R^2 = 0.42$ for ENT). If SAB Creek and Beach sites were removed from overall analysis, the fit was improved further and a strong linear relationship

Table 5 Statistical analysis of viable (Cov-IMS/ATP and SYBR-PMA) and non-viable methods (HF183 SYBR) compared with standard FIB measurements (EC and ENT by IDEXX

Status type	Assay comparison	R^2	P	Slope	SE
Viable vs viable	HF183 PMA-SYBR vs EC IDEXX	0.43	0.01	0.72	0.21
Viable vs viable	HF183 PMA-SYBR vs ENT IDEXX	0.55	0.00	0.70	0.16
Viable vs viable	ENT Cov-IMS vs ENT IDEXX	0.71	0.00	0.59	0.14
Non-viable vs viable	HF 183 SYBR vs EC IDEXX	0.33	0.01	0.70	0.25
Non-viable vs viable	HF 183 SYBR vs ENT IDEXX	0.36	0.01	0.63	0.21
Non-viable vs viable	ENT1A vs ENT IDEXX	0.09	0.08	0.36	0.21

P values greater than 0.05 are determined as significant

was observed ($R^2 = 0.73$) when comparing qPCR and ENT measurements.

Further, HF183 SYBR qPCR was unable to distinguish between when the SAB wastewater treatment plant functioned properly versus when effluent had viable fecal waste from inadequate sewage treatment. The HF183 SYBR marker was consistently detected, likely due to presence of treated, dead cellular DNA. Alternative rapid methods to qPCR are needed for assessing water quality in sampling locations receiving treated wastewater effluent. Addition of a PMA treatment step to qPCR can improve the relationship of molecular source markers to FIB and the ability to detect recent sewage spills. In this work, qPCR measurements with PMA treatment showed a strong correlation to measurements made by culture-based methods. Low FIB levels (<10MPN/100 mL) at the SAB discharge point coincided with low levels of the human-associated marker (HF183 SYBR < LOD) measured with PMA-qPCR on two different dates. In addition, a spike in FIB (>24916 MPN/100 mL) coincided with a spike for the human marker measured by PMA-qPCR (Fig. 3). Samples analyzed with qPCR only and with PMA treatment exhibited similar values (between 10^3 and 10^5 copies/100 mL) on Feb 19th, 2010, suggesting that viable cells were contributing a higher portion of the human-marker.

Previous studies support the need of viability-based methods for detection of viable cells and for accurately assessing wastewater effluent (Nocker et al. 2007; Bae and Wuertz 2009a; Sassourbre et al. 2012a, b). Bae and Wuertz (2009a) used a PMA-qPCR assay and measured

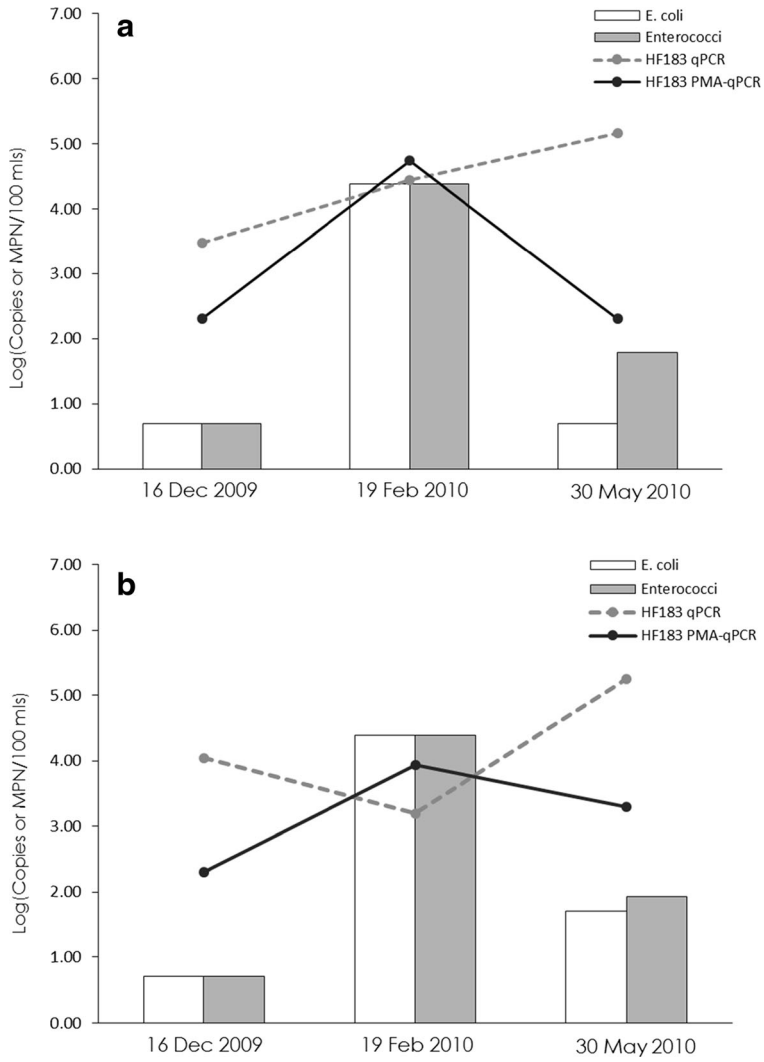

Fig. 3 Concentrations of FIB, qPCR, and PMA-qPCR are shown for **a** SAB Creek and **b** SAB Beach for three different sample dates. PMA-qPCR samples that did not amplify (non-detects ND) were set to the limit of detection for the assay of 200 copies/mL

