EXHIBIT K

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Sent: Tuesday, October 13, 2009 12:00 PM

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Subject: Supplemental Information for Malibu

Attachments: SCCWRP 2005 Study.pdf; Malibu Well Location Map.pdf; Malibu Hydrographs Final.pdf; Malibu Supp Response Letter.10.13.09.pdf

Ms. Erickson,

Please see attached and confirm receipt.

Thank you,
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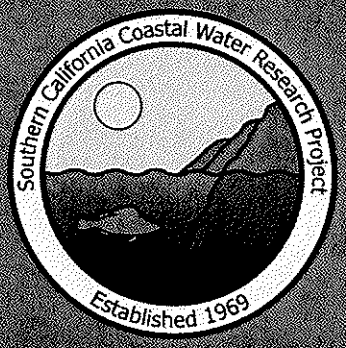
Technical Report 446
February 2005

Multi-Tiered Approach Using Quantitative Polymerase Chain Reaction For Tracking Sources of Fecal Pollution to Santa Monica Bay, California



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ABSTRACT

The ubiquity of fecal indicator bacteria such as *Escherichia coli* and enterococcus make tracking sources in urban watersheds extremely challenging. In this study, 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), CA. A mass-based design at six mainstem sites and four major tributaries was used to quantify the flux of enterococcus and *E. coli* using traditional culture-based methods, and three additional indicators including enterococcus, *Bacteroides* sp. and enterovirus, using quantitative polymerase chain reaction (QPCR). Sources and concentrations of fecal indicator bacteria were ubiquitously high throughout Ballona Creek and no single tributary appeared to dominate the fecal inputs. The flux of enterococcus and *E. coli* averaged 10^9 to 10^{10} cells/hr and were as high at the head of watershed as they were at the mouth prior to its discharge into SMB. In contrast, the site furthest upstream had the most frequent occurrence and generally the greatest concentrations of enterovirus. Ninety-two percent of the samples that tested positive for enterovirus also tested positive for *Bacteroides* sp. A similar survey in Malibu Creek, a nearby non-urban watershed, found low levels of traditional fecal indicator bacteria and no detectable enterovirus or *Bacteroides* sp. The influent and effluent from three structural best management practices (BMPs) were evaluated for removal efficiency. Results indicated that those with ultraviolet (UV) treatment worked better than a constructed treatment wetland for reducing enterococcus concentrations using culture-based methods, but also degrading its DNA based on QPCR measurements.

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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 coliform or *E. coli*, and enterococcus, particularly in areas impacted by urban runoff. Thirteen percent of the shoreline mile-days in SMB exceeded water quality thresholds between 1995-2000 with over 50% of these exceedences located near storm drains (Schiff et al 2003). In contrast, sewage spills were relatively rare accounting for less than 0.1% of the water quality exceedences and subsequent warnings to swimmers. Moreover, swimming near storm drains in SMB can lead to an increased risk of swimming-related illnesses. Haile et al (1999) 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 risk 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 on which the water quality thresholds were derived are not specific to humans. These fecal indicator bacteria can be shed from any warm-blooded organism including wild and domesticated animals (Geldreich 1978). 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 the environment. Unlike many of the pathogens of interest, fecal indicator bacteria may survive and even grow in the environment. For example, fecal indicator bacteria were able to persist in beach wrack impacting beaches in Cape Cod, MA (Weiskel et al. 1996).

Viruses are one tool that could prove useful in source tracking studies because they are the pathogen of interest. 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 growth-based endpoints that are much too slow to be effective source tracking tools. Recently developed molecular techniques, such as Quantitative Reverse Transcriptase Polymerase Chain Reaction (QRTPCR) can detect and quantify viral genetic material directly from water samples. Results of tests conducted previously in Southern California (Noble and Fuhrman, 2000; Tsai et al., 1993; Tsai et al., 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. The major drawback to

using viruses as source tracking tools, however, is their dependence on a large human population in order to have sufficient numbers for detection (Noble et al. 2003).

A different approach would be to use alternative bacterial indicators for source tracking that might be much more abundant in fecal waste discharges. This alternative approach could prove useful if host specific bacterial indicators could be found. Of the facultative anaerobic organisms common in human fecal flora, enterococci have been found in almost all subjects with a mean level of $\log_{10}$ 8.9 per gram feces (Klessen et al, 2000). Another option would be *Bacteroides* sp., which make up approximately one-third of the human fecal microflora, considerably outnumbering the fecal coliforms. *Bacteroides* sp. belongs to a group of nonspore forming 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, Bernhard and Field 2000b).

Both virus and alternative bacterial indicators such as *Bacteroides* sp. have been shown to be potentially useful source tracking tools. Griffith 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. *Bacteroides* sp. correctly identified human sources of fecal pollution when present in mixed water samples delivered blind to the laboratory. Likewise, human enterovirus measurements had virtually no false positives, a problem that plagued many other methods in that study. However, the human marker identified in *Bacteroides* sp. may be present in additional hosts or the primers used to detect the human marker may cross react with species from nonhuman hosts (Kreader 1995). Similarly, enterovirus consistently and correctly detected human sewage when present, but had difficulty determining human sources when only one or a few likely uninfected individuals contributed fecal material. Since no method has all of the traits to be the consummate bacterial source tracking tool, a multi-tiered multi-indicator approach has been recommended by some investigators (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.

This 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 newly developed methods for enterococcus, *Bacteroides* sp., and enterovirus. All of these newly developed methods rely on QPCR or QRTPCR, which has not been applied previously for source tracking studies in urban watersheds until now. The multi-tiered approach was applied using a mass-based design to quantify inputs and flux through an urban watershed to the beach. A subsidiary objective included using the multi-tiered approach through a relatively undeveloped watershed. Finally, the multi-tiered approach was used to determine the effectiveness of a variety of structural best management practices (BMPs) that were aimed at reducing bacterial inputs from urban watersheds.

MATERIALS AND METHODS

This study was conducted in three phases. The first phase quantified inputs of flow, bacteria concentrations and virus particles, 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). The second phase quantified bacteria concentrations and virus particles in the Malibu Creek watershed, the second largest tributary to SMB. Malibu Creek is only 12% developed and has a large lagoon system at its terminus prior to discharging across the beach to the world famous Surfrider Beach. Although no flow was measured in Malibu Creek to provide flux estimates, this system provided the opportunity to measure concentrations at several points through the lagoon and as it enters the ocean to assess shoreline mixing and dilution. The third phase examined the effectiveness of three BMPs to reduce bacteria and virus concentrations. The three BMPs, only two of which were located in the Santa Monica Bay watershed, included a multimedia filtration system with inline ultraviolet (UV) treatment, a filtration-aeration system with an inline UV treatment system, and a constructed wetland. For each of the BMPs, an influent-effluent approach was used to estimate treatment effectiveness.

