
The association of fecal indicator bacteria with human viruses and microbial source tracking markers at coastal beaches impacted by nonpoint source pollution

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ABSTRACT

Water quality was assessed at two marine beaches in California (USA) by measuring culturable fecal indicator bacteria (FIB) concentrations, library-independent microbial source tracking (MST) methods targeting human-associated microbes (human polyomaviruses PCR and qPCR, *Methanobrevibacter smithii* PCR, *Bacteroides* HF183 PCR) and a human pathogen (adenovirus by nested PCR). FIB levels periodically exceeded regulatory thresholds at Doheny and Avalon Beaches for enterococci (28.5 and 31.7% of samples, respectively) and fecal coliforms (20 and 5.8%). Adenoviruses were detected at four of five sites at Doheny Beach, and were correlated with detection of human polyomaviruses and human *Bacteroides* HF183; however, adenoviruses were not detected at Avalon. The most frequently detected human source marker at both beaches was *Bacteroides* HF183, in 27% of samples. Correlations between FIBs and human markers were much more frequent at Doheny Beach than at Avalon Beach, e.g. adenovirus was correlated with human polyomaviruses (HPyVs) and HF183. Human sewage markers and adenoviruses were routinely detected in samples meeting FIB regulatory standards. The “toolbox approach” of FIB measurement coupled with analysis of several MST markers and human pathogens used here demonstrated that

human sewage is at least partly responsible for the degradation of water quality, particularly at Doheny Beach, and resulted in a more definitive assessment of recreational water quality and human health risk than reliance on FIB concentrations alone could have provided.

INTRODUCTION

The emerging paradigm in assessment of recreational water quality includes the concept that knowledge of the dominant source(s) of microbial contamination is crucial for protection of human and ecosystem health, for accurate risk assessment, and for remediation of water bodies with impaired water quality. Determination of whether water is contaminated by human sources, such as municipal sewage or onsite disposal (septic) systems, is a first, and important step in assessing human health risk and devising appropriate remediation strategies for a given water body

Bacteroides spp. are gram-negative, strictly anaerobic, non-spore forming bacilli that out-number conventional fecal indicator bacteria (FIB), such as coliforms and enterococci, in both human and animal feces, i.e., they occur at concentrations of 10^9 to 10^{11} organisms•g⁻¹ in feces (Holdeman *et al.* 1976, Wexler 2007), and 10^9 *Bacteroides*•100 ml⁻¹ in sewage (Converse *et al.* 2009). In contrast,

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conventional FIB concentrations in untreated sewage are orders of magnitude lower, e.g. approximately 10^7 CFU•100 ml⁻¹ for total coliforms, and 10^6 CFU•100 ml⁻¹ for fecal coliforms and enterococci (Harwood *et al.* 2005). Due to its relatively great sensitivity (Harwood *et al.* 2009) and its position as one of the first library-independent MST methods directed against human fecal sources, the human-associated *Bacteroides* assay has been widely used to assess pollution sources in both polymerase chain reaction (PCR) and quantitative-polymerase chain reaction (qPCR) formats (Bernhard and Field 2000, Boehm *et al.* 2003, Dick and Field 2004, Dick *et al.* 2005, Seurinck *et al.* 2005, Layton *et al.* 2006, Gourmelon *et al.* 2007, Kildare *et al.* 2007, Okabe *et al.* 2007, Ahmed *et al.* 2008, Jenkins *et al.* 2009).

M. smithii is the most prominent methanogen in the human gastrointestinal tract, and has been found at concentrations of 10^7 to 10^{10} organisms•g⁻¹ in feces (Bond *et al.* 1971, Lin and Miller 1998). The use of the *nifH* gene of *M. smithii* to identify human-associated fecal pollution has been limited but successful in MST studies conducted to date (Ufnar *et al.* 2006, Harwood *et al.* 2009), as it is relatively host-specific compared to some other MST markers (Harwood *et al.* 2009, Johnston *et al.* 2010, Ahmed *et al.* 2012).

Adenovirus type 40 and 41 are etiological agents of viral gastroenteritis. These viruses have been utilized to indicate human fecal pollution in water (Pina *et al.* 1998, Chapron *et al.* 2000, Jiang *et al.* 2001). Because they are pathogens, these viruses directly inform risk assessment models for human health. In contrast, human polyomaviruses are generally nonpathogenic, and are excreted in the urine and feces of healthy individuals (Vanchiere *et al.* 2005, Wong *et al.* 2009). The HPyVs PCR and qPCR methods used in this study targeted the species JCV and BKV (McQuaig *et al.* 2006, McQuaig *et al.* 2009), which are both widespread in sewage (Markowitz *et al.* 1993, Agostini *et al.* 1996, Bofill-Mas *et al.* 2000, Polo *et al.* 2004). Both viruses are genetically stable, distributed worldwide, and maintain high seropositive rates in the human population (Bofill-Mas *et al.* 2000, Agostini *et al.* 2001, Stolt *et al.* 2003, Del Valle *et al.* 2004, Takasaka *et al.* 2004, Pavesi 2005, Zhong *et al.* 2009). The use of PCR methods that target both JCV and BKV in order to detect human sewage pollution has been successful in a number of laboratory and field studies (Bofill-Mas *et al.* 2006;

McQuaig *et al.* 2006, 2009; Harwood *et al.* 2009; Ahmed *et al.* 2010).

Research to define the most useful microbial source tracking (MST) method(s) for human and other pollution sources is on-going, and several researchers have suggested a “toolbox” or multi-tooled approach (Noble *et al.* 2003, 2006; Santo Domingo *et al.* 2007; Vogel *et al.* 2007). Studies using the toolbox approach incorporate multiple indicators or markers to assess water quality and pollution source (Noble *et al.* 2003, Vogel *et al.* 2007). To date, several studies have been conducted in which non-point or point source contributions to water quality degradation have been determined utilizing a MST toolbox approach (Harwood *et al.* 2000, Boehm *et al.* 2003, Hundesa *et al.* 2006, McQuaig *et al.* 2006, Noble *et al.* 2006).

This study incorporated data from two West Coast (USA) beaches with various potential fecal inputs and differing hydrology: Doheny State Beach (Dana Point, CA) and Avalon Beach (Catalina Island, CA). See Methods section for more detail. Established and emerging methods for detecting sewage contamination from human sources were employed here, including PCR for human-associated *Bacteroides* HF183 (Bernhard and Field 2000), *Methanobrevibacter smithii* PCR (Ufnar *et al.* 2006), nested PCR for adenovirus (Pina *et al.* 1998), and PCR and qPCR for HPyVs (McQuaig *et al.* 2006, 2009) at Doheny and Avalon beaches. The goals of this study were to: assess the presence and absence of several MST markers targeting human sources, enumerate FIB by conventional, culture-dependent methods, determine any correlations among indicators, markers, and pathogens, and examine the differences in occurrence of indicators and markers among the locations. We hypothesized that the amalgamation of bacterial, viral, and methanogen-based MST data would allow for a more complete perspective of microbial contamination sources and a better interpretation of water quality and human health risks at beaches thought to be impacted by nonpoint source pollution.

