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Bacterial pathogens in Hawaiian coastal streams—Associations with fecal indicators, land cover, and water quality

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ARTICLE INFO

Article history:
Received 7 January 2011
Received in revised form
23 February 2011
Accepted 16 March 2011
Available online 12 April 2011

Keywords:
Salmonella
Campylobacter
Staphylococcus aureus
Vibrio
Fecal indicator
Tropical streams

ABSTRACT

This work aimed to understand the distribution of five bacterial pathogens in O'ahu coastal streams and relate their presence to microbial indicator concentrations, land cover of the surrounding watersheds, and physical–chemical measures of stream water quality. Twenty-two streams were sampled four times (in December and March, before sunrise and at high noon) to capture seasonal and time of day variation. *Salmonella*, *Campylobacter*, *Staphylococcus aureus*, *Vibrio vulnificus*, and *V. parahaemolyticus* were widespread —12 of 22 O'ahu streams had all five pathogens. All stream waters also had detectable concentrations of four fecal indicators and total vibrio with log mean ± standard deviation densities of 2.2 ± 0.8 enterococci, 2.7 ± 0.7 *Escherichia coli*, 1.1 ± 0.7 *Clostridium perfringens*, 1.2 ± 0.8 F^+ coliphages, and 3.6 ± 0.7 total vibrio per 100 ml. Bivariate associations between pathogens and indicators showed enterococci positively associated with the greatest number of bacterial pathogens. Higher concentrations of enterococci and higher incidence of *Campylobacter* were found in stream waters collected before sunrise, suggesting these organisms are sensitive to sunlight. Multivariate regression models of microbes as a function of land cover and physical–chemical water quality showed positive associations between *Salmonella* and agricultural and forested land covers, and between *S. aureus* and urban and agricultural land covers; these results suggested that sources specific to those land covers may contribute these pathogens to streams. Further, significant associations between some microbial targets and physical–chemical stream water quality (i.e., temperature, nutrients, turbidity) suggested that organism persistence may be affected by stream characteristics. Results implicate streams as a source of pathogens to coastal waters. Future work is recommended to determine infectious risks of recreational waterborne illness related to O'ahu stream exposures and to mitigate these risks through control of land-based runoff sources.

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doi:10.1016/j.watres.2011.03.033

1. Introduction

Each year exposures to marine waters contaminated by microbes cause an estimated 120 million gastrointestinal infections (GIs), 50 million acute respiratory infections (ARIs) (Shuval, 2003), and numerous skin infections (Yau et al., 2009). One source of microbial pollution to coastal waters is land-based runoff that discharges from rivers, streams and culverts to the nearshore. Runoff may contain pathogens from leaking sewage infrastructure, wild and domestic animal excreta, and other poorly understood environmental reservoirs such as soils and sands; in sum these are non-point sources of pollution (Boehm et al., 2009a).

Several epidemiology studies have investigated health effects from recreational exposure to land-based runoff and the results are equivocal. Haile et al. (1999) found increased risks of GI and ARI for swimmers recreating near storm drains at a southern Californian marine beach and a correlation between risk and fecal indicator bacteria (FIB) concentrations. In contrast, Calderon et al. (1991) found no statistically significant association between swimmers' illness risk and FIB in a freshwater pond contaminated by agricultural runoff. Dwight et al. (2004) found that Southern Californian surfers exposed to urban runoff had higher illness rates than Northern Californian surfers exposed to rural runoff. In these epidemiology studies, pathogen data were not readily available or limited and the exact source of fecal indicator organisms in the runoff was not known. Data on pathogens in runoff and insight into the factors that modulate their concentrations in runoff, would improve our ability to calculate and understand risks from exposure to terrestrial runoff.

Most research on fecal pollution and risks from recreational swimming has been conducted in temperate climates. Both US and WHO standards for recreational water quality were promulgated using data collected in temperate zones (Boehm et al., 2009a). This is despite the fact that US tropical beaches receive more visitors than all temperate beaches combined (Leeworthy and Wiley, 2001). Furthermore, numerous studies find enterococci and *Escherichia coli*, the indicators used to assess water quality around the globe, in tropical soils and streams (Hardina and Fujioka, 1991; Hazen, 1988); there is concern that the presence of these organisms in tropical recreational waters may not indicate contamination or presence of pathogens. To improve waterborne pathogen surveillance in the tropics, researchers suggest monitoring alternative indicators, like *Clostridium perfringens* and F+ coliphages (Fung et al., 2007; Luther and Fujioka, 2004). However, pathogen data are needed to corroborate whether or not an association exists between FIB, alternative indicators, and pathogens in the tropics.

In the present study, we measured human bacterial skin and GI pathogens in tropical coastal streams discharging to marine waters, and tested their association with traditional and alternative indicator organisms, surrounding land cover, and physical–chemical water quality. Specifically, we documented the occurrence or concentrations of *Salmonella*, *Campylobacter*, *Staphylococcus aureus*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* in 22 tropical streams of O'ahu, Hawai'i using a combination of culture-based and molecular methods. One goal was to test pathogen associations with indicators including *E. coli*, enterococci, *C. perfringens*, F+ coliphage, and total vibrio to determine which indicators have predictive power of bacterial pathogens. A second goal was to build multivariate statistical models to understand how land cover and physical–chemical stream parameters controlled pathogens and indicators. The modeling is premised on a conceptual model where bacterial concentrations in streams (1) increase due to microbial fluxes from the surrounding land; fluxes are affected by land cover, and (2) change in response to physical–chemical characteristics of the stream that affect organism persistence. The work presented here is unique in that it investigates the distribution of both GI and non-GI pathogens in a tropical climate, two research needs specifically mandated in the US BEACH Act of 2000.

2. Materials and methods

2.1. Sampling sites

Twenty-two streams were identified on O'ahu, Hawai'i for sampling (Table 1). Streams were selected because they discharge to coastal waters adjacent to popular swimming beaches. We also selected streams that drained watersheds with diverse land covers. In all cases, there were no known sewage point sources to the streams; all watersheds had separate storm and sewage conveyances. One site (Kūhiō) was a storm drain.

2.1.1. Land cover

Land cover was determined for stream watersheds using ArcGIS (ESRI, Redlands, CA) and Hawai'i Land Cover 2001 data (NOAA 2001). The data consist of Landsat Enhanced Thematic Mapper data at 30 m resolution for 18 land coverage classes. These classes were aggregated into four broad categories: urban, agriculture, forested, and other (unclassified, unconsolidated shore, water, and bare land). Watershed boundary data were obtained from the Hawai'i Statewide GIS program (Hawaii, 2010). The fraction of each watershed that was urban, agricultural, and forested was calculated by normalizing the areas, respectively, by the total watershed area.

