Spatial and hydrologic variation of *Bacteroidales*, adenovirus and enterovirus in a semi-arid, wastewater effluent-impacted watershed

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ABSTRACT

Bacteroidales and viruses were contemporaneously measured during dry and wet weather conditions at a watershed-scale in a semi-arid watershed impacted by a mixture of agricultural runoff, municipal wastewater effluent and municipal runoff. The results highlight the presence of municipal wastewater effluent as a confounding factor for microbial source tracking (MST) studies, and thus data were segregated into groups based on whether they were impacted by wastewater effluent. In semi-arid environments such as the Calleguas Creek watershed, located in southern California, the relative contribution of municipal wastewater effluent is dependent on hydrology as storm events lead to conditions where agricultural and municipal stormwater dominate receiving waters (rather than municipal wastewater, which is the case during dry weather). As such, the approach to data segregation was dependent on hydrology/storm conditions. Storm events led to significant increases in ruminant- and dog-associated *Bacteroidales* concentrations, indicating that overland transport connects strong non-human fecal sources with surface waters. Because the dataset had a large number of non-detect samples, data handling included the Kaplan–Meir estimator and data were presented graphically in a manner that reflects the potential effect of detection limits. In surface water samples with virus detections, *Escherichia coli* concentrations were often below (in compliance with) the recreational water quality criteria. In fact, sites downstream of direct inputs of municipal wastewater effluent exhibited the lowest concentrations of *E. coli*, but the highest concentrations of human-associated *Bacteroidales* and highest detection rates of human viruses. The toolkit,

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comprised of the four *Bacteroidales* assays and human virus assays used, can be successfully applied to inform watershed managers seeking to comply with recreational water quality criteria. However, care should be taken when analyzing data to account for the effect of non-detect samples, sources with differing microbial viability, and diverging hydrologic conditions.

1. Introduction

Over 12,000 waterbodies in the United States are categorized as impaired by fecal indicator bacteria (FIB) discharges, and have been subject to total maximum daily loads (TMDLs), which describe the water quality improvement strategy to address FIB sources in the watershed (USEPA, 2009). Compliance with recreational water quality (REC) criteria in developed watersheds, both in the U.S. and elsewhere, represents a significant challenge to responsible agencies, as a myriad of non-point bacteria sources contribute to impairment. Some watersheds that are only subject to natural bacteria sources (e.g., birds) have been found to exceed REC criteria (Tiefenthaler et al., 2008), and some waterbodies have been subject to extensive remediation efforts yet exceedances of criteria persist (POLA, 2006). During storm events in urbanized watersheds, which may represent >99% of the annual bacteria discharge (Reeves et al., 2004), loading rates can be extraordinarily high – several times greater than the equivalent daily fecal loading from the entire human population within the watershed (Surbeck et al., 2006). The United States Environmental Protection Agency (USEPA) recently conducted extensive research including epidemiological studies and adopted revised federal REC criteria (Wade et al., 2006; USEPA, 2012). The revised criteria underscore the importance of the type of fecal source when evaluating potential REC health risks. Health risks associated with recreating in waters impacted by non-human sources can be orders of magnitude less than those with human sources (Colford et al., 2007; Soller et al., 2010).

Given the immense challenges involved with complying with REC criteria, and the importance of fecal source type to the level of risks, watershed managers often desire data regarding the fecal sources that are driving levels of FIB. Collectively referred to as microbial source tracking (MST), a plethora of methods have been developed to characterize the contribution of fecal discharges from different host populations to surface waters and are applied throughout the world (Field and Samadpour, 2007; Santo Domingo et al., 2007; Boehm et al., 2013). The most widely-applied and tested of these approaches targets host-associated 16S rRNA genes of the *Bacteroidales*, and assays based on quantitative PCR (qPCR) can be used to estimate genomic concentrations (Kildare et al., 2007; Shanks et al., 2008, 2009). Multiple comparison studies have tested and confirmed that, while not 100% sensitive or specific, many *Bacteroidales* markers are sufficiently sensitive and specific for detecting host-associated contamination (Boehm et al., 2013; Layton et al., 2013; Raith et al., 2013; Schriewer et al., 2013), are repeatable/reproducible (Ebentier

et al., 2013), and the stable populations required for marker-based MST are present around the globe (Reischer et al., 2013).

Statistical and modeling approaches have been evaluated for using ratios of host-associated to universal *Bacteroidales* markers to quantify the contribution of human versus non-human sources on levels of FIB in watersheds (Stoeckel and Harwood, 2007; Wang et al., 2010, 2013; Russell et al., 2013). Applications of these ratios, which should account for differences in fate and transport characteristics along with the fact that MST assays are imperfect, are emerging as a tool for quantitative MST. Ratios and concentrations are interpreted differently; all host-associated concentrations represent the potential impact of that host population on downstream waters, while host-associated:universal ratios highlight the effect of that host population on the total *Bacteroidales* loading at the monitored site. Suppose that a runoff site has very high levels of the human marker BacHum (when compared to other sites) but a very low ratio of BacHum:BacUni. In this case, the site might pose an elevated risk to recreational users who come into contact with a waterbody impacted by human fecal sources, but on the other hand an agency that is responsible for remediating that site should also target potential non-human sources.

To support REC risk assessment, MST assays can be coupled with pathogen assays, particularly those for human viruses (McBride et al., 2013; Harwood et al., 2014). Virus assays with qPCR have been shown to be highly specific for mixed human fecal sources (Harwood et al., 2013), though they are often absent in individual fecal samples (Noble et al., 2003). Enterovirus, a single-stranded RNA virus, has been readily detected with qPCR during several studies of the coastal ocean and coastal watersheds in the western U.S. (Fuhrman et al., 2005; Noble et al., 2006; Viau et al., 2011). Adenovirus, a double-stranded DNA virus, is often detected in these same environments (Choi and Jiang, 2005; Sassoubre et al., 2012), and has been reported to have prolonged survival time and increased resistance to UV treatments (Nwachuku et al., 2005). Prior to this study, no known studies have contemporaneously measured *Bacteroidales* and viruses over the long-term at watershed-scale in waterbodies impacted by a mixture of agricultural runoff, municipal wastewater and municipal stormwater.

