San Diego County Enterococcus Regrowth Study

Final Report

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Southern California Coastal Water Research Project
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Prepared for the Municipal Stormwater Copermittees of the County of San Diego

by

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EXECUTIVE SUMMARY

Southern California beaches experience elevated levels in enterococci bacteria more frequently as compared to other fecal indicator bacteria (FIB), such as total coliforms and fecal coliforms (Noble, *et al.*, 2003). This may in part be due to the fact that enterococci include non-fecal or "natural" strains that live and grow in water, soil, plants and insects (Mundt *et al*., 2001; Kuzina *et al*., 2001; Jay *et al*., 2005; Cox and Gilmore 2007). Thus, elevated levels of enterococci in water could be related to input from natural sources. There is a general lack of knowledge about the abundance of enterococci among natural sources, including the ability of these bacteria to grow in the environment, and how these factors are related to their occurrence in water.

When FIB levels in beach water exceed contact water recreation (REC-1) beneficial use water quality objectives (WQO) and AB 411 public health standards in California, local agencies and municipalities are mandated to identify and reduce sources of FIB, including enterococci. It has been hypothesized that the storm drain system, comprised of surface features, underground pipes, and open channels, is a corollary source of FIB and specifically enterococci due to natural sources. A focused study of storm drains as potential source of enterococci is essential to the efficient and effectual management of this problem.

In 2008, the 21 Municipal Stormwater Copermittees of the County of San Diego, in cooperation with the Regional Water Quality Control Board, San Diego Region, supported the combined efforts of SCCWRP, the City of Encinitas and the City of San Diego to conduct a microbiological study to investigate storm drains as a potential source of *Enterococcus* bacteria to San Diego's coastal waters during dry weather. Moonlight State Beach in Encinitas and Rock Pile Beach in La Jolla (the City of San Diego) were selected as study sites based on a regional survey of beaches impacted by storm drains or creeks historically demonstrating high levels of *Enterococcus.* The goals of this study were to determine the ability of enterococci to grow in the storm drain environment and to identify potential natural sources of these bacteria in storm drains and beach water. The approaches used to address these goals included:

- Placement of sterilized concrete coupons to in drains to determine if enterococci in storm water attach to and grow on storm drain surfaces,
- Prioritizing sources of enterococci by quantifying densities in storm drain flows, beach water, vegetation, sand, seawrack, and insects,
- Correlating levels of enterococci with water quality parameters such as nutrient levels, temperature, pH, turbidity, etc.,
- Determining relationships between enterococcal species and biotypes (sub-species) in runoff flows, beach water and surface biofilm to that of fecal (bird and sewage) and natural sources,
- Testing creek/storm drain water samples for *Bacteroidales* human marker (HF183) to rule out human fecal contamination.

Sampling at both study sites began in May 2010 and ended in October 2010. Samples were collected weekly or every other week depending on the parameters being measured.

The results of this study showed that the freshwater plants, algae, decaying organic material and seawrack in Cottonwood Creek harbored high densities of enterococci, indicating that they are potential sources to Moonlight State Beach. Greater than 80% of enterococci isolates from

Cottonwood Creek were identified as *E. casseliflavus* or *E. mundtii*: species primarily found on plants and in soil and not considered common inhabitants of the gastrointestinal tracts of humans. Moreover, the distribution of enterococci species and strains found in the creek and the storm drain system during the 22 week sampling period were phenotypically most similar to species and strains found among natural sources as compared to those present in sewage. Finally, enterococci were shown to colonize concrete coupons placed in the storm drain, demonstrating that enterococci in Cottonwood Creek were capable of growing on concrete storm drain surfaces.

At La Jolla (Sample Site CSD037), enterococci appeared to be even better adapted to growing on concrete surfaces in the enclosed storm drain. Similar to Moonlight State Beach, up to 87% of enterococci isolated from flows in CSD037 were identified as *E. casseliflavus* or *E. mundtii* and results of the coupon study at this site showed higher densities of *Enterococcus* per unit of surface area than was observed at Moonlight State Beach.

While only a small number of water samples were tested, none of the creek/storm water or beach water samples tested for the human *Bacteriodales* marker at either beach had concentrations indicative of human fecal contamination. These results suggest that human fecal contamination may not have been a significant source of enterococci to either storm drain during the study period.

This study demonstrated that storm drains are themselves a potential source of enterococci to beach water. Enterococci were shown to be able to colonize and grow on concrete surfaces and high concentrations of bacteria were found associated with vegetation, algae and in settled organic material on the creek bottom at Moonlight State Beach. In the enclosed drain at La Jolla, concentrations of enterococci attached and growing on concrete were higher than those seen on coupons at Moonlight State Beach, indicating that these bacteria were better able to grow and thrive in this environment than in the more exposed location at Moonlight State Beach. Further, greater than 80% of enterococci isolated from beach water at both sites were the same strains found growing in the storm drains.

Despite the apparent link between enterococci growing in storm drains and that found in beach water, it is unclear how and when enterococci make their way to the beach or if the rate at which enterococci slough off of biofilm growing in enclosed drains is great enough to cause exceedances of water quality standards. Future work will focus on characterizing reservoirs of enterococci in watersheds and the fate and transport of enterococci growing on drain surfaces and other upstream sources to the beach..

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1. INTRODUCTION

Total coliform, fecal coliform, *E. coli* and *Enterococcus* are fecal indicator bacteria (FIB) which serve as surrogates for human pathogens that can cause gastrointestinal illness. This relationship has been established through epidemiology studies of swimming related illness at sewage impacted beaches. In 1986, California adopted a water quality standard for *Enterococcus* to gauge the sanitary quality of marine recreational water. Enterococci are normal inhabitants of the intestinal tracts of humans and animals. However, multiple studies have shown that levels of enterococci in marine water are not well-correlated with the presence of pathogens. One reason this may be so is that the *Enterococcus* group also includes "natural" strains that are adapted to grow in the environment or that primarily exist in extra-intestinal habitats. Current water testing methods do not distinguish between fecal and natural strains of *Enterococcus*. Thus, presupposing that all gauging enterococci measured in environmental water samples are of fecal origin may lead to erroneous conclusions regarding microbial water quality and associated health risk to humans.

Contaminated water flowing in storm drains conveys enterococci from upstream sources, ultimately impacting coastal waters. However, if enterococci persist and grow on and shed from surfaces within storm drains, it is possible for a storm drain to not only be a conveyance, but a source of enterococci to the beach. One way bacteria persist and grow in the natural environment is to develop and attach to surfaces by forming biofilm (i.e., layers of slime produced by bacteria). Enterococci are known to produce and thrive in biofilm (Tendolkar *et al*. 2004). Biofilm provides enterococci access to nutrients, protects them from ultraviolet light, disinfectants and desiccation and allows them to remain in a viable but non-culturable state for extended periods (Lleò *et al*. 2005). When environmental conditions are favorable for growth, bacteria in biofilm can replicate to high levels. As the biofilm ages, bacteria on the outermost layers may be sloughed off by forces such as flowing water and transported to other locations where biofilm formation may be re-established. Thus, it has been hypothesized that enterococci both form and attach to biofilm on surfaces in storm drains as well as on sand particles, rocks, plants, algae, decaying organic material and even insects. Once the biofilm matures, this enterococci may then be shed or resuspended into the water, becoming an intermittent or persistent source of bacteria to beaches.

One way to better understand the effect of natural enterococci on beach water quality is to quantify and identify enterococci at the species and strain level. These methods are helpful in distinguishing natural versus fecal inputs of enterococci and for establishing relationships between enterococci in storm and beach water. This study followed the development of biofilm containing enterococci on stormdrain surfaces and used species identification and a broad-based strain typing method (phenotyping) as a tool for preliminary comparison of enterococci found among fecal and natural sources compared to those present in receiving waters.

1.1 Study Objectives

The main objectives of this study were:

1. To determine if biofilms that form on the surfaces of storm drains support the growth of enterococci.

2. To determine if enterococci growing on drain surfaces are numerous enough to be considered a potential source of bacteria to beach water and sand.

3. To determine if enterococci growing on drain surfaces may be differentiated from those of fecal origin.

A secondary objective was to assess potential reservoirs of *Enterococcus* in Cottonwood Creek.

1.2 Description of Sampling Sites

1.2.1 Moonlight State Beach – City of Encinitas

The City of Encinitas lies on the northern coast of San Diego County, 21 miles north of the City of San Diego. Moonlight State Beach is situated in a residential neighborhood at the bottom of a hill which gently slopes to the Pacific Ocean. A wide, sandy beach offers recreational activities that include swimming, surfing and fishing. The beach receives urban runoff from Cottonwood Creek, which conveys nuisance flows and groundwater from residential and commercial areas upstream. About 85% of the dry weather creek flows are treated at an on-site Ultra Violet (UV) treatment facility approximately 230 yards upstream of Moonlight State Beach (Figure 1). A 15% by-pass of the UV treatment facility was required by the San Diego Regional Water Quality Control Board to maintain biological connectivity. Combined flow (UV treated and by-pass creek water) travels through a densely vegetated section of Cottonwood Creek before flowing through three storm drain pipes that discharge directly into the receiving water at Moonlight State Beach (Figures 2 & 3). The pipes are 72 in. oblong and stretch 284 ft. from the creek channel to the ocean outfall. The pipes are constructed of corrugated galvanized steel and are tar lined to prevent corrosion. Flow rates are typically 141-150 gpm (velocity is 0.68ft./sec).

Figure 1: Sampling Stations at Moonlight State Beach

Figure 2. Cottonwood Creek (Upstream)

Figure 3. Cottonwood Creek Outfall at Moonlight State Beach (Downstream)

1.2.2 Rock Pile Beach (La Jolla) - City of San Diego

Rock Pile Beach in La Jolla is located below a storm drain identified as CSD037. Enterococcal regrowth was assessed inside the drain pipe which is constructed of concrete and runs 8 ft. below ground (Figure 5). Dry weather flow consists of nuisance flows and ground water intrusion from nearby residential and commercial areas. Upstream flows were collected below the manhole at the corner of Midway Street and Chelsea Avenue (Figure 4). Downstream flows were obtained at the outlet of the pipe located above the tidal prism (Figure 6). Flow rates are typically 11 – 13.5 gpm (velocity is 2.9ft./sec). Flows are generally constant except when runoff is released from the sump upstream leading to sudden increases in flow. Under typical dry weather conditions, flows discharged at the outlet seep under cobble rock and are not observed flowing across Rock Pile Beach. While the beach is considered a recreational area, due to limited accessibility to this site, it is rarely used for swimming.

Figure 4. Sampling stations for storm drain CSD037 in La Jolla

Figure 5. CSD037 (upstream, below manhole)

Figure 6. CSD037 (downstream, outlet at Rock Pile Beach, La Jolla)

1.3 Sample Collection

Field sampling at Moonlight State Beach in Encinitas and CSD037 in La Jolla commenced on May 19, 2010 and ended on October 5, 2010. Samples at Moonlight State Beach were collected by personnel from the City of Encinitas Clean Water Program. Storm drain CDS037 was sampled by the City of San Diego Storm Water Department-Pollution Prevention staff. The sampling protocols are available in Appendix B.

A total of 426 samples were collected from both locations (Table 1). At Moonlight State Beach, samples included creek water upstream and downstream, UV treated creek water, UV bypass creek water, groundwater, beach water, concrete coupons, plants, algae, beach sand, insects and seawrack. Insects (brine flies and springtails) found on the wrack and underlying sand were also analyzed as transport vectors of enterococci. Samples from CDS037 consisted of upstream and downstream water, concrete coupons and beach water (when pipe flow was observed flowing into the ocean).

Beach and creek/storm water samples for microbiological analyses were collected on a weekly basis from weeks 2 through 14 and every other week following week 14. Coupons and natural samples (plants, sand, etc.) were collected every other week from week 2 through week 20.

Storm water samples for chemical analysis were collected every other week and transported to contract laboratories within 2 hours of collection. In total, 22 storm water samples (11 samples each from CSD037 in La Jolla and Cottonwood Creek in Encinitas) were collected and analyzed for nutrients. There were 185 analyses for ammonia, total nitrogen, nitrate, nitrites, phosphates and total organic carbon which were analyzed by EnviroMatrix for all samples collected in Encinitas and Test America Laboratories for all samples collected in La Jolla.

Table 1. Inventory of Samples Collected

	Moonlight State Beach	**Rock Pile Beach - La Jolla**
Water Samples		
Beach Water	17	3*
Upstream Creek/Storm Drain Water	17	17
Downstream Creek/Storm Drain Water	17	17
Creek Upstream of UV Facility Water	17	
UV Treatment Facility Treated Water	9	
Groundwater	1	
Street Runoff	1	
Plant Samples		
Algae/Decomposed Vegetation	13	
Freshwater Plants	16	
Seawrack	13	6
Other Samples		
Concrete Coupons	44	44
Cobble Rock		4
CSD037 Drain Biofilm		3
Beach Sand	15	
Bird Stools	22	
Insects	130	
Total No. Samples	**332**	**94**

*Samples only collected when storm drain observed flowing into beach

2.0 METHODS

Table 2. Summary of Study Objectives and Corresponding Sample Type and Analysis Methods.

Objective	Sample Type	Analysis Method
Identify Natural Sources	Birds, Beach Sand, Freshwater Plants, Algae/Decomposed Vegetation, Seawrack, Insects	Enterococcus Enumeration
Identify Other Source Inputs	Groundwater Inflows to Cottonwood Creek, Street Runoff Adjacent to Cottonwood Creek	Enterococcus Enumeration
Assess Spatial and Temporal Variability of Enterococci Levels in Impacted Waters	Cottonwood Creek Water (Upstream and Downstream), Moonlight State Beach water, CSD037 Water (Upstream and Downstream), Rock Pile Beach Water	Enterococcus Enumeration
Investigate Enterococcal Growth by Measuring Density of Enterococci Attached to Coupons; Look for Clonal Biotypes	Concrete Coupons	Enterococcus Enumeration, Enterococcus Speciation and Biotyping
Correlate Enterococci Densities in Creek/Drain Water and on Coupons with Nutrients and Other Water Quality Parameters	Cottonwood Creek Water, CSD037 Water, Coupons	Nutrients, Temperature, pH, Conductivity, Dissolved Oxygen, Total Organic Carbon, Turbidity
Characterize Enterococcus Species and Biotype Distributions Representative of Impacted Waters, Sources, and Coupons	All	Enterococcus Speciation and Biotyping
Compare Relatedness of Enterococcus Biotypes Representative of Impacted Waters, Sources, and Coupon	All	Multivariate Analysis of Enterococcus Biotypes (Community Analysis)
Assess Possible Human Fecal Contamination in Impacted Waters	Cottonwood Creek water, Moonlight State Beach water, CSD037 water, Rock Pile Beach Water	Bacteroidales Human HF183 Marker

2.1 Identifying Natural & Other Source Inputs

2.1.1 *Enterococcus Enumeration*

All samples were enumerated in duplicate for enterococci using IDEXX Enterolert, resulting in 722 tests (see Appendix C4 "Bacteriological and Chemistry Results" spreadsheet). A subset of samples (N=358) was also enumerated using EPA Method 1600 to obtain isolates for species identification. (Note: although MPN values are based on statistical probability and CFU are observed colony counts, for regulatory purposes the units are considered equivalent).