2.1.2. Field sampling

Two water sampling campaigns were conducted at the 22 sites over five days in December 2009 (14–18 Dec 2009) and March 2010 (28 Mar–3 Apr 2010). Daily precipitation during and prior to the field campaigns was obtained from a centralized rain gauge on O'ahu (Moanalua USGS site 212359157502601). It should be noted that rainfall is spatially variable over the island, so data from this gauge represent approximate rainfall in the studied watersheds. The streams have very limited USGS stream gauge coverage, so flow data were not available.

During each campaign, the 22 sites were visited twice (once before the sun rose and once at high noon); in sum each stream was sampled four times. Twenty-liter water samples were collected in triple rinsed, 10% HCl-washed plastic containers. Water was sampled at an accessible location near the intersection of the stream and coastal ocean (Table 1).

Table 1 – O'ahu Stream Survey Site Description, Land Use. Hawai'i department of health water quality standards are provided where relevant. Stream values in exceedance of these standards are bolded.

Location			Watershed land cover			Ancillary Measurements							
Stream	Latitude/ Longitude of sample collection	Associated beach	FOR	URB	AG	TEMP (°C)	Salinity	DO (mg/l)	CHL*a* (μg/L)[c]	TURB (NTU)[b,c]	NO_2+NO_3 (μg-N/L)[c]	DIN[d] (μg-N/L)[c]	PO_4 (μg-P/L)[c]
Ala Wai[a]	21.288°N, 157.839°W	Ala Moana	18%	79%	0%	24.0	25.2	5.3	2.1	2.3	**319**	388	16
Kūhiō Storm Drain[a]	21.271°N, 157.824° W	Kūhiō	18%	79%	0%	24.5	33.2	6.5	0.2	1.2	52	72	8.1
Kapakahi	21.270° N, 157.778°W	Wai'alae	54%	45%	0%	23.7	30.6	4.5	1.6	10	22	141	11
Wai'alae Golf Course[a]	21.273°N, 157.771°W	Kahala	67%	31%	0%	24.1	23.6	7.1	1.3	15	47	92	14
Wailupe[a]	21.278°N, 157.750°W	Wailupe	67%	31%	0%	24.6	22.4	6.2	1.1	7.9	29	83	12
Moanalua	21.333°N, 157.894°W	Ke'ehi lagoon	79%	21%	0%	25.0	33.5	4.6	4.0	**7.7**	45	133	7.1
Kalihi	21.332°N, 157.891°W	Ke'ehi lagoon	72%	28%	0%	24.0	23.5	5.3	2.6	5.1	**76**	159	15
Mākua	21.530°N, 158.229°W	Mākua	97%	2%	0%	23.9	37.3	6.3	7.9	4.4	3.4	4.5	1.4
Kaupuni	21.448°N, 158.193°W	Poka'i Bay	83%	13%	2%	24.7	30.0	6.0	1.1	4.7	*186*	218	25
Mā'ili'ili[a]	21.429°N, 158.180°W	Ma'ili	78%	16%	2%	23.6	34.3	7.0	0.7	1.8	*117*	146	2.8
Mā'ili[a]	21.409°N, 158.177°W	Ma'ili	78%	16%	2%	24.6	30.1	6.5	0.6	3.3	*2128*	*2163*	4.2
Nānākuli	21.376°N, 158.140°W	Nānākuli	85%	13%	0%	23.5	20.9	5.0	6.1	6.7	5.9	55	7.2
Mālaekahana	21.673°N, 157.936°W	Māleakahana	90%	3%	6%	23.7	0.6	3.5	0.5	5.7	**5011**	*5143*	7.6
Waimea	21.641°N, 158.063°W	Waimea	98%	0%	2%	23.6	7.8	6.4	1.4	3.5	32	87	4.0
Anahulu	21.594°N, 158.103°W	Hale'iwa	80%	3%	17%	23.0	14.9	6.6	0.5	1.8	**1310**	*1339*	88
Paukauila	21.580° N, 158.117° W	Kaiaka	38%	24%	37%	23.1	11.0	4.7	3.6	5.4	**1142**	*1250*	39
Kiikii	21.579° N, 158.120° W	Kaiaka	27%	29%	42%	22.8	10.2	7.9	7.4	6.7	350	358	4.8
Waimānalo	21.365° N, 157.709° W	Waimānolo	81%	14%	3%	24.4	17.2	5.0	1.8	3.9	**809**	922	33
Ka'elepulu	21.398° N, 157.726° W	Kailua	47%	52%	0%	24.3	17.5	6.9	3.3	1.8	8.6	34	2.3
Kawainui	21.426° N, 157.741° W	Kailua	72%	20%	0%	24.5	11.7	5.3	2.2	2.8	10	22	14
Kahana	21.556° N, 157.869° W	Kahana	98%	0%	0%	22.1	4.8	5.3	0.3	1.9	26	52	5.6
Punalu'u	21.579° N, 157.885° W	Punalu'u	95%	1%	4%	21.7	1.2	7.4	0.3	1.8	33	49	9.7
All Stream Average						23.8	20.1	5.9	1.5	3.9	26	41	9.4
Hawai'i DOH Water Quality Standards[e]						–	–	>5	–	<5	<70	<250[f]	<50[f]

a Ala wai/Kūhiō, Mā'ili/Mā'ili'ili and Wai'alae Golf Course/Wailupe have shared watersheds.
b NTU = Nephelometric Turbidity Units.
c Values represent geometric mean of $n = 4$.
d DIN = dissolved organic nitrogen, which is the sum of NO_2^-, NO_3^-, and NH_4^+.
e Hawai'ian Department of Health (2001) water quality standard during rainy season for geometric mean.
f Standards for total nitrogen and phosphorus—note that stream values do not include organic nitrogen or phosphorus so are not directly comparable.

Field sampling controls were taken every other day by performing sampling procedures with distilled water—field blanks were assayed concurrently with water samples to confirm sterility of sampling procedures for all microbial assays.

At the stream, salinity and temperature were measured using a sensor (YSI85, YSI Inc., Yellow Springs, OH), dissolved oxygen (mg/l) was measured with an optical probe (ProODO, YSI Inc.), and turbidity was measured using a benchtop turbidity meter (HF Scientific DRT-15CE, Fort Myers, FL).

2.2. *Laboratory analyses*

Water samples were stored on ice during transport to the laboratory and processed within 6–8 h of collection in accordance with EPA and standard methods for water sampling (AWWA 2005, USEPA 2006).

