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Dissolved Inorganic Nitrogen, Soluble Reactive Phosphorous, and Microbial Pollutant Loading from Tropical Rural Watersheds in Hawai'i to the Coastal Ocean During Non-Storm Conditions

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Abstract This study quantifies dissolved inorganic nitrogen (DIN), soluble reactive phosphorous (SRP), and microbial pollutant inputs to a tropical embayment, Hanalei Bay, Kaua'i, Hawai'i from rural watersheds during two field excursions during non-storm conditions. We employ land cover analysis and a suite of nucleic acid fecal source tracking markers (host-specific *Bacteroidales* and human enterovirus) to identify sources of pollutants to the bay. The highest concentrations of DIN and SRP are in streams draining watersheds with large areas of cultivated land, suggesting fertilizer is a source of these nutrients to the streams and coastal waters. Pollutant areal loading correlates with the fractions of urban and cultivated land cover. Microbial source tracking indicates the presence of human, pig, and ruminant feces in the streams. This work provides preliminary evidence that human development affects loading of DIN, SRP, and microbial pollutants to tropical coastal waters; further study is needed to confirm this. Additionally, results point to a mix of microbial pollutant sources.

Keywords Microbial pollution · Microbial source tracking · Tropical streams · Inorganic nitrogen · Phosphorous · Pollutant loading · Pollutant flux · *Bacteroidales* · Enterovirus · Hanalei Bay · Hawai'i · Rural tropical watersheds · Hawaii · Enterococci

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Introduction

Bacterial and nutrient pollution are two of the greatest threats to coastal waters (Pew oceans commission 2003). In 2008, there were over 20,300 days of closures or advisories at US beaches due to high levels of fecal indicator bacteria (FIB), up from 6,200 in 1999 (Dorfman and Rosselot 2009). Twenty percent of the Clean Water Act 303d listings for total maximum daily loads for US waters are for bacterial pollution and 18% are for nutrients (USEPA 2010). Bacterial and nutrient pollution endanger human and ecosystem health, respectively, and create economic losses for coastal communities by decreasing revenue and property values (Given et al. 2006; National Research Council 2000).

A major obstacle in the remediation of contaminated shorelines is a lack of knowledge about the sources of microbial and nutrient contamination. FIB, particularly enterococci (ENT) and *Escherichia coli* (EC), can be found in a number of fecal (Ashbolt et al. 2001; Harwood et al. 2000; Layton et al. 2010; Parveen et al. 1999) and non-fecal (e.g., in soil and on plants) (Hardina and Fujioka 1991; Yamahara et al. 2007; Yamahara et al. 2009) sources, many of which are diffuse or

non-point in nature. Recently, a number of microbial source tracking tools have been developed for use in watersheds (Field and Samadpour 2007; Stoeckel and Harwood 2007). *Bacteroidales* bacteria are an increasingly popular microbial source tracking tool and have been applied in a number of studies in temperate and subtropical regions (Boehm et al. 2003; Rosario et al. 2009; Santoro and Boehm 2007; Shanks et al. 2006), but only limited work with *Bacteroidales* has been conducted in the tropics (Betancourt and Fujioka 2006). *Bacteroidales* groups appear to be more source specific than other microbial source tracking tools (Gawler et al. 2007; Griffith et al. 2003).

To date, there are *Bacteroidales* DNA markers for human (Bernhard and Field 2000a), ruminant (Shanks et al. 2010), swine (Dick et al. 2005), and other feces such as dog and horse (Dick et al. 2005; Kildare et al. 2007). The bacteria that contain these source-specific DNA markers have not yet been cultivated, so the markers can only be measured using culture-independent methods such as polymerase chain reaction (PCR). Another microbial source tracking target is human virus. The presence of human viruses in coastal waters is strong evidence of a human contamination source and a health threat. Accordingly, enteroviruses—single-stranded, positive-sense, RNA viruses—have been used throughout the developed world to assist in microbial source tracking (Boehm et al. 2003; Boehm et al. 2009; Mocé-Llivina et al. 2005; Noble and Fuhrman 2001).

Nutrient sources to impacted waters can also be difficult to identify. Dissolved ammonium and nitrate inputs may be assimilated by flora and fauna or removed via coupled nitrification and denitrification. Dissolved soluble reactive phosphorous (SRP) may also be assimilated or removed abiotically via sorption to sediments. Excess nutrient inputs can overwhelm the assimilatory and removal capacity of a waterbody and lead to elevated concentrations and loading to receiving waters. The source of excess nitrogen can sometimes be discerned using stable isotopes of nitrogen (Cole et al. 2004; Kendall 1998; Umezawa et al. 2002); however, isotopic source signatures can be obscured by fractionation if nitrogen cycling occurs within the waterbody. An alternate approach for identifying sources is to link nutrient loading and concentrations to particular land cover and uses (Choi et al. 2007; Cole et al. 2006; Knee et al. 2010). The latter approach is indirect and is inferred from statistical associations.

According to the Hawai'i Department of Health's routine water quality sampling, beaches in Hanalei Bay, Kaua'i, Hawai'i next to the Hanalei River have some of the worst microbial water quality in the state. Forty-three percent of the water samples taken there in 2005 were in violation of bacterial water quality criteria (data not shown). Nutrient pollution is also a concern in the bay, particularly for coral reef health. Inputs of excess nitrogen and phosphorous to waters surrounding coral reefs can result in decreased coral cover due to increases in fleshy macroalgae (McCook 1999; Stimson et al. 2001).

The present study measures concentrations and loading of FIB, dissolved inorganic nitrogen (DIN), and SRP from each of the four major watersheds draining to Hanalei Bay, Kaua'i, Hawai'i during two snapshot, non-storm sampling events. We perform land cover analysis to gain insight into potential contaminant sources. Further, we apply a suite of *Bacteroidales* source tracking markers as well as the human enterovirus marker to identify likely sources of FIB contamination. We assess which of the watersheds contribute the highest loading of contaminants to Hanalei Bay during the conditions studied and identify potential point (lo'i ditches, culverts, animal enclosures, waste storage systems) and non-point (runoff, soils) sources (Table 1) for further study and/or remediation.