The objectives of this study were to (i) evaluate the abundance of four validated fecal *Bacteroidales* genetic markers (universal [BacUni], human- [BacHum], dog- [BacCan], and ruminant-associated [BacCow]) in treated and untreated municipal wastewater, (ii) compare quantitative data on host-associated fecal source identifiers based on *Bacteroidales* and human enteroviruses and adenoviruses with FIB measurements in surface waters, and (iii) utilize the spatial and

hydrologic variations of these quantitative MST markers to elucidate the predominant FIB in Calleguas Creek Watershed (CCW), a multi-use coastal watershed in southern California. We hypothesized that concentrations of *Bacteroidales* and viruses would relate to certain types of discharges in the watershed (e.g., agricultural runoff, urban runoff, and municipal wastewater), and expected our results to assist stakeholders with development and implementation of a bacteria TMDL.

To test this hypothesis, the four *Bacteroidales* specific assays (BacUni, BacHum, BacCow, and BacCan), *Escherichia coli* and human-associated viruses (enteroviruses and adenoviruses) were monitored at multiple CCW sites for one year. To our knowledge, this was the first long-term, watershed-scale study to quantitatively measure *Bacteroidales* and human viruses in water samples. Our approach consisted of combining MST and pathogen methodologies. First, we filtered large volume (100-L) samples and spiked water samples with surrogates in order to increase the accuracy of quantitation by accounting for DNA losses that occur during filtration and extraction (Rajal et al., 2007a). Then we used qPCR to quantify genomic concentrations of human viruses — adenovirus and enterovirus (Rajal et al., 2007b) — and universal and host-associated *Bacteroidales* markers and their ratios to the universal marker (Kildare et al., 2007). Our approach to data synthesis incorporates tools not often used by MST studies including application of a Monte Carlo model to account for imperfect MST assays and statistical approaches that robustly account for datasets that are dominated by non-detect results.

2. Methods and materials

2.1. Watershed description

Calleguas Creek watershed is subject to a mixture of land uses including agricultural (25%), urban land use (25%) and open space (50%) (Ventura County, 2014). Three subwatersheds of the CCW were monitored: Arroyo Simi, Conejo Creek, and Revolon Slough (Fig. 1). Arroyo Simi and Conejo Creek were investigated with transects, each having three sampling sites, while Revolon Slough was investigated with a single site. Each of the sampled sites is listed as "impaired" by the State of California due to impacts from *E. coli* sources, meaning that a TMDL will be developed for these sites under federal requirements. For both investigated transects, the predominant land uses in the immediate vicinity of the three sampled sites, from upstream to downstream, ranged from open space (limited development) to urban (residential, commercial and industrial land uses) to agricultural (row crops and orchards). Tertiary-treated, chlorine-disinfected effluent ("effluent") from municipal wastewater treatment plants (WWTPs) is discharged at three locations within CCW, upstream of the intermediate site of the Arroyo Simi transect, upstream of the most upstream site of the Conejo Creek transect, and upstream of the intermediate site of the Conejo Creek transect. During dry weather, a majority of the flow rate at locations downstream of the WWTP outfalls is effluent. The land use of Revolon Slough is predominantly irrigated agriculture, though

discharges of urban runoff are also present. While there is potential for seepage from wastewater collection systems to flow through storm drains into receiving waters (Haile et al., 1999; Sercu et al., 2009), the wastewater and stormwater systems in CCW are separate and there were no reported sewage spills during sampling events.

2.2. Sample collection

2.2.1. Collection of samples from surface waters and weather definition

Samples consisting of 100 L of surface water were collected in five autoclaved, rinsed, 20-L polypropylene carboys for pathogen analysis and microbial source tracking. A total of 73 grab samples were generally collected monthly from the seven surface water sites between June 2004 and May 2005 (Kundu et al., 2013). Samples were transported on ice and processed for ultrafiltration as stated below.

In southern California, wet weather is traditionally defined as days with greater than 0.1 inches plus the three following days. For this study, all wet weather samples were collected during active storm events when it was raining and flows were elevated. Dry weather samples were collected after at least one week of non-rain days.

2.2.2. Collection of primary influent and disinfected effluent samples from municipal wastewater treatment plants

Primary influent (minimally-treated sewage at the headworks) samples were collected in sterile 250-mL bottles, and transported on ice to the laboratory on the same day. Samples of disinfected effluent were collected in 2-L bottles. A total of 14 samples were collected each of primary influent and disinfected effluent.

2.3. Traditional indicator and chemical methods

E. coli concentrations [most probable number per 100 mL, MPN per 100 mL] were measured according to Standard Method 9223, which is based on chromogenic substrate (IDEXX Colilert). An additional water sample was collected at each site and analyzed for total suspended solids according to Standard Method 2540D [milligram per liter, mg/L].

The following parameters were measured at the time of water collection: water temperature, turbidity, conductivity, dissolved oxygen, and pH (Hach Quanta, Loveland, CO). When measurable but not hazardous (e.g. storm) flow conditions were present, flow rate measurements were performed with an electromagnetic flow meter.