Sample Collection and Filtration

Ballona Creek

Samples were collected at six mainstem and four of the major tributaries to the Ballona Creek system. The six mainstem sites extended from where the system daylights at Cochran Avenue to Inglewood Avenue, which is 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. Doppler area-velocity sensors 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 at each site between 8:00 AM and 2:00 PM 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, 2004). Four liter 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 6 hours at 10 different sites.

Table 1. Ballona Creek sampling sites.

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

Malibu Creek

One hour composite samples were collected at five sites along the mainstem of Malibu Creek (Table 2). The sites stretched from the Cold Creek tributary to the head of the lagoon, near the mouth of the lagoon, in the discharge across the beach, and in the wave wash immediately in front of the discharge across the beach. Composite samples were collected in a similar fashion as Ballona Creek with the following exceptions. A single composite sample was collected at each site daily on three consecutive days (November 10, 11 and 12, 2004) coinciding with low tide to ensure that the flow direction was from the lagoon, across the beach, and into the wave wash. No flow information was collected since most of the sites were hydrologically unrateable.

Table 2. Malibu Creek sampling sites.

Site	Description	GPS Coordinates (NAD 83 datum)
Bridge at Cold Creek	Mainstem	34 04.865N 118 42.262W
Bridge at Cross Creek	Mainstem	34 02.578N 118 41.052W
Head of Malibu Lagoon	Lagoon	34 02.154N 118 41.036W
Mouth of Malibu Lagoon	Lagoon	34 01.920N 118 40.810W
Malibu Creek Wavewash	Mixing Zone	34 01.920N 118 40.810W

BMPs

Three BMPs were selected for sampling. The three sites included: the Santa Monica Urban Runoff (SMURRF) treatment facility located in Santa Monica; the Clear Creek, Inc. MURF™ pilot treatment facility located in Paradise Cove; and a constructed wetland (WET CAT) located in Laguna Niguel. The SMURRF consists of a grit screen to remove debris and trash, a dissolved aeration system to separate the oil and grease, and a microfiltration system to remove solids inline with a UV treatment device. The MURF system uses a combination of proprietary multi-media filtration and UV treatment. The WET CAT is a 2.1 acre constructed wetland with no other in line treatment. Grab samples were collected from the influent and effluent at each BMP.

Filtration

After collection, samples were placed on ice in a cooler and transported immediately to the University of Southern California for processing. For each composite sample, 200-600 ml of sample volume was vacuum filtered through replicate 47mm 0.45µm polycarbonate filters (Poretics, Inc.) using a filter funnel and receiver (Millipore, Inc.) for bacterial marker analysis by QPCR. In addition, replicate filtrations were conducted using 47 mm diameter 0.7 µm nominal pore size, type GF/F microporous filters (Whatman, Inc.), and replicate Type HA Millipore mixed ester cellulose acetate/nitrate, 0.45 um pore size filters (for subsequent enterovirus analysis). The polycarbonate filters were immediately placed into a 1.5 ml screw-cap tube and placed on dry ice until storage at -80°C. Type HA filters were either placed into a Whirl-pak bag for analysis by the Fuhrman laboratory (EnteroA), or into a 1.5 ml screw-cap tube for subsequent analysis by the Noble laboratory (EnteroB). Type GF/F filters were cut into quarters, each quarter placed in a 1.5 ml screw cap tube and placed on dry ice until storage at –80°C. .

Indicator Bacteria Analyses Using Chromogenic Substrate

Concentrations of *E. coli* and enterococcus were measured by chromogenic substrate methods using kits supplied by IDEXX Laboratories, Inc. (Westbrook, ME). *E. coli* was measured using the Colilert-18® reagents, while enterococci were measured using Enterolert™ reagents. Both tests used the Quanti-Tray/2000 for enumeration of cells. 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).

Enterovirus Analyses Using QRTPCR

Samples were analyzed for enteroviruses using two separate, but similar methods conducted in two separate laboratories, EnteroA (Fuhrman laboratory) and EnteroB (Noble laboratory). For EnteroA, filters were extracted using the RNeasy mini kit (Qiagen Cat. No.74106) and QIAvac 24 vacuum manifold (Qiagen Cat. No.19403). The extraction protocol was modified from the manufacturer's instructions as follows: 1ml lysis buffer RLT (with 10µl β-mercaptoethanol) was added directly into each Whirl-Pak bag, allowed to soak the filter for ten minutes, and the resulting extracts (lysates) were carefully removed by pipet into 2 ml microcentrifuge tubes (droplets hanging in the bag and water clinging to the filter were first squeezed to the bottom corner of the bag by manually applying pressure to the outside of the bag). If there was visible filter or sample debris, the particulate matter was removed by brief centrifugation. Then one volume of 70% ethanol (usually 1 ml) was added to the extract and mixed by pipetting. Samples were transferred to the RNeasy spin columns, filtered through with the QIAvac at approximately 500 mm Hg vacuum, and were washed on the manifold once with 700 µl RW1 solution, and twice with 500 µl RPE solution to remove contaminants. The columns were cleared of remaining droplets of buffer by centrifugation into a 2 ml collection tube (14,000 rpm, Eppendorf 5415 microfuge, 2 minutes), and the buffer discarded. The RNA

was eluted from the columns into a 1.5 ml collection tube with 50 µl volumes of RNase free water by centrifugation (Eppendorf 10,000 rpm, 2 min.), after allowing the water to stay in the column 1 min. This filter extraction step typically took up to two hours for 15 samples.