Natural samples (i.e., sand, seawrack, plants, etc.), coupons, and water (beach and storm water) samples were analyzed for enterococci by the City of San Diego Environmental Monitoring Department (EMD). Protocols for processing and analyzing samples are described in Appendix C.

2.2 *Enterococcus* Speciation and Biotyping

A total of 913 presumptive enterococci isolates were identified to genus/species level from most sample types (N=85) collected during the beginning (weeks 2 & 6), middle (weeks 10, 13, & 14) and end of the study period (weeks 16 and 20). An average of 10 isolates were typed per sample. *Enterococcus* species identification was done using Vitek II (an automated bacterial identification system) and additional biochemical testing (see protocols in Appendix D3) (Ferguson *et al*. 2005). In addition to genus/species identification, the biotype number obtained using Vitek II was utilized to indicate phenotypic strain types (i.e. biotypes). The biotype numbers were also used to explore similarities and differences between enterococci communities in beach/creek/storm water and those of potential sources.

2.3 Analyzing Bacteria Sources

2.3.1 Freshwater Plants, Seawrack/Kelp and Algae

To determine enterococcal densities among plant sources at Moonlight Beach, vegetation including freshwater plants and algae was collected from Cottonwood Creek and seawrack/kelp was collected near the outfall at the beach. In Cottonwood Creek, samples included the roots and leaves of *Ditichilis spicata*, the most abundant plant type and algae from the bottom of the pipe drain. Fresh and decomposed seawrack/kelp samples collected at the ocean outfall were predominantly *Macrocystis* species. All plants were collected by hand using disposable gloves, rinsed in creek water to remove attached sediment, and placed into ziplock bags. In all, 16 freshwater plant, 13 algae and 14 seawrack/kelp samples were collected during the study period.

Ditichilis spicata

Macrocystis

Algae

All samples were processed at the laboratory within 2 hours of collection. About 25 - 50g of plant material was transferred to clean ziplock bags. Phosphate buffered saline (PBS) was added to achieve a 1:10 dilution. The bags were shaken vigorously for about 10 – 20 seconds followed by gentle massaging for 2 min. to remove attached enterococci. The eluant was poured into sterile Nalgene bottles and processed similarly to the water samples for enterococci using both IDEXX Enterolert and EPA Method 1600 (Appendix B).

2.3.2 Beach Sand

A total of 14 sand samples were collected at Moonlight State Beach during the study period. The top 2 cm of beach sand in the intertidal zone was collected using sterile 50 cc centrifuge tubes. All samples were processed at the laboratory within 2 hours of collection. PBS was added to the samples and followed by vigorous hand shaking for 2 minutes as per Boehm *et al*. (2009). Eluants were processed similar to water samples using IDEXX and EPA Method 1600 (Appendix B).

2.3.3 Bird Stools

At Moonlight State Beach, bird stools, mostly from seagulls were also assessed as a natural fecal source of enterococci. This work was done as part of a separate project funded by the City of Encinitas to complement the bacteria regrowth study. A total of 55 bird stool samples (comprised of 5 individual stools collected every other week) were obtained from the sand near the outfall at Moonlight State Beach. All samples were processed at the laboratory within 2 hours of collection. Enterococci were isolated from the samples and enumerated similar to the methodology used to analyze sand (sediment) samples (Appendix B).

2.3.4 Brine flies and Springtails

Springtails (*Collembola*) and brine flies (*Ephydidrae*), which were abundant on seawrack and beach sand at Moonlight State Beach, were examined as transport vectors and/or reservoirs of enterococci. Brine flies were collected by scooping an insect net around swarms flying above seawrack that was present on the beach. The flies were transferred into zip lock bags. Piles of sand containing springtails (arthropods known as *Collembolla*) were also scooped into zip lock bags. The flies and springtails were transported to the laboratory where they were immobilized and separated from sand by placing them into the freezer for 15 minutes. A total of 80 springtails and 50 brine flies were analyzed for enterococci. The outside surfaces as well as the guts were processed using previously published methods (Kuzina et al., 2001; Cox and Gilmore 2007) and enumerated for enterococci using Enterolert and EPA Method 1600.

2.3.5 Concrete Coupons

Fifty sterilized virgin concrete coupons were placed on the bottom surfaces of each of the storm drain pipes in La Jolla at CSD037 (Figure 5) and in Cottonwood Creek (Figure 7) on May 19, 2010. The coupons measured 2 x 3 x 1 inches and were strung together using a stainless steel wire attached to the storm drain to keep coupons immobile and submerged under flowing water. After a 2-week incubation period, two coupons were removed weekly from each site until the end of the study. Each time a coupon was removed it was immediately placed in a 2 L bottle containing 500 ml PBS and transported to the City of San Diego's Marine Microbiology Laboratory, Environmental Monitoring Department (EMD). Three different extraction methods were employed to examine the level of enterococci cell attachment to the coupons using: (1) swirling to remove loosely attached cells; (2) shaking to remove moderately attached cells; and (3) sonication (using a Branson Sonifer 450 for three minutes at 30% output (i.e. 120 Watt) to remove firmly attached cells as per Ferguson *et al*. (2005). Eluants resulting from each of these fractions (F1=loosely attached cells; F2=moderately attached; F3=firmly attached) were analyzed in duplicate similarly to the methodology used to analyze water samples (Enterolert for enumeration and EPA Method 1600 to obtain isolates for speciation). Since enterococci in the "loose" fraction could have also included enterococci that had settled out of water, only enterococci extracted by shaking and sonication, i.e. attached cells (measured for F2 plus F3) were considered to be biofilm related.

Figure 7. Collecting Concrete Coupons in Cottonwood Creek

2.4 *Enterococcus* Community Analysis

The community structure of *Enterococcus* species and strains representative of the various sources were compared using phenotyping methods based on the physical characteristics and biochemical reactions of bacteria. First, the distributions (i.e., abundance) of *species communities* representative of water and source samples were determined to make cursory comparisons between source types. This was followed by a more detailed level of analysis of *Enterococcus biotype communities* using detrended correspondence analysis (DCA), which is a multivariate ordination analysis technique first demonstrated by Cao *et al* (in publication) as a viable approach for relating fecal sources of bacterial pollution to storm water runoff.

2.5 Chemical Parameters

Turbidity, temperature, pH, dissolved oxygen and conductivity of storm water samples (N=185) were measured weekly at both study locations. Other water quality parameters, including nutrients (i.e., phosphorus, nitrogen, ammonia and total organic carbon) of storm water were measured every other week. A complete list of the chemical constituents and analytical methods used are listed in Appendix B. Grab samples were obtained in bottles, reagents and sample collection instructions provided by EnviroMatrix and Test America. All water quality parameters were compared with enterococcal densities (in water and on coupons) to assess possible correlations related to persistence and growth.

2.6 *Bacteroidales* Human Marker

The presence/absence of human fecal bacteria in 20 samples including beach water, creek water and storm drain flow was analyzed by SCCWRP using the *Bacteroidales* HF183 human marker method as described by Shanks *et al* (2008).

2.7 Genetic Analyses Planned for Future Testing

Approximately 400 samples collected from both study sites were archived for possible future testing, including microbial community analysis using terminal restriction fragment length polymorphism (TRFLP).

The definition of "clonal strains" in this study was based on testing the metabolic functions (i.e., biotype) and physical traits of bacteria, which are the most commonly used methods in hospital and clinical laboratories to identify bacteria. Bacteria with similar metabolic profiles may be closely related but may not be genetically identical. Bacteria strains descendant from the same parent (i.e. cells resulting from growth) should be 99.5% - 100% similar in genetic make-up. It is anticipated that future DNA typing and virulence testing will be performed to determine how enterococci obtained from this study compare with human and animal strains. This will be done to confirm the relationships of strains based on comparing their biotypes. The genetic similarity of selected enterococci isolates that were identified as "clonal" biotypes based on phenotypic methods will be confirmed as being genetically clonal (i.e. descendants from the same parent cell) using pulsed-field gel electrophoresis (PFGE), a DNA fingerprinting method. This work will be done as part of Donna Ferguson's doctoral work to complement this study.

2.8 Statistical Analysis

Correlations in enterococci concentrations were determined using Microsoft Excel Software and SigmaStat, version 2 (Systat, Chicago, IL). Multivariate analysis was conducted using Canoco for Windows 4.5 (Microcomputer Power, Ithaca, NY).

3.0 RESULTS

3.1 Results at Moonlight State Beach

3.1.1 Enterococci Levels in Beach Water

Concentrations of enterococci at Moonlight State Beach exceeded water quality standards more than 40% of the time during the study period and were positively correlated with those in Cottonwood Creek ($p < 0.001$). *Enterococcus* densities at Moonlight State Beach ranged from 10 to 2,420 MPN based on testing with Enterolert and 4 to 360 CFU per 100 ml based on EPA Method 1600 (Table 3).

Table 3. Enterococci Densities in Beach Water (per 100 ml)

	Enterolert[a]		EPA Method 1600[a]	
	Mean (± std. error)	**Range**	**Mean (± std. error)**	**Range**
Moonlight State Beach	398 (±182)	10 - 2420	50 (±22)	4 - 360
Rock Pile Beach	20 (±3)[b] 350 (±330)[c]	18 – 1011	5 (±4)[b] 40 (±35)[c]	2 – 111

[a]averages based on replicates
[b]dry weather
[c]wet weather (1 event)

During the study, there were three rain events (with levels greater than 0.01 inches) that led to increased enterococci levels in creek and beach water (Figure 8). On July 6, 2010 (week 7) there was 0.07 inches of rain; during week 20, there was 1.55 inches on October 4, 2010 and 0.06 inches the next day, which was also the last day of sampling.

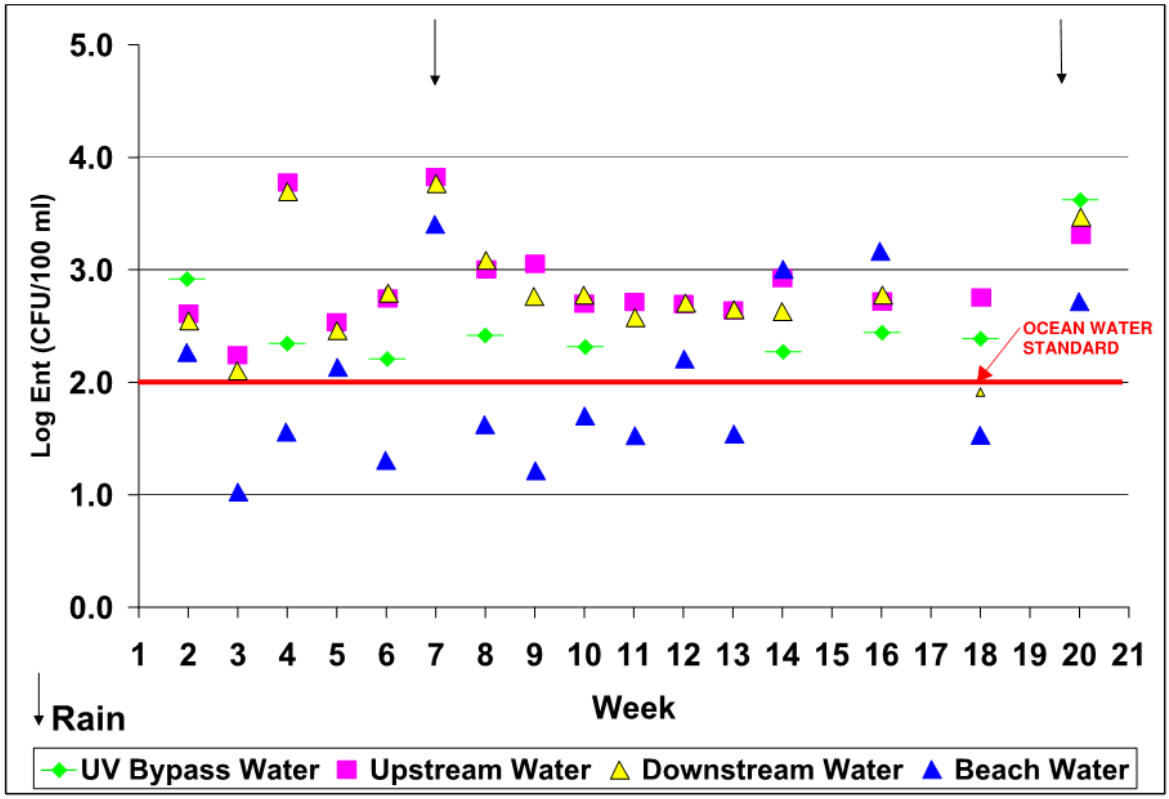


Figure 8. ***Enterococcus*** **Densities in Cottonwood Creek and Moonlight State Beach**

3.1.2 Enterococci Levels in Cottonwood Creek

The UV disinfection system appeared to be highly effective in reducing enterococci levels. Enterococci levels in Cottonwood Creek flows before UV treatment averaged 700 CFU/100 ml, but were mostly below the detection limit post-treatment with only 1 of 9 samples having 1 CFU/100 ml. However, downstream of the UV treatment facility, enterococci concentrations rebounded to an average 1400 CFU/100 ml: higher than those in UV bypass water.($p<0.05$) (Table 4).

3.1.3 Enterococci Levels in Upstream Sources

Enterococci densities for sources impacting Cottonwood Creek include untreated creek flows, street runoff, freshwater plants, algae, and seawrack (Table 2). During dry weather, there was very little flow discharged from pipes draining upstream areas into the creek. However, street runoff entering the creek below the UV treatment facility was identified as a possible upstream source by exploratory sampling. Enterococci levels ranged from 530 to 1195 MPN/100 ml in a street runoff sample (Table 4) from a residential area across from the creek as shown in Figures 9 & 10.

Figures 9 & 10 Residential Runoff into Cottonwood Creek

Significant volumes of groundwater were observed flowing into the creek from a drain pipe located just below the UV treatment facility. However, little to no enterococci were detected in groundwater samples analyzed during the study period.

