Quantification of pathogens and markers of fecal contamination during storm events along popular surfing beaches in San Diego, California

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A B S T R A C T

Along southern California beaches, the concentrations of fecal indicator bacteria (FIB) used to quantify the potential presence of fecal contamination in coastal recreational waters have been previously documented to be higher during wet weather conditions (typically winter or spring) than those observed during summer dry weather conditions. FIB are used for management of recreational waters because measurement of the bacterial and viral pathogens that are the potential causes of illness in beachgoers exposed to stormwater can be expensive, time-consuming, and technically difficult. Here, we use droplet digital Polymerase Chain Reaction (digital PCR) and digital reverse transcriptase PCR (digital RT-PCR) assays for direct quantification of pathogenic viruses, pathogenic bacteria, and source-specific markers of fecal contamination in the stormwater discharges. We applied these assays across multiple storm events from two different watersheds that discharge to popular surfing beaches in San Diego, CA. Stormwater discharges had higher FIB concentrations as compared to proximal beaches, often by ten-fold or more during wet weather. Multiple lines of evidence indicated that the stormwater discharges contained human fecal contamination, despite the presence of separate storm sewer and sanitary sewer systems in both watersheds. Human fecal source markers (up to 100% of samples, 20-12440 HF183 copies per 100 ml) and human norovirus (up to 96% of samples, 25-495 NoV copies per 100 ml) were routinely detected in stormwater discharge samples. Potential bacterial pathogens were also detected and quantified: Campylobacter spp. (up to 100% of samples, 16-504 gene copies per 100 ml) and Salmonella (up to 25% of samples, 6-86 gene copies per 100 ml). Other viral human pathogens were also measured, but occurred at generally lower concentrations: adenovirus (detected in up to 22% of samples, 14-41 AdV copies per 100 ml); no enterovirus was detected in any stormwater discharge sample. Higher concentrations of avian source markers were noted in the stormwater discharge located immediately downstream of a large bird sanctuary along with increased Campylobacter concentrations and notably different Campylobacter species composition than the watershed that had no bird sanctuary. This study is one of the few to directly measure an array of important bacterial and viral pathogens in stormwater discharges to recreational beaches, and provides context for stormwater-based management of beaches during high risk wet-weather periods. Furthermore, the combination of culture-based and digital PCR-derived data is demonstrated to be valuable for assessing hydrographic relationships, considering delivery mechanisms, and providing foundational exposure information for risk assessment.

1. Introduction

Coastal southern California, with its Mediterranean climate patterns receives >95% of its precipitation during the winter season, with 70% of the precipitation occurring between January and March (NRC Report, 1990; Ackerman and Weisberg, 2003). Urban

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stormwater runoff in southern California is known to contain high concentrations of fecal indicator bacteria (FIB) such as total and fecal coliforms and *Enterococcus*, which has been shown to have median concentrations ranging from 100-100,000 MPN per 100 ml (Griffith et al., 2010; Schiff and Kinney. 2001; Gannon and Busse, 1989; Brownell et al., 2007; Tiefenthaler et al., 2011; Parker et al., 2010). The result is an observed increase in FIB concentrations at marine bathing beaches from median Enterococcus concentrations of 10−100 MPN per 100 ml during dry weather and 10-10,000 MPN per 100 ml following storm events (Ackerman and Weisberg, 2003; Noble et al., 2003).

Concerns about exposure to pathogens and subsequent adverse human health effects (Given et al., 2006) have culminated in routine advisories against body contact with recreational waters from coastal Public Health Departments for up to 72 h following rain events (Thoe et al., 2014). Yet, in southern California, surfers regularly enter the ocean following rainstorms despite the well-advertised warnings of illness from public health officials because that is often when the best surf conditions occur. Even though stormwater is known as a major conduit of fecal contamination in southern California, relatively few previous studies exist that fully quantified bacterial and viral pathogens associated with fecal contamination (e.g. Jiang et al., 2001). Of those that have been published, little or no hydrographic information was available to provide context.

Monitoring FIB at marine beaches is useful for assessing public health risk because FIB concentrations have been previously determined to be related to illness rates of swimmers (e.g. Haile et al.,1999, Wade et al. 2010). FIB are also relatively easy and inexpensive to measure compared to actual pathogens. However, total coliforms, fecal coliforms, *E. coli,* and *Enterococcus spp.* are rarely the causative agents of illness. Instead, FIB co-occur with a wide range of pathogens found in human feces that may cause illness, including viruses, pathogenic bacteria, and/or protists (Prüss, 1998; Fong and Lipp, 2005); however, the relationships between presence of FIB and actual human pathogens in environmental waters are unpredictable (e.g. Bosch. 1998; Griffin et al., 2003; Fong and Lipp, 2005; Wu et al., 2011; McQuaig et al., 2012; Corsi et al., 2014). This is especially important in areas where storm drainage systems are separate from sanitary sewer systems, such as southern California, that present no *a priori* expectation to find human pathogens in stormwater runoff.

Several studies have been conducted to assess the impact of human noroviruses (NoV) and other human viral pathogens in sewage and on coastal waters in the context of sewage discharge and other potential anthropogenic sources (Eftim et al., 2017; Hassard et al., 2017). However, to our knowledge, there are very few reported calculations of human viral pathogen load from storm events, and likely none for stormwater discharge proximal to a high use recreational location. Therefore, identifying the sources of fecal contamination, and full quantification of pathogens are important steps toward understanding the risk to surfers using receiving waters for recreation following storm events (Soller et al., 2010, 2017).

Stormwater discharges (also called freshwater outlets in some previous documents) in southern California have previously been found to contain human fecal contamination confirmed through the presence of human viral pathogens such as enterovirus, NoV, and adenovirus (AdV) throughout the region (e.g. Noble et al., 2006; Noble and Fuhrman, 2001; Jiang et al., 2001). Several of the existing published studies measured the pathogen concentrations in stormwater samples from a range of locations, but techniques available at the time limited the ability to precisely quantify the pathogens (e.g. Jiang et al., 2001). New technological applications of droplet digital polymerase chain reaction (digital PCR) have enhanced the ability to measure microbial source tracking (MST) markers of host organisms and viral and bacterial pathogens present in stormwater runoff while being robust to inhibitory substances (Cao et al., 2015a; Coudray-Meunier et al., 2015).

The main objective of this study was to investigate viral and bacterial pathogen dynamics in the stormwater of two urban watersheds that discharge to high-use swimming/surfing beaches in southern California. To accomplish this, we used state-of-the-science quantitative analyses to determine concentrations of important viral and bacterial pathogens, along with MST markers selected for their high specificity and sensitivity (Boehm et al., 2013) and FIB quantification during multiple storms that occurred during winter 2014 and 2015. The microbial contaminant information was paired with available hydrographic information in order to assess emergent hydrographic relationships that drive illness risk to surfers during wet weather conditions. This exercise was conducted in tight coordination with a concurrent epidemiology study (Arnold et al., 2017) and QMRA modeling effort (Soller et al., 2017). We specifically selected these two distinct watersheds that discharge to popular surfing beaches in San Diego, CA to compare our findings in the context of land use, watershed size, and discharge impacts. Surfers at the beaches at the end of these watersheds reported a combined 4088 surfing days in wet and dry weather during the study (Arnold et al., 2017). A final objective of the study was to utilize principal component analysis and other relational statistical approaches to assess relationships across MST markers and pathogen types, yielding potentially valuable information for subsequent mitigation efforts.

