DETECTION OF ENTEROVIRUS IN MARINE RECREATIONAL WATERS OF SOUTHERNS CALIFORNIA USING QUANTITATIVE AND NESTED PCR TECHNIQUES

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Abstract

ROBERT ALBURY: Detection of Enterovirus in Marine Recreational Waters of Southern California using Quantitative and Nested PCR techniques
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Contamination of recreational waters with enteric viruses poses a risk to swimmer's health. To protect health, new methods are needed for virus detection. This project focused on the detection of enteric viruses in marine recreational waters of Southern California as part of coordinated epidemiology studies conducted at recreational beaches from 2008 to 2009. A new procedure was developed for the detection of enterovirus and norovirus using a rapid and high through-put polymerase chain reaction (PCR) technique. Additionally a limited comparison of quantitative and nested PCR was conducted. This study found a significant difference in the detection of viruses among the three study beaches. Additionally, the nested PCR protocol detected a statistically greater occurrence of enteric viruses than quantitative PCR. The findings of this study suggest that rapid PCR-based methods may be developed and standardized for routine monitoring of enteric viruses from recreational beaches.

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Table of Contents

List of Tables

List Figures

1 Introduction

The Environmental Protection Agency (EPA) standard guidelines for recreational marine waters are currently based on measurement of fecal indicator bacteria (FIB). FIBs are easily identified and tracked biological organisms that are known to occur in association with pathogenic agents that are less easily identified. These bacteria are detectable with standard methods, including quantal methods employing liquid culture media, enumerative methods using membrane filtration, and molecular analysis techniques. Fecal coliforms and enterococci are examples of FIB. Both of these FIB can be easily detected with standard methods and their primary source in the environment is from fecal contamination.

Detection of FIB may not be adequate to indicate the extent of pathogen presence in contaminated waters. For example, FIB may not accurately reflect the presence and environmental fate of pathogenic bacteria, viruses or protozoan parasites. Therefore, there is the potential for recreational waters to test safe by the EPA guidelines FIB but to still pose a risk to human health. This limitation with the measurement of FIB is one reason for the development of new water quality tools, such as methods for the direct measurement of pathogenic viruses.

This potential failing of FIB to reliably represent pathogen presence and risks is not the only problem with the standard system now recommended by EPA. There is also the potential that FIB may not be adequate to indicate the presence and risks in marine recreational waters from nonpoint sources pollutants. These guidelines may be an

effective measure of human health risk from waters impacted by point sources such as sewage treatment plant discharge in recreational waters. However, as waste water treatment has improved, the amount of water contaminated by point sources of pollution has decreased. The extent to which the current EPA recreational water quality guidelines predict the presence and risks from non-point sources is inadequately documented and therefore uncertain. New methods are needed to evaluate FIB and pathogens associated with non-point source contamination and to track non-point source contamination due to its potential effects on human health (Boehm et al 2009).

Another potential failing of FIBs is their inability to directly monitor the presence of enteric viruses in marine recreational waters. Some enteric viruses are known to cause illness in humans, such as enteroviruses, noroviruses, rotaviruses, hepatitis A virus, and adenoviruses (Haile et al 1999). Recent research has shown that enteric viruses are present in marine recreational waters and that virus pose a potential health risk to recreational water users (Soller et al. 2010). To better protect public health, methods are needed that can reliably and accurately detect the presence of enteric viruses, such as Enteroviruses and Noroviruses.

The primary focus of this study was the development and implementation of tools to assess the presence of Enteroviruses and Noroviruses in marine recreational waters. In 2007-2009, the Southern California Coastal Water Research Project (SCCWRP) coordinated epidemiological studies at recreational beaches in southern California to help inform EPA's efforts toward updated recreational water quality criteria. These epidemiological studies were set up using a prospective cohort design in which beach goers were monitored for water exposure while at the beach and then surveyed for

possible bathing-associated illness up to two weeks subsequent to their beach visit. Participants were asked about gastrointestinal illness, upper respiratory illness, rash, eye ailments and earaches. Researchers also collected water samples to be analyzed for a number of microbiological agents that may be predictive of public health risks. Initial analysis for enteric viruses resulted in a large number of negative samples. However, the epidemiological data indicated illness reports that were consistent with viral infections, and which were not predicted by analysis of fecal indicator bacteria.

The approach of this study included the utilization of high through-put, automated techniques for analysis of viral RNA from archived filter membranes used to collect microbes from water. Additionally, this project included two forms of polymerase chain reaction (PCR) analysis, nested-PCR and quantitative PCR, for a subset of samples. A comparison of the potential use of these two methods to detect enteroviruses and noroviruses is included. Additionally, this thesis will detail the procedures developed for analysis of viruses in these marine water samples. The results of these tests will be used to associate the presence of enteric viruses in coastal recreational waters to reported health outcomes of recreational water users in an associated epidemiological survey. The microbiological-epidemiological comparison and association aspects of the greater SCCWRP project will be conducted by a different research team at a later date.

1.1 Objectives

The goal of this study was to evaluate water samples collected during bathing beach epidemiological studies in southern California in 2008-2009 for the presence of enteric viruses using more sensitive nucleic acid-based detection methodology than had previously been applied. Because enteric viruses are known to cause illness in humans there is a need for studies linking the occurrence of these viruses in the environmental

recreational waters to illness in exposed recreational swimmers. This project focused on the presence of Enteroviruses and Noroviruses, two groups of enteric viruses of public health concern. Archived water samples analyzed for this study were limited to a morning collection period, 0800 hours, at three recreational beaches in Southern California during the summers of 2008 and 2009. All three beaches were selected for this study because they are impacted by non-point source pollution and have extensive recreational use.

2. Review of the Literature

Coastal marine waters have the potential to cause illness among recreational users when the water has been contaminated by point sources, such as waste water treatment plant effluent, or non-point sources, such as urban runoff (Haile et al 199, Fleisher et al 1996). The overall health effects from exposure to contaminated recreational waters have been established in a series of epidemiological studies conducted since the 1970's (Table 2-1). These studies have focused on monitoring the levels of indicator bacteria at the recreational beaches (Kamizoulis et al 2004).

2.1 Epidemiological History of Recreational Water Related Illness

The study of health effects from physical exposure to contaminated fresh and marine waters began in 1950's with the work of Albert Stevenson. This study examined a mixture of aqueduct environments, including freshwater, tidal streams and artificial waters. Stevenson examined the occurrence of gastrointestinal illness (G.I.) in swimmers and non-swimmers, and found that swimmers were more likely to show symptoms of G.I. than non-swimmers along with a doubling of illness rates for those less than 10 years of age as compared those greater than 10 years of age. Stevenson failed to find any differences in rates of illness in swimmers based on the contamination level of contaminated water exposure (Stevenson 1953). His research demonstrated the need to conduct specialized experiments to better characterize the resources needed to implement effective public health standards.

The implementation of appropriate water standards for public health requires field research to determine levels of fecal indicator bacteria (FIB), along with the models

needed to interpret the FIBs sampling data. FIBs are easily identified and tracked biological organisms that are known to occur in association with pathogenic agents that are less easily identified. FIBs function as indicators for possible exposure to environmental contaminants, and are the main tool for reducing illness among marine recreational users.

Fifty percent of the world's population lives within 200 km of marine beaches. (Shuval 2005). This number is increasing, with the potential to directly and indirectly affect human health and well-being. Humans can be affected by poor water quality directly through risk of illness and indirectly through the effects reduced commerce can have on a community. Past research into the relationship between perception and water quality management of inland waters found that people respond to visible and olfactory indicators of water quality (Ilbery et al 1986). Ilbery's review of contemporary research showed that people are more likely to react negatively to inland waters that have undergone eutrophication, suggesting that water which appears dirty would be naturally avoided. This provides an important consideration when developing new measures of water quality that would be able to show contamination of recreational waters that is not directly detectable on macroscopic level.

Contamination of marine waters can be attributed in part to anthropogenic factors. 10,000 million cubic meters of wastewater are released each year by domestic water treatment facilities (2.3 trillion Gallons) (Shuval 2005). The waste flows into lakes, rivers and coastal waters, presenting a possible public health risk. Even with the current regulations regarding the quality of effluent being released there is a small chance of an

accidental contamination event occurring. These events can occur when there is a sewage spill or a failure in the treatment system.

The federal requirements under the Beaches Environmental Assessment and Coastal Health (BEACH) Act recognize the need for new tools to monitor coastal waters. The BEACH Act requires the development of an effective public health system that can reliably determine the water quality of our coastal marine environments. The goal is to keep the occurrence of illness down to acceptable levels, currently defined as 19 cases of G.I. illness per 1,000 swimmers (EPA 1986). This acceptable level was developed as part of a translation from the 1960's criteria to the thresholds sets in the 1980's. Epidemiological studies were then conducted to determine exposure levels where the acceptable levels were violated, data that was used to set exposure thresholds for the current EPA Guidelines.

2.1.1 General Health Effects

Health effects of contaminated water commonly include gastrointestinal (G.I.) illness. Skin, ear, and eye infections, are also possible, as are neurological damages, infections of open wounds, and respiratory tract infections. Respiratory tract infections include mild sinus infections, upper respiratory tract infections (AFRI) and lower respiratory tract infections (LRTI) that have proven to be fatal to the immune-compromised (Castor et al 2004, Prieto et al 2001). There is a strong collection of epidemiology studies that have reviewed the health impacts associated with exposure to contaminated recreational waters and have established the risk for different illness. These studies have demonstrated the link between contaminated marine recreational waters and illness (See Table 2-1 for listing of the major studies).

Table 2-1: Review of Epidemiological Studies on Illness Associated with Coastal Marine Recreational Waters

Article	Year	Key Findings	Study Type	Notes
Colford	2007	Water contact: G.I. =1.4(1.0, 1.8); Skin Rash =2.3(1.6, 3.2). Swallowing water: G.I. =1.9(1.3, 2.7); Skin Rash =2.1 (1.4, 2.2)	cohort	Findings presented as odd ratios
Shuval	2005	Attributable Risk: G.I. 5%; AFRI 1-5% (Mean=2%)	Review	Point Source contamination,
Dwight	2004	OR during high run-off: Any symptom 1.85 (1.36,2.52); HCGI 2.32 (1.27,4.25); Skin infection 1.93(1.12,3.33)	Cross-sectional surveys	Urban run-off, surfers
Prieto	2001	Risk of G.I. illness with increasing exposure: Seabathing P=0.45; Total coliforms P=0.004; Faecal coliforms P=0.13; Faecal streptococci P=0.042; *Staphylococus aureus* P=0.43; *Pseudomonas aeruginosa* P=0.072	Cohort Study	Conducted in Spain
Haile	1999	Proximity to storm drains increased risk of illness Respiratory Disease (distance zero m) RR 1.78 (1.29-2.45) Respiratory Disease (distance 51-100 m) RR 1.18 (0.94-1.49)	cohort	Urban run-off, exposure from storm drains
Pruss	1998	18 prospective, 2 retrospective, 2 randomize controlled trial Common Illness: G.I.; eye infections; skin complaints; ear, nose and throat infections; respiratory illness Health may differ between swimmers and non-swimmers Airsolization of pathogens may effect non-swimmers	Review	
Fleisher et al	1996	Thresholds: Acute febrile respiratoy illness (AFRI); 60/100 mL of fecal streptococci Ear aliment; 100/100 mL of fecal coliform	RCT: 4 regional groups, random assignment of exposure	
Kay et al	1994	Thresholds: Faecal streptococci >30/100 mL for G.I. illness	RCT: 4 regional study sites, lasted 4 years	UK study, used to define UK & EU standards
Corbett	1993	Illness rate: 24% of participants reported symptoms Swimmers 2* likely than non-swimmers to report symptoms	Cohort	
Scyfried	1985	Illness rate: 70.6% of swimmers 30.6% of non-swimmers	Family unit cohort	
Cabelli et al	1982	Thresholds: Enterococcus & *Escherichia coli* conc. > 10/100mL fot G.I. attack rates great than 10 per 1000 person	Prospective study, multi-year and site	Set the standard for the field and used to set first EPA standards
Stevenson	1953	Incidence Rates: G.I. < 10 YOA: 2.3 >=10 YOA: 1.2 Nose & Throat < 10 YOA: 7.0 >=10 YOA: 3.2 Skin < 10 YOA: 1.4 >=10 YOA: 0.5 Eye, Ear, other < 10 YOA: 2.4 >=10 YOA: 0.9	Cohort	First study in US, both fresh water and tidal