2.4. Processing of samples for Bacteroidales and virus analysis

Details regarding sample processing methods can be found in the Supplemental information. Viruses and bacteria in 100 L water samples were concentrated by ultrafiltration using two sequential hollow fiber modules as described previously (Rajal et al., 2007a). Real-time QPCR for surrogate PP7, adenovirus and enterovirus was performed as described in Rajal et al. (2007b). Real-time QPCR for the fecal *Bacteroidales* assays (universal) BacUni, (human-associated) BacHum, (ruminant-
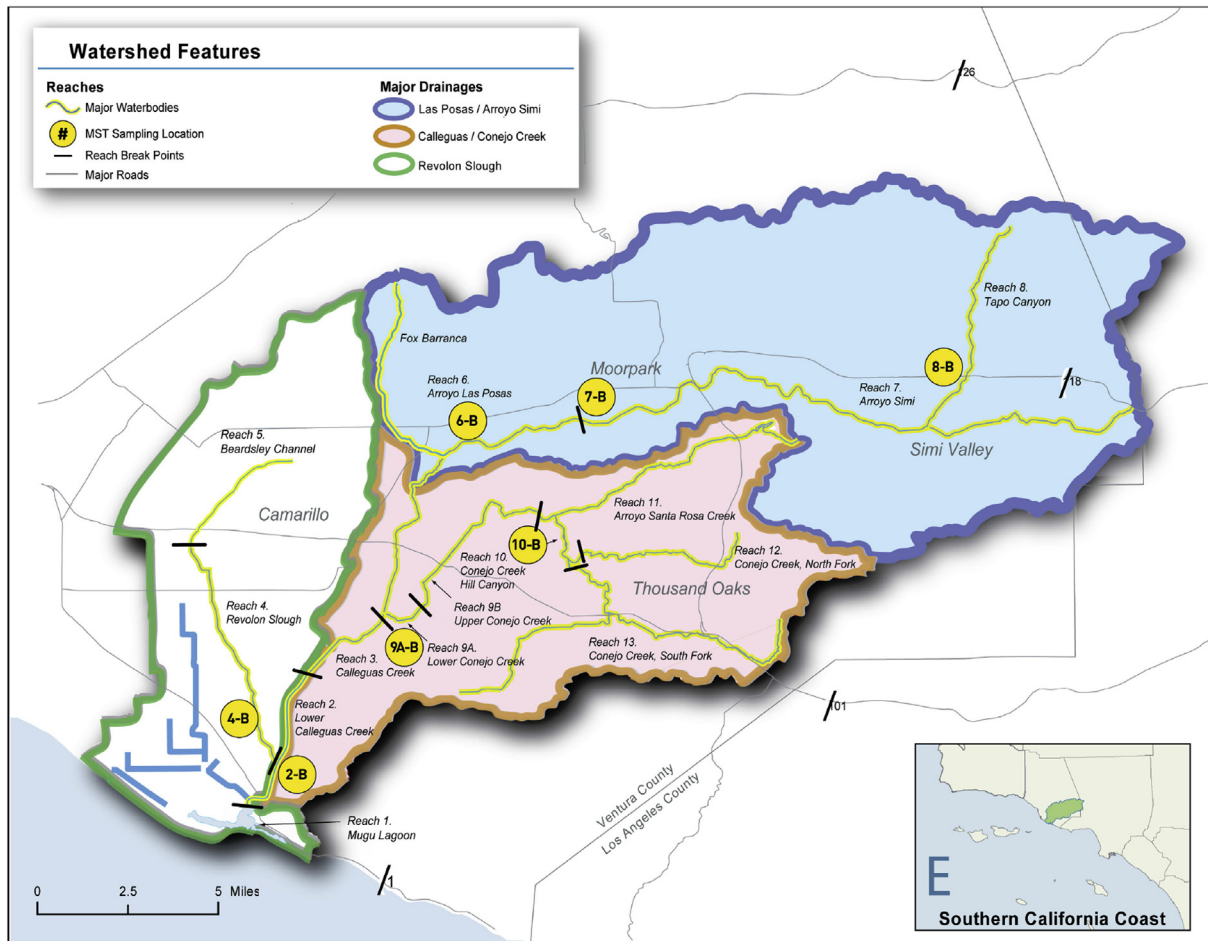
Fig. 1 − Map of the Calleguas Creek Watershed and monitoring locations (yellow circles). Waterbodies are shown with yellow lines, and the three subwatersheds analyzed along transects during this study are highlighted (pink border, Conejo Creek; blue border, Arroyo Simi; green border, Revolon Slough). The State of California designates 10 distinct reaches in the watershed, which are shown with black bars. Revolon Slough and Conejo Creek do not mix prior to discharge to the estuary. Treated WTP effluent discharges occur upstream of sites 10-B, 9A-B and 7-B. The blue lines in southwestern portion of watershed show major agricultural drainage ditches. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

associated) BacCow, and (dog-associated) BacCan was performed as described in Kildare et al. (2007).

Detection of target nucleic acids by real-time QPCR (which was based on TaqMan assays) was found to be strongly affected by the presence of inhibitors, and the multiple dilution approach was used to address inhibition in all wastewater and surface water samples, as described previously (Rajal et al., 2007a). For each sample, a unique sample limit of detection (SLOD) was calculated that accounts for varying inhibition, concentration factors, and filtration recovery (Fig. 2).