For each PCR reaction, 5 µl of the 50 µl RNA was analyzed by QRTPCR on a Mx3000P Thermal Cycler (Stratagene, Inc.). The PCR protocol was modified from the single-tube RT-PCR method previously developed for sludge samples by Monpoeho et al. (2001). Primers and probe, not changed from that original published method (except for the BHQ quencher), were reverse primer Ev1 [5'-GATTGTCACCATAAGCAGC-3'], forward primer Ev2 [5'-CCCCTGAATGCGGCTAATC-3'], synthesized by Qiagen and Ev-probe [5'-FAM-CGGAACCGACTACTTTGGGTGTCCGT-BHQ-Phosphor-3'], synthesized by Sigma Genosys. A GenBank BLAST search done on 3 June 2004 revealed that only human (not other animal) enteroviruses matched all three primer and probe sequences. Each PCR reaction contained 5 µl RNA extract and 20 µl master mix, each 20 µl master mix contained: 1X Taq gold buffer (ABI), 5.5mM $MgCl_2$ (ABI), 500uM dNTPs (ABI), 6% glycerol (Sigma Chemical Co.), 2% PVP 40 (polyvinylpyrrolidone, av. MW 40,000, Sigma Chemical Co.), 500nM Ev1, 400nM Ev2, 120nM Ev-probe, 1.5µg T4 gene 32 protein (Ambion), 10 units of RNAsin (ABI), 2.5 units of AmpliTaq gold (ABI) and 5 units MULV reverse transcriptase (ABI). Each RNA extract was analyzed in duplicate. Enterovirus RNA was transcribed into cDNA at 50°C for 45 minutes, the cDNA was amplified by PCR, after a 95°C 10 minute hot start, for 50 cycles at 94°C for 15 sec and 60°C for 1min. Fluorescence measurements were made during the extension step, every cycle at 60°C. Calculations for quantification were done by the Stratagene QPCR software in real time, with raw data saved for possible reanalysis. Parameters (e.g. fluorescence threshold) were set manually after PCR was done to generate a standard curve with optimal statistics (usually r^2>0.95, slope around 3.3) and unknowns were calculated based on that standard curve. Standards were prepared using the poliovirus stock described above. Standards used in the high concentration set were 10-fold dilutions ranging from high to low concentration. For EnteroB, a similar approach was used. Samples were extracted using the RNeasy mini kit (Qiagen Cat. No.74106), with additional of 2.0% polyvinylpyrrolidone (PVP)-40 (final concentration) and the filter fully homogenized in the screw cap tube. After homogenization, approximately 700µl of the RLT/filter slurry was applied to a QiaShredder column (until the Qiashredder was full) and spun at max speed, $\geq$ 8000 x *g*, for 2 minutes. It was often necessary to perform two spins to ensure the entire volume of RLT/filter slurry was shredded. The supernatant fluid was then carefully removed and placed into a new 1.5 ml tube. The volume of solution in each tube was estimated by pipetting and 0.4 volumes of potassium acetate were added. Tubes were mixed by inversion and incubated on ice for 15 minutes. The mixture was then spun at 4°C for 15-30 minutes and the supernatant transferred to a new 1.5 ml microfuge tube. Following this, the protocol for the Qiagen RNeasy Plant and Fungi RNA isolation was followed starting at step 5. Five µl of extracted RNA from the previous procedure was added to 5X RT Buffer, 6 mM MgCl2, 500 nM dNTPs (final concentration), 700 nM EV1 Reverse primer, 700 nM EV1 Forward primer, and 300 nM EV-BHQ TaqMan probe, 10 units of RNAsin, 2.5 units of Taq polymerase, and 5 units MULV reverse transcriptase. The Cepheid Smart Cycler® was programmed to: 1 hour

RT at 37°C followed by a 15 minute hold at 95°C for *Taq* activation, then 45 cycles of 94°C 15 seconds (denature), 60°C 1 minute (anneal/extension-optics on).

QRTPCR results were available three hours after the start of analysis, making the total PCR preparation and analysis time less than 5 hours for 15 samples. Results are reported as equivalent virus particles per unit sample volume, meaning that this is where the QRTPCR calculation indicated the sample appeared relative to the standard curve prepared from poliovirus standards.

Bacterial Analyses Using QPCR

The polycarbonate filters were processed for DNA extraction using the UltraClean™ Fecal DNA Isolation Kit (MoBio Laboratories, Inc., 12811-50) as per manufacturer's alternative protocol. Eluted DNA extracts were stored at –20°C until use.

Table 3. Primer and probe sequences for PCR detection of enterococci

Sequence Name	Nucleotide Sequence 5' to 3'	Length	GC (%)	T_m (°C)	Detection System*
ECST748F[1]	5'aga aat tcc aaa cga act tg-3'	20mer	35	51.2	
ENC854R[1]	5'-cag tgc tct acc tcc atc att-3'	21mer	47.6	57.9	
GPL813TQ[1]	5'Cy3-tgg ttc tct ccg aaa tag ctt tag ggc ta-BHQ-2-3'	29mer	44.8	65.3	Taqman

[1]Ludwig and Schleifer, 2000.

Total enterococci primers and probe were constructed using the rDNA regions around the target site of a well established enterococci group specific primer (ENC854R) (Table 3). The primer ECST748F targets enterococci, lactococci, and several clostridia. The target site of the probe GPL813TQ is present in rDNA from a variety of representatives of gram-positive bacteria with a low G+C DNA content (Ludwig and Schleifer, 2000).

Table 4. Master mix using individual reagents

Reagents	Final conc. (µM)	Initial vol (µl)
Water		3.9
10X Taq buffer (Mg^{++} free)	1	2.5
250mM MgCl2	5	0.5
10mM DNTPs	0.5	1.25
10µM ENC854R	1	2.5
10µM ECT748F	1	2.5
10µM GPL813 TQ Cy3 Probe	0.08	0.2
5U/u Taq polymerase	0.05	0.5

The Master Mix of reagents (Table 4) yields a final volume of 20 µl, to which 5 µl of sample (either DNA extract from an environmental sample, 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 min, and 45 cycles of denaturation (94°C) for 15 seconds, and annealing (60°C) for 30 seconds, the optics were turned on during the annealing step. The Cepheid Smart Cycler 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. For quality control, combined *E. faecalis* and *E. faecium* were used as our calibration strain for the total enterococci primer and probe set. Control bacteria preparations were prepared by boiling bacteria for 5 minutes, centrifuging 1 min at 12,000 rpm in a Beckman Microcentrifuge, and immediate storage on ice. *E. faecalis* and *E. faecium* cells were enumerated using either SYBR Green I epifluorescence microscopy (Noble and Fuhrman, 1998) and/or using Enterolert® or the EPA 1600 methods (APHA 1992). This yielded information on both the cell numbers in the sample, and the number of metabolically active cells present in the sample. Serial dilutions of the standards were made in duplicate in DEPC-treated sterile water, and four point standard curves are run in concert with the unknown samples on the Smart Cycler II instrument. Total enterococci primers were tested with all 19 validly described species of the genus enterococci, and demonstrated amplification of rDNA of all strains, with varying efficiencies.

