Widespread detection of human- and ruminant-origin *Bacteroidales* markers in subtidal waters of the Salish Sea in Washington State

Zack S. Oyafuso, Anne E. Baxter, Jason E. Hall, Sean M. Naman, Correigh M. Greene and Linda D. Rhodes

ABSTRACT

Rising populations around coastal systems are increasing the threats to marine water quality. To assess anthropogenic fecal influence, subtidal waters were examined monthly for human- and ruminant-sourced *Bacteroidales* markers at 80 sites across six oceanographic basins of the Salish Sea (Washington State) from April through October, 2011. In the basins containing cities with individual populations >190,000, >50% of sites were positive for the human marker, while in the basins with high densities of dairy and cattle operations, ~30% of sites were positive for the ruminant marker. Marker prevalence was elevated in spring (April and May) and fall (October) and reduced during summer (June through September), corresponding with seasonal precipitation. By logistic regression, the odds of human marker detection increased with percentage of adjacent catchment impervious surface, dissolved nitrate concentration, and abundance of low nucleic acid bacteria, but decreased with salinity and chlorophyll fluorescence. The odds of ruminant marker detection increased with dissolved ammonium concentration, mean flow rate for the nearest river, and adjacent shoreline length. These relationships are consistent with terrestrial to marine water flow as a transport mechanism. Thus, *Bacteroidales* markers traditionally used for identifying nearby sources can be used for assessing anthropogenic fecal inputs to regional marine ecosystems.

Key words | anthropogenic indicators, marine waters, molecular source tracking, Puget Sound

Zack S. Oyafuso
Joint Institute for the Study of the Atmosphere and Ocean,
University of Washington,
PO Box 355672, Seattle, WA 98195,
USA
Current address: Hawaii Institute of Marine Biology,
University of Hawaii at Manoa,
PO Box 1346, Kaneohe, HI 96744,
USA

Anne E. Baxter
Jason E. Hall
Correigh M. Greene
Linda D. Rhodes (corresponding author)
Northwest Fisheries Science Center,
National Marine Fisheries Service,
NOAA, 2725 Montlake Boulevard East,
Seattle, WA 98112,
USA
E-mail: *linda.rhodes@noaa.gov*

Sean M. Naman
Frank Orth and Associates, Under Contract to Northwest Fisheries Science Center,
National Marine Fisheries Service,
NOAA, 2725 Montlake Boulevard East,
Seattle, WA 98112,
USA
Current address: Department of Zoology,
University of British Columbia,
#4200-6270 University Boulevard,
Vancouver, BC,
Canada V6T 1Z4

INTRODUCTION

Coastal marine waters of the USA are recipients of runoff and wastewater effluent from adjacent lands. Human activities and land use can dramatically affect marine water quality through discharge of chemicals, changes in physical qualities (e.g., temperature), or release of biological products such as feces. The US portion of the Salish Sea in northwestern Washington State encompasses coastal waters extending over 280 km in length and more than 4,000 km of shoreline, and a substantial portion is contained in the largest fjord in the continental USA, Puget Sound. Approximately 4.3 million people live within 50 miles of Puget Sound (2011 estimate, US Census Bureau), and its associated shoreline is involved in a variety of human activities ranging from industrial to urban to agricultural uses. In Washington State, routine monitoring for fecal contamination at the marine shoreline is jointly managed by state and local jurisdictions, primarily through the Beach Environmental, Assessment, Communication and Health program (http://www.ecy.wa.gov/programs/eap/beach/) and shellfish testing programs (http://www.doh.wa.gov/CommunityandEnvironment/Shellfish). In states such as California, monitoring attention has extended toward the

doi: 10.2166/wh.2015.253

sources of fecal contamination through programs such as the Clean Beaches Initiative (http://www.waterboards.ca.gov/water_issues/programs/beaches/cbi_projects/) to develop standardized protocols for sampling, analyses, and interpretations (Griffith *et al.* 2013).

Although determining the source of fecal material for regulatory purposes can rely on culture-based methods (US Environmental Protection Agency 2005), molecular techniques focusing on alternate fecal markers have enabled good sensitivity and source identification (e.g., Kildare *et al.* 2007; Layton *et al.* 2013). Detection of *Bacteroidales* genetic markers has been widely used to identify fecal contamination, as well as focal sources of contamination such as combined sewer overflows (CSOs) or specific agricultural operations (e.g., Shanks *et al.* 2006; Newton *et al.* 2011; Tambalo *et al.* 2012; Lee *et al.* 2014) in relatively restricted systems such as tributaries or nearly enclosed bays. Current marine monitoring efforts are directed toward beach sand, intertidal water (e.g., Russell *et al.* 2013; Heaney *et al.* 2014), and watersheds draining into confined bays (Shanks *et al.* 2006). Whether *Bacteroidales* markers can be indicators in larger ecosystem regimes, such as oceanographic basins of the Salish Sea, is unknown. The relatively short persistence of *Bacteroidales* markers (Dick *et al.* 2010; Schulz & Childers 2011) and complex hydrodynamic mixing in the Salish Sea (Khangaonkar *et al.* 2011; Sutherland *et al.* 2011) suggest that the presence of these markers in deeper subtidal waters away from point sources would be unlikely. Because subtidal waters can be integrators of both point and non-point sources, detection of *Bacteroidales* markers at deeper water locations would suggest their utility as indicators of larger scale anthropogenic effects.

In 2011, we undertook an extensive survey of neritic subtidal water quality as part of a larger assessment of the pelagic ecosystem in the US portion of the Salish Sea. In addition to collecting and analyzing environmental DNA from subtidal water for the presence of human and ruminant *Bacteroidales* markers, we collected physical oceanographic measurements (e.g., temperature, salinity, pH) and analyzed for dissolved nutrients (e.g., nitrate, phosphate). Eighty sites distributed over six oceanographic basins were chosen to represent a range of adjacent land uses and geomorphic types. The occurrence of the *Bacteroidales* signal was then modeled with calculated land use parameters and a suite of biological, chemical, and physical variables that were measured at sample collection. Our findings demonstrate that this approach can provide evidence for anthropogenic effects in subtidal locations that are reflective of land uses, and that fecal source tracking may serve as a useful monitoring indicator.