Materials and Methods

Study Site There are four main water bodies that discharge into Hanalei Bay: Hanalei River, Wai'oli stream, Waikoko stream, and Waipā stream (Fig. 1). These drain steep watersheds covered with tropical rain forests with small farms and urban areas underlain by basalt. There are numerous potential point and non-point sources of microbial pollutants and nutrients in each watershed. All local residences utilize on-site wastewater disposal, including both cesspools and septic systems. Agriculture is practiced widely. Taro, the most common crop, is grown in paddy fields or lo'i, where a constant flow of cool water is applied to the crop and then discharged into nearby streams via ditches. Lo'i are typically fertilized and attract waterfowl. Domesticated animals such as cows, goats, pigs, horses, and chickens are kept in enclosures adjacent to some of the cultivated lands, and feral pigs and goats roam forested portions of the watersheds. Finally, tropical soils are documented reservoirs of fecal indicator bacteria (Hardina and Fujioka 1991) where they are thought to proliferate due to the high moisture, nutrient content, and temperatures. These sources, partitioned into the types of land cover where they might be found, are summarized in Table 1.

Land Cover Analysis Land cover analysis was performed using ArcGIS (ESRI, Redlands, CA). The Hawai'i Land Cover 2001 data set (National Oceanographic and Atmospheric Administration Coastal Services Center; http://www.csc.noaa.gov/crs/lca/hawaii.html), consisting of analyzed, field-validated Landsat Enhanced Thematic Mapper data from 2001, was employed for land use analysis. The land cover data set was the most recent available at the time

Table 1 Potential point and non-point sources of fecal indicator bacteria in watersheds included in this study, separated by land cover

Cultivated		Urban		Forested	
Point	Non-point	Point	Non-point	Point	Non-point
Lo'i ditches	Runoff	Cesspools	Runoff		Wildlife
Animal enclosures	Wildlife	Septic systems	Soils		Soils
	Soils	Storm drains			

of our study. The data set has a 30-m resolution and divides land cover into 18 categories, which we aggregated into four groups: urban (including high- and low-intensity developed land), cultivated, forested (including all forest, grassland, and wetland classifications), and other (including unclassified, unconsolidated shore, water, and bare land). Watershed boundary data were obtained from the Hawai'i Statewide GIS Program (http://www.state.hi.us/dbedt/gis/); details of how boundaries were generated can be found in the meta-data provided by the State of Hawai'i Planning Office (http://www.state.hi.us/dbedt/gis/data/wshedpy_n83.txt).

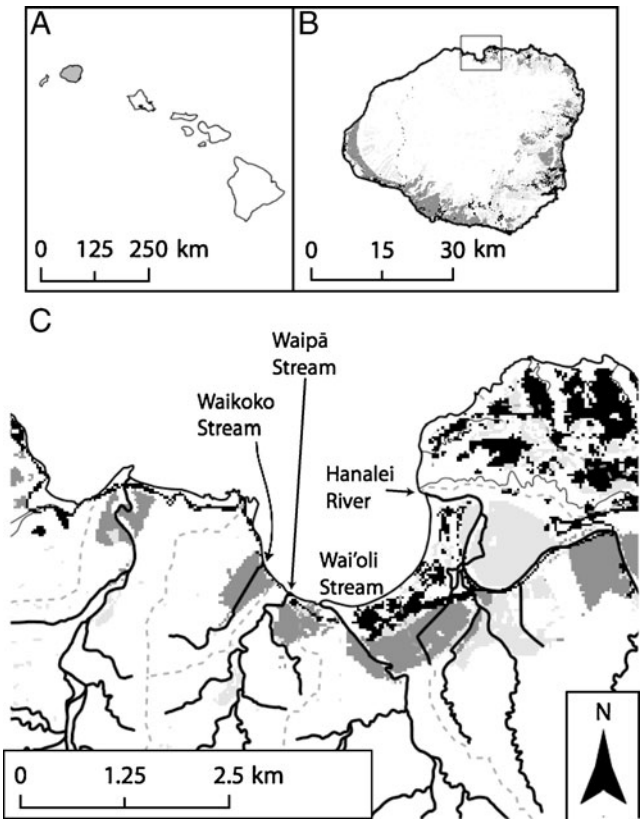

Fig. 1 Map of study sites, adapted with permission from Knee et al. (2008). **a** Hawaiian archipelago with the island of Kaua'i highlighted in *gray*. **b** Island of Kaua'i. *Shading* indicates land use, *black* for urban development, *dark gray* for agriculture, *light gray* for grassland, *white* for forested land and water. The *rectangle* indicates the study area, including northward-facing, semicircular Hanalei Bay. **c** Study area. Land use is the same as in panel **b**. Rivers and streams are indicated by *black lines* with the five rivers/streams included in the study in *bold lines*. *Dashed lines* indicate watershed boundaries. Land use data are from the National Oceanographic and Atmospheric Administration Coastal Services Center (www.csc.noaa.gov/crs/lca/hawaii.html); all other geographic data were obtained from the Hawai'i Statewide GIS program (http://www.state.hi.us/dbedt/gis)

Field Sampling Water collection was conducted in the morning and/or afternoon on 5 days in 2008 (26–30 March 2008) and 4 days in 2009 (14–18 March 2009) at the mouths of the Hanalei River (22°12.874′N, 159°29.813 W), Waipā stream (22°12.249′ N, 159°30.834 W), Wai'oli stream (22°12.170′N, 159°30.617 W), and Waikoko stream (22°12.428′ N, 159°31.010 W; Fig. 1). This resulted in the collection of seven and six samples from each water body in 2008 and 2009, respectively. During each sampling event, a single sample was collected at the mouth of the stream of water flowing into the ocean. This sample is representative of water draining into Hanalei Bay and also integrates loading from the entire watershed. With the exception of the Hanalei River, the streams were fairly shallow and less than 5 m in width, so a depth-integrated sample was collected by hand from the middle of the stream. At the Hanalei River, samples of flowing water were collected approximately 3 m from the river bank towards the midline of the river. The width of the river is approximately 10 m at this location. Care was taken to stand downstream of the sample collection site to avoid contamination.