Bacteroides sp. Using Conventional PCR

Amplification of the human-specific Bacteroides/Prevotella marker generally followed the procedure of Bernhard and Field (2000), with PCR primers that amplify partial 16S rRNA from the human fecal (HF) specific group. DNA was extracted a MoBio Ultra Clean fecal extraction kit. A range of extracted DNA quantities (2 – 5 µl, representing 1-70 ng per assay, with most samples in the range of 5-20 ng) was tested to avoid problems with inhibition. DNA was amplified with Bacteroides-Provotella specific primers Bac708r CAATCGGAGTTCTTCGTG and HF183f ATCATGAGTTCACATGTCCG. Each 50-µl PCR mixture contained the following reagents: 1 X Taq polymerase buffer (Promega), each primer at a concentration of 1µM, each deoxynucleoside triphosphate at a concentration of 200µM, 1.25U of Taq polymerase (Promega), 0.64 µg of bovine serum albumin (Sigma) per µl and 1.5mM $MgCl_2$. The thermal cycler was run under the following conditions, 2 min 95°C, then 25 cycles of 95°C for 30 sec, 60°C for 30 sec and 72 °C for 30 sec followed by a 5-min extension at 72°C. Then 1µ of each PCR product was re amplified using the same conditions as above for another 25 cycles. PCR products were visualized in a 2% agarose gel stained with 1X SYBR Gold (Molecular Probes) and compared to a 100bp DNA ladder (Promega). Positive results had 525 bp products. The positive control was human fecal sample extracted with a QIAamp stool kit. Negative controls use water instead of sample. All negative samples are spiked (in a second PCR

run) with 0.1 ng of positive control to determine possible inhibition. Inhibited samples are re-run with less DNA.

RESULTS

Ballona Creek

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 daylights 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.

The flux of fecal indicator bacteria remained relatively constant moving downstream in Ballona Creek (Figure 3). The average flux of *E. coli* ranged from 1.1×10^{10} to 5.3×10^{10} cells/hr at the six mainstem sites. The average flux of enterococcus ranged from 6.6×10^{8} to 1.4×10^{9} cells/hr at the six mainstem sites. In both cases, there was no discernable increase in bacterial flux; no two mainstem sites were significantly different from one another for either *E. coli* or enterococcus.

The flux of fecal indicator bacteria decreased over time (Figure 4). The average flux of enterococcus was highest at 9:00 AM (2.9×10^{9} cells/hr) and monotonically decreased throughout the study period. The lowest flux was measured at 2:00 PM (3.0×10^{9} cells/hr). Similar patterns were observed for *E. coli* (data not shown). In contrast to the culture-based methods, the QPCR method for measuring enterococcus did not decrease over time. The flux of enterococcus ranged from 2.7×10^{10} to 4.7×10^{10} cells/hr with the 9:00 AM and 2:00 PM samples being nearly equivalent.

The relative pattern of enterococcus 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 *E. coli*. The flux of enterococcus from Benedict tributary ranged from 4.1×10^{9} to 1.4×10^{10} cells/hr throughout the sampling period while the flux of enterococcus from Adams tributary ranged from 3.7×10^{5} to 4.4×10^{6} cells/hr. On average, Benedict tributary contributed 81% of the enterococcus loading from all four tributaries.

The hourly flux of enterococcus (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 its nearest downstream site. The mainstem showed virtually no response to any of these tributary inputs, including Adams. Enterococcus flux remained virtually unchanged from upstream to downstream of each of the tributary inputs (Figure 5, Figure 3).

Measurements of *Bacteroides* sp. and enterovirus indicated the presence of human fecal contamination throughout the system (Table 5). *Bacteroides* sp. was present in 12 of 36 mainstem samples (33%). Enterovirus was present in 14 of 36 mainstem samples (44%). The concordance among these measurements was nearly complete; almost every location that detected *Bacteroides* sp. was also positive for enterovirus. Only two samples were positive for enterovirus and not *Bacteroides* sp. These two samples were furthest downstream or latest in the day.

Table 5. Number of enterovirus genomes (per 100 ml) detected.

Distance Upstream (km)	Time of Day					
	9:00	10:00	11:00	12:00	1:00	2:00
6.3	106*	71*	93*	70*	67*	
5.4		41*	19**	25*		
4.7		17*		113*	51	
2.6				79*		
1.5				13*		39
0						

* Human Bacteroides marker also detected.
** PCR reaction for human Bacteroides marker inconclusive due to inhibition.

Spatial and temporal patterns in enterovirus concentration were evident in the Ballona Creek system (Table 3). 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 Ave., where 89% of the samples contained measurable levels of enterovirus. In addition, the highest concentrations of enterovirus were measured at Cochran Ave. during four of the six time periods. A general pattern in enterovirus detection was observed during the course of the day. Enterovirus was detected earliest in the day at upstream sites. Enterovirus was detected most frequently late in the day at the downstream sites. The 12:00 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 high concentrations in any of the tributaries; only Adams tributary had any detectable enterovirus.

Malibu Creek

Malibu Creek had a similar pattern of fecal indicator bacteria concentrations at each sampling event throughout the study period (Figure 6). Concentrations decreased along the mainstem as it flowed from Cold Creek to Cross Creek, then increased as it flowed through the estuary until it discharged into the ocean at Malibu Beach. The increase in fecal indicator bacteria through the lagoon averaged 10^2 MPN/100 ml for both

enterococcus and *E. coli*. The dilution factor from the discharge to the shoreline as a result of wave induced mixing averaged 0.86 for *E. coli* and 0.34 for enterococcus. Despite the increase in fecal indicator bacteria concentrations, none of the Malibu samples were positive for enteroviruses or *Bacteroides* sp.

BMP's

Both of the BMPs that incorporated UV treatment systems were more effective than the constructed wetland at removing fecal indicator bacteria (Table 6). Albeit concentrations were low in the influent, the constructed wetland did not reduce concentrations of *E. coli* or enterococcus using either culture-based or QPCR methods in the effluent. Both UV treatment systems, however, reduced influent concentrations of *E. coli* and enterococcus in the effluent to near or below method reporting levels. Enterococcus concentrations by QPCR were reduced by an order of magnitude, which was not as great as the culture-based method. No enterovirus or *Bacteroides* sp. were detected in any of the BMP influent or effluent samples analyzed.

Table 6. BMP effectiveness for indicator bacteria removal measured by culture-based (chromogenic substrate, CS) or quantitative polymerase chain reaction (QPCR) methods.