2. Materials and Methods

2.1. Study design and water sample collection

The basic study design had two elements: ocean receiving waters and stormwater discharges. The ocean receiving water element focused exclusively on cultured FIB measurements, but was sampled at multiple sites at differing distances from the stormwater discharge point daily from January−March 2014 and December-March 2015, with the exception of Dec. 24, 25, and 31, 2014 and January 1, 2015. In this way, we captured the spatial and temporal influence of the stormwater discharges on the beach receiving water environment.

The stormwater discharge element focused on multiple microbial targets, but was limited to a single sampling location at the end of each watershed just before discharging to the beach, and exclusively during wet weather. Wet weather was defined to be consistent with the County of San Diego Public Health Department rain advisory; the day of rain ≥2.54 mm (≥0.1 inch), plus 72 h (3 days). The additional measurements (in addition to the same FIB measured in the ocean) included host specific MST markers (human, avian, canine), viral pathogens (NoV genogroups I and II, enterovirus, AdV), and bacterial pathogens (*Campylobacter, Salmonella*).

2.1.1. Beaches
Daily ocean water samples were collected from January 15, 2014 to March 5, 2014 and from December 2, 2014 to March 31, 2015 at a total of six sites from two California beaches: Tourmaline Surfing Park (N = 2) and Ocean Beach in San Diego, CA (N = 4) (Fig. 1). Beach sampling details are described in the Supplementary Materials and Methods.

2.1.2. Watersheds
Tourmaline Creek is a small highly urbanized watershed (Fig. 1, S1). The watershed is approximately 3.3 km^2 and 86% developed
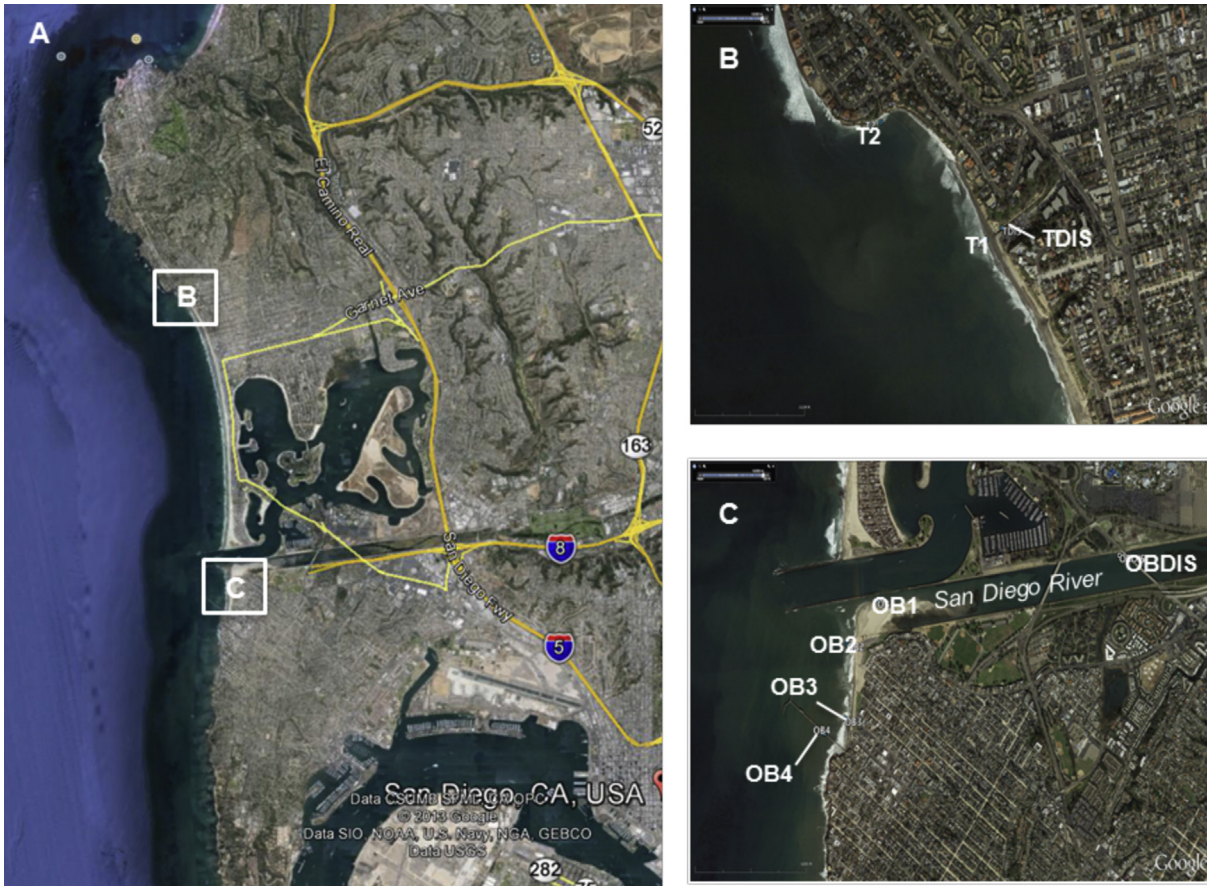
Fig. 1. Map of (A) two popular surfing beaches in San Diego, CA with insets of (B) Tourmaline Surfing Park and (C) Ocean Beach showing study sampling locations, including stormwater discharges for Tourmaline Creek (TDIS) and San Diego River (OBDIS).

land use, the majority of which (62%) is urban residential and commercial (Table 1). The San Diego River is a much larger and more diverse watershed (Fig. 1, S2). In total, the San Diego River is 1124 km^2, but two major dams are located on this system, and neither dam discharged during the study period. The watershed area below the dams is 451 km^2, and 53% is developed land use (Table 1). The development is composed of urban residential, commercial and industrial land uses, with a relatively small proportion of agricultural area, especially in the lower floodplain. A bird sanctuary is located along the lower 1.5 km estuarine portion of the San Diego River. Both Tourmaline Creek and the San Diego

River are located within a large urban, coastal population center and serve as storm drainage separate from the sanitary sewer system.

2.1.3. Discharges

Six storms were sampled from Tourmaline Creek and San Diego River immediately upstream from the Tourmaline Surfing Park and Ocean Beach, respectively (Fig. 1). Time-weighted composite samples were collected which comprised of grab samples every 30 min until flow decreased below levels at which samples could be collected at Tourmaline Creek or salinity rose above 22 ppt at San

Table 1
Watershed characteristics and storm flow for the San Diego River and Tourmaline Creek.

	San Diego River	Tourmaline Creek
Area	451 km^2	3.9 km^2
Land Use Rank		
1	Open Space (41%)	Single Family Residential (62%)
2	Single Family Residential (20%)	Roads (19%)
3	Roads (8%)	Open Space (13%)
Sewer Pipe Material Rank		
1	Vitrified Clay (61%)	Vitrified Clay (63%)
2	Polyvinyl Chloride (30%)	Polyvinyl Chloride (35%)
3	Concrete Pipe (2.5%)	Concrete Pipe (1.7%)
Total Storm Flow		
12/2- 12/5/2014	1.15 Million m^3	not measured
12/12- 12/15/2014	1.12 Million m^3	not measured
1/11- 1/14/2015	0.36 Million m^3	not measured
2/23- 2/26/2015	0.23 Million m^3	not measured
3/1-3/4/2015	0.96 Million m^3	0.029 Million m^3

Diego River (indicating high tide), or 6 h had elapsed, whichever occurred first. If rainfall persisted after 6 h, a second time-weighted composite was started to sample from 6 to 12 h. At both sites, composite samples were collected in clean, pre-sterilized and site rinsed 20 L high density polyethylene or polypropylene containers. In addition, at both discharge sites, 20 L grab samples were collected each day over the next three days consistent with the wet weather definition. Further details are described in the Supplementary Materials and Methods.