METHODS

Construction of Recombinant Plasmid for Human Polyomaviruses (HPyVs) Control and qPCR Standard Curve

BK virus (ATCC VR-837) was obtained from the American Type Culture Collection (Manassas, VA),

and propagated in HEL-299 cells (ATCC CCL-137). The cell line was grown in Eagle minimum essential medium (Sigma, St. Louis, Mo.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Inc., Carlsbad, CA). Cell lines were maintained in Eagle minimum essential medium containing 2% FBS. DNA was extracted from 0.1 ml of the BK virus culture using DNeasy Blood & Tissue Kit (Qiagen, Inc., Valencia, CA), and used as template in the HPyVs PCR assay (McQuaig *et al.* 2006). The resulting 176-bp amplicon was purified using QIAquick PCR Purification Kit (Qiagen, Inc.) and then cloned into pCR®4-TOPO vector (Invitrogen, Inc.). The vector was then transferred into *E. coli* One Shot® chemically competent cells, and plated on LB agar containing 100 µg•ml⁻¹ ampicillin. Recombinant plasmids with a single copy of the amplicon were purified using GenElute™ Five-Minute Plasmid Miniprep Kit (Sigma, St. Louis, MI) following manufacturer's instructions. Purified recombinant plasmid DNA containing the insert was quantified using a Qubit™ fluorometer (Invitrogen, Inc., Carlsbad, CA). DNA quantification was performed in triplicate and averaged to determine the estimated total DNA concentration. Insert copy numbers were estimated by multiplying the average DNA concentration by Avogadro's number then dividing by the product of the entire plasmid and average weight of a base pair (Yun *et al.* 2006).

Positive PCR Controls

All primers and probes used in this study are described in Table 1. To construct clones for use as positive controls, a specific gene fragment for each MST marker (human-associated 525-bp region of the 16S rRNA gene of *Bacteroides* or 221-bp region of the *mnif* gene of *M. smithii*) was amplified using the primers described below. The 525-bp and 221-bp amplicons were then cloned into a pCR®4-TOPO vector and transformed into *E. coli* One Shot® chemically competent cells as described above. Recombinant plasmids were propagated and purified as described above. Plasmids containing inserts were confirmed by sequencing at MacroGen USA (Rockville, MD). All sequences were subjected to BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST>) for comparison with published sequences. All constructed plasmids used as PCR or qPCR positive controls contained the correct sequence.

Negative Controls

The absence of contamination in each DNA extraction was tested using sterile, DNA-free water, which was processed in parallel with all samples through DNA extraction and PCR or qPCR. In addition, contamination of water samples during collection or filtration was ruled out using field and method blanks. Method blanks were processed in parallel with all water samples starting with filtration

Table 1. Primers and probe sequences used in this study.

Assay	Primers and Probe	Sequence	Reference
Human <i>Bacteroides</i> PCR	HF183	5'-ATC ATG AGT TCA CAT GTC CG-3'	(Bernhard and Field 2000)
	Bac708R	5'-CAA TCG GAG TTC TTC GTG-3'	
<i>M. smithii</i> PCR	Mnif-342f	5'-AAC AGA AAA CCC AGT GAA GAG-3'	(Ufnar <i>et al.</i> 2006)
	Mnif-363r	5'-ACG TAA AGG CAC TGA AAA ACC-3'	
Adenovirus nested PCR	hexAA1885	5'-GCC GCA GTG GTC TTA CAT GCA CAT C-3'	(Pina <i>et al.</i> 1998)
	hexAA1913	5'-CAG CAC GCC GCG GAT GTC AAA GT-3'	
	nehexAA1893	5'-GCC ACC GAG ACG TAC TTC AGC CTG-3'	
	nehexAA1905	5'-TTG TAC GAG TAC GCG GTA TCC TCG CGG TC-3'	
Human polyomavirus PCR/qPCR*	SM2	5'-AGT CTT TAG GGT CTT CTA CCT TT-3'	(McQuaig <i>et al.</i> 2006, 2009)
	P6	5'-GGT GCC AAC CTA TGG AAC AG-3'	
	KGJ3	5'-(FAM)-TCA TCA CTG GCA AAC AT-(MGBNFQ)-3'	

*The SM2 and P6 primers were used in both the PCR and qPCR assays.

of sterile buffered water, through to DNA extraction and PCR or qPCR. All DNA extraction blanks and method blanks were negative in all assays.

Human Polyomavirus qPCR and Standard Curve

The HPyVs qPCR mixtures were prepared using SM2 and P6 primers and KGJ3 probe (Table 1). Previously published reaction mixture and thermocycling conditions were used (McQuaig *et al.* 2009). To produce a standard curve, the recombinant plasmid DNA was serially diluted in nuclease-free reagent grade water to a final concentration ranging from 10^2 to 10^6 gene copies $\cdot\mu\text{l}^{-1}$. Five microliters of each dilution were used as template in the Taqman[®] real time standard curve PCR reactions. Each dilution was run in duplicate. A standard curve was run with every qPCR assay. Applied Biosystems default settings for the threshold cycle (C_T) were used for data analysis. The C_T values were plotted against copy number to generate the standard curve. Linear regression was used to assess the relationship between C_T values and copy number.

Doheny Beach Sites and Sampling Schedule

All samples analyzed in this study were collected as part of a larger epidemiological study in southern California (Colford 2012). This study incorporated data from two West Coast (USA) beaches with various potential fecal inputs and differing hydrology: Doheny State Beach (Dana Point, CA) and Avalon Beach (Catalina Island, CA). Fecal indicator bacteria (FIB) concentrations in the waters of Doheny State Beach frequently exceed regulatory standards for microbial water quality (104 and 400 CFU/100 ml for enterococci and fecal coliforms, respectively; Natural Resources Defense Council 2009). The poor water quality has been attributed to several factors, including limited water circulation caused by a jetty located at the north-west end of the beach, high-density seagull populations releasing fecal matter into the water and sediments, and urban runoff from the San Juan Creek watershed (Orange County 2003, Natural Resources Defense Council 2009). Occasionally, under high tide or high flow conditions in San Juan Creek, the San Juan Creek lagoon breaches a confining berm of sand that forms during summer and discharges into the Pacific Ocean (Moore *et al.* 2009). Water quality monitoring around the outflow area has reported consistently higher concentrations of FIB in the creek compared

