Multi-tiered approach using quantitative polymerase chain reaction for tracking sources of fecal pollution to Santa Monica Bay, California

Rachel T. Noble[1], John F. Griffith, A. Denene Blackwood[1], Jed A. Fuhrman[2], Jason B. Gregory[1], Ximena Hernandez[2], Xiaolin Liang[2], Angie A. Bera[3] and Kenneth Schiff

Abstract

The ubiquity of fecal indicator bacteria such as *Escherichia coli* (*E. coli*; EC) and *Enterococcus* sp. (ENT) in urban environments makes tracking fecal contamination extremely challenging. A multi-tiered approach was used to assess sources of fecal pollution in Ballona Creek, an urban watershed that drains to Santa Monica Bay (SMB) near Los Angeles, CA. A mass-based design at six mainstem sites and four major tributaries over a six hour time period was used to assess: Tier 1) the flux of ENT and EC using culture-based methods; Tier 2) ENT using quantitative PCR (QPCR), and detection and/or quantification of additional markers of human fecal contamination including *Bacteroides* sp. human specific marker and enterovirus, using quantitative reverse transcriptase PCR (QRTPCR); and Tier 3) the specific types of enteroviral genomes found via sequence analysis. Sources and concentrations of fecal indicator bacteria were ubiquitously high throughout Ballona Creek, with no single tributary dominating fecal inputs. The flux of ENT and EC averaged 10^{9} to 10^{10} cells h^{-1} and was as high at the head of watershed as at the mouth prior to discharge into SMB. In addition, there was a consistent detectable signal of the *Bacteroides* human specific marker, with 86% of the samples taken over the extent over the study period testing positive. Enteroviruses were quantifiable in 14 of 36 samples (39%), with the highest concentrations at the site furthest upstream, Cochran Avenue. These results indicated the power of using multiple approaches to assess and quantify fecal contamination in freshwater conduits to high use, high priority recreational swimming areas.

Introduction

Santa Monica Bay (SMB), California, is home to some of the most popular beaches in the world. It is located adjacent to metropolitan Los Angeles where more than 50 million beachgoers visit SMB shorelines every year, which is more than all other beaches in California combined (SMBRC 2005). However, there are serious concerns about beach water quality because of continued exceedences of water quality thresholds based on fecal indicator bacteria such as total coliforms, fecal coliforms or *Escherichia coli* (*E. coli*; EC) and *Enterococcus* (ENT), particularly in areas impacted by urban runoff. Thirteen percent of the shoreline mile-days in SMB exceeded water quality thresholds between 1995 and 2000 with over 50% of these exceedances located near storm drains (Pina *et al.* 1998). The public health risk associated with urban runoff has been directly demonstrated through epidemiology studies. Haile *et al.* (2003) demonstrated that swimmers near storm drain discharges in SMB had a higher likelihood of respiratory and/or gastrointestinal symptoms compared to swimmers more than 400 m from a storm drain.

Despite the impairments to water quality and risks to human health, identifying and eliminating the sources of bacteria responsible for the beach warnings remains elusive. The difficulty in identifying and eliminating the sources of bacteria results from two important factors. First, the traditional indicators of fecal pollution upon which the water quality thresholds were developed are not specific to humans. These fecal indicator bacteria can be shed from any warm-blooded organism, including wild and domesticated animals (Geldreich 1978).

[1]*University of North Carolina, Chapel Hill, NC*
[2]*University of Southern California, Los Angeles, CA*
[3]*Santa Monica Baykeeper, Santa Monica, CA*

Therefore, source tracking turns into a challenging scenario when these diffuse and frequently intermittent or episodic fecal releases occur. The second difficulty when identifying and eliminating sources of fecal indicator bacteria is their ubiquity in urban environments. Finally, unlike many of the human pathogens of concern, fecal indicator bacteria may survive and even grow in the environment (e.g., Kinzelman *et al.* 2004, Solo-Gabrielle ett al. 2000, Weiskel *et al.* 1996).

Viruses are one tool that could prove useful in source tracking studies because they include many pathogens of concern, and they are generally species-specific. Viruses are known to cause a significant portion of waterborne disease from water contact, mostly from ingestion of sewage contaminated water and seafood (Fogarty *et al.* 1995). Until recently, however, methods for virus detection and quantification have relied on cell culture based approaches that are much too slow to be effective source tracking tools. Recently developed molecular techniques, such as Quantitative Reverse Transcriptase PCR (QRTPCR) can detect and quantify viral genetic material directly from water samples. Results of tests conducted previously in southern California (Fuhrman *et al.* 2005, Noble and Fuhrman 2001a, Tsai *et al.* 1993, 1994), in Florida (Griffin *et al.* 1999, Rose *et al.* 1997), and Europe (Pina *et al.* 1998) using conventional RTPCR or PCR have detected a host of genetic material from human specific viruses including enterovirus, hepatitis A virus, rotavirus, and adenovirus in urban runoff discharges or seawater samples.