A significant natural reservoir of enterococci identified in the creek was *Ditichilis spicata*, the most abundant plant species growing in Cottonwood Creek. Also known as salt grass, the roots and leaves of samples analyzed for enterococci yielded an average of 763 CFU/g (wet weight). The majority of isolates (64%) identified from this plant were *E. mundtii,* which is known to be associated with plants. The biotypes in this species group were also found in the creek water samples. Interestingly, a single biotype dominated these bacteria. This low diversity among bacteria species and the high density of *E. mundtii* species is indicative of potential regrowth or possibly, species/strain selectivity for this plant.

The highest concentrations of enterococci (1.9×10^4 CFU/g wet weight) among plants were found among algae and decomposed vegetation that grew or settled on the surface of the outfall pipe draining Cottonwood Creek to Moonlight State Beach. This algae was located immediately above sediment and seawrack, thus it is possible that there was some interaction between these sources in terms of enterococci distribution. Algae samples at this location were not collected on a regular basis because algal growth was highly variable over the sampling period.

3.1.4 Enterococci Levels Found Among Downstream Sources

At the mouth of Cottonwood Creek, seagulls were found to be the highest density source of enterococci. Mean concentrations of enterococci in bird stools averaged 1.8×10^7/g. Seagulls were commonly seen foraging on the beach and in creek flows in the inter-tidal zone. During the study period, bird densities (within 75 feet of the outfall flow) ranged from 0 to approximately 150 birds.

Another significant downstream reservoir of enterococci was kelp/seawrack, which had densities of enterococci averaging 430 CFU/g (wet weight). Levels of enterococci on wrack varied, with wrack that was partly decomposed having higher densities as compared to fresh kelp more recently washed up on the beach.

The amount of seawrack on the beach varied temporally and spatially. During spring tides, there were higher abundances of wrack on the beach and in the creek, particularly at the creek mouth and in the drainage pipes.

Beach sand in the intertidal zone contained low densities (average of 6 CFU/g) of *Enterococcus*, suggesting that enterococci carried in creek flows were not retained and therefore, not likely growing in sand.

A lesser, but interesting source of enterococci observed through the study were brine flies and springtails. Hundreds of these insects and arthropods were commonly found among seawrack and beach sand and low levels of enterococci were extracted from their outer surfaces in the laboratory. The origin of enterococci associated with brine flies and springtails was not determined. Levels of enterococci found on the surfaces of brine flies averaged 2 CFU enterococci, which was similar to those reported in an earlier study conducted at Pacific Beach, San Diego (McCoy and Aumand 2006). An average of 18 CFU enterococci were found per springtail. Altogether, these results suggest that insects and arthropods found on beach sand may serve as mechanical vectors of *Enterococcus*.

Table 4. Densities of Enterococci Among Probable Sources* at Moonlight Beach and Impact on Creek and Beach

Source	Mean Densities	Range	Units	Source Impact to Storm Drain*
CSD037 Upstream Flow	3312	172 – 52,987	100 ml	NA
CSD037 Downstream Flow	2427	100 - 6373	100 ml	NA
Storm Drain Surface**	1830	173 - 3487	in sq	Medium
Coupon**	983	173 - 7465	per coupon	Medium
Cobble Rock	270	3 - 369	in sq	NA
Seawrack/Kelp	117	0 - 259	1 g (wet wt)	NA

*Source impact was based on enterococci densities and community analysis
NA = not applicable
*Loosely attached plus firmly attached enterococci

3.1.5 ***Enterococcus Species and Biotypes at Moonlight State Beach: Distribution and Persistence***

A total of 569 isolates among 49 samples were identified to species and biotype (subspecies) level. The majority (81%) of enterococci isolates from Cottonwood Creek were identified as *E. casseliflavus* or *E. mundtii* (Figure 11). These species primarily exist on plants and soil and are not considered common inhabitants of the gastrointestinal tracts of humans. Further, *E. casseliflavus* are not known to colonize the intestinal tracts of animals but may be present in hosts as transients, i.e. "passing through" (Aarestrup *et al*. 2002; Jay *et al*., 2005). The majority of *E. casseliflavus* and *E. mundtii* came from plants (*Ditichilis spicata*) collected in Cottonwood creek. This plant was the most abundant natural source identified in the lower reaches of the creek. Another significant result was that most of the *E. mundtii* biotypes clustered in one large group, suggesting that this plant serves as a natural reservoir of this enterococcal species.

The distribution of *E. casseliflavus* and *E. mundtii* and also, *E. faecalis* found among seawrack at Moonlight and La Jolla were similar, suggesting geographic stability of species composition among wrack (Figure 11).

The most common species isolated from bird stools at Moonlight State Beach was *E. faecalis*, which is consistent with an earlier study characterizing enterococci in bird stools at Doheny State Beach in Dana Point, CA (Ferguson *et al*., 2010). *E. faecalis* was also predominant among brine flies and springtails commonly found on beach sand and also feeding on decaying vegetation/algae. *E. faecalis* was also dominant among isolates from beach sand and algae. However, despite the abundance of *E. faecalis* among sources spatially related to beach sand, only 4% of enterococci in beach water were identified as *E. faecalis*. The most abundant species (38%) in beach water was *E. casseliflavus*, which was predominant in Cottonwood Creek water and plants.

Bird stools collected on the beach during the study period (adjacent to the creek) had variable concentrations of enterococci ranging from 2.8×10^1 to 4.9×10^7 CFU per gram. The estimated number of birds seen within 75 feet of the creek outfall during the sampling period ranged from 0 to 150, or an average of 25 birds per day. The majority of enterococci (80%) from bird stools were identified as *E. faecalis*, which was also predominant in sand and beach water.

Among all the natural samples analyzed for enterococci species and subspecies, springtails, fresh water plants (*Ditichilis spicata*) and sea wrack had the highest percentages of "clonal" biotypes (93.3%, 88.0% and 80.6% respectively). In this study, clonality is defined as biotypes with identical biochemical (i.e. metabolic) profiles. Bacterial strains in the environment are highly diverse. Thus, finding clonal (non-diverse) enterococcal biotypes in environmental samples is highly indicative of adaptation, colonization (growth) or selection of specific enterococcal species/subspecies. The largest "clonal" group was comprised of a single *E. mundtii* biotype found among plants, Cottonwood Creek water, coupons and beach water. The fact that 93% (28 of 30) of isolates from a creek plant sample were clonal biotypes of *E. mundtii*, a plant-associated species, is highly indicative that this species is capable of growing on the surfaces of this plant.

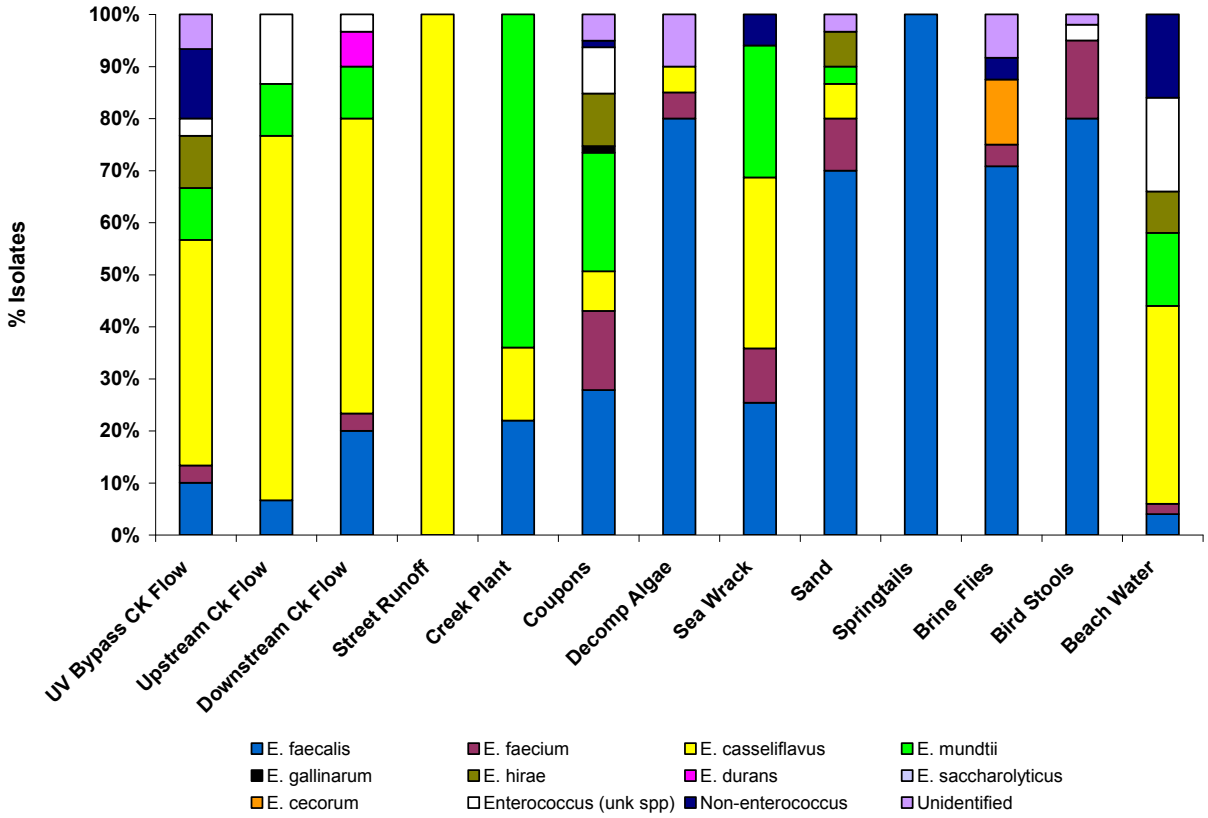


Figure 11. Overall Distribution of *Enterococcus* Species at Moonlight State Beach.

The *Enterococcus* species distribution representative of the samples from sampling week 2, 6 and 20 varied between weeks (Figure 12). Week 2 samples were predominantly non-*Enterococcus* species identified as *Streptococcus*, which is known to cause "false positive" results for *Enterococcus*. However, it is notable that when enterococci levels exceeded standards, about half of the isolates in beach water were *E. casseliflavus*. DNA typing of these isolates (to be conducted by Donna Ferguson) would be useful for determining whether these strains genetically match the isolates from natural upstream sources, such as freshwater plants and seawrack. This analysis could help to establish a spatial link between *E. casseliflavus* from upstream sources to that of beach water.

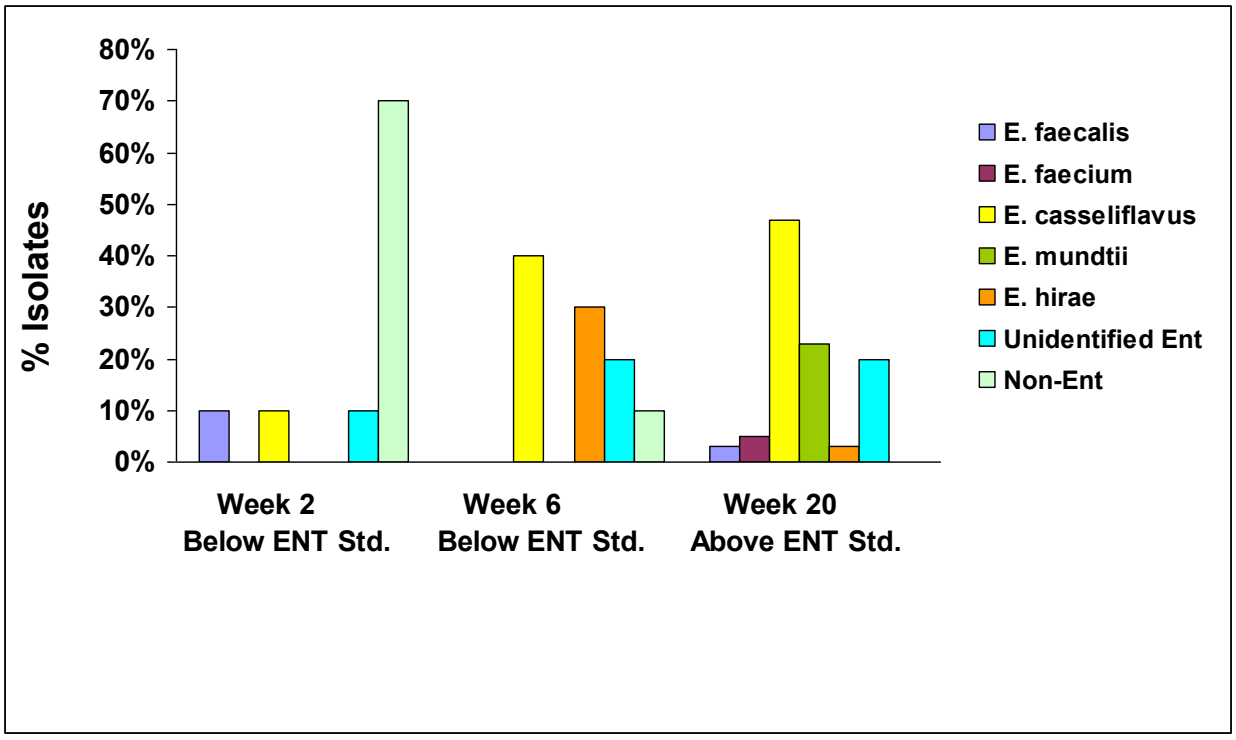


Figure 12. Comparison of *Enterococcus* Species Distribution in Moonlight State Beach Water When Levels Were Below and Above *Enterococcus* Standards

3.1.6 Moonlight State Beach Enterococcus Community Analysis

Description of Multivariate Analysis (using Detrended Correspondence Analysis (DCA). The biotype data obtained by the Vitek testing were used to indentify enterococci "strain" types and to establish enterococcal community profiles. The distribution of biotypes shared across sources was analyzed using DCA, a type of ordination analysis. The *Enterococcus* biotype community profile (variable 1) among various source samples (variable 2) used to derive "profiles" or biotype patterns is shown in the ordination scatter plot (Figure 13). The variation of *Enterococcus* biotypes across various sources are ordered on an imaginary line or axis, which in this case, are spatial gradients. Each axis is an eigenvector; the direction of the axes (left versus right, up versus down) is arbitrary and does not affect the interpretation of the results. In the ordination plot, proximity implies similarity. Thus, sources that are closer in proximity indicate that the biotypes associated with these sources are likely to occur more often, or in higher relative abundance than in sources that are more distant. The ordination plot below (Figure 13) shows that the enterococci biotypes in the beach water samples taken from Moonlight State Beach were most similar to biotype communities representative of Cottonwood Creek (UV bypass water and downstream flow, freshwater plants and algae in the creek and seawrack. Enterococcal communities among sand, bird stools and insects were less similar, indicating a lower level of relatedness to enterococci in beach water.