2.1.2 Rates of Illness

Gastrointestinal illness, and other associated diseases, can occur without exposure to contaminated recreational water as part of the normal burden of disease for a population. Epidemiological studies work to quantify incidence of disease that is attributable to contact recreation. A study of LA and Orange County found a background yearly rate of G.I. symptoms between 627,800 and 1,479,200 reports cases (Turbow 2009). A review of the literature demonstrated that there was an average of 5% of G.I. illness in water with acceptable quality standards (50 fecal streptococci per L). This review continued by tabulating the average yearly clinical cases with an average of 4 days of illness; G.I. 120,312,000 cases per year, AFRI 48,125,000 cases per year, and LRTI 1,636,250 cases per year (Shuval 2005). Another case study examined the occurrence of outbreak events and beach closures, and found that there were only 65 recorded outbreaks with 2536 total illness events reported for the US in 2001-2002. The study also found that there had been 24,853 beach closures/advisory days in 2004, a 3% increase from 2003 and the highest in 15 years (NRDC 2005). These studies show that is there is burden of gastrointestinal illness among population with potential exposure to recreational waters.

2.2 Role of Viruses in Recreational Water Management

Recent epidemiological studies conducted in recreational marine waters in Southern California (SCCWRP) have seen a potential occurrence of human health effects unrelated to bacterial contamination (John Griffith, SCCWRP, personal communication). This situation presents an opportunity to assess a potential relationship between human health effects from exposure to recreational water and occurrence of enteric viruses. The US EPA has identified a need to assess the role of enteric virus in human health effects

from using marine recreational waters (EPA 2011); this project can provide needed data on the environmental occurrence of enteric virus in marine recreational waters.

Enteric viruses are widely believed to be the main etiological agents of recreational water illness (WHO 2003). As a group, enteric viruses are also believed to be responsible for a significant percentage of waterborne outbreaks where the etiological agents are unknown. Viral outbreaks have been difficult to study for a long time due to the relatively low densities of viruses in environmental samples and the lack of effective cell culture systems for the detection of the viruses that cause gastroenteritis like Noroviruses and Enteroviruses. The advent of PCR-based methods targeting viral nucleic acids has increased virus detection capabilities. There is currently an opportunity to consider risks of enteric viral infections in water quality plans.

Noroviruses are the most common cause of viral gastroenteritis worldwide and are commonly implicated in waterborne outbreaks (Kageyama et al. 2004, Hoebe et al. 2004, Nygard et al. 2004, Yoder et al. 2008). Based on recent studies, Noroviruses account for 94% of the reported outbreaks of non-bacterial gastroenteritis that have been examined over a 4.5-year period in the US (Fankhauser et al. 2002) and 87% of outbreaks reported in The Netherlands (Vinjé et al. 1997). Members of the family Caliciviridae, Noroviruses are non-enveloped viruses, 27–35 nm in diameter, possessing a single-stranded RNA genome of 7.5–7.7 kb (Green et al. 2001, Atmar and Estes 2001). Noroviruses are transmitted via the fecal-oral route. The infection is self-limiting to the epithelial cells of the small intestine, causing fever, diarrhea, and explosive vomiting that usually lasts for two days. Re-arrangements in Norovirus capsid antigenic properties

appear to have contributed to the prevalence of this virus in the human population, evolving by antigenic drift in the face of human herd immunity (Lindesmith et al. 2008).

Enteroviruses are members of the Picornaviradae family and include poliovirus, echovirus, and Coxsackie A and B viruses. Human enteroviruses are one of the most commonly detected viruses in polluted waters (Rusin et al. 2000). Furthermore, enteroviruses are estimated to cause 30 million to 50 million infections per year with 30,000 to 50,000 of these resulting in meningitis hospitalizations (Oberste et al. 1999). Enteroviruses have been found in activated sludge, sewage outfalls, and fresh and marine waters associated with human fecal contamination (Kopecka et al. 1993, Reynolds et al. 1998, Griffin et al. 1999, Noble et al. 2001, Jiang et al. 2001). Also, enteroviruses are relatively stable in the environment. Enteroviruses are resistant to chlorine (Keswick et al. 1984, Payment et al. 1985), UV disinfection (Batigelli et al. 1993), and are tolerant of a wide range of temperatures and salinities (Skraber et al. 2004, Wetz et al. 2004). Due to viral replication in the gastrointestinal tract, an infected individual may shed enteroviruses from the stool for up to 16 weeks (Romero 1999), with densities as high as 10^6 viruses per gram of feces (Melnick and Rennick 1980).

2.2.1 Characteristics of an Ideal Indicator Organism

Monitoring of marine recreational waters for contamination of biological hazards is critical in managing health risk to recreational water users. The historical concern for recreational water quality has been the contamination of the near shore waters by untreated or under treated effluent from waste water treatment plants, but increasing standards and improved technology has reduced this risk. In recent years a new risk for near shore water contamination has been identified as non-point sources of pollution, such as urban runoff.

The direct measurement of pathogens in recreational waters has not been practical. Effective monitoring of water quality for recreational marine settings has instead relied on indicators. An ideal indicator organism would adequately predict the occurrence of human illness among recreational users. Such an indicator organism would meet many of factors listed below, though not all factors need to be addressed with each indicator. An ideal indicator organism should:

- Be present whenever the pathogens are present;
- Be present only when the presence of pathogens is an imminent danger (i.e., they must not proliferate to any greater extent in the aqueous environment);
- Occur in much greater numbers than the pathogens;
- Grow readily, on simple media (if bacterial);
- Be randomly distributed in the sample to be examined, or it should be possible to obtain a uniform distribution by simple homogenization procedures;
- Not be seriously inhibited in their growth by the presence of other bacteria;
- Be non-pathogenic to humans; and
- Be readily detected in low numbers reliably, rapidly, and at low cost.

(National Research Council of the National Academies, 2004)

The selection of an indicator organism also requires knowledge of the etiology of illness among recreational users who are exposed to fecal contaminated waters. This information would allow for the selection of an indicator organism that would behavior similarly to the potential pathogen in the environment. These standards have led to the selection of fecal bacteria that would make for the best indicator organism, such as fecal coliforms and with a current focus on concentrations of enterococci for recreational waters. These indicators have been effective at managing the occurrence of bacterial related illness such as skin rashes, ear and eye infections, and gastrointestinal illness (EPA 2004). While enterococci may be effective for predicting the occurrence of illness from bacterial sources, recent research has shown that not all occurrences of illness

among recreational users can be directly predicted by the presence of enterococci (John Griffith, SCCWRP, personal communication).

Finally the bacterial detection of coliphages has been proposed as indicators of enteric viruses in water, which is due to the similar lifecycle between coliphages and enteric viruses. This method provides an indirect measurement of enteric virus based on the amount of detectable viruses that can infect *E. coli*. This method is does not allow direct detection of viruses that are pathogenic to humans.

2.2.2 Current Methods for Assessing Quality of Recreational Waters

Current water quality standards for the assessment of recreational waters were defined by the EPA in 1986. These standards were focused on the detection of enterococci as the FIB and provide flexibility in the sampling schedule to provide the most relevant data to the management agencies. For example, the State of North Carolina Department of Environment and Natural Resources (NCDENR) has implemented a management plan for recreational waters that is based on a variable sampling rate, determined by the seasonality and the amount of recreational users. Sampling beaches are divided into three different tiers, based on proximity to popular recreational areas, storm drains and waste water treatment plant outflows. Additionally the sampling regimen is determined by seasonality of the sampling period, which includes division of season (April through September), shoulder season (October) and off season (November through March). This approach allows the highest rate of sampling to occur during the summer months at beaches popular for recreational activity. During this period samples are taken on a weekly basis and tested for the presences of Enterococcus as the primary FIB (NCDENR 2009).

The current method for testing the presence of enterococcus used by NC DENR and many other states is the Idexx Laboratory Enterolert® Quanti-Tray® (NCDENR 2009). This method is based on a one-time use tray that is divided into multiple equal value wells, which are filled with marine water sampled from the recreational site mixed with the proprietary media. The tray is then sealed with the Idexx Laboratory proprietary tray sealer and incubated at 42^{o}C for 24 hours. The number of positive wells are then counted and used to calculate a MPN for the sample. This product requires low skill but provides a slow method for the detection of FIB in marine samples. While this method is an improvement upon the ease of use compared to older multiple tube fermentation and membrane filtration techniques it is still a time consuming method. There is a potential for improvement upon these time limitations with the use of reverse transcriptase PCR for the detection of enteric viruses.

2.2.3 Criteria for the Development of New Viral Indicators

A viral indicator for recreational water quality would need to meet similar standards as do traditional FIB, such that a dose-response relationship can be established between the environmental concentration of viral RNA in marine waters and the occurrence of related illness in a feasible time scale among exposure through recreational activity. The testing of the feasibility of Norovirus and enterovirus as recreational water quality indicators would require environmental samples that are linked with epidemiology studies of recreational users with known health outcomes data. A case-control study would then need to be conducted to determine if there is scientifically significant correlation between the occurrence of relevant illness and exposure to marine waters contaminated with a potential viral indicator. This project will focus on Enterovirus and Norovirus Genotype I and II.

This project will provide the needed environmental data for the assessment of entrovirus and norovirus. Results will be shared with collaborators to determine whether occurrence of these viruses in water correlate with health outcomes.

3. Materials and Methods

3.1 Study design

This study was designed to test the feasibility of detecting enteric virus RNA in coastal recreational waters collected from three Southern California recreational beaches (Figure 3-1). Water samples were collected by SCCWRP as part of a larger research project during the summers of 2008 for Doheny and Avalon beaches and 2009 for Malibu Beach. Up to 500 mL of the samples were filtered on to 45µm nitrocellulose filters, using standard aseptic techniques. The filters were then rolled and placed into labeled 3 ml tubes, which were then grouped by beach study site and stored at -80^{o}C for archiving. Samples were stored for up to 2.5 years at -80^{o}C until analysis for this study began in November of 2010 at the UNC-CH Microbiome Core Facility. During the storage period, samples were twice transported on dry ice and did not thaw.

This study focused on samples collected during the 8am sample period for all three study beaches. The sampling period for Doheny Beach was from 7/24/2008 through 9/14/2008. Avalon Beach samples were collected from 6/26/2008 through 8/31/2008, and the Malibu Beach samples were collected from 5/23/2009 through 9/20/2009. All transects from each of the sample beaches were tested in this study: 5 transects for both Doheny and Malibu Beaches and 4 transects for Avalon Beach. A total of 358 sample extractions were processed in this study, additionally the blank samples collected during the field study were examined in parallel to insure there was no systematic contamination of the samples. Due to the large number of samples collected for this project, methods were developed to allow for high through-put molecular analysis. All of the procedures

were performed in a 96-well plate format and used automated equipment including the use of robotic apparatus when possible.