In 2009, six samples were obtained from the culvert discharging into Wai'oli stream approximately 300 m from the mouth. The culvert runs the length of Hanalei town where it collects urban and storm runoff (Fig. 1). A depth-integrated sample was collected from the center of the flowing discharge just before it entered the Wai'oli stream.

One-liter water samples were collected in triple rinsed, 10% HCl-washed plastic containers. A water quality sensor (YSI 85, YSI Inc., Yellow Springs, OH, USA) was used to record salinity and temperature. In 2009 only, turbidity was measured in each water sample using a benchtop turbidity meter (HF Scientific DRT-15CE, Fort Myers, FL). A handheld flow meter (Flow Probe Model FP100, Global Water, Instrumentation, Inc., Gold River, CA) was used to obtain a depth- and cross-sectional average flow velocity during each sampling event at the storm drain and Wai'oli, Waikoko, and Waipā streams. The velocity was multiplied by the appropriate cross-sectional area to estimate volu-

metric flow rates. Daily average discharge measurements for the Hanalei River were obtained from a U.S. Geological Survey (USGS) flow gauge (http://waterdata.usgs.gov/, site 16103000). To characterize the rainfall during and prior to the study, daily rainfall was obtained from the USGS (http://waterdata.usgs.gov, site 221101159280801).

Fecal Indicator Bacteria Ten milliliters of water sample was added to 90 mL of Butterfield's buffer (Weber Scientific, Hamilton, NJ), to which Enterolert (to enumerate ENT) or Colilert-18 (to enumerate EC) reagent was added (IDEXX, Westbrook, MN). These mixtures were poured into QuantiTray/2000 (IDEXX, Westbrook, MN) and processed following the manufacturer's directions. Bacterial concentrations are reported as most probable number (MPN)/100 mL.

Nutrients Thirty milliliters of water were filtered through a 0.2-μm pore size PES syringe filter (Millipore, Billerica, MA) and stored at −20°C until analysis. The concentrations of SRP, nitrate, nitrite, and ammonium were measured by standard methods with a nutrient autoanalyzer (Lachat QuikChem 8000, Loveland, CO). The detection limits are 0.2 μM nitrate, 0.1 μM nitrite, 0.1 μM SRP, and 0.1 μM ammonium. DIN is reported as the sum of nitrate, nitrite, and ammonium.

Molecular Analysis–Filtrations and Nucleic Acid Extractions For each water sample, 500 mL was filtered through an HA 0.45 μm pore size filter (Millipore, Billerica, MA) to collect the bacterial and viral fractions (Fuhrman et al. 2005). In a few cases when the water was especially turbid, only 400 mL was filtered because of clogging. Filters were stored in 2 oz Whirl-Pak bags (Nasco, Fort Atkinson, WI) at −80°C until analysis. DNA and RNA were extracted and purified from filters using modifications of the AllPrep™ DNA/RNA Micro Kit (Qiagen, Valencia, CA). Filters were thawed and 1 ml of RLT plus buffer (containing 10 μl β-mercaptoethanol and 20 ng of carrier RNA) was added directly to each Whirl-Pak bag and filters were allowed to soak for 10 min. Lysates were removed from Whirl-Pak bags by pipetting into 2 ml microcentrifuge tubes and 1 ml of 70% ethanol was added to the lysate. Samples were then added to AllPrep spin columns and processed from this point forward using the manufacturer's protocols. Purified DNA was eluted in 50 μl EB buffer and purified RNA was eluted in 14 μl of RNase-free water. DNA and RNA extracts were stored at −20°C and −80°C, respectively.

Molecular Analysis–Host-Specific Bacteroidales PCR Conventional PCR was used to detect the host-specific *Bacteroidales* fecal marker in humans (HF183) (Bernhard and Field 2000a, b), ruminants (CF128, CF193) (Bernhard and Field 2000a), and swine (PF163) (Dick et al. 2005) in DNA extracted from water samples. All conventional PCRs for host-specific *Bacteroidales* utilized 2 μl of template DNA. This corresponds to DNA from 25 mL of water. HF183, CF128, and CF193 assays were performed with the following PCR chemistry: 1X Takara Ex Taq PCR buffer, 200 μM Takara Ex Taq dNTPs, 0.2 μM forward (HF183, CF128 or CF193) primer, 0.2 μM reverse (Bac708) primer, 0.08% bovine serum albumin fraction V (GIBCO, Carlsbad, CA), and 0.025 U/μl of Takara Ex Taq. The PF163 assay was performed using the following PCR chemistry: 1X PCR Gold Buffer (Applied Biosystems, Foster City, CA), 1.5 mM PCR Gold $MgCl_2$, 200 μM Takara Ex Taq dNTPs, 0.2 μM forward (PF163) primer, 0.2 μM reverse (Bac708) primer, 0.08% bovine serum albumin fraction V (GIBCO, Carlsbad, CA), and 0.025 U/μl of Takara Ex Taq. Thermal cycling conditions consisted of 2 min of initial denaturation at 95°C, followed by 35 cycles of 45 s at 95°C, 45 s at the appropriate annealing temperature, 45 s at 72°C, and a final extension of 7 min at 72°C. Annealing temperatures were as follows: 63°C for HF183, 62°C for CF128 and CF193, and 59°C for PF163. Visualization of amplified DNA products was performed by electrophoresis in 1.5% agarose gels stained with 0.5 μg/mL ethidium bromide. Positive PCR reactions produced DNA fragments of 525 bp (HF183), 585 bp (CF128), 515 bp (CF193), and 545 bp (PF163). The detection limit for the PCR amplification was approximately 40 copies of target. Given the volume of template run in each reaction, this corresponds to a detection limit per volume of sample water of 160 copies per 100 mL.