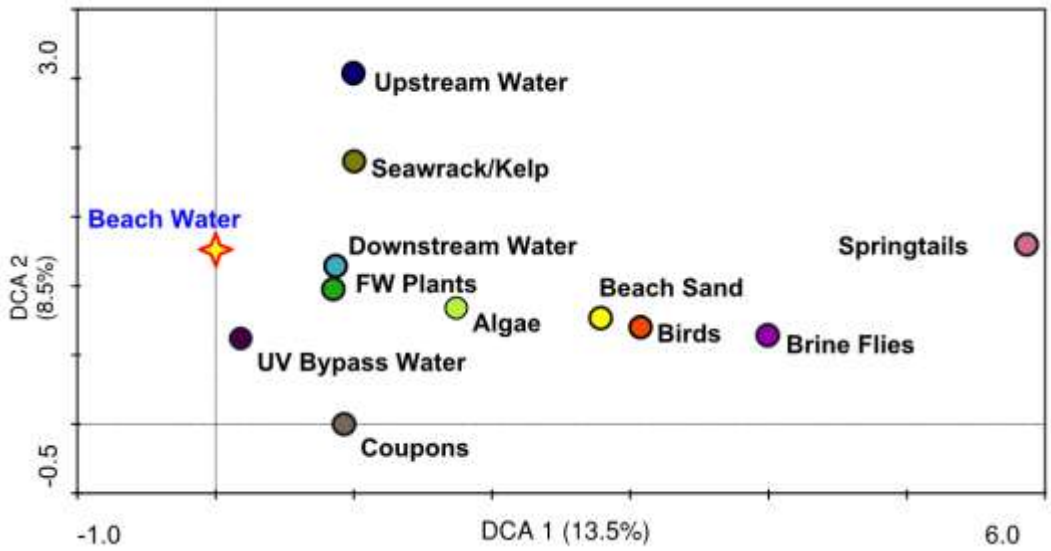


Figure 13. Multivariate analysis of Biotype Composition of Moonlight State Beach Samples for Relating Enterococci to Potential Sources

Enterococcus species and biotypes from sources influencing Cottonwood Creek (freshwater plants, seawrack and algae) were also found in beach water, which was not surprising given the continuous flow into the ocean. However, the presence of unrelated enterococci found in beach water suggests the possibility of additional sources of enterococci that were not evaluated in this study. Also shown in Figure 12, the enterococci communities in beach sand and bird stools were highly related, while the community structure among springtails was more distantly related to the wrack and beach sand, suggesting selectivity of specific *E. faecalis* strains on the surfaces of these insects.

3.2 Results for CSD037 Storm Drain and Rock Pile Beach, La Jolla

3.2.1 Enterococci Levels in CSD037 Flows

Enterococci concentrations in storm drain CSD037 flow averaged 3,312 MPN/100 upstream and 2427 MPN/100 ml at the outlet (Table 5). During two sampling occasions, conductivity levels increased from an average level of 7.44 S/cm to 11.17 S/cm and 11.64 S/cm, indicating that saltwater intrusion may have accounted for slightly lower enterococci levels at the outlet downstream.

Rain events at La Jolla occurred on the same days as Encinitas, but with lower levels of precipitation. On July 6, 2010 (week 7) there was 0.01 inches of rain; during week 20, there was 0.03 inches. Enterococci levels in CSD037 flows did not appear to be influenced by precipitation, as was observed 20 miles north at Cottonwood Creek (Figure 8) in Encinitas.

3.2.2 Enterococci Levels in Rock Pile Beach Water

Since runoff discharged at the beach typically went underground, enterococci concentrations in drain flows could not be correlated to those of beach water. Only 2 beach samples were collected during the study period; the mean densities 350.4 (± 330.4) MPN/100 ml based on Enterolert and 40.3 (± 35.4) CFU/100 ml by EPA Method 1600 (Figure 14).

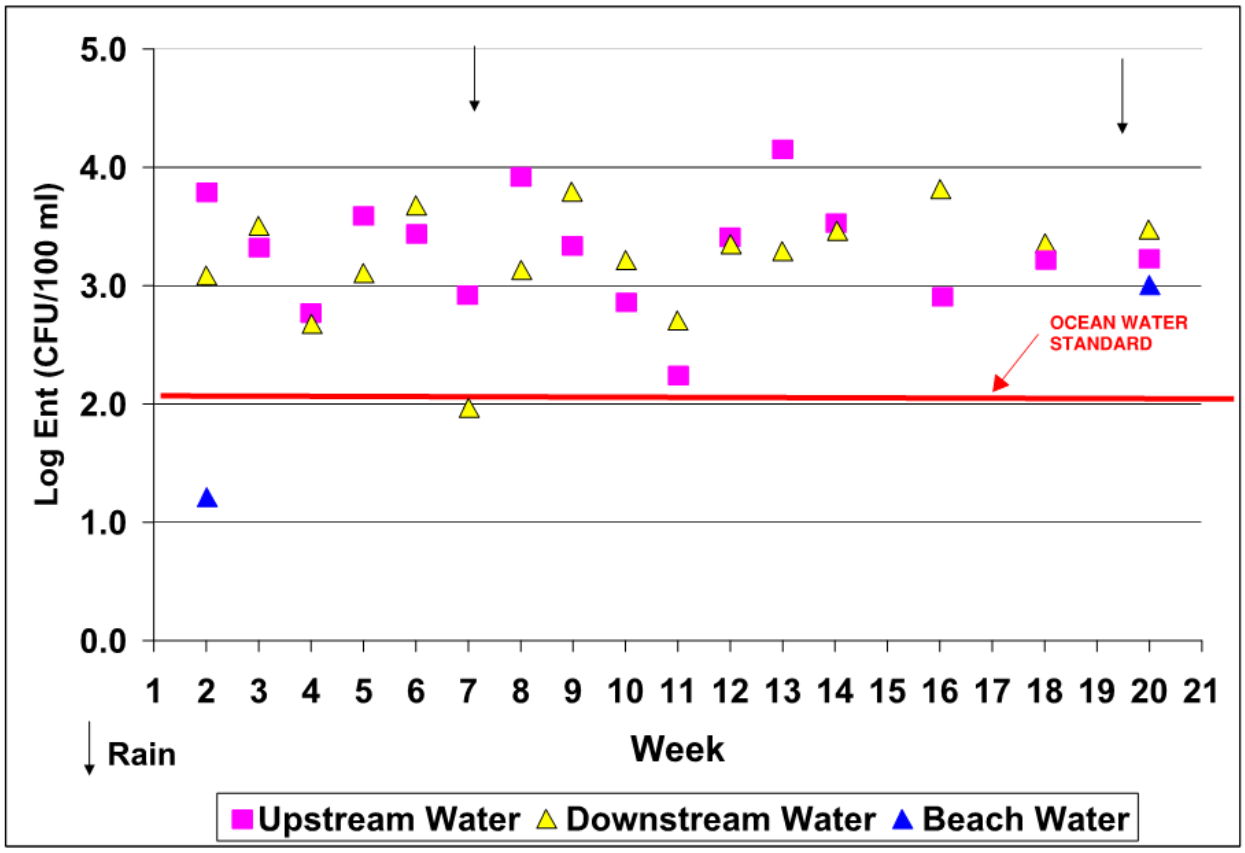


Figure 14. ***Enterococcus*** **Densities in Storm Drain (CSD037) Flows and Rock Pile Beach**

3.2.3 Enterococcus Levels of CSD037 Sources

Prior to commencing this study, City of San Diego field staff hypothesized that algal growth on the bottom of the storm drain at CSD037 was a probable source of enterococci at the outfall. However, no algae were observed in the drain during this study period. While enterococci concentrations for upstream flows in CSD037 were somewhat correlated to turbidity ($p = 0.19$), there was no sediment observed in the storm drain where samples were collected.

Enterococci regrowth on storm drain surfaces was also assessed as a source of *Enterococcus* to runoff using concrete coupons as described in Section 2.3.4. Regrowth associated with biofilm on CSD037 pipe surfaces was also investigated by chipping off small pieces from the bottom of the pipe and extracting enterococci using the same method to analyze the concrete coupons. The mean density of enterococci found was 1830 CFU/in sq of pipe surface (Table 5).

Cobble rock located between the CSD037 pipe outlet and the beach was also examined as a source of biofilm related enterococci. Rocks were processed similarly to concrete coupons. The densities of loosely attached enterococci ranged from 220 – 329 CFU/in sq of rock surface (Table 5).

Seawrack on the beach had enterococci levels that ranged from 0 – 259 MPN/g (wet weight) (Table 5). Few enterococci (per gram) were found on wrack that appeared fresh, i.e. recently washed up on the beach. Higher levels were obtained from older wrack that was partially dry and decomposed.

Table 5. Densities of Enterococci at CSD037 (Upstream to Downstream)

Source	Mean Densities	Range	Units	Source Impact to Storm Drain*
CSD037 Upstream Flow	3312	172 – 52,987	100 ml	NA
CSD037 Downstream Flow	2427	100 - 6373	100 ml	NA
Storm Drain Surface**	1830	173 - 3487	in sq	Medium
Coupon**	983	173 - 7465	per coupon	Medium
Cobble Rock (below outlet)	270	3 - 369	in sq	NA
Seawrack/Kelp (below outlet)	117	0 - 259	1 g (wet wt)	NA

*Source impact was based on enterococci densities and community analysis
NA = not applicable
** Includes loosely attached and firmly attached enterococci

3.2.4 Enterococcus Species and Biotypes at La Jolla

A total of 344 presumptive enterococcal isolates from 36 samples of varying source types were identified to species and biotype level. *E. casseliflavus* was the most abundant species identified among all samples analyzed, including CSD037 flows, coupons, biofilm on CSD037, cobble rock and beach water (Figure 13). *E. mundtii* was the second most predominant species found among CSD037 flows and beach water. Thus, 87%, 82% and 62% of enterococci were identified as plant related species (*E. casseliflavus* and *E. mundtii*) among upstream water, downstream water and coupons, respectively (Figure 13). Also, the distribution of enterococcal species in CSD037 upstream flows was similar to that of downstream flows and storm drain biofilm. The species distribution among coupons, beach water, cobble, seawrack at Rock Pile Beach was more diverse than what was observed at Moonlight State Beach in Encinitas, suggestive of species selectivity, geographic variability or differences in source inputs between the two study sites.

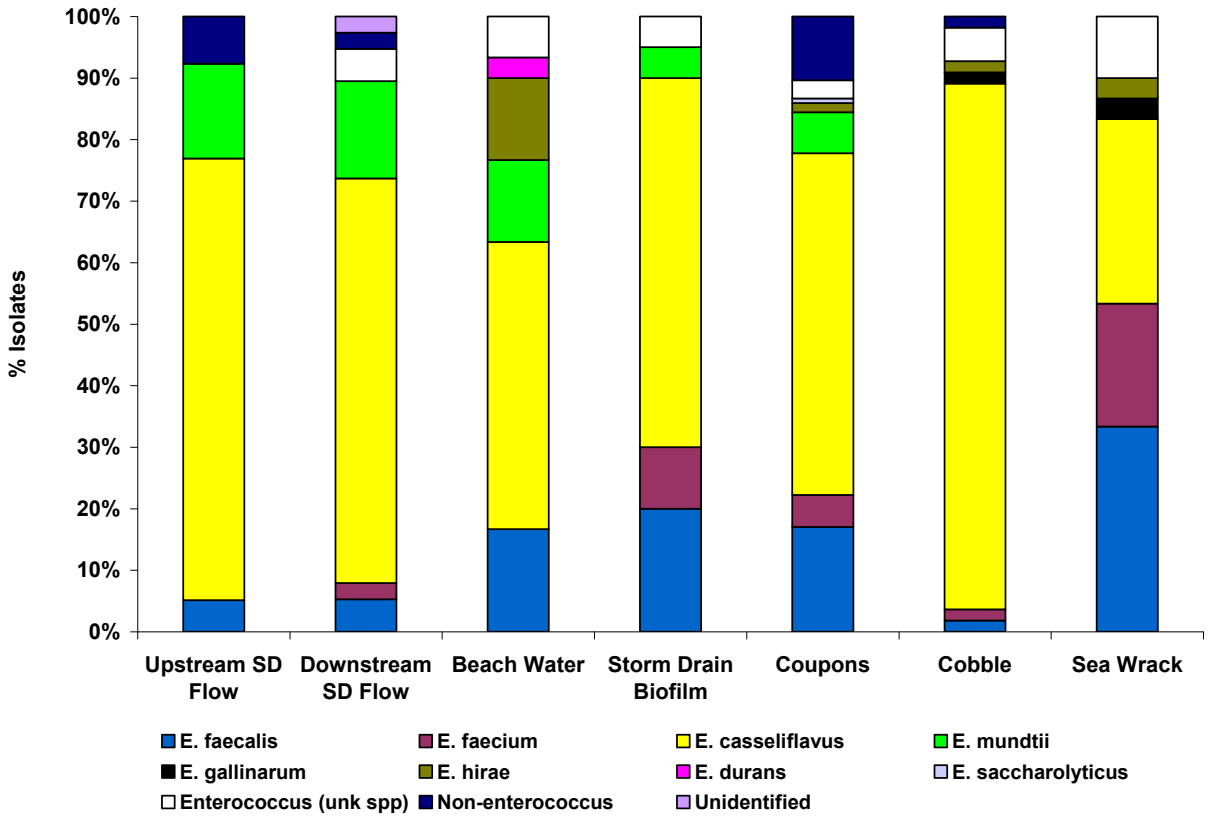


Figure 15. Distribution of *Enterococcus* species at for CDS037 Storm Drain (SD) and Rock Pile Beach.

3.2.5 Rock Pile Beach and CDS037 Enterococcus Community Analysis

Enterococcus biotypes shared between various source types (i.e., beach water versus storm water, plants, coupons, etc.) was compared using multivariate analysis to assess relationships between sources and water. The results indicated that the enterococcal biotype community representative of upstream storm water was similar to that of the downstream community (Figure 16). Interestingly, the enterococcal biotype community of beach water was more similar to that of concrete coupons and storm pipe biofilm, suggesting that the enterococcal population of beach water populations may be influenced by persistent strains of *E. casseliflavus*. DNA strain typing may further elucidate this relationship.