A different approach would be to use alternative bacterial indicators for source tracking that might be much more abundant in urban discharges. For example, *Bacteroides* sp., makes up approximately one-third of the human fecal microflora, considerably outnumbers the fecal coliforms, EC, and ENT. *Bacteroides* sp. are obligate anaerobes, so there is little concern over persistence or regrowth in the environment. More importantly, human specific *Bacteroides* sp. markers have been developed, increasing the value of this potential indicator (Bernhard and Field 2000a, b; Dick and Field 2004).

Both virus and alternative bacterial indicators such as *Bacteroides* sp. have been shown to be potentially useful source tracking tools. Griffith *et al.* (2003) and Noble *et al.* (2003) concluded that genetic based methods, such as PCR consistently provided the best information when attempting to conduct source tracking on mixed source samples. To date, however, no single method has all of the traits to be the consummate source-tracking tool. Therefore, a multi-tiered multi-indicator approach has been recommended by some investigators (Boehm *et al.* 2003, Stewart *et al.* 2003). By using multiple tools, investigators can utilize the strengths of each to ascertain inputs and track fates that will ultimately lead to successful management solutions.

The objective of this study was to identify the contributions and quantify the loading of fecal contamination to the SMB using a multi-tiered approach. The first tier included traditional fecal indicator bacteria measurements. The second tier included molecular assays developed and conducted for ENT, *Bacteroides* sp .human specific marker, and enterovirus. These methods rely on conventional PCR, QPCR, or QRTPCR, which have not been previously applied in conjunction with one another for source tracking studies in urban watersheds. The third tier was to sequence the enterovirus from the field samples with the greatest concentrations to determine the likely type of enterovirus amplified in the assay. The multi-tiered approach was applied using a mass-based design to quantify inputs and flux through an urban watershed to the beach. The multi-tiered approach was applied using a mass-based design to quantify inputs and flux through an urban watershed to the beach.

Methods

This study quantified inputs of flow, bacteria concentrations and virus genomic equivalents, then tracked them through an urban watershed over time. This mass-based design was applied in the Ballona Creek watershed, the largest tributary to SMB. Ballona Creek is over 85% developed and currently has the largest inputs of fecal indicator bacteria to SMB (Figure 1).

Samples were collected at six mainstem, and four major tributaries, to Ballona Creek. The six mainstem sites extended from Cochran Avenue (where the system daylights from the underground storm drainage system) to Inglewood Avenue (located at the head of tide just prior to discharge into SMB; Table 1). The four tributaries represented the four largest hydrodynamic inputs to the system and were located in reaches between each of the mainstem sampling sites. Flow was calculated as the product of flow rate and wetted cross-sectional area

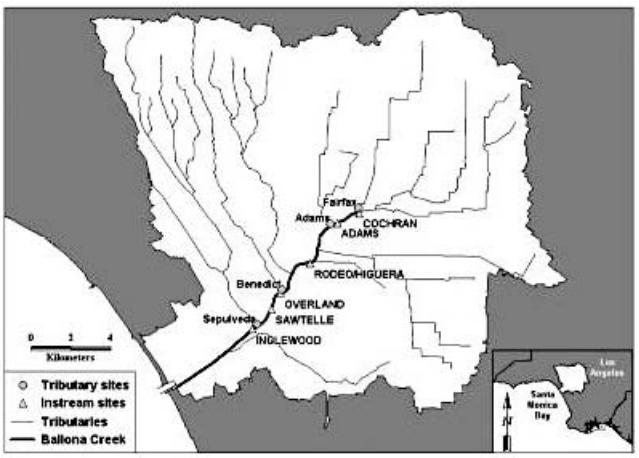

Figure 1. Map of the Ballona Creek watershed in Los Angeles, Calif. Tributary and main-stem sampling sites for the water quality study are indicated. (Inset) Santa Monica Bay, in Southern California.

(Viessman *et al.* 1989). Doppler area-velocity sensors (Teledyne ISCO, Los Angeles, CA) were used to measure flow rate . Pressure transducers that measure stage, along with verified as-built cross sections, were used to estimate wetted cross-sectional area. One minute instantaneous flow was logged electronically during the entire six hour sampling period. Both the area-velocity sensors and pressure transducers were calibrated prior to sampling.

One hour composite water samples were collected immediate downstream of flow measurement devices at each site (see GPS coordinates Table 1) between 8:00 a.m. and 2:00 p.m. on August 26, 2004. The six hour sampling period corresponds to the approximate hydrodynamic travel time from Cochran Avenue to Inglewood Avenue (Ackerman *et al.* 2003). The hourly 4-L composite samples at each site were created after combining ten individual 400-ml grab samples collected every 6 minutes into a single container. In total, 60 composite samples were collected at Ballona Creek as a result of sampling for 6 hours at 10 different sites.

After collection, samples were placed on ice and transported immediately to the University of Southern California for processing. For each composite sample, 100 ml of water was devoted to indicator bacteria analysis, and 200-600 ml of sample volume was vacuum filtered through replicate 47-mm 0.4-ìm polycarbonate (PC) filters (Poretics, Inc., Livermore, CA) using a filter funnel and receiver (Millipore, Inc., Bedford, MA) for ENT analyses by QPCR or *Bacteroides* by conventional PCR, as suggested by Haugland *et al.* (2005). In addition, replicate filtrations were also conducted using 47-mm Type HA (Millipore, Inc., Bedford, MA) mixed cellulose ester, 0.45-µm pore size filters for enterovirus analysis as suggested by Fuhrman *et al.* (2005). For each filter, the volume filtered was the maximum filterable within ca. 10 minutes of starting the filtration. The total volume filtered was dependent on the location and turbidity of each individual sample, and the filter volumes were carefully recorded to the nearest 1 ml.

