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Human-Associated *Bacteroides* spp. and Human Polyomaviruses as Microbial Source Tracking Markers in Hawaii

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ABSTRACT

Identification of sources of fecal contaminants is needed to (i) determine the health risk associated with recreational water use and (ii) implement appropriate management practices to mitigate this risk and protect the environment. This study evaluated human-associated *Bacteroides* spp. (HF183TaqMan) and human polyomavirus (HPyV) markers for host sensitivity and specificity using human and animal fecal samples collected in Hawaii. The decay rates of those markers and indicator bacteria were identified in marine and freshwater microcosms exposed and not exposed to sunlight, followed by field testing of the usability of the molecular markers. Both markers were strongly associated with sewage, although the cross-reactivity of the HF183TaqMan (also present in 82% of canine [$n = 11$], 30% of mongoose [$n = 10$], and 10% of feline [$n = 10$] samples) needs to be considered. Concentrations of HF183TaqMan in human fecal samples exceeded those in cross-reactive animals at least 1,000-fold. In the absence of sunlight, the decay rates of both markers were comparable to the die-off rates of enterococci in experimental freshwater and marine water microcosms. However, in sunlight, the decay rates of both markers were significantly lower than the decay rate of enterococci. While both markers have their individual limitations in terms of sensitivity and specificity, these limitations can be mitigated by using both markers simultaneously; ergo, this study supports the concurrent use of HF183TaqMan and HPyV markers for the detection of sewage contamination in coastal and inland waters in Hawaii.

IMPORTANCE

This study represents an in-depth characterization of microbial source tracking (MST) markers in Hawaii. The distribution and concentrations of HF183TaqMan and HPyV markers in human and animal fecal samples and in wastewater, coupled with decay data obtained from sunlight-exposed and unexposed microcosms, support the concurrent application of HF183TaqMan and HPyV markers for sewage contamination detection in Hawaii waters. Both markers are more conservative and more specific markers of sewage than fecal indicator bacteria (enterococci and *Escherichia coli*). Analysis of HF183TaqMan (or newer derivatives) is recommended for inclusion in future epidemiological studies concerned with beach water quality, while better concentration techniques are needed for HPyV. Such epidemiological studies can be used to develop new recreational water quality criteria, which will provide direct information on the absence or presence of sewage contamination in water samples as well as reliable measurements of the risk of waterborne disease transmission to swimmers.

The recently revised recreational water quality criteria (1) continue to use concentrations of fecal indicator bacteria (FIB), such as enterococci and *Escherichia coli*, to determine risks to swimmers and to establish recreational water quality standards. However, major limitations in the implementation of the current recreational water quality standards are related to the fact that the approved FIB measurement methods do not determine their sources, and only proven sewage sources of FIB have been shown to reliably predict the risk of disease transmission to swimmers (2). Moreover, many studies have provided evidence that in many situations, FIB are poor indicators of human health risk. Both enterococci and *E. coli* can grow in various environments outside the human host (3–10) and therefore are not good proxies for sewage-borne pathogens. Enterococci and *E. coli* can originate from various animal sources other than humans, further complicating the link between FIB and human health. The health risk associated with these proxy organisms is expected to differ depending on their source (11, 12). To determine the actual human health risk associated with recreational water use and to implement appropriate management practices and remediation strategies, the identification of contamination sources(s) is needed.

Microbial source tracking (MST) methods are aimed at identifying sources of enterococci and *E. coli* and have been extensively reviewed elsewhere (12–17). The molecular marker-based approach, which utilizes PCR technology, has become one of most widely used MST methods to identify contamination source(s).

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PCR is easy to use, and a wide variety of host-specific markers are available.

Enteric microbial fauna is expected to vary over regional scales, as diet, hygiene, and environment significantly affect host microbiomes (18). Therefore, there is a need to validate molecular PCR markers for host specificity and for sensitivity (i.e., prevalence in the host population) in populations in the specific geographic area under study (12, 14).

Although several evaluation studies of molecular PCR markers have been conducted on the U.S. mainland (19–21) and elsewhere (22–24), no marker validation studies have been conducted in Hawaii or on other Pacific Islands. These validation studies have demonstrated that bacterial MST markers can be found in species of which they are not indicative, although at less frequently and at lower concentrations. Therefore, it would be incorrect to use markers validated elsewhere in Hawaii (or in any other location, for that matter) without prior marker validation studies. More importantly, the cross-reactivity of bacterial MST markers suggests that the simultaneous use of more specific markers (such as viruses) in parallel provides stronger evidence for determining the source of contamination.

To understand the distribution and relevance of molecular markers in relation to a contamination event, such as a sewage spill, concentrations of the markers in sewage as well as decay rates of the markers must be considered and compared to those of FIB and human pathogens. Environmental differences, such as sunlight, water temperatures, etc., vary on the regional scale. While studies of decay rates of different molecular source-specific markers are starting to emerge (25–32), relatively little is known about the decay rates of markers in tropical waters. Human-associated *Bacteroides* spp. (when determined using the HF183TaqMan assay, as proposed earlier [33]), have been identified as one of the best-performing bacterial markers to detect human-associated fecal contamination based on an evaluation of 41 bacterial source tracking markers (20), while human polyomavirus has been demonstrated to be a highly specific sewage marker in other studies (21, 34–36). In an earlier study, Betancourt and Fujioka (37) demonstrated that a human-associated *Bacteroides* (HF183) assay, as proposed in another study (38), has great promise in Hawaii due to the speed and sensitivity of the assay and the prevalence of the marker in sewage. Our study focused on human-associated *Bacteroides* spp. (HF183TaqMan) (33) and human polyomaviruses (HPyV) (34) as promising PCR markers of human sewage contamination.

In Hawaii, enterococcal concentrations routinely exceed water quality standards in most streams. As enterococci grow in Hawaiian soils and are shed by animals (3, 39), we cannot tell whether the source of the enterococci found in water is environmental or a sewage leak. Therefore, there is an immediate need for a method that would reliably determine the source of microbial contaminants in Hawaii's recreational waters.

The goal of our study was to determine whether the combination of two human fecal molecular markers (HF183TaqMan and HPyV) can be reliably used to determine if recreational waters in Hawaii are contaminated with sewage. To achieve this goal, the following activities were completed: (i) a comparison of the concentrations of the two markers to concentrations of viable enterococci and *Clostridium perfringens* in fecal and sewage samples, (ii) validation of host sensitivity and specificity of the two markers for human and selected animal feces, (iii) determination of concentrations of the two markers in raw sewage and sewage treated to various degrees from two wastewater treatment plants, (iv) determination of the stability of the two markers in fresh and marine water samples at ambient temperature (25°C) with and without exposure to sunlight, (v) determination of concentrations of the two markers in a freshwater stream and at two coastal marine sites which historically receive nonpoint source discharges from a highly urbanized area and from an unpopulated area with high rainfall, and (vi) development of recommendations on the suitability of these two molecular markers to reliably detect the absence or presence of sewage contamination in Hawaii's recreational waters based on the data obtained.