2.5.   Statistical analysis

Statistical analyses were performed using R software version 2.12.0 and the NADA library. Tests were selected based on the fact that our MST and pathogen datasets were highly censored (large number of non-detect samples). In general, non-parametric tests were used that can handle varying detection limits (without substitution). For summary statistics, estimates were generated using Kaplan−Meir statistics (Kaplan and Meier, 1958), which are commonly used in survival analysis and readily-adaptable to environmental statistics to handle datasets with a large numbers of non-detects, as described by Helsel, 2005 and Helsel, 2012. To highlight the effect of test selection, summary statistics were also generated using regression-on-order statistics using a jackknife procedure based on SLOD for non-detect samples (Shumway et al., 2002) and maximum likelihood estimation (MLE). For comparison of water quality among sites, the Mann−Whitney−Wilcoxon test was used to determine if any of the sites exhibited distributional differences. For all detected significant differences among groups at $p < 0.05$, the Kruskal−Wallis test was also reported $p < 0.05$. The pairwise p-values were corrected with a correction factor to determine the individual error rate. Both the Bonferroni (highly
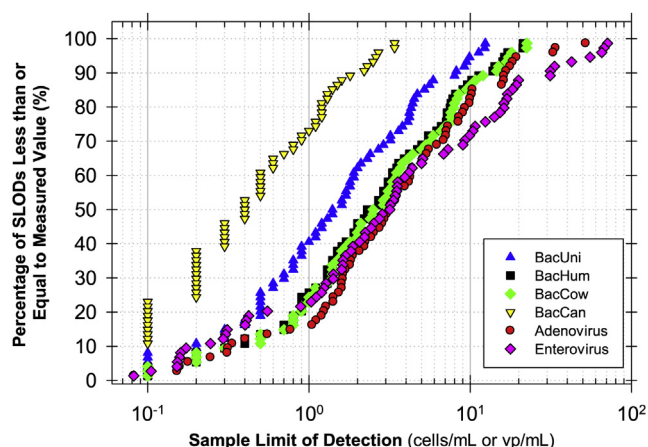
Fig. 2 – Distribution of sample limits of detection for samples collected from CCW, specific to qPCR markers (blue triangle up, BacUni; black square, BacHum; green diamond, BacCow, yellow triangle down, BacCan; red circle, adenovirus; pink diamond, enterovirus). All samples analyzed by qPCR were 100 L. The 50% value of the y-axis axis represents the study median. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

conservative) and Benjamin & Hocklenberg (B&H; less conservative) correction factors were applied (Helsel, 2012). Correlation analyses were based on tests of Kendall's tau.

A Monte Carlo model developed by Wang et al. (2010) was used to calculate "true" ratios of BacHum:BacUni, BacCow:BacUni, and BacDog:BacUni. These true ratios are referred to as Hum_{ratio}, Cow_{ratio}, and Dog_{ratio}, respectively. The Monte Carlo model accounts for the fact that the markers are not 100% specific and sensitive. The model also accounts for the fact that the raw ratios are not equal to unity for feces and sewage (e.g., BacHum:BacUni is less than one in sewage because there are *Bacteroidales*-specific markers in human feces other than BacHum). Note the fecal samples used for model validation in Wang et al. (2010) were collected from the Calleguas Creek watershed and also used to validate the *Bacteroidales* assays by Kildare et al. (2007) that are applied herein. As such, application of the Monte Carlo model for this study is well-vetted.

3. Results

3.1. Municipal wastewater

As described in the Supplemental information, analysis of untreated and treated wastewater samples provided data regarding baseline levels and ratios of *Bacteroidales* in illicit discharges (untreated wastewater) and just downstream of WWTP outfalls (treated wastewater). The BacHum:BacUni ratio was found to be geographically-dependent but relatively stable within a region, and levels of *Bacteroidales* in tertiary-treated, disinfected effluent were found to be relatively high compared to ambient surface water.

3.2. Surface waters

3.2.1. Prevailing rates in surface waters

The prevailing rate, or positive detection frequency, allows for a simple assessment of the predominance of investigated sources. The microbial indicators that were assayed with qPCR during this study varied widely in their prevailing rates (Table 1). The universal *Bacteroidales* marker (BacUni) was detected in all 74 surface water samples (detection frequency of 100%), while enterovirus and adenovirus were only detected in one and eight samples (1% and 11%), respectively. Of the host-associated *Bacteroidales* markers, the human-associated marker (BacHum) was detected most frequently (detection frequency of 90%) and the cow-associated marker (BacCow) least frequently (55%). BacCow was only detected in two of eight samples (20%) from Revolon Slough (4-B), which is dominated by agriculture (row crops, not livestock).

The climate of Calleguas Creek watershed is arid, with storms generally being limited to the winter and spring seasons. Annual rainfall is approximately 15 inches. The prevailing rates of all host-associated *Bacteroidales* markers were higher during wet weather, and one of eight (12.5% detection frequency) adenovirus detections occurred during wet weather. The mean estimated percent recovery of each *Bacteroidales* marker from wet weather CCW samples was not significantly different [p = 0.50] from dry weather samples (data not shown). The wide range of detection frequencies for qPCR targets suggests that our corresponding estimates of sample-specific limits of detection (SLOD; Fig. 2 shows the SLODs for each marker) were important to ensure data analysis and interpretation reflects varying SLODs. The ubiquity (i.e., high frequency of detection) of BacUni, BacHum, and BacCan suggests that qualitative (presence/absence) PCR would not provide much insight with regards to the impact of these bacteria sources on CCW.

The fact that a large portion of the collected samples were non-detect suggests that data handling of non-detects can effect report summary statistics. The potential effect of data handling is illustrated in the reported summary statistics for BacCow during dry weather, which was not detected in 39% of samples, for three different approaches: Kaplan−Meier, ROS, and MLE (Table 2). The estimated mean and median by the different tests differ by up to a factor of 4.1 (median of Kaplan−Meier versus ROS). The effect of non-detects should also be considered when graphically presenting datasets; the cumulative distribution plots in Fig. 3 present the potential range of non-detect samples.

3.2.2. Spatial and hydrologic variations in abundance

Variations over space (site-by-site) and hydrology (wet versus dry weather) were assessed to elucidate the characteristics of FIB sources that are impacting the CCW. Concentrations of BacUni, BacCow, BacCan, and *E. coli* were significantly higher during wet weather (p < 0.005). For surface water data, rather than using the "raw" host-associated:universal marker ratios, a statistical model described by Wang et al. (2010) was used to generate "true" ratios, referred to as Hum_{ratio}, Cow_{ratio}, and Dog_{ratio}, and $Other_{ratio}$. These true ratios reflect conditional probabilities that incorporate the rate of false positives and

Table 1 – Summary of Kaplan–Meier statistics for Bacteroidales and adenovirus concentrations measured in the CCW, grouped by hydrologic condition (dry versus wet weather).