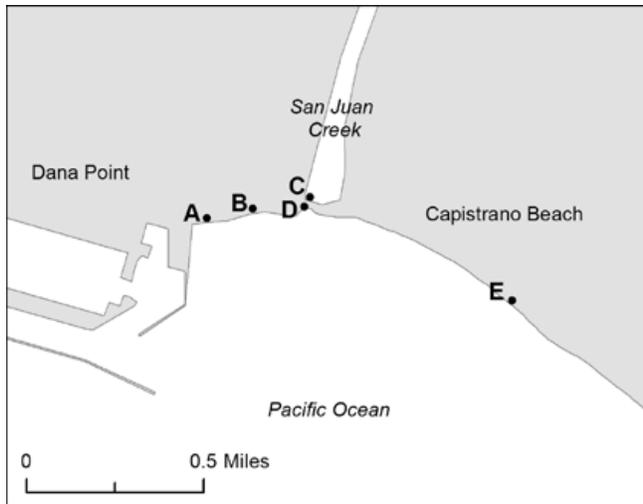
to the ocean (Natural Resources Defense Council 2009), and higher enterococci levels at the beach when the berm is open compared to when it is closed (Colford 2012). In a 2003 study, antibiotic resistance analysis and ribotyping of *E. coli* and enterococci in the area suggested that the high concentrations of indicator bacteria were due to intervening storm drains, direct fecal contamination by avian sources (e.g. seagulls), and survival and proliferation of fecal bacteria adapted to the secondary habitat (Orange County 2003). A wastewater treatment plant is located near the San Juan Creek less than a mile upstream of Doheny Beach; however the effluent is discharged into the San Juan Creek Ocean Outfall (Ktagi *et al.* 2008). The Ocean Outfall is located approximately 2.1 km off shore in a southwest direction from Doheny Beach at San Juan Creek and has a flow rate of 19.1 mgd (South Orange County Wastewater Authority 2005, 2007).

Water was collected every weekend at five sites along Doheny Beach over a four-month period (May-September 2008). Sites were designated A, B, C, D and E (Figure 1a). Sites A, B, D and E samples were collected along the beach. Site A samples were collected adjacent to the jetty. Site B samples were collected at the north beach between Site A and D. Site C samples were collected in the San Juan Creek Lagoon approximately 50 m prior to its discharge across the beach into the ocean. Site D samples were collected on the north end of the south beach adjacent to lagoon. Site E samples were collected at the south end of the south beach.

Avalon Beach Sites and Sampling Schedule

The FIB concentrations in the waters of Avalon Beach frequently exceed regulatory standards (Natural Resources Defense Council 2009). However, in contrast to Doheny Beach, Avalon Beach waters circulate relatively freely (Jones and Bogucki 2002). Sewer lines run parallel to and within 20 m of the beach, and stormwater runoff is channeled into the sewer lines using low-flow diverters (Grant *et al.* 2002, Boehm *et al.* 2003). When the low-flow diverters reach maximum capacity, runoff enters small drains that discharge into the ocean through the sand (Grant *et al.* 2002.). By way of these drains, untreated runoff and sewage can enter the ocean and degrade water quality. In addition, Avalon suffers from aging sewer infrastructure, much of which dates from the early twentieth century. The situation is exacerbated by the limited availability of freshwater

a)



b)

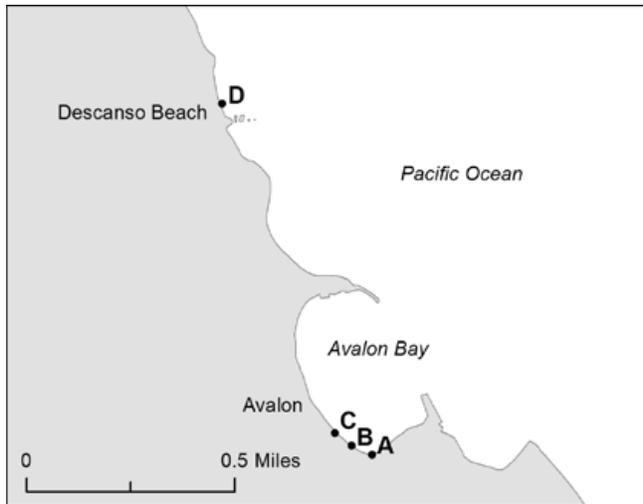


Figure 1. Sample sites: Site designation at Doheny State Beach and San Juan Creek Lagoon (a). Site A and B was located north of San Juan Creek, Site C was the San Juan Creek lagoon, Site D was located on the beach side of the San Juan Creek lagoon, and Site E was located south of the lagoon. Site designations at Avalon Beach at Catalina Island (b). Site A, B, and C were located along Avalon Beach. Site D was located north of Avalon at Descanso Beach. Images courtesy of the US Geological Survey; <http://terraserver-usa.com>.

on the island, which necessitates the use of seawater to run the sanitary collection system and makes iron and steel sewer pipes more susceptible to failure due to corrosion. Large numbers of sea gulls and pigeons accumulate around restaurants near the beach and may also contribute to fecal bacteria inputs (Boehm *et al.* 2003). In addition, the area is frequented by boat traffic with many boats anchoring off the beach for hours and even days.

Water was collected every Thursday through Sunday at 4 sites along Catalina Island over a 2-month period (June-August 2008). Sites were designated A, B, C, and D (Figure 1b). Samples from sites A, B, and C were collected along Avalon Beach inside Avalon Harbor. Site A samples were collected at the south beach in the southernmost corner of the beach. Site B samples were collected on the south side of the pier (located between sites B and C). Site C samples were collected on the north side of the pier. Avalon Beach site D (Descanso Beach), which is north of Avalon Beach and outside Avalon Harbor, was used as a control site due to historically low levels of fecal bacteria there (Natural Resources Defense Council 2009).

Sample Collection

Samples were collected between 8:00 and 9:00 a.m. All samples were taken at 0.5 meter depth. Samples were collected in sterile polyethylene buckets and transported to the laboratory on ice. Water samples were processed within three hours of collection. All samples were analyzed for enterococci, total coliforms, fecal coliforms, HPyVs (quantified by qPCR and presence/absence detection by PCR), human-associated *Bacteroides*, *M. smithii*, and adenovirus (as described below).

Enumeration of Culturable Indicator Organisms

Culturable concentrations of all indicator organisms were obtained using standard methods. Enterococci were enumerated by membrane filtration on mEI agar, with incubation at $41 \pm 0.5^\circ\text{C}$ for 24 hours (USEPA 1997). Fecal coliform concentrations were determined by membrane filtration using mFC agar, with incubation at $44.5 \pm 0.5^\circ\text{C}$ for 24 hours (APHA 1998). Total coliform concentrations were enumerated by membrane filtration on mEndo agar, with incubation at $35 \pm 0.5^\circ\text{C}$ for 24 hours (APHA 1998).

Concentration of Microbes for PCR

Bacteria, methanogens, and viruses from 500 ml samples were concentrated simultaneously on a 0.45 μm pore size nitrocellulose filter. Bacteria and methanogens were concentrated by physical capture on the filter. To concentrate the viruses, the pH of the water was adjusted to 3.5 using 2.0 N HCl prior to filtration (American Public Health Association 1998, McQuaig *et al.* 2006, Harwood *et al.* 2009). The low

pH does not affect the concentration and detection of the bacteria and methanogens (Harwood *et al.* 2009). After filtration, each filter was placed into a 2 ml microcentrifuge tube. The tube containing the filter was placed on dry ice and shipped from California to the University of South Florida lab (Tampa, Florida). DNA was extracted from the filter using a modified MoBio and Qiagen DNA extraction protocol as described in the Supplemental Information (SI) available at ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2012AnnualReport/ar12_23SI.pdf, and was used as template for human-associated *Bacteroides* PCR, *M. smithii* PCR, adenovirus nested PCR, HPyVs PCR and HPyVs qPCR assays.