MATERIALS AND METHODS

Field sites and site-associated information

Sites were visited once per month for seven consecutive months (April through October) in 2011, with a regression design to focus on sites with varying shoreline and catchment land uses. To capture a broad representation of neritic waters in six oceanographic basins of Puget Sound and adjacent Salish Sea, the following parameters were used for site selection: bathymetry (minimum 10 m depth), shoreline units based on the Puget Sound Nearshore Ecosystem Restoration Project drift cell framework (http://www.pugetsoundnearshore.org/esrp/nearshore.html), and geomorphic habitat types based on the Salmon and Steelhead Habitat Inventory Assessment Program (http://nwifc.org/about-us/habitat/sshiap/; http://apps.wdfw.wa.gov/salmonscape/). Site selection also employed land use variables that were determined by calculating the percentage or area of the catchment or shoreline buffer currently placed into agriculture use, impervious surface, or development using shoreline units based on the Puget Sound Nearshore Ecosystem Restoration Projects process unit (http://www.pugetsoundnearshore.org/pugetsound.html). The locations of sampled sites are displayed in Figure 1, and the number of sites within each basin were: Rosario = 16; Whidbey = 17; Admiralty = 6; Hood Canal = 14; Central = 13; South = 14. Sampling was scheduled to occur during weeks of neap tides to reduce influence of tidal variations.

Sample and field data collection

Water samples were collected with a 5 L Niskin bottle from a depth of 6 m and the temperature of the sample was immediately measured with a digital thermometer. For total DNA

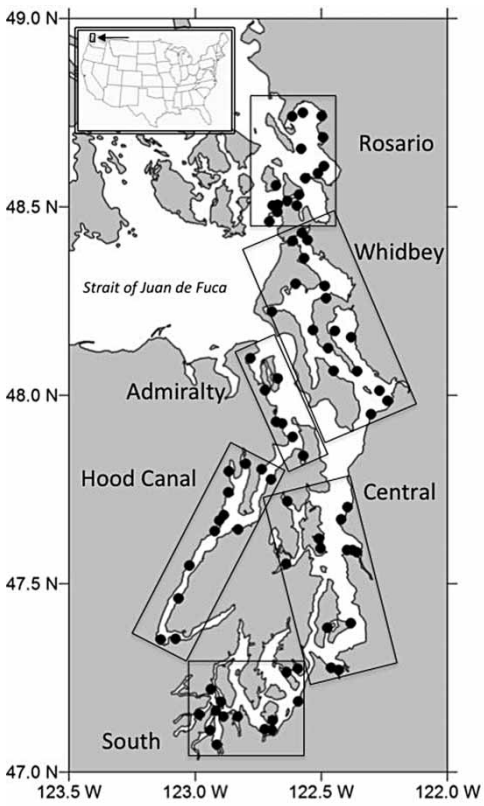

Figure 1 | Locations of sites sampled each month from April to October 2011. Boxes delineate sites contained within each of six oceanographic basins: gray, land; white, marine waters. Inset shows relative position of map within the continental USA.

extraction, 500 mL aliquots were sequentially filtered through 5 μm and 0.2 μm polyethersulfone filters, flash frozen in liquid nitrogen, and subsequently stored at −80 °C until processed. For flow cytometry, 3 mL aliquots of whole water were preserved in paraformaldehyde (final concentration 2%), flash frozen in liquid nitrogen, and subsequently stored at −80 °C. For heterotrophic production analysis, 15 mL aliquots were removed and immediately placed on ice until assayed. For dissolved inorganic nutrients, 60 mL aliquots were filtered through 0.45 μm surfactant-free cellulose acetate filter syringe into polyethylene bottles, and placed on ice until storage at −20 °C.

Water column profile data were collected with a Sea-Bird SEACAT sonde (SBE19plusV2) that included an integral instrument package measuring depth, temperature, conductivity, density, dissolved oxygen, pH, turbidity, photosynthetically active radiation, and chlorophyll fluorescence. Descent and ascent rates were approximately the same at 0.3 m s^{-1}, and measurements were collected, processed, and then binned into 0.5 m increments (Sea-Bird SBE Data Processing Software, V 7.23.1).

Nutrient and microbial abundance analyses

Nutrient assays and calibrations were performed on a Technicon AutoAnalyzerII according to World Ocean Circulation Experiment protocols by the University of Washington Marine Chemistry Laboratory (http://www.ocean.washington.edu/story/Marine+Chemistry+Laboratory). Chlorophyll *a* analyses were performed by the method of Welschmeyer (1994) and samples read on a Turner 10-AU fluorometer. Microbial abundance was measured by flow cytometry by the methods of Sherr *et al.* (2006) using a Becton-Dickinson FACSCalibur that was calibrated to determine flow rate, sizing, and fluorescence scaling. Bacterial/archeal abundance was defined by cytometry as high nucleic acid and low nucleic acid subsets according to the methods of Sherr *et al.* (2006).

DNA extraction and quantitative amplification of *Bacteroidales* 16S rDNA

Total DNA was extracted from filters (0.2 and 5.0 μm) by the method of Boström *et al.* (2004), and precipitated DNA was passed through a membrane-based filter (Qiagen, Valencia, CA, USA) to remove inhibitors. The concentration of each DNA sample was measured by the Quant-iT PicoGreen dsDNA assay (Life Technologies, Grand Island, NY, USA). Quantitative PCR TaqMan assays were performed on a Stratgene Mx3000P QPCR System (Santa Clara, CA, USA). The human-associated *Bacteroidales* assay followed procedures by Newton *et al.* (2011), using HF183f (5′-ATC ATG AGT TCA CAT GTC CG-3′) (Bernhard & Field 2000), BacHum241r (5′-CGT TAC CCC GCC TAC TAT CTA ATG-3′), and BacHum193p (5′-6-FAM/TCC GGT AGA /ZEN/CGA TGG GGA TGC GTT-3′ IABkFQ) (Kildare *et al.* 2007) as the forward and reverse primers and probe, respectively (Integrated DNA Technologies). The *Bacteroidales* primer and probe set from Layton *et al.* (2006) was originally designed and described as bovine-associated, but extensive inter-laboratory comparisons have shown that other bovine-associated assays exhibit varying degrees of cross-reactivity with other ruminants such as