Statistic	BacUni (cell eq/mL)		BacHum (cell eq/mL)		BacCow (cell eq/mL)		BacCan (cell eq/mL)		Adenovirus[a] (genomes/mL)	
	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry
N[b]	15	59	14	59	15	59	15	59	15	58
N detected	15	59	13	53	13	36	15	47	1	8
% detected	100	100	92.9	89.8	86.7	61.0	100	79.7	6.7%	13.8%
Median	2747	1111	95	43	2.5	0.3	36	1.0	NA[c]	NA
Mean	12,019	3673	300	500	57	1.6	47	1.7	NA	NA
Std. dev.	19,092	11,353	680	2211	151	3.5	55	2.7	NA	NA
10th %ile	384	67	46	0.0001	0.7	0.02	5	0.030	NA	NA
25th %ile	1649	320	66	7.0	1.5	0.03	17	0.120	NA	NA
50th %ile	2747	1111	95	43	2.5	0.33	36	1.0	NA	NA
75th %ile	14,284	3089	147	162	44	1.6	59	3.0	NA	NA
90th %ile	40,096	6439	341	957	64	4.6	67	4.0	1432	613

a Enterovirus was detected in 1 of 58 (1.7%) dry weather samples and zero wet weather samples.
b Number of samples.
c Not applicable because of insufficient number of detects to reliably estimate summary statistic.

negatives inherent in MST assays, and provide a more quantitative MST framework compared to the raw ratios. During wet weather (Table 3), Hum_{ratio} was significantly lower [p = 0.02] compared to dry weather while Cow_{ratio} and Dog_{ratio} were significantly higher [p < 0.018] (Fig. 3 shows the distributions of measured concentrations during dry and wet weather while accounting for non-detects in the dataset).

Due to the significant differences in detection frequencies and abundance during wet versus dry weather, and lower number of samples available for the wet weather condition, spatial variations were only assessed for the dry weather condition (Fig. 4). The only statistically significant spatial difference in MST marker abundance among sites was for BacHum and Hum_{ratio} at the intermediate Conejo Creek site (9A-B), with at least one being significantly higher [p < 0.05 with B&H correction] than all other sites except the upstream Conejo Creek site (10-B). None of the other marker-site or ratio-site combinations exhibited significant differences. Virus detections were too rare to reliably assess spatial differences; adenovirus was only detected more than once at the upstream and intermediate sites along the Conejo Creek transect (10-B and 9A-B). Concentrations of adenovirus were significantly higher during wet weather [p < 0.001]. The only enterovirus detection was at the Revolon Slough site (4-B).

3.2.3.   Relationships among measurements

Correlations among the measured parameters were evaluated using the 59 samples collected during dry weather. The 15 wet weather samples were not included because the concentrations of most of the Bacteroidales targets, E. coli and TSS were significantly higher during wet weather, possibly leading to dry and wet weather "clusters" that could induce less-meaningful correlations. Correlations were based on tests of Kendall's τ, which incorporates SLODs.

During dry weather, the Bacteroidales measurements were weakly correlated to one another [τ > 0.275, p < 0.001], but not to E. coli or TSS. The strongest correlation among the Bacteroidales markers was for BacUni and BacHum [τ = 0.596, p < 0.001]. The fact that Bacteroidales markers correlated with one another, but not with E. coli, is likely a reflection of the differences in both organism ecology (e.g., facultatively anaerobic versus anaerobic) and quantification methodology (e.g., viability-versus genome-based methods). In addition, this suggests that sites along our transects were subject to discharges from multiple source types simultaneously (e.g., inputs from both cow and human sources occurred). Adenovirus concentrations were not correlated to any other variable.

4.   Discussion

This is the first study known to contemporaneously analyze on a watershed-scale Bacteroidales and human virus concentrations in flowing freshwater impacted by municipal wastewater. Overall, our study design was based on evaluating relative differences in universal and host-associated Bacteroidales and human virus concentrations over space and time (or weather condition), as elevated host-associated Bacteroidales concentrations were assumed to be due to fecal discharges from that host population (e.g., BacCow is due to

Table 2 – Comparison of Kaplan–Meier, ROS, and MLE summary statistics for concentrations of BacCow measured in the CCW during dry weather.

Statistic	Statistical method		
	Kaplan–Meier	Regression on order statistics	Maximum likelihood estimator
	(Cell eq/mL)	(Cell eq/mL)	(Cell eq/mL)
N[a]	59	59	59
N detected	36	36	36
% detected	61.0	61.0	61.0
Median	0.33	0.08	0.23
Mean	1.6	1.5	3.5
Std. dev.	3.5	3.5	53
10th %tile	0.02	0.02	0.01
25th %tile	0.03	0.04	0.05
50th %tile	0.33	0.08	0.2
75th %tile	1.6	1.6	1.1
90th %tile	4.6	3.9	4.5

[a] Number of samples.

impacts by cows in the watershed). Such an assumption is warranted based on the efforts taken to develop and validate the applied MST markers and ultrafiltration method (Kildare et al., 2007; Rajal et al., 2007a, 2007b), but as discussed below, there are a number of confounding factors, such as decay rates and viability, that should be considered when designing, conducting, and analyzing the results of MST studies.

Significantly elevated concentrations and ratios of BacCow and BacCan during wet weather, along with significantly lower concentrations and true ratios of BacHum during wet weather (Fig. 3, Tables 1 and 3), indicate that non-human sources may be responsible for the significantly elevated BacUni, and perhaps *E. coli*, concentrations that occur during storm events in the CCW. The non-human sources responsible for elevated *Bacteroidales* loading during storm events in CCW are likely contributing to the corresponding exceedances of *E. coli* criteria and should be an important consideration for local stakeholders during TMDL implementation.