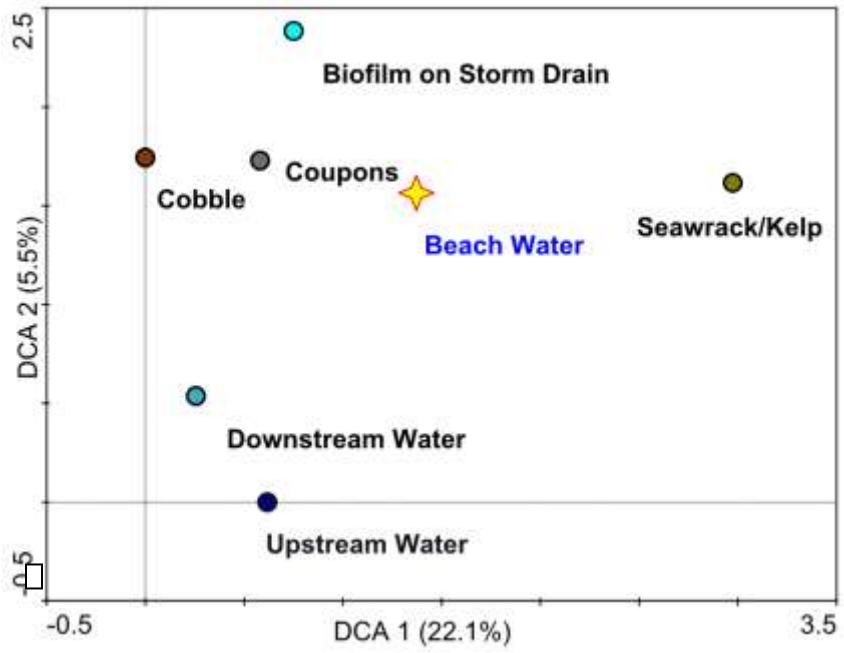


Figure 16. Multivariate analysis of Biotype Composition of Rock Pile Beach and CDS037 Samples

Moreover, the *E. casseliflavus* biotypes found in CSD037 were very similar, suggestive of enterococcal growth, a common source origin or species/strain selectivity in this environment. This finding is important because bacteria with identical biotypes metabolize the same nutrients, indicating that selectivity of similar biotypes in this environment may be related to the nutrients here. Also, bacteria that are descended from the same parent cell will have identical or highly similar metabolic profiles. Thus, finding similar biotypes in CSD037 also suggests persistence and growth of enterococci that may be adapted to this environment.

3.3 Assessing Enterococcal Growth As A Source to Moonlight State Beach and Rock Pile Beach Flows Using Concrete Coupons

The coupon studies at both study locations showed that enterococci were capable of attaching to concrete coupons. After two weeks of incubation, enterococci attached to coupons at Cottonwood Creek (Encinitas) were 158 (±122) or approximately 7 cells per square inch of coupon surface area. Densities on coupons in CSD037 (La Jolla) had higher densities at 2182 (±1250) or 91 cells per square inch (Table 6).

Table 6. Mean Densities of Enterococci Attached to Concrete Coupons (± std dev)

	Cottonwood Creek		CDS037	
Level of Attachment	**Range of Counts per Coupon**	**Range of Counts per Coupon Surface Area (in^2)**	**Range of Counts per Coupon**	**Range of Counts per Coupon Surface Area (in^2)**
Loose	54 (± 13) - 2415 (± 89)	2 - 101	311 (± 208) - 6160 (± 8401)	13 - 257
Firm	26 (± 10) - 140 (± 30)	1 - 6	173 (± 101) - 7465 (± 5566)	7 - 311

The densities of enterococci extracted from coupons at both locations were highly variable and did not continue to increase with each passing week, as first hypothesized. However, the biomass of biofilm on coupon surfaces did continue to increase over time (Figure 16). During filtration, increased amounts of biofilm resulted in clogged filters and high background levels of particulates on filters which interfered with counting colonies and likely led to under estimates of cell density when using EPA Method 1600. This problem was resolved by using Enterolert for enumerating enterococci in turbid samples

Figure 16 . Biofilm Build Up on Concrete Coupons from Cottonwood Creek and CSD037 at Week 4 and Week 12

One of the most interesting results of the coupon study was the notable differences in how enterococci attachment and species distribution differed between the study sites. There were significantly higher levels of *loosely* attached enterococci on Cottonwood Creek coupons as compared to more firmly attached, i.e. biofilm related cells in CSD037. Also, whereas enterococci from Cottonwood Creek coupons were highly diversified, CSD037 coupons were consistently dominated by *E. casseliflavus*, suggesting species selectivity.

At both sites, particularly at Cottonwood Creek, the levels of firmly attached enterococci were lower than expected considering the high levels found in overlying water. However, clonal biotypes were consistently recovered from the coupons over a 14-week period. In fact, the majority of biotype groups, 68% and 78% among coupons from Cottonwood Creek and CSD037, respectively included more than 1 "clone" (nearly identical biotype) indicating that these strains may have originated from the same source, represent persistent strains and/or resulted from growth on coupon surfaces.

At both study sites, the same biotypes extracted from the coupons were also identified in runoff flows and in beach water. This was unexpected at La Jolla because outlet flows from CSD037 were not observed flowing directly into beach water. These results suggest that it is possible that drain flows under the cobble are reaching the beach and/or that there are persistent *E. casselfilavus* strains in the drain and beach water that are phenotypically similar.

3.4 Correlation Between Enterococci on Coupons and Water Quality Parameters

The densities of enterococci on coupons were also compared to water quality parameters measured in creek/storm water flows at both study locations. The water samples for chemical analyses were collected at the same time as coupons. The densities of enterococci attached to coupons from Cottonwood Creek were highly correlated with total organic carbon (TOC) and dissolved oxygen (DO) ($p<0.05$). At La Jolla, enterococci densities were correlated with total

nitrogen and phosphorus (ortho-P) ($p<0.05$) (Table 7). Surprisingly, there was no correlation between enterococci densities on coupons and water temperature, which might be expected since bacterial growth is temperature dependent; however, average temperatures at both study sites during the study period were lower than temperatures recorded in previous years.

Table 7. Water Quality Parameters and Correlation to Densities of Enterococci Firmly Attached to Concrete Coupons (*statistically significant p < 0.05)

Parameter	Cottonwood Creek		CSD037	
	Mean	Range	Mean	Range
Turbidity	6.8	1.7-28.3	6.22	0-33.2
Temperature (°C)	20.1	17.6-21.0	20.9	17.3-23.4
pH	7.3	6.39-8.75	8.02	6.64-8.38
Coductivity (S/cm)	4.6	1.04-9.00	7.90	5.29-11.64
DO (mg/L)	**7.0***	0.53-11.79	8.68	8.38-9.13
NH4 mg/L	0.10	0-0.24	NA	NA
NO3 mg/L	26.3	23.6-32.7	NA	NA
NO3/NO2 mg/L	26.4	21.1-32.8	NA	NA
NO2 mg/L	0.02	0-0.06	0.08	0-0.9
TKN mg/L	0.8	0.8-2.0	0.29	0-0.7
Total N mg/L	27.2	13.2-33.6	**2.6***	0-13.2
Ortho-P mg/L	0.2	0.05-0.44	**0.07***	0-0.40
Total P mg/L	0.3	0.06-0.53	0-0.16	0-0.65
TOC mg/L	**4.5***	3.9-9.2	6.8	1.3-12.0

3.5 *Bacteroidales* Human Marker (HF183) Testing

The focus of this study was to investigate natural inputs and growth of enterococci in runoff from nonpoint sources. However, since runoff had consistently high levels of *Enterococcus*, it was important to examine potential contamination from human fecal sources.

A total of 20 samples were collected to test for the *Bacteroidales* Human Marker (HF183), a highly specific marker of human fecal contamination. Five samples (two beach water samples from Moonlight State Beach, one beach water sample from Rock Pile Beach and two CSD037 storm water samples) were not tested due to insufficient quantities of DNA. The 15 samples successfully tested included one beach water sample from Moonlight State Beach and eight creek water samples from Cottonwood Creek and six CSD037 samples collected during sampling weeks 3, 6, 13 and 20. Only very low levels of the marker were observed and no conclusively positive samples were detected. However, it is also important to keep in mind that

these results are representative of 20 samples, of which only 15 had sufficient amounts of DNA for testing.

Table 8. Summary of Microbiological Analyses

Analysis	Method	No. Samples	Laboratory
Enterococci Enumeration	IDEXX Enterolert	722	EMD MML
Enterococci Isolation From Insects	Kuzina et al., 2001; Cox and Gilmore 2007	50 Brine Flies, 80 Springtails	SCCWRP
Enterococci Extraction From Coupons	Ferguson et al., 2005	64	EMD MML
Enterococci Speciation	(1) EPA Method 1600, (2) Vitek II, Motility, Pigment and Additional Biochemicals	(1) 358, (2) 903	EMD MML
Enterococci Community Analysis	Multivariate Analysis	CSD037= 45 Biotypes, Moonlight State Beach = 55 Biotypes	SCCWRP
Bacteriodales Human Marker	HF 183 (Shanks, et al. 2008)	20	SCCWRP

4. DISCUSSION

This study was designed to assess whether enterococci were capable of growing in two different types of storm drain systems impacting beaches downstream. The study sites included a largely open natural creek (Cottonwood Creek) at Moonlight State Beach and an enclosed concrete-lined storm drain (CSD037) at Rock Pile Beach in La Jolla. Both drainage systems convey runoff flows originating from residential/commercial areas. Because the natural creek site was heavily vegetated, a secondary objective of examining other potential natural reservoirs of *Enterococcus* such as plants, seawrack, algae, insects and birds was added for this site.

The results of the coupon study at Cottonwood Creek showed that enterococci in creek water were indeed capable of attaching to and growing on concrete coupon surfaces. The same enterococci biotypes found on the coupons were repeatedly isolated over 14 weeks. The highest concentrations of enterococci were loosely attached to coupons. Thus, turbulent flows could resuspend or scour enterococci from drain surfaces, plant leaves, wrack, etc., leading to elevated levels in creek water flowing to the beach water.

Clonal biotypes were commonly detected among coupons (at both study sites), in CDS037 storm water and among vegetation at Cottonwood Creek. These findings are significant because most natural habitats are typically highly diversified, i.e., comprised of multiple species and strain types. Thus, finding a low diversity of species and strains in high densities is indicative of growth, adaptation and/or selectivity.

At La Jolla, we focused on investigating enterococci persistence and growth in an enclosed concrete storm drain (CSD037). Most of the enterococci in CSD037 flows were consistently identified as *E. casseliflavus*. Since we did not observe plants, algae or sediment in the drain pipe, isolates identified as *E. casseliflavus* may have originated from upstream sources related to plants and soil, including irrigation runoff. Also, increased levels of enterococci in CSD037 flows were correlated with turbidity, nitrogen and phosphorus, which may indicate mobilization of soil particles and nutrients from lawn fertilizers.

The concrete coupons in La Jolla from CSD037 consistently yielded higher densities of enterococci as compared to the coupons from Cottonwood Creek. CSD037 coupons also had a greater buildup of biofilm, which could be related higher flow velocities and/or differences in the biofilm communities. A previous study showed that increased flow velocities were related to (1) increased biofilm formation and (2) higher bacteria levels in biofilm in water distribution pipes (Lehtola *et al*., 2006). Another notable difference that could account for differing growth rates was related to the amount of sunlight exposure. Enterococci are known to survive for longer periods in dark environments (Lleò et al. 2005). Coupons in CSD037 were exclusively in the dark, but at Cottonwood Creek coupons were partially exposed to sunlight. Previous studies on *Enterococcus* growth in biofilm have shown that other environmental factors such as nutrients and osmolarity (e.g. concentration of sodium chloride) also influence biofilm formation and persistence of certain *E. faecalis* strains (Mohamed and Huang, 2007).

At both locations, enterococcal biotypes identified in storm/creek runoff during the early weeks of the study were repeatedly identified throughout the study period, suggesting that these enterococci may represent persistent strains. DNA typing could further elucidate these findings.

We hypothesized that natural reservoirs of enterococci might contain high densities of enterococci, due to the ability of these bacteria to grow in the environment. Since bacteria are typically adapted to particular niches or habitats, we expected to observe differences between the abundance of enterococcal species and strains found in environmental versus fecal sources. For example, with the exception of animals with plant-based diets, *E. casseliflavus* and *E. mundtii* are more commonly associated with plants and soil (Ott *et al*., 2001; Aarestrup *et al*., 2002; Jay *et al*., 2005) and less abundant in fecal material. Thus, an abundance of plant associated species in these urbanized watersheds is more likely representative of non-fecally derived enterococci. As a result, our approach for characterizing environmental reserviors was based on the abundance and proximity of sources to creek/storm flows and beach water, the densities of enterococci recovered from these sources and the relatedness of enterococcal communities to that of runoff and beach water.

Runoff in Cottonwood Creek contained higher levels of enterococci downstream of the UV facility as compared to upstream, indicating that there were downstream contributions and/or growth of enterococci occurring in the creek post UV treatment. Since plants were an abundant potential natural source in the creek, enterococcal species and strains found in creek flows were compared to those found in the plants. The majority (over 70%) of enterococci isolated from creek flows were identified as *E. casseliflavus* and *E. mundtii*, which is consistent with the species found in creek vegetation. Algae and seawrack in the creek were also heavily populated with the same species. While *E. faecalis* and *E. faecium* were also present at low levels (4% and

2%, respectively among total number of isolates), it is important to note these species, which are predominant in human fecal matter, also include strains that are considered environmental (Ott *et al.* 2001).

Although this study was focused on investigating regrowth of enterococci, a limited number of samples from both study sites were also tested for the human *Bacteroidales* marker to assess the possibility of human fecal contamination in water. Beach water, Cottonwood Creek water and CSD037 runoff flows were analyzed using the *Bacteroidales* HF183 qPCR method described by Shanks, et al. 2008. All samples tested (with sufficient DNA yields) indicated very low or non-detectable levels of the human marker, indicating that these samples had little or no evidence of human fecal material.

5. SUMMARY

Despite UV disinfection the flows from Cottonwood Creek continue to be a source of enterococci to Moonlight State Beach. Elevated levels in beach water likely occur due to enterococcal input from untreated creek flows, street runoff, vegetation in the creek and seawrack at the creek mouth. Long-term persistence and growth of enterococci on drain surfaces was successfully demonstrated using concrete coupons placed in the drain outlet to Cottonwood Creek. Thus, in addition to enterococci contributions from upstream flows and natural sources, the capability of enterococci to grow on biotic (e.g. plants) and abiotic (e.g. concrete) surfaces in the creek has the potential to provide a net input of enterococci to the creek. Moreover, the majority of enterococci found among source inputs to Cottonwood Creek were identified as *E. casseliflavus and E. mundtii*. These species are most commonly associated with vegetation and soil, although they can be present in fecal waste of hosts that consume vegetation. In addition to creek related sources, bird stools were determined as an important secondary source. Beach sand was not found to be an important reservoir of enterococci at Moonlight Beach; there were consistently low levels of enterococci in sand collected at the mixing zone, suggesting a low likelihood of growth and entrainment of enterococci here.