Indicator	Constructed Wetland		Filtration+UV		Filter+DAF+UV	
	Influent	Effluent	Influent	Effluent	Influent	Effluent
***E. coli* (CS) (MPN/100 ml)**	<10	36	58	20	147	<10
Enterococcus (CS) (MPN/100 ml)	<10	47	184	15	42	<10
Enterococcus (QPCR) (Cells/100 ml)	163	110	5004	729	No Data	No Data
Enterovirus (genomes/100 ml)	-	-	-	-	No Data	No Data

* no *Bacteroides* sp. detected
- no enterovirus detected

DISCUSSION

The Ballona Creek watershed is a system severely 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 discharges 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, including 100% of the samples just upstream of SMB, exceeded the water quality thresholds established by the State of California. 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.

Our 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.

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^3/s discharge furthest downstream at Inglewood Ave. 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 Ave. 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 Ave. Since Cochran Ave. had the most frequent occurrence and highest concentrations of enterovirus, this source would appear to be the most likely candidate for future management actions. The co-occurrence of the human *Bacteroides* sp. marker at most of the locations and time periods where enterovirus was quantified, most notably in all of the Cochran Ave. samples, provides 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; Leguyader et al., 1994), and viruses have sometimes been found in oysters without coliform contamination (Goyal et al., 1984; Yamashita et al., 1992). 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. Noble *et al* (2000) measured virus and fecal indicator bacteria in dry weather urban runoff in drains along 300 km of shoreline from Santa Barbara to San Diego. Despite 46% of the storm drains containing detectable enterovirus, there was no correlation with fecal indicator bacteria concentrations.

The results of this study indicated that Ballona Creek presents a greater risk to human health than Malibu Creek. There were no enterovirus or *Bacteroides* sp. detected in any sample from the Malibu Creek watershed. The bacterial concentrations were lower at Malibu Creek than at Ballona Creek; none of the Malibu Creek samples from the bottom of the watershed (at Cross Creek) exceeded water quality thresholds established by the State of California. Interestingly, fecal indicator bacteria concentrations increased as water flowed through the lagoon at the base of the Malibu watershed. No enterovirus or *Bacteroides* sp. was detected in these samples either, although other studies have detected human specific sources of viruses in this discharge (John Griffith *personal communication*).

The use of QPCR to measure fecal indicator bacteria presents unique opportunities and challenges. The advantage of QPCR for measuring fecal indicator bacteria is speed potentially providing measurements in less than four hours (Griffith et al 2004). 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 enterococcus using culture-based methods generally decreased as the day progressed, most likely as the result of degradation from sunlight (Noble et al. 2004). Ballona Creek is a 40m 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 even though the enterococci were not viable.

The data from this study suggested the UV treatment systems were more effective than the constructed wetland at reducing fecal indicator bacterial concentrations. We suspected the UV treatment system would be effective since this method is a well-known mechanism for degrading bacteria (Fujioka et al. 1981, Davies and Evison, 1991, Davies-Colley et al. 1994, Noble et al 2004). Not only did the UV system reduce concentrations of enterococci using culture-based techniques , but it also degraded its DNA as shown by large reductions in enterococcus by QPCR. On the other hand, the effectiveness of the treatment wetland remains incompletely quantified. Although levels of fecal indicator bacteria were similar before and after flowing through the wetland, concentrations were very low to begin with. Monitoring by others at this treatment wetland suggest that it has been effective at reducing fecal indicator bacteria concentrations using culture-based methods (Nancy Palmer, City of Laguna Niguel *personal communication*). More study, particularly with the QPCR, will be needed before the wetland effectiveness can be fully quantified.

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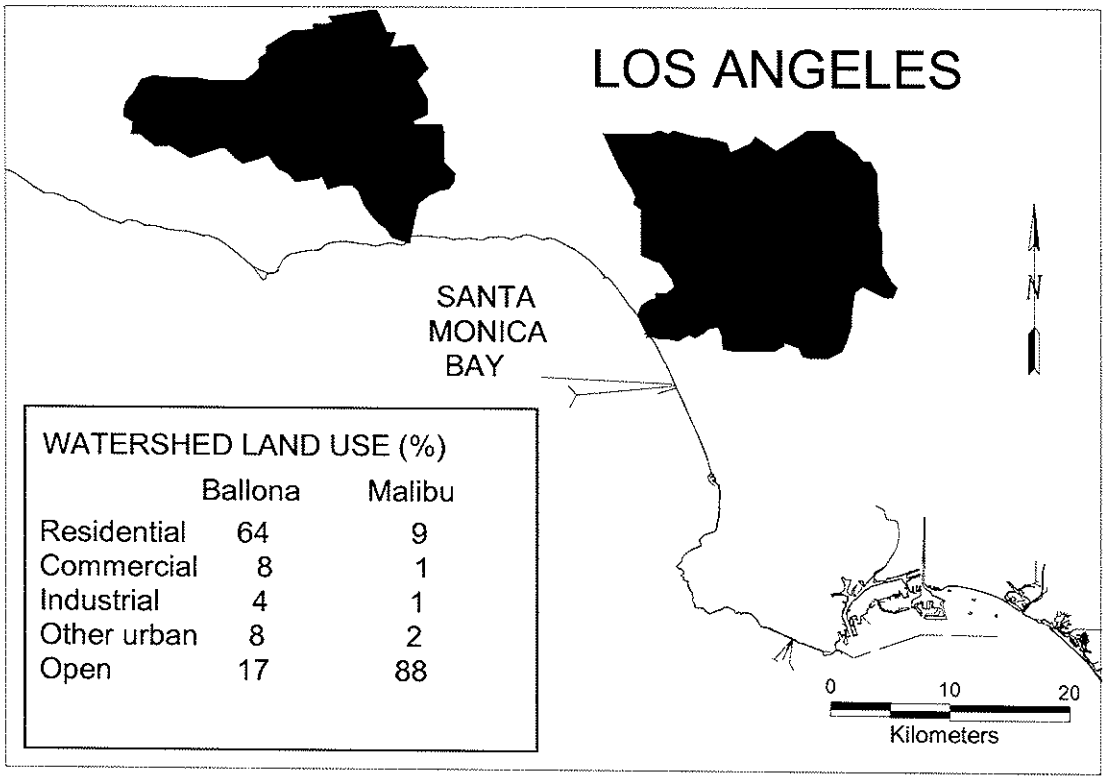


Figure 1: Map of Santa Monica Bay, CA indicating the locations and land use distribution for Ballona Creek and Malibu Creek watersheds.