Detection of Human-Associated *Bacteroides*

Previously published primers specific for a region of the 16S rRNA gene of human-associated *Bacteroides* (Bernhard and Field 2000), including the HF183 forward primer, were used in a touchdown PCR (Harwood *et al.* 2009; Table 1). PCR reactions were prepared as previously published (Harwood *et al.* 2009). The touchdown PCR reaction conditions were as follows: DNA polymerase activation at 95°C for 3 minutes, followed by 43 cycles of DNA melting at 94°C for 45 seconds, then annealing for 45 seconds, and extension at 72°C for 30 seconds. Annealing temperatures ranged from 65 to 55°C. Cycles were performed twice at temperatures 65 to 63°C, once at temperatures 62 to 56°C, and finally 30 times at 55°C; followed by a final elongation at 72°C for 5 minutes (Eppendorf Mastercycler Thermocycler). A plasmid containing the 525 bp target fragment of the 16S rRNA gene was used as the PCR positive control. PCR products were separated and visualized using SYBR gold staining.

Detection of Human-Associated *M. smithii*

Previously published primers specific for the *nifH* gene of human-associated *M. smithii* (Ufnar *et al.* 2006) were used in the touchdown PCR (Harwood *et al.* 2009; Table 1). PCR reactions were prepared as previously published (Harwood *et al.* 2009). The touchdown PCR reaction conditions were the same as previously described above for the human-associated *Bacteroides* assay. A plasmid containing the 221 bp target fragment of the *nifH* gene was used as the PCR positive control. PCR products were separated and visualized as above.

Adenovirus Nested PCR

Previously published primers specific for the hexon gene of human adenoviruses were used in the nested PCR (Pina *et al.* 1998; Table 1). Initial amplification was carried out in a 50- μ l reaction mixture containing 25 μ l of GoTaq Green Master Mix (Promega Corporation), 0.8 μ M concentrations of each primer (hexAA 1885 and hexAA 9113), and 5 μ l of template DNA. In both PCR reactions, the first round of denaturation was carried out for 4 minutes at 94°C followed by 30 cycles of denaturing at 94°C for 90 seconds, annealing at 55°C for 90 seconds, and extension at 72°C for 120 seconds, followed by a final elongation at 72°C for 5 minutes. For the nested PCR, the 50 μ l reaction mixture contained 25 μ l of GoTaq Green Master Mix (Promega Corporation), 0.4 μ M concentrations of each primer (nehexAA 1893 and nehexAA 1905), and 1 μ l of template from the first round of PCR.

Detection of Human Polyomaviruses

Primers specific for a partial region of the HPyVs T-antigen gene were used for PCR (McQuaig *et al.* 2009; Table 1). PCR reaction preparations and thermocycling conditions were performed as previously published (Harwood *et al.* 2009). A plasmid containing the 173 bp target fragment of the T antigen gene was used as the PCR positive control. PCR products were separated and visualized as above.

Quantification of Human Polyomaviruses in Beach Samples

All qPCR reactions were carried out in a 25 μ l volume, essentially as described in McQuaig *et al.* (2006). Amplification conditions and standard curve material were as described above for PCR. Standard curve reactions were run in duplicate for each qPCR run performed over a range from x-y, and the average R^2 was 0.9879 ± 0.0103 .

Statistical Analysis

Summary statistics were computed for variables of interest using GraphPad InStat version 3.00 (GraphPad Software, San Diego, CA). Bacterial concentrations and HPyVs copy numbers were \log_{10} transformed and differences among concentrations and copy numbers were determined using paired or unpaired t-tests. Means were considered significantly different at the alpha level of 0.05. Relationships

between indicators and markers were determined by calculating Pearson correlation coefficients. Differences were considered significant when $P < 0.05$, and two-sided tests were performed for all analyses. Observations of human associated markers were converted to binary data, and binary logistic regression models (SPSS version 17.0) were used to assess relationships between HPyVs or FIB concentrations and presence or absence of human-associated markers. Nagelkerke's R square, which can range from 0.0 to 1.0, denotes the effect size (the strength of the relationship); stronger associations have values closer to 1.0. Relationships were considered significant when the P value for the model chi square was ≤ 0.05 and the confidence interval for the odds ratio did not overlap 1.0. The odds ratio is the measure of the effect size and an estimation of the probability of the same response of the two variables. Fisher's exact test was used to assess significant differences in the frequency of observation of binary marker data. Differences were considered significant when an alpha level of 0.05.

RESULTS

Bacterial Water Quality Indicator Concentrations at Doheny Beach Sites

One hundred thirty samples were collected from five Doheny Beach sites over the study period (26 samples per site). The average \log_{10} -transformed concentrations of all fecal indicator bacteria (FIB) at each Doheny site are summarized in Figure 2a. The average concentration of each FIB at site C was significantly greater than at any other Doheny site ($P < 0.001$). Enterococci at site C exceeded regulatory standards for one-time sampling (104 CFU/100 ml) in 88.5% of the samples (Natural Resources Defense Council 2009). Fecal coliform concentrations at site C exceeded regulatory standards (400 CFU/100 ml) in 76.9% of the samples (Natural Resources Defense Council 2009). California has a total coliform regulatory standard of 10,000 CFU/100 ml, and at site C total coliform concentrations exceeded the California standard in 10.0% of the samples.

The average concentrations of both enterococci and fecal coliforms at site D were significantly larger than those at sites A, B, and E ($P < 0.05$). FIB in water samples at site D exceeded enterococci and fecal coliform regulatory standards in 30.8 and 15.4% of the samples, respectively. The average concentrations of enterococci and fecal coliforms

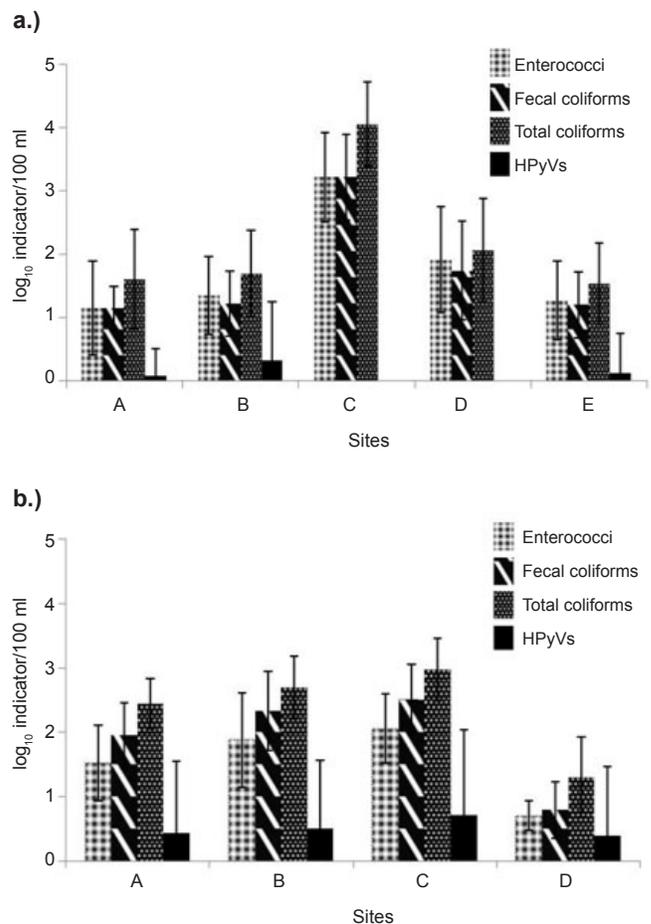


Figure 2. The average \log_{10} -transformed concentration of enterococci, fecal coliforms, total coliforms, and human polyomaviruses (HPyVs) at the Doheny Beach sites (a) and Avalon Beach sites (b). Error bars represent standard deviation. Indicator bacteria are reported as \log_{10} colony forming units (CFU) $\cdot 100 \text{ ml}^{-1}$. HPyVs are reported as \log_{10} copy numbers $\cdot 100 \text{ ml}^{-1}$.