At La Jolla, levels of enterococci in storm drain CSD037 consistently exceeded water quality standards throughout the study period. The most predominant species of *Enterococcus* isolated from biofilm found in the drain and on cobble rocks below the drain outlet was *E. casseliflavus*, which is commonly found in plants and soil. Enterococci densities were also significantly correlated with nitrogen and phosphorus (commonly found in soil) as well as turbidity. Hence, elevated levels of enterococci in storm drain flows could be caused stimulated growth due to abundant nutrients or by input of enterococci from irrigation/lawn runoff or resuspension and/or detachment of enterococci in storm drain biofilm due to changing flow patterns.

The results of this study suggest that enterococci in these storm drain systems may be predominantly from natural sources: including strains capable of growing on drain surfaces. While this study demonstrated the potential for a continuous supply of enterococci produced by biofilms to reach beaches, it did not quantify how much enterococci is shed by storm drain biofilm or what impact this might have on beach water quality. Further studies involving controlled laboratory experiments will be necessary to establish rates of bacterial loading to beaches from drain surfaces colonized by enterococci in biofilm.

5.1 Recommendations

5.1.1 Moonlight State Beach

- Conduct a study using a mass-balance approach to bacterial loading to determine relative proportions of enterococci from storm drain surfaces and other natural sources.
- Reduce irrigation and street runoff flows to Cottonwood Creek.
- Consider removing seawrack at creek mouth to beach area above tide line.
- Conduct a more comprehensive source identification study using multiple human marker methods to rule out human fecal inputs
- Conduct DNA typing and virulence testing on enterococci archived from this study to confirm findings based on phenotyping results and to assess the genetic relatedness of strains in water, fecal waste and natural sources to human strains known to cause infections to assess potential health risks.

5.1.2 La Jolla

Reduce irrigation runoff flows to CSD037.

- Determine loading of creek water due to shedding of enterococci from biofilm through laboratory experiments.
- Investigate sources of enterococci upstream of CSD037 such as lawn grass, soil and plants.
- Assess fluxes in enterococci concentrations relative to increases in runoff flow volumes, velocities and turbidity levels related to releases from sump pumps upstream.
- Conduct DNA typing and virulence testing on enterococci archived from this study to confirm findings based on phenotyping results and to assess the genetic relatedness of strains in water, fecal waste and natural sources to human strains known to cause infections to assess potential health risks.

6. References

Aarestrup, F. M., P. Butaye and W. Witte. 2002. Nonhuman reservoirs of enterococci. In The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance. Ed. Gilmore, M.S., D. B. Clewell, P. Courvalin, G. M. Dunny, B. E. Murray, L.B. Rice. ASM Press, Washington D.C.,

Boehm, A. B., Griffith, J., McGee, C., Edge, T. A., Solo-Gabriele, H.M., R. Whitman, Y. Cao, M. Getrich, J.A. Jay, D. Ferguson, K.D. Goodwin, C.M. Lee, M. Madison, and S. B. Weisberg. 2009. Faecal indicator bacteria enumeration in beach sand: a comparison study of extraction methods in medium to coarse sands. J. Appl. Microbiol. 107:1740-1750.

Cao, Y., L. Van De Werfhorst, J. Murray, and T. Holden. 2011. Application of an integrative community analysis approach for microbial source tracking in a coastal creek. (Submitted for publication).

Cox, C. R., and M. S. Gilmore. 2007. Native microbial colonization of *Drosophila melanogaster* and its use as a model of *Enterococcus faecalis* pathogenesis. Inf. Imm. 75:1565-1576.

Ferguson, D. M., D. F., Moore, M. A. Getrich and M. H. Zhowandai. 2005. Enumeration and speciation of enterococci found in marine and intertidal sediments and coastal water in southern California. J. Appl. Micro. 99:598-608.

Ferguson, D. M., Cao, Y. and Griffith, J. F. 2010. *Enterococcus* spp. composition analysis to assess fecal pollution at Doheny Beach, California. Presented at ASM 3rd Int. Conf. on Enterococci, Portland, Ore.

Jha, A. K., H. P. Bias and J. M. Vivanco. 2005. *Enterococcus faecalis* mammalian virulence-related factors exhibit potent pathogenicity in the Arabidopsis thaliana plant model. Inf. Imm. 73:464-475.

Jay, J.M., M. J. Loessner, and D. A. Golden. 2005. Modern Food Microbiology. Springer, New York, N.Y. p. 483.

Kuzina, L. V., J. J. Peloquin, D. C. Vacek, and T. A. Miller. 2001. Isolation and identification of bacteria associated with adult laboratory Mexican fruit flies, *Anastrepha ludens* (*Diptera: Tephritidae*). Curr. Microbiol. 42:290-294.

Lehtola, M. J., M. Laxander, I. T. Miettinen, A. Hirvonen, T. Vartiainen, P. J. Martikainen. 2006. The effects of changing water flow velocity on the formation of biofilms and water quality in pilot distribution system consisting of copper or polyethylene pipes. Wat. Res. 40:2151-2160.

Lleò, M. M., B. Bonato, D. Benedetti and P. Canepari. 2005. Survival of enterococci species in aquatic environments. FEMS Microbiol. Ecol. 54:189-196.

McCoy, D., and L. Aumand. 2006. The amplification of indicator bacteria occurring as a result of kelp mound incubation along a protected beach in southern California. Tech. Paper #0609. Weston Solutions, Carlsbad, CA.

Noble, R.T., D., F., Moore, M.K. Leecaster, C.D. McGee and S.B. Weisberg. 2003. Comparison of total coliform, fecal coliform, and Enterococcus bacterial indicator response for ocean recreational water testing. Wat. Res. 37:1637-1643.

Ott, E.-M., Müller, T., Müller, M., Fran, C. M. A. P., Ulrich, A., Gabel, M. and Seyfarth, W. 2001. Community dynamics and antagonistic potential of enterococci colonizing the phyllosphere of grasses. J. Appl. Microbiol. 91:54-66.

Shanks, O. C. , E. Atikovic, A. D. Blackwood, J. Lu, R. T. Noble, J. Santo Domingo, S. Seifring, M. Sivaganesan, and R. A. Haugland. 2008. Quantitative PCR for detection and enumeration of genetic markers of bovine fecal pollution. Appl Environ. Microbiol. 74:745-752.

Tendolkar PM, Baghdayan AS, Gilmore MS and Shankar N (2004) Enterococcal surface protein, Esp, enhances biofilm formation by *Enterococcus faecalis*. Inf Imm 72: 6032-6039

APPENDIX A

Table 1. Summary of Analyses Conducted and Number of Tests

Analysis	Number of Tests
Enterococci Detection and Enumeration by Enterolert (IDEXX)	722
Enterococci Detection and Enumeration by EPA Method 1600	358
Filtration for Molecular Analyses	~400
Enterococci Speciation	903
Water Quality Parameters	217
Nutrient Analyses	185

Table 2. Nutrient Analysis

Constituent	Units	Method	
		EnviroMatrix	Test America
Ammonia as N	mg/l	SM4500 NH3 B,C	SM4500 NH3 D
Nitrate as N	mg/l	SM4500 NO3 E	EPA 300.0
Nitrate/Nitrite as N	mg/l	SM4500 NO3 E	EPA 300.0
Nitrite as N	mg/l	SM4500 NO2 B	EPA 300.0
Total Kjeldahl Nitrogen	mg/l	SM4500 N C	SM4500 N C
Total Nitrogen	mg/l	Calculation	Calculation
Orthophosphate as P	mg/l	SM4500 P E	EPA 300.0
Phosphorus, Total	mg/l	SM4500 P B, E	EPA 300.0
Total Organic Carbon	mg/l	SM5310 C	SM5310 B

APPENDIX B

SCCWRP PROTOCOL
FOR
ANALYZING SAN DIEGO STORM DRAIN STUDY SAMPLES
May 18, 2010

OVERVIEW

The storm drains selected for study include EH 420 at Moonlight State Beach and CSD037 in La Jolla. The main objective of this study is to assess the occurrence of enterococcal growth in storm drains and whether this leads to urban runoff contributing enterococci to beach waters. Enterococcal growth will be assessed using concrete coupons placed on the bottom surfaces of storm drains known to have high fecal indicator bacteria levels. The hypothesis is that enterococci in urban runoff will attach to concrete coupons, form biofilm and multiply, leading to increased densities of enterococci over time.

A total of 40 concrete coupons will be placed in each storm drain (SD) for a 20 week period. After an initial 2 week "incubation" period (week #2), 2 coupons will be removed from each SD. A SD water sample will also be collected upstream and downstream of coupons. Water samples will be analyzed for chemical parameters (TOC, TKN, Total P, Ammonia, Ortho-P, Nitrate-N, Nitrite-N) every other week. Chemical analyses for Moonlight State Beach will be conducted by Enviromatrix; La Jolla samples will be analyzed by the City lab.

At Moonlight State Beach, plants and algae, beach water and sediment samples will also be collected starting on week #2. All samples will be tested in the laboratory for enterococci densities. After week #2, these samples will be collected every other week. Natural samples such as plants, algae and sediments will be analyzed for enterococci levels to determine enterococci growth and possible relationships between the populations associated with these material to those found in storm drain water and in beach water.

SAMPLE COLLECTION

Field Equipment and Supplies
Chain of custody forms
Field recording sheets
Clip board, pens and sharpies
Sterile sampling containers
Bacteriological Testing:
2L polypropylene bottles, sterile
1L polypropylene bottles, sterile
50 cc centrifuge tube
Chemical Analyses:
TOC: 250 ml amber bottle with HCL
TKN, Total P, Ammonia: 500 ml poly w/ H_2SO_4
Ortho-P, Nitrate-N, Nitrite-N: 500 ml poly unpreserved
60 cc syringes
Zip lock bags – gal.
Portable pH/conductivity/temp/DO meter
Paper towels
Gloves
Waste bag for gloves, paper towels, etc.
Plastic scraper
Coolers with blue ice packs

Water Quality Parameters
Before collecting samples, measure pH, temp, etc. of storm drain water and beach water.

Sample Collection Order

Moonlight State Beach/Cottonwood Creek:
UV Bypass water
UV Treated water
Beach water
Sediment at beach outlet
Seawrack, downstream of coupons
Algae, downstream of coupons
SD water, immediately downstream of coupons
Concrete coupons
SD water upstream of coupons
Plant, upstream of coupons

La Jolla (CSD037):
SD water downstream of coupons
Concrete coupons
SD water upstream of coupons

SAMPLE COLLECTION PROCEDURES

Note: Store all samples in cooler for transport to the laboratory.

UV Bypass Water

1. Put on clean gloves
2. Collect Bypass water using sterile 1L pp bottle

UV Treated water

1. Change gloves
2. Collect UV Treated water using sterile 1L pp bottle

Beach Water

1. Wear gloves
2. Use a 1L pp bottle to collect approx. 1L sample at ankle depth, in mixing zone, on incoming wave.

Sediment Sample

1. Change to clean pair of gloves
2. Collect 3 tubes of sediment samples near the beach water collection site.
3. Collect approx. 50g sediment, i.e. fill a 50 cc centrifuge tube. Collect only the top 2 cm. of wet sediment.
4. Pour off any excess water.

Seawrack Sample

1. Change gloves
2. Collect handful of seawrack and store in ziplock bag.

Algae Sample

1. Change gloves
2. Collect approx. 50g algae sample downstream of coupons. Note: Algae may or may not be present in the SD water. Algae can be free floating or attached to plants or bottom surfaces. Collect handful of algae and store in zip lock bag. If algae is attached to the storm drain surface, use a bottle or 50cc centrifuge tube to scrape algae from surface and collect into bottle/tube.

Storm Drain Water-Downstream

1. Change gloves
2. Collect SD water using sterile 1L pp bottle without touching bottom surface of storm drain**;** for very low flows, use a 60 cc syringe to collect the water and fill 1L pp bottle.

Concrete Coupons

1. Change gloves
2. Remove one coupon from wire basket.
3. Gently place coupon into 2L polypropylene (pp) bottle containing 500ml sterile PBS.
4. Remove second coupon from basket and place into separate 2L bottle with PBS.
5. Place bottles into cooler with ice packs and transport to laboratory.

Storm Drain Water Upstream

1. Change gloves

2. Collect SD water using sterile 1L pp bottle without touching bottom surface of storm drain**;** if the flow level is low, use a 60 cc syringe to collect the water and fill 1L pp bottle.

Plant Sample

1. Change gloves
2. Select one type of plant in the creek, below the coupons.
3. Collect a handful of plant (roots included) and store in ziplock bag.
4. Different types of plants can be selected during succeeding sampling events.

Table 1. Sample Types and Numbers

	No. Samples/Week	**No. Lab Replicates**	**No. weeks**	**Total No. Samples**
Moonlight State Beach/Cottonwood Creek				
Concrete coupons	2	3*	20	120
SD water - DS	1	2	20	40
SD water - US	1	2	20	40
Beach Water	1	2	20	40
UV Bypass water	1	2	10	20
Sediment	1	2	10	20
Plant	1	2	10	20
Algae	1	2	10	20
Seawrack	1	2	10	20
SUB-TOTAL	10			340
La Jolla/CSD037				
Concrete coupons	2	3*	20	120
SD water - DS	1	2	20	40
SD water - US	1	2	20	40
SUB-TOTAL	4			200
TOTAL				**540**

SD = storm drain
DS = downstream
US = upstream
* F1, F2, F3

Table 2. Sample Collection Containers and Volumes/Weights

Sample Type	Sample Volume/Weight	Sample Collection Containers	No. of Containers/Sampling Event	
			Moonlight Beach	La Jolla
Concrete coupons	NA	2L pp bottles	2	2
Storm drain water (US & DS), UV Bypass	1L (each)	1L pp bottles	3	2
Beach water	1L	1L pp bottle	1	0
Sediment	~50g/tube	3 each 50 cc centrifuge tubes	1	0
Plants, seawrack & algae	~50g each	ziplock bags , 50 cc tubes or 1L bottle	3	0
TOC	250 ml	250 ml, amber w/ HCl	1	1
TKN, Total P, Ammonia	500 ml	500 ml pp w/ H_2SO_4	1	1
Ortho-P, Nitrate-N, Nitrite-N	500 ml	500 ml pp	1	1

SAMPLE PROCESSING

Equipment and Supplies

Bronson sonicator
Membrane filtration equipment and supplies
90 ml & 99 ml Di water (for dilutions)
PBS
Graduated cylinders
Gloves
1L pp bottles
250 ml pp bottles
50 cc centrifuge tubes
1 L pp beakers
Weigh balance
Metal weigh boats
Plastic weigh boats (large)
Disposable spatulas
Drying oven
Quantitrays
Enterolert powder
125 ml IDEXX bottles

EXTRACTING ENTEROCOCCI

Enterococci will be extracted from concrete, plants, algae and sediment using shaking or sonication methods. The eluents will then be tested by membrane filtration and Enterolert.