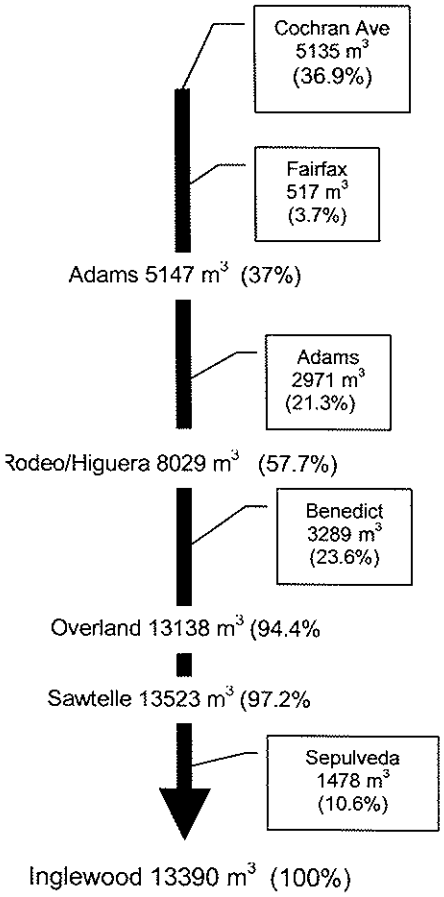


Figure 2. Schematic diagram depicting additive flow in main channel and percent contribution from each tributary.

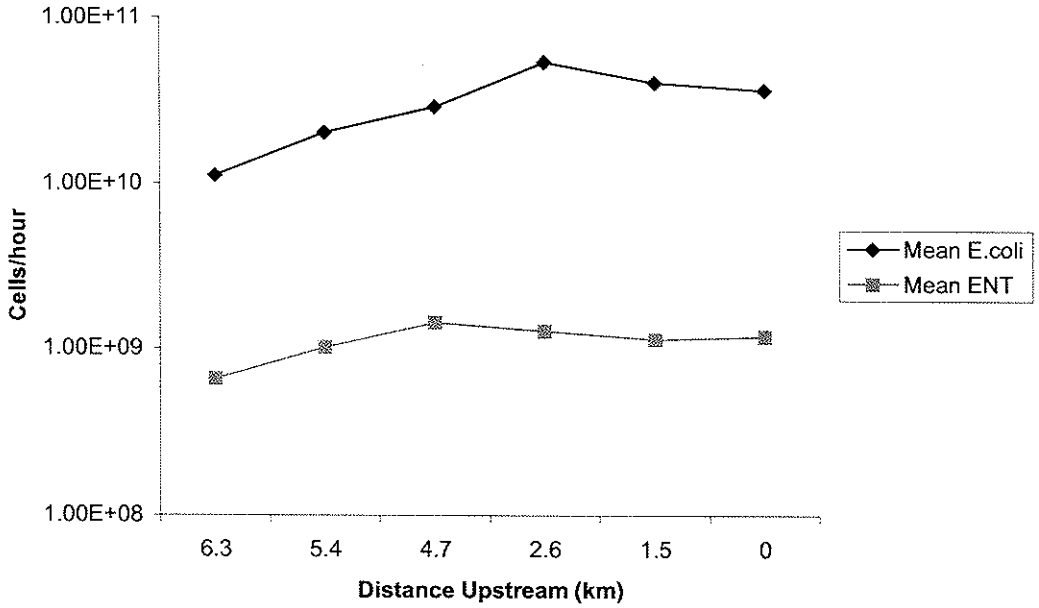


Figure 3. Mean hourly flux of *E. coli* and enterococcus at each station in main channel of Ballona Creek measured using the IDEXX™ method.

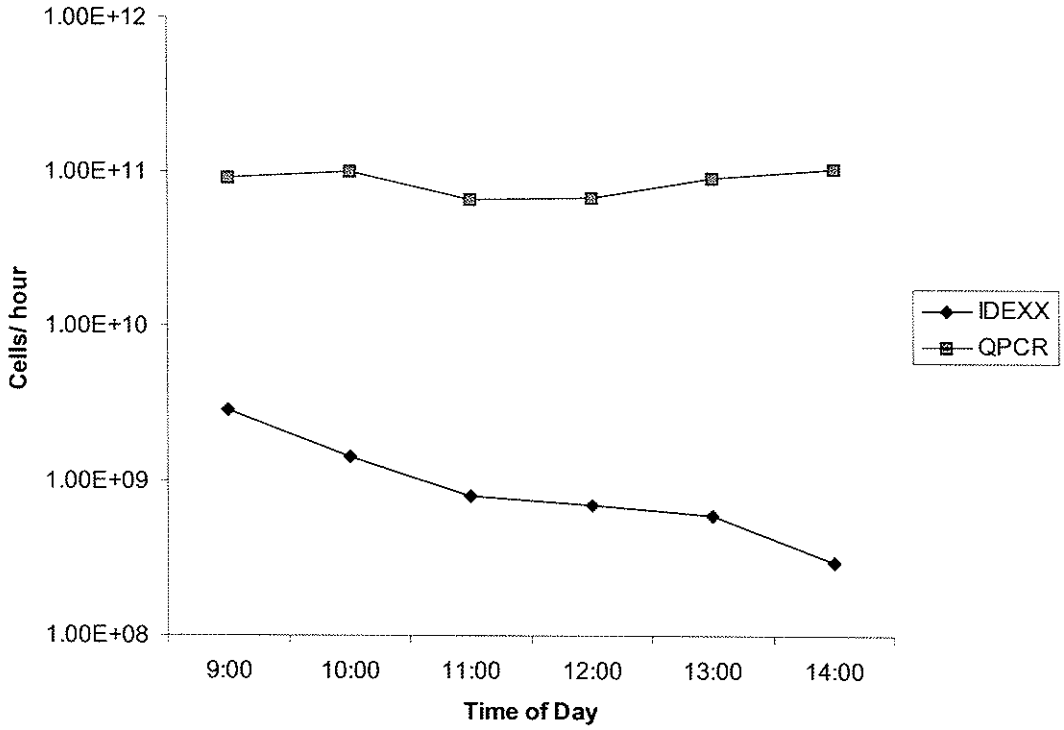


Figure 4. Mean hourly flux of enterococcus along the main channel of Ballona Creek as measured using both IDEXX the QPCR methods.