deer (e.g., Boehm *et al.* 2013). Consequently, we hereafter refer to this reaction as ruminant-associated. The ruminant-associated assay used forward primer BoBac367f (5′-GAA GRC TGA ACC AGC CAA GTA-3′), reverse primer BoBac467r (5′-GCT TAT TCA TAC GGT ACA TAC AAG-3′), and hydrolysis probe BoBac402 (5′-6-FAM/TGA AGG ATG /ZEN/AAG GTT CTA TGG ATT GTA AAC TT-3′ IABkFQ) (Integrated DNA Technologies). Each reaction was 25 μL total volume (final concentrations: 1 × Taqman Gene Expression master mix (Life Technologies), 0.2 mg mL^{-1} bovine serum albumin, 1 μM of each primer and 0.08 μM probe (human assay) or 0.3 μM of each primer and 0.1 μM probe (ruminant assay), and 10 ng of extracted DNA). Samples were run in duplicate and standards were run in quadruplicate. The standards consisted of serial dilutions from 1.5×10^6 to 1.5 copies of HBac plasmid (Newton *et al.* 2011) for the human assay and 2.5×10^7 to 25 copies of TNBo1-4 (Layton *et al.* 2006) for the ruminant assay. The thermal regime for both assays consisted of 50 °C for 2 min, 95 °C for 10 min and 45 cycles at 95 °C for 15 s and 60 °C for 1 min.

A detectable signal was defined as a C_q value lower than the mean C_q for the lowest concentration of standard on the plate. Samples that had at least one detectable signal were subjected to a repeat assay. The presence of inhibitors in extracted samples was assessed in separate reactions using a random selection of ~25% of the samples ($n = 121$) that were spiked with an internal amplification control (Nordstrom *et al.* 2007), but no evidence of inhibition was observed. Standard curves were acceptable if R^2 of the curve exceeded 0.990. Concentration values were reported as target copies per 100 mL, correcting for the volumes of seawater filtered and the resultant DNA/RNA product, the DNA/RNA concentration in each reaction, and the relationship of the fluorescence of the qPCR amplification product to the standard curve. Replicate intra-assay variation was relatively low, with an average coefficient of variation of 20.1% for the human marker and 6.6% for the ruminant marker. The no template and blank controls consistently generated no C_q.

Statistical analyses

Deviance goodness-of-fit tests were conducted for the *Bacteroidales* signal among basins and sampling month. Differences in the proportion of signals for a given month or site were defined as statistically significant from the overall proportion of the *Bacteroidales* signal by the G-test. The presence of the *Bacteroidales* signal in relation to various biological, chemical, physical, and land use parameters was modeled using a logistic regression. A stepwise approach was used in the model selection, using Akaike Information Criterion (AIC) as the evaluative metric for variables, with inclusion of a variable resulting in the maximum decrease in AIC value greater than 2.0 as the decision rule (Burnham & Anderson 2002). Because no positive samples were detected for the ruminant *Bacteroidales* marker in July, data for July and August were combined for regression analysis. Mann–Whitney–Wilcoxon testing for differences between *Bacteroidales*-positive and -negative sites was performed on parameters that were not retained in the final logistic regression models. Statistical analyses were performed with R software (R Developmental Core Team 2011) and Stata v12.1 (StataCorp, College Station, TX, USA).

RESULTS

Quantitative abundance and distribution of *Bacteroidales* markers

The mean copy numbers of targets per 100 mL of seawater were relatively low for both markers. The mean for the human marker was 18 copies per 100 mL (median = 3, range = 1 to 723, $n = 200$) and for the ruminant marker was 65 copies per 100 mL (median = 32, range = 3 to 701, $n = 86$). Among positive sites, the copy number varied by sampling month and by basin. For the human marker, mean copy number was highest in April and lowest in August (Figure 2). Although sites in the Central basin dominated the total copy numbers for the human marker for most months, mean copy numbers from sites in the Rosario basin were high in May and July. In contrast, mean ruminant copy numbers were elevated in both April and May (i.e., exceeded a mean of 200 copies per 100 mL seawater), and sites in the Whidbey basin were dominant in both earlier and later months (Figure 2). Mean copies of the ruminant marker were

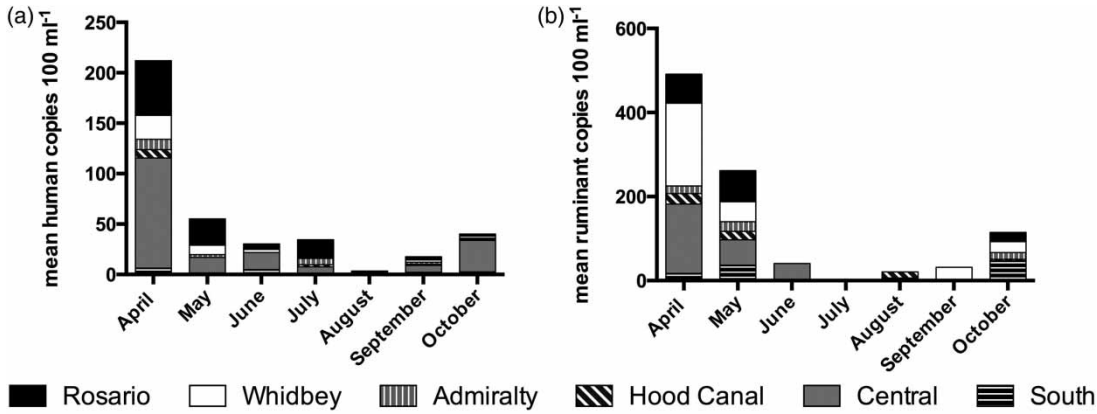

Figure 2 | Mean target copies per 100 mL of seawater by month for (a) the human marker and (b) the ruminant marker for the six basins.

also prominent at sites in the Central and Rosario basins during most of the months.