The fact that sites 9A-B and 10-B along Conejo Creek receive direct inputs of treated WWTP effluent increases the likelihood that non-viable (disinfected) cells may be responsible for the elevated concentrations of BacHum and the detections of adenovirus at these sites. Evidence of the influence of non-viable cells is provided by that fact that sites 9A-B and 10-B exhibited relatively high concentrations of BacHum (genome-based measurement) but relatively low concentrations of *E. coli* (viability-based measurement) (Fig. 4).

Detection frequency of MST and pathogen marker can be quite low, as reflected by adenovirus, enterovirus and BacCow in this study. As shown with the simple comparison of summary statistics produced by Kaplan–Meier, ROS and MLE methods, the handling of these non-detects can affect findings and conclusions regarding sources. In the field of MST, the handling of non-detects has generally been rudimentary, for example, often replacing non-detect values with one-half the LOD. This study demonstrates that statistical methods and graphical procedures from the field of survival analysis can be readily employed to handle the high rate of non-detects and sample-specific LODs (Helsel, 2005).

4.1. Applicability of host-associated-to-universal *Bacteroidales* ratios

Bacteroidales concentrations were analyzed both individually and with respect to the true ratios of the host-associated-to-universal marker (Table 3). However, additional research is needed before host-associated:universal *Bacteroidales* ratios can be used in a truly quantitative manner (e.g., cows versus dogs) to assess the dominant source(s) to collected water samples (Wang et al., 2013).

To use host-associated:universal *Bacteroidales* ratios for fecal load allocations the following three relationships should be evaluated, discussed further below: (i) the environmental persistence of the host-associated marker when compared to the universal marker and other host-associated markers, (ii) the value of the host-associated to universal ratio and its variability (i.e., stability) in fecal sources, and (iii) if the ratios are to be used for source apportionment of FIB and/or pathogens, then the relative abundance and environmental persistence of *Bacteroidales* versus FIB and/or pathogens. In addition, the specificity and sensitivity of the applied MST assays should be incorporated, which was the purpose of generating true ratios Hum_{ratio}, Cow_{ratio}, and Dog_{ratio} with the Monte Carlo model.

With regards to (i), the persistence of the universal and host-associated *Bacteroidales* markers used for this study is known to be comparable among the four markers studied here in both freshwater and seawater environments. The markers were previously evaluated using flow-through, open-air microcosms in seawater and freshwater under dark and sunlit (diurnal cycle) conditions (Bae and Wuertz, 2009, 2012). It was concluded that decay rates among universal (BacUni) and host-associated *Bacteroidales* markers (BacHum, BacCow, and BacCan) were not significantly different, suggesting that differential persistence is not a limiting factor for quantifying relative source contribution.

Relationship (ii) was partially addressed in the present study for untreated sewage discharges; the BacHum:BacUni ratio appeared to be relatively stable in regional sewage (Table S1), suggesting that it can be used as a "signature" of
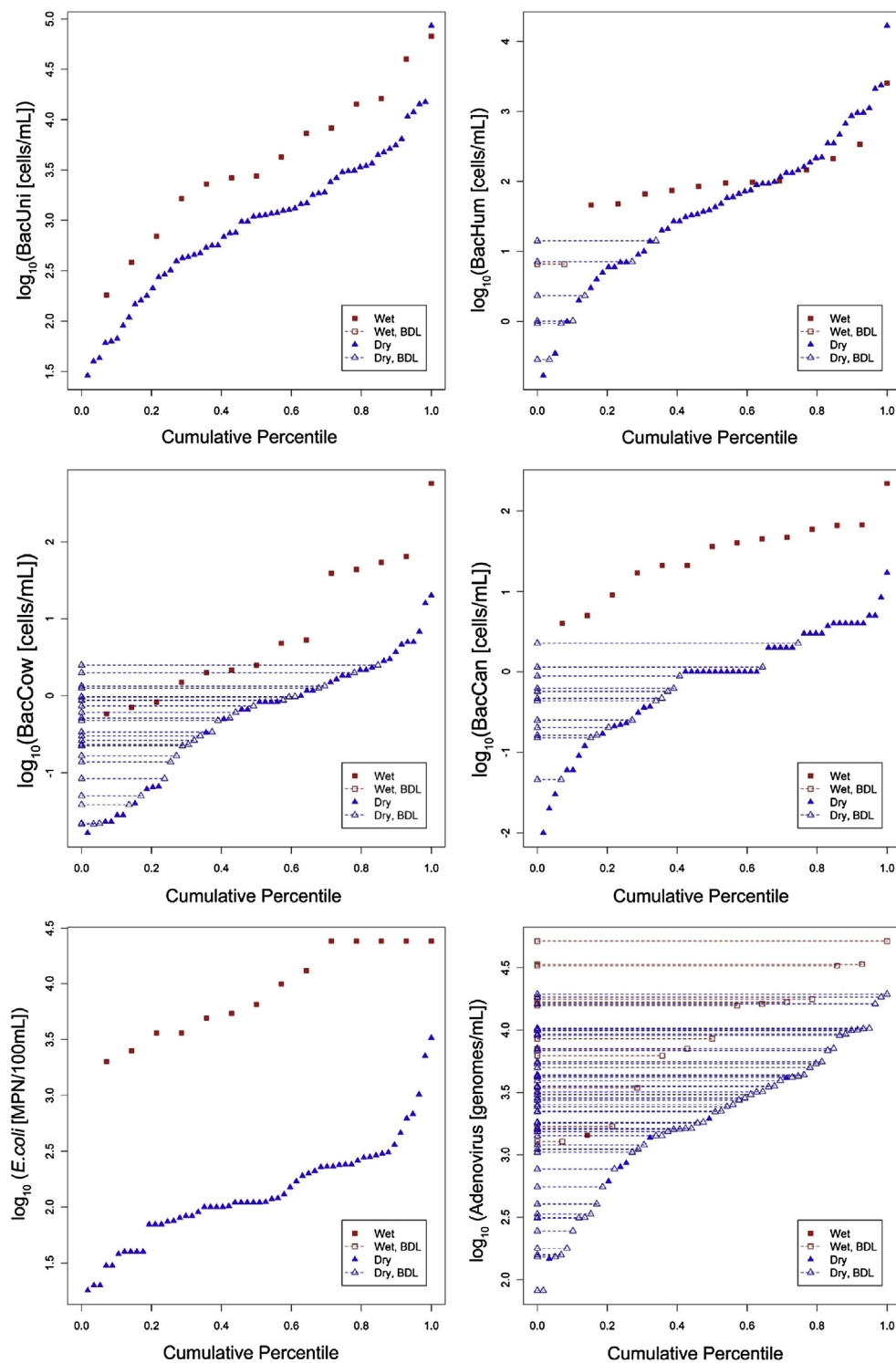
Fig. 3 − Cumulative distribution plots of *Bacteroidales*, adenovirus, and *E. coli* concentrations measured during dry weather (blue triangles) and wet weather (red squares). Non-detect (BDL) samples are plotted at the SLOD without fill and the dotted lines show the potential range of percentiles for the sample (samples with low SLODs have a smaller range than samples with high SLODs). The dotted lines in these figures transparently show the potential effect of non-detect samples on estimated percentiles (and summary statistics). The 50th percentile value of the x-axis represents the study median. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3 – Summary of Kaplan–Meier statistics for host-associated to universal *Bacteroidales* ratios measured in the CCW, grouped by hydrologic condition (dry versus wet weather).