MATERIALS AND METHODS

Sample collection. (i) Fecal samples. Fecal samples from various animals were collected to determine differences in concentrations and ratios of FIB and molecular markers between humans and other animals. The data obtained allow us to make deductions about the sensitivity and specificity of the various markers. Single stool samples were provided by 10 volunteers age 33 to 59 years (1:1 female-to-male ratio) between 24 April and 24 June 2013, who collected specimens according to the protocol approved by the institutional review board of the Human Studies Program, University of Hawaii (notice CHS 21096) using aseptic stool specimen collection kits. Fecal samples originating from 10 individual cats (*Felis catus*) were collected around the University of Hawaii Mānoa campus in Honolulu, HI, between 25 March 2013 and 22 March 2014. Ten individual rat (*Rattus rattus*) and 10 individual mongoose (*Herpestes auropunctatus*) fecal samples were collected between 5 March and 2 May 2013 on Hawaii Island. Eleven individual domestic dog (*Canis lupus* subsp. *familiaris*) samples were collected from various sites on the island of Oahu (Aiea, Kaimuki, Kalihi, Kaneohe, Mānoa, Waipahu, and downtown Honolulu), and five chicken (*Gallus gallus*) samples were collected near the University of Hawaii at Mānoa lower campus in May 2015. Ten feral pig (*Sus scrofa*) samples were collected on 30 October 2015 in a forest adjacent to the laboratory of the Hawaii Department of Health at Waimano Home Road on Oahu.

(ii) Wastewater samples. These samples were used to determine the concentrations of the two molecular markers in comparison to FIB in sewage treated to various levels. Wastewater samples were collected from two treatment plants on Oahu three times between 28 May and 1 June 2013 at the Sand Island Wastewater Treatment Plant (SIWWTP) and three times between 13 and 17 October 2013 at the Kailua Wastewater Treatment Plant (KWWTP) (see Fig. S1 in the supplemental material). At the SIWWTP, samples were collected from the influent, after primary treatment (before UV disinfection), and after UV disinfection. At the KWWTP, samples were taken from the influent, after primary treatment, and after secondary treatment. All wastewater samples were transported to the laboratory on ice and analyzed within 3 h. The wastewater at both treatment plants was characterized by relatively high salinity (average 5.9 ppt at SIWWTP and 8.3 ppt at KWWTP).

(iii) Stream samples. These samples were used to determine and compare concentrations of FIB and molecular markers in water from two sites in Mānoa Stream. Samples were collected monthly between 1 May 2013 and 30 April 2014 from the upper section of Mānoa Stream on Oahu, above Harold L. Lyon Arboretum (site MS1, $n = 12$ samples) (21°20′3.70″N, 157°48′1.08″W) where the stream is surrounded by tropical rainforest, and from the middle section (site MS2, $n = 12$ samples) (21°17′26.29″N, 157°48′53.40″W) of the stream where it has flowed 4.7 km through sewered residential neighborhoods (see Fig. S1 in the supplemental material).

(iv) Marine coastal samples. These samples were used to determine and compare the concentrations of FIB and molecular markers at two beach sites on Oahu. Marine coastal samples were collected monthly at two sites, Kahana Bay (KB1) (Storet identification [ID] 000230;

TABLE 1 Primers and probes used in molecular tests

Assay	Forward primer	Reverse primer	Probe[a]	Reference
Sketa	5′-GGTTTCCGCAGCTGGG	5′-CCGAGCCGTCCTGGTC	5′-FAM-AGTCGCAGGCGGCCACCGT-BHQ	44
HF183-TaqMan	5′-ATCATGAGTTCACATGTCCG	5′-CGTAGGAGTTTGGACCGTGT	5′-FAM-CTGAGAGGAAGGTCCCCCACATTGGA-BHQ	33
HPyV	5′-AGTCTTTAGGGTCTTCTACCTTT	5′-GGTGCCAACCTATGGAACAG	5′-FAM-TCATCACTGGCAAACAT-MGBNFQ	34

[a] FAM, 6-carboxyfluorescein; BHQ, black hole quencher.

21°33′21.3″N, 157°52′21.7″W) (n = 13 samples), and Keehi Lagoon (KL1) (Storet ID 000342; 21°19′50.3″N, 157°53′40.6″W) (n = 13 samples) over a 1-year period (1 May 2013 to 30 April 2014) (see Fig. S1 in the supplemental material). Kahana Bay is located on the windward side of Oahu in one of the wettest areas on the island, with a mean annual rainfall ranging from 25.2 cm in the lower sections to 61.5 cm in the upper sections of the valley (40). The watershed associated with Kahana Bay is undeveloped and is predominantly covered by lush rainforest penetrated by few roads. Keehi Lagoon, in contrast, is located in a heavily urbanized area of Oahu with surrounding watersheds predominantly characterized by industrial or residential land use.

Survival and persistence studies. (i) Decay of cultivable microorganisms and molecular MST markers in the absence of light and native biota. To explore the decay of FIB and markers, water was collected from Mānoa Stream next to the University of Hawaii Mānoa campus (salinity <2 ppt, pH 7.84), and seawater was collected from the middle section of Ala Moana Beach on Oahu (salinity = 33.0 ppt, pH 8.16) on 3 June 2013. Twelve liters of each water type was filtered through Supor-200 membrane filters (0.2-μm pore size) (Pall Corporation, Ann Arbor, MI) to remove native microbial fauna, thus enabling us to study the decay of sewage-borne microorganisms independently of the effects of site-specific flora and fauna. The filtered samples were spiked with 500 ml of raw sewage from the SIWWTP. Both of the filtered/spiked samples were shaken twice for 30 s by hand to evenly distribute the sewage and then were equally divided into three sterile polypropylene containers (4-liter final volumes). The containers with the spiked freshwater (salinity, 0.3 ppt; pH 7.35) and seawater (salinity, 32.3 ppt; pH 7.96) were loosely closed, placed in the dark at 22°C, and continuously agitated using magnetic stirrers. All six microcosms were tested for concentrations of enterococci and *C. perfringens* (culture-based methods) and molecular markers (HF183TaqMan and HPyV) at days 0, 1, 3, 5, 8, 11, 15, 19, 24, 30, and 40. The sampling frequency was determined based on the initial observed decay rates of FIB.