2.1.4. Environmental observations

Water temperature and salinity were measured at the time of sampling using a handheld YSI Pro30 temperature and conductivity meter (YSI, Yellow Springs, Ohio). Tidal data were obtained from the NOAA observation station in Quivira Basin, San Diego (NOAA number TWC0413, 32.7667N, 117.2333W), located in between the two study beaches. Wind speed and direction were measured at the time of sampling. Observations were recorded at the time of sampling for wave height, number of surfers in the water, number of dogs on the beach, and number of birds on the beach or in the water. Precipitation data was taken from the National Weather Service station at the San Diego Lindbergh International Airport (KSAN).

2.2. Culture methods

2.2.1. FIB membrane filtration

For both beaches and stormwater discharges, *Enterococcus* concentrations were measured using standard culture-based methods: EPA Method 1600 (USEPA, 2006). Briefly, a 100 ml sample of water and 1:10 and 1:100 dilutions with Sterile phosphate buffered saline (PBS) were filtered through a sterile 47 mm diameter. type HA 0.45 μm (Millipore, Bedford, MA, USA). The filter was placed on mEI agar media. The plate is inverted and incubated at $41.0 \pm 0.5\,°C$ for 24 ± 2 h. Analyses met quality control objectives for absence of background contamination (blanks) and minimum precision (duplicates) of $\leq 10\%$. Detailed methods are described in the supplemental material.

2.3. Molecular methods

Very brief methods are presented below. Detailed methods are described in the Supplementary Materials and Methods. Primer and probe sequences for all assays are described in detail in Table S1.

2.3.1. Filtration for bacteria and viruses

To collect bacterial DNA, 100 ml of seawater or stormwater was filtered on a vacuum manifold through 47 mm dia. 0.4 μm polycarbonate filters (Millipore, Bedford, MA, USA). To collect viruses, an adsorption method using mixed cellulose ester (type HA; Millipore, Bedford, MA, USA) filters was employed to filter 200-500 ml brackish and fresh stormwater samples amended to a final concentration of 25 mM $MgCl_2$ and a pH < 3.5 (Katayama et al., 2002; Conn et al., 2012).

2.3.2. DNA/RNA extraction

Filters for bacterial DNA analyses were extracted using commercial kits (DNA EZ RWO4, GeneRite, Mammoth Junction, NJ, USA) following previously published methods (Cao et al., 2015a; Boehm et al., 2013; Layton et al., 2013).

Virus filters were extracted by one of two methods over the course of the study. Method A: PowerViral Environmental RNA/DNA Extraction Kit (formerly MoBio Laboratories, Carlsbad, CA, presently AllPrep PowerViral DNA/RNA kit, QIAGEN, Germantown,

MD). Method B: modified Qiagen RNEasy Plus Universal Mini kit (Qiagen, Germantown, MD, USA).

2.3.3. Digital PCR

Human, avian, and canine-associated MST markers, *Campylobacter, Salmonella,* human AdV, human NoV genogroups I and II, and pan-enterovirus were quantified using digital PCR assays and described below and in the Supplementary Materials and Methods.

2.3.4. Microbial source tracking markers

Microbial source tracking markers were quantified using previously published digital PCR assays or qPCR assays adapted to digital PCR for human markers (HF183 Cao et al., 2015a), avian markers (Lee et al., 2013; Sinigalliano et al., 2013), and canine markers (DG3, Green et al., 2014).

2.3.5. Bacterial pathogens

Bacterial pathogens were quantified using digital PCR assays adapted from previously published qPCR assays for *Campylobacter* (Cao et al., 2016; Lund et al., 2004; LaGier et al., 2004; He et al., 2010; Vondrakova et al., 2014) and *Salmonella* (González-Escalona et al., 2009; Malorny et al., 2008).

2.3.6. Viral pathogens

Viral pathogens were quantified using digital PCR assays adapted from previously published qPCR assays for human AdV (Jothikumar et al., 2005; Cao et al., 2015b), human NoV genogroups I and II (da Silva et al., 2007), and pan-enterovirus (Gregory et al., 2006).

2.4. Statistical analyses and watershed calculations

Spatial distributions of FIB relative to the stormwater discharge location at both surfing beaches were analyzed and contrasted in dry versus wet weather. *Enterococcus*, human pathogens, human source markers, and animal source markers were analyzed for detection frequency. For those assay targets with sufficient detection frequency, geometric mean concentrations were compared between watersheds and among storm events. Concentrations of *Enterococcus,* human pathogens, human source markers, and animal source markers were compared to storm event characteristics and correlated to one another. Principal components analysis was performed on the pathogen and source marker data to explore patterns among these variables.

All data were transformed as the $\log_{10}$ of the measured concentrations. Samples that were below detection were assigned a value of 0 for the analyses, although we cannot rule out a low concentration of the bacteria or viruses. To calculate storm flow, instantaneous rates from the discharge data were converted volumes from the increase 10% above baseline until the end of the day of the final sample or the return to 10% of baseline, whichever came first. For detailed statistical analyses please see the Supplemental Material and Methods.

3. Results

3.1. Study success

In total, 1160 beach samples were collected for FIB analysis from Tourmaline Surfing Park and Ocean Beach; 67 stormwater discharge samples were collected during six storm events (Table 2) from Tourmaline Creek and San Diego River immediately upstream from the Tourmaline Surfing Park and Ocean Beach, respectively. This represents data from all but one storm during the sampling period that met our wet weather definition.

Table 2
Storm date, precipitation amounts, and microbial water quality measurements (X) in the stormwater discharge from each of the sampled events.

Storm Dates	Precipitation in mm (inches)	Storm number	Fecal indicator bacteria	Human pathogens	Microbial source markers
2/28-3/5/2014	48.3 (1.90)	0	X		
12/2- 12/5/2014	64.3 (2.53)	1	X	X	X
12/12- 12/15/2014	26.7 (1.05)	2	X	X	X
1/11- 1/14/2015	9.4 (0.37)	3	X	X	X
2/23- 2/26/2015	4.8 (0.19)	4	X	X	X
3/1-3/4/2015	26.2 (1.03)	5	X	X	X

The sampled storm events ranged from 4.8 to 64.3 mm precipitation (Table 2), straddling the long-term average for single storm precipitation in the region (ca. 13 mm). Interestingly, both of the sample years were below the long-term annual average rainfall (ca. 300-350 mm per year): 129 mm annual precipitation in the October 2013-September 2014 water year and 303 mm annual precipitation in the October 2014-September 2015 water year.

3.2. Overall watershed and storm dynamics

San Diego River and Tourmaline Creek were selected for this study based upon a single major commonality, but they have several major differences. The single common factor between the two watersheds is their proximity to famous, high-use surfing beaches in southern California. Major differences include watershed size, total stormwater flows, dominant land use type, and conveyance type (e.g. Table 1). In the reaches closest to the beach receiving waters, San Diego River is a soft-bottom drainage canal type conveyance, while Tourmaline Creek is a concrete lined creek-like conveyance discharging directly to the beach. Both watersheds are dominated by vitrified clay sewage lines, of which many exist from prior to 1950 (Table 1). The dominant land use type for the San Diego River watershed is open space (41%), while for Tourmaline Creek, single-family residential land-uses dominate the landscape (62%, Table 1). While each storm studied differed in total precipitation and rainfall amount (Table 2), examination of the hydrographs associated with San Diego River clearly show that the falling limb and tail of the storms extended for longer periods of time prior to return to baseline, typically 5-8 days, whereas Tourmaline Creek is much "flashier", and return to trickle or no-flow occurs almost always in less than 5 days (as shown in the March 1-4, 2015 storm, Fig. S3) and sometimes in less than 3 days. Given the differing land-use characteristics between the two watersheds this is not surprising. Discharge data for San Diego River is available from a USGS stream gauge that is approximately 8.5 km from the beach. However, flow data from the beach discharge locations is limited. Estimated stormwater flows to Ocean Beach (from San Diego River) are roughly 10-50 times those from Tourmaline Creek (Table 1).