Table 1. Sampling sites along the mainstem and major tributaries of Ballona Creek.

Site	Description	GPS Coordinates (NAD 83 datum)
Cochran Ave.	mainstem	34° 02.662" N 118° 21.237" W
Fairfax Drain	tributary	34° 02.298" N 118° 22.136" W
Adams Ave.	mainstem	34° 02.009" N 118° 22.494" W
Adams Drain	tributary	34° 02.009" N 118° 22.494" W
Rodeo/Higuera	mainstem	34° 01.305" N 118° 22.693" W
Benedict Box Channel	tributary	34° 00.925" N 118° 23.432" W
Overland Ave.	mainstem	33° 00.429" N 118° 23.771" W
Sawtelle Ave.	mainstem	33° 59.816" N 118° 24.164" W
Sepulveda Channel	tributary	33° 59.512" N 118° 24.693" W
Inglewood Ave.	mainstem	33° 59.394" N 118° 24.696" W

All filters were placed in microcentrifuge tubes and stored at –80°C for further analysis.

Concentrations of EC and ENT were measured by Defined Substrate Technology™ using kits supplied by IDEXX Laboratories, Inc. (Westbrook, ME) according to the manufacturer's instructions. Briefly, 10-fold and 100-fold dilutions of the water samples were made with deionized water containing the appropriate media and sodium thiosulfate, mixed to dissolve, dispensed into Quanti-Tray®/2000, and heat sealed. *E. coli* was measured using the Colilert-18® reagents, while ENT was measured using Enterolert™ reagents. Samples were incubated overnight per the manufacturer's instructions and inspected for positive wells. Conversion of positive wells from these tests to a most probable number (MPN) was done following Hurley and Roscoe (1983).

The polycarbonate filters were processed for DNA extraction using the UltraClean™ Fecal DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) as per manufacturer's alternative protocol for maximum yield. Eluted DNA extracts were stored at –20°C until use.

The concentration of the extracted DNA was measured using Quant-iT™ Picogreen® dsDNA reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Standard curves were generated in duplicate using Lambda DNA standards between 2.5 ng/ml and 600 ng/ml and a negative a negative control (0 ng/ml). Fluorometric measurements were made using a Bio-Rad VersaFluor fluorometer.

Total ENT primers and probe are described by Ludwig and Schleiffer (2000) and were constructed using the 23S rDNA regions around the target site of a well-established ENT group specific primer (ENC854R). The primer ECST748F targets ENT, lactococci, and several clostridia. The target site of the probe GPL813TQ is present in 23S rDNA from a variety of representatives of gram-positive bacteria with a low G+C DNA content (Ludwig and Schleifer 2000).

The master mix contained 1X Taq buffer, 4 mM MgCl2, 3 mM dNTPs, 2.5U Ex Taq R polymerase (RPCR kit for quantitative PCR kit, TaKaRa Mirus Bio, Madison, WI), 1ìM ENC853R, 1ìM ECT748F, 0.1ìM GPL813 TaqMan® Cy3 Probe (synthesized by MWG Biotech, High Point, NC) and nuclease free water yielding a final volume of 20 µl, to which 5 µl of sample (either DNA extract from an environmental sample, ranging from 1 to 76 ng genomic DNA), or 5 µl of lysed cell suspension or genomic equivalents) was added for a final volume of 25 µl. The samples were run under the following optimized assay conditions for PCR: 1 cycle initial hold at 95°C for 2 minutes, and 45 cycles of denaturation at 94°C for 15 seconds, and annealing/extension at 60°C for 30 seconds, the optics were turned on during the annealing step. The Cepheid Smart Cycler® II was set with the following specific parameters for this assay. The Dye Set was set for FCTC25. The Ct analysis mode was set for growth curve (linear) analyses, with a manual threshold typically set at between 5 and 15 fluorescence units. The background subtract level was set at a minimum of 12 and a maximum of 40. The BoxCar averaging feature was set at 0. The assay was previously optimized for Taq, Mg++, and dNTP concentrations as well as all cycling parameters (data not shown). For quality control, combined *Enterococcus faecalis* (*E. faecalis*; ATCC 29212) and *Enterococcus faecium* (*E. faecium*; ATCC 35667) were used as our calibration strains for the total ENT primer and probe set. Control bacteria preparations were prepared by boiling bacteria for 5 minutes, centrifuging 2 minutes at 12,000 x g at 4°C, and immediate storage on ice. *E. faecalis* and *E. faecium* cells were enumerated using SYBR Green I epifluorescence microscopy (Noble and Fuhrman 2001). Serial dilutions of the standards were made in DEPC-treated sterile water, and four point standard curves were run in duplicate in concert with the unknown samples on the Smart Cycler II instrument. Total ENT primers were tested with all 19 validly described species of the genus *Enterococcus*, and demonstrated amplification of rDNA of all strains, with varying efficiencies (Ludwig and Schleifer 2000).