Enterovirus RT-PCR Reverse transcription PCR was used to detect enteroviruses in RNA extracted from water samples (Noble and Fuhrman 2001). Briefly, 5 μl of RNA extract was used as template in 25 μl reactions with the following composition: 1X Qiagen OneStep RT-PCR Buffer, 400 μM Qiagen dNTP mix, 0.6 μM EV upstream primer, 0.6 μM EV downstream primer, and 1X Qiagen OneStep RT-PCR Enzyme Mix. Five microliters of template corresponds to RNA from 70 mL of water. Thermal cycling conditions consisted of a reverse transcription step at 50 C for 30 min and an initial PCR activation step at 95°C for 15 min, followed by 40 cycles of 30 s at 95°C, 30 s at 59°C, 1 min at 72°C, and a final extension of 10 min at 72°C. Visualization of amplified DNA products was performed by electrophoresis in 1.5% agarose gels stained with 0.5 μg/mL ethidium bromide. Positive RT-PCR reactions produced DNA fragments of 196 bp. The detection limit of the RT-PCR is approximately 40 copies, or in terms of water volume, 57 copies per 100 mL.

Host Specificity of Bacteroidales Markers Eighteen fecal samples were collected in Hanalei in December 2009 to

assess the specificity of the host-specific *Bacteroidales* markers. A total of five chicken, five swine, three horse, and five cow fecal samples were collected from farms located with Hanalei watersheds using sterile plastic centrifuge tubes and frozen until analysis. Chicken feces were included because of the large number of domestic and feral chickens that roam the watersheds. DNA was extracted from fecal samples using a modified bead beating and DNeasy Blood and Tissue kit. Using sterile forceps, 0.02 to 0.16 g of each fecal sample was inserted into 2 ml screw cap microcentrifuge tubes containing 0.3 g of acid-washed glass beads (Sigma, St. Louis, MO, catalog #G-1277). Five hundred microliters of guanidine isothiocyanate buffer (5 M guanidine thiocyanate, 100 mM EDTA, 0.5% *N*-lauroyl sarcosine) were added to each bead beating tube. Bead beating tubes were milled for 5 min at maximum rate in a Mini-BeadBeater-1(Biospec Corp., Bartlesville, OK). Tubes were centrifuged at 12,000×g for 1 min to pellet the glass beads and debris. Lysates were transferred to new sterile 2-ml microcentrifuge tubes containing 500 μl of buffer AL and 500 μl of 100% ethanol and briefly vortexed. Lysates were transferred to DNeasy DNA spin columns and were processed from this point forward using manufacture's protocols. Purified DNA was eluted in 50 μl EB buffer.

In 2008, two samples were collected from a cesspool located within Hanalei town and analyzed for *Bacteroidales* source tracking markers as well as the EV marker. A small volume (10 mL) was filtered through an HA filter. Filters were processed as described for water samples to extract both DNA and RNA. DNA and RNA were analyzed using the same PCR protocols as described above.

Quality Control During sampling, filtration blanks were run to check for cross contamination. During nucleic acid extractions, extraction blanks were run. No-template controls were run during all PCR runs. In all cases, results indicated no cross contamination.

Data Analysis Bacterial concentrations measured below the detection limit of 10 MPN/100 mL (n=2 of 116 measurements) were assigned a concentration of half the detection limit. Instantaneous fluxes of DIN, SRP, and bacteria were determined by multiplying contemporaneously measured flow rates and concentrations. Watershed-specific EC, ENT, DIN, and SRP areal loads were determined by dividing the average flux at each site by the area of the watershed. Bacterial concentrations, fluxes, and areal loads, as well as turbidity were $\log_{10}$-transformed to achieve normality prior to any analyses. Analyses of variance (ANOVA) were used to determine how year, location, and occurrence of markers controlled concentrations and occurrence of targets. Pearson's correlation coefficient (r_p) was used to examine covariation of parameters; generalized estimating equations (GEE) (Liang and Zeger 1986) were used to test for associations between land cover and concentrations, fluxes, and loading. GEE account for the fact that there is just one measure of land cover per site, yet multiple measures of concentration, flux, and loading. Contingency tables compared the occurrence of the molecular markers between sites. Statistical results with $p<0.05$ were deemed statistically significant. When errors are reported, they represent standard deviations. All analyses were carried out using PASW Statistics Release 18.0.0 (SPSS Inc., Chicago, IL).

Results

Land Cover The watersheds are small, less than 100 km^2, with variable land cover (Table 2). The watershed with the most cultivated land is Hanalei (1.4 km^2); however, this represents a small fraction (2.3%) of the total watershed area. Waikoko has the greatest percent of its watershed in cultivation (13.5%) representing 0.25 km^2. Watershed size and cultivated, urban, and forested area are all highly correlated among the watersheds ($r_p>0.98$, $p<0.05$), as are the percentages of each watershed that are urban and cultivated ($r_p=0.81$, $p<0.05$). This indicates that larger watersheds have more of each type of land cover, and that urban and cultivated land cover percentages tend to covary. Because urban land cover is less than 2.5% for all watersheds, they are considered rural (Kay et al. 2008).

Rainfall The Hawai'ian Islands experience a wet and dry season. During the wet season, which lasts from October to April, the study area often receives 50 cm or more of rain per month (1.7 cm/day) according to the rain gauge at the USGS, Hanalei station. Rainfall averaged 0.1 cm/day and 0.2 cm/day, respectively, during the 2008 and 2009 studies. The fact that these values are below the average rainfall of 0.7 cm/day for 1 April 2007 to 30 March 2009 and the absence of storms during the study periods or the 5 days preceding them confirm that the work was completed during non-storm conditions.

Salinity and Temperature The salinity of water was measured to determine if there was a significant fraction of seawater in the sampled discharge. Mean salinities were all less than 2, indicating that stream discharge is primarily freshwater (seawater salinity at this site is 34, data not shown). Therefore, salinity was not further considered in the analysis. Water temperatures were between 21°C and 22°C.