3.3. Microbial contaminants in stormwater discharge

Regardless of beach, and regardless of which site at either beach, wet weather concentrations of *Enterococcus* were 0.5−1.5 log higher during wet weather than during dry weather (Fig. 2). At Ocean Beach, there was a decreasing gradient of *Enterococcus* concentrations from sites closest to the wet weather discharge to sites furthest from the discharge. At Tourmaline Surfing Park, where the total storm flows are smaller, and the surf zone mixing is less distinct, the gradient in *Enterococcus* concentrations between sites was less apparent (Fig. 2). This gradient in concentration following storms is also apparent in the daily Ocean Beach and Tourmaline Surfing Park beach sites *Enterococcus* concentrations, however the *Enterococcus* concentrations are more variable during dry weather (Figs. S4, S5). *Enterococcus* concentrations were lower

in smaller storms in Tourmaline Creek and the San Diego River as well as at most beach sites (Fig. S6), and the patterns in *Enterococcus* concentrations tended to decrease over the duration of storms (Figs. S3−S5).

3.3.1. Human pathogens in stormwater discharge
3.3.1.1. Stormwater norovirus concentrations. Concentrations of NoV in stormwater discharges from the San Diego River were significantly greater than those from Tourmaline Creek (Fig. 3, Table S3; geometric means by storm t = 2.4, p = 0.049; all samples, t = 2.5 p = 0.02). Across all five storms, average combined NoV concentrations ranged from 19.2 to 175.6 gene copies per 100 ml in San Diego River stormwater discharges (grand geometric mean 97.3 gene copies per 100 ml). Tourmaline Creek stormwater discharge average combined NoV concentrations ranged from 5.2 to 110.9 gene copies per 100 ml (grand geometric mean 33.9 gene copies per 100 ml).

3.3.1.2. Stormwater campylobacter concentrations. Overall, *Campylobacter* concentrations were higher in stormwater discharges from the San Diego River than Tourmaline Creek (Fig. 4, Table S3). Presumably, this was largely due to the presence of *C. coli* and *C. lari*. Average concentrations of *C. coli* in the San Diego River ranged from 23.4 to 116.2 (grand geometric mean 61.4) gene copies per 100 ml, while average *C. coli* concentrations in Tourmaline Creek ranged from 3.0 to 5.33 (grand geometric mean 3.7) gene copies per 100 ml. Likewise, average concentrations of *C. lari* in the San Diego River ranged from 15.2 to 30.9 (grand geometric mean 18.7) gene copies per 100 ml, while *C. lari* average concentrations in Tourmaline Creek ranged from 3.8 to 7.2 (grand geometric mean 4.7) gene copies per 100 ml. The concentrations of *C. jejuni* were more comparable between watersheds. Average concentrations of *C. jejuni* in stormwater discharges from San Diego River ranged from below detection to 8.0 (grand geometric mean 3.5) gene copies per 100 ml, while *C. jejuni* average concentrations in stormwater discharges from Tourmaline Creek ranged from below detection to 11.36 (grand geometric mean 5.0) gene copies per 100 ml.

3.3.1.3. Frequency of pathogen detection in stormwater. The pathogen detection frequency in stormwater varied among the different pathogens measured in discharges from San Diego watersheds (Table 3). NoV was the most commonly detected viral pathogen. All but one sample contained NoV in the San Diego River stormwater discharges, and three-quarters of the discharge samples contained NoV in Tourmaline Creek. Of the two strains measured, NoV genogroup II dominated the detection frequency in San Diego River stormwater discharge samples (Table 3). In contrast, enterovirus was not detected in any stormwater discharge sample (Table 3). AdV was only marginally detected, being quantified in roughly one of five stormwater discharge samples from San Diego River and nearly one of ten samples from Tourmaline Creek.

Campylobacter was the most commonly detected bacterial pathogen (Table 3). Every stormwater discharge sample from San
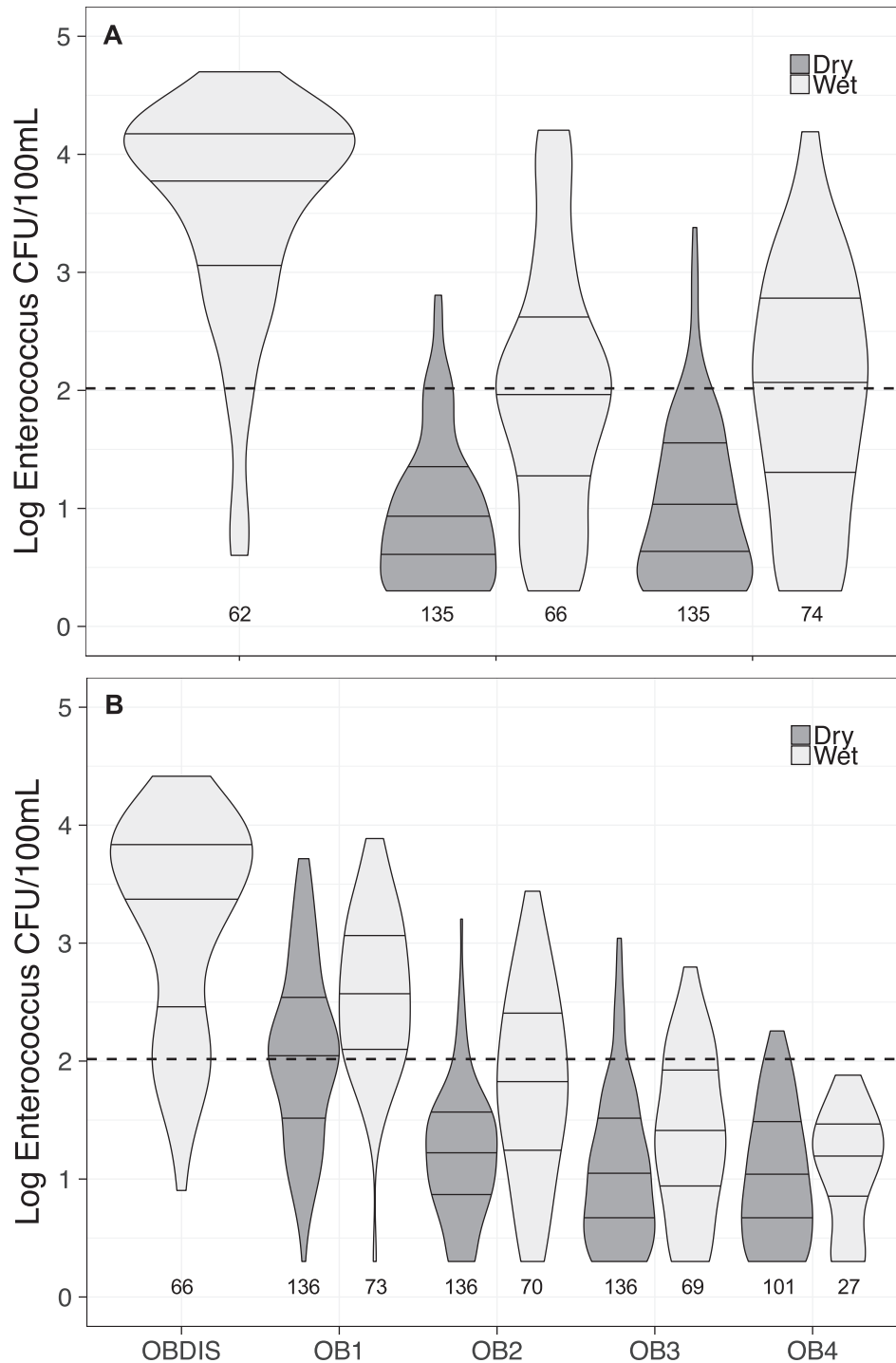
Fig. 2. Violin plots of cultured *Enterococcus* concentrations in wet versus dry weather at (A) Tourmaline Surfing Park and (B) Ocean Beach during the 2013-14 and 2014-15 wet season. Lines in the violin plots represent the range, median, 25th and 75th percentile. Wet weather was defined by the County Health Department as >2.5 mm precipitation in 24 h plus three days. Dashed line represents the single sample maximum enterococcus exceedence level of 104 CFU/100 ml. The concentrations are in Log_{10} scale. The number of samples are displayed beneath the plots. See Fig. 1 for sampling site locations.