Amplification of the human-specific *Bacteroides*/Prevotella marker generally followed the procedure of Bernhard and Field (2000a), with PCR primers that amplify partial 16S rRNA from the human fecal (HF) specific group. A range of extracted DNA quantities (2 - 5 ml, representing 1 - 70 ng per assay, with most samples in the range of 5 - 20 ng) was tested to avoid problems with inhibition of the PCR. DNA was amplified with *Bacteroides-Prevotella* specific primers from Bernhard and Field (2000a). The PCR mixture was exactly that as described by Bernhard and Field (2000a), except, that only 1 µm of each primer was used. The PCR conditions were specifically optimized for this study and differed from the original

publication: 2 minutes 95°C, then 25 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72 °C for 30 seconds followed by a 5-minute extension at 72°C. Then 1 µl of each PCR product was re-amplified using the same conditions as above for another 25 cycles. PCR was performed on a 3000MX Thermalcyvler (Stratagene). PCR products were visualized in a 2% agarose gel stained with 1X SYBR Gold (Molecular Probes, Eugene, OR) and compared to a 100 bp DNA ladder (Promega). Positive results yielded 525 bp amplicons. The positive control was human fecal sample extracted with a QIAamp DNA Stool Kit (QIAGEN, Valencia, CA). Negative controls contained water instead of sample. All samples were initially run with 5 µl of extracted material. After the initial analyses, all negative samples (14 of 60) were spiked with 0.1 ng of positive control DNA and reanalyzed to determine possible inhibition. Three of the 14 negative samples: 2 from Benedict Box Channel and 1 from Adams Drain exhibited inhabitation. Inhibited samples were reanalyzed using 2 µl of sample, but all three remained negative.

Samples, along with negative controls, were extracted using a modified RNeasy Plant Mini Kit (QIAGEN Plant and Fungi RNA isolation protocol). The supplied RLT homogenization buffer was supplemented with poly vinyl-pyrrolidone (PVP)-40 at a final concentration of 2%. Prior to the extraction fresh ß-mercaptoethanol (Sigma Chemical Co.) was added to the extraction buffer in the exact concentration recommended by the manufacturer. Filters (Type HA) were manually homogenized with a pipet tip and 700 µl of the RLT/filter slurry was applied to a QiaShredder column (QIAGEN) and spun at max speed, ≥8000 x g, for 2 minutes to aid in viral lysis as well as separate filter particles from the filtrate (Fuhrman *et al.* 2005). The filtrate was then carefully removed without disturbing the pelleted material and placed into a new 1.5 ml tube. The volume of solution in each tube was estimated by pipetting and 0.4 ml volumes of 5M potassium acetate (pH 6.5) were added. Tubes were mixed by inversion and incubated on ice for 15 minutes. The mixture was then spun (12,000 x g) at 4°C for 15 - 30 minutes and the supernatant transferred to a new 1.5 ml microfuge tube. Subsequently, the Plant and Fungi RNA isolation protocol was followed starting at step 5. The RNA was eluted with 50 µl of the supplied RNase free water. A one-step TaqMan® qRT-PCR was performed on the extracted RNA, with final reaction volumes of 25 µl, using a Qiagen One-Step RT-PCR kit (Qiagen). Five µl of extracted RNA was added to 20 µl of master mix containing 1X RT Buffer, 6 mM MgCl2, 500 nM dNTPs, 700 nM EV1 reverse primer [5'- TGTCACCATA AGCAGCCA-3'], 700 nM EV1 Forward primer 5'-[CCCTGAAT-GC GGCTAAT-3'], 30 µg Bovine Serum Albumin, 20 units of recombinant RNAsin (Promega Corp.), 1.5% PVP-25 (Monpoeho *et al.* 2001; Sigma Chemical Co.), 100 genomic equivalent units of a competitive internal positive control (CIPC) developed in house (Gregory *et al.* 2006) following the general approach of Kleiboeker (2003), plus 300 nM CIPC probe 5'[Cy5]- TGTGCTGCAAGGCGAT-TAAGTTGGGT-[BHQ2]-3', and 300 nM EV-BHQ probe 5' [FAM]-ACGGACACCCAAAGTAGTCG-GTTC-[BHQ-1] 3', and one µl of enzyme mix (containing both reverse transcriptase and DNA polymerase). Probe and primers were synthesized by MWG Biotech, Inc. The Cepheid Smart Cycler® II was programmed to: 1 hour reverse transcription step at 50ºC followed by a 15 minute hold at 95ºC for DNA polymerase activation, then 45 cycles of 94ºC for 15 seconds (denature), 60ºC for 1 minute (anneal/extension-optics on).

QRTPCR results were available three hours after the start of analysis, making the total RNA extraction, QRTPCR preparation, and analysis time less than five hours. After the first analyses, those samples that appeared to have inhibition of the QRTPCR reaction (as indicated by the CIPC) had RNA sample volumes reduced in half and re-run. No inhibition was observed at this lower RNA concentration. Standard curves were generated using a synthetic enteroviral transcript that was quantified using fluorometric analysis and sample genome concentrations were interpolated from the standard curve using the manufacturer's curve fitting software. Quantitative results are reported per liter sample volume.