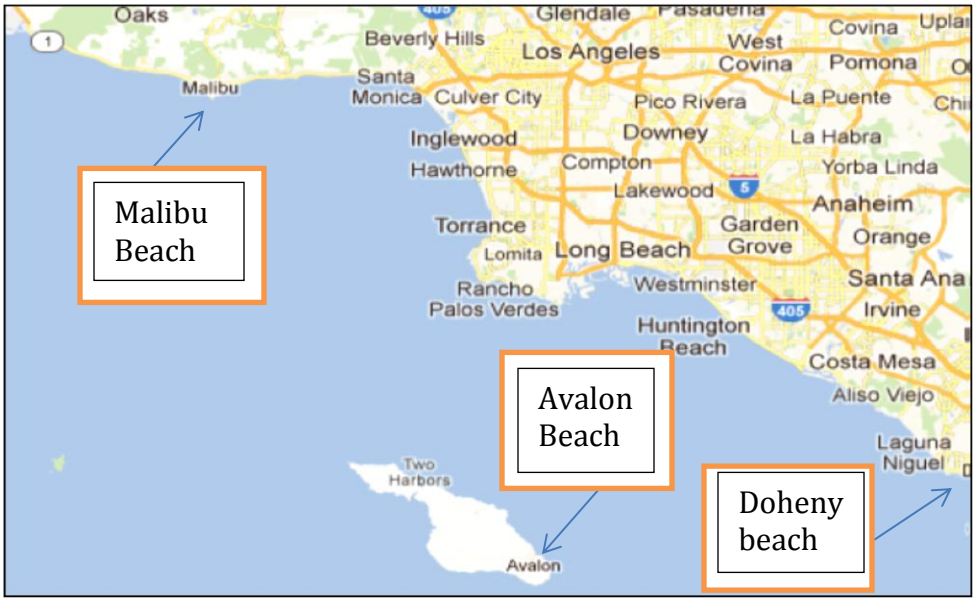


Figure 3-1: Map of Coastal Southern California, study sites are marked

3.2 Experimental Methods

This project had three major sample processing steps that needed to be conducted; the extraction and isolation of viral RNA from filter membranes, the timely analysis of nearly 500 samples, and the assessment of the presence and genotyping of viral RNA. Both quantitative and qualitative quality control steps were included in each of the major steps.

3.2.1 Extraction and Isolation of viral RNA from Samples

The extraction and isolation of viral RNA samples occurred through three steps, in which the nitrocellulose filters with material collected from water samples were first physically broken down, the RNA from the pieces of filter material was then chemically extracted, and finally the RNA extracts were isolated from the extraction buffer on a Qiagen BioRobot. This process was tested using known sample concentrations to determine the recovery rate of both and enterovirus and representative noroviruses of

genogroups I and II. Positive controls were filtered through 45µm nitrocellulose filters, folded into an eppendorf tube, and frozen to -80^{o}C for one day. These positive controls where then used to fine tune these procedures.

The initial stage of this process was conducted in a biological safety cabinet that had been cleaned using 75% ethanol spray and RNase AWAY spray on all working surfaces and tools used through this procedure. All work was performed over an ice try, in which the samples were only removed when being processed. The tools used for this procedure included a single edge stainless steel razor blade, two forceps, a sterilized and RNase free disposable weigh discs was used as a cutting board, along with ChemWipes that are used in the cleaning of the tools. The filter disc was cut into pieces less than 1cm x 1cm using the above razor blades. These pieces were then loaded into a marked sample block, called an S-Block, and a record of each sample location was recorded. The sample block is a deep-well 96-well plate, allowing for approximately 50 µl of sample volume. Samples were spilt between two different wells due to the volume limitation of each well.

The filter samples were removed from their sample tubes and place on a cleaned weigh disc via the use of forceps. The rolled filters where then held in place via the forceps and cut into pieces less than 1cmx 1cm squares with the razor blades, half of each filter was loaded in the sample wells. There was some variance in the size of these pieces but no piece was larger than the above stated size. It was determined during the validation that the pieces need to be covered in the elution buffer. The pieces where then loaded into the 2 wells of the S-Block. It was determine that filter pieces should be split between two wells do to volume limitations. The sample locations were recorded on load sheets based on column numbers and row letters. Once all of the wells were loaded with pieces of the

filters, the block was sealed using pre-sized sealing tape. The sealed blocks were then labeled with sample beach and year, along with date range of samples. The sealed and labeled blocks where then stored in the -80^{0}C freezer until all samples were processed and loaded into sample blocks.

The next stage of this sample preparation process was the extraction of RNA from the filter paper. This was performed by submerging the pieces of the sample filters in an extraction buffer, the extraction buffer was composed of components taken from Qaigen® AllPrep RNA/DNA extraction and isolation kit, these components are referred to by their names used by Qaigen. This buffer was composed of ATL buffer, 350 µl per loaded well, AL buffer, 350 µl per loaded well, and protease, 20µl per loaded well. All of the components were part of a Qiagen tissue lysis kit. These buffers were mixed in 50 reaction batches that were then loaded into the S-Block via a Thermo Scientific 8-Channel pipette. Once all of the wells had been loaded for the S-Block, the block was again sealed using the pre-sized sealant tape. The sealed S-Block was then centrifuged for 10 minutes at 3000 RPM, and the sample block was then loaded into a sonication water bath set to 60^{0}C for 30 minutes. The S-Block was then removed from the sonication water bath and centrifuged for a second time for 10 minutes at 3000 RPM. The block was then unsealed and the buffer was aspirated via an eppendorf manual P-200 8-channel pipette. A total volume of approximately 500 µl of buffer was removed, taking care that none of the filter pieces were transferred with the buffer.

The extraction buffer was then loaded into the Qiagen Biorobot S-Block location. The robot was setup to perform an RNA isolation procedure, which was developed by

Qiagen as part of the AllPrep DNA/RNA 96 Kit (Qiagen 2008). This process involved multiple buffer washes that allowed for collection viral RNA into a 96 well plate.

3.2.2 RNA Isolation Quality Assurance

This stage of the procedure was composed of two independent steps for the determination of quantitative and qualitative measures of the RNA isolation procedure. These processes required a total of 7µl of RNA isolated from each sample well. Each of the duplicates was examined to insure that it was usable. The initial step was to scan 2µl of the RNA isolate in a Tecan infinite M200 plate reader with a Tecan NanoQuant Plate (16 well format). The Tecan was standardized using 2µl of the AVE Buffer used during the RNA isolation step as the final buffer which viral RNA is isolated. This provided a background light absorbance reading for all of the other samples. The samples where then loaded onto the NanoQuant plate, which was cleaned with 75% ethanol before and after each run. The Tecan infinite M200 would then compare the absorbance of each sample to the AVE buffer standard to determine the amount of RNA in the sample that was then expressed in units of ng/µl. The Tecan infinite M200 has internal settings for the detection RNA, DNA, and Protein based on their absorbance of light. This data was then saved in a spreadsheet for analysis at a later date.

Gel electrophoresis was used to determine the quality of the RNA through a qualitative measure based on the clarity of nucleotide banding on the gel. The standard used for this assessment was the distention of bands that could be viewed on the gel, without any concern for the size and number of bands. This process used the remaining 5µl of RNA isolate set aside for this process. The RNA was mixed with 1µl of 6x loading dye to stabilized the RNA and improve the ease of loading the gel. The gel used for this process was poured 30 minutes prior to use through the use of a 100 well gel mold. This

process required the use of 200 mL of 1% AGAR TAE gel, which was produced by mixing 2 g of Agarose in 200mL of 1x TAE and heating for two minutes in a lab microwave at maximum power. The TAE buffer used in this process is mixture a of Tris base, acetic acid and EDTA made by Fisher Scientific, and the Agarose was made by Fisher Scientific. The mixture was allowed to cool on a lab countertop for 20 minutes, at which point 2µl of Life Technologies' SYBR® Safe DNA was mixed into the gel media, to provide ultraviolet visualization of PCR products. The gel was allowed to setup for an additional twenty minutes. Once the gel had set it was transferred into a Bio-Rad Sub-Cell® Model 96 Cell and the apparatus was filled with 1x TAE buffer. The wells where then loaded in order by column of the 96 well sample plate via a Thermo Scientific Matrix Equalizer Electronic multichannel pipette (1-30µl range), such that the top row of the gel had sample "A1-H6" and the bottom row had samples "A7-H12". The four corner wells were loaded with 10kb DNA ladders. The gel was then run for 35 minutes at 125 volts, while the apparatus had two ice packs placed on it to prevent the gel from overheating and melting. Once the gel had finished running it was transferred to a UV light box with an attached digital camera, which is controlled by a desktop computer. The gel was positioned via the visible light capabilities of the camera, and the UV light source was turned on for a few seconds. While the UV light was on both a physical and digital image of the gel were produced for analysis at a later date.

3.2.3 cDNA Synthesis

The production of the cDNA products for the reverse transcriptase presented two issues. The initial issue was whether to use general primers or specie specific primers. It was determined that the use of specie specific primers would improve the detection of viral RNA in the samples. The primers used in the reverse transcriptase production of

cDNA are detailed in section 3.5 *cDNA Synthesis*. The other issue was the verification of product quality. It was determine that a two-step process would be needed to ensure quality. This process used both a quantitative assessment of the amount of DNA present in the sample post reverse transcription, along with an estimate of product size through gel electrophoreses with DNA ladders.

The RNA isolates were taken out of the -80^{0}C freezer, placed on ice, and allowed to completely thaw. This process was also performed in the 96 well formats with the sample order of the RNA isolates maintained. All of the RNA isolates were processed in triplicates in order to produce enough cDNA products for use in the later stages of this project. The master mix for this project was as follows per reaction: 1µl of primer 1, 1µl of primer 2, 2µl of Buffer, 4.2µl of autoclaved microbiological grade water, 1 µl of polymerase, and 0.8µl of dNTPs. The polymerase, Buffer and dNTPs used in this synthesis were from an Applied Biosciences High Capacity Reverse cDNA synthesis kit. The primers used for the cDNA synthesis were specific to each of the three virus strains examined for this project (TABLE 3-1).

Table 3-1: cDNA Synthesis Primers for Enterovirus and Norovirus

Virus	Primer	References
Enterovirus	ENV-F, ENV-R	Afonina et al 2007
Norovirus GI	JJVIF, JJVIR	Jothikumar 2005
Norovirus GII	JJV2R, GOG2R	Jothikumar 2005

Because the synthesis was performed in triplicate, a total of 300 reactions were mixed for each sample plate of RNA isolates. The master mix was prepared in three separate batches and 10µl of master mix was loaded into each well of the synthesis plates via an Eppendorf Repeater Stream pipet. Once the plate was loaded with the master mix, a 10µl volume of each RNA isolate was loaded into its corresponding well, and the filled plated was then sealed using pre-sized sealing tape. Each of these synthesis plates would

only have a third of the isolated RNA of each sample. Each synthesis plate was loaded into a Veriti 96-Well Thermocycler and set to run on a cycle of 10 minutes at 25^0C, 4 hour at 37^0C, and 5 seconds at 55^0C. The Veriti thermocyclers would maintain the sample plates at 4^0C until the procedure was complete. Once the DNA synthesis cycles of all three of the plates had finished, they were removed from the Veriti Thermocycler and unsealed. The triplicate synthesis products of each sample were then pooled into a single well of the cDNA synthesis storage plate. Once all cDNA synthesis products are pooled into a single plate, the plate was labeled the same as the RNA isolates and the plate was sealed with both sealant tape and a hard plastic cover, which was parafilmed in place. The sealed plates were then stored in a -20^0C freezer until the cleanup of cDNA products.