Concrete Coupons (CC1 and CC2)

Enterococci will be removed from coupon using 3 different extraction methods resulting in 3 separate fractions (F) of bacteria. The methods include: swirling the bottle containing the coupon to remove loosely attached cells (F1); shaking to remove moderately attached cells (F2); and sonication to remove firmly attached cells (F3). The three fractions will be processed and analyzed separately.

1. Put on clean gloves
2. Gently swirl original sample bottle containing coupon 3 times (F1).
3. Remove coupon from bottle and place into a clean 2L bottle labeled "F2"; add 500ml PBS and cap; shake bottle containing coupon vigorously for 2 minutes.
4. Remove coupon from bottle and place in a clean 1L pyrex beaker; add 500 ml PBS; sonicate coupon for 3 minutes at 30% output. Be sure to move beaker around so sound waves make contact with all sides of the coupon.
5. Pour off sonicated water into 1L bottle labeled "F3".
6. Rinse the probe with 100% EtOH between samples. Rinse off EtOH with sterile PBS.

Plants (P), Seawrack (W) and Algae (A)

1. Wear gloves
2. Tare balance.
3. Weight bag containing sample and remove sample as needed to achieve approx 50g sample (plus the weight of the bag).
4. Record weight of sample + bag on bench sheet.
5. Add enough PBS to bag to achieve approx. 1:10 dilution.
6. Double bag the sample + PBS to prevent leaking and shake/massage for 2 min.
7. Pour off eluent into another bottle.

Sediment (S)

1. Allow tubes to sit upright in a rack for ~10min, then pour off any excess water on the top of the sediment.
2. Put the tubes labeled for molecular analysis and moisture content in the -80C.
3. Wear gloves
4. Tare a 500ml bottle
5. Use a flame-sterilized spatula to scoop sediment out of the tube labeled "Entero Extraction" into tared 500ml bottle.
6. Weight out 50g +/- 5g, record weight on bench sheet,
7. Add 250 ml of PBS
8. Shake vigorously for 2 min by hand, over an arc of about 10 cm
9. Allow sediment & eluent to settle 30 sec
10. Decant eluent into the second sterile bottle by pouring, taking care to leave the sediment behind
11. Add an additional 200 ml of PBS to the sediment and gently swirl for 10 s

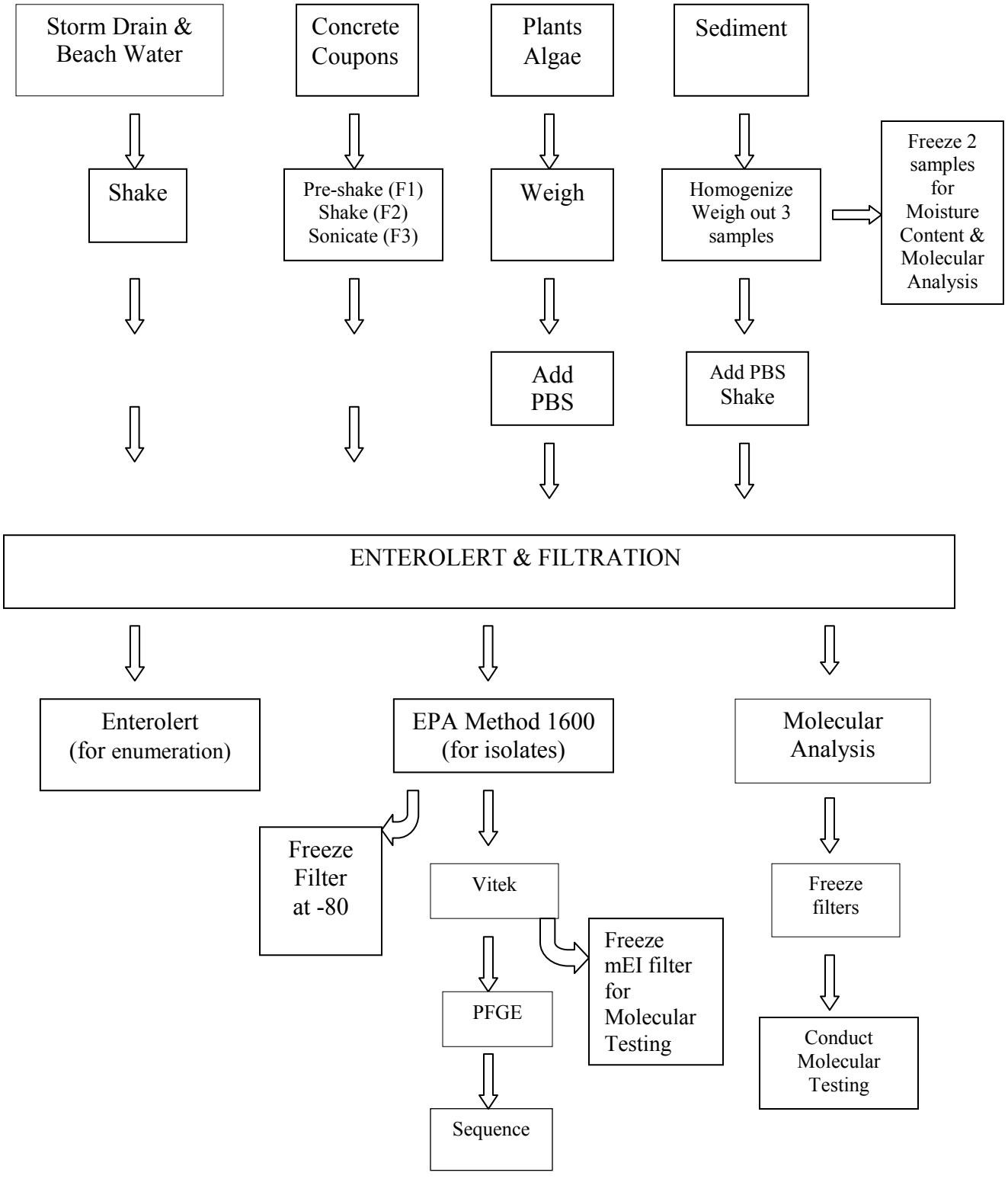
12. Allow contents to settle for 30 s, and then pour eluent into the same bottle used for first rinse

Sediment Moisture Content

The moisture content of sediment samples is determined in order to report the enterococci densities per gram of dry weight of sediment.

1. Tare balance and place metal weigh boat on scale. Record weight on bench recording and calculation sheet in Part B
2. Add ~10 grams of sample to weigh boat, spread the material out in the boat. Record actual weight of boat and sand on bench recording and calculation sheet in Part B
3. Place weigh boat and sample in drying oven overnight at 103-105 ^{o}C
4. Remove sample from oven and Allow to cool until it can be handled safely (do not allow sample to sit out for >1 hour)
5. Re-weigh boat and sand, record new weight on bench recording and calculation sheet in Part B

Figure 1. Summary of Sample Processing



Note: Save all samples and remaining elutriates in refrigerator.

MEMBRANE FILTRATION AND ENTEROLERT

1. Pre-label filtration and Enterolert supplies (funnels, mEI plates, Enterolert bottles and QT) with sample ID numbers and sample volumes
2. Process using volumes shown in Table 3
3. Repeat above for replicate

Table 3. Summary of Filtration Volumes (ml) and No. Replicates

Sample	Enterolert	Reps	EPA Method 1600	Reps	Molecular Analysis	Reps
Beach water	1 dilution	2	2 dilutions	1	200	2
UV Bypass water	2 dilutions	2	2 dilutions	1	200	2
Storm drain water US DS	2 dilutions	 2 2	 2 dilutions 2 dilutions	 1 1	 200 200	 2 2
Concrete coupon (F1, F2, F3)	F1-1 dilution F2 -1 dilution F3- 1 dilution	2 2 2	2 dilutions 2 dilutions 2 dilutions	1 1 1	>25 >25 >25	1 1 1
Sediment	1 dilution	2	2 dilutions	1	>25	1
Plants, seawrack and algae	1 dilution	2	2 dilutions	1	>25	1

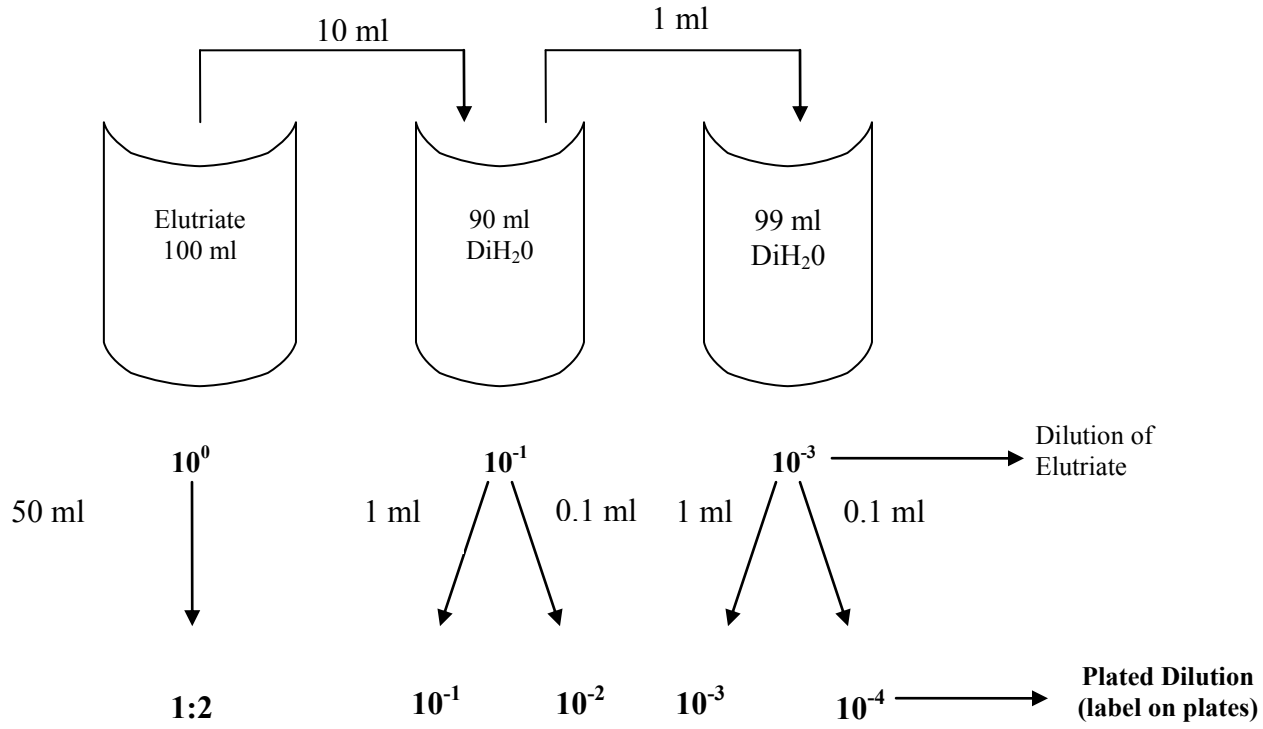
* Volumes and dilutions may vary depending on enterococci levels

****Please also run Enterolert and mEI on the UV treated water then store****
**********in 4C for later analysis**********

DILUTIONS

As enterococci counts on coupons increase, dilutions of elutriates from F1, F2, & F3 may be required.

Figure 2. Dilutions for Elutriates



Enumeration of Enterococci

1. Enterococci densities for F1, F2 and F3

$$\frac{\text{Enterococci CFU/100 ml x vol. elutriate}}{\text{24 sq in (area of coupon)}} = \text{Enterococci CFU/sq in.}$$

2. Enterococci densities for plant and algae samples

$$\frac{\text{Enterococci CFU x DF}}{\text{Wet weight (g)}} = \text{Enterococci CFU/g wet weight}$$

DF = 10 if 1:10 dilution used
Ex. DF = 50g/ 500 ml PBS = 1:10

3. Enterococci densities for sediment

$$\frac{\text{Enterococci CFU x DF}}{\text{Dry weight (g)}} = \text{Enterococci CFU/g dry weight}$$

DF = 10 if 1:10 dilution used

4. Report results using units shown in Table 5

Table 5. Reporting Units for Enterococci CFU (Enterolert)

Sample Type	**CFU Reporting Units**
Storm water	Per 100 ml
Beach water	Per 100 ml
Concrete coupons	Per 58 cm^2
Algae, Plant, Seawrack	Per g **wet** wt
Sediment	Per g **dry** wt

Processing Bird Stools for *Enterococcus*

Sample Collection

1. Scoop bird stool off sand using sterile 50 cc tube; avoid collecting sand.
2. Collect 5 stool samples per tube.
3. Store samples in a cooler containing blue ice.
4. Process within 2 hours of collection.

Laboratory Analysis

1. Obtain weight of bird stool by subtracting the weight of an empty 50 cc tube from the weight of the 50 cc tube containing the bird stool.
2. Add 25 ml sterile PBS to the tube containing the bird stool.
3. Vortex the stool for 1 minute
4. Emulsify the stool using a sterile stick or tongue blade and repeat vortex step.
5. Prepare a 1:100 dilutions of bird stools using PBS
    a. Aliquot 9 ml of PBS into sterile tubes (glass or plastic 15 ml cc tubes)
    b. Prepare a 1:10 dilution of original suspension. Transfer 1 ml from 25 ml suspension and put into 9 ml PBS tube.
    c. Vortex diluted suspension
    d. Transfer 1 ml of 1:10 dilution to another 9 ml tube of PBS (1:100)
    e. Vortex diluted suspension
6. Filter 1 ml (1:100) and 0.1 ml (1:1000) volumes of the 1:100 dilution onto HAWP filters.
7. Note: If there are no counts using these dilutions, filter 5 ml from the 1:10 dilution (1:2).
8. Place filters onto mEI agar and incubate at 41°C for 24h
9. Count the number of colonies presumptive for Enterococcus
10. Multiply the number of colonies by the dilution factor (25) and by the volume dilutions; report the number of colonies per gram of stool
    a. Example: Number of colonies is 30 for 10^{-1} dilution volume filtered; stool = 0.2 gram

$$30 \text{ CFU} \times 25 \times 10^{3} / 0.2 = 7.5 \times 10^{-6} \text{ CFU/g}$$

FILTRATION FOR MOLECULAR ANALYSIS

Background

The filtration procedure for molecular analysis is similar to the membrane filtration method used for fecal indicator bacteria. The primary difference is that bacteria captured on filters are analyzed by DNA extraction, amplification and detection of targeted regions. Since environmental samples frequently contain enzymes that degrade DNA, the filters must be frozen immediately in liquid nitrogen to prevent loss of DNA.