human marker in influent and effluent samples from a wastewater treatment plant, effectively distinguishing viable fecal pollution. This study shows PMA-qPCR better captures dynamic changes in fecal indicator concentrations relating to treatment of wastewater effluent. This is the first study to our knowledge to document the successful application of the HF183 SYBR PMA-qPCR assay for monitoring sewage treatment at a wastewater discharge point.

PMA-qPCR may need to be optimized on a per assay basis for different molecular host markers. In this study, several PMA-qPCR samples gave measurements that were higher than the same samples measured without PMA-treatment. Possibly, this is a result of using an optimized BacHum PMA-qPCR assay when comparing directly to HF183 SYBR marker measurements. Although outside the scope of this work, future research is needed to evaluate the performance of PMA-qPCR for a suite of human-associated and animal host markers. In addition, further optimization may be required for use of the PMA-qPCR assay in molecular source tracking and environmental field studies. Future work to investigate the widespread use of PMA-qPCR will also require testing the application of this viability-based method in less contaminated watersheds.

Other alternative rapid methods besides PMA-qPCR may assist in detecting recent fecal contamination events. For example, in this study, a second alternative method, the covalently linked immunomagnetic separation/adenosine triphosphate (Cov-IMS/ATP), was evaluated for performance in rapidly assessing water quality near point-sources of pollution. Cov-IMS/ATP exhibited a strong linear relationship with IDEXX, when all sample locations were included in analysis.

The use of viability-based, rapid methods for detection of sewage contamination may greatly benefit regions with frequent infrastructure failure. SAB Creek, which is made up of mostly treated effluent from the SAB wastewater treatment plant, had FIB concentrations in exceedance of USEPA and Mexico water quality standards in three of 12 sampling events (25% of samples). Rapid detection of fecal contamination is critical for remediation efforts and to identify problems associated with sewage treatment at that facility. In addition, same day detection and notification of a sewage spill would help prevent exposure of swimmers and beach visitors to contaminated waters. The city of Tijuana collects and treats 81% of the sewage generated in the region, the remainder of which has the potential to enter and contaminate the environment (BECC 2009b). Work has been completed to improve collection and treatment with the addition of three new reclamation plants, aimed to reduce the overall burden on SAB wastewater treatment plant. Nonetheless, inadequate sewage treatment from the SAB plant was evident. Therefore, it is critical for appropriate monitoring methods to be used in assessing coastal water quality in waters impacted by wastewater effluent, especially in regions prone to infrastructure failures. PMA-qPCR and Cov-IMS/ATP both show promise in augmenting source tracking studies to accurately determine the presence and magnitude of recent inputs of human fecal contamination.

Laboratory studies have shown that samples treated with PMA can distinguish between live and dead *Bacteroidales* originating from human feces, yet use of PMA has been understudied in field investigations for assessing fecal contamination in environmental samples (Varma et al. 2009; Bae and Wuertz 2009a; Nocker et al. 2006). Therefore, it is important to understand the relationship between qPCR samples treated with and without PMA and their relationship to other viability- and culture-based methods. Although host-associated microbial source tracking markers can be helpful in identifying sources of contamination for targeted remediation efforts, detection of the human-associated marker with qPCR alone may not be appropriate for assessing sewage treatment and coastal water quality near wastewater discharge points. Instead, alternative viability-based methods can assist water quality monitoring and remediation efforts to detect recent fecal contamination events originating from inadequate sewage treatment or faulty infrastructure. In addition, this study of point and non-point sources serves as an important positive control for non-point source pollution studies, which are common in the United States.

5 Conclusion

- The San Antonio de los Buenos sub-watershed is impaired for FIB, human- and dog-associated markers, indicating widespread fecal contamination, particularly within creek and storm drain sites.
- Pretreatment with propidium monoazide (PMA) was found to successfully inhibit DNA resulting from dead cells in a complex environmental matrix.

- PMA-qPCR samples were more comparable to a standard culture-based method (IDEXX) than to qPCR samples without the PMA treatment for quantification of environmental creek waters impacted with sewage inputs.
- Viability-based methods such as PMA-qPCR and Cov-IMS/ATP can assist monitoring efforts to rapidly detect recent fecal contamination events and sewage spills resulting from point-sources and to differentiate between treated effluent (dead target DNA) and viable inputs.

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