The cDNA synthesis products were then cleaned up using the following procedure on the Qiagen BioRobot using the QIAquick kit and protocols, which is described in detail in the QIAquick® Multiwell PCR Purification Handbook (Qiagen). Once the purified samples were removed from the BioRobot, 7µl of each well was reserved for quantification and quality assurance protocols. The plates were then labeled and sealed and placed in the -20^0C freezer for short term storage.

3.2.4 Quantification of cDNA products

The quantification of cDNA products was based on the same procedure that was explained in subsection B above for quantitative quality assurance of the RNA isolates. The major change in this process was the use of a different buffer in the clean-up of the cDNA synthesis products. The Buffer EB (11mM Tris·Cl, pH 8.5) was use as the blanking agent for the Tecan infinite M200 plate reader. The analysis and data recording of the purified cDNA products were recorded in the same process as detailed above in subsection B above. Additionally, gel electrophoresis was also run

on purified cDNA product to assess fragmentation of the cDNA products; this followed the same protocol and data recording methods as detailed in *3.2.2 RNA Isolation Quality Assurance.*

3.2.5 Taqman® Quantitative PCR

The initial project design was to solely use a quantitative Taqman assay for the detection of Enterovirus and Norovirus genotype 1 and genotype 2. After the initial development of the methods was completed, including the validation of internal standards, a small scale test was done on Doheny beach samples. The results of this initial run proved less than satisfactory and it was determined that a nested PCR technique would be used, based on previous results by Dr. Sobsey's lab group at University of North Carolina that compared quantitative PCR to nested PCR analysis for noroviruses and adenoviruses in concentrated seawater samples from the same beaches. However, it was not possible to rerun all of the Doheny samples again by nested PCR, due to variable use of cDNA products and variable output of the sample extraction and isolation procedure.

A limited run of quantitative PCR was initially performed on the Doheny 2008 samples. The purified cDNA products were allowed to fully thaw while on ice. Each of these samples was run in triplicate with a 20µl reaction volume for a sample total of 60µl. The master mix was prepared to account for this increased sample volume. The master mix composition was 2x Buffer: 1.5 Primer1: 1.5 Primer2: 1.5 Probe: 0.5 sterile DI water, once the total needed volume was known the volume for each component was calculated for each reaction. Each well was loaded with a standardized concentration of cDNA products, for a goal of 5ng/1µl in a 60µl total volume. This was achieved from

consideration of the data collected during the cDNA product quantification protocol in subsection D. An example of the calculation for cDNA products follows, for cDNA product concentration 6.51ng/1µl:

$$C_1V_1 = C_2V_2$$
$$(6.51ng/1\mu L) \times V_1 = (5.0ng/1\mu L) \times (60\mu L)$$
$$(6.51ng/1\mu L) \times V_1 = 300ng$$
$$V_1 = 300ng \times (1\mu L/6.51ng)$$
$$V_1 = 46.08\mu L$$

The above equation shows that 46.08µl of this sample was analyzed. The sample was mixed with 13.92µl of sterile DI water in a total reaction volume of 60µl and a standardized concentration of 5ng/1µl. This resulted in a different volume being processed for each sample. This process was repeated for the positive control and all samples allocated to this 96-well plate. Once all wells had been loaded the plate was sealed with pre-sized sealing tape and loaded in the Applied Biosystems 7500 Fast Real-Time PCR Machine. All samples were loaded in optically clear plastic plates. The plates were run for 35 cycles of 95^0C for 30 seconds, 55^0C for 30 seconds, and 72^0C for 1 minute. Additionally 12 of 65 samples where spiked with known concentrations of salmon sperm DNA as internal control, which showed no major presence of sample inhibition. The detailed results can be found in table A-2 located in Appendix A. Finally, water blanks were used as negative controls to show the lowest level of detection for the run. Any positive results below this level were disregarded. The results for each of the triplicates were examined independently, and then if the results were found to be similar they were averaged for the final concentration shown in Table 3-2 in the Results section.

Table 3-2: q-PCR Primers and Probes for Enterovirus and Norovirus

Viruses	Primers	Probes	References
Enterovirus	ENV-F, ENV-R	EV Probe	Gregory 2006, Afonina 2007
Norovirus GI	JJVIF, JJVIR	Ring1b	Jothikumar 2005
Norovirus GII	JJV2F, COG2R	Ring2	Jothikumar 2005

3.2.6 Nested PCR

In order to improve the sensitivity with which enteric viruses could be detected, a nested PCR protocol was adopted. It was decided to limit this protocol to Enterovirus and Norovirus GII cDNA products in order to limit costs. This procedure required three subsequent stages; the initial PCR stage uses a more general primer set. The second stage products of this initial PCR stage were then used in a second stage of PCR with more specific primer sets. These second sets of primers are nested to amplify the products of the first sets of primers, an approach that increases the sensitivity of detection compared to single stage PCR. The table below details the primers used in this procedure, including the sequence of the primers, the sources for the development of these primers, and the size of the PCR products. The final stage of this procedure was the use of gel electrophoreses to identify samples with the second stage PCR products.

Table 3-3: Primers used in Nested PCR for Enterovirus and Norovirus

Virus type	Amplification Reaction	Primer	Sequence	Product Size (bp)
Enterovirus	First	Ent1	5'-CGGTACCTTTGTACGCCTGT-3'	534
		Ent2	5'-ATTGTCACCATAAGCAGCCA-3	
	Nested	neEnt1	5'-TCCGGCCCCTGAATGCGGCTA-3'	138
		neEnt2	5'-GAAACACGGACACCCAAAGTA-3'	
Norovirus G2	First	MJV 12/13	5' TAY CAY TAT GAT GCH GAY TA 3'	327
		REG A	5' CTC RTC ATC ICC ATA RAA IGA 3'	
	Nested	REG A	5' CTC RTC ATC ICC ATA RAA IGA 3'	317
		MP 290	5' GAY TAC TCY CSI TGG GAY TC 3'	

1. Roberto personal communication

The initial stage of Nested PCR was conducted in single reactions only, with each 96 well plate having both a positive and negative control. The positive controls used for the nested PCR procedure were based on manufactured controls that were known positives for both Enterovirus and Norovirus; these samples were taken through both the

RNA extraction and isolation and cDNA synthesis and cleanup. This was to insure that positive controls would have undergone the same treatment as the samples, which would prevent any potential problem associated with the isolation and extraction process.

The initial PCR reaction used the following master mix for both the enterovirus and the Norovirus genogroup 2 procedures. Each well of the 96-well plates was filled with 20µl of the master mix, with an 8-channel auto pipette to insure even distribution of the master mix among all wells. Once all wells were loaded with the master mix 5 µl of the samples and controls were then loaded into the corresponding wells. Once all wells were loaded with sample and master mix the plate was sealed with sealing tape and loaded into a Veriti 96-well thermocycler.

The following cycle was used for the initial and the nested PCR reaction (Lee et al 2005):
- Stage 1: 95^0C for 4 minutes
- Stage 2: 35 cycles of 95^0C for 30 seconds, 55^0C for 30 seconds, and 72^0C for 1 minute
- Stage 3: 72^0C for 7 minutes
- Stage 4: 4^0C until the plate is removed from the thermocycler

The initial PCR master mix is as follows, listed per reaction:
- 12.5µl of Applied Biosystems AmpliTaq Gold® PCR master mix
- 1 µl of primer 1
- 1 µl of primer 2
- 5.5 µl of autoclaved microbiological grade water

The nested PCR reaction used the following reaction mixture 48 µl of Nested PCR master mix and 2 µl of initial PCR reaction product. The 96-well plate was loaded in the same manner described in the above paragraph for the initial PCR reaction. The master mix used for the nested PCR reaction differed from the master mix used for the initial PCR, with the following listed ingredients per reaction:

-25 µl of Applied Biosystems AmpliTaq Gold® PCR master mix
- 1 µl of primer 1
- 1 µl of primer 2
- 21 µl of autoclaved microbiological grade water

Once the entire plate had been loaded with the master mix, 2 µl volumes of the initial PCR reaction product were loaded into the corresponding wells. The nested PCR reaction used the same thermocycler protocol as did the initial PCR reaction.

The final step of this protocol was the use of gel electrophoresis to separate the amplified DAN products of the PCR reactions. The gels produced during this protocol were imaged using a UV light box and digital camera as described in section B of Methods. The enterovirus nested PCR required the use of a 2% TAE Agar gel, which was made by mixing 4g of Agarose in 200mL of 1x TAE. This mixture was used because the nested PCR products for enterovirus are 137 bp in size. Due to this small size of the PCR products a denser gel needed to be used to separate out the amplified target DAN products from the free nucleotide bases.

3.3 Statistical Analysis

The results of the Taqman® Assay and Nested PCR were examined using chi-square tests to determine if (1) there was any variance between the three sample beaches and their sample stations; and (2) to examine the differences between the positive results for nested PCR and the Taqman® Assay, to determine if one method is more sensitive. All results were recorded in a binary format for the statistical analysis, with results being scoured as positive or negative. The rational for these tests will be described in detail in their representative section, with an explanation of their interpretation. All computerized statistical testing was done using SPSS software, and an alpha value was set at 0.05.

4. Results

The goal of this project was to analyze archived filter concentrates of recreational water samples for enterovirus and norovirus RNA using high throughput molecular techniques for nucleic acid amplification by quantitative RT-PCR and nested RT-PCR. A total of 356 water samples were analyzed, with 69 of the samples positive for entrovirus sby nested PCR methods and 8 sample positive for enterovirus with a Taqman® PCR assay. These tests had a positive percentage of 19.3% for enterovirus positives and 0% for norovirus genogroup I and II. When these results for enterovirus and norovirus RNA detection by nucleic acid amplification are examined by study beaches, Doheny had 22 positive samples (55%) out of 40 test samples, Avalon Beach had 19 positive samples (16%) out of 119 tested samples, and Malibu Beach had 28 positive samples (14.4%) out of 195 tested samples. The rest of this section will detail these virus positive results, including the description of their distribution by sampling location and statistical analysis. The descriptive analysis will examine the distribution of these results on the basis of sampling locations both within the recreational beaches and among the recreational beaches, any potential patterns of these, while the statistical analysis will determine if the relationships reported in the previous sections are informative as by an analysis of their differences in positive occurrence.

4.1 Descriptive Analysis

The primary results for this project are detailed in Table A-1 found in Appendix A, with the results grouped by the nested PCR and Taqman® assay protocols. The results are identified by the TSIndex as provided by SCCWRP, with the sampling date, day,

study beach, sampling station and additional factors. All samples examined in this project were from 8am morning sampling periods. Due to the size of this table this section will only contain a summary table detailing the number distribution of positive samples detected by both Nested PCR and Taqman® Assay methods. Table 4-1 lists the number of positive samples per study beach and the distribution by sampling site per beach for the nested PCR enterovirus test. Table 4-2 details the results of Taqman® Assay for enterovirus, showing both the distribution the quantitative results by sample site.