2.2.1. *Fecal indicator and total vibrio enumeration*

Samples were assayed for both traditional and alternative indicators, including *E. coli*, enterococci, *C. perfringens*, F+ coliphages, and total vibrio. In all cases, membrane filtration was used; water was filtered through 0.45 μm pore-size mixed cellulose ester filters (S-Pak Type HA, Millipore, Billerica, MA). Filtration blanks were run during each day of sampling for all assays and in all cases indicated no cross contamination.

E. coli were enumerated in 1 ml, 10 ml, and 100 ml volumes with MI media (Difco Laboratories Inc, Detroit, MI), and incubated at 35 °C for 24 h according to EPA method 1604 (lowest detectable concentration (LDC) = 1 colony forming unit (CFU)/100 ml) (USEPA 2002). The same volumes were used to quantify enterococci by EPA Method 1600 with mEI agar (EMD Chemicals, Gibbstown, NJ) incubated at 41 °C for 24 h (USEPA 2006) (LDC = 1 CFU/100 ml). For *C. perfringens*, the Hawai'i Department of Health procedure was used for detection in 10 ml, 100 ml, and 500 ml water samples with mCP agar (see supporting materials (SM)) (LDC = 0.2 CFU/100 ml). Total vibrio were assayed in 0.1 ml, 1 ml and 10 ml volumes with TCBS agar (EMD) following Hsieh et al. (2008). Filters on TCBS were incubated for 24 h at 35 °C and yellow/green colonies were counted as total vibrio (LDC = 10 CFU/100 ml).

F+ coliphages were enumerated following Boehm et al. (2009b). $MgCl_2$ was added to a final concentration of 0.05 M in the water sample (Victoria et al. 2009) and 10 ml, 100 ml, and 500 ml were membrane filtered. Filters were placed on 0.3 ml of sterile 50% 1× PBS/50% glycerol and frozen at −80 °C. Samples were assayed by a modified double agar layer method (USEPA 2001a) as described in the SM (LDC = 0.2 plaque forming units (PFU)/100 ml).

2.2.2. *Culture-dependent pathogen detection*

In each sample, the presence/absence of *Salmonella*, *Campylobacter*, and *S. aureus* was measured while *Vibrio vulnificus* and *V. parahaemolyticus* were quantified. The quantification of *Salmonella*, *Campylobacter*, and *S. aureus* was not possible due to finite time and staffing available during the field work. Presumptive isolates were confirmed with molecular methods using the respective primers and probes listed in Table 2—only pathogen isolates positive by both culture- and PCR-based confirmation methods are reported (quantification cycle < 35 were considered positive). Further details of pathogen assays are provided in the SM.

Salmonella (SAL) presence/absence from 1 L water samples (LDC = 1 most probable number (MPN)/L) was determined following Shellenbarger et al. (2008). Presumptive positives were confirmed using Salmonella-genus-specific PCR (Table 2).

Campylobacter (CAMPY) presence/absence was determined for 1 L of water with a two-step enrichment procedure modified from Khan and Edge (2007) (LDC = 1 MPN/L). Presumptive CAMPY isolates (two isolates for each positive sampling event) were first confirmed with Campylobacter genus-specific PCR followed by further typing of isolates as *C. jejuni* or *C. coli* using endpoint Taqman PCR (Table 2).

S. aureus (STAPH) detection followed Goodwin and Pobuda (2009) (LDC = 1.2 CFU/100 ml). Water volumes up to 80 ml were filtered and filters were placed on CHROMagar™ Staph aureus (BD, Franklin Lakes, New Jersey) at 37 °C for 24 h. Putative STAPH were restreaked to isolation to confirm morphology followed by *S. aureus*-specific PCR (Table 2).

V. vulnificus (VVUL) and *V. parahaemolyticus* (VPARA) were quantified by filtering 0.1 ml, 1 ml, and 10 ml volumes and placing filters on CHROMagar™ Vibrio (BD). Plates were incubated at 40 °C for 24 h (LDC = 10 CFU/100 ml) (further details can be found in SM). Presumptive isolates were confirmed by endpoint Taqman PCR (Table 2).

2.2.3. *Nutrients and chlorophyll a*

For nutrients analyses, 30 ml of water were filtered through 0.2 μm pore-size PES syringe filters (Millipore) and stored at −20 °C. Phosphate, nitrate, nitrite, and ammonium were measured by standard methods with a nutrient autoanalyzer (Lachat QuikChem 8000, Loveland, CO). Lowest detectable concentrations were 0.5 μg/L PO_4^{3-}-P/L, 2.8 μg NO_3^--N/L, 1.4 μg NO_2^--N/L, and 1.4 μg NH_4^+-N/L.

To measure chlorophyll *a*, water was filtered in the dark through GF/F filters until pale green was seen (60–240 ml) and immediately frozen. Chlorophyll *a* was determined following EPA method 445 (USEPA 1997).

2.3. *Statistical analyses*

Statistical analyses were carried out using Minitab 15.0 or SPSS 18.0. For continuous microorganism data, non-detects were substituted with lowest detectable concentrations (Harwood et al., 2005) and raw/transformed data (e.g., $\log_{10}$, square-root) were evaluated for normality with quantile–quantile plots. Bivariate pairwise associations between bacterial pathogens were assessed using contingency tables, one-way analyses of variance (ANOVA), and Pearson's correlation coefficient (r_p). The same methods were used to investigate time of sampling effects. Bivariate associations between pathogens and microbial indicators were tested using generalized estimating equations (GEEs), with binomial logistic regression models for dichotomous dependent variables and linear regression models for continuous variables. Multivariate GEE models were developed for microorganisms as a function of land cover and physical–chemical water quality

Table 2 – PCR primers and probes used in the study.

Target Group (gene)	Primer and probe sequences (5′-3′)	Size (bp)	Reference
Salmonella (*invA* gene)	F-primer: GTGAAATTATCGCCACGTTCGGGCAA R-primer: TCATCGCACCGTCAAAGGAACC	284	(Malorny et al., 2003)
Campylobacter (16S rRNA)	F-primer: CTAGAGTACAAACTAATAAGTCTC R-primer: ATTCTAAAACGCATCACTTCCTTG	650–800	(Khan and Edge, 2007)
Campylobacter jejuni (*hipO*)	F-primer: TGCTAGTGAGGTTGCAAAAGAATT R-primer: TCATTTCGCAAAAAAATCCAAA Probe: FAM-ACGATGATTAAATTCACAATTTTTTTCGCCAAA-BHQ	100	(LaGier et al., 2004)
Campylobacter coli (*glyA*)	F-primer: CATATTGTAAAACCAAAGCTTATCGG R-primer: AGTCCAGCAATGTGTGCAATG Probe: FAM-TAAGCTCCAACTTCATCCGCAATCTCTCTAAATTT-BHQ	133	(LaGier et al., 2004)
Staphylococcus aureus (*clfA*)	F-primer: GCAAAATCCAGCACAACAGGAAACGA R-primer: CTTGATCTCCAGCCATAATTGGTGG	638	(Mason et al., 2001)
Vibrio vulnificus (*vvhA/B*)	F-primer: TGCCT(AG)GATGTTTATGGTGAGAAC R-primer: TCGACTGTGAGCGTTTTGTC Probe: FAM-TAGCCGAGT(AG)GCATCCGATCGTTGTT-BHQ	179	(Wetz et al., 2008)
Vibrio paraheomolyticus (*gyrB*)	F-primer: TGAAGGTTTGACTGCCGTTGT R-primer: TGGGTTTTCGACCAAGAACTCA Probe: FAM-TTCTCACCCATCGCCGATTCAACCGC-BHQ	148	(Cai et al., 2006)