Diego River contained *Campylobacter*, but just less than half of the discharge samples contained *Campylobacter* in Tourmaline Creek. *Salmonella* was detected in only one-quarter and one-tenth of the stormwater discharge samples from the San Diego River and Tourmaline Creek, respectively.

The two watersheds also differed in their detection frequency among *Campylobacter* species (Table 3). *C. coli* (87%) and *C. lari* (78%) were the most frequently detected species of *Campylobacter* in stormwater discharges from the San Diego River. In contrast, *C. lari* (48%) and *C. jejuni* (29%) were the most commonly detected species of *Campylobacter* in Tourmaline Creek.
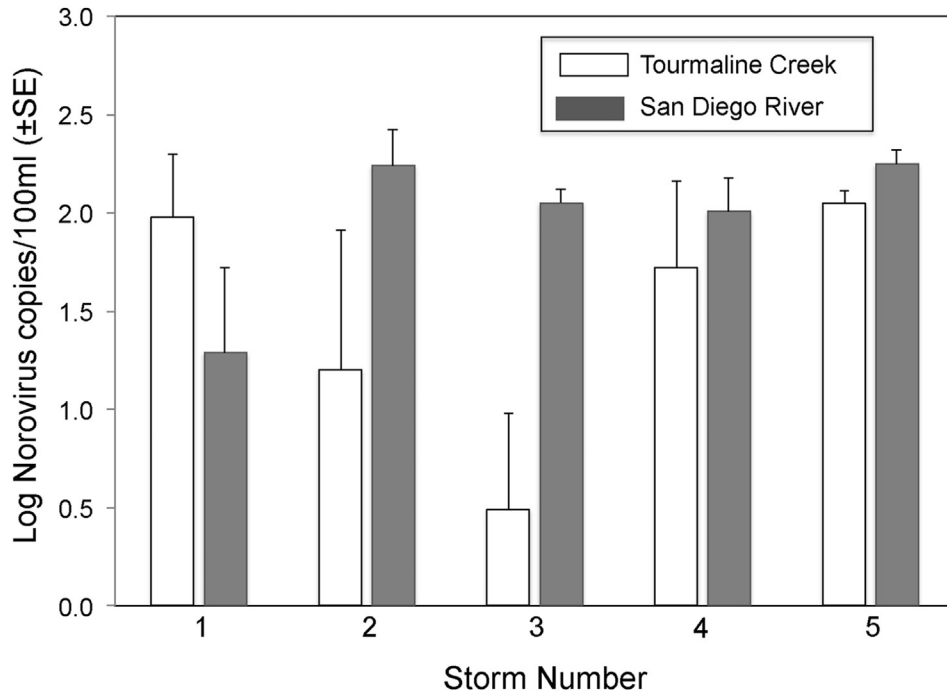
Fig. 3. Norovirus concentrations measured by digital PCR in stormwater discharges from San Diego River and Tourmaline Creek during multiple storm events of the 2014-15 storm season. See Table 2 for the dates and rainfall associated with the storm numbers.

3.3.2. Human MST markers in stormwater

Even though we noted NoV concentrations that were higher in San Diego River discharge than at Tourmaline Creek, the opposite trend was true for the HF183 human fecal marker (Fig. 5, Table S3). The concentrations of HF183 in stormwater discharges were significantly greater in Tourmaline Creek compared to the San Diego River (Fig. 5; t = 3.08, p = 0.004). Average concentrations of HF183 ranged from 20 to 175 (grand geometric mean 82.4) gene copies per 100 ml in stormwater discharges from the San Diego River, and 282 to 904 (grand geometric mean 525.5) gene copies per 100 ml in stormwater discharges from Tourmaline Creek. HF183 concentrations were significantly positively correlated to *Enterococcus* concentrations as measured by both culture and by digital PCR, but were significantly negatively correlated to avian marker and *Campylobacter* spp. HF183 concentrations showed no correlation with other bacterial or viral concentrations in stormwater (Table S4).

Consistent with the frequent presence of human pathogens, the presence of the human MST marker HF183 was also frequent (Table 3). HF183 was detected in every stormwater discharge sample from the San Diego River and nearly every sample from Tourmaline Creek. Neither San Diego River nor Tourmaline Creek has an NPDES permit for discharge from a publicly owned wastewater treatment plant.

3.3.3. Animal MST markers in stormwater

There were significant differences in concentrations of avian markers and apparent, but not statistically significant, differences in concentrations of canine markers in stormwater discharges between the San Diego River and Tourmaline Creek (Fig. 5; Gull: t = 12.2 p < 0.001; Dog: t = 1.8, p = 0.08). However, the concentration patterns of the animal markers were opposite that of HF183 marker. The avian marker was negatively correlated to HF183 and to *Enterococcus* measured by digital PCR while it was positively correlated to *Campylobacter* spp. and *Campylobacter coli* (Table S4). The canine marker was not significantly correlated to FIB,

pathogens, or MST markers. Concentrations of avian markers were two orders of magnitude greater, and the canine marker one order of magnitude greater, in stormwater discharges from the San Diego River compared to Tourmaline Creek.

Similar to HF183, animal MST markers were also detected frequently in San Diego stormwater discharges (Table 3). The avian MST marker was detected more frequently in the stormwater discharge from the San Diego River than Tourmaline Creek (100% vs. 83%, respectively). The estuary of the San Diego River upstream of the sample site is a protected bird sanctuary. Similarly, the canine MST marker was detected more frequently in the stormwater discharge from the San Diego River than Tourmaline Creek (83% vs. 57%, respectively). Both watersheds have large residential land use components.

3.3.4. Fecal indicator bacteria in stormwater

Enterococci were detected in every sample by cultivation and by digital PCR (Table 2). *Enterococcus* concentrations from culture and *Enterococcus* concentrations determined from digital PCR were significantly correlated to one another (Fig. S7, Table S4), although the slope diverged from 1 (slope = 0.596, r^2 = 0.7, p < 0.001, Fig. S7).