Sequencing of Enteroviral QRTPCR Positives

Enteroviral QRTPCR positive samples from the enterovirus analyses were sequenced to assess the specificity and fidelity of our enteroviral QRTPCR and elucidate the identities of the enteroviral genomes being amplified by the assay. Following the initial enteroviral analysis of the Ballona Creek samples, RNA samples identified as positive for enteroviral genomes were again amplified using the QRTPCR protocol. However, after numerous repeated freezing and thawing of the extracted RNAs for various analyses, only Cochran Avenue 9:00 a.m.,

10:00 a.m., and 11:00 a.m. samples contained amplifiable enteroviral genetic material (Fuhrman *et al.* 2005). The 144 bp enteroviral QRTPCR product was distinguished from the 126 bp internal positive control product using a 10% polyacrylamide, 1x Tris Borate EDTA, gel. The gel was stained with ethidium bromide and visualized with a 254 nm ultraviolet light. The larger 144 bp enteroviral QRTPCR products were excised, homogenized in 50 µl of 1x QIAGEN OneStep RTPCR buffer using a microfuge pestle, and incubated overnight at 37°C while shaking. The enteroviral products were purified (Wizard® SV gel and PCR Clean Up System, Promega), cloned into the 3.9-kilo base pCR® 2.1 TOPO®Vector (TOPO TA cloning kit, Invitrogen, Carlsbad, CA), transformed into TOP10 chemically competent *E. coli* and plated on Luria-Bertani agar plates containing 100 µg/ml of ampicillin. Bacterial clones were screened using QPCR and positive colonies from each of the 3 sites were selected and grown individually in 3 mls of 2X YT media overnight at 37°C. Plasmid DNA was isolated (PerfectPrep® Plasmid Mini Kit, Eppendorf®, Westbury, Conn.) and the DNA concentration was calculated using UV spectrophotometry. Plasmid DNA was sequenced bidirectionally by MWG Biotech and the chromatograms were inspected using Sequencher™, Version 4.2 (Gene Codes Corp., Ann Arbor, Mich.). The sequences were aligned to sequences in the NCBI GenBank® using BLAST, and are available by searching nucleotide accession numbers DQ196482 through DQ196487.

Calculations and statistical analyses

Data analysis was comprised of four steps. First, the hydrologic budget was evaluated to determine if the majority of flow was sampled. This evaluation was conducted by comparing the volumetric inputs from each of the tributaries to the the volumetric discharges along the mainstem of Ballona Creek. The second step was to examine temporal and spatial trends in fecal indicator bacterial flux. Flux of indicator bacteria was calculated by multiplying the indicator bacteria concentration (per dl or 100 ml) by the flow rate (in dl per hour), arriving at a flux of indicator bacteria cells per hour. The mean hourly flux (temporal analysis) was calculated by averaging the flux of indicator bacteria at all mainstem locations for each hourly interval (N = 6). The mean flux at each site (spatial analysis) was calculated by averaging the flux at each mainstem or tributary site for all hourly samples (N = 6). Statistical analysis of the differences in bacterial flux between hourly time periods or, alternatively, between mainstem sites was conducted using analysis of variance (ANOVA; Zar 1984). The third data analysis step was to examine spatial and temporal patterns in the frequency of *Bacteroides* detection. The *Bacteroides* method used in this study was a presence/absence endpoint. This examination was conducted by tabulating the locations and time periods of *Bacteroides* detection to detect patterns moving downstream, adjacent to tributaries, or over time. The fourth data analysis step was to examine the spatial and temporal extent of enterovirus concentrations. Unlike *Bacteroides*, the presence of enterovirus was quantified so the magnitude of enterovirus concentrations was tabulated among the different locations across the different time periods. Similar to the *Bacteroides* data analsysis, patterns moving downstream, adjacent to tributaries, or over time were examined.

Results

Total volume discharged from Ballona Creek during the six-hour sampling period was 13,390 m^3 (Figure 2). Of this volume, 97% was attributed to monitored inputs from Cochran, Fairfax, Adams and Benedict, and Sepulveda tributaries. The largest volume was contributed at Cochran Avenue where the creek emerges into daylight from beneath downtown Los Angeles. Flow remained relatively stable over the study period at all sites with little variation or pattern in discharge. For example, the coefficient of variation for flow at the most downstream site, Inglewood Avenue, was less than 8%, approaching the resolution of our flow monitoring devices.

There was no observed spatial trend in fecal indicator bacteria flux in Ballona Creek during this study (Figure 3). The average flux of EC ranged from 1.1×10^{10} to 5.3×10^{10} cells $hour^{-1}$ at the six mainstem sites. The average flux of ENT ranged from 6.6×10^{8} to 1.4×10^{9} cells $hour^{-1}$ at the six mainstem sites. In both cases, there was no discernable increase in bacterial flux as one moved downstream; no two mainstem sites were significantly different from one another for either EC or ENT (ANOVA, $p > 0.05$).