characteristics. GEEs were necessary because observations at streams represented repeated measurements and this technique accounts for data clustering amongst sites (Hardin and Hilbe, 2003; Walters et al., 2011). Statistics were deemed significant if $P \leq 0.05$; some marginally significant associations ($0.05 < P \leq 0.1$) were also discussed.

3. Results

Two sampling campaigns were designed to capture broad seasonal (December, March) and time of day (before sunrise-AM, high noon-PM) differences in stream water quality during the Hawai'i rainy season (Nov 1–Apr 30). Average rainfall during sample collection days was 0.1 cm/d in December and 0.3 cm/d in March, and was lower than the 1.5 cm/d overall average rainfall during the 2009–2010 rainy season. Thus, conditions were relatively dry.

All data were evaluated for normality and transformed if necessary. Raw data were used for salinity, temperature (TEMP), and dissolved oxygen in mg/l (DO). Log_{10}-transformations were employed for all microorganisms, turbidity (TURB), chlorophyll *a* (CHL*a*), and nutrients (PO_4^{3-}, NO_2^-, NO_3^-). Ammonium (NH_4^+), forested (FOR), urban (URB), and agricultural (AG) land cover fractions were normalized by square-root transformations.

3.1. Land cover and physical–chemical properties of streams

Land cover within the watersheds was largely tropical forest cover (FOR), but 18 of 22 streams had between 1% and 79% urban land cover (URB, Table 1). Agricultural land cover (AG) was present in 10 of 22 stream watersheds ranging from 2 to 42% of the total watershed area. URB and FOR land covers were strongly, negatively correlated ($r_p = -0.918$, $P < 0.001$), so URB is used hereafter to represent both variables.

Mean stream salinity, TEMP, TURB, CHL*a*, and nutrient concentrations are reported in Table 1. Salinity ranged from 0.6 to 34.3, illustrating that streams spanned a range of marine influence. DO in 4 of 22 streams was below the state rainy season standard of 5 mg/l. Numerous streams exceeded water quality criteria for TURB and nutrients. Only TEMP and DO varied significantly between AM and PM samples (PM samples had higher TEMP by 2.8 °C and DO by 1.5 mg/L, ANOVA, $P \leq 0.001$). Other physico-chemical parameters showed no AM/PM fluctuations but varied significantly between streams (ANOVA, $P = 0.001$). PO_4^{3-} was the only parameter that differed significantly between DEC/MAR (ANOVA, $P = 0.011$) with PO_4^{3-} higher in MAR by 1.8 μg P/L relative to DEC. All collected water quality data are provided in Table S1.

TEMP, salinity, and DO were significantly correlated ($r_p = 0.32$ for DO-TEMP *and* salinity-TEMP, $P < 0.005$), so TEMP was used to represent all three variables in multivariate models. Nutrient concentrations were positively correlated ($0.40 < r_p < 0.95$, $P < 0.001$), so PO_4^{3-} was used to represent nutrients. Additionally, TURB and CHL*a* were correlated ($r_p = 0.35$, $P = 0.001$) so TURB was used to represent both parameters.

3.2. Pathogens

The presence of at least one pathogen, including *Salmonella* (SAL), *Campylobacter*, (CAMPY), and/or *S. aureus* (STAPH) was detected in 21 of the 22 streams (Fig. 1). *V. vulnificus* (VVUL) and *V. parahaemolyticus* (VPARA) were also widespread (Table S2).

SAL was present in 15 of 22 streams (Fig. 1a) and was detected in two or more samples in 8/22 streams. There were no significant AM/PM or DEC/MAR differences in SAL occurrence ($\chi 2$ test, $P > 0.05$).

CAMPY were present in 18 of 22 streams (Fig. 1b). While no DEC/MAR differences were found in CAMPY ($\chi 2$ test, $P > 0.05$), CAMPY was detected at a significantly higher frequency in AM compared to PM samples ($P < 0.001$). Confirmed CAMPY isolates from each of 25 samples were further typed to distinguish *C. jejuni* (CJEJ) and *C. coli* (CCOLI). In all but two samples, the confirmed CAMPY isolates were typed as the same species. In four of the 25 samples CCOLI were detected, in 7 CJEJ was detected, in 2 both CJEJ and an unknown CAMPY species were detected, and in the remaining 12 samples unknown CAMPY species were detected. In summary, 4 streams had more than one type of CAMPY isolated either in a single or multiple samples, while CAMPY isolated from 5 and 2 streams, respectively, were identified as CJEJ and CCOLI exclusively (Fig. 1b).

STAPH was present in all but three streams and detected in 14/22 streams at least twice (Fig. 1c). STAPH was detected at a higher frequency in MAR than DEC ($\chi 2$ test, $P = 0.001$) with a maximum detection in 31% of the samples in DEC versus 68% in MAR; no differences were observed between AM/PM ($P > 0.05$). During DEC sampling, all samples were also tested for methicillin resistant *S. aureus* (MRSA) (methods in SM) but only one stream, Mā'ili'ili, had a positive isolate so further analyses were not undertaken in MAR.

Concentrations of VVUL and VPARA averaged 2.3 ± 0.9 and 2.4 ± 0.7 log CFU/100 ml, respectively. Reported densities include both pathogenic and non-pathogenic subspecies since PCRs did not target specific VVUL and VPARA virulence genes. No variations were noted between AM/PM or DEC/MAR samples but differences between streams were significant for both pathogens (Table S2, $P < 0.05$).

Twelve of 22 streams were positive for SAL, STAPH, CAMPY, VVUL, and VPARA, but there were only a few significant associations between these organisms. SAL was detected at a higher frequency when STAPH was present ($\chi 2$ test, $P = 0.012$). VVUL was higher when SAL was present (ANOVA, $P = 0.012$) and when CAMPY was present (ANOVA, $P = 0.017$). VVUL concentrations were significantly correlated to VPARA ($r_p = 0.42$, $P < 0.001$).