Spatial and temporal prevalence of positive sites

The proportion of all sites over all months that were positive for either human- or ruminant-origin *Bacteroidales* was 0.381 ($n = 525$) and 0.177 ($n = 485$), respectively. The proportion of positive sites was not uniformly distributed across the oceanographic basins for either marker (G-test, $p < 0.006$, df = 5). Values were significantly elevated in the Rosario ($p = 0.002$), Central ($p < 0.001$), and South ($p = 0.033$) basins for the human marker and in the Whidbey ($p < 0.001$) basin for the ruminant marker. In contrast, values for the human marker were lowest for sites in Hood Canal (Figure 3). The proportion of positive sites exhibited significant monthly variations for each marker (G-test, $p < 0.00001$, df = 6), with higher proportions occurring in spring (April and May) and fall (September and October). The lowest proportion of sites that were positive for the human marker occurred in August, while very low proportions were observed from June through September for the ruminant marker (Figure 4).

Logistic regression for physical, nutrient, microbial, and land use effects

The best model for the human marker included two factors (basin, month) and five variables (nitrate concentration, concentration of low nucleic acid content bacteria,

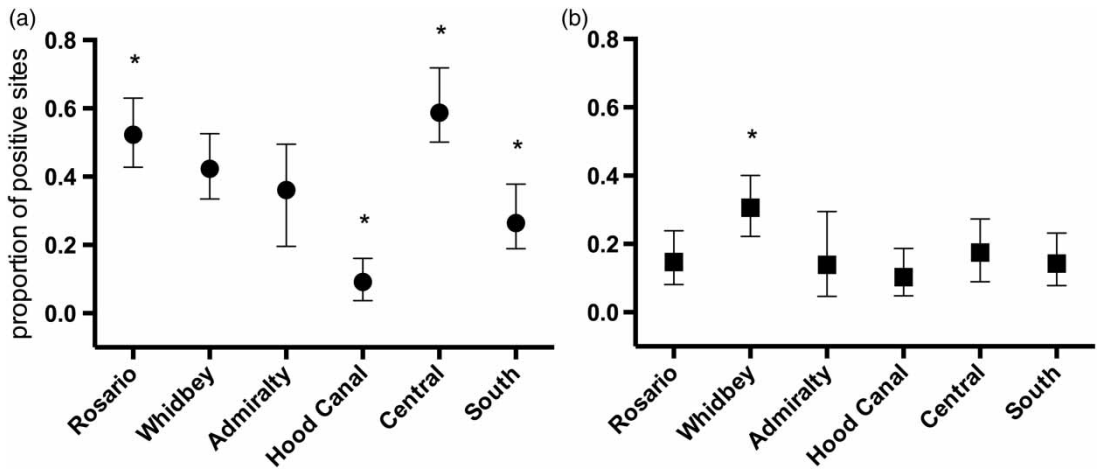

Figure 3 | Proportion of sites (±95 exact binomial confidence interval) positive for (a) the human marker and (b) the ruminant marker by basin. * indicates significant difference from overall proportion of positive site (G-test, goodness-of-fit, $p < 0.05$).

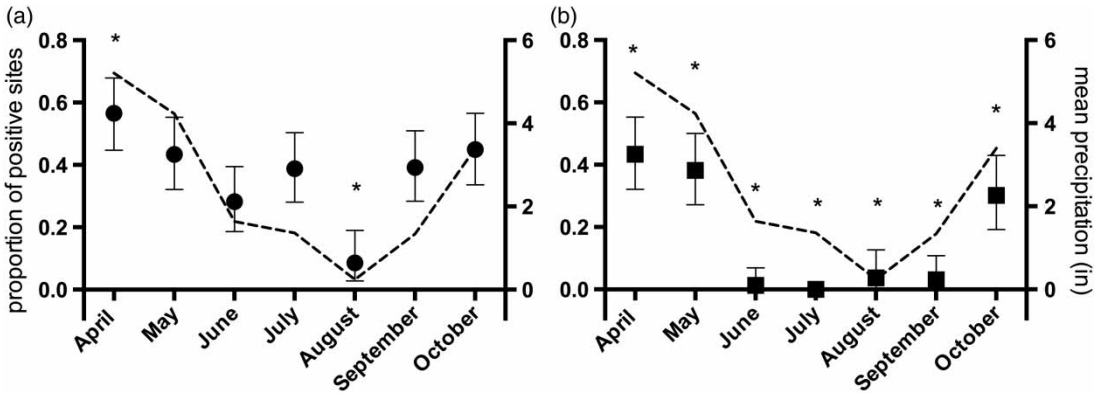

Figure 4 | Proportion of sites (±95 exact binomial confidence interval) positive for (a) the human marker and (b) the ruminant marker by month. * indicates significant difference from overall proportion of positive site (G-test, goodness-of-fit, $p < 0.05$). Dashed line on each graph is the mean monthly precipitation (inches) recorded for the Puget Sound Lowlands for 2011 (NOAA National Climactic Data Center Time Series).

percentage of catchment covered with impervious surface, salinity, and chlorophyll fluorescence). The model revealed a 90% lowered odds of detecting a positive sample in Hood Canal, and an 86% lowered odds of detecting a positive sample in August relative to April (Table 1). Two environmental factors increased the odds for the human marker, with a 10% increase for each micromolar rise in nitrate concentration and a 4% increase for each percentage rise in impervious surface in the associated catchment (Table 1). Conversely, for every unit rise in salinity, the odds of detecting the human marker decreased by 25%. For the microbial variables, the odds of detecting the human marker increased 2% as concentration of low nucleic acid bacteria rose by 10^5 cells mL^{-1}, while detection odds decreased by 63% for each increase in chlorophyll fluorescence units (Table 1). This model had a positive

Table 1 | Significant factors and variables for logistic regressions of the human or ruminant marker by site ($p < 0.05$) and percent change in odds relative to reference factor or per variable unit with corresponding odds ratio and p-value