Nutrient and FIB Concentrations Average DIN, ammonium, and SRP concentrations for the streams are provided in

Table 2 Watershed area and land cover (square kilometers) within each watershed, with the percent of each watershed occupied by the land cover indicated in parentheses

Watershed	Area (km^2)	Urban (km^2)	Cultivated (km^2)	Forested (km^2)	Other (km^2)
Hanalei	61.2	0.5 (0.8%)	1.4 (2.3%)	58.3 (95.2%)	1.1 (1.8%)
Wai'oli	14.1	0.2 (1.1%)	0.4 (2.9%)	13.5 (95.7%)	0.04 (0.3%)
Waipā	6.4	0.01 (0.2%)	0.1 (2.0%)	6.3 (97.8%)	0 (0%)
Waikoko	1.9	0.03 (1.6%)	0.3 (13.5%)	1.6 (84.4%)	0.01 (0.4%)

Table 3. There were no significant differences in nutrient concentrations at any of the four Hanalei sites between years (ANOVA, $p>0.05$). When data from both years were aggregated, the four Hanalei Bay sites differed significantly in terms of their SRP, DIN, and ammonium concentrations (ANOVA, $p<0.05$). SRP was less than 1 μM on average. The highest SRP concentrations were observed at Waikoko (0.7±0.6 μM) where it was, on average, 0.3–0.4 μM higher than all other streams (Tukey's post hoc test, $p<0.05$). DIN was highest at the Wai'oli (6.2±0.4 μM) and Waikoko (8.6±4.5 μM) streams. DIN at these sites was approximately 2–5 μM higher than levels in the Hanalei River and Waipā stream ($p<0.05$, Tukey's post hoc test). In general, ammonium levels were elevated in the streams, consistently accounting for over half the total DIN. The average ammonium levels in the Waikoko stream (5.9±3.0 μM) were approximately three times higher than at other sites. SRP, DIN, and ammonium concentrations at the four Hanalei Bay sites were positively associated with the percent of the watershed that is urban (GEE, $p<0.05$) and cultivated (GEE, $p<0.05$).

The log mean EC and ENT were 2.7 and 2.2 logMPN/100 mL, respectively, across all samples collected from the four waterways. FIB concentrations varied among Hanalei watersheds and between years. Both EC and ENT were higher in 2008 than 2009 by approximately 0.3 and 0.4 log units, respectively ($p<0.05$, ANOVA). Of the two indicator groups, only EC varied significantly among Hanalei sites when data from both years were aggregated (Table 3). The highest EC levels were measured at the Waipā stream, which was only statistically different from the Hanalei River (higher by 0.5 log units, Tukey's post hoc test, $p<0.05$). ENT was significantly correlated to ammonium and DIN ($r_p=0.28$, $p<0.05$ for *both*) as well as EC ($r_p=0.67$, $p<0.05$). Land cover was not associated with EC or ENT concentrations among Hanalei Bay sites (GEE, $p>0.05$). Turbidity, measured only in 2009, was positively correlated to ENT and EC in Hanalei Bay streams (ENT, $r_p=0.49$; EC, $r_p=0.53$; $p<0.05$ for both).

FIB and Nutrient Loading The flow rate of water during the study varied among waterways; Hanalei River had the highest mean discharge rate of 1.2×10^4 m^3/h while the Waikoko stream had the lowest at 490 m^3/h (Fig. 2). Flow rate also varied between years with the exception of flow at Wai'oli which was approximately the same in 2008 and 2009. The mean flow at the Hanalei River was 9×10^3 m^3/h and 15×10^3 m^3/h in 2008 and 2009, respectively, approximately equal to the 25th and 75th percentile of flow rates over the 20-year USGS discharge record. Flow at Waikoko was 300 and 700 m^3/h in 2008 and 2009, respectively; at Waipā, it was 300 and 1,300 m^3/h in 2008 and 2009, respectively. There are no records or previous flow measurements available for the three smaller streams for comparison; however, based on observations at the Hanalei River, it is reasonable to assume that the discharge conditions observed are representative of midrange, non-storm flow conditions.

Two-way ANOVAs, considering the effects of stream and year, indicated no significant variation in ENT and EC flux explained by year ($p>0.38$) despite the variations in flow but significant stream effects ($p<0.05$). ENT and EC flux data from 2008 to 2009 were aggregated to estimate the FIB flux into Hanalei Bay during the midrange flow, non-storm conditions examined herein (Fig. 2).

The total loading of EC and ENT into the bay, estimated by adding the log mean of fluxes from the Wai'oli, Waikoko, Waipā, and Hanalei streams was 10.8 logMPN/h EC (i.e., $10^{10.8}$ MPN/h), and 10.1 logMPN/h ENT. For EC, the largest flux into the bay was from the Hanalei River where the log mean flux is 10.6 logMPN/h, representing

Table 3 Mean and standard deviation (in parentheses) of nutrients and $\log_{10}$-transformed EC and ENT for each stream

	DIN (μM)	NH_4^+ (μM)	SRP (μM)	log EC (MPN/100 mL)	log ENT (MPN/100 mL)
Waipā ($n=13$)	3.2 (1.0)	2.2 (0.8)	0.2 (0.03)	3.0 (0.4)	2.4 (0.6)
Waikoko ($n=13$)	8.6 (4.5)	5.9 (3.0)	0.7 (0.6)	2.7 (0.4)	2.4 (0.4)
Wai'oli ($n=13$)	6.2 (0.4)	2.9 (0.2)	0.3 (0.03)	2.7 (0.3)	2.1 (0.7)
Hanalei ($n=13$)	3.7 (0.7)	2.5 (0.7)	0.3 (0.08)	2.5 (0.3)	1.8 (0.5)