There was an observed temporal trend in fecal indicator bacteria flux in Ballona Creek during this study (Figure 4). The average flux of ENT was highest at 9:00 a.m. (2.9×10^{10} cells $hour^{-1}$) and monotonically decreased throughout the study peri-

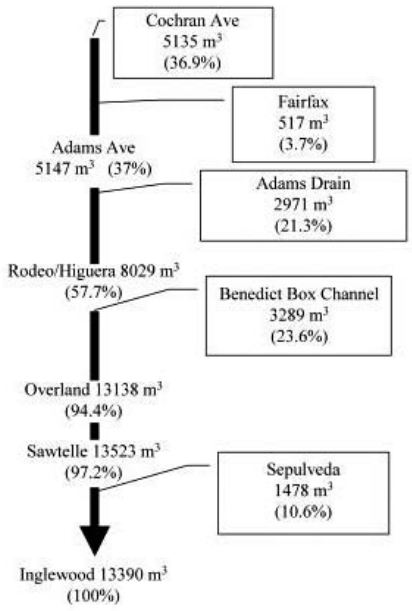

Figure 2. Schematic diagram depicting additive flow in the main channel of Ballona Creek and the percentage contributed by each tributary sampled.

od. The lowest flux was measured at 2:00 p.m. (3.0 x 10^9 cells $hour^{-1}$). Similar patterns were observed for EC (data not shown). In contrast to the culture-based methods, the QPCR results for ENT did not decrease over time. The flux of ENT ranged from 2.7 x 10^{10} to 4.7 x 10^{10} cells $hour^{-1}$ with the 9:00 a.m. and 2:00 p.m. samples being nearly equivalent.

The relative pattern of ENT contributions between tributaries was similar at all time periods (Figure 5). Benedict tributary always had the greatest flux of fecal indicator bacteria followed by Sepulveda, Fairfax and Adams tributaries. A similar pattern was also observed for EC. The flux of ENT from Benedict tributary ranged from 4.1 x 10^9 to 1.4 x 10^{10} cells $hour^{-1}$ throughout the sampling period while the flux of ENT from Adams tributary ranged from 3.7 x 10^5 to 4.4 x 10^6 cells $hour^{-1}$. On average, Benedict tributary contributed 81% of the ENT loading from all four tributaries.

The hourly flux of ENT (using culture-based methods) from each of the four main tributaries approximated the load being passed down Ballona Creek (Figure 5). Regardless of hour, the flux from each of the tributaries was within a factor of 10^1 compared to its nearest downstream site on the mainstem of Ballona Creek. The only exception was the Adams tributary, which was as much as four orders of magnitude less than the nearest downstream site. The mainstem showed virtually no response to any of these tributary inputs, including Adams. The flux of ENT remained virtually unchanged from upstream to downstream of each of the tributary inputs (Figures 3 and 5).

The *Bacteroides* human specific marker was positively detected throughout the mainstem of Ballona Creek. The *Bacteroides* sp. human specific marker was confirmed to be present in 31 of the 36 mainstem samples tested (86%; Table 2). A positive signal was observed at all sites during early morning hours, but during periods of heightened UV (midday and late afternoon), a decrease in the number of positive results for the *Bacteroides* human specific marker was observed. For example, only 3 of 6 samples at 2:00 p.m. were positive for the *Bacteroides* human specific marker and exceeded the water quality threshold for EC.

Enteroviruses were detected and quantified in 14 of 36 mainstem samples tested (39%; Table 2). Moreover, spatial and temporal patterns in enterovirus concentration were also evident in the Ballona Creek system. Main channel locations in the upper reaches of the study area were more likely to be positive for enteroviruses than downstream sites. The most consistently positive site was Cochran Avenue, where 89% of the samples contained measurable levels of enterovirus. In addition, some of the highest concentrations of enterovirus concentrations were measured at this site during four of the six time periods (ranging from 1563 to 4257 enterovirus genomes per liter). A general pattern in enterovirus detection was observed during the course of the day. Enterovirus was detected during early and mid-day at upstream sites. Enterovirus was detected most frequently late in the day at the downstream

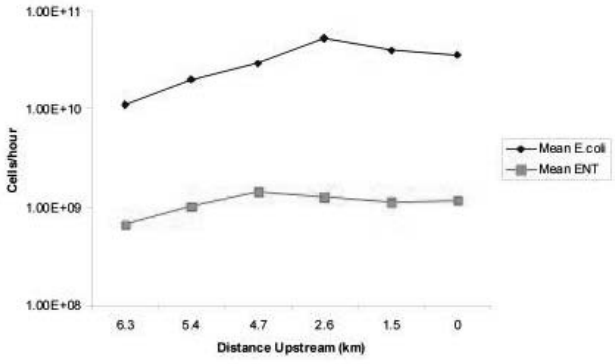

Figure 3. Mean flux of *E. coli* and *Enterococcus* cells (expressed as cells per hour) at main-channel sampling sites of Ballona Creek (26 August 2004).