Human marker		Ruminant marker	
Factor or variable (variable unit)	Percent change in odds (Odds ratio, p-value)	Factor or variable (variable unit)	Percent change in odds (Odds ratio, p-value)
Basin (reference = Rosario): Hood Canal	−89.8 (0.1019, $p < 0.001$)	–	–
Month (reference = April): August	−85.8 (0.1425, $p = 0.003$)	Month (reference = April): June	−99.8 (0.0076, $p < 0.001$)
		July–August	−97.4 (0.0069, $p < 0.001$)
		September	−98.2 (0.0188, $p < 0.001$)
		October	−62.0 (0.3963, $p = 0.026$)
Nitrate (μM)	10.0 (1.1004, $p < 0.001$)	Ammonium (μM)	41.1 (1.4122, $p = 0.035$)
Low nucleic acid content bacteria (cells mL^{-1})	2.2 (1.00002, $p = 0.001$)	–	–
Percent impervious surface in catchment (%)	4.3 (1.0426, $p < 0.001$)	Mean river flow rate ($10^3 \times ft^3\ s^{-1}$)	8.3 (1.0001, $p = 0.004$)
–	–	Shoreline length (km)	1.9 (1.00002, $p = 0.007$)
Salinity (ppt)	−25.2 (0.7481, $p = 0.014$)	–	–
Fluorescence (FU)	−63.3 (0.3665, $p < 0.001$)	–	–

Due to a lack of samples in July for the ruminant assays, the July and August samples were pooled together for the analysis of the ruminant indicator.
– indicates empty cells.

predictive value of 74.4%, a negative predictive value of 81.6%, and overall correctly classified 79.1% of the sites.

For the ruminant marker, the best logistic regression model included one factor (month) and three variables (ammonium concentration, mean river flow, length of associated shoreline). This model showed that the odds of detecting the ruminant marker was reduced by more than 97% during June through September and by 62% in October (Table 2), relative to April. However, inclusion of all three environmental variables increased the odds of detecting the ruminant marker. Detection odds increased 41% for each micromolar rise in ammonium concentration, 8% for each $10^3 \times ft^3\ s^{-1}$ increase in 7-day mean river flow rate, and nearly 2% for each km increase in shoreline length (Table 2). This model had a positive predictive value of 70.3%, a negative predictive value of 86.7%, and overall correctly classified 85.1% of the sites.

Recognizing that stepwise procedures may mathematically remove variables with high covariation, we also tested variables that were not retained in the best regression models for an association with the occurrence of the human or ruminant *Bacteroidales* markers. Precipitation and the percentage of impervious area in the adjacent catchment area was significantly higher at sites where either marker was detected (Table 2). For the human marker, the percentage of shoreline that was developed or impervious and the concentration of dissolved oxygen was higher at positive sites, but pH and microbial heterotrophic production were lower at positive sites. For the ruminant marker, dissolved oxygen concentrations were lower at positive sites (Table 2).

Table 2 | Comparison of values for environmental, biological, or land use parameters for sites with detectable *Bacteroidales* marker, relative to sites where the marker was not detected

	Human marker present	Ruminant marker present
Precipitation	↑	↑
Area of catchment with impervious surface	↑	↑
Percentage of shoreline developed or with impervious surface	↑	–
Dissolved oxygen	↑	↓
pH	↓	–
Heterotrophic production	↓	–

↑ indicates significantly higher and ↓ indicates significantly lower values at positive sites (Mann–Whitney test, $p < 0.05$).

DISCUSSION

Microbial source tracking (MST) is a widely applied tool for locating point sources of fecal contamination, and its ability to assign host origins of fecal contamination is used for risk management. As the number of studies rises, additional applications for source tracking have emerged. We explored the use of source tracking markers as indicators of land use or anthropogenic impact by examining subtidal marine waters within the context of associated oceanographic parameters and a quantitative range of land uses. Although the *Bacteroidales* markers have relatively short environmental persistence (Bell *et al.* 2009; Dick *et al.* 2010; Tambalo *et al.* 2012), there was widespread detection of both the human and ruminant markers at an offshore depth of 6 m. Because lower water temperatures and higher salinity can increase *Bacteroidales* duration up to two-fold (Schulz & Childers 2011), subtidal *Bacteroidales* may persist longer than surface bacteria. Nonetheless, the spatial and temporal patterns of detections support the utility of these markers as indicators of land use and human impacts.

We were able to identify statistically significant environmental associations with *Bacteroidales* markers. Seasonal variation influenced both target copy number (Figure 2) and the proportion of positive sites (Figure 4), suggesting environmental drivers or contributors. From 1991 to 2011, mean monthly rainfall in the Puget Sound lowland region was lowest in August (9 of 20 years, NOAA National Climatic Data Center), and the pattern of monthly precipitation reflected the prevalence patterns for both markers (Figure 4). The 7-day cumulative precipitation at the nearest USGS station for each site was higher at positive sites for each marker (Table 2), suggesting that rainfall is an important component of the appearance of either marker in subtidal waters. Impervious surface, either as a percentage of the catchment area or shoreline buffer or as the actual area of the catchment, was associated with marker detection (Tables 1 and 2). Rainfall contributes to terrestrial run-off, and impervious surfaces increase both the rate and volume

of run-off. Thus, rainfall is a likely driver for the monthly effects and the effects of impervious surface, salinity, and mean river flow detected by the logistic regression analyses (Table 1) and parameter comparisons (Table 2).

For the ruminant marker, transport by river may be as important as direct run-off for mobilizing fecal material into Puget Sound. The odds of detecting the ruminant marker increased with increases in the mean river flow rate (Table 1), and positive sites had greater percentages of impervious surface in the associated catchment (Table 2), which contribute to run-off and stronger terrestrial flow to marine waters. Distances between terrestrial sources and sampling sites can be large (tens of kilometers) and dilution by marine water volumes and tidal fluxes may contribute to small odds ratios for mean river flow rates (Table 1). The ~40% increase in odds of detecting the ruminant marker with increasing ammonium concentration and the lower dissolved oxygen at positive sites suggests a physical association with unprocessed waste, which could move quickly from an originating site into marine waters by riverine transport. Alternatively, source sites located close to marine waters could directly discharge waste, a possibility that is consistent with the increased odds of detection at sites with greater shoreline length (Table 1).