(ii) Decay of FIB and molecular MST markers in the presence and absence of sunlight with native biota. A second set of experiments was conducted to study the decay of microorganisms in microcosms exposed and not exposed to sunlight. For these experiments, 4.5 liters of freshwater collected from Mānoa Stream next to the University of Hawaii Mānoa campus and 4.5 liters of seawater collected at the middle section of Ala Moana Beach were used. These unfiltered water samples were spiked with 500 ml of fresh raw sewage (pH 7.37; turbidity, 88.5 nephelometric turbidity units [NTU]; salinity, 4.7 ppt) collected from the SIWWTP. The final freshwater matrix had a salinity of <1 ppt, turbidity of 10.5 NTU, and pH 7.6, while the final seawater matrix had a salinity of 28.4 ppt, turbidity of 18.8 NTU, and pH 8.17. Both freshwater and marine samples were mixed well and divided into four 1-liter laboratory-grade glass flasks. One flask of each fresh and saltwater sample was covered with aluminum foil to prevent sunlight penetration. The flasks were submerged in a water bath placed on a multiposition magnetic stirrer (Bellco Glass, Inc., Vineland, NJ) and placed outdoors in full sunlight between 9:00 AM and 3:00 PM on 22 June (freshwater experiment) and 24 June 2015 (seawater experiment). The water in the flasks was agitated throughout the experiment using magnetic stirrers, and the water bath temperature was kept constant at 24 to 25°C by adding ice and removing excess water from the bath as needed. Illuminance (in kiloluxes) and UV intensity (in milliwatts per centimeter squared) (see Fig. S2 in the supplemental material) were measured at the water surface every 30 s using a TR-74Ui Illuminance UV recorder (T&D Corporation, Nagano, Japan) placed in the middle of an empty flask placed in water. Water samples (120 ml) were analyzed before the start of the experiment and every hour thereafter. A total of seven samples were collected from each flask. All four microcosms, three light and one dark, were tested for enterococci, *Escherichia coli*, *C. perfringens* (culture-based methods), and the molecular markers under study, HF183TaqMan and HPyV.

Microbiological analyses for cultivation-based tests. (i) Fecal samples. For the fecal samples, 1 g of fecal material (each species) was mixed into 100 ml of phosphate-buffered dilution water (41). These diluted fecal samples were further diluted as needed to recover FIB using the cultivation-based methods described below.

(ii) Sewage, decay studies, and water samples. The sewage samples and samples from the decay study microcosms were serially diluted as needed. Ten-milliliter samples from each dilution were analyzed. Ten-milliliter aliquots of the undiluted marine (KB1 and KL1) and freshwater (MS1 and MS2) samples were analyzed.

For all samples, enterococci and *E. coli* concentrations were determined using the Enterolert and Colilert-18 systems, respectively, in Quanti-Tray/2000 format (Idexx Laboratories, Inc., Westbrook, ME). Concentrations of *C. perfringens* spores were determined using a membrane filter-based method (42), which involved heat shock of 100-ml aliquots at 65°C for 15 min, followed by filtration and incubation of the filters on mCP medium in a GasPak EZ anaerobe pouch system (BD Diagnostics, Franklin Lakes, NJ) at 42°C for 24 h. The phosphatase test, using ammonium hydroxide vapors, was used to confirm positive colonies (variations of pink, red, and magenta reactions after 20 s of exposure). Concentrations of enterococci and *C. perfringens* were determined in all the samples collected, while *E. coli* concentrations were determined in the decay experiments only.

Microbiological analyses for molecular tests. (i) Fecal samples. DNA extractions were performed on homogenized undiluted fecal material (0.3 g [wet weight]) using the same DNA extraction protocol as for water and sewage. All DNA samples were obtained using a final 100-μl elution step.

(ii) Sewage, decay studies, and water samples. Subsamples of all samples were first adjusted to a pH of 3.5 to ensure the adsorption of human polyomaviruses due to the electrostatic interaction between the viral capsid and the filter (34, 43). Twenty-five milliliters of each raw sewage sample was filtered through a mixed cellulose ester membrane filter (Pall Corporation, Ann Arbor, MI) for DNA extractions. The filtration volume for sewage samples collected before UV disinfection and from effluent was 50 ml. One hundred-milliliter aliquots from all the microcosms of the decay studies were filtered. A 300-ml volume of each marine and freshwater sample was filtered for DNA extraction.

DNA was extracted from microbiological material retained on each filter using the PowerSoil DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA), according to the manufacturer's protocol, except that we incorporated 2 min of bead beating at maximum speed on a Mini-Beadbeater (BioSpec Products, Inc., Bartlesville, OK) instead of vortexing for 10 min.

Concentrations of HF183TaqMan and HPyV markers were determined using SsoAdvanced universal probes supermix chemistry (Bio-Rad Laboratories, Inc., Hercules, CA) and primers (Table 1) synthesized by the Integrated DNA Technologies (Coralville, IA). Each 25-μl PCR mixture contained 5 μl of sample, bovine serum albumin (0.2 mg · ml^{-1} final

TABLE 2 Fecal sample values of indicator bacteria (enterococci and *C. perfringens*) and molecular MST markers (human-associated *Bacteroides* and human polyomaviruses) concentrations

Sample type	Sample no.	Enterococcal MPN · g^{-1} [a]			*C. perfringens* CFU · g^{-1}			Human-associated *Bacteroides* (gc · g^{-1}) [b]			Human polyomaviruses (GM) (gc · g^{-1})
		GM	Min	Max	GM	Min	Max	GM	Min	Max	
Humans	10	7.10×10^3	3.10×10^2	1.20×10^6	1.38×10^2	<10	4.10×10^3	9.62×10^6 (8)	$<3.3 \times 10^2$	2.08×10^9	ND
Dogs	11	1.99×10^4	4.10×10^2	1.99×10^6	1.82×10^4	<10	3.28×10^5	4.42×10^3 (9)	$<3.3 \times 10^2$	9.56×10^5	ND
Cats	10	2.76×10^5	1.75×10^5	$>9.68 \times 10^5$	<10	<10	<10	Detected (1)	$<3.3 \times 10^2$	$<3.3 \times 10^2$	ND
Rats	10	4.10×10^5	6.05×10^3	$>2.92 \times 10^7$	<10	<10	<10	ND	ND	ND	ND
Mongooses	10	1.51×10^5	1.73×10^3	$>2.42 \times 10^7$	<10	<10	<10	2.00×10^3 (3)	$<3.3 \times 10^2$	1.05×10^4	ND
Feral pigs	10	3.74×10^4	1.31×10^3	2.42×10^7	2.15×10^4	3.00×10^3	1.10×10^5	ND	ND	ND	ND
Chickens	5	1.07×10^5	3.46×10^4	3.60×10^6	2.1×10^3	2.1×10^3	2.1×10^3	ND	ND	ND	ND

[a] MPN, most probable number; GM, geometric mean; Min, minimum; Max, maximum.
[b] Number of samples positive for MST markers is indicated in parentheses in GM column. gc, gene copies; ND, not detected.

concentration), forward and reverse primers (500 nM final concentration each), probe (80 nM final concentration), and SsoAdvanced universal probes supermix (diluted to 1× final concentration). Each sample was tested in duplicate. The cycling parameters in each PCR run were as follows: initial polymerase activation for 2 min at 95°C, followed by 40 denaturation cycles for 10 s at 95°C and annealing-extension for 30 s at 60°C. Linearized plasmid, containing a target specific sequence insert, was quantified using a double-stranded DNA (dsDNA) high-sensitivity (HS) assay kit on a Qubit 2.0 fluorometer (Life Technologies; Carlsbad, CA) and serially diluted as the quantification standard. Triplicate reactions of each dilution were included in each quantitative PCR (qPCR) run. Samples containing PCR inhibitors were determined by challenging PCR mixtures containing equal concentrations of salmon testes DNA (Sigma-Aldrich, St. Louis, MO) with 5 μl of an unknown sample as in a study by Haugland et al. (44) (Sketa assay, Table 1). Samples for which the threshold cycle was delayed by more than three PCR cycles compared to reactions challenged by molecular-grade water (corresponding roughly to 1-log underestimate of initial concentration) were considered inhibited and subjected to a 10-fold dilution with molecular-grade water.