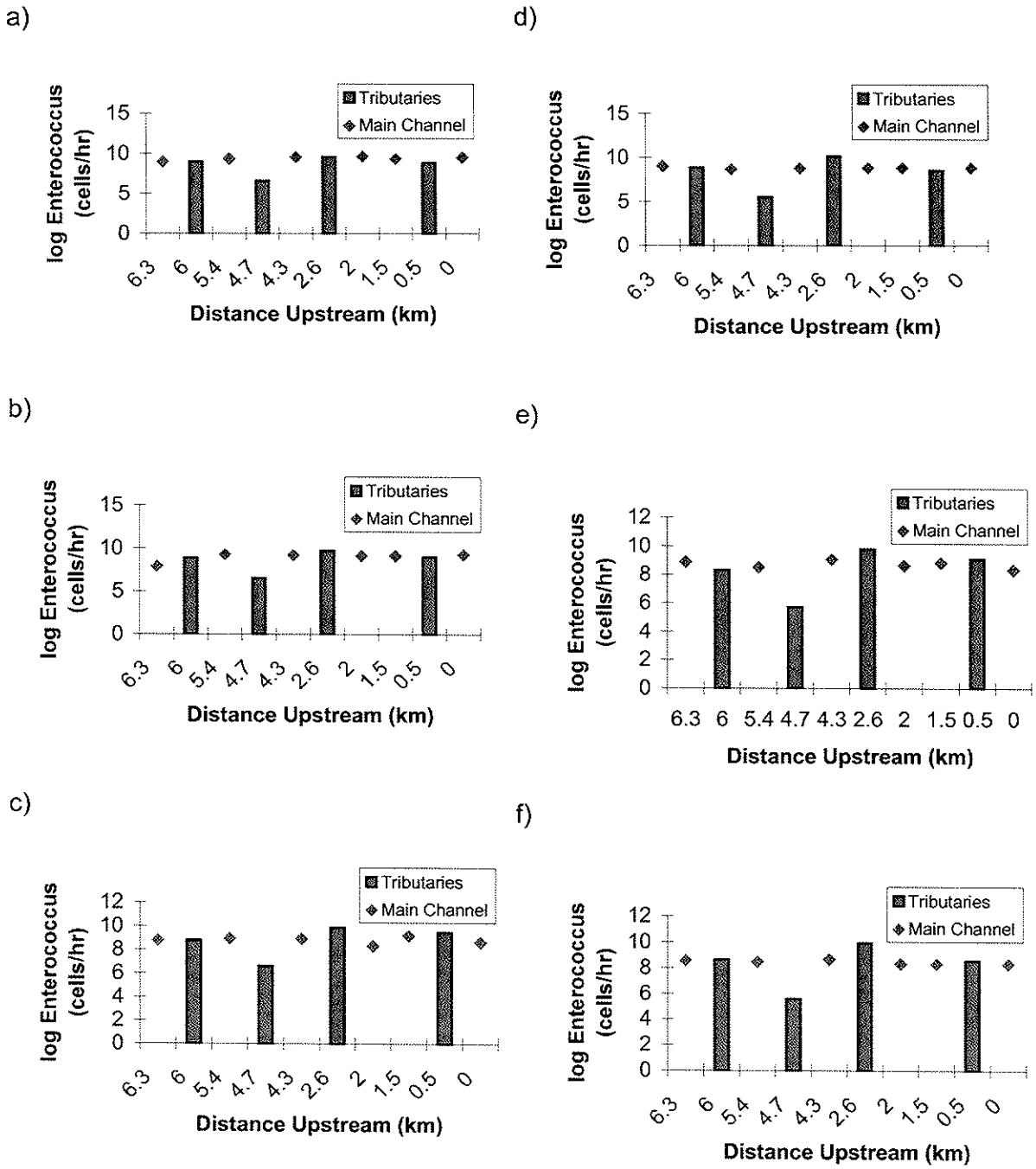
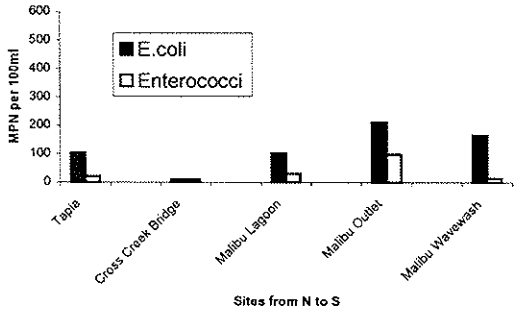


Figure 5. Enterococcus loading in main channel and tributaries of Ballona Creek at a) 9:00, b) 10:00, c)11:00, d) 12:00, e)13:00, f)14:00.

a)



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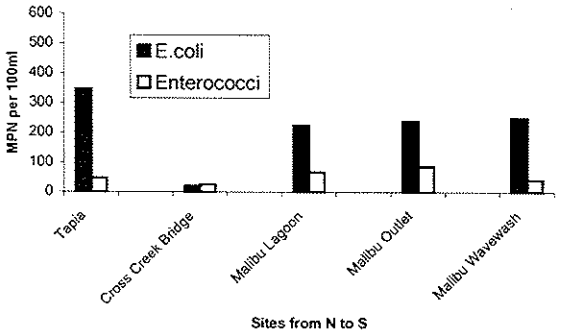

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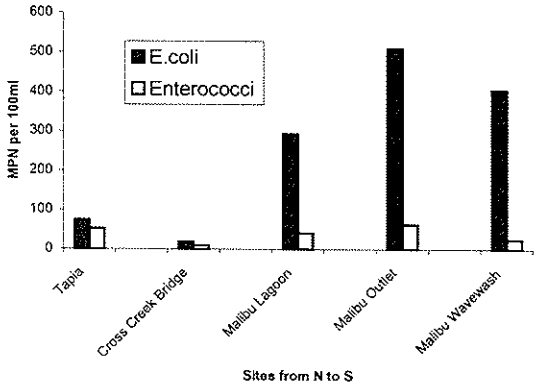

Figures 6. Fecal indicator bacteria concentrations in Malibu Creek on a) 11/10/04 b) 11/11/04 and c) 11/12/04

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Malibu Downtown Area
Well Location Map

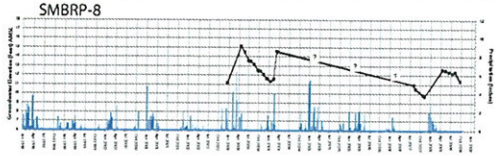
0 200 400 600 800 1000
US survey feet

0 50 100 150 200 250 300
Meters

Aerial Photo: USGS High Resolution
State Orthoimagery for Los Angeles
County, California, 2006