The most likely sources of the ruminant marker detections are cattle and cow operations, which include the beef rearing industry, the dairy industry, and cattle feeding/finishing industry. For example, ruminant-associated *Bacteroidales* TaqMan assays have been able to detect the effects of cattle exclusion from streams (Wilkes *et al.* 2013). In counties adjacent to Puget Sound, there were 3,072 beef cattle and cow operations, 392 dairy cattle and cow operations, and 105 cattle feed operations in 2007 (National Agricultural Statistics Service, USDA). Since colonization by European and non-indigenous Americans, at least 55% of wetlands (~44.3 km^2) in the Puget Sound Basin have been eliminated by tidal barriers, often to drain and convert for agricultural purposes (Schlenger *et al.* 2011). These modifications place agricultural operations closer to marine waters as well as remove wetlands that can slow and modify run-off. Commercial dairy operations tend to be clustered in several areas, principally in the Rosario and Whidbey basins and along the Puyallup River that flows in the Central basin (Washington Department of Agriculture 2011). Although Whidbey basin has a lower density of dairy farms than Rosario and Central basins, their close proximity to the shoreline in Whidbey basin may contribute to the stronger ruminant signal found there. Waste management from dairy and feeding/finishing operations receives greater regulatory oversight through the EPA's National Pollutant Discharge Elimination System permit program than beef rearing operations that do not concentrate animals and are not required to participate in that program. Consequently, the contribution of beef rearing operations to detection of the ruminant marker cannot be assessed.

The human marker shared a temporal pattern with the ruminant marker, but differed in spatial distribution and potential contributing factors. Hood Canal had significantly lower odds of the human marker, compared to other basins. While Hood Canal receives substantive input from four rivers draining from the Olympic Mountains, there are no major cities or larger scale agricultural activities along Hood Canal. In contrast, the strongest human signals occurred at sites proximal to the three largest cities adjacent to Puget Sound: Seattle (population = 612,100), Tacoma (population = 198,900), and Everett (population = 103,100) (2011 populations, WA State Office of Financial Management). The increased odds of detection associated with higher percentages of impervious surface in adjacent catchments and the higher percentage of developed or impervious shorelines at positive sites may reflect the loss of terrestrial modulation of surface water and groundwater. Alternatively, these land use variables may relate to intensification of sewage disposal in areas of high population density. Although site selection was not predicated on the locations of wastewater treatment plants (WWTPs) and CSO outfalls, four of the seven sites within 1 km of a discharge outfall were in the Central basin, and the two highest marker counts were at sites located near a discharge. While the decreased odds with salinity suggest an association with a freshwater source such as a CSO, terrestrial run-off, or rivers, the increased odds with nitrate concentration and the higher dissolved oxygen at positive sites are more consistent with processed, rather than raw, wastewater. An assessment of dissolved inorganic nitrogen loads in Puget Sound estimated an annual average loading of 54,800 kg d^{-1}, with 59% from WWTPs, 14% from human nonpoint sources in rivers, and 27% from natural (non-human) sources, with

strong seasonal variations in river sources and very little variation in inputs from WWTPs (Mohamedali *et al.* 2011). The strong seasonal pattern, associated nutrient and physical factors, and the linkage to human density and modified environment indicate that water sources containing human markers may undergo nitrification prior to discharge but the discharges are driven by rainfall (e.g., Dillon & Chanton 2005). On-site septic systems that do not complete the nitrification–denitrification process can release nitrates during periods of high rainfall and ground saturation, especially when located near streams or shorelines. Alternatively, direct CSO discharge into marine waters may undergo *in situ* nitrification. Based on our observations, extension of this type of study into months of greater rainfall (November through March) are likely to yield greater prevalence of positive sites and higher quantitative detection of both markers.

The relationship of low nucleic acid bacteria to the human marker appears correlated rather than causal, and the effect on the odds of detection was small. Flow cytometry and sorting have shown that low nucleic acid bacteria can have lower metabolic activity than high nucleic acid bacteria (Sherr *et al.* 2006), consistent with the lower heterotrophic production at sites where the human marker was detected (Table 2). Nucleic acid subsets may represent distinct taxonomic communities, as shown in an assessment of high and low nucleic acid subsets from Mediterranean waters, but that study found *Bacteroidetes* were associated primarily with the high nucleic acid population (Vila-Costa *et al.* 2012).

Because our sampling occurred in waters with significant tidal fluxes, it would be difficult to attempt identification of specific sources of markers without higher resolution spatial sampling over a wider seasonal scale. Marker studies including land use variables have typically focused on streams, where there are clear directional effects between source and MST detections (e.g., Wilkes *et al.* 2013; Lee *et al.* 2014). In some cases, the larger effect of land use relative to seasonal factors was used to identify sources such as on-site septic systems (Peed *et al.* 2011; Gentry-Shields *et al.* 2012).

Detecting a fecal marker in a particular body of water is a function of several factors: marker source, transport from terrestrial to the aquatic system, environmental persistence, and retention within the water body. The last factor is dependent on water residence time, which can be estimated from hydrodynamic models. A hindcast stimulation for 2006 employing the Regional Ocean Modeling System estimated that median residence in the Whidbey basin ranged between 15 and 33 days, in the Central basin ranged between 37 and 50 days, and in the South basin ranged between 49 and 50 days, whereas median residence in Hood Canal ranged between 81 and 146 days (Sutherland *et al.* 2011; http://faculty.washington.edu/pmacc/MoSSea/images/Tr_means_Psound.jpg). In spite of shorter residence times in the Whidbey basin, the ruminant marker was prevalent in April and May. Conversely, both markers were rarely detected in Hood Canal, suggesting a lack of sources. Higher spatial resolution modeling of residence time and simulations incorporating annual variations in circulation may be useful for identifying points of origin, because particle transport can be relatively swift in some parts of Puget Sound (Yang & Khangaonkar 2007).