Initial DNA concentrations in the samples tested were determined using the baseline subtracted curve fit method on the CFX Manager 3.1 software (Bio-Rad Laboratories, Inc., Hercules, CA). The manufacturer's default parameters were accepted only after inspecting the standard curve for outliers and upon consideration of the efficiency and regression coefficient statistics. A PCR efficiency in the range of 90 to 110% and R^2 exceeding 0.990 were considered acceptable. Three no-template controls (NTC) were included with each PCR run. When efficiency or R^2 parameters were not met or any of the NTC was positive, the whole run was repeated. The lower limit of quantification (LLQ) was five gene copies (gc) per 25-μl PCR, which translates to 100 gc in the DNA extract (per sample). Samples that were identified as positive by duplicate PCR but were below the LLQ were identified as detected. Although rare, samples found positive by one only of the duplicate reactions were retested. The performance data of the qPCR tests are provided in the supplemental material.

Statistical analyses of fecal samples. Marker sensitivity was determined as the percentage of marker-positive samples in the human fecal samples (14, 17). Specificity of the marker was determined as the percentage of marker-negative samples in the nonhuman fecal samples (14, 17).

One-way analysis of variance (ANOVA) was used to compare log-transformed concentrations of enterococci and HF183TaqMan in the fecal samples. Because an assumption of equal variance was not valid for the log-transformed bacterial data, we used Kruskal-Wallis ANOVA on ranks to evaluate differences among the samples. The Dunn's test was used for pairwise multiple comparisons, and the differences were considered significant when the P value was <0.05. This analysis was performed using SigmaPlot 12 software (Systat Software, Inc., San Jose, CA).

Freshwater and marine water microcosm decay experiment. Microbial decay rates were based on the Chick-Watson first-order decay model (45, 46) as follows: the geometric mean of concentrations in triplicate microcosms was calculated and the data normalized by dividing the mean at each time point by the mean of microcosm triplicates at the beginning of the experiment (C/C_0). Normalized data were transformed with the natural logarithm [$\ln(C/C_0)$]. Decay rates (k) and their 95% confidence intervals were calculated using a linear curve fit between $\ln(C/C_0)$ and time. Data points below the lower quantification limits were excluded from the rate calculation. The time to inactivate 90% of a given microbial population (T_{90}) was calculated from the relationship T_{90} (in days) = $\ln(10/k)$ Analyses of covariance (ANCOVA) followed by the Tukey test were used to compare slopes among the different samples to identify the effect of sunlight, water type, and type of microorganism on the decay rate. ANCOVA was performed using Minitab 17 (Minitab, Inc., State College, PA).

Correlation analysis was used to identify Pearson product-moment coefficient (R) to explore the strength of the association between salinity and log-transformed microbiological parameters (enterococci and *C. perfringens* concentrations). A correlation was considered to be significant when the P value was <0.05.

RESULTS

Concentrations of FIB and molecular markers in fecal and sewage samples. Enterococci were detected in all fecal samples tested, while *C. perfringens* was detected in seven out of 10 human, 10 out of 11 canine, and one out of five chicken fecal samples. *C. perfringens* was not detected in the samples collected from cats, rats, or mongooses. Enterococcal concentrations were highly varied among human, canine, feral pig, mongoose, and chicken fecal samples but were relatively consistent in cat feces (Table 2). Likewise, *C. perfringens* concentrations, when detected, varied between individual fecal samples. Among all fecal samples positive for FIB, the concentrations of both FIB were lowest in human samples (Table 2).

The concentrations of HF183TaqMan exceeded the concentrations of enterococci and *C. perfringens* in the human fecal samples tested, indicating that this marker can be a more sensitive proxy for sewage contamination (Table 2). The highest concentrations of HF183TaqMan were associated with human samples, although concentrations varied vastly among individuals (Table 2). In human fecal samples, the concentrations of HF183TaqMan exceeded concentrations of enterococci 1,355:1. This ratio is high compared to that seen in the cross-reactive animal feces (HF183TaqMan-to-enterococci ratio for dog, 0.222:1, and mongoose, 0.013:1). Nevertheless, relatively high concentrations of this marker in the dog and mongoose samples can be a concern when using this marker alone for source tracking purposes.

TABLE 3 Wastewater geometric means of indicator bacteria (enterococci and *C. perfringens*) and molecular MST markers (HF183TaqMan and HPyV) concentrations

Sample type	Sample size	Enterococcal MPN ($100\ ml^{-1}$)	*C. perfringens* CFU ($100\ ml^{-1}$)	HF183TaqMan (no. of positive samples) ($gc \cdot g^{-1}$)	HpyV (no. of positive samples) ($gc \cdot g^{-1}$)
SIWWTP					
Influent	3	1.66×10^6	7.22×10^4	1.59×10^6	3.18×10^3
Primary treated	3	2.52×10^5	1.50×10^4	7.58×10^5	2.56×10^3
Effluent	3	2.45×10^4	4.34×10^3	5.98×10^5	3.66×10^2
KWWTP					
Influent	3	1.14×10^6	9.59×10^4	6.35×10^5	9.97×10^3
Primary treated	3	6.36×10^5	2.90×10^4	9.38×10^3	1.60×10^2
Secondary treated effluent	3	2.30×10^3	2.10×10^3	4.18×10^1	Detected, $<1 \times 10^2$

Human polyomaviruses were not detected in any of the fecal samples tested.

Host sensitivity and specificity of the human-associated markers in human feces and selected animal feces. HF183TaqMan appears to be highly prevalent in the human population in Hawaii. The marker was detected in all wastewater samples (100% sensitivity) and in eight out of 10 individual human fecal samples (80% sensitivity). Although this highly human-specific marker (34) is highly prevalent in the human population, it was also detected in nine out of 11 dog samples (81.8%), in three out of 10 mongoose samples (30.0%), and in a single cat sample (10%). Therefore, the specificity of this marker based on our limited sample size was 78%.

In this regard, it needs to be considered that concentrations of HF183TaqMan in human fecal samples were significantly higher than those in animal samples exhibiting cross-reactivity with this marker ($P < 0.05$ for all pairwise comparisons). Also importantly, this marker was not detected in feral pigs, chickens, or rats, which are numerous in Hawaiian watersheds.

The HPyV marker was detected in all the sewage samples tested (Table 3) but not in any of the fecal samples tested (Table 2).

FIB and molecular MST markers in sewage. Concentrations of enterococci decreased by 98.5% over the treatment train at the SIWWTP, which performs primary treatment followed by UV disinfection. Concentrations of enterococci decreased by 99.8% at the KWWTP, which performs primary and secondary treatment but no disinfection (Table 3). Concentrations of *C. perfringens* decreased by 94.0% and 97.8% at the SIWWTP and KWWTP, respectively, suggesting that *C. perfringens* was more resistant to treatment than were enterococci (Table 3).