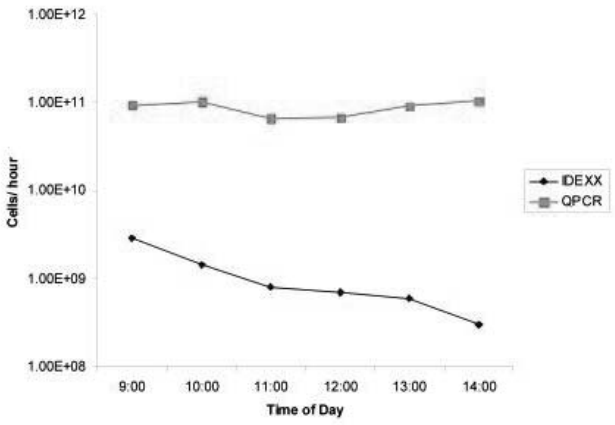

Figure 4. Mean hourly flux of *Enterococcus* spp. (expressed as cells per hour) along the main channel of Ballona Creek, measured by using either an IDEXX chromogenic substrate (Enterolert) or QPCR methods on 26 August 2004.

sites. The 12:00 p.m. sampling interval had the most frequent detection of enterovirus with the highest concentrations observed at the middle sites in the watershed. Enterovirus was not detected in nearly all of the tributary samples (data not shown); only the Adams Drain tributary had any detectable enterovirus.

The highest sequence homology observed from the three enterovirus positive Cochran Avenue samples that were sequenced (>95%) was with human *Coxsackievirus* A22 (GenBank® Accession no. AF499643), human *Coxsackievirus* A19 (Accession no. AF499641), and human *Enterovirus* 90 (Accession no. AY773285). The 144 bp QRT-PCR product corresponded to nucleotides 453-596 of human *Coxsackievirus* A22, nucleotides 457-600 of human *Enterovirus* 90, and nucleotides 454-597 of human *Coxsackievirus* A19.

Discussion

The Ballona Creek watershed is a system impacted by fecal pollution. The flux of fecal indicator bacteria was as high at the head of the watershed as it was at the mouth of the creek where it dis-

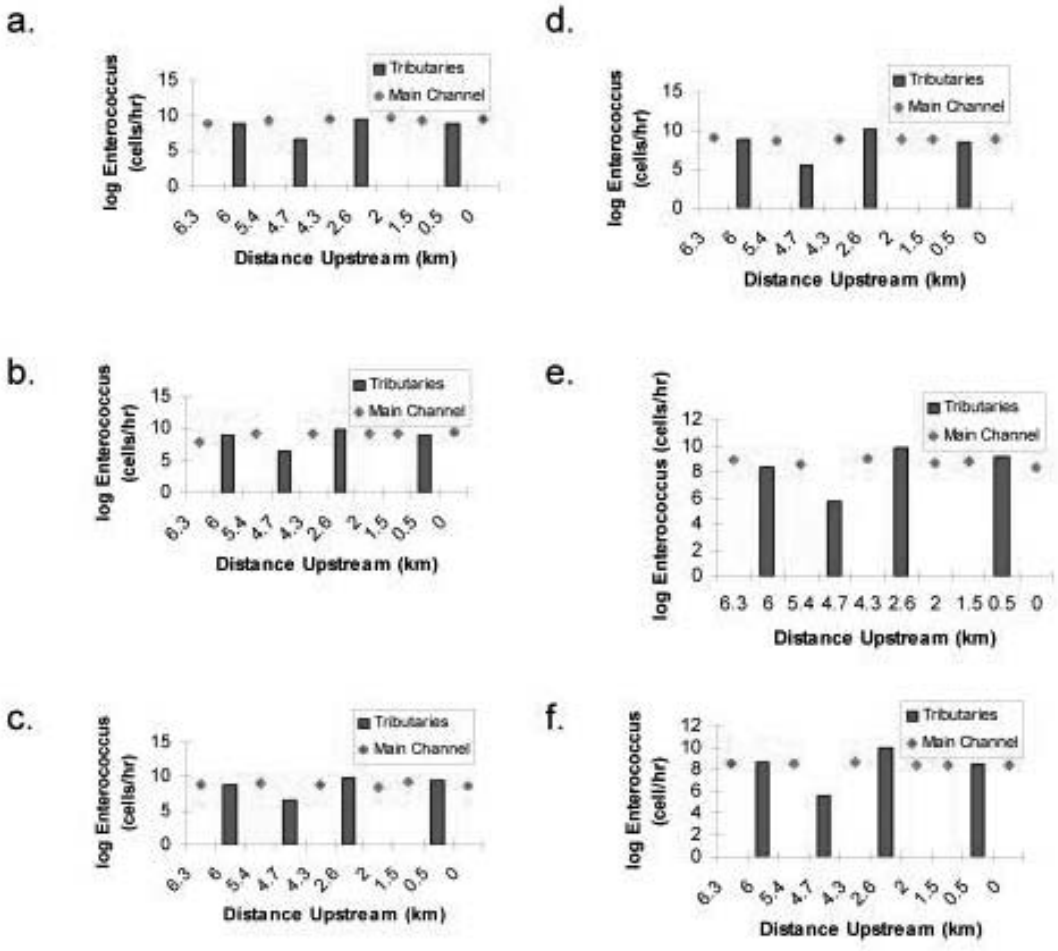

Figure 5. Loading of *Enterococcus* spp. (expressed as cells per hour) in the main channel and tributaries of Ballona Creek at 9:00 (a), 10:00 (b), 11:00 (c), 12:00 (d), 13:00 (e), and 14:00 (f) on 26 August 2004.

charges into SMB. Although we focused on flux of these fecal indicator bacteria, it is important to note that 92% of all samples collected from Ballona Creek in this study exceeded the water quality thresholds established by California State Assembly Bill 411. The presence of human enterovirus and human specific markers of *Bacteroides* sp. further documents the fecal inputs and should increase an environmental manager's awareness of the possible human health risks associated with these discharges.