Our application of *Bacteroidales* markers as indicators of anthropogenic fecal input to larger marine ecosystems has implications for similar assessments of other coastal areas impacted by human activities. The strong seasonal patterns indicate that sampling throughout the year, particularly during periods of higher rainfall, should be a design strategy. The influence of salinity and association of river flow rate on detection suggest that freshwater gradients are also important design considerations. While we employed detailed information about land use adjacent to each site, the detection patterns also corresponded to basin-wide land use patterns. This suggests that *Bacteroidales* markers can reflect the integrative nature of spatially and hydrodynamically complex marine regions.

CONCLUSIONS

The Salish Sea is a coastal waterway system that spans two countries and contains the largest fjord in the continental USA. Immigration to the region surrounding the Salish Sea is placing pressure on marine water quality, and major efforts are underway to reduce or mitigate human impacts. Fecal contamination is one of the oldest effects of humans on water quality, and this study found evidence of anthropogenic fecal contamination in pelagic subtidal locations that are not proximal to obvious point sources. Both land use

factors, such as impervious surface or developed shoreline, and seasonal factors, such as precipitation and river flow rate, are implicated as possible sources or transport mechanisms for *Bacteroidales* source markers to deeper waters. Additional contributory factors such as elevated nitrate or ammonium concentrations suggest partially treated or untreated sources, while lower salinity and pH are consistent with a freshwater influence. Although this type of survey is not designed for identifying specific geographic origins of contamination, it demonstrates that host source tracking markers can be applied as indicators for monitoring regional shifts of anthropogenic fecal inputs into the Salish Sea.

ACKNOWLEDGEMENTS

This work was supported by US Environmental Protection Agency funding and the National Marine Fisheries Service (National Oceanographic and Atmospheric Administration, Department of Commerce). Z.S.O. was supported by the Joint Institute for the Study of the Atmosphere and Ocean (JISAO) under NOAA Cooperative Agreement NA10OAR4320148, Contribution No. 2367. We thank S. McLellan (University of Wisconsin-Milwaukee) for the plasmid control for the human marker assay, A. Layton (University of Tennessee-Knoxville) for the plasmid control for the ruminant marker assay, and H. Imaki (Northwest Fisheries Science Center) for invaluable GIS support in the design phase. This study involved a large number of participants including staff from the Northwest Fisheries Science Center, Tribal partners (the Squaxin Tribe, the Port Gamble S'Klallam Tribe, the Skagit River System Cooperative), and vessel operators J. King, S. Bold, and D. Lomax. Mention of trade names or commercial products is solely for providing specific information and does not imply recommendation or endorsement.

REFERENCES

Bell, A., Layton, A. C., Mckay, L., Williams, D., Gentry, R. & Sayler, G. S. 2009 Factors influencing the persistence of fecal bacteroides in stream water. *J. Environ. Qual.* **38**, 1224–1232.

Bernhard, A. E. & Field, K. G. 2000 A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA. *Appl. Environ. Microbiol.* **66**, 4571–4574.

Boehm, A. B., Van De Werfhorst, L. C., Griffith, J. F., Holden, P. A., Jay, J. A., Shanks, O. C., Wang, D. & Weisberg, S. B. 2013 Performance of forty-one microbial source tracking methods: a twenty-seven lab evaluation study. *Water Res.* **47**, 6812–6828.

Boström, K. H., Simu, K., Hagstrom, A. & Riemann, L. 2004 Optimization of DNA extraction for quantitative marine bacterioplankton community analysis. *Limonol. Oceanogr.* **2**, 365–373.

Burnham, K. P. & Anderson, D. R. 2002 *Model Selection and Multi-modal Inference: A Practical Information-theoretic Approach*. Springer-Verlag, New York, USA.

Dick, L. K., Stelzer, E. A., Bertke, E. E., Fong, D. L. & Stoeckel, D. M. 2010 Relative decay of *Bacteroidales* microbial source tracking markers and cultivated *Escherichia coli* in freshwater microcosms. *Appl. Environ. Microbiol.* **76**, 3255–3262.

Dillon, K. S. & Chanton, J. P. 2005 Nutrient transformations between rainfall and stormwater runoff in an urbanized coastal environment: Sarasota Bay, Florida. *Limnol. Oceanogr.* **50**, 62–69.

Gentry-Shields, J., Rowny, J. G. & Stewart, J. R. 2012 Hubac and nifH source tracking markers display a relationship to land use but not rainfall. *Water Res.* **46**, 6163–6174.

Griffith, J. F., Layton, B. A., Boehm, A. B., Holden, P. A., Jay, J. A., Hagedom, C., McGee, C. D. & Weisberg, S. B. 2013 *The California Microbial Source Identification Manual: A Tiered Approach to Identifying Fecal Pollution Sources to Beaches*. Southern California Coastal Water Research Project, Southern California Coastal Water Research Project Commission, Costa Mesa, California, USA, Technical Report 804, p. 83.

Heaney, C. D., Exum, N. G., Dufour, A. P., Brenner, K. P., Haugland, R. A., Chern, E., Schwab, K. J., Love, D. C., Serre, M. L., Noble, R. & Wade, T. J. 2014 Water quality, weather and environmental factors associated with fecal indicator organism density in beach sand at two recreational marine beaches. *Sci. Total Environ.* **497–498**, 440–447.

Khangaonkar, T., Yang, Z., Kim, T. & Roberts, M. 2011 Tidally averaged circulation in Puget Sound sub-basins: Comparison of historical data, analytical model, and numerical model. *Estuarine Coast. Shelf Sci.* **93**, 305–319.

Kildare, B. J., Leutenegger, C. M., McSwain, B. S., Bambic, D. G., Rajal, V. B. & Wuertz, S. 2007 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal *Bacteroidales*: A Bayesian approach. *Water Res.* **41**, 3701–3715.

Layton, A., McKay, L., Williams, D., Garrett, V., Gentry, R. & Sayler, G. 2006 Development of *Bacteroides* 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. *Appl. Environ. Microbiol.* **72**, 4214–4224.