among sites A, B, and E were not significantly different. The individual enterococci concentrations at sites A, B, and E exceeded regulatory standards in only 11.5, 7.7, and 3.8% of the samples, respectively. Fecal coliform concentrations at sites B and E exceeded regulatory standards in only 3.8% of the samples, while no samples with concentrations that exceeded regulatory standards were detected at site A. Aside from site C, total coliform concentrations did not exceed 10,000 CFU/100 ml at any site.

Correlations of Bacterial Indicators among Doheny Beach Sites

Throughout this manuscript, correlations are only noted when $P < 0.05$. The concentrations of enterococci at site A were positively and

significantly correlated with those at site C ($R^2 = 0.1692$) and site E ($r = 0.6390$, $R^2 = 0.4084$). Enterococci concentrations at site E were also positively correlated with those at site B ($R^2 = 0.2076$). Moreover site C was positively correlated with site D ($R^2 = 0.3407$). The concentrations of fecal coliforms at site D were positively correlated with site B ($R^2 = 0.2225$), site C ($R^2 = 0.1748$), and site E ($R^2 = 0.1699$). The concentrations of total coliforms were positively correlated between all sites ($R^2 = 0.2449 - 0.4330$) except sites C and E ($P = 0.1237$). In addition, enterococci, fecal coliforms, and total coliforms were positively correlated among each other at each site ($R^2 = 0.2489 - 0.7310$).

qPCR Detection of Human Polyomaviruses (HPyVs) at Doheny Beach Sites

HPyVs were rarely detected by qPCR at any Doheny site (Figure 2a). HPyVs were not detected at sites C and D, were detected once at site A and E, and detected three times at site B. Quantities of HPyVs detected ranged from 125 to 2,884 copy numbers $\cdot 100 \text{ ml}^{-1}$. There were no significant differences in the concentrations of HPyVs copy numbers detected among the sites. In addition, there were no significant correlations of HPyVs copy numbers between sites where HPyVs were detected.

PCR Detection of Human-Associated Water Quality Indicators at Doheny Beach Sites

The frequency of human marker detection at each site is summarized in Figure 3a. HPyVs were detected by PCR in four samples at site A, three samples at site B, one sample at sites D and E, and were not detected at site C. Human-associated *Bacteroides* were detected in nine samples at site C, eight samples at site D, four samples at site B, and two samples at both sites A and E. The *M. smithii* marker was detected in Doheny Beach only once at site C. Adenovirus was detected in three samples at both sites B and D, two samples at site A, one sample at site C, and was not detected at site E. The frequency of human-associated *Bacteroides* detection was significantly more frequent than *M. smithii* detection ($P < 0.01$). All other detection frequencies were not significantly different.

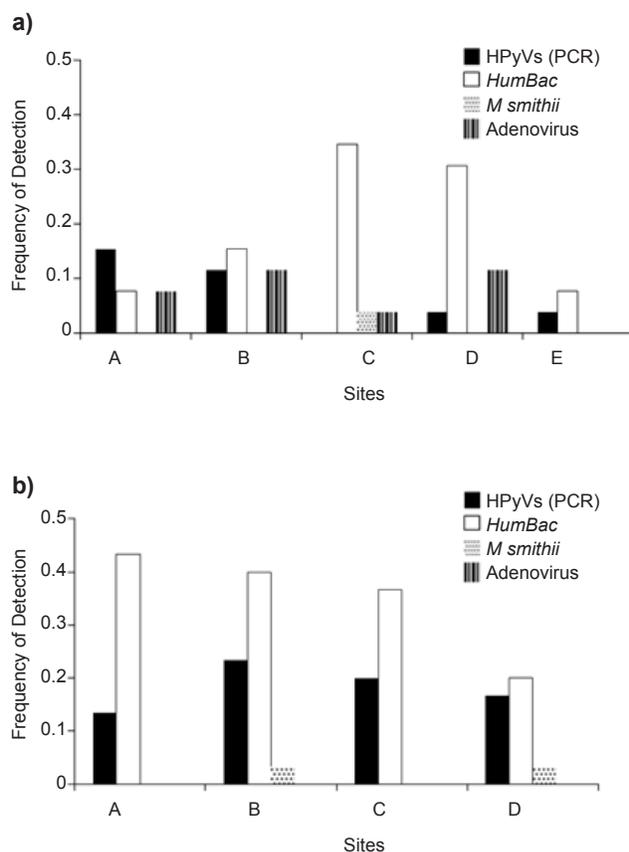


Figure 3. Frequency of detection of human-associated MST markers and adenoviruses at the Doheny Beach sites (a) and Avalon Beach sites (b). HumBac - human-associated *Bacteroides* marker.

Relationships among Indicators and Markers at Doheny Beach

Correlation between analytes at each site was frequently, but not always noted. At site A, PCR detection of HPyVs and the concentrations of fecal coliforms were strongly correlated (Nagelkerke's $R^2 = 0.567$, $P < 0.001$, odds ratio = 246.100, $P = 0.039$). At site B, the concentrations of HPyVs were moderately correlated with the presence of adenovirus (Nagelkerke's $R^2 = 0.413$, $P < 0.013$, odds ratio = 3.653, $P = 0.025$). In addition, the detection of HPyVs was significantly correlated with the occurrence of adenovirus (odds ratio 15.333, $R^2 = 0.3884$, $P = 0.0269$) at site B. Several positive correlations were documented at site C, including enterococci concentrations and the occurrence of the human-associated *Bacteroides* HF183 marker (Nagelkerke's $R^2 = 0.204$, $P = 0.041$), *M. smithii* (Nagelkerke's $R^2 = 1.000$, $P = 0.004$), and adenovirus (Nagelkerke's $R^2 = 1.000$, $P = 0.004$). The human-associated *Bacteroides* marker was also significantly correlated with fecal

coliform concentrations (Nagelkerke's $R^2 = 0.191$, $P = 0.049$). No significant relationships were observed at site D or E.