The Taqman® Assay detected only 8 samples out of a total of 65 samples with any concentrations of Enterovirus cDNA. Furthermore, there was no detection of cDNA for either Norovirus Group1 or Group2 of the same 65 samples. Only one of the positive results for the Taqman® Assay was also positive by Nested PCR, Doheny Beach 2008 sample A8-581. The Nested PCR protocol found a total of the 69 samples with presence of Enterovirus cDNA and no presence of Norovirus Group2 cDNA in any of the samples. Due to the limited availability of isolated RNA product from the Doheny samples, due to the use of the samples for Taqman® Assay, only some samples were analyzed in the Nested PCR protocol (40 of the original 65 samples were available for assay). The samples examined for the Nested PCR were limited to the collection period from 8/1/2008 through 9/14/2008, whereas the samples available for Taqman® assay were collected from 7/4/2008 through 9/14/2008.

Table 4-1: Number of Samples Positive for Enterovirus using Nested PCR by Study Beach and Sample Station

Doheny 2008 (22 out of 40 Total)					Avalon 2009 (19 out of 119 Total)				Malibu 2009 (28 out of 195 Total)				
A	B	C	D	E	A	B	C	D	A	B	C	D	E
6	0	6	6	4	3	6	3	7	7	11	7	1	2

Table 4-2: Distribution of Enterovirus Positivity by Taqman® Assay by Sample Station at Doheny Beach

Doheny 2008 (8 out of 65 Total)				
A	B	C	D	E
3	0	2	2	1

The data presented above show some potential for differences in enterovirus-positivity not only among the sample beaches but also among the sampling sites at each beach. These potential differences are explored in greater detail in the following section. Additionally, the estimated enterovirus concentrations of enterovirus-positive samples in ng/L (SD) at each sample site by Taqman® Assay were as follows: Site A was 40.9(26.7), 194 (70.8), and 197 (20.2); Site C 48.2 (26.6) and 27.5 (4.51); Site D was 44.2 and 31.29 (1.29); and Site E was 40.9. There was no detection of Norovirus Genogroup I or Genogroup II for both Nested PCR and Taqman® Assay. There is no known relationship between these concentrations and virion units, these is merely a measure of PCR product concentration and not a measure of viral density.

4.2 Statistical analysis

The data were examined using chi-square tests to determine if (1) there was any difference the percentage of positive viral detection between the three sample beaches and their sample stations; and (2) to examine the differences between the frequencies of virus-positive results by nested PCR and the Taqman® Assay in these samples. The rationales for these tests will be described in detail in their representative sections, with an explanation of their meanings.

4.2.1 Comparison of virus positivity of samples among Study Beaches

The comparison between the beaches was conducted by examining the differences in the occurrence of sample positivity for Enterovirus Nested PCR results for each beach. Since these results are binary in nature this data can be described as non-parametric and would require the use of s statistical test that would account for the inability to define or

specify a particular frequency distribution of enterovirus-positivity. A chi-square test, with post-hoc analysis, was used to determine the statistical significance in the differences of frequencies of enterovirus positivity within and between the study beaches. This test required the calculation of percentages of positive results of each study beach and their sampling sites as frequencies of enterovirus positivity. This statistical analysis was performed as two different comparisons of frequencies of enterovirus-positivity of samples, the inter-beach comparison and the intra-beach comparison. Both comparisons of frequencies of enterovirus-positivity were conducted with post-hoc analysis if the chi-square test showed a significant difference.

4.2.1.1 Inter-beach Comparison

A chi-square test was conducted on data from the three study beaches to determine if there was a significant difference in frequencies of enterovirus positivity of samples among the beaches. This test is based on the null hypothesis that each beach had the same occurrence of percentage positive samples for entrovirus by Nested PCR and Taqman® Assay. If the null hypothesis was disproven by an α of at least 0.05 the alternative hypothesis that there was a difference in the frequency of virus positivity between beaches would be proven. The chi-square test was conducted by the following formula in Equations 4-1 below. The Figure below (Figure 4-1) shows the method used to calculate the expected values used in the Chi-Square Goodness of Fit test, this is based on the assumption of equal proportions. Additionally Figure 4-2 shows the methods used to calculate the observed percentage of positive results. The results of these calculation and the observed percentages for each study site are presented in the Table 4-3, these values were then used to calculate the χ^2 value in Tables 4-4 and 4-5.

Figure 4-1: Chi-Square test

$$\chi^2 = \sum_{i=1}^{n} \frac{(O_i - E_i)^2}{E_i}$$

Where $O_i = the\ observed\ percentage$

$E_i = the\ expected\ percentage\ based\ on\ an\ uniform\ distribution$

Table 4-3: Study Beach Comparison, Enterovirus Positive and Negative Results for Seawater Samples Analyzed by Nested PCR

	Doheny	Avolan	Malibu	Total
Positive	22	19	28	69
Negative	18	104	167	289
Total	40	123	195	358

Figure 4-2: Calculation of Expected Values, as Percentage of Both Positive and Negative Values for Virus Positivity of Seawater Samples, by Beach

	Doheny	Avalon	Malibu
Positive	$\left[\left(\frac{\left(T_D \times \frac{T_p}{T_t}\right)}{T_D}\right) \times 100\right]$	$\left[\left(\frac{\left(T_A \times \frac{T_p}{T_t}\right)}{T_A}\right) \times 100\right]$	$\left[\left(\frac{\left(T_M \times \frac{T_p}{T_t}\right)}{T_M}\right) \times 100\right]$
Negative	$\left[\left(\frac{\left(T_D \times \frac{T_n}{T_t}\right)}{T_D}\right) \times 100\right]$	$\left[\left(\frac{\left(T_A \times \frac{T_n}{T_t}\right)}{T_A}\right) \times 100\right]$	$\left[\left(\frac{\left(T_M \times \frac{T_n}{T_t}\right)}{T_M}\right) \times 100\right]$

Where T_t= Is the total number samples

T_p= Is the total number of positive samples

T_n = Is the total number of negative samples

T_D = Is the total number of Doheny samples

T_A = Is the total number of Avolan samples

T_M = Is the total number of Malibu samples

Figure 4-3: Calculation of Observed Percentages of Virus Positivity of Seawater Samples, by Beach

	Doheny	Avolan	Malibu
Positive	$\left[\left(\frac{D_p}{T_D}\right) \times 100\right]$	$\left[\left(\frac{A_p}{T_D}\right) \times 100\right]$	$\left[\left(\frac{M_p}{T_D}\right) \times 100\right]$
Negative	$\left[\left(\frac{D_n}{T_D}\right) \times 100\right]$	$\left[\left(\frac{A_n}{T_D}\right) \times 100\right]$	$\left[\left(\frac{M_n}{T_D}\right) \times 100\right]$

Where T_D = Is the total number of Doheny Samples

D_p = Is the number of positive Doheny Samples

D_n = Is the number of negative Doheny Samples

T_A = Is the total number of Avolan Samples

A_p = Is the number of positive Avolan Samples

A_n = Is the number of negative Avolan Samples

T_M = Is the total number of Malibu Samples

M_p = Is the number of positive Malibu Samples

M_n = Is the number of negative Malibu Samples

Table 4-4: Percentages of Observed and Expected Virus Positive Seawater Samples, by Beach

	Doheny	Avolan	Malibu
Observed	55.00 %	15.45%	14.36%
Expected	19.27 %	19.27%	19.27%

Based on the above formula and tables the chi-square value was calculated to be: $X^2(2, N=358) = 36.999, p=1*10^{-8}$

This result demonstrates that there is a statistically significant difference among the observed frequency of occurrence of enteroviruses in seawater by study beach. The source of the differences in the occurrence of enterovirus sample positivity among the three study beaches was determined through the use of Crosstabs analysis of SPSS software. Crosstabs analysis was set up to perform a chi-square goodness of fit comparison among the three study beaches. Additionally this analysis was set up to perform standard post-hoc tests for chi-square, as the determination of residuals for each of the beaches. Residuals are a measure of how much the observed percentage differs from the expected values, and these values are assigned signs which represent how (in what direction) they differ from the expected values. Table 4-5 below shows the results of this analysis.

Table 4-5: Post-Hoc Analysis of Inter-beach Comparison for Enterovirus Positivity

			Nested PCR		Total
			negative for entrovirus	positive for entrovirus	
Beach	Avalon	Count	104_a	19_a	123
		Std. Residual	.5	-1.0	
	Doheny	Count	18_a	22_b	40
		Std. Residual	-2.5	5.1	
	Malibu	Count	167_a	28_b	195
		Std. Residual	.8	-1.6	
Total		Count	289	69	358

Each subscript letter denotes a subset of Nested PCR categories whose column proportions do not differ significantly from each other at the 0.05 level.

The above table shows that Doheny and Malibu Beach are different from Avalon Beach on the basis of frequency of enterovirus positivity of seawater samples, but not from each other. These results suggest the potential for differences in frequencies of enterovirus positivity among beaches.

4.2.1.2 Intra-beach Comparison

The second stage of this assessment was the examination of the differences in frequencies of sample enterovirus positivity among different sample stations within each of the study beaches. This was done by comparing the frequency of enterovirus positive results across sample transects within each study beach. This analysis required the same process of statistical as described above for the separate data of each of the three different beaches. The observed and expected percentages of positive Nested PCR for Enteroviruses in seawater samples was calculated using the SPSS Crosstabs analysis with Chi-Square and configured to calculate standardize residuals. Each beach was analyzed individually, and the results of the chi-square tests and residuals comparisons are shown in Tables 4-6 through 4-11.

Table 4-6: Post-Hoc Analysis of Enterovirus Positive and Negative Frequencies in Samples for Doheny Beach, by Sample Station

			Nested PCR		Total
			negative for entrovirus	positive for entrovirus	
Station	A	Count	2_a	6_a	8
		Std. Residual	-.8	.8	
	B	Count	8_a	0_b	8
		Std. Residual	2.3	-2.1	
	C	Count	2_a	6_a	8
		Std. Residual	-.8	.8	
	D	Count	2_a	6_a	8
		Std. Residual	-.8	.8	
	E	Count	4_a	4_a	8
		Std. Residual	.2	-.2	
Total		Count	18	22	40

Each subscript letter denotes a subset of Nested PCR categories whose column proportions do not differ significantly from each other at the 0.05 level.

Table 4-7: Chi-square Results for Doheny Intra-Beach Comparison of Positive Samples for Enteroviruses, by Sample Station

	Value	Df	Asymp. Sig. (2-sided)
Pearson Chi-Square	13.737[a]	4	.008
Likelihood Ratio	16.969	4	.002
N of Valid Cases	40		

a. 10 cells (100.0%) have expected count less than 5. The minimum expected count is 3.60.

The above two tables show that there are significant differences in sample enterovirus positivity among the sampling locations (stations) at Doheny beach. The comparison of residual for each of the sampling locations showed that only station B stands out as being different in enterovirus positivity from the other stations and is the source of differences detected in the chi-square results.

Table 4-8: Post-Hoc Analysis of Enterovirus Positive and Negative Frequencies in Samples for Intra-Beach Comparison at Avalon Beach, by Sample Station

			Nested PCR		Total
			negative for entrovirus	positive for entrovirus	
Station	A	Count	27_a	3_a	30
		Std. Residual	.4	-.8	
	B	Count	23_a	6_a	29
		Std. Residual	-.3	.6	
	C	Count	27_a	3_a	30
		Std. Residual	.4	-.8	
	D	Count	23_a	7_a	30
		Std. Residual	-.4	1.0	
Total		Count	100	19	119

Each subscript letter denotes a subset of Nested PCR categories whose column proportions do not differ significantly from each other at the .05 level.