The number of samples (n) for each site is provided

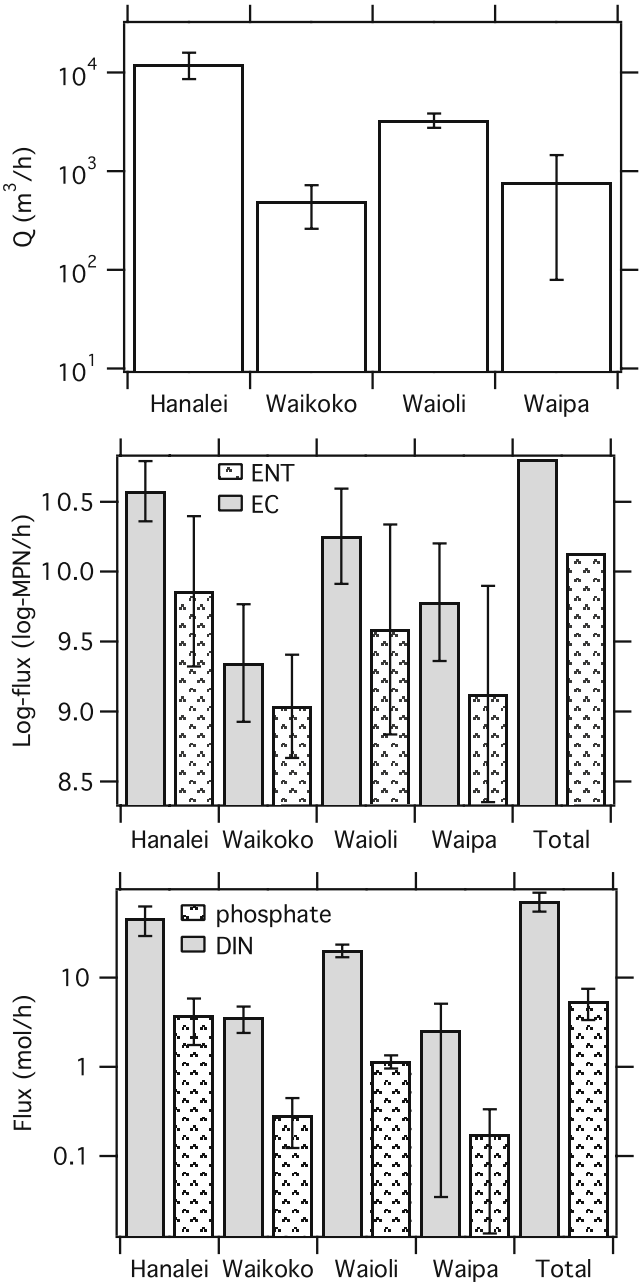

Fig. 2 Mean flux of water (*Q*, top), microbial pollutants (*middle*), and inorganic nutrients (*bottom*) to Hanalei Bay averaged over the two snapshot sampling events in 2008 and 2009. *Error bars* represent standard deviations obtained from comparing 13 measurements or calculations from each site. *Total* is the arithmetic sum of all fluxes to the bay. Errors on total were calculated by propagating error on individual stream estimates

approximately 60% of the total flux. The flux from Wai'oli was of the same order of magnitude as that from the Hanalei River and represented approximately 30% of the total EC flux into the bay. Waipā contributed 10% of the total EC flux to the bay while Waikoko contributed a small fraction (3% of the total). For ENT, the largest flux into the bay was from the Hanalei River (log mean flux of 9.9 log MPN/h, representing 50% of the total flux). The next largest flux was from Wai'oli (log mean flux of 9.6 log MPN/h) accounting for 30% of the total flux. The remaining 20% of the total flux comes from the Waikoko and Waipā streams which each accounted for approximately 10% of the total flux.

For the nutrient fluxes, two-way ANOVAs indicated a significant variation in DIN and SRP flux explained by year ($p<0.05$) and stream ($p<0.05$). DIN and SRP flux from the Waikoko and Wai'oli streams were approximately the same in 2008 and 2009, but were smaller from the Hanalei River and Waipā stream in 2008 than 2009 (Hanalei River: DIN flux of 33 vs 61 mol/h, SRP flux of 2 vs 6 mol/h; Waipā: DIN flux of 0.9 vs 4.5 mol/h, SRP flux of 0.07 vs 0.3 mol/h). Recall that streams generally had lower water discharge in 2008 than 2009.

DIN and SRP data from 2008 to 2009 were combined to estimate mean fluxes into Hanalei Bay during the midrange, non-storm conditions. Mean DIN fluxes from the Hanalei River, Wai'oli, Waikoko, and Waipā were 46, 20, 4, and 3 mol/h, respectively. Adding these to obtain an estimate of the mean total DIN flux from the river and streams to the bay gives 72 mol/h (equivalent to 1 kgN/h). Mean SRP fluxes from Hanalei River, Wai'oli, Waikoko, and Waipā were 4, 1, 0.3, and 0.2 mol/h, respectively. Total SRP flux to Hanalei Bay is estimated at 5 mol/h (equivalent to 0.16 kgP/h).

The Wai'oli stream receives runoff from a culvert draining runoff (urban and storm) from Hanalei town; the average flow rate from the culvert during the study was 200±6 m^3/h. On average, the storm drain contributed 35%, <1%, 2%, and 3% of the mean DIN, SRP, EC, and ENT loading from the Wai'oli stream, respectively.

Fluxes of FIB, SRP, and DIN were positively associated with watershed size (GEE, all $p<0.05$) as well as to cultivated, urban, and forested areas (associations not shown, a result of land cover variables being correlated). Watershed-specific EC, ENT, DIN, and SRP areal loading rates were determined by dividing the mean flux at each site by the area of the watershed (Table 4). Mean loading of EC and ENT were approximately 10^9 MPN/h/km^2 and 10^8 MPN/h/km^2, respectively, for each watershed with the highest loading at Wai'oli and Waikoko. Mean DIN areal loading rates varied from a minimum of 0.4 to a maximum of 2 mol/h/km^2 at Waipā and Waikoko, respectively. Mean SRP areal loading rates varied from a minimum of 0.03 to a maximum of 0.15 mol/h/km^2 at Waipā and Waikoko, respectively. ENT, DIN, and SRP loading rates were positively associated with the fraction of the watershed that is urban and cultivated (GEE, $p<0.05$) and negatively correlated to the fraction forested (GEE, $p<0.05$); EC loading was positively associated with the urban fraction of the watershed (GEE, $p<0.05$).

Table 4 Mean areal loading rates for EC, ENT, DIN, and SRP from each watershed averaged over the two snapshot sampling events in 2008 and 2009

River/stream	EC (MPN/h/km^2)	ENT (MPN/h/km^2)	DIN (mol/h/km^2)	SRP (mol/h/km^2)
Hanalei	6×10^8	1×10^8	0.75	0.06
Wai'oli	13×10^8	3×10^8	1.43	0.08
Waipā	9×10^8	2×10^8	0.40	0.03
Waikoko	12×10^8	6×10^8	1.9	0.15

Occurrence of Molecular Markers When challenged with fecal DNA from target and non-target hosts within the watersheds, the *Bacteroidales* markers—with the exception of CF128—performed well (Table 5). There was no cross-reactivity between markers. The presence of the markers in the target feces varied. The HF183 and enterovirus markers were present in 100% of cesspool samples. The PF163 marker was present in three of five pig feces (60%). The CF193 marker was present in two of five cow feces (40%). The CF128 marker was not detected in the ruminant (cow) feces. These results suggest that all markers, except for CF128, may be used for source tracking, but that we may be underestimating the presence of feces. These performances of the markers are not unexpected and are comparable to those reported by others (Shanks et al. 2010).