Statistic	Hum_{ratio}		Cow_{ratio}		Can_{ratio}	
	Wet	Dry	Wet	Dry	Wet	Dry
N[a]	14	59	15	59	15	59
N detected	13	53	13	36	15	47
% detected	92.9	89.8	86.7	61.0	100	79.7
Median	0.02	0.05	0.01	0.002	0.07	0.01
Mean	0.03	0.08	0.04	0.02	0.19	0.03
Std. dev.	0.03	0.07	0.07	0.04	0.25	0.08
10th %tile	0.0001	0.002	0.0001	0.0001	0.017	0.001
25th %tile	0.001	0.01	0.005	0.0001	0.04	0.003
50th %tile	0.018	0.05	0.015	0.002	0.07	0.01
75th %tile	0.04	0.13	0.04	0.01	0.30	0.02
90th %tile	0.08	0.20	0.15	0.07	0.61	0.05

a Number of samples.

human fecal impacts, but the ratio might vary geographically. For fecal discharges from individual humans, however, the BacHum:BacUni ratio was highly variable, possibly limiting its utility for areas subject to individual as opposed
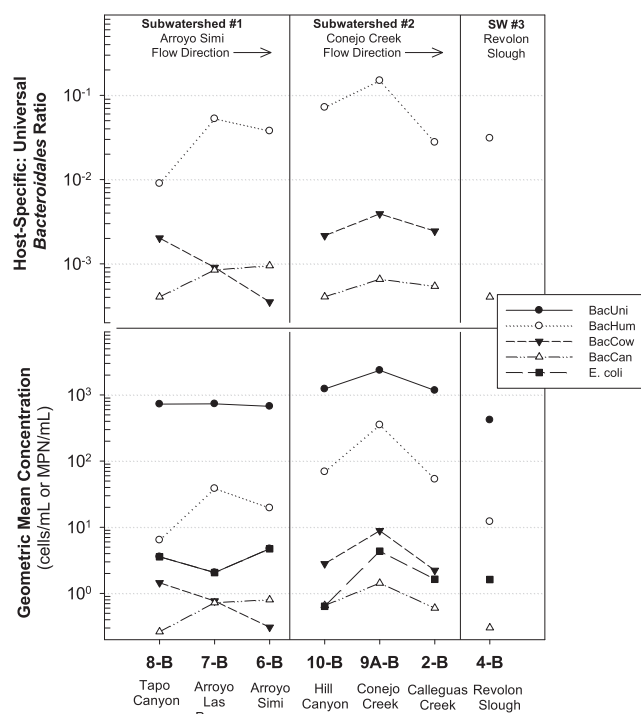

Fig. 4 – Geometric mean *Bacteroidales* and *E. coli* concentrations (bottom plot) and host-associated to universal *Bacteroidales* ratios (top plot) measured during dry weather along three transects in CCW (Arroyo Simi, left; Conejo Creek, center; Revolon Slough, right). Ratios were calculated for each sample as the host-associated *Bacteroidales* marker concentration (open circle, BacHum; filled triangle, BacCow; open triangle, BacCan) divided by the BacUni concentration (filled circle, BacUni). *E. coli* concentrations are shown in the bottom plot (filled square). Error bars are not shown to allow for plotting within a single figure.

to mixed human fecal sources (e.g., areas with homeless persons).

Finally, relationship (iii) is especially critical for studies related to TMDLs – the linkage between *Bacteroidales* and FIB hinges on the relative abundance of *Bacteroidales* in fecal sources and the relative persistence. *Bacteroidales* may be relatively abundant in the fecal samples from a given host, while *E. coli* are relatively low. Based on the fecal samples analyzed during the watershed-specific validation of the qPCR markers applied herein, this is likely the case for seagulls (Kildare et al., 2007), which are more amendable to MST with *Catellicoccus* (Sinigalliano et al., 2013). With regards to relative environmental persistence, the most critical relationship for human risk assessment is relative decay rates of pathogens versus *Bacteroidales*. Walters and Field (2006) found that BacHum exhibited similar survival characteristics to infectious enteroviruses in a sunlight-exposed, sewage-derived microcosm, with both being detected through 8 days of experiment. Bae and Wuertz (2012) found that *Bacteroidales* and *Campylobacter* cells exposed to sunlight exhibited similar survival rates, and host-associated *Bacteroidales* DNA and waterborne pathogen DNA were degraded at comparable rates.