Bacterial indicator concentrations and marker detection at each Doheny site were compiled for all sites combined (Table 2). Enterococci concentrations were strongly correlated with fecal coliforms ($r = 0.8620$, $R^2 = 0.7430$), total coliforms ($r = 0.8480$, $R^2 = 0.7180$), and *M. smithii* presence (Nagelkerke's $R^2 = 1.000$). Fecal coliforms were also strongly correlated with total coliforms ($r = 0.8780$, $R^2 = 0.771$). Total coliforms were moderately correlated with *M. smithii* (Nagelkerke's $R^2 = 0.466$, $P = 0.021$). The human-associated *Bacteroides* marker was weakly correlated with enterococci (Nagelkerke's $R^2 = 0.192$, $P = 0.004$), fecal coliforms (Nagelkerke's $R^2 = 0.190$, $P < 0.001$, odds ratio = 2.383, $P < 0.0005$), and total coliforms (Nagelkerke's $R^2 = 0.137$, $P = 0.001$, odds ratio = 1.858, $P = 0.001$). HPyVs detected by PCR and HPyVs copy numbers were significantly correlated (Nagelkerke's $R^2 = 0.521$, $P < 0.001$). The presence of adenovirus was correlated with both HPyVs concentrations (Nagelkerke's $R^2 = 0.087$, $P < 0.033$)

and detection of human-associated *Bacteroides* (Likelihood ratio = 2.307, $R^2 = 0.1078$, $P = 0.0016$).

Overall, human-associated *Bacteroides* were the most frequently detected marker of human sewage pollution at all the Doheny sites ($n = 25$). The *M. smithii* marker was the least frequently detected marker ($n = 1$). Adenovirus and HPyVs were detected by PCR were detected in nine samples. HPyVs were detected by qPCR in five samples. The distribution of the various markers in discrete samples is summarized in Table 3. For most comparisons, presence-absence results matched in over 80% of samples (Table 3). More co-observations occurred for human-associated *Bacteroides* and HPyVs than other combination of markers, and human-associated *Bacteroides* and adenoviruses were co-observed in 4.6% of all samples at Doheny Beach.

Bacterial Water Quality Indicator Concentrations at Avalon Beach Sites

One hundred twenty samples were collected from Avalon Beach over the entire study period,

Table 2. Correlations of microbial targets at Doheny Beach and Avalon Beach (data from all sites combined). Comparisons between quantitative data were performed using Pearson's correlation coefficient and are reported as r , comparisons between quantitative data set and binary data set were performed using binary logistic regression and are reported as Nagelkerke's R^2 . Only correlations in which $P < 0.05$ are noted; all non-significant correlations are denoted NS. No significant relationships were observed with Adenovirus in the Avalon Beach samples.

Beach and Microbial Target (Correlation Type)	Total Coliforms	Fecal Coliforms	Enterococci	HPyVs	H-Bac	<i>M. smithii</i>
Doheny Beach						
Fecal Coliforms	$r = 0.8780$					
Enterococci	$r = 0.8480$	$r = 0.8620$				
HPyVs ^a	NS	NS	NS			
H-Bac ^b	$R^2 = 0.1370$	$R^2 = 0.1900$	$R^2 = 0.1920$	NS		
<i>M. smithii</i>	$R^2 = 0.4660$	NS	$R^2 = 1.000$	NS	NS	
Adenovirus	NS	NS	NS	$R^2 = 0.0870$	$R^2 = 0.1078$	NS
Avalon Beach						
Fecal Coliforms	$r = 0.8926$					
Enterococci	$r = 0.6277$	$r = 0.7282$				
HPyVs	NS	NS	NS			
H-Bac	$R^2 = 0.061$	$R^2 = 0.074$	NS	NS		
<i>M. smithii</i>	NS	NS	NS	NS	NS	
^a HPyVs, human polyomaviruses detected by qPCR						
^b H-Bac, human-associated <i>Bacteroides</i> marker						

Table 3. The co-occurrence of human-associated markers in the Doheny and Avalon Beach samples.

Beach	Marker A	Marker B	Matching Results (%)	Marker A Positives	Marker B Positives
Doheny	HPyVs # ^a	<i>M. smithii</i>	95.4	5	1
	HPyVs #	HPyVs ^b	93.9	5	9
	<i>M. smithii</i>	Adenovirus	93.8	1	9
	HPyVs	<i>M. smithii</i>	92.3	9	1
	HPyVs #	Adenovirus	90.8	5	9
	HPyVs	Adenovirus	89.2	9	9
	H-Bac ^c	Adenovirus	83.1	25	9
	H-Bac	<i>M. smithii</i>	81.5	25	1
	HPyVs	H-Bac	80	9	25
	HPyVs #	H-Bac	78.5	5	25
Avalon	<i>M. smithii</i>	Adenovirus	98.3	2	0
	HPyVs #	HPyVs	87.5	21	22
	HPyVs #	Adenovirus	82.5	21	0
	HPyVs	Adenovirus	81.7	22	0
	HPyVs	<i>M. smithii</i>	81.7	22	2
	HPyVs #	<i>M. smithii</i>	80.8	21	2
	H-Bac	Adenovirus	65	42	0
	H-Bac	<i>M. smithii</i>	65	42	2
	HPyVs	H-Bac	56.7	22	42
	HPyVs #	H-Bac	55.8	21	42

^aHPyVs #, human polyomaviruses detected by qPCR

^bHPyVs, human polyomaviruses detected by PCR

^cH-Bac, human-associated *Bacteroides* marker

with 30 samples collected from each of the sites. The average concentrations of all fecal indicator bacteria (FIB) at each Avalon site are summarized in Figure 2b. Enterococci, fecal coliform, and total coliform concentrations at site C were significantly greater than at site A and D ($P < 0.01$). Enterococci at site C exceeded regulatory standards in 60.0% of the samples. Fecal coliform concentrations at site C exceeded regulatory standards in 10.0% of the samples. Enterococci and total coliform concentrations at sites A and B were not significantly different; however fecal coliform concentrations at site B were significantly larger than at site A ($P < 0.05$). Enterococci and fecal coliforms at site B exceeded regulatory standards in 46.7% and 13.3% of samples, respectively. Total coliforms exceeded 10,000 CFU•100 ml⁻¹ in 6.7% of the samples. At site A, fecal and total coliform concentrations did not exceed regulatory limits; while enterococci exceedances were only observed in 20.0% of the samples. The average concentrations of all FIB at site D were significantly lower than all other Avalon

sites ($P < 0.001$). Exceedances were not noted for any bacterial indicators at site D.

Correlations of Bacterial Indicators among Avalon Beach Sites

The concentrations of enterococci at site D were negatively correlated with those at site A ($r = -0.4813$, $R^2 = 0.2316$). No significant correlations were found for fecal coliforms among the Avalon sites. Total coliform concentrations were positively correlated at sites B and C ($r = 0.4171$, $R^2 = 0.1739$). In addition, enterococci, fecal coliforms, and total coliforms were positively correlated among each other at sites A, B, and C ($r = 0.5431 - 0.9239$, $R^2 = 0.3404 - 0.5315$). At site D, only fecal and total coliforms were significantly correlated ($r = 0.6211$, $R^2 = 0.3858$).

qPCR Detection of HPyVs at Avalon Beach Sites

HPyVs were detected by qPCR at every Avalon site (Figure 3b). HPyVs were detected seven times at site C, six times at site B, and four times at both sites A and D. Quantities of HPyVs detected ranged from 50 to 35,481 copy numbers•100 ml⁻¹. There were no significant differences in the concentrations of HPyVs copy numbers detected among the sites. In addition, there were no significant correlations of HPyVs copy numbers among the sites.