Layton, B. A., Cao, Y., Ebentier, D. L., Hanley, K., Balleste, E., Brandao, J., Byappanahalli, M., Converse, R., Farnleitner, A. H., Gentry-Shields, J., Gidley, M. L., Gourmelon, M., Lee, C. S., Lee, J., Lozach, S., Madi, T., Meijer, W. G., Noble, R., Peed, L., Reischer, G. H., Rodrigues, R., Rose, J. B., Schriewer, A., Sinigalliano, C., Srinivasan, S., Stewart, J., Van De Werfhorst, L. C., Wang, D., Whitman, R., Wuertz, S., Jay, J., Holden, P. A., Boehm, A. B., Shanks, O. & Griffith, J. F. 2013 Performance of human fecal anaerobe-associated PCR-based assays in a multi-laboratory method evaluation study. *Water Res.* **47**, 6897–6908.

Lee, D. Y., Lee, H., Trevors, J. T., Weir, S. C., Thomas, J. L. & Habash, M. 2014 Characterization of sources and loadings of fecal pollutants using microbial source tracking assays in urban and rural areas of the Grand River Watershed, Southwestern Ontario. *Water Res.* **53**, 123–131.

Mohamedali, T., Roberts, M., Sackmann, B. & Kolosseus, A. 2011 Puget Sound Dissolved Oxygen Model. Nutrient Load Summary for 1999–2008. Department of Ecology, S.o.W. (ed.), p. 144, Washington State Department of Ecology, Olympia, WA, USA.

Newton, R. J., Vandewalle, J. L., Borchardt, M. A., Gorelick, M. H. & McLellan, S. L. 2011 Lachnospiraceae and Bacteroidales alternative fecal indicators reveal chronic human sewage contamination in an urban harbor. *Appl. Environ. Microbiol.* **77**, 6972–6981.

Nordstrom, J. L., Vickery, M. C., Blackstone, G. M., Murray, S. L. & DePaola, A. 2007 Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters. *Appl. Environ. Microbiol.* **73**, 5840–5847.

Peed, L. A., Nietch, C. T., Kelty, C. A., Meckes, M., Mooney, T., Sivaganesan, M. & Shanks, O. C. 2011 Combining land use information and small stream sampling with PCR-based methods for better characterization of diffuse sources of human fecal pollution. *Environ. Sci. Technol.* **45**, 5652–5659.

Russell, T. L., Sassoubre, L. M., Wang, D., Masuda, S., Chen, H., Soetjipto, C., Hassaballah, A. & Boehm, A. B. 2013 A coupled modeling and molecular biology approach to microbial source tracking at Cowell Beach, Santa Cruz, CA, United States. *Environ. Sci. Technol.* **47**, 10231–10239.

Schlenger, P., MacLennan, A., Iverson, E., Fresh, K., Tanner, C., Lyons, B., Todd, S., Carman, R., Myers, D., Campbell, S. & Wick, A. 2011 *Strategic Needs Assessment: Analysis of Nearshore Ecosystem Process Degradation in Puget Sound*. Puget Sound Nearshore Ecosystem Restoration Project. 2011-02, p. 458. http://www.pugetsoundnearshore.org/technical_papers/strategic_needs_assessment_final.pdf.

Schulz, C. J. & Childers, G. W. 2011 Fecal *Bacteroidales* diversity and decay in response to variations in temperature and salinity. *Appl. Environ. Microbiol.* **77**, 2563–2572.

Shanks, O. C., Nietch, C., Simonich, M., Younger, M., Reynolds, D. & Field, K. G. 2006 Basin-wide analysis of the dynamics of fecal contamination and fecal source identification in Tillamook Bay, Oregon. *Appl. Environ. Microbiol.* **72**, 5537–5546.

Sherr, E. B., Sherr, B. F. & Longnecker, K. 2006 Distribution of bacterial abundance and cell-specific nucleic acid content in the Northeast Pacific Ocean. *Deep-Sea Res. I* **53**, 713–725.

Sutherland, D. A., MacCready, P., Banas, N. S. & Smedstad, L. F. 2011 A model study of the Salish Sea estuarine circulation. *J. Phys. Oceanogr.* **41**, 1125–1143.

Tambalo, D. D., Fremaux, B., Boa, T. & Yost, C. K. 2012 Persistence of host-associated Bacteroidales gene markers and their quantitative detection in an urban and agricultural mixed prairie watershed. *Water Res.* **46**, 2891–2904.

US Environmental Protection Agency 2005 *Microbial Source Tracking Guide Document*. US EPA, Washington, DC, USA, p. 131.

Vila-Costa, M., Gasol, J. M., Sharma, S. & Moran, M. A. 2012 Community analysis of high- and low-nucleic acid-containing bacteria in NW Mediterranean coastal waters using 16S rDNA pyrosequencing. *Environ. Microbiol.* **14**, 1390–1402.

Washington Department of Agriculture 2011 *Washington Dairies and Digesters*. Washington State Department of Agriculture, Olympia, WA, USA, p. 21.

Welschmeyer, N. A. 1994 Fluorometric analysis of chlorophyll *a* in presence of chlorophyll *b* and phaeopigments. *Limnol. Oceanogr.* **39**, 1985–1992.

Wilkes, G., Brassard, J., Edge, T. A., Gannon, V., Jokinen, C. C., Jones, T. H., Marti, R., Neumann, N. F., Ruecker, N. J., Sunohara, M., Topp, E. & Lapen, D. R. 2013 Coherence among different microbial source tracking markers in a small agricultural stream with or without livestock exclusion practices. *Appl. Environ. Microbiol.* **79**, 6207–6219.

Yang, Z. & Khangaonkar, T. 2007 *Development of a Hydrodynamic Model of Puget Sound and Northwest Straits*. US Department of Energy. http://www.pnl.gov/main/publications/external/technical_reports/PNNL-17161.pdf.

First received 26 October 2014; accepted in revised form 6 January 2015. Available online 18 February 2015