3.3.5. Comparison of composite pathogens (PCA) to molecular MST markers

Principal component analysis on the $\log_{10}$ transformed pathogens revealed potential relationships among the pathogens and between the pathogens and the MST markers (Fig. S8). The first two principal component axes explained 56% of the variance in the data with the first axis (which had the largest contributions from *Campylobacter coli*, *Campylobacter lari*, and human NoV genogroup II, Table S5) explaining 38% of the variance. Enterococcus 23S rRNA (Ent dPCR), HF183, and avian (Gull) marker were significantly correlated to the first two axes, with Enterococcus and HF183 negatively correlated to the first principal component and avian (Gull) marker positively correlated to the axis. Bacterial pathogens and bacterial source markers grouped together in the ordination
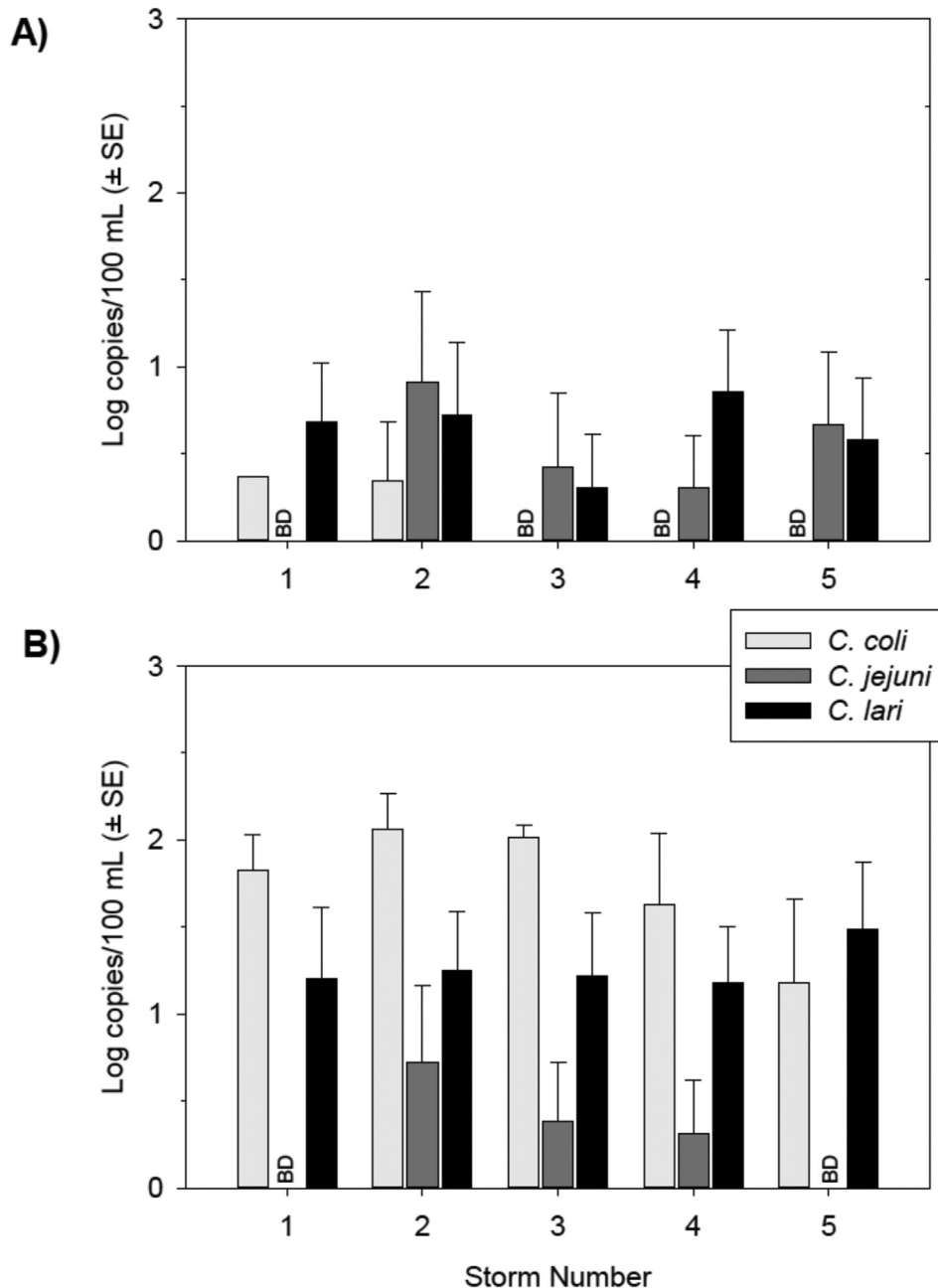
Fig. 4. Concentrations of *Campylobacter coli*, *Campylobacter jejuni*, and *Campylobacter lari* measured by digital PCR in stormwater discharges from (A) Tourmaline Creek and (B) San Diego River. BD = below detection limit. See Table 2 for the dates and rainfall associated with the storm numbers.

(Fig. S8). *Campylobacter coli* and *Campylobacter lari* correlated most strongly with the avian (Gull) MST marker. Campylobacter jejuni was more closely correlated to HF183 while enterococcus 23S rRNA by ddPCR was not well correlated to the pathogens. The pathogen PCA also distinguished between the storm discharge samples clustering nearly all of the San Diego River samples separately from the Tourmaline Creek samples.

3.3.6. Inhibition of digital PCR and RT-PCR assays

Only 5% of San Diego River and 10% of Tourmaline Creek stormwater discharge samples were inhibited for DNA-based digital PCR, and these samples showed minimal inhibition as measured using controlled additions of salmon testes DNA. In contrast, 100% of samples from both watersheds were at least

partially inhibited for digital RT-PCR, as measured using controlled additions of mouse lung RNA. Inhibition ranged from 100% to 67% (Fig. S9) and was not correlated to concentration ($r^2 < 0.014$; $p > 0.10$, Fig. S10). In both DNA and RNA assays, no adjustment for inhibition was made and quantifiable samples were taken at face value.

4. Discussion

This study focused on a detailed, quantitative characterization of stormwater discharge from two watersheds in southern California. The study was intensive, including analysis of six total storms over two winter storm seasons, with over 1000 FIB analyses conducted. Stormwater discharges from both the San Diego River and
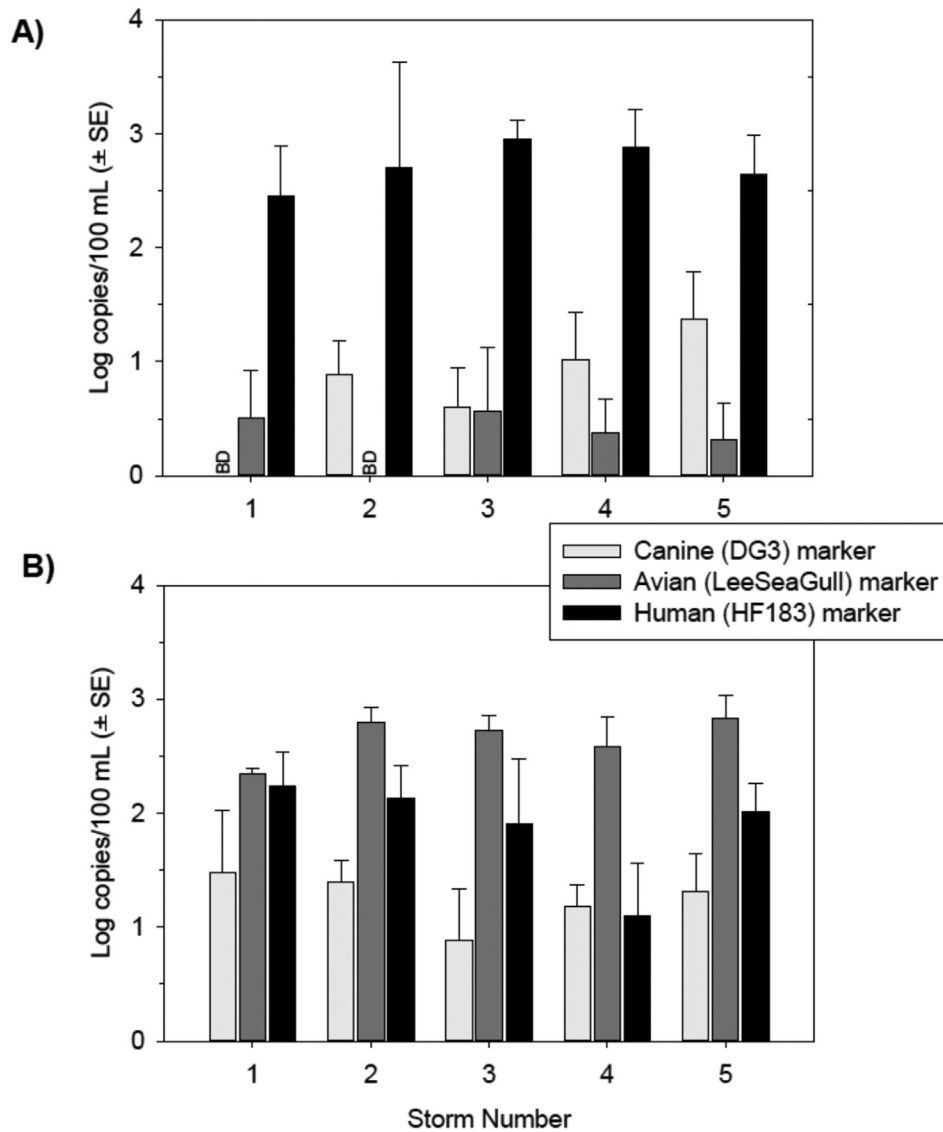
Table 3
Pathogen and source marker detection frequency by watershed.