PCR Detection of Human-Associated Water Quality Indicators at Avalon Beach Sites

The frequency of human marker detection at each site is summarized in Figure 3b. HPyVs were detected by PCR in seven samples at site B, 6 samples at site C, 5 samples at site D, and 4 samples at site A. Human-associated *Bacteroides* were detected in 13 samples at site A, 12 samples at site B, 11 samples at site C, and 6 samples at site D. The *M. smithii* marker was detected once at sites B and D, and was not detected at either site A or D. Adenovirus was not detected at any Avalon Beach site. The frequency of human-associated *Bacteroides* detection was significantly more frequent than HPyVs, *M. smithii*, and adenovirus detection ($P < 0.01$). The frequency of HPyVs detection was significantly more frequent than *M. smithii* and adenovirus detection ($P < 0.01$).

Relationships among Indicators and Markers at Avalon Beach

Unlike the Doheny Beach sites, there were no significant relationships between indicators or human-associated markers at any of the sites at Avalon Beach. When FIB concentrations and marker detection at all Avalon Beach sites were compiled, enterococci concentrations were strongly correlated with fecal coliforms ($r = 0.7282$, $R^2 = 0.5303$) and total coliforms ($r = 0.6277$, $R^2 = 0.3940$). Fecal coliforms were also strongly correlated with total coliforms ($r = 0.8926$, $R^2 = 0.7967$). The presence of the human-associated *Bacteroides* marker was weakly correlated with fecal coliforms (Nagelkerke's $R^2 = 0.074$, $P = 0.010$, odds ratio = 1.827, $P = 0.014$) and total coliforms (Nagelkerke's $R^2 = 0.061$, $P = 0.020$, odds ratio = 1.774, $P = 0.027$). HPyVs detected by PCR and HPyVs copy numbers were significantly correlated (Nagelkerke's $R^2 = 0.361$, $P < 0.001$, odds ratio = 2.777, $P < 0.0005$). All correlations among analytes are summarized in Table 2.

Overall, human-associated *Bacteroides* were the most frequently detected at all the Avalon sites ($n = 42$). HPyVs were detected by PCR were detected in 22 samples, *M. smithii* was detected in 2 samples, and adenoviruses were not detected at any site. The co-occurrence of human markers and pathogens is summarized in Table 3.

Comparison of Doheny and Avalon Indicators and Markers

The concentrations of enterococci, fecal coliforms, and total coliforms were not significantly different between Doheny and Avalon Beaches. In contrast, the concentration of HPyVs copy numbers was significantly larger at Avalon Beach ($P = 0.0006$). In addition, the frequency of PCR detection of HPyVs was significantly higher at Avalon Beach sites ($P = 0.0181$). The frequency of human-associated *Bacteroides* and *M. smithii* detection was not significantly different between Avalon and Doheny Beaches. Adenovirus was not detected at any Avalon Beach site and therefore statistical analysis could not be performed; however it was detected in 9 out of 130 samples at Doheny Beach, indicating a higher frequency of occurrence than at Avalon Beach.

For samples in which MST markers were detected, the frequency of samples meeting FIB regulatory

standards was calculated (Table 4). Both enterococci and fecal coliform concentrations were consistently below regulatory standards (104 CFU/ml and 400 CFU/ml, respectively) when human markers were detected in samples. In Doheny samples, fecal coliforms concentrations met regulatory standards in 60-100% of the samples in which adenovirus, HPyVs, and human-associated *Bacteroides* were detected; whereas, enterococci concentrations met regulatory standards in 36 to 80% of the samples positive for the three human markers. When *M. smithii* was detected at Doheny Beach ($n = 1$), FIB levels in the sample exceeded regulatory standards for both enterococci and fecal coliforms. In Avalon samples, fecal coliforms concentrations met regulatory standards in 76 to 100% of the samples that were positive for HPyVs, human-associated *Bacteroides*, or *M. smithii*. Enterococci concentrations met regulatory standards in a smaller percentage (57 - 100%) of the samples positive for at least one human marker (Table 4).

DISCUSSION

This study is among the first to compare the magnitude and frequency of observation of culturable fecal indicator bacteria (FIB), multiple

Table 4. Frequency of samples positive for human-specific markers and adenoviruses in which water quality met fecal indicator bacteria (FIB) regulatory standards.

Beach	Marker	Frequency of Detection in Samples Not Exceeding Regulatory Standards	
		Enterococci (<104 CFU/ml)	Fecal Coliforms (<400 CFU/ml)
Doheny	Adenovirus	0.44	0.78
	H-Bac ^a	0.36	0.6
	HPyVs ^b	0.78	1
	HPyVs # ^c	0.8	1
	<i>M. smithii</i>	0	0
Avalon	Adenovirus	N/A ^d	N/A
	H-Bac	0.62	0.76
	HPyVs	0.59	0.89
	HPyVs #	0.57	0.81
	<i>M. smithii</i>	1	1

^aH-Bac, human-associated *Bacteroides* marker

^bHPyVs, human polyomaviruses detected by PCR

^cHPyVs #, human polyomaviruses detected by qPCR

^dN/A, marker was not detected in any samples

MST markers and a viral pathogen in recreational waters. Among the most important findings were the correlations of human-associated MST markers with each other and with adenovirus detection. The Doheny State Beach sites were distinguishable in terms of FIB concentrations, and frequency of MST marker and pathogen detection. The San Juan Creek lagoon (site C) just upstream of the discharge to the ocean (site D) consistently had the highest concentrations of indicator bacteria, which were highly correlated with the nearest beach site (D) observations. Despite the high concentrations of FIB at sites C and D, adenoviruses and human polyomaviruses (HPyVs) were detected at a higher frequency at the northern beach sites (A and B), and the presence and concentration of the HPyVs marker was most strongly correlated with adenoviruses at site B. In addition, the presence of human-associated *Bacteroides*, HPyVs, and adenovirus co-occurred in 2 of the 26 samples collected at site B, but not at any other Doheny site.

The nearshore water sites at north Doheny Beach are shallow and commonly used by children. Craun *et al.* (2005) compiled sources of recreational water contamination leading to water-borne illness outbreaks and reported that 25% of the outbreaks were attributed to children in diapers and 34% were due to bather overcrowding (Craun *et al.* 2005), which could be a factor in the more frequent detection of viruses at sites A and B. On the other hand, *Bacteroides* HF183 was detected most frequently at sites C (lagoon) and the adjacent beach site, D. The epidemiology study conducted in conjunction with this study found that *Enterococcus* concentrations at Doheny Beach were higher when the berm that periodically blocks the lagoon's entry to the Ocean was open (Colford *et al.* 2012, and that more relationships between the frequency of highly credible gastrointestinal illness and various methods for enumerating FIB (including qPCR) were found when the berm was open. These findings certainly implicate San Juan Creek as an important, but not the only source of FIB and pathogens at Doheny Beach.