N

SMBRP-8



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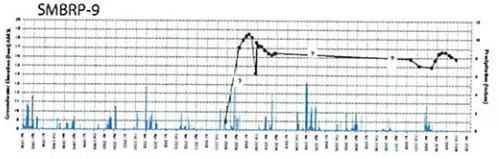

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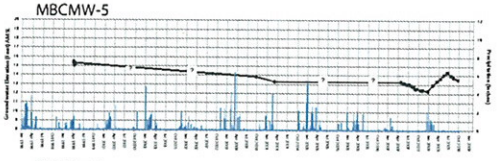

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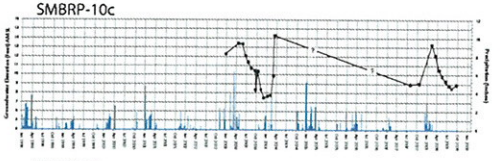

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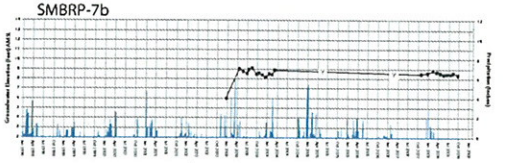
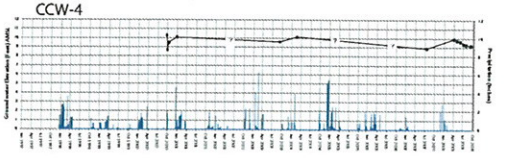


CCW-4



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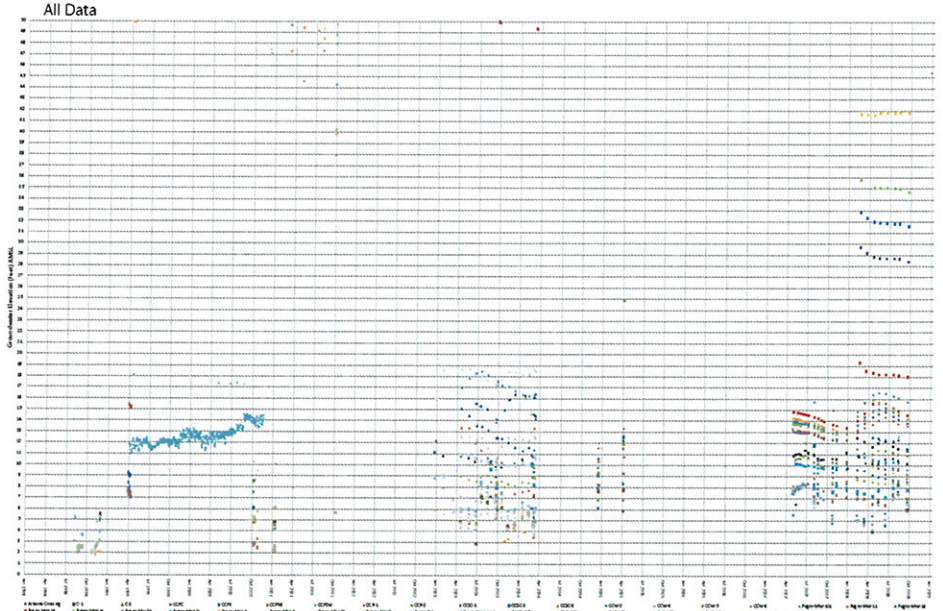
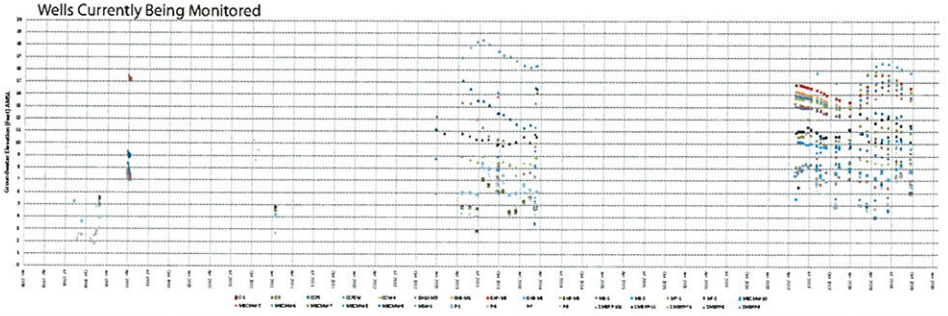


Wells Currently Being Monitored



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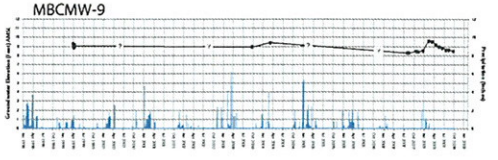

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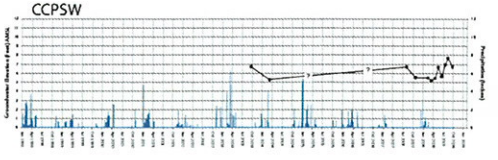

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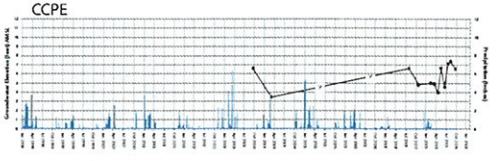

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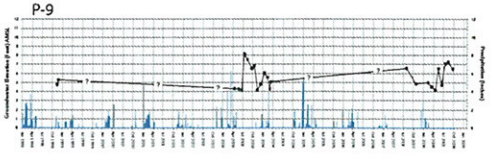

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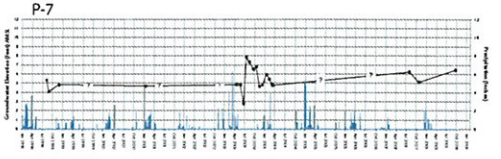

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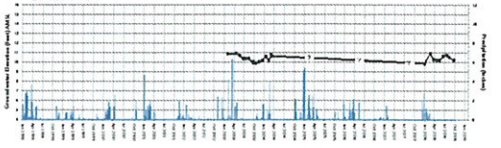

Earth Consultants International

STONE ENVIRONMENTAL INC

McDonald Morrissey ASSOCIATES Inc.

Malibu Downtown Area
Historical Hydrographs 1998-2008

Notes:
First water level measurement taken for SMBRP-7b,8,9, and 10c may be inaccurate due to procedures during the installation process.

Precipitation data compiled from hourly rain gage MLB located in the hills above Malibu and maintained by Los Angeles County Fire Dept. in cooperation with the California Department of Water Resources.

Groundwater data compiled from:
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