	Proportion of samples above detection limit	
	San Diego River (N = 23)	Tourmaline Creek (N = 21)
Pathogens		
Norovirus	0.96	0.72
Norovirus I	0.09	0.05
Norovirus II	0.96	0.72
Adenovirus	0.22	0.09
Enterovirus	0.00	0.00
Campylobacter sp.	1.00	0.45
C. coli	0.87	0.10
C. jejuni	0.17	0.29
C. lari	0.78	0.48
Salmonella	0.25	0.10
Source markers		
Human (HF183)	1.00	0.95
Avian (LeeSeagull)	1.00	0.83
Canine (DG3)	0.83	0.57

Tourmaline Creek had a dramatic impact on FIB concentrations at the study beaches in San Diego (Fig. 2, S4, S5). The FIB concentrations in the stormwater at the beach sites closest to the discharge locations were often five-to ten-fold higher during wet weather as compared to dry weather. This increase occurred consistently across storms and across both watersheds, and this trend is not limited to just the two San Diego beaches in this study. Similar increases have been observed at most other beaches in southern California (Noble et al., 2003). Other studies, such as those completed by Corsi et al. (2014), Stumpf et al. (2010), and Ahn et al. (2005) have shown similar patterns associated with stormwater discharge into receiving waters.

Several patterns emerged from *Enterococcus,* NoV, and HF183 concentrations in the stormwater from both Tourmaline Creek and the San Diego River showing differences in their response during storm flows. First, although our total number of storms assessed was low (n = 6), there appeared to be a relationship that emerged between storm size (in total precipitation amount) and mean

Fig. 5. Concentrations of source markers for canine, avian, and human hosts measured by digital PCR in stormwater discharges from (A) Tourmaline Creek and (B) San Diego River. BD = below detection limit. See Table 2 for the dates and rainfall associated with the storm numbers.

stormwater discharge *Enterococcus* sp. concentration. While we do not have detailed flow measurements explicitly from the discharge locations for the two watersheds for every single storm, we can make inferences using the storm size and the days following the storm. There was a clear decreasing pattern in *Enterococcus* concentrations with storm size (Fig. 2, S6) and days since rainfall for both watersheds (e.g. Table S3, Figs. S4, S5). Interestingly, when examining the NoV patterns and comparison with days since rainfall, San Diego River seemed to have a more persistent falling limb and tail, while Tourmaline Creek NoV concentrations dropped to below the detection limit in the same period. Furthermore, within individual storms, there is a persistent human contamination signal in the tail of the storm, shown by NoV and HF183 concentrations in both watersheds (Fig. S3). This suggests that measures such as controlling the first inch of the storm may not adequately control the delivery of the human contamination. The size of the storm also had little effect on the pathogen or the MST marker concentration in the stormwater discharge, except for HF183 in the San Diego River (Figs. 3−5). This decoupling of the *Enterococcus* from the pathogens and MST markers (except HF183 in the San Diego River) across storm size implies different origins or fates than the Enterococcus. While this could be, in part, due to small sample size, it adds to the evidence that we cannot assume for any given storm that stormwater based delivery of human pathogens will mirror that of FIB.

While qPCR has proved useful in previous microbial contaminant assessments in southern California (Choi and Jiang, 2005; Jiang et al., 2007) and in other regions of the United States (Fong et al., 2010), the results from these studies combined with the present study indicate that combined use of qPCR and dPCR technology for detailed stormwater assessments might be fruitful for the future. For example, a comprehensive study of stormwater, hydrological variation and viral pathogen quantification was conducted in 2007−2008 and published in 2014 by Corsi et al. Corsi et al. (2014) prominently feature a flow weighted sampling approach for both dry and wet flow conditions, combined with detailed analyses for a range of human and bovine pathogens. With this stormwater assessment in southern California, the flow-weighted sampling approach was not possible for every storm sampled due to infrastructure issues and tidal influence. However, flow values for the San Diego River measured upstream indicate peak discharge rates ranging from 1.01 to 20.7 m^3 per second with estimated storm volumes from to 2.3×10^5 to 1.5×10^6 m^3 across each of the five storms (Table 1). At Tourmaline, during the March 1-4, 2015 storm, the peak discharge rate was 1.13 m^3 per second with a storm volume of 2.9×10^4 m^3 (Table 1, Fig. S3). Combining these flow estimates with concentrations allows a broad loading calculation. Estimated combined NoV load in the San Diego River was 6.4×10^{11} gene copies in the Dec 2-5, 2014 storm and 1.8×10^{12} gene copies in the March 1-4, 2015 storm. In Tourmaline Creek the estimated NoV load was 3.3×10^{10} gene copies. While we suggest that a flow weighted sampling approach would have been optimal here in order to model the potential exposure of surfers to microbial contaminants, we note that by using measured concentrations and estimating dilution, the exposure of surfers was still successfully modeled (Soller et al., 2017). Clearly, flow-weighted and automated sampling approaches are a necessary next step for characterizing the dynamics of stormwater discharge to prominent surfing and recreation areas during wet weather.

Another overall finding is that, in spite of the differences in watershed size and characteristics (Table 1), the differences in the observed concentration of pathogens and FIB were small. Instead, it appeared that microbial source played a more important role in the water quality patterns observed during wet weather. For example, avian source marker and *Campylobacter* species assemblages differed between watersheds, likely due to the bird sanctuary and potentially the greater amount of open space along the San Diego River watershed, but not the Tourmaline Creek watershed. In contrast, there was little difference between watersheds in detection of human specific virus, which suggests similar human sources. However, although the observed concentrations of pathogens were somewhat similar, due to the difference in volume the total number of pathogens discharged to surfing beaches were estimated to be roughly 100-fold different.