3.3. Indicators

E. coli (EC), *Enterococcus* (ENT), *C. perfringens* (CPERF), and F+ coliphages (F + PHAGE) as well as total vibrio (TOTVIB) were detected in all streams (Fig. 2). There were no significant differences in EC, CPERF, or TOTVIB between AM/PM or MAR/DEC samples (ANOVA, $P > 0.05$). Seasonal fluctuations were significant for ENT ($P = 0.048$) and F + PHAGE ($P = 0.001$), with MAR densities being lower than DEC by 0.25–0.5 log CFU/PFU per 100 ml. ENT concentrations also showed time of day

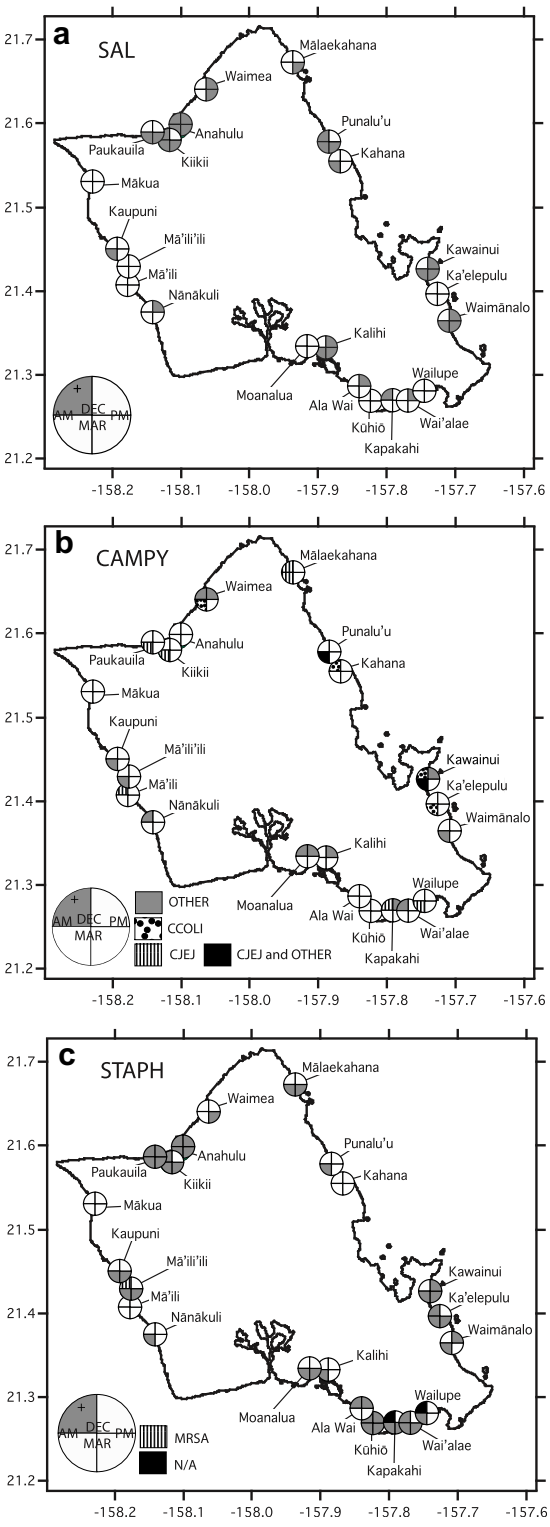

Fig. 1 – Presence/absence of a) *Salmonella*, b) *Campylobacter*, and c) *Staphylococcus aureus* in 22 O'ahu coastal streams (shaded = present, white = absent) by time of day (AM/PM) and season (DEC/MAR). Circles denote DEC with top half and MAR samples with bottom half. AM presence is on the left and PM presence is on the right. For CAMPY (1b), positive *C. jejuni* are lines, positive *C. coli* are dots, positive *C. jejuni* and other CAMPY are black, while other CAMPY are shaded. For STAPH (1c), positive MRSA in March is indicated by stripes while STAPH analyses not completed are black in that part of the circle.

differences ($P = 0.001$) with AM values averaging 0.5-log higher than PM values.

3.4. *Pathogen associations with indicators*

Each pathogen tested (except STAPH and CCOLI) showed a positive, significant association ($P \leq 0.05$) to at least one indicator based on GEE models (Table 3). SAL, CAMPY, and *CJEJ* were positively associated with ENT concentrations—ENT was 0.6–1 log higher when one of these pathogens was present compared to when it was absent (ANOVA, $P < 0.05$). ENT was marginally positively associated with STAPH ($P < 0.1$). CAMPY was marginally positively associated with CPERF – CPERF was 0.3 log unit higher when CAMPY was detected (ANOVA, $P = 0.09$). SAL was also marginally associated with CPERF ($P < 0.1$). VVUL was positively associated with all indicators tested, while VPARA was positively associated with TOTVIB, CPERF, and EC.

3.5. *Multivariate models of microorganisms*

Multivariate models of microorganisms were created using GEE models (Table 4, Fig. S1). The land cover variables URB, AG and physico-chemical characteristics TEMP, TURB, and PO_4^{3-} were included as independent variables.

SAL occurrence was marginally associated with AG, URB, TEMP, and PO_4^{3-} ($P < 0.10$)—associations were positive for all variables except URB. CAMPY occurrence was negatively associated with TEMP ($P < 0.001$) and marginally negatively associated with AG ($P = 0.1$). STAPH was positively associated with both AG and URB ($P < 0.05$). No significant water quality or land use associations were found for VVUL while VPARA was marginally positively associated with URB and TURB ($P < 0.1$).

For indicators, ENT, TOTVIB, and F + PHAGE were significantly associated with more than one independent variable. ENT had a positive association with TURB and a negative association with TEMP ($P < 0.05$). While only of marginal significance ($P < 0.1$), ENT was also positively associated to both AG and PO_4^{3-}. TOTVIB showed a significant negative relationship to AG and positive relationship to TEMP ($P < 0.05$). F + PHAGE was negatively associated with AG ($P < 0.05$) and TEMP (P=0.1), and positively associated with PO_4^{3-} ($P < 0.1$). One stream parameter was significant for each of the other indicators—EC was positively related to PO_4^{3-} ($P < 0.05$) and CPERF was positively associated with TURB ($P < 0.05$).