In the influent of the two wastewater treatment plants, the concentrations of HF183TaqMan ranged from 5.98×10^5 to 1.66×10^6 $gc \cdot 100\ ml^{-1}$ (geometric mean, 1.59×10^6 $gc \cdot 100\ ml^{-1}$) and from 3.81×10^5 to 1.08×10^6 $gc \cdot 100\ ml^{-1}$ (geometric mean, 6.35×10^5 $gc \cdot 100\ ml^{-1}$) at the Sand Island and Kailua plants, respectively. Concentrations of HF183TaqMan decreased 62.5% during the wastewater treatment process at the SIWWTP and 99.99% at the KWWTP (Table 3), and HPyV concentrations decreased 88.5% and >99.7%, respectively, during treatment at the treatment plants. While the removal of HF183TaqMan was comparable to the removal of enterococci when primary treatment was followed by secondary treatment at the KWWTP, the primary treatment followed by UV disinfection at the SIWWTP was not as effective in removing both markers from the wastewater.

It should be noted that the geometric means of HF183TaqMan concentrations in KWWTP influent samples were roughly an order of magnitude lower than those in human fecal samples. Concentrations of HF183TaqMan were similar to the levels of enterococci in the sewage (HF183TaqMan-to-enterococci ratio in SIWWTP influent, 0.96, and in KWWTP influent, 0.56) and exceeded this ratio in cross-reactive animals.

Decay of FIB and MST markers in the absence of sunlight and native biota (marine and freshwater microcosms). In the absence of sunlight and native aquatic biota, the concentrations of the two human-associated markers decreased over time at rates similar to those of enterococci both in freshwater and seawater, while the concentration of *C. perfringens* changed little (Fig. 1 and Table 4). Although HF183TaqMan could be quantified until day 11 in freshwater and day 15 in seawater, and HPyV until day 11 (both water types), both markers remained detectable for twice as long. HF183TaqMan was detected in two out of three microcosms of both water types at day 30 and HPyV in a single microcosm of both water types at day 24. Concentrations of HPyV declined below the quantification and detection limits faster than HF183TaqMan. The decay rate of both markers was significantly different in the freshwater and seawater at alpha level of 0.10 but not at 0.05 (ANCOVA, $P = 0.071$ for freshwater and $P = 0.052$ for seawater). Despite the apparent difference in estimates of T_{90} (time to decrease concentrations by 90% in days) (Table 4), the decay rates of both molecular markers were not significantly different from the decay rates of enterococci (ANCOVA for HF183TaqMan, $P = 0.921$ for freshwater and $P = 0.214$ for seawater; for HPyV, $P = 0.186$ for freshwater and $P = 0.242$ for seawater).

Decay of *C. perfringens* was extremely slow over the study period (average concentrations decreased 55% in freshwater and 70% in marine water over the 40-day period), and no T_{90} could be determined. The decay of *C. perfringens* was significantly slower than the decay of enterococci and the molecular markers (ANCOVA, $P < 0.001$ for all pairwise comparisons).

Concentrations of enterococci and markers tended to decrease more slowly in seawater than in freshwater, although this difference was not statistically significant for any of the microorganisms compared (ANCOVA; $P = 0.863$ for enterococci, $P = 0.935$ for *C. perfringens*, $P = 0.866$ HF183TaqMan, and $P = 1.000$ for HPyV). The relatively small number of samples in which the markers could be quantified limits the significance test.

Decay of FIB and MST markers in sunlight (unfiltered marine and freshwater microcosms). The decay of FIB and molec-

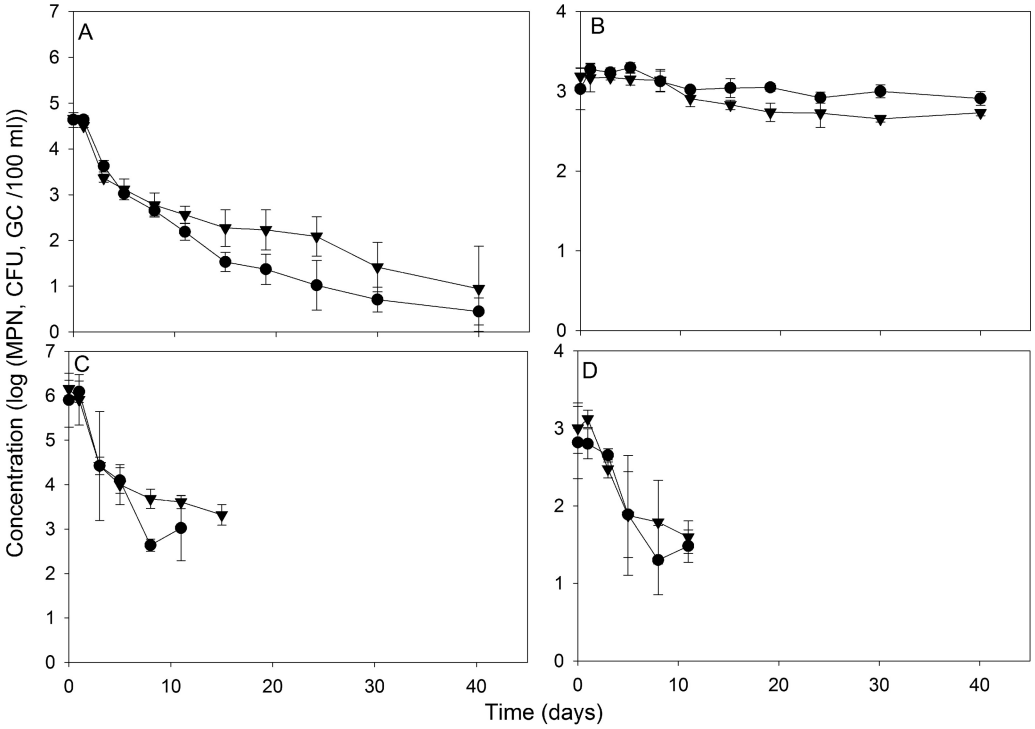

FIG 1 Survival in dark without native aquatic biota: indicator bacteria and MST marker concentrations by culture-based methods (enterococci [A] and *C. perfringens* [B]) or molecular methods (HF183TaqMan [C] and HPyV [D]) in freshwater (●) and marine (▼) microcosms over 40 days. Data points represent geometric means and 95% confidence intervals of concentrations in freshwater ($n = 3$) and seawater ($n = 3$) microcosms. Only results above the quantification limit were plotted.

ular MST markers was faster in the sun-exposed microcosms than in unexposed microcosms (Table 4). Although native aquatic biota was present in these unfiltered samples, no decay of any of the microorganisms was observed in the dark container over the 6-h period of the study. In the sun-exposed microcosms, the decay rates of all the microorganisms were significantly higher than those observed in the dark microcosms for both water types (ANCOVA; in freshwater, $P < 0.001$ for enterococci, $P < 0.001$ for *E. coli*, $P = 0.046$ for *C. perfringens*, $P = 0.007$ for HF183TaqMan, and $P < 0.001$ for HPyV; in seawater, $P < 0.001$ for enterococci, $P < 0.001$ for *E. coli*, $P < 0.001$ for *C. perfringens*, $P = 0.001$ for HF183TaqMan, and $P < 0.001$ for HPyV) (Fig. 2).