Because of these remaining data gaps (and others), the *Bacteroidales* ratios calculated herein were only used in a "within-host" framework among sites and weather conditions, just as with the corresponding concentrations, instead of attempting to quantify the relative contribution of fecal discharges from the different host populations (e.g., Bac-Cow:BacUni is not compared to BacHum:BacUni). Furthermore, it is acknowledged that the *Bacteroidales* ratios do not necessarily reflect the relative abundance of sources of FIB.

4.2. Influence of treated WWTP effluent on qPCR-based MST

Our results demonstrate that the relatively high concentrations of *Bacteroidales* and human virus cells in WWTP effluent confound qPCR-based MST efforts. MST with qPCR does not distinguish between treated and untreated sources of human feces, which is disconcerting for stakeholders seeking to identify sources of bacteria in an attempt to reduce human

health risks in recreational waters. Source trackers should either (a) segregate sites that do and do not receive treated WWTP effluent during statistical analyses of the relative values of BacUni, BacHum and BacHum:BacUni or (b) apply laboratory or field techniques that remove/attenuate non-viable cells from water samples prior to performing qPCR assays.

With regards to approach (a), MST study designs and data analysis should evaluate samples collected downstream of WWTP effluent discharges separately from samples collected either upstream of the WWTP discharge or from untreated discharges to the waterbody (e.g., urban runoff). For instance, considering the sites within the CCW that do not receive treated WWTP effluent, it should be disconcerting to watershed managers that site 4-B had higher levels of BacHum and Human$_{ratio}$ (and a higher virus detection rate) when compared to site 8-B. However, for most MST applications the concentrations measured at site 8-B should not be directly compared to site 10-B, which receives treated WWTP effluent.

For approach (b) above, the use of propidium monoazide (PMA) with qPCR (PMA-qPCR) has been found to show promise for distinguishing between viable and non-viable *Bacteroidales* cells in sewage and treated WWTP effluent (Bae and Wuertz, 2009). That PMA-qPCR approach was optimized using the four assays applied during this study, and concentrations of BacUni, BacHum, BacCow, and BacCan measured by PMA-qPCR decayed much more rapidly in freshwater and seawater when compared to concentrations reported by qPCR (Bae and Wuertz, 2009, 2012). Future MST and pathogen studies of watersheds influenced by WWTP effluent should consider the application of PMA-qPCR. The CCW study was performed prior to optimization of the PMA-qPCR approach, and thus future applications of this dataset for source assessment should rely on approach (a) describe above (data segregation).

4.3.   Occurrence of human viruses in surface waters

In CCW, prevailing rates of adenovirus and enterovirus are much lower (11% and 1%, respectively) when compared to those for *Bacteroidales* (Table 1). The much higher detection rate of *Bacteroidales* when compared to human virus may be expected, as *Bacteroidales* are abundant in the feces of a majority of hosts (Menaja et al., 1996), while viruses are only shed by hosts that are infected. The presumed low abundance of human virus was the motivation for collecting 100-L samples during this study; *Bacteroidales* could be readily detected using much smaller samples volumes (Dick and Field, 2004). As in this study of the CCW, other viral studies in southern California have detected adenovirus more frequently than enterovirus (Jiang and Chu, 2004; Choi and Jiang, 2005). However, these studies detected human virus more frequently during the winter months, while six of nine (67%) detections in CCW were during the summer months. Like previous viral studies that demonstrated the lack of relationship among virus occurrence and compliance with microbial water quality criteria (Gerba et al., 1979; Jiang et al., 2001; Noble and Fuhrman, 2001; Griffin et al., 2003), eight of nine (89%) human virus detections in CCW occurred when *E. coli*

concentrations were below the single sample criteria of 235 MPN/100 mL.

As with human *Bacteroidales*, the presence of treated WWTP effluent may confound attempts to identify high-risk human virus sources. Other studies have shown human virus genomes to be readily detected in treated WWTP effluent, while corresponding viable virus titers were typically quite low (Boehm et al., 2005). In the present study, seven of nine (78%) human virus detections occurred in waters dominated by treated WWTP effluent discharges but the viability/infectivity of these viruses are unknown. A recent QMRA study based on the adenovirus concentrations in the CCW reported here, which assumed various proportions of detected viruses were infectious, estimated that human health risks associated with primary and secondary water contact were lower than acceptable thresholds by USEPA (Kundu et al., 2013).

5.   Conclusions

This study combined (i) large-volume hollow fiber ultrafiltration of surface water samples using a multiple replicate dilution approach and incorporating estimates of SLODs based on spiked surrogates, (ii) quantification of multiple serotypes of adenovirus and enterovirus, (iii) application of four validated probe-based *Bacteroidales* assays, and (iv) data analysis with a Monte Carlo model and statistical routines that account for non-detects and sample-specific LODs.

The results demonstrate that MST based on *Bacteroidales* assays can inform watershed managers seeking to develop strategies to comply with REC criteria, but it is critical to handle non-detects with appropriate statistical methods and to acknowledge the underlying assumptions of qPCR-based MST. While MST shows promise for providing quantitative source apportionment, there are still data gaps including relative decay rates of FIB, *Bacteroidales* and pathogens in effluent-impacted surface waters and lack of qPCR assays for viruses that reflect viable/infective concentrations (e.g., using PMA). Eventually, MST markers may support not only source apportionment but also risk assessment, given additional epidemiological data and/or empirical descriptions of pathogen—*Bacteroidales* relationships.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2015.02.023.

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