This study is not the first to examine the presence of viruses in urban runoff entering shorelines in SMB and other southern California urban watersheds. For example, Gold *et al.* (1990) and Gold *et al.* (1992) found viruses in repeated samples from multiple storm drains to SMB using both cell culture and RTPCR techniques. Haile *et al.* (1999) detected human specific viruses in all three storm drains tested in their epidemiology study of SMB. Noble and Fuhrman (2001) found human enteric virus genomes in the nearshore marine waters of SMB. Jiang *et al.* (2001) found human adenovirus in samples collected at 12 sites between Malibu and the Mexican border, and Fuhrman *et al.* (2005) previously found human enterovirus genomes in Ballona Creek.

The multi-tiered approach used in this study can assist watershed managers in determining sources and efficiently abating the most significant inputs of fecal indicator bacteria. If managers relied solely on the patterns in fecal indicator bacteria from Ballona Creek, then the only option would be to treat the entire 37 m^3s^{-1} discharge furthest downstream at Inglewood Avenue because the flux of fecal indicator bacteria was similar from all sources. The use of multiple tools, however, allows managers to prioritize the most important sources. In this case, the presence of human enterovirus was greatest from the Cochran Avenue site, where the system daylights from the underground storm drain system beneath Los Angeles and the discharge volume is one-third the volume at Inglewood Avenue Previous studies of southern California storm drains have detected a human pathogenic virus signal (Jiang *et al.* 2001; Jiang and Chu 2004; Noble and Fuhrman 2001, 2001a). Since Cochran Avenue had the most frequent occurrence and highest concentrations of enterovirus, plus a consistent co-occurrence of the *Bacteroides* sp. human specific marker, this source would appear to be the most likely candidate for future management actions. The sequencing results that confirmed the presence of several potential risks to human health (human Coxsackie and enteroviruses) should provide the reassurance most managers would need before planning future management steps.

The lack of correlation between bacterial indicator levels and levels of human pathogenic viruses has been observed in previous studies (Dufour 1984, Elliott and Colwell 1985) and demonstrates the value of a multi-tiered approach used herein for source identification. For example, analysis of wild shellfish from the Atlantic coast of France indicated no significant correlation between fecal coliforms and enteroviruses or Hepatitis A virus (LeGuyader *et al.* 1993, 1994), and viruses have sometimes been found

Table 2. Number of enterovirus genomes (per liter) along the mainstem of Ballona Creek in Los Angeles, CA. Asterisks indicate positive results for Bacteroides human-specific marker.

Kilometers Upstream from Santa Monica Bay (Site)	Time of Day					
	9:00	10:00	11:00	12:00	13:00	14:00
6.3 (Cochran)	3255*	1391*	1714*	1440*	1336*	*
5.4 (Adams)	*	630*	200[a]	290*	*	*
4.7 (Rodeo/Higuera)	*	96*	*	1641*	579	
2.6 (Overland)	*	*	*	926*	*	*
1.5 (Sawtelle)	*	*	*	61*	*	384
0 (Inglewood)	*	*	*	*	*	*

*PCR reaction inhibited for Bacteroides human-specific marker

in oysters without coliform contamination (Goyal *et al.* 1984). Noble and Fuhrman (2001) detected enterovirus in 35% of the 50 shoreline samples they examined over a five year period and no significant statistical relationship to any of the standard bacterial indicators was found. Virus and fecal indicator bacteria were measured in dry weather urban runoff in drains along 300 km of shoreline from Santa Barbara to San Diego, CA (Noble and Fuhrman 2001). Despite 40% of the storm drains containing detectable enterovirus, there was no correlation with fecal indicator bacteria concentrations. It is also possible that differential rates of degradation of viruses and bacteria can explain much of the discordant relationship between viral pathogens and indicator bacteria (e.g., Noble *et al.* 2003).

The use of QPCR to measure fecal indicator bacteria presents unique opportunities and challenges. An advantage of QPCR for measuring fecal indicator bacteria is speed, potentially providing measurements in less than three hours (Griffith *et al.* 2003). However, culture-based methods only quantify viable bacteria, while QPCR measures the DNA from both cultivable and noncultivable microbes. This was most apparent in the temporal trends from Ballona Creek. Samples of ENT using culture-based methods generally decreased as the day progressed, most likely as the result of cell photoinactivation from sunlight (Davies and Evison 1991, Davies-Colley *et al.* 1994, Noble *et al.* 2004). Ballona Creek is a 40-m wide concrete-lined channel concentrating solar energy into the shallow creek in the channel invert. The QPCR results, however, remained steady indicating that the bacterial DNA was still intact and detectable, even though the ENT were not viable.

Overall, the use of multiple approaches provided convincing evidence of the extent and types of microbial contamination in this urban watershed. It is believed that such studies will provide invaluable information for researchers and managers trying to balance regulatory burdens and public safety.

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