Table 4-9: Chi-Square Results for Intra-Beach Comparison of Frequencies of Sample Positivity for Enteroviruses, by Sample Station at Avalon Beach

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	3.288[a]	3	.349
Likelihood Ratio	3.333	3	.343
N of Valid Cases	119		

a. 4 cells (50.0%) have expected count less than 5. The minimum expected count is 4.63.

The above two tables show that there was no significant difference in frequencies of enterovirus positive seawater samples among the different sampling locations at Avalon Beach.

Table 4-10: Post-Hoc Analysis of Enterovirus Positive and Negative Frequencies in Samples for Intra-Beach Comparison for Malibu Beach, by Sample Station

			Nested PCR		Total
			negative for entrovirus	positive for entrovirus	
Station	A	Count	32_a	7_a	39
		Std. Residual	-.2	.6	
	B	Count	28_a	11_b	39
		Std. Residual	-.9	2.3	
	C	Count	32_a	7_a	39
		Std. Residual	-.2	.6	
	D	Count	38_a	1_b	39
		Std. Residual	.8	-1.9	
	E	Count	37_a	2_a	39
		Std. Residual	.6	-1.5	
Total		Count	167	28	195

Each subscript letter denotes a subset of Nested PCR categories whose column proportions do not differ significantly from each other at the .05 level.

Table 4-11: Chi-Square Results for Intra-Beach Comparison of Frequencies of Sample Positivity for Enteroviruses, by Sample Station at Malibu Beach

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	14.012[a]	4	.007
Likelihood Ratio	15.561	4	.004
N of Valid Cases	195		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 5.60.

The above two tables show that there are significant differences among the frequencies of enterovirus positivity for sampling stations of Malibu Beach, with X^2=14.012. An examination of residual values for the sampling stations at Malibu Beach shows that the source of these differences is station B and D, with station B having more positives than the other stations of the beach and D having fewer positives than the other stations of the beach.

4.2.2 Comparison between Taqman® and Nested PCR Results

Another major comparison of this project was the determination of differences in beach water sample positivity for enteroviruses between the Taqman® and Nested PCR tests. This analysis was just limited to samples only from Doheny Beach 2008. This limitation was due to a lack of availability of extracted and isolated cDNA samples, as the Taqman® assay required a variable but often relatively large amount of the isolated cDNA product as part of the quantitative PCR protocol. Because of this sample availability problem, only 48 of the 75 samples processed in the Taqman® PCR assay were tested in the nested PCR protocol. Table 4-15 below details the difference between the Taqman® and Nested PCR protocol results for enterovirus positivity of beach water samples.

To test the differences between the results of the Taqman® and Nested PCR protocols a McNemar's test was used, since the samples are paired non-parametric, binary data. The test uses a 2 × 2 contingency table with a dichotomous trait (enterovirus positivity of water samples), with matched pairs of subjects (Taqman analysis and Nested PCR analysis), to determine whether the row and column marginal frequencies are equal. This test was used to compare the number of sample enterovirus positive results from both protocols, while accounting for the different number of tested samples. The test statistic is like the chi-square distribution with 1 degree of freedom. Figure 4-3 below details the formula f the statistic and test construction and the associated table 4-15 details the data used in the test. The McNemar's test assumes a Null Hypothesis where the count in cells b and c, table 4-14 below, will approximate each other. Cells b and c refer the cases were the results of the test do not agree between the two test. A significant

deviation between these two values will result in a large χ^2 value above 3.841, as a scientifically significant finding at α of 0.05.

Figure 4-4: Formula for McNemar's Test

$$\chi^2 = \frac{(b-c)^2}{b+c}$$

Table4-12: McNemar's Test for Comparison between Nested PCR and Taqman® Assay for Frequency of Enterovirus Positivity and Negativity of Beach Water Samples

	Nested PCR Positive	Nested PCR Negative	Row Total
Taqman® Positive	1	1	2
Taqman® Negative	21	17	38
Column Total	22	18	*40*

McNemar's test result is $\chi^2(1, N=40) = 18.2, p<0.05$

The results of the above McNemar's test provides statistical that there is significant difference between the results for frequency of enterovirus positivity of the Nested PCR and Taqman® assays. Based on the greater amount of Nested PCR enterovirus positive results it would appear that Nested PCR is better at detecting enterovirus in these seawater samples than is the Taqman® assay.

4.3 Summary of Results

The results of this study show that there is a difference between the occurrence of positive enterovirus results with nested PCR by Beach Study Site and Study Site Stations for Enterovirus, there was no detection of either Norovirus Genogroup. The above data would suggest that Doheny Beach had a greater occurrence of positive tests than the other two study sites, with Doheny Beach having 55% positive results which was significantly above the observed percentage for both Avalon Beach (15.45% positive) and Malibu Beach (14.36% positive).

The analysis of difference within each study beach by sample transect (station) found that both Doheny and Malibu Beach had a significant differences in the percentage of positive enterovirus results among the sample transects (stations). The source of

significant difference at Doheny Beach was sample site D, and the source of significant difference at Malibu Beach was from both sample sites B and D.

In addition, the results from the analysis of frequencies of enterovirus positive marine beach water samples by McNemar's Test showed that there was a significant difference between the Taqman® assay and Nested PCR methods. Only one of the samples enterovirus positive by Nested PCR assay was also enterovirus positive by Taqman® assay. This agreement in enterovirus positivity by both assays was for sample ID A8-581. However 6 of the positive samples by Taqman® were not analyzed by the Nested PCR method, which limits the potential comparison between the two assay methods. Even when accounting for this potential lack of analysis by both methods for some samples, it would appear that there is a potential for Nested PCR method to be more sensitive in the detection of enterovirus than the Taqman® assay. This is based on the observed statistical difference between the two methods shown in sub section *4.2.2*.

5. Discussion

Results of this study demonstrate that sensitive, high through-put molecular techniques for nucleic acid extraction and amplification can be successfully applied to detect enteric viruses from concentrated samples of recreational waters. The detection of enterovirus varied among the three different Southern California beaches analyzed, which was also observed with two of beach's transect (sample station along the beach shoreline). An additional major finding of this study is that a nested PCR protocol proved more sensitive than use of a quantitative Taqman® assay in the detection of enterovirus in these environmental coastal marine seawater samples.

The samples analyzed for this study were collected as part of a larger environmental epidemiological study designed to provide data on microbial occurrence in bathing water and health effects in bathers to aid in the setting of new water quality guidelines as exposure guidelines for recreational marine waters. Doheny Beach was found to yield significantly greater detection of enterovirus than the other study beaches. An initial epidemiological review of that data from the larger SCCWRP study found an increase in human health effects among recreational swimmers at Doheny Beach. The measured effects were not correlated to traditional FIB methods (John Griffith, SCCWRP, personal communication) and viruses may have been causative agent of the illnesses reported. The findings of this study will be used in a combined epidemiological health effects and microbial water quality analysis to determine if exposure to enterovirus in bathing water was associated with the occurrence of adverse health effects. If so, enterovirus could be considered as an alternative indicator of microbial water quality and

exposure risk for the management of marine recreational waters. Additionally based on the findings of this project norovirus would not be recommended as alternative indicator.

This study also documented significant difference of enterovirus positive results among transects of sample stations at Doheny and Malibu Beaches. These differences in sample enterovirus positivity across transects at these beaches suggest that virus occurrence can be highly variable across space at a single beach. This would require that special attention be paid to the magnitude of virus positivity by location of sampling sites for long term monitoring plans, and may even require an initial sanitary and microbial water quality assessment study to determine which sampling locations would be the most representative of the sources of exposure risk for the recreational site in question. Additionally, the differences in frequencies of sample positivity for enteroviruses among different stations at the beach level may prove to limit the effectiveness of using a single grab sampling scheme for monitoring beaches for water and waterborne pathogen exposure risk.

Another major finding of this study was the difference between the detection of Enterovirus in Doheny beach samples with the Nested PCR and Taqman® assays. This study found a statically significant difference between the results for enterovirus positivity using these two assays, with Nested PCR detecting more enterovirus positive samples than the Taqman® Assay. The comparison of these two different tests only had one overlapping positive sample, with the remainder of the samples testing positive with only one of the two methods, usually the nest PCR method.

Multiple comparison studies between real-time, quantitative PCR and Nested PCR have been conducted across different applications to the analysis of the microbial

quality of different clinical and environmental samples. A study comparing these two methods in their ability to detect *Mycobacterium avium* subsp. *paratuberculosis* in Bovine Fecal Samples found no statistically significant differences, though the overlap of positive and negative results was not perfect (Fang et al 2002). Another study comparing the ability of Nested PCR and real-time PCR to detect Herpes simplex virus type 1 (HSV-1), Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) from serum samples of HIV patents, found comparable sensitivity and specificity for all three viruses with only two false positives (Drago et al 2004). Additional studies have also found the real-time PCR methods will detect positive samples that were also detected by Nested PCR, for Herpes simplex virus from patents suspected of having neonatal HSV infections and HSV encephalitis (Kawada 2004). These reports support the findings of other research on the comparison of Taqman® Assay and Nested PCR methods for the detection entrovirus in clinical samples of infected persons, which also found a comparable sensitivity and specificity for virus detection (Watkins-Riedal et al 2002). However, these pervious clinical study reports are in direct conflict with the findings of this study, probably owing to the differences in virus levels in clinical and environmental samples (with higher virus levels in clinical than in environmental samples) and the difficulty in analyzing complex seawater samples for enteric viruses due to the PCR inhibitory effects of constituents in the sample matrix. These difficulties in analyzing complex seawater samples has been supported by recent research in the detection of enteric virus in seawater samples from similar study sites (Rodriguez and Thie et al 2012). This paper supports the findings of this project and also support the suggested for future research that is needed.

The findings of this study suggest that Taqman® qPCR assays can under-detect the actual presence of enterovirus in environmental samples, as documented by more frequent detection of enterovirus positive samples by nested PCR analysis of the same samples. This could be a major problem for reliable monitoring of recreational waters resulting in a systematic under-detection of enteric virus in samples. False negative measurements could prevent needed public health measures from being undertaken when they would appear to have been needed, due to the evidence for virus presence by a more effective detection method.

With improved methods, molecular detection of enteric viruses may allow implementation of enteric virus monitoring programs. While the nested PCR methods are not directly quantitative like the Taqman® assay and other quantitative real-time PCR methods, nested PCR could still provide a sensitive, reliable method for the detection of enteric viruses in less than 24 hours. Nested PCR can also be made more quantitative by analyzing different quantities of sample per reactions to detect the endpoint of sample CR positivity (an extinction dilution assay format). Additionally, there is the potential through procedural optimization and automation to reduce the turnaround time of this method to hours. Finally, nested PCR, or a different optimized molecular protocol, could be adapted for not only enteroviruses but for other pathogens as well, allowing direct detection of other important pathogens in waters from recreational beaches.

6. Future Research

There are two major avenues for further research for this project. The first path is the more in-depth exploration of the environmental variability in the occurrence of enteric viruses, including enterovirus in seawater bathing samples of different beaches having different risks of enteric pathogen contamination. The effective implementation of enteric virus monitoring programs would require greater understanding on how concentrations can differ in space and vary over time in environmental samples and if these variations are site specific or if some kind of pattern can be established that would allow for predictive modeling or development of generalizable monitoring plans.