An important component of this study was the ability to quantify FIB, pathogens, and MST markers to simultaneously assess sources of fecal contamination and public health risk (Soller et al., 2017) in the context of stormwater dynamics. The application of molecular methods such as qPCR for assessment of MST markers and pathogens in a stormwater discharge setting is not new (e.g. Sauer et al., 2011). However, a major difference between the study that we report here, and previous studies is the relevance of the discharge to public health risk of the surfing population. Our study corroborates the findings presented by Sauer et al. (2011) where characterization of a larger number locations (45 stormwater outfalls) was conducted in Milwaukee, Wisconsin. In both studies, human fecal contamination as determined by the presence of multiple *Bacteroides*-based markers, was prevalent across all stormwater samples. Our study demonstrated a strong and positive relationship between HF183 and Enterococcus concentrations, but the Sauer et al. (2011) study did not see discernible statistical relationships between the human MST marker concentrations and FIB concentrations. One main difference of the Sauer et al. (2011) study was that concentrations of human enteric viruses were only determined in a subset of samples that were shown to have high concentrations of human specific Bacteroides-based markers. Even though hundreds of samples for Sauer et al. (2011) were analyzed for the human specific Bacteroides-based markers, only one was further subjected to intensive viral pathogen analyses. In the study presented here, while fewer discharge locations were studied, the viral and bacterial pathogen concentrations were determined for all of the samples collected for five storms.

Multiple lines of evidence point to chronic sources of human fecal contamination, potentially in the form of sewage, despite the municipal storm sewer system being separate from the sanitary sewer system. First, there was consistent detection, at relatively high concentrations, of human specific pathogens such as NoV. NoV has been suggested as one of the primary etiologic agents associated with gastrointestinal illness in the United States (Teunis et al., 2008; Mead et al., 1999; Scallan et al., 2011) and can be contracted via stormwater or exposure to sewage (Ueki et al., 2005; Westrell et al., 2006). Since inhibition of the NoV quantification was prevalent but not adjusted for throughout this study, the concentrations that are reported are conservative estimates of the true values. In spite of this, the concentrations that we report in the stormwater discharges to the two surfing beaches studied were, on average, higher than those previously reported by other studies, such as the in-stream investigation by Corsi et al. (2014). Second, there was consistent detection, sometimes at high concentrations, of HF183 across both watersheds, across storms and across the duration of storms (Figs. 5 and S3). HF183 is known to be both sensitive and specific to human sources of fecal contamination (Boehm et al., 2013, 2015; Layton et al., 2013), and was positively correlated to *Enterococcus* concentrations in the stormwater (Table S4). AdV were detected and quantified in the stormwater discharge at both locations, but at lower concentrations than observed for NoV.

Animal fecal sources were noted in the stormwater discharges over the course of the study. One nearly ubiquitous animal fecal source was avian, based on the observed concentrations of the avian MST marker, especially downstream of the bird sanctuary in

San Diego River. This is consistent with the increased frequency of detection, increased concentrations, and greater abundance of *Campylobacter lari* in the San Diego River stormwater discharge samples compared to the Tourmaline Creek discharge. The avian marker was positively correlated to the genus-specific *Campylobacter* assay and *Campylobacter coli* and *Campylobacter lari* is a pathogen known to be found in marine birds, including seagulls (Lu et al., 2011). The canine MST marker was also found consistently, but not ubiquitously in both watersheds. Both avian and canine hosts have the capacity to contribute large quantities of FIB to runoff sources (Sinigalliano et al., 2013; Schriewer et al., 2013), although we note that in this study, the avian marker was negatively correlated to HF183 and *Enterococcus* concentrations as measured by digital PCR. Relational statistical analyses were conducted in order to better examine the relationships across pathogens, FIB, and MST marker given their biogeochemical and survival characteristics and differences.

Although there was little correlation between the individual pathogen concentrations and the indicators or the MST markers (Table S4), generating composite pathogen variables via principal component analysis found significant correlations between HF183, avian (Gull) Marker, and *Enterococcus* (Ent dPCR) and the composite pathogen axes (Fig. S8). Within the PCA-space human NoV genogroups and human AdV were correlated, with human NoV genogroup II having the stronger signal. While the correlation of *Campylobacter coli*, *Campylobacter lari,* and *Salmonella* spp. with avian (Gull) marker was expected due to the prevalence, the closer relationship of *Campylobacter jejuni* with HF183 suggests an anthropogenic origin for *Campylobacter jejuni* in these watersheds. The separation of viral from bacterial pathogens, and grouping of viruses and bacteria, in the PCA-space suggests different fate and transport for these pathogens in stormwater systems possibly due to the biochemical and physical differences between viruses and bacteria and their interaction with the stormwater matrix.

While the application of qPCR for stormwater discharge assessments is not novel (e.g. Sauer et al., 2011), the use of digital PCR for combined quantification of FIB, source-specific MST markers and human viral pathogens is relatively new. While a direct qPCR/ digital PCR comparison was not the feature of this study, digital PCR may offer advantages over qPCR, especially for quantification of human enteric viral pathogen targets, which tend to occur at much lower copy numbers than MST-markers. Digital PCR essentially partitions a single PCR into tens of thousands of individual nanoliter-sized droplets (Huggett et al., 2013, Cao et al., 2015a, 2016). While the primers and probes are identical between traditional qPCR and digital PCR, the partitioning allows for absolute quantification via Poisson statistics, while at the same time potentially increasing the analytical sensitivity over traditional qPCR because multiple reactions can be merged. Human viral pathogen quantification by digital PCR is not always more sensitive than qPCR (Coudray-Meunier et al., 2015), but has repeatedly been demonstrated to be less prone to interference from inhibitory substances, more robust, and more reproducible than qPCR (Coudray-Meunier et al., 2015; Cao et al., 2015a).

Stormwater discharge is a difficult matrix for any type of PCR. This is because of interference with the nucleic acids and requisite amplification enzymes by the typically high levels of total suspended solids, dissolved compounds, and turbidities associated with overland flow (Schiff et al., 2016). Fine sediment particles and high molecular weight compounds, such as humic acids, are especially deleterious to the reverse transcriptase steps associated with RNA targets (e.g. Cao et al., 2015a). Even with digital RT-PCR, we still experienced inhibition in most stormwater samples. However, the increased sensitivity of digital RT-PCR helped when using dilution to overcome inhibition and, when detected, digital

RT-PCR enabled the quantification of RNA targets rarely seen in previous studies when using qRT-PCR (Noble et al., 2006; Sauer et al., 2011). Although we used RNA virus controls to account for losses, ultimately, we chose not to adjust results for inhibition. Instead, we reported the results directly as a conservative, but precise measure of the RNA-based pathogen concentrations in stormwater.

Even though the Corsi et al. (2014) study revolutionized the currently available information at the time for viral pathogens in stormwater discharge, the study serves as a reminder of the difficulties associated with viral pathogen quantification in such a highly variable matrix. Corsi et al. (2014) reported a 63% inhibition rate across all virus analyses. A low percentage of our DNA target analyses were inhibited (5-10%), but for the RNA-based viral targets, such as NoV and enterovirus, the rates of inhibition were higher (up to 100%). However, with digital PCR these samples were still analyzed and provided quantitative data, yielding useful, but conservative information. Continued optimization of stormwater sample processing approaches will hopefully alleviate these issues for the future.

5. Conclusions

- The study demonstrates the impact of stormwater discharge on surfing beaches as observed by increasing dilution of *Enterococcus* concentrations away from the discharge location.
- Multiple lines of evidence reveal the presence of human fecal contamination in both stormwater discharges across all storms suggesting chronic contamination, potentially from sanitary sewer infrastructure.
- Even given expected dilution patterns, the human viral pathogens concentrated from stormwater discharge samples are an infrastructure and public health concern.
- Principal components analyses indicated correlation among viral pathogens and correlation among bacterial pathogens, potentially reflecting their physical and biochemical differences and differential transport in storm drain systems.
- The quantitative data that was generated by digital PCR for direct pathogen and source marker measurements show consistent quantification over storms ranging in size and duration, indicating the potential for digital PCR to play a more influential future role for stormwater mitigation.
- In combination, these results and this approach can be used by beach managers to inform decisions about public health and water quality management.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2018.01.056.

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