3.6. *Comparison between ENT and CPERF as pollution indices*

Some researchers suggest that ENT found in pristine tropical soils interfere with their ability to predict pollution from fecal sources (Boehm et al., 2009a). Fujioka and colleagues suggest that CPERF may be a better indicator of fecal pollution than ENT for tropical waters and that CPERF concentrations can discern pollution sources based on a "Fung/Fujioka scale" of pollution (Fung et al., 2007). According to this scale, when CPERF is greater than 100 CFU/100 ml, sewage is the pollution source. When CPERF is between 10 and 100 CFU/100 ml, non-point pollution is the source. Finally, waters are considered

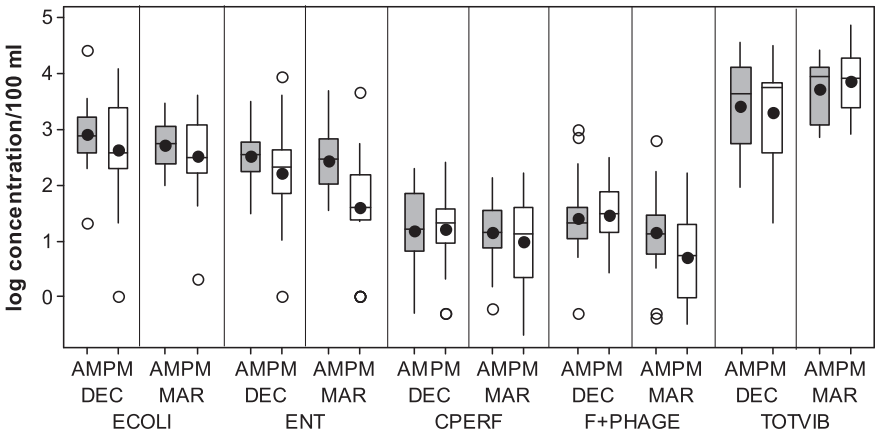

Fig. 2 – Log fecal indicator and total vibrio concentrations in 22 O'ahu coastal streams by time of day (AM/PM) and season (DEC/MAR)—indicators include *E. coli* (CFU), enterococci (CFU), *C. perfringens* (CFU), F+ coliphages (PFU), and total vibrio (CFU). Box- and-whisker plots represent the median (inner box line), 25th and 75th percentiles (lower and upper outer box lines), whiskers cover 10th and 90th percentiles and data outliers are represented by open circles. Geometric means are indicated with a black circle ($n = 22$).

uncontaminated when CPERF is present at less than 10 CFU/100 ml. We plotted CPERF versus ENT (Fig. 3) to gain insight on how the CPERF pollution scale compares to the USEPA ENT standard of 104 CFU/100 ml. Using the Fung/Fujioka scale, 53 of 88 samples indicated non-point source pollution while 6 samples indicated sewage pollution. Forty-four of these 59 "contaminated" samples (e.g. CPERF≥10 CFU/100 ml) also exceeded the ENT standard. Agreement between the Fung/Fujioka scale for contamination and single-sample exceedance for ENT, as well as the pairwise correlation between ENT and CPERF ($r_p = 0.50$, $P < 0.05$) suggests that similar information can be obtained from ENT as CPERF in these tropical streams.

4. Discussion

4.1. Bacterial pathogens

Salmonella, *Campylobacter*, *S. aureus*, *V. vulnificus*, and *V. parahaemolyticus*, are implicated in recreational water and shellfish outbreaks (USEPA 2009b). In this study, multiple isolations of these organisms in a given stream were frequent (Fig. 1, Table S2). STAPH and *Vibrio* spp. were most commonly detected (STAPH was present in 19/22 streams, while *Vibrio* spp. were detected in all streams) followed by CAMPY (18/22) and SAL (15/22); all pathogens were isolated from 12/22 streams.

Salmonella, a leading cause of gastroenteritis in the US (USEPA 2009b), are frequently isolated from surface waters (Haley et al., 2009; Walters et al., 2011; Wilkes et al., 2009). We found SAL in the majority of O'ahu streams at concentrations greater than 1 MPN/1 L. SAL occurrence was positively associated with higher temperature (thus higher DO and salinity as well), nutrients, and AG; while negatively associated with URB (Table 4). Relationships between water quality and SAL may imply increased *Salmonella* persistence in warm, eutrophic, relatively saline water. Previously, *Salmonella* in microcosms showed decreased persistence in warm relative to cool waters and no real trends in persistence in variable saline waters (Evison, 1988; Wait and Sobsey, 2001), while Evison (1988) reported increased *Salmonella* persistence in waters

Table 3 – Bivariate pathogen–indicator relationships. GEE logistic regression (logit function) was used for dichotomous variables while GEE linear regression was applied for continuous variables. β is the parameter coefficient; P-value is provided.

Microorganism	Enterococci		*E. coli*		*C. perfringens*		F+ coliphage		Total Vibrio	
	β	*P*-value	β	*P*-value	β	*P*-value	β	*P*-value	β	*P*-value
Salmonella	1.09	0.012[a]	−0.02	0.948	0.609	0.095[b]	0.321	0.187	−0.06	0.828
Campylobacter	1.12	<0.001[a]	0.19	0.458	0.64	0.030[a]	0.60	0.075[b]	0.15	0.688
C. jejuni	2.23	<0.001[a]	0.27	0.354	0.88	0.130	0.66	0.124	−0.08	0.877
C. coli	0.10	0.791	0.45	0.514	0.50	0.275	0.12	0.761	−0.16	0.743
S. aureus	0.49	0.097[b]	0.35	0.245	−0.09	0.804	−0.35	0.119	0.35	0.315
V. vulnificus	0.43	0.001[a]	0.24	0.037[a]	0.49	<0.001[a]	0.26	0.019[a]	0.32	0.026[a]
V. parahaemolyticus	0.16	0.193	0.26	0.004[a]	0.32	0.003[a]	0.18	0.122	0.41	<0.001[a]

a $P \leq 0.05$.
b $0.05 < P < 0.1$.

Table 4 – Pathogen/indicator multivariate models with stream physico-chemical water quality parameters and watershed land use.