Over the 2-year study, 52 samples were collected and analyzed from the four rivers/streams discharging into Hanalei Bay for the occurrence of the *Bacteroidales* human-specific (HF), the two ruminant-specific (CF193 and CF128), and swine-specific (PF) markers, as well as the enterovirus (EV) marker. Of these, 11.5% (n=6) were positive for the HF marker, 0% for the CF128 marker, 7.7% (n=4) for the CF193 marker, 21.2% (n=11) for the PF marker, and 17.3% (n=9) for the EV marker. The occurrence of the markers did not vary between years, with the exception of the EV marker, which was detected only in 2008. The occurrence of the HF, CF129, CF193, and EV markers did not vary among the sites (χ^2 test, $p>0.05$); however, the occurrence of the PF marker did (χ^2 test, $p<0.05$) (Table 6). The PF marker was detected in nine of 13 (70%) samples collected from the Waipā stream compared to one of 12 (8%) samples in Hanalei River, one of 12 (8%) in the Wai'oli stream, and zero of 13 samples from Waikoko. Additionally, six water samples from the culvert discharging storm water to the Wai'oli stream were tested for source tracking markers. One of these six was positive for the HF marker. No other markers were present.

When samples from the four water bodies discharging into Hanalei Bay were considered in aggregate, log-EC and log-ENT concentrations were not significantly different in samples that were positive and negative for the HF, CF128, CF193, or EV markers; however, log EC and log ENT were statistically higher in samples that were positive for the PF marker compared to those that were negative (by approximately 0.5 log units for both indicators; ANOVA, $p<0.05$). There was no difference in turbidity between samples that were positive and negative for fecal markers.

Discussion

ENT concentrations in streams discharging into Hanalei Bay during the two 4 to 5 day snapshot, non-storm sampling events were elevated compared to the ENT geometric mean standard of 35 MPN/100 mL (1.5 log MPN/100 mL; Table 3). There is no EC standard in Hawai'i, but EC concentrations were higher than ENT, and higher than the US freshwater standard of 126 MPN/100 mL (2.1 logMPN/100 mL; Table 3). There were no associations between EC and ENT concentrations and land cover variables; however, FIB concentrations measured from a more pristine watershed (Lumaha'i) during our 2009 study were significantly lower than those measured in the Hanalei streams (data not shown). The lack of association between concentrations and urban and cultivated land covers suggests FIB inputs from unmanaged soils and wildlife may be important in these watersheds (Table 1).

When considered from a loading perspective, the Hanalei River provided the majority of the EC and ENT loading to the bay during the conditions studied (Fig. 2). This suggests

Table 5 Number of samples of a particular fecal source positive for each of the five sources of tracking markers defined in the text

Source tracking marker	Cesspool (n=2)	Swine (n=5)	Horse (n=3)	Cow (n=5)	Chicken (n=5)
Swine (PF)	0	3	0	0	0
Ruminant I (CF128)	0	0	0	0	0
Ruminant II (CF193)	0	0	0	2	0
Human (HF)	2	0	0	0	0
Enterovirus (EV)	2	na	na	na	na

na indicates that a test was not performed

Table 6 The number of samples from each site that were positive for each of the five sources of tracking markers

"Culvert" is the culvert draining urban runoff from Hanalei town to Wai'oli stream

Marker	Hanalei (n=13)	Culvert (n=6)	Waikoko (n=13)	Wai'oli (n=13)	Waipā (n=13)
HF	1	1	3	0	2
CF128	0	0	0	0	0
CF193	1	0	1	0	2
PF	1	0	0	1	9
EV	2	0	3	1	3

that locations within the Hanalei River watershed should be a priority target for remediation and implementation of best management practices in order to reduce loading of indicator bacteria to Hanalei Bay during midrange flow conditions, like those examined here; however, the smaller streams, in aggregate, contribute an approximately equal load of the EC and ENT to the bay as the Hanalei River. Thus, their contributions to the bay cannot be ignored.

Average concentrations of DIN and SRP in the nearshore waters of Hanalei Bay reported by Knee et al. (2008) are 1.4 μM and 0.1 μM, respectively. The concentrations of DIN and SRP in the streams of Hanalei were at least twice the nearshore concentration indicating that the streams are important nutrient sources to Hanalei Bay. DIN and SRP concentrations were highest in streams draining watersheds with the highest fraction of urban and cultivated land, pointing to the importance of associated sources (Table 1). The fluxes of DIN and SRP were highest from the Hanalei River, accounting for over 50% of the total stream flux to Hanalei Bay. This suggests, as for the FIB, that the Hanalei River should be a target for remediation if DIN and SRP loading to the bay is to be reduced during midrange flow, non-storm conditions. A likely source of the DIN and SRP is fertilizer, particularly given the high ammonium concentrations observed (Table 3) and the association between concentrations and cultivated land cover. Previous work in Hanalei Bay using stable isotopes of nitrogen in macroalgal tissue and particulate organic matter suggested that fertilizer is a major source of biologically available nitrogen in the bay (Derse et al. 2007). It is important to acknowledge that other forms of nitrogen and phosphorous (e.g., organic dissolved forms or particulate forms) are likely important nutrient sources in these watersheds. Future work at this site should include these measures, if feasible, to further understand total nutrient inputs to coastal waters.

ENT and EC areal loading rates from the present study were on the order of 10^8 and 10^9 MPN/km^2/h, respectively. This compares to 4.9×10^7 ENTCFU/km^2/h and 2.9×10^8 fecal coliform CFU/km^2/hr reported by Kay et al. (2008) for 125 rural catchments in the United Kingdom. Note that the UK areal loads were determined by sampling every 2–3 days for months at a time and included storm event sampling, an approach that differs from the one used herein. The order of magnitude higher loading rates observed in the tropical compared to temperate watersheds could be explained by FIB contributions from tropical soils (Hardina and Fujioka 1991); however, temperate soils have also been shown to harbor FIB (Yamahara et al. 2009). The correlation between turbidity and FIB concentrations in the Hanalei streams supports the notion that soil may indeed act as an FIB source in the tropical watersheds.