The findings of this study regarding the potential under detection of entrovirus by Taqman® Assay is not supported by the literature, but does beg the very important question of the validity of real-time PCR effectiveness as a monitoring tool for environmental water samples. Based on the findings of this study it would appear that there is need for additional studies comparing both the sensitivity and specificity of qPCR methods in the detection of enteroviruses and other enteric viruses in environmental samples to that of other methods, such as Nested PCR. These studies should focus on testing simulated as well as real environmental samples with known concentrations of viruses spiked into natural water samples.

7. Conclusions

Enteric viruses in marine waters are detectable using molecular techniques, such as the nested PCR methods described in this thesis. These methods would be useful in the management of recreational for the prevention of enteric virus related diseases, although these methods would require additional work as described in this thesis to determine safe exposure thresholds and the development of sampling protocols for the recreational site being monitored. These findings show that PCR techniques can be a useful tool in recreational management with the additional research laid out in this thesis.

Appendix

Table A-1: Total PCR Results

TSIndex	Date	Day	Beach	Station	Description	Time Point	Sample ID	Nested PCR (1=occurrence)			Taqman Assay ng/ml (SD)		
								Entrovirus	Norovirus G1	Norovirus G2	Entrovirus	Norovirus G1	Norovirus G2
230	7/4/2008	Friday	Doheny	A	Jetty	8am	A8-230		N/A		40.89 (26.73)		
232	7/4/2008	Friday	Doheny	C	Pond	8am	C8-232		N/A		48.19 (26.59)		
233	7/4/2008	Friday	Doheny	D	S Beach1	8am	D8-233		N/A		44.23		
234	7/4/2008	Friday	Doheny	E	S Beach2	8am	E8-234		N/A		40.86		
298	7/19/2008	Saturday	Doheny	D	S Beach1	8am	D8-298		N/A		31.29 (1.29)		
310	7/20/2008	Sunday	Doheny	C	Pond	8am	C8-310		N/A		27.53 (4.51)		
311	7/20/2008	Sunday	Doheny	D	S Beach1	8am	D8-311		N/A				
312	7/20/2008	Sunday	Doheny	E	S Beach2	8am	E8-312		N/A				
321	7/26/2008	Saturday	Doheny	A	Jetty	8am	A8-321		N/A				
322	7/26/2008	Saturday	Doheny	B	N Beach	8am	B8-322		N/A				
323	7/26/2008	Saturday	Doheny	C	Pond	8am	C8-323		N/A				
324	7/26/2008	Saturday	Doheny	D	S Beach1	8am	D8-324		N/A				
325	7/26/2008	Saturday	Doheny	E	S Beach2	8am	E8-325		N/A				
334	7/27/2008	Sunday	Doheny	A	Jetty	8am	A8-334		N/A				
335	7/27/2008	Sunday	Doheny	B	N Beach	8am	B8-335		N/A				
336	7/27/2008	Sunday	Doheny	C	Pond	8am	C8-336		N/A				
337	7/27/2008	Sunday	Doheny	D	S Beach1	8am	D8-337		N/A				
338	7/27/2008	Sunday	Doheny	E	S Beach2	8am	E8-338		N/A				
347	8/2/2008	Saturday	Doheny	A	Jetty	8am	A8-347		N/A				
348	8/2/2008	Saturday	Doheny	B	N Beach	8am	B8-348		N/A				
349	8/2/2008	Saturday	Doheny	C	Pond	8am	C8-349	1	N/A				
350	8/2/2008	Saturday	Doheny	D	S Beach1	8am	D8-350	1	N/A				
351	8/2/2008	Saturday	Doheny	E	S Beach2	8am	E8-351	1	N/A				
360	8/3/2008	Sunday	Doheny	A	Jetty	8am	A8-360	1	N/A				
362	8/3/2008	Sunday	Doheny	C	Pond	8am	C8-362	1	N/A				
363	8/3/2008	Sunday	Doheny	D	S Beach1	8am	D8-363	1	N/A				
3	8/3/2008	Sunday	Doheny	E	S Beach2	8am	E8-364	1	N/A				
373	8/9/2008	Saturday	Doheny	A	Jetty	8am	A8-373		N/A				

374	8/9/2008	Saturday	Doheny	B	N Beach	8am	B8-374		N/A	
375	8/9/2008	Saturday	Doheny	C	Pond	8am	C8-375		N/A	
376	8/9/2008	Saturday	Doheny	D	S Beach1	8am	D8-376		N/A	
377	8/9/2008	Saturday	Doheny	E	S Beach2	8am	E8-377		N/A	
386	8/10/2008	Sunday	Doheny	A	Jetty	8am	A8-386		N/A	
387	8/10/2008	Sunday	Doheny	B	N Beach	8am	B8-387		N/A	
388	8/10/2008	Sunday	Doheny	C	Pond	8am	C8-388		N/A	
389	8/10/2008	Sunday	Doheny	D	S Beach1	8am	D8-389		N/A	
390	8/10/2008	Sunday	Doheny	E	S Beach2	8am	E8-390		N/A	
399	8/16/2008	Saturday	Doheny	A	Jetty	8am	A8-399		N/A	
400	8/16/2008	Saturday	Doheny	B	N Beach	8am	B8-400		N/A	
401	8/16/2008	Saturday	Doheny	C	Pond	8am	C8-401		N/A	
402	8/16/2008	Saturday	Doheny	D	S Beach1	8am	D8-402		N/A	
403	8/16/2008	Saturday	Doheny	E	S Beach2	8am	E8-403		N/A	
412	8/17/2008	Sunday	Doheny	A	Jetty	8am	A8-412		N/A	
413	8/17/2008	Sunday	Doheny	B	N Beach	8am	B8-413		N/A	
414	8/17/2008	Sunday	Doheny	C	Pond	8am	C8-414		N/A	
415	8/17/2008	Sunday	Doheny	D	S Beach1	8am	D8-415		N/A	
416	8/17/2008	Sunday	Doheny	E	S Beach2	8am	E8-416		N/A	
425	8/23/2008	Saturday	Doheny	A	Jetty	8am	A8-425		N/A	
426	8/23/2008	Saturday	Doheny	B	N Beach	8am	B8-426		N/A	
427	8/23/2008	Saturday	Doheny	C	Pond	8am	C8-427		N/A	
428	8/23/2008	Saturday	Doheny	D	S Beach1	8am	D8-428		N/A	
429	8/23/2008	Saturday	Doheny	E	S Beach2	8am	E8-429		N/A	
438	8/24/2008	Sunday	Doheny	A	Jetty	8am	A8-438		N/A	
439	8/24/2008	Sunday	Doheny	B	N Beach	8am	B8-439		N/A	
440	8/24/2008	Sunday	Doheny	C	Pond	8am	C8-440		N/A	
441	8/24/2008	Sunday	Doheny	D	S Beach1	8am	D8-441		N/A	
442	8/24/2008	Sunday	Doheny	E	S Beach2	8am	E8-442		N/A	
451	8/30/2008	Saturday	Doheny	A	Jetty	8am	A8-451	1	N/A	
452	8/30/2008	Saturday	Doheny	B	N Beach	8am	B8-452		N/A	
453	8/30/2008	Saturday	Doheny	C	Pond	8am	C8-453		N/A	
454	8/30/2008	Saturday	Doheny	D	S Beach1	8am	D8-454	1	N/A	
464	8/31/2008	Sunday	Doheny	A	Jetty	8am	A8-464	1	N/A	
466	8/31/2008	Sunday	Doheny	C	Pond	8am	C8-466	1	N/A	
467	8/31/2008	Sunday	Doheny	D	S Beach1	8am	D8-467	1	N/A	
468	8/31/2008	Sunday	Doheny	E	S Beach2	8am	E8-468		N/A	
477	9/1/2008	Monday	Doheny	A	Jetty	8am	A8-477		N/A	193.36 (70.80
478	9/1/2008	Monday	Doheny	B	N Beach	8am	B8-478		N/A	
479	9/1/2008	Monday	Doheny	C	Pond	8am	C8-479	1	N/A	
480	9/1/2008	Monday	Doheny	D	S Beach1	8am	D8-480		N/A	
481	9/1/2008	Monday	Doheny	E	S Beach2	8am	E8-481		N/A	
490			Doheny	A	Jetty	8am	A8-490		N/A	

491			Doheny	B	N Beach	8am	B8-491		N/A	
492			Doheny	C	Pond	8am	C8-492		N/A	
493			Doheny	D	S Beach1	8am	D8-493		N/A	
494			Doheny	E	S Beach2	8am	E8-494		N/A	
503			Doheny	A	Jetty	8am	A8-503		N/A	
504			Doheny	B	N Beach	8am	B8-504		N/A	
505			Doheny	C	Pond	8am	C8-505		N/A	
506			Doheny	D	S Beach1	8am	D8-506		N/A	
507			Doheny	E	S Beach2	8am	E8-507		N/A	
516			Doheny	A	Jetty	8am	A8-516		N/A	
517			Doheny	B	N Beach	8am	B8-517		N/A	
518			Doheny	C	Pond	8am	C8-518		N/A	
519			Doheny	D	S Beach1	8am	D8-519		N/A	
520			Doheny	E	S Beach2	8am	E8-520		N/A	
529			Doheny	A	Jetty	8am	A8-529		N/A	
530			Doheny	B	N Beach	8am	B8-530		N/A	
531			Doheny	C	Pond	8am	C8-531		N/A	
532			Doheny	D	S Beach1	8am	D8-532		N/A	
533			Doheny	E	S Beach2	8am	E8-533		N/A	
542			Doheny	A	Jetty	8am	A8-542	1	N/A	
543			Doheny	B	N Beach	8am	B8-543		N/A	
544			Doheny	C	Pond	8am	C8-544	1	N/A	
545			Doheny	D	S Beach1	8am	D8-545		N/A	
546			Doheny	E	S Beach2	8am	E8-546		N/A	
555			Doheny	A	Jetty	8am	A8-555		N/A	
556			Doheny	B	N Beach	8am	B8-556		N/A	
557			Doheny	C	Pond	8am	C8-557		N/A	
558			Doheny	D	S Beach1	8am	D8-558		N/A	
559			Doheny	E	S Beach2	8am	E8-559		N/A	
568			Doheny	A	Jetty	8am	A8-568		N/A	
569			Doheny	B	N Beach	8am	B8-569		N/A	
570			Doheny	C	Pond	8am	C8-570		N/A	
571			Doheny	D	S Beach1	8am	D8-571		N/A	
572			Doheny	E	S Beach2	8am	E8-572		N/A	
581	9/13/08	Sunday	Doheny	A	Jetty	8am	A8-581	1	N/A	196.64 (20.24)
582	9/13/08	Sunday	Doheny	B	N Beach	8am	B8-582		N/A	
583	9/13/08	Sunday	Doheny	C	Pond	8am	C8-583		N/A	
584	9/13/08	Sunday	Doheny	D	S Beach1	8am	D8-584	1	N/A	
585	9/13/08	Sunday	Doheny	E	S Beach2	8am	E8-585	1	N/A	
594	9/14/08	Monday	Doheny	A	Jetty	8am	A8-594	1	N/A	
595	9/14/08	Monday	Doheny	B	N Beach	8am	B8-595		N/A	
596	9/14/08	Monday	Doheny	C	Pond	8am	C8-596	1	N/A	