Equipment and Supplies

Polycarbonate HTTP membrane, 47mm, 0.4um
Manifold setup for membrane filtration (adaptor, vacuum pump and flask etc.)
Disposable filtration funnels
Pre-labeled 2 ml microtube (i.e., bead beating tubes)
Microtube rack
Freezer boxes, pre-labeled
Filter forceps (2 pairs per station)
100% ethanol
Beaker
Alcohol lamp, tea candle or Bunsen burner
PBS rinsing buffer
Liquid nitrogen tank
Dewar flask for liquid nitrogen
Bench recording sheet (operator name, sample name, etc) and pen
Safety items: latex gloves, lab coat, safety glasses, freezer gloves
Ultra low freezer (-70 to -80 C)
Bench recording sheets

Samples

Beach water
Storm drain water
Elutriates from Concrete Coupons (F1, F2 & F3)
Elutriates from Sediment, Plants and Algae

Filtration Procedure

1. Set up membrane filtration apparatus
2. Check vacuum pressure. Turn on the pump and close all valves. Then adjust the vacuum pressure using the knurled knob on the vacuum inlet of the pump (*<20 inch Hg, or <0.6 atm*)
3. Light the candle, alcohol lamp or Bunsen burner (for flame sterilization)
4. Wear gloves and use aseptic technique from now on

5. Place corresponding 2 ml microtubes into microtube rack; loosen tube caps
6. Soak one pair of forceps in a beaker with 100% ethanol
7. Mount the filtration funnel and label with sample name/number
8. Remove the funnel from the base and place it upside down on the bench on top of the lid
9. Take one pair of forceps, flame sterilize and allow it to cool a little; remove the grid filter from the filter housing and discard it (*be careful not to damage the supporting filter underneath the grid filter*)
10. Use the same forceps to carefully pick up one polycarbonate membrane (*clear grayish membrane in-between paper separators; do not mistake the paper separator as the membrane filter*), and carefully place it onto the center of the supporting filter (*to replace the grid filter discarded in step 10*)
11. Put the forceps back into the beaker with ethanol
12. Mount the filtration funnel back onto the housing to secure the polycarbonate membrane; *make sure there is no gap between the edge of polycarbonate membrane and the bottom of the filtration funnel and that the edge of the membrane is not folded, i.e. no liquid should go through the housing/support without passing through the polycarbonate membrane first.*
13. Fill the funnel with desired volume of water sample (*depending on turbidity of the water, one may need to start with a smaller volume)*, open the vacuum valve and start filtering. (This volume may need to change depending on the biomass density in the water and desired [DNA] recovery)
14. Filter water. Record volume filtered; if filter clogs before designated volume is reached, filter a smaller volume; record actual volume filtered for each filter on both the bench recording sheet and on the microtube itself (use permanent marker).
15. After all the water passes through the membrane filter, close vacuum valve and remove the funnel.
16. Using a pair of flame sterilized (*and cooled)* forceps, carefully roll up the polycarbonate membrane filter on the housing. First, fold one edge of the membrane onto itself (about ¼ of the diameter of the membrane) and hold in place with second forceps. Then, using each pair of forceps alternately, roll the membrane to crate a tube. Place into the corresponding 2ml microtube (*check the label on the tube*) and screw the cap securely.
17. Place microtube into dewar flask containing liquid nitrogen until the sample can be placed into pre-labeled freezer box and stored in a -80C freezer.
18. Repeat steps 9 – 17 for replicate filters).

APPENDIX C1

Number of Filters to Archive for Molecular Analysis

Moonlight State Beach

Storm drain water:	2 samples x 1 rep x 20 weeks = 40
UV Bypass water	1 sample x 1 rep x 10 weeks = 10
Beach water:	1 sample x 1 rep x 20 weeks = 20
Coupons:	2 samples x 3 fractions x 1 reps x 20weeks = 120
Algae:	1 sample x 1 rep x 10weeks = 10
Plant:	1 sample x 1 rep x 10 weeks = 10
Seawrack:	1 sample x 1 rep x 10 weeks = 10
Sediment:	1 sample x 1 rep x 6 weeks = 10
Sub-total	230

City of San Diego

Storm drain water:	2 samples x 2 reps x 20 weeks = 80
Coupons:	2 samples x 3 fractions x 1 reps x 20 weeks = 120
Sub-total:	200

TOTAL: 430

APPENDIX C2

Number of mEI plates

Moonlight State Beach

Beach water: 1 sample x 2 dilns x 1 rep x 20 weeks = 40
SD Water: 2 samples x 2 dilns x 1 rep x 20 weeks = 80
UV Bypass: 1 sample x 2 dilns x 1 rep x 10 weeks = 20
Coupons: 2 samples x 3 fractions x 2 dilns x 1 rep x 20 weeks =240
Algae: 1 sample x 2 dilns x 1 rep x 10 weeks = 20
Plant: 1 sample x 2 dilns x 1 rep x 10weeks = 20
Sediment: 1 samples x 2 dilns x 1 rep x 10 weeks= 20
Sub-total: 440

City of San Diego

SD Water: 2 samples x 2 dilns x 1 reps x 20 weeks = 80
Coupons: 2 samples x 3 fractions x 2 dilns x1 rep x 20 week = 240
Sub-total: 320

TOTAL: 760

Number of Quantitrays/Enterolert powder

Beach water: 1 sample x 1 diln x 2 reps x 20 weeks = 40
SD Water: 2 samples x 2 dilns x 2 reps x 20 weeks = 160
UV Bypass: 1 sample x 1 diln x 2 reps x 10 weeks = 20
Coupons: 2 samples x 3 fractions x 2 reps x 20 weeks =240
Algae: 1 sample x 2 dilns x 2 rep x 10 weeks = 40
Plant: 1 sample x 2 dilns x 2 reps x 10 weeks = 40
Seawrack: 1 sample x 2 dilns x 2 reps x 10 weeks = 40
Sediment: 1 samples x 2 dilns x 2 reps x 10 weeks = 40
Sub-total: 620

City of San Diego

SD Water: 2 samples x 2 dilns x 2 reps x 20 week = 160
Coupons: 2 samples x 3 fractions x 1 reps x 20 week =120
Sub-total: 320

TOTAL: 900

APPENDIX C3

PROTOCOL FOR ENTEROCOCCUS SPECIATION
Updated 10/25/10

Purpose and general overview

1.1 Purpose: To identify *Enterococcus* and other closely related species isolated on mEI media using the Vitek II. The Vitek II is an automated biochemical testing system used to identify colonies or isolates to genus and species level.

1.2 Overview: A single colony from mEI media presumptive for enterococci are subcultured to Blood agar plates (BAPs) to establish purity. If the 24 h culture appears pure and resembles *Enterococcus*, then Vitek can be performed. If you are uncertain about Enterococcus morphology, perform a gram stain. A pigment test is also performed on 24h cultures on BAP. After Vitek and pigment testing, the isolates are frozen for future use. Isolates that are not definitively identified by Vitek may require further purification, gram staining and/or additional biochemical testing as described below.

Materials and supplies

1.3 Sterile loops and needles
1.4 Microscope slides, Gram stain reagents & DI water
1.5 Blood agar plates (TSA with 5% sheep's blood) (Northeast Labs)
1.6 TSA (t-soy agar) slants (NEL)
1.7 Sterile cotton swabs
1.8 Vitek supplies, including Gram positive (GP) Vitek Identification cards, PBS
1.9 2 ml cryovials
1.10 Brucella broth with 15% glycerol (freezing media) (make in house)
1.11 Freezer boxes
1.12 Heart Infusion broth (make in house)
1.13 Purple broth w/ sucrose, mannitol and arabinose (NEL)
1.14 Motility media w/ TTC (NEL)
1.15 Methyl-alpha-D-glucopyranoside (in house)

Isolation of enterococci for speciation

1.16 Allow all refrigerated media to reach room temperature prior to subculturing. Check expiration dates on all culture media prior to use.

1.17 Select up to 10 colonies per mEI plate presumptive for enterococcus (i.e. colonies with blue halo, regardless of colony size); pick different sized colonies that are representative of the total population; if there are only a few colonies on the mEI plate, sub-culture all colonies present.

1.18 Subculture a single, well isolated colony from a 24 h mEI culture onto a BAP and incubate at 35ºC for 35.0 ± 0.5°C for 18 - 24 hours.

1.19 Cultures on BAP should be pure and resemble enterococci (i.e., colonies are small to medium sized, greyish-white, slightly yellow in color and alpha hemolytic (green in color) or non-hemolytic.

1.20 If colonies on BAP appear mixed, prepare a "purity plate" as follows: transfer a single, well-isolated *Enterococcus*-like colony onto BAP using a needle or 1 ul loop. Label this BAP as "pp" for "purity plate" and incubate for 24 h. If there are no well isolated colonies, use a 1 ul loop to streak for isolation. If colonies on BAP pp appear **atypical** for *Enterococcus*, conduct gram stain from BAP. *Enterococcus* are gram-positive cocci that occur singly, in pairs, or as short chains. They may also occur as coccobacilli. Record gram stain reaction onto worksheet. Do not VITEK gram-negative organisms.

1.21 If Viteking cannot be performed at 24h, the culture may be subcultured to BAP, incubated as before and refrigerated for up to 1 week;TSA slants may be used for more long term storage. Use a sterile loop or needle to transfer a single, well-isolated colony from mEI to BAP or TSA slant. Incubate at 35°C for 24 hours. Store the original mEI plates in the refrigerator. After 24h, check the BAPs or TSA slants for growth. If there is growth on the TSA slant, tighten tube cap and store at 4°C until Vitek can be performed. At this point, the stored mEI plates can be discarded into a biohazard bag.

1.22 Do not leave BAPs or TSA slants at 35°C for more than 24 hours; store in the refrigerator.

1.23 For Vitek, use 24h cultures grown on BAP.

1.24 Remember: if cultures have been refrigerated, they need to be subcultured to a fresh BAP (24h) prior to Vitek.

Vitek and pigment testing

1.25 Perform Vitek as per the laboratory's SOP.

1.26 Record VITEK identification on Vitek printouts into electronic database. File hard copies of Vitek print-outs.

1.27 After Vitek, perform pigment test using 24h BAP used for Vitek. Swab a few colonies and examine for yellow coloration under a bright lamp. Record degree of yellow pigment, i.e., "no pigment", "slight yellow" or "yellow" onto worksheet.

1.28 Species identified with other than "excellent" or "very good" identification should be identified using additional biochemical testing such as gram stain and pigment, **motility** and **carbohydrate fermentation** tests. Gram stain cultures from 24h BAP and record result on worksheet. Refer to gram stain procedure. In some cases, identifications are less than "very good" due to the following: culture was mixed, organism is gram negative, organism is not in the database, the ID must be based on tests that are not part of the Vitek method, such as pigment or motility.

1.29 To discriminate between **E. gallinarum vs. E. casseliflavus (** i.e., isolates with "low discrimination" ID by Vitek) rely on pigment test; also conduct motility test because non-motile isolates identified by E. gallinarum may be E. mundtii. Also, test all non-motile E. gallinarum isolates with methyl-alpha-D-glucopyranoside (MADG) to differentiate from E. mundtii.

1.29.1 Typical reactions

E. gallinarum: no yellow pigment or slight yellow, motility (+), MADG (+)
E. casseliflavus: definite yellow pigment, motility (+)
E. mundtii: definite yellow pigment, motility (-), MADG (-)

1.29.2 **Motility test**: use needle to inoculate motility agar. Stab needle at least half way into center of agar. Incubate 35.0 ± 0.5°C for 18 - 24 hours. Growth away from stab line = positive (+).

1.30 **To identify "low discrimination" E. faecium vs E. durans vs E. hirae, or to identify E. mundtii,** perform carbohydrate fermentation tests (arabinose, sucrose and mannitol). Choose the carbohydrate that will discriminate between the species.

1.30.1 T**ypical reactions**

E. faecium: no yellow pigment, arabinose (+), mannitol (+)
E. durans: sucrose (-), arabinose (-), mannitol (-)
E. hirae: sucrose (+), arabinose (-), mannitol (-)
E. mundtii: yellow pigment, sucrose (+), arabinose (+), mannitol (+)

Arabinose, sucrose,mannitol and MADG tests: first inoculate tube of heart infusion broth (HIB) with a small loop-full 24 hour old culture grown on BAP. Incubate HIB at 35.0 ± 0.5°C for 18 - 24 hours. Take 5 drops of culture and inoculate into purple broth media containing sucrose, arabinose or mannitol. Incubate for 24 h as before and read for color change (acidification): Yellow = (+); no color change = (-).

1.31 **IMPORTANT: repeat the pigment test if Vitek identification and pigment results do not match up** (i.e., a yellow pigmented E. gallinarum or E. faecium and non-pigmented E. casseliflavus).

Species Identification

1.32 If the Vitek identification is of low discrimination, unacceptable, or E. gallinarum, Tthe **final species identification** should be based on the biochemical results in addition to the Vitek result. Record the biochemical reactions in the Vitek database under "Comments".

1.33 References useful for species identification

1.33.1 Facklam, R.R. and Collins, M.D. 1989. Identification of *Enterococcus* species isolated from human infections by a conventional test scheme. J. Clin. Microbiol. 27:731-734. http://www.pubmedcentral.nih.gov/picrender.fcgi?artid=267406&blobtype=pdf

1.33.2 Teixeira, L.M. and Facklam, R.R. 2003. *Enterococcus*, p. 422-433. *In* P.R. Murray, E.J. Baron, J.H. Jorgensen, M.A. Pfaller and R.H. Yolken (ed.), Manual of clinical microbiology, 8th ed. American Society for Microbiology, Washington, D.C.

Freezing isolates

1.34 Freeze all isolates from a 24h BAP using freezing media. Remove cryovials containing freezing media from the refrigerator and allow media to reach room temperature.

1.35 Label the cryovial with the sample identification number.

1.36 Take a loopful of organism and emulsify in freezing media.

1.37 Place cryovial into freezer box and freeze at -80°C.

1.38 Record the freezer box number and date frozen onto the worksheet.

Media formulation

1.39 **Freezing media** (1 liter)

1.39.1 28 g Brucella broth

1.39.2 1 L DI water

1.39.3 150 ml glycerol

1.39.4 Heat to dissolve. Transfer to 1 liter bottle and autoclave 121°C for 30 min.

1.39.5 Allow media to cool. Dispense 1 ml into sterile 2 ml cryovials and store in refrigerator until used.

1.40 **Heart infusion broth** (1 liter)

1.40.1 25 g Heart infusion broth

1.40.2 1 L DI water

1.40.3 Heat to dissolve.

1.40.4 Dispense into test tubes w/ screw caps (10 ml per tube); label test tube rack with the date made and your initials.

1.40.5 Autoclave 121°C for 15 min.

1.40.6 Tighten caps when the media has cooled.

1.40.7 Refrigerate until used.