Pathogen	GEE Model			Indicator	GEE Model		
	Parameter	Coefficient	P-value		Parameter	Coefficient	P-value
Salmonella	Intercept	−7.727	0.024[b]	Enterococci	Intercept	5.427	<0.001[b]
QICC = 102.097[a]	AG	3.044	0.084[c]	QICC = 52.416	AG	0.473	0.086[c]
	URB	−1.848	0.061[c]		URB	−0.188	0.479
	TEMP	0.249	0.076[c]		TEMP	−0.163	<0.001[b]
	TURB	−0.044	0.934		TURB	0.543	0.006[b]
	PO_4^{3-}	1.436	0.061[c]		PO_4^{3-}	0.338	0.087[c]
Campylobacter	Intercept	9.645	0.001[b]	*E. coli*	Intercept	3.105	0.003[b]
QICC = 106.064	AG	−1.464	0.100[c]	QICC = 52.573	AG	0.314	0.236
	URB	−0.707	0.506		URB	−0.087	0.739
	TEMP	−0.435	<0.001[b]		TEMP	−0.040	0.394
	TURB	0.397	0.572		TURB	0.109	0.566
	PO_4^{3-}	−0.114	0.843		PO_4^{3-}	0.450	0.004[b]
S. aureus	Intercept	−1.798	0.576	*C. perfringens*	Intercept	0.376	0.629
QICC = 112.386	AG	4.993	0.046[b]	QICC = 48.224	AG	−0.217	0.579
	URB	3.719	0.005[b]		URB	−0.246	0.491
	TEMP	−0.018	0.903		TEMP	0.006	0.832
	TURB	−0.311	0.704		TURB	0.904	<0.001[b]
	PO_4^{3-}	0.298	0.644		PO_4^{3-}	0.169	0.413
V. vulnificus	Intercept	2.38	0.051[c]	F+ coliphages	Intercept	2.498	0.003[b]
QICC = 78.53	AG	−0.059	0.905	QICC = 58.575	AG	−0.692	0.007[b]
	URB	−0.075	0.874		URB	−0.132	0.710
	TEMP	−0.016	0.750		TEMP	−0.066	0.100[c]
	TURB	−0.109	0.681		TURB	0.078	0.742
	PO_4^{3-}	0.404	0.171		PO_4^{3-}	0.322	0.075[c]
V. parahaemolyticus	Intercept	1.18	0.177	Total vibrio	Intercept	1.620	0.050[b]
QICC = 50.306	AG	−0.472	0.109	QICC = 48.826	AG	−0.951	0.014[b]
	URB	0.790	0.053[c]		URB	0.243	0.621
	TEMP	0.023	0.543		TEMP	0.072	0.041[b]
	TURB	0.348	0.084[c]		TURB	0.376	0.116
	PO_4^{3-}	0.245	0.132		PO_4^{3-}	0.035	0.810

a Corrected Quasi likelihood under Independence Model criterion—information criteria are in small-is-better form.
b $P \leq 0.05$.
c $0.05 < P < 0.1$.

with high nutrients. The positive AG and negative URB land cover associations suggest SAL sources related to agricultural activities (e.g. animal rearing, fertilizers) and within forested land covers (e.g. feral pigs, goats) may be important. This finding contrasts with Walters et al. (2011) who reported positive SAL associations with urban land cover in central California, but is consistent with frequent *Salmonella* isolations in studies of agricultural watersheds (Wilkes et al., 2009) and rural watersheds with known animal inputs (Haley et al., 2009).

C. jejuni and *C. coli* are the most important human pathogens of the 17 *Campylobacter* species (USEPA 2009b), and results indicate they were prevalent in O'ahu streams, with *C. jejuni* in 8/18 streams and *C. coli* in 4/18 streams. CAMPY incidence was greater in AM compared to PM samples and was negatively associated with TEMP (thus also DO and salinity). Increased CAMPY occurrence when DO, TEMP, and salinity were lower is consistent with CAMPY being sensitive to oxygenated conditions and having increased persistence in low temperature, low salinity waters (Buswell et al., 1998). CAMPY detection in low salinity waters may also be explained by CAMPY-rich freshwater runoff being diluted by marine water. CAMPY was largely isolated in AM samples suggesting sensitivity to sunlight. Sinton et al. (2007) found CAMPY were more sensitive to sunlight than SAL and EC. Finally, no correlations to land cover were found for CAMPY, but previous studies found that both human and animal sources contributed to surface water contamination (e.g. Vereen et al., 2007) so further work should determine sources of *Campylobacter* in O'ahu streams.

STAPH was ubiquitous in O'ahu streams. Frequent isolation of *S. aureus* in coastal waters around O'ahu (Charoenca and Fujioka, 1993) and other recreational waters is common (Yau et al., 2009). Charoenca and Fujioka (1993) suggest that beach-goer shedding is the source of STAPH to O'ahu coastal waters. Our results suggest that STAPH may also emanate from coastal streams. Presence of STAPH was associated with URB and AG land covers suggesting that sources within those land covers contribute STAPH to streams. Future work is needed to pinpoint STAPH sources in streams and determine whether environmental reservoirs exist. *S. aureus* infections are a serious problem in Hawai'i—the state leads the US in annual deaths from STAPH and MRSA infections (HHIC 2007) and an epidemiology study at Kūhiō Beach found *S. aureus* infections were four times more likely with marine water exposures (Charoenca and Fujioka, 1995).

Vibrio vulnificus and *V. parahaemolyticus* are leading causes of shellfish-related illness (Iwamoto et al., 2010) and the two

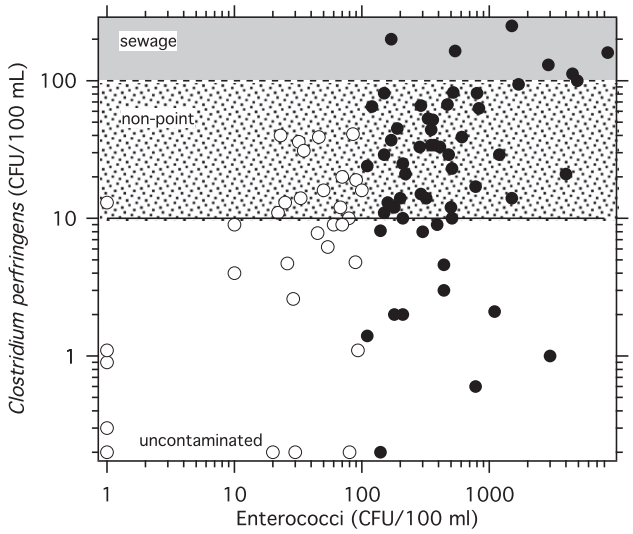

Fig. 3 – *C. perfringens* versus enterococci ($n = 88$). Black symbols indicate when enterococci were over 104 CFU/100ml, while white symbols indicate when under. Horizontal lines show 10 CFU and 100 CFU/100 ml of *C. perfringens*, which delineates non-point and sewage sources according to the Fung/Fujioka scale.

vibrio pathogens most frequently associated with US recreational water outbreaks (Dziuban et al., 2006)—this study found that both organisms were widespread in O'ahu streams. It should be noted that PCRs used to confirm *Vibrio* species did not target virulence genes, so reported densities likely over-represent pathogenic strains. *V. vulnificus* concentrations observed here were comparable to those measured in the Gulf of Mexico by Panicker et al. (2004) and during a tropical storm by Wetz et al. (2008) while *V. parahaemolyticus* levels were higher than those recorded by DePaola et al. (1990) in marine waters off the continental US.