In general, the viability of culturable FIB decreased at a higher rate than the decay of the molecular markers' signals. Over the 6-h experiment, inactivation of enterococci, *E. coli*, and *C. perfringens* was rapid, as indicated by a 90% reduction in cultivable cell fraction within 0.8 to 2.1 h (Table 4 and Fig. 2), while a 90% reduction in molecular signal was not observed in either water type. In both freshwater and seawater microcosms exposed to sunlight, the decay rates of enterococci, *E. coli*, and *C. perfringens* were not signif-

TABLE 4 Decay rates and respective 95% confidence intervals of indicator bacteria and molecular markers in freshwater and marine microcosms[a]

Indicator by microcosm type	Freshwater		Marine	
	Decay rate (k) (95% CI)	T_{90}	Decay rate (k) (95% CI)	T_{90}
Microcosms not exposed to sunlight				
Enterococci	−0.226 (−0.306 to −0.146)	10.2	−0.170 (−0.224 to −0.115)	13.6
C. perfringens	−0.022 (−0.032 to −0.012)	>40	−0.033 (−0.049 to −0.019)	>40
HF183TaqMan	−0.602 (−0.859 to −0.345)	3.8	−0.347 (−0.657 to −0.038)	6.6
HPyV	−0.356 (−0.657 to −0.056)	6.5	−0.328 (−0.608 to −0.049)	7.0
Microcosms exposed to sunlight				
Enterococci	−1.985 (−2.278 to −1.692)	1.2	−1.109 (−1.387 to −0.831)	2.1
E. coli	−1.684 (−2.128 to −1.239)	1.4	−2.939 (−4.288 to −1.590)	0.8
C. perfringens	−1.163 (−2.361 to −0.035)	2.0	−1.636 (−3.846 to −0.574)	1.4
HF183TaqMan	−0.031 (−0.232 to −0.294)	>6	−0.138 (−0.410 to −0.135)	>6
HPyV	−0.111 (−0.256 to −0.034)	>6	−0.365 (−0.538 to −0.193)	>6

[a] In microcosms not exposed to sunlight, decay rate and T_{90} are measured in day^{-1} and days, respectively. In microcosms exposed to sunlight, decay rate and T_{90} are measured in hour^{-1} and hours, respectively. CI, confidence interval.

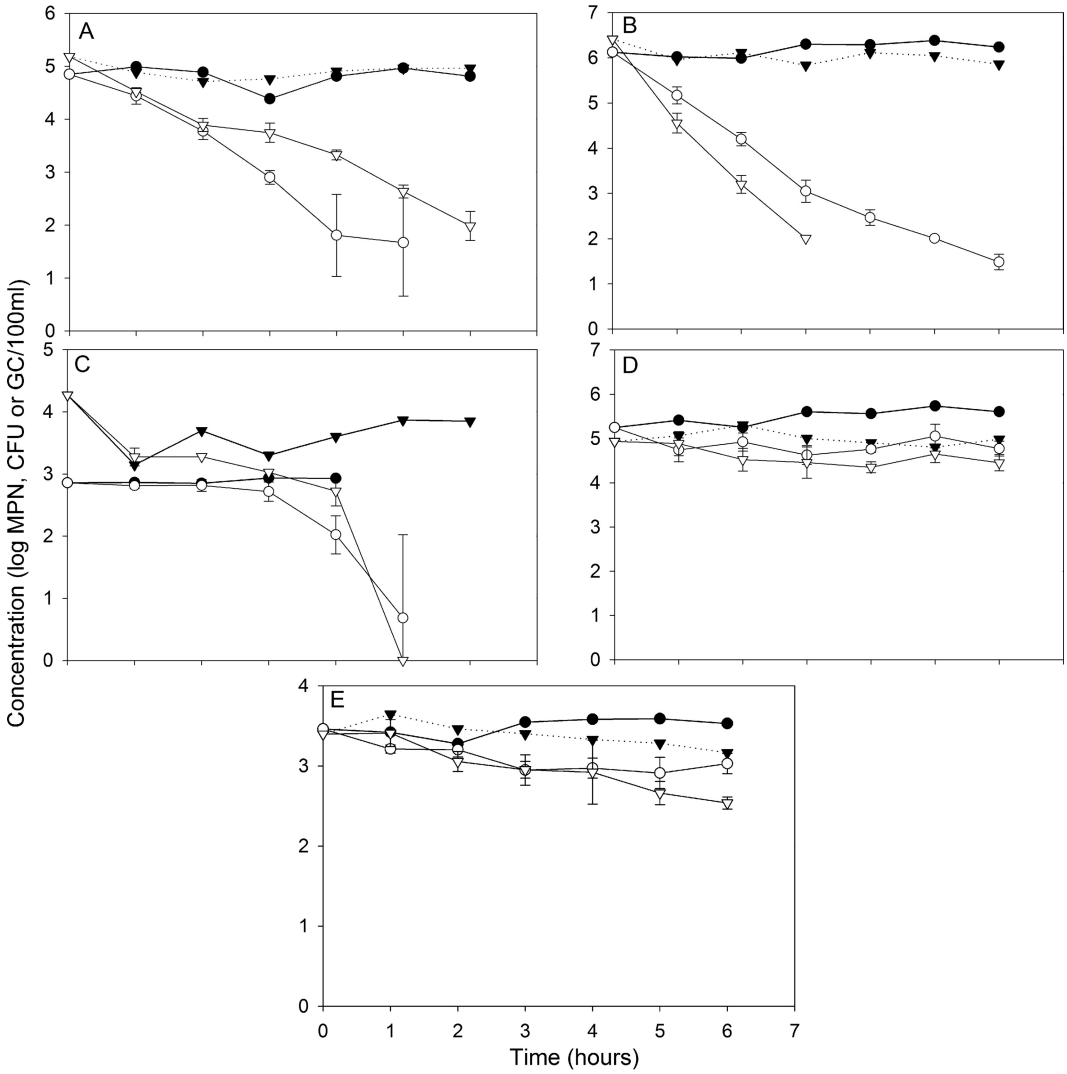

FIG 2 Survival with native aquatic biota: indicator bacteria and MST marker concentrations by culture-based methods (enterococci [A], *E. coli* [B], and *C. perfringens* [C]) and molecular methods (HF183TaqMan [D] and HPyV [E]) in freshwater (circles) and seawater (triangles) microcosms over 6 h in dark (closed symbols) or sunlight (open symbols). Data points represent geometric means and 95% confidence intervals of concentrations in freshwater and seawater microcosms. Only results above the quantification limit were plotted.

icantly different (ANCOVA, $P \geq 0.08$ for all pairwise comparisons). Also, the decay rates of HF183TaqMan and HPyV were comparable to each other in both the freshwater and seawater microcosms (ANCOVA, $P = 1.00$ for both matrixes). When the decay rates of enterococci and *E. coli* were compared to those of HF183TaqMan and HPyV, the differences were always significant (ANCOVA; $P \leq 0.009$ for freshwater comparisons and $P \leq 0.037$ for seawater comparisons). The decay of *C. perfringens* did not fit well to the linear decay model ($R^2 = 0.69$ in freshwater and 0.74 in seawater), as there was a sudden drop in concentration after 4 h when concentrations decreased rapidly below the detection limit; hence, a larger uncertainty is associated with the decay estimates for *C. perfringens*. Nevertheless, over 6 h, a 100-fold reduction in *C. perfringens* concentrations was observed in all sunlight-exposed microcosms.