Areal loading rates of FIB, DIN, and SRP were compared between watersheds with different land cover. Because the watersheds are rural and have large forested areas, the overall urban and cultivated fractions are low. Therefore, the reported conclusions regarding the impact of urban and cultivated fractions should be regarded as preliminary. Loadings were high in watersheds with high urban and cultivated fractions. This suggests sources within cultivated and urban lands such as managed soils, agricultural and urban runoff, animal enclosures, wildlife, lo'i ditches, cesspools, septic tanks, and storm drains may be significant contributors of FIB, DIN, and SRP to coastal streams. Additional work in more highly developed, tropical watersheds is needed to further explore the relationship between land cover and pollutant loading.

The conclusion from the land cover analyses that sources associated with cultivated and urban land cover including managed soils, agricultural, and urban runoff (point and non-point), and domesticated and feral animals (Table 1) may contribute FIB to streams is supported by occurrence of the microbial source tracking markers. Ruminant and pig-specific fecal markers were found in the Hanalei streams (Table 6); feral and domesticated ruminants and pigs are present in the cultivated portions of the watersheds. EC and ENT concentrations were higher in samples containing the pig marker. Interestingly, DIN was also higher in samples containing the PF marker (ANOVA, $p<0.05$). At the same time, many stream samples contained FIB but did not contain source tracking markers. The absence of markers in these samples indicates contributions of FIB from other types of feces (e.g., avian or horse) or non-fecal sources such as watershed soils (Hardina and Fujioka 1991) or the markers were at concentrations below the assay detection limits.

At least one sample from each Hanalei Bay stream was positive for a human fecal marker (Table 6). Thus, to some extent, the on-site wastewater management systems (cesspools

and septic tanks) are likely contributing human waste to the streams draining into Hanalei Bay. While water from the culvert that discharges runoff from Hanalei town into the Wai'oli stream contributed an insignificant fraction of FIB load to the stream (<3%), it contained the human fecal marker. The presence of the human marker in the culvert suggests exfiltrated septage or cesspool discharge in Hanalei town can make its way to the Wai'oli stream via runoff where it may discharge to coastal waters. Presence of the enterovirus marker is suggestive of human enterovirus present in the streams (Table 6). It should be noted that presence of the RNA marker does not prove that infectious virus is present.

The high prevalence of the pig fecal marker (nine of 13 samples) in Waipā stream suggests that managed activities occurring close to the stream and near its mouth can have influences on the water quality. During the time of our studies, pigs were being raised in enclosures close to Waipā stream (within 10 m), a practice not occurring close to the other streams. The high prevalence of the PF163 marker suggests pig feces from the enclosures was entering the stream and affecting its water quality. This result also suggests that land use activities in close proximity to streams may have a more substantial impact on stream water quality than activities far from the streams.

The CF128 marker was not detected in cow fecal samples during the testing of bovine feces (Table 6) and was also undetected in all water samples. Shanks et al. (2010) report the differential prevalence of CF128 and CF193 ruminant markers among cattle herds across the United States, so it is possible that ruminants in the Hanalei region do not carry the CF128 marker.

The occurrence of EV exclusively in 2008 was confirmed in follow-up re-analysis of select RNA extracts from both years by a second technician (data not shown). The absence of EV during 2009 could be a result of higher rains in 2009 compared to 2008, essentially diluting a rare molecular target in more runoff. ENT and EC concentrations were significantly higher in 2008 when the EV marker was detected compared to 2009 when it was not.

The reported flux and areal loading rates should be viewed as estimates and not be used to compute annual pollutant loads. Flux and loading estimates were made using data from two snapshot multi-day sampling events during midrange discharge, non-storm conditions. Ideally, more observations that extend over a variety of discharge conditions, including storm events, should be used to estimate pollutant loading (Endreny et al. 2005; Sprague 2001). Even with a large number of observations, there still exists a great deal of uncertainty in loading calculations (Smart et al. 1999). Furthermore, at this particular field site, stream flow may become blocked by a sand berm (formed by deposition of sediments by waves) or by high tides under some conditions. The loading of pollutants to the nearshore during storm events may be orders of magnitude greater than during the ambient, non-storm conditions described here and thus deserve attention and quantification. As an example, Kay et al. (2008) found that FIB areal loadings during storm flow in UK catchments were significantly higher than during base flow. Similar reports exist for nutrients (Lewis et al. 1999).

Conclusions

Instantaneous fluxes of dissolved inorganic nitrogen, soluble reactive phosphorous, and microbial pollutants from tropical rural watersheds to the coastal ocean were determined during two field sampling "snapshots" representing midrange, non-storm conditions, and their sources investigated using microbial source tracking methods as well as land cover analysis. Spatial variation in concentrations and areal loading rates to the bay indicated that cultivated and urban land cover may be sources of nutrients to coastal waters. Bacterial and nutrient areal loading rates were highest for watersheds with the largest urban and cultivated fractions, pointing to increased loading from the sources within managed lands. Fecal source tracking marker occurrence implicates feral and domesticated animals (ruminants and pigs) and humans as sources of microbial pollutants.

The Hanalei River provides the largest flux of fecal indicator bacteria and dissolved inorganic N and P forms to Hanalei Bay and should be the focus of remediation efforts and best management practices to reduce loadings during conditions like those investigated herein. However, more research should be done on a finer geographic resolution within this watershed to identify and quantify localized point and non-point sources of pollutants (Table 1), including soils that are generating the largest pollutant yields within the basin. Once identified, these localized hotspots will represent areas where best management practice implementation is likely to be cost-effective. Changes in wastewater, runoff, and animal management are possible remediation strategies for the watershed. The areal loading rates reported here can be used as benchmarks to measure the impact of future best management practices; however, care should be taken in the extrapolation of the loading rates to different hydrologic conditions.

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