At Avalon Beach, fecal coliform concentrations did not show any geographic relationship (correlation of values between proximal sites). The same observation was true of enterococci. This lack of correlation suggests a separate or disproportionate fecal input(s) that affects each site individually, and/or a greater level of mixing of the waters compared to Doheny Beach. At least two of the three human

markers were frequently detected at the Avalon Beach sites (sites A, B, and C). The frequency of human marker detection at these sites strongly suggests contamination from a human source(s). While adenoviruses were not detected at any Avalon Beach site, the relatively small volume of sample analyzed (500 - 1,000 ml) may have limited the detection frequency. Jiang *et al.* (2001) suggest concentrating adenoviruses from 20 L of water for a representative analysis of indirect anthropogenic input (e.g., leaking sewers). However, the absence of the adenovirus marker and the infrequent detection of the *M. smithii* marker may be due to differential decay rates of the organisms in environmental conditions, and/ or may indicate human fecal pollution from non-communal source (e.g. septic tanks or boat discharge), because both adenoviruses and *M. smithii* are excreted by a minority of the population and are less likely to be detected in non-community wastes (McQuaig *et al.* 2009).

The Avalon control site (site D), chosen for its historically low levels of fecal bacteria concentrations, maintained low levels of culturable FIBs, which did not exceed regulatory standards. However, human-associated *Bacteroides*, *M. smithii*, and HPyVs were detected by PCR at this site. Moreover, all three markers were detected in one of the samples simultaneously. The detection of the human-associated markers coupled with the low levels of culturable bacterial indicators indicates the disconnect between FIB levels and human sewage contamination in certain conditions. The low levels of FIB and frequent human-associated marker detection may indicate minimal fecal contributions from wildlife and other natural sources, and a strong human fecal input(s). However, recent sewage contamination would result in high levels of IO concentrations. The relatively low FIB concentrations may be caused by the general inability of fecal bacteria to persist in a culturable state in conditions of high salinity and exposure to solar radiation (Anderson *et al.* 2005).

Overall, the human-associated *Bacteroides* HF183 marker was the most frequently detected indicator of human sewage at both Doheny and Avalon sites. This marker also showed the highest co-occurrence with the adenovirus marker detected at the Doheny sites; however since the human-associated *Bacteroides* marker was detected at the highest frequency the co-absence with adenovirus was the lowest among all the markers (Table 3).

Recent studies have documented the detection of the human-associated *Bacteroides* marker (HF183) in a small percentage of animal fecal samples, including chickens, cats, and particularly dogs, indicating incomplete specificity of this marker (Harwood *et al.* 2009, McQuaig *et al.* 2009, Shanks *et al.* 2010). Although dogs are not allowed on the beach, Doheny State Beach Park is dog-friendly and also maintains a large seagull population near San Juan Creek (personal communication, John F. Griffith). However, the levels of human-associated *Bacteroides* spp. in raw sewage are several orders of magnitude higher than the other human-associated markers used here, giving the marker a potentially higher sensitivity (Harwood *et al.* 2009). The presence of the human-associated *Bacteroides* marker should therefore be interpreted carefully, preferably in a quantitative format (Layton *et al.* 2006, Kildare *et al.* 2007, Converse *et al.* 2009) and in conjunction with other human-associated markers.

Recent studies have also reported that the human specificity of the *M. smithii* assay is less than 100% (although it is well over 90%; Harwood *et al.* 2009, McQuaig *et al.* 2009). Despite this caveat the *M. smithii* marker is ubiquitous in sewage, for instance Harwood *et al.* (2009) reported the detection of *M. smithii* in 10^{-3} and 10^{-4} dilutions of sewage, and in all sewage samples collected from south Florida, northwest Florida, and Mississippi (Harwood *et al.* 2009). Throughout this study the *M. smithii* marker was infrequently detected. Compared to the human-associated *Bacteroides* marker which has been detected in sewage diluted as low as 10^{-6} (Harwood *et al.* 2009), the *M. smithii* marker is relatively insensitive. However, the *M. smithii* and adenovirus marker had the highest percent of matching results at both beaches, although the majority of the matching results were co-absences.

The presence of HPyVs detected by PCR and the quantity of HPyVs by qPCR were highly correlated at both Doheny and Avalon Beaches, which is not surprising considering both assays utilize the same primers. However, agreement among the methods in terms of presence or absence was less than 100%. We hypothesize that the discrepancies in detection frequency can be attributed to the different template volumes used in each assay (2 μ l was used in the qPCR assay and 5 μ l was used in the PCR assay). The interpretation of HPyVs data for recreational water quality assessment may be influenced by the fact that these viruses are excreted in urine; however

the high percentage of analogous results with adenovirus at Doheny Beach (90.2%), and correlation with adenovirus detection, suggests that HPyVs can be predictive of human health risks.

The presence of adenoviruses is a direct assessment of human health risks; however, only a small percentage of the population excretes these viruses (Jiang 2006), which can lead to inconsistent detection in sewage-impacted waters. The lack of adenovirus detection at the Avalon Beach sites when the three human MST markers were present demonstrates that the absence of adenoviruses does not necessarily imply lack of human health concerns. Therefore, it is recommended that the adenovirus marker be used in conjunction with other pathogen assays and MST markers for detection of sewage contamination.

The potential of HPyVs to be excreted in urine of swimmers and the incomplete specificity of both the human-associated *Bacteroides* and *M. smithii* marker can mean ambiguous results when only one marker is detected. However, the predictive power of each marker is increased when more than one marker is detected at the same site. For all markers utilized in this study, an epidemiological study assessing the human health risks associated with the presence or absence of the marker would more precisely define the usefulness of each assay. In addition, determining the concentrations of each marker by qPCR in future studies may provide a better understanding of relationships of one marker with other MST markers and waterborne pathogens and ultimately provide a better perspective on the proportion of microbial contamination from human sources.

This study has provided insight on the usefulness of standard, culture-dependent methods for measurements of FIB and several human-associated microbial source tracking markers to assess water quality at beaches impacted by non-point and point source pollution. Determining water quality is a complex assessment of various indicators, and careful consideration of the location, climate, historical water quality data, and possible sources of contamination should be made before water quality indicators are selected. In addition, we strongly recommend the use of a multi-indicator “toolbox” approach when assessing water quality.

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ACKNOWLEDGEMENTS

We would like to thank all the Southern California Coastal Research Project (SCCWRP) personnel involved with the sample collection. We would also like to thank Dr. John Paul for the use of the ABI 7500 instrument. In addition, we would like to thank Jenny Delaney, Dave John, Lauren McDaniel, Robert Ulrich, Beth Young, and Brian Zielinski for technical and logistical support. Funding for this study was provided in part by USEPA Gulf of Mexico Alliance Regional Partnership Projects MX-96478707-0.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2012AnnualReport/ar12_23SI.pdf