No statistically significant differences between the decay rates of microorganisms in either the freshwater or seawater matrixes were observed (ANCOVA, $P \geq 0.456$ for all pairwise comparisons). This was also supported by T_{90} estimates, as there was overlap between the 95% confidence interval estimates for freshwater and seawater matrixes (Table 4).

Field testing in stream and coastal samples. (i) Stream samples. In Mānoa Stream, concentrations of enterococci ranged from 74 to 15,531 most probable number (MPN) · 100 ml^{-1} (geometric mean, 831 MPN · 100 ml^{-1}), and *C. perfringens* concentrations ranged from <10 to 540 CFU · 100 ml^{-1} (geometric mean, 42 CFU · 100 ml^{-1}).

Log-transformed concentrations of enterococci and *C. perfringens* were significantly different between the two sites studied ($P = 0.014$ and <0.001, respectively). At the upstream site MS1, above Lyon H. Arboretum, concentrations of enterococci MS1 varied from 74 to 4,570 MPN · 100 ml^{-1} (geometric mean, 405 MPN · 100 ml^{-1}), and concentrations of *C. perfringens* varied from <10 to 40 CFU · 100 ml^{-1} (average, 12 CFU · 100 ml^{-1}). Overall,

samples collected at this site complied with Hawaii's two-part water quality standard, as although 75% of the samples exceeded the statistical threshold value (STV) for enterococci, no sample exceeded the *C. perfringens*-based water quality guideline.

Concentrations of enterococci and *C. perfringens* at downstream site MS2 by the University of Hawaii varied from 373 to 15,531 MPN · 100 ml^{-1} (geometric mean, 1,706 MPN · 100 ml^{-1}) and from 20 to 540 CFU · 100 ml^{-1} (geometric mean, 70 CFU · 100 ml^{-1}), respectively. The *Enterococcus*-based standard (STV) was exceeded in all the samples from this site. The *C. perfringens*-based guideline was exceeded in eight out of 12 samples (67%) tested.

HF183TaqMan was detected in 25% of the samples collected at upstream site MS1, but HPyV was not detected at this site. In contrast, HF183TaqMan was detected in 83.3% and HPyV in 58.3% of the samples collected at site MS2, suggesting sewage input in the residential section of the watershed. HF183TaqMan could be quantified in only two samples from site MS1 (795 and 16,919 gc · 100 ml^{-1}), but it was quantified in 10 out of 12 samples collected at site MS2, where the concentrations varied from 533 gc · 100 ml^{-1} to 43,047 gc · 100 ml^{-1} (geometric mean, 4,317 gc · 100 ml^{-1}). HPyV could be quantified in 28% of the samples from site MS2 but in none from site MS1.

(ii) Coastal samples. At both coastal sites, Keehi Lagoon (KL1) and Kahana Bay (KB1), salinity and microbial FIB concentrations fluctuated extensively over the study period. The average salinity in KL1 was 33 ppt, while at KB1, the average salinity was 23 ppt, indicating strong freshwater input from Kahana Valley. This was corroborated by visual inspection, as the bay water was mostly brown when sampled, suggesting a high influx of terrestrial sediment. There was significant negative correlation between salinity and enterococcal concentrations ($R = -0.577$, $P = 0.002$).

Concentrations of enterococci were not significantly different between the two coastal sites studied ($P = 0.084$). In nonurbanized KB1, concentrations of enterococci varied from <10 to 389 MPN · 100 ml^{-1} (geometric mean, 61 MPN · 100 ml^{-1}) and exceeded those at KL1, at <10 to 369 MPN · 100 ml^{-1} (geometric mean, 59.3 MPN · 100 ml^{-1}), which is located in a heavily urbanized area. *C. perfringens* concentrations were significantly different between the two sites ($P < 0.043$), ranging from <10 to 20 CFU · 100 ml^{-1} (geometric mean, 5 CFU · 100 ml^{-1}) at KB1 and from <10 to 96 CFU · 100 ml^{-1} at KL1 (geometric mean, 10 CFU · 100 ml^{-1}). The STV for enterococci was exceeded in two samples collected from both sites (KB1 and KL1). One of those samples (KL1) also exceeded the 50-CFU · 100 ml^{-1} guideline set for *C. perfringens* (96 CFU · 100 ml^{-1}). Both of the STV exceedances in KL1 were found in low-salinity samples (salinity 20.6 and 19.5ppt), while the salinity in the rest of the samples exceeded 29 ppt. There was significant negative correlation between salinity and enterococcal concentrations at KL1 ($R = -0.716$, $P = 0.006$, $n = 13$), linking elevated FIB levels to freshwater inputs from streams and canals.

HF183TaqMan was found in five out of 13 coastal samples (38%) collected at KL1, where the concentrations varied from 130 to 4,910 gc · 100 ml^{-1} (geometric mean, 341 gc · 100 ml^{-1}). HF183TaqMan was not detected in any of the samples collected at KB1. The HPyV marker was not detected in any of the coastal samples tested. Frequent recovery of the HF183TaqMan at KL1 probably indicates an intermittent human contamination issue in the lagoon, while high levels of enterococci in KB1 appear to originate from environmental sources.

DISCUSSION

In this study, we detected HF183TaqMan in dogs, mongooses, and cats, while an earlier study (20) conducted in the continental United States detected this marker in deer, dogs, pigeons, and farmed pigs. In a more recent study conducted in Florida, HF183 was identified at high levels and frequently in deer feces (47). While cross-reactivity of HF183 may hamper data interpretation, the average concentrations of HF183TaqMan in source-specific samples (human stool and sewage) exceeded by more than two logs the concentrations seen in dogs, mongooses, and cats, a finding which is very similar to those reported for samples collected in the continental United States (20). Nevertheless, the cross-reactivity of the marker needs to be considered in source tracking studies conducted in Hawaii, as water samples not impacted by sewage can be positive for HF183TaqMan. Therefore, testing for other more specific markers, such as human viruses, in parallel is warranted. It also needs to be considered that concentrations of the HF183TaqMan marker in wastewater were roughly two logs lower than concentrations of different HF183 markers in Australia (48). This may suggest that an evaluation of alternative HF183 markers, such as the recently described HF183/BacR287 assay (49), is warranted in Hawaii.

We did not detect HPyV in any of the tested human fecal samples, likely because these viruses are predominantly shed in urine (50, 51). While absent from fecal samples, HPyV were detected in all wastewater samples tested. However, HPyV concentrations in sewage were roughly two logs lower than those found elsewhere (34, 48). As concentrations of HPyV in raw sewage and effluent were lower than concentrations of other indicator organisms, such as enterococci and *C. perfringens*, testing of large sample volumes would be advisable.