Various studies (e.g. Hsieh et al., 2008; Johnson et al., 2010) have shown that temperature and salinity were most important in modulating both *V. vulnificus* and *V. parahaemolyticus* concentrations—optimal environments for both pathogens include warmer temperatures (15–30 °C) and mesosaline waters (5–25). No temperature or salinity associations were found here for either VVUL or VPARA. The lack of temperature correlation could be due to all streams being within optimal temperature ranges for vibrios. The lack of a linear salinity correlation could be due to the fact that mesosaline conditions are optimal (Johnson et al., 2010; Wetz et al., 2008). VPARA showed a marginal positive association with turbidity, consistent with Johnson et al. (2010) who found VPARA correlated to turbidity/chlorophyll *a* in the Gulf of Mexico. No land cover associations were found for VVUL, but VPARA were marginally positively related to URB land cover. Since vibrios are indigenous it is unlikely that this organism emanates from sources within URB land covers. Association does not prove causation, so the relationship between vibrios and URB land cover may arise due to an unmeasured variable which covaries with URB land cover. For example, perhaps vibrio growth or persistence is affected by DOC (not measured herein) which could be higher in streams draining URB land covers. Future work will need to further investigate this association.

4.2. *Pathogen–indicator relationships*

A goal of this study was to understand which indicators were best at predicting pathogens in tropical streams. Currently, the Hawai'i Department of Health (DOH) monitors both enterococci and *C. perfringens* in waters to dictate pollution advisories. F+ coliphages are also suggested as a more conservative indicator of sewage pollution in the tropics due to increased persistence compared to ENT and EC (Fujioka and Yoneyama, 2002; Luther and Fujioka, 2004).

No one indicator was associated with all pathogens, as has been reported previously in studies of temperate waters (Harwood et al., 2005; Wilkes et al., 2009). ENT correlated with SAL, CAMPY, CJEJ, and VVUL (and marginally with STAPH) while CPERF was associated with CAMPY, VVUL, and VPARA (and marginally with SAL) (Table 3). Strong relationships between ENT and CAMPY were reported previously in temperate stream waters (Wilkes et al., 2009) but the same study found EC was a better surrogate for SAL and parasites. In the current study, EC was only associated with VVUL. F+ coliphages were also significantly associated with VVUL while only marginally correlated with CAMPY ($P < 0.1$). TOTVIB was a good indicator of both VVUL and VPARA. Overall, ENT was the best surrogate for bacterial pathogens in tropical stream waters, but CPERF was also a good predicator. ENT and CPERF concentrations covaried and the 'Fung–Fujioka Scale' of pollution was consistent with the current USEPA enterococci marine water standards for monitoring fecal pollution. Further quantification of bacterial and viral pathogens in streams could improve associations found here and may also show that F+ coliphages are indicative of viral pathogens.

Multivariate analyses of indicators highlight a number of possible reasons for pathogen/indicator correlations in these tropical streams (Table 4). For ENT and SAL, both had marginal relationships to AG land cover and nutrients suggesting that both can derive from agricultural land sources. ENT and CAMPY associations may be due to their negative correlations to the temperature/salinity/DO water quality parameters and increased ENT concentration/CAMPY occurrence in samples taken prior to sunrise; these findings suggest that both organisms are light sensitive and/or persist under similar water conditions. Finally, relationships between CPERF and VPARA could be due to their concurrent associations with turbidity/chlorophyll *a*.

4.3. *Limitations and future work*

There are several limitations to this study. Associations between land cover and several microbial targets suggest that sources within specific land covers contribute microbes to streams. However, further work using host-specific source tracking markers is needed to fully understand pathogen sources in streams. Statistical associations between microbes and physical–chemical water quality variables were observed in this study. While these results are consistent with such variables affecting microbial persistence or fate in streams, laboratory experiments are needed to establish causal

relationships. Finally, it is important to note that this study was carried out during two field campaigns conducted under similar, relatively dry climatic conditions. Additional work should investigate the effects of rainfall and storm events on pathogen loadings in these coastal streams. In particular, the effect of stream discharge rate on microbial occurrence should be investigated. Such work may be best undertaken using temporally intensive sampling at a limited number of sites.

The present study established widespread distribution of bacterial pathogens in streams discharging to coastal waters, insinuating the potential for recreational waterborne illness upon exposure to stream runoff. In order to determine potential risks, a quantitative microbial risk assessment (QMRA) must be performed; this requires knowledge of pathogen concentration. Measurements of concentration were only possible with vibrio pathogens in the present study. Future work should quantify pathogen concentrations when feasible. Additionally, etiologies of recreational waterborne illness are believed to be predominantly viral (USEPA 2009a), so future work should assess human viruses concentrations in these streams. Finally, tropical streams are known to harbor *Leptospira interrogans*, the etiological agent of leptospirosis. Hawai'i has the highest rates of leptospirosis in the US (USEPA 2009b), thus future investigations into the distribution of this pathogen are of interest.

5. Conclusions

1. *Salmonella*, *Campylobacter* (including *C. jejuni*), *S. aureus*, *V. vulnificus*, and *V. parahaemolyticus* were widespread in O'ahu streams that discharge to coastal waters, indicating land-based activities and thus runoff as a source of pathogens.
2. Of the five indicators tested, enterococci were significantly associated with the most bacterial pathogens in O'ahu coastal streams.
3. Positive associations between specific land covers and *Salmonella* (AG, FOR), enterococci (AG), and *S. aureus* (AG, URB) suggest sources within these land covers contribute microbes to streams. Further microbial source tracking using, for example, molecular host-specific markers is needed to pinpoint specific microbial sources.
4. Significant associations between microbial targets and physical–chemical stream water quality (i.e., temperature, nutrients, turbidity) suggest that organism persistence is affected by stream water quality. Further work should investigate causal mechanisms.
5. *Campylobacter* incidence and enterococci concentrations were greater in samples collected before sunrise suggesting these organisms are sensitive to sunlight.

Acknowledgments

The authors are indebted to the field sampling team in Hawai'i, including Alissa Rogers, Patie Boehm, Ari Mesa, Mike Cooke, Mark Mench, and Shimi Rii. Two anonymous reviewers improved the manuscript. This work was funded by NSF Oceans and Human Health: Pacific Research Center for Marine Biomedicine Grant No. OCE/0910491.

Appendix. Supplementary material

The supplementary data associated with this article can be found in the on-line version at doi:10.1016/j.watres.2011.03.033.

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