It is well accepted that a single source-specific MST marker provides little assurance of accurate results; hence, the utilization of a marker "toolbox" is advocated (12, 16). When the HF183TaqMan and HPyV markers are used in tandem, the individual limitations of sensitivity and specificity of the markers are reduced. Furthermore, our study suggests that combining bacterial markers with source-specific viral markers represents a very powerful approach that can be used to determine relative health risk in the following manner. If both human molecular markers are detected, it is a reliable indication of sewage-related impairment, which represents higher risk to bathers than microbial contaminants originating from nonpoint sources. On the other hand, when no marker is detected, enterococci in the samples are likely not from sewage and represent a lower risk to bathers. When only HF183TaqMan is detected, further evidence should be acquired to confirm sewage-based impairment and/or explore other contamination sources. Detection of only human polyomaviruses is very unlikely, as their concentration in raw sewage is roughly 1,000-fold lower than that of HF183TaqMan.

Sunlight has a critical role in determining the fate of microorganisms (52, 53). In this study, as in many others (28, 30, 54–58), inactivation of FIB exposed to sunlight is significantly faster than that of bacteria kept in the dark in both freshwater and marine water matrixes. In our sunlight-exposed microcosms, concentrations of enterococci and *E. coli* were reduced 90% in ≤2.1 h. The observed inactivation rates were higher than those in studies conducted in temperate climates in the United States and New Zealand (28, 30, 57, 59) but similar to those observed in subtropical

regions of Australia (25), indicating that regional differences exist. Greater solar intensity and higher water temperatures are likely the dominant contributing factors.

Decay of the molecular microbial source tracking markers, HF183TaqMan and HPyV, was significantly lower than the decay observed in cultivation-based measurements of enterococci, *E. coli*, and *C. perfringens* in the sunlight-exposed microcosms. This is in agreement with other studies (30, 60). The decay rates of molecular markers in sunlight-exposed microcosms in this study appear to be lower than those in other studies (30, 57, 59, 60) that have examined similar but not identical source-specific markers. While the markers targeted were certainly different and contributed to this discrepancy between the studies, it is possible that a 6-h experiment is not reliable to determine the decay of molecular markers, as a longer period is needed for the onset of molecular damage that is measurable by quantitative PCR, especially when concentrations are determined based on short amplicons (61).

Predation and other natural stressors can play a significant role in the decay of FIB and molecular markers (32). It has been demonstrated that the persistence of enterococci is greatly extended in the absence of light and grazing in seawater (62). Higher decay rates of molecular markers are frequently observed in freshwater environments than those in marine environments (27, 30). This has been attributed to the decrease in grazing activity under less-favorable conditions (27), and it was likely the case in our study with dark microcosms, as indigenous flora and fauna were removed from those microcosms. We observed higher decay rates, although not to a statistically significant degree, of HF183TaqMan and enterococci in the dark freshwater microcosms than those in the dark saltwater microcosms. As natural biota was removed by filtration before this experiment, the freshwater-associated grazers present in the raw sewage added to the microcosms were likely able to thrive and contribute to the decay of enterococci and molecular markers in the dark freshwater microcosms.

We applied the concept of using HF183TaqMan and HPyV for the detection of human contamination in freshwater and seawater samples on Oahu, HI. In Mānoa Stream, we detected HF183TaqMan in 83.3% and HPyV in 58.3% of the samples collected at site MS2 with predominantly residential land use, but no HPyV were detected at the upstream site MS1 in a nonurbanized area. Detection of HPyV, coupled with elevated *C. perfringens* concentrations in the residential section, indicates possible sewage-related impairment in the middle section of Mānoa Stream. The detection of HF183TaqMan in three samples (25%) collected from the upstream site was likely due to cross-reactivity with local wildlife, as no human source should exist in this area, and HPyV was not detected. Our coastal sampling demonstrated that higher concentrations of enterococci can be detected in nonurbanized coastal water environments in Hawaii, such as Kahana Bay. While high concentrations of enterococci in Keehi Lagoon were linked to human sources through HF183TaqMan, high enterococcal levels in Kahana Bay, which exceeded those found in Keehi Lagoon, are likely not from human sources. Although we did not test for animal-associated MST markers in Kahana Bay, the most likely source of enterococci in the bay is the stormwater runoff and resuspension of soil and sediment. Although larger sample volumes and better concentration techniques may be helpful, the scope of our analyses of coastal waters demonstrates that these human-associated markers can be detected and utilized in Hawaii.

A determination of ambient indicator and human marker levels should be made before sewage spills occur so the impact of the spill and related health risk can be better determined. While we did not detect HPyV at either coastal site, this marker was detected in Mānoa Stream, where it provided strong evidence of sewage-related contamination. HF183TaqMan was detected at both sites in Mānoa Stream. Hence, the application of these two complementary molecular assays (HF183TaqMan and HPyV) simultaneously appears to be well suited in situations where sewage contamination originates from a spill event or ambient contamination source (leaking sewer lines or cesspool) near the site. In those situations, molecular marker concentrations can be linked to likelihood of disease transmission based on the known concentrations of markers and FIB in raw sewage. However, interpretation of the results might be more complicated when contamination is from treated sewage, as treatment processes, such as UV disinfection, can reduce FIB concentrations and, presumably, viable pathogen levels to a greater degree than it can molecular markers.

In summary, this study recommends parallel use of the HF183TaqMan and HPyV markers to determine whether water is contaminated by sewage. Human sewage is the primary source of these two molecular markers in Hawaii, and these markers have far fewer nonsewage sources than FIB (*E. coli*, enterococci, and *C. perfringens*). In contrast to FIB, *Bacteroides* spp. (HF183TaqMan) are unlikely to multiply under extraenteric environmental conditions, and it is impossible for the HPyV to do so. The molecular MST markers are more stable under various environmental conditions, including primary treatments, secondary treatments, and disinfection of sewage, than are cultivable FIB. Therefore, these two molecular markers are more conservative and more specific markers of sewage than are FIB. Of these two markers, the polyomavirus marker (HPyV) is more specific but less sensitive to sewage contamination, because the concentrations of HPyV in sewage are relatively low compared to FIB or HF183TaqMan. Although the cross-reaction of the HF183TaqMan marker with several animal species feces (dogs, mongooses, and cats) was determined, it may not be a serious limitation, because the ratio of HF183TaqMan to enterococci in human feces was about 1,000-fold greater than the ratio in animal feces. Therefore, our results support the application of HF183TaqMan for detecting sewage contamination in Hawaiian environmental waters even when animal fecal contamination is suspected, with the caveat that one or more human-specific markers should be used to support findings of human sewage contamination.

This study also recommends the use of the HF183TaqMan marker or its newer derivatives, such as HF183/BacR287 (49), in epidemiological studies, so that measured health risks to swimmers can be correlated to concentrations of this marker in recreational water samples. Parallel application of more-human-specific viral markers, such as HPyV, is also warranted in epidemiological studies, although better concentration techniques are needed. If successful, the results of such epidemiological studies can be used to develop new recreational water quality criteria based on measurements of a sufficiently sensitive and specific molecular marker of sewage, which will provide direct information on the absence or presence of sewage contamination in recreational water samples as well as a reliable measurement for risk of waterborne disease transmission to swimmers.

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