597	9/14/08	Monday	Doheny	D	S Beach1	8am	D8-597	1	N/A			
598	9/14/08	Monday	Doheny	E	S Beach2	8am	E8-598	1	N/A			
	6/26/2008		Avolan	A		8am	T1		N/A	N/A	N/A	N/A
	6/26/2008		Avolan	B		8am	T2		N/A	N/A	N/A	N/A
	6/26/2008		Avolan	C		8am	T3		N/A	N/A	N/A	N/A
	6/26/2008		Avolan	D		8am	T4		N/A	N/A	N/A	N/A
	6/27/2008		Avolan	A		8am	325		N/A	N/A	N/A	N/A
	6/27/2008		Avolan	B		8am	326		N/A	N/A	N/A	N/A
	6/27/2008		Avolan	C		8am	327		N/A	N/A	N/A	N/A
	6/27/2008		Avolan	D		8am	328		N/A	N/A	N/A	N/A
	6/28/2008		Avolan	A		8am	345		N/A	N/A	N/A	N/A
	6/28/2008		Avolan	B		8am	346		N/A	N/A	N/A	N/A
	6/28/2008		Avolan	C		8am	347		N/A	N/A	N/A	N/A
	6/28/2008		Avolan	D		8am	348		N/A	N/A	N/A	N/A
	6/29/2008		Avolan	A		8am			N/A	N/A	N/A	N/A
	6/29/2008		Avolan	B		8am			N/A	N/A	N/A	N/A
	6/29/2008		Avolan	C		8am			N/A	N/A	N/A	N/A
	6/29/2008		Avolan	D		8am			N/A	N/A	N/A	N/A
	7/4/2008		Avolan	A		8am	355		N/A	N/A	N/A	N/A
	7/4/2008		Avolan	B		8am	356		N/A	N/A	N/A	N/A
	7/4/2008		Avolan	C		8am	357		N/A	N/A	N/A	N/A
	7/4/2008		Avolan	D		8am	358		N/A	N/A	N/A	N/A
	7/5/2008		Avolan	A		8am	365		N/A	N/A	N/A	N/A
	7/5/2008		Avolan	B		8am	366		N/A	N/A	N/A	N/A
	7/5/2008		Avolan	C		8am	367		N/A	N/A	N/A	N/A
	7/5/2008		Avolan	D		8am	368		N/A	N/A	N/A	N/A
	7/10/2008		Avolan	A		8am	375		N/A	N/A	N/A	N/A
	7/10/2008		Avolan	B		8am	376		N/A	N/A	N/A	N/A
	7/10/2008		Avolan	C		8am	377		N/A	N/A	N/A	N/A
	7/10/2008		Avolan	D		8am	378		N/A	N/A	N/A	N/A
	7/11/2008		Avolan	A		8am	385		N/A	N/A	N/A	N/A
	7/11/2008		Avolan	B		8am	386		N/A	N/A	N/A	N/A
	7/11/2008		Avolan	C		8am	387		N/A	N/A	N/A	N/A
	7/11/2008		Avolan	D		8am	388		N/A	N/A	N/A	N/A
	7/12/2008		Avolan	A		8am	395		N/A	N/A	N/A	N/A
	7/12/2008		Avolan	B		8am	396		N/A	N/A	N/A	N/A
	7/12/2008		Avolan	C		8am	397		N/A	N/A	N/A	N/A
	7/12/2008		Avolan	D		8am	398		N/A	N/A	N/A	N/A
	7/13/2008		Avolan	A		8am	405		N/A	N/A	N/A	N/A
	7/13/2008		Avolan	B		8am	406	1	N/A	N/A	N/A	N/A
	7/13/2008		Avolan	C		8am	407	1	N/A	N/A	N/A	N/A
	7/13/2008		Avolan	D		8am	408	1	N/A	N/A	N/A	N/A
	7/18/2008		Avolan	A		8am	415		N/A	N/A	N/A	N/A

7/18/2008	Avolan	B	8am	416		N/A	N/A	N/A	N/A
7/18/2008	Avolan	C	8am	417		N/A	N/A	N/A	N/A
7/18/2008	Avolan	D	8am	418	1	N/A	N/A	N/A	N/A
7/19/2008	Avolan	A	8am	425	1	N/A	N/A	N/A	N/A
7/19/2008	Avolan	B	8am	426		N/A	N/A	N/A	N/A
7/19/2008	Avolan	C	8am	427		N/A	N/A	N/A	N/A
7/19/2008	Avolan	D	8am	428	1	N/A	N/A	N/A	N/A
7/25/2008	Avolan	A	8am	445		N/A	N/A	N/A	N/A
7/25/2008	Avolan	B	8am	446		N/A	N/A	N/A	N/A
7/25/2008	Avolan	C	8am	447		N/A	N/A	N/A	N/A
7/25/2008	Avolan	D	8am	448		N/A	N/A	N/A	N/A
7/26/2008	Avolan	A	8am	455	1	N/A	N/A	N/A	N/A
7/26/2008	Avolan	B	8am	456	1	N/A	N/A	N/A	N/A
7/26/2008	Avolan	C	8am	457		N/A	N/A	N/A	N/A
7/26/2008	Avolan	D	8am	458		N/A	N/A	N/A	N/A
7/27/2008	Avolan	A	8am	465		N/A	N/A	N/A	N/A
7/27/2008	Avolan	B	8am	466		N/A	N/A	N/A	N/A
7/27/2008	Avolan	C	8am	467		N/A	N/A	N/A	N/A
7/27/2008	Avolan	D	8am	468	1	N/A	N/A	N/A	N/A
8/1/2008	Avolan	A	8am	475		N/A	N/A	N/A	N/A
8/1/2008	Avolan	B	8am	476		N/A	N/A	N/A	N/A
8/1/2008	Avolan	C	8am	477	1	N/A	N/A	N/A	N/A
8/1/2008	Avolan	D	8am	478		N/A	N/A	N/A	N/A
8/2/2008	Avolan	A	8am	485		N/A	N/A	N/A	N/A
8/2/2008	Avolan	B	8am	486	1	N/A	N/A	N/A	N/A
8/2/2008	Avolan	C	8am	487		N/A	N/A	N/A	N/A
8/2/2008	Avolan	D	8am	488		N/A	N/A	N/A	N/A
8/3/2008	Avolan	A	8am	495		N/A	N/A	N/A	N/A
8/3/2008	Avolan	B	8am	496		N/A	N/A	N/A	N/A
8/3/2008	Avolan	C	8am	497		N/A	N/A	N/A	N/A
8/3/2008	Avolan	D	8am	498	1	N/A	N/A	N/A	N/A
8/8/2008	Avolan	A	8am	505		N/A	N/A	N/A	N/A
8/8/2008	Avolan	B	8am	506	1	N/A	N/A	N/A	N/A
8/8/2008	Avolan	C	8am	507		N/A	N/A	N/A	N/A
8/8/2008	Avolan	D	8am	508		N/A	N/A	N/A	N/A
8/9/2008	Avolan	A	8am	515		N/A	N/A	N/A	N/A
8/9/2008	Avolan	B	8am	516	1	N/A	N/A	N/A	N/A
8/9/2008	Avolan	C	8am	517		N/A	N/A	N/A	N/A
8/9/2008	Avolan	D	8am	518		N/A	N/A	N/A	N/A
8/10/2008	Avolan	A	8am	525	1	N/A	N/A	N/A	N/A
8/10/2008	Avolan	B	8am	526	1	N/A	N/A	N/A	N/A
8/10/2008	Avolan	C	8am	527		N/A	N/A	N/A	N/A
8/10/2008	Avolan	D	8am	528		N/A	N/A	N/A	N/A

8/15/2008	Avolan	A	8am	535		N/A	N/A	N/A	N/A
8/15/2008	Avolan	B	8am	536		N/A	N/A	N/A	N/A
8/15/2008	Avolan	C	8am	537		N/A	N/A	N/A	N/A
8/15/2008	Avolan	D	8am	538		N/A	N/A	N/A	N/A
8/16/2008	Avolan	A	8am	545		N/A	N/A	N/A	N/A
8/16/2008	Avolan	B	8am	546		N/A	N/A	N/A	N/A
8/16/2008	Avolan	C	8am	547		N/A	N/A	N/A	N/A
8/16/2008	Avolan	D	8am	548	1	N/A	N/A	N/A	N/A
8/17/2008	Avolan	A	8am	555		N/A	N/A	N/A	N/A
8/17/2008	Avolan	B	8am	556		N/A	N/A	N/A	N/A
8/17/2008	Avolan	C	8am	557		N/A	N/A	N/A	N/A
8/17/2008	Avolan	D	8am	558	1	N/A	N/A	N/A	N/A
8/23/2008	Avolan	A	8am	575	1	N/A	N/A	N/A	N/A
8/23/2008	Avolan	B	8am	576	1	N/A	N/A	N/A	N/A
8/23/2008	Avolan	C	8am	577		N/A	N/A	N/A	N/A
8/23/2008	Avolan	D	8am	578		N/A	N/A	N/A	N/A
8/24/2008	Avolan	A	8am	585		N/A	N/A	N/A	N/A
8/24/2008	Avolan	B	8am	586		N/A	N/A	N/A	N/A
8/24/2008	Avolan	C	8am	587		N/A	N/A	N/A	N/A
8/24/2008	Avolan	D	8am	588		N/A	N/A	N/A	N/A
8/29/2008	Avolan	A	8am	595		N/A	N/A	N/A	N/A
8/29/2008	Avolan	B	8am	596		N/A	N/A	N/A	N/A
8/29/2008	Avolan	C	8am	597	1	N/A	N/A	N/A	N/A
20-Jul	Avolan	A	8am	435		N/A	N/A	N/A	N/A
20-Jul	Avolan	B	8am	436		N/A	N/A	N/A	N/A
20-Jul	Avolan	C	8am	437		N/A	N/A	N/A	N/A
20-Jul	Avolan	D	8am	438		N/A	N/A	N/A	N/A
8/	Avolan	A	8am	565		N/A	N/A	N/A	N/A
8/	Avolan	B	8am	566		N/A	N/A	N/A	N/A
8/	Avolan	C	8am	567		N/A	N/A	N/A	N/A
8/	Avolan	D	8am	568		N/A	N/A	N/A	N/A
8/29/2008	Avolan	D	8am	598		N/A	N/A	N/A	N/A
8/30/2008	Avolan	A	8am	605		N/A	N/A	N/A	N/A
8/30/2008	Avolan	B	8am	606		N/A	N/A	N/A	N/A
8/30/2008	Avolan	C	8am	607		N/A	N/A	N/A	N/A
8/30/2008	Avolan	D	8am	608		N/A	N/A	N/A	N/A
8/31/2008	Avolan	A	8am	615		N/A	N/A	N/A	N/A
8/31/2008	Avolan	B	8am	616		N/A	N/A	N/A	N/A
8/31/2008	Avolan	C	8am	617		N/A	N/A	N/A	N/A
8/31/2008	Avolan	D	8am	618		N/A	N/A	N/A	N/A
5/23/2009	Malibu	A	8am	1		N/A	N/A	N/A	N/A
5/23/2009	Malibu	B	8am	2	1	N/A	N/A	N/A	N/A
5/23/2009	Malibu	C	8am	3	1	N/A	N/A	N/A	N/A

5/23/2009	Malibu	D	8am	4		N/A	N/A	N/A	N/A
5/23/2009	Malibu	E	8am	5		N/A	N/A	N/A	N/A
5/24/2009	Malibu	A	8am	10		N/A	N/A	N/A	N/A
5/24/2009	Malibu	B	8am	11	1	N/A	N/A	N/A	N/A
5/24/2009	Malibu	C	8am	12		N/A	N/A	N/A	N/A
5/24/2009	Malibu	D	8am	13		N/A	N/A	N/A	N/A
5/24/2009	Malibu	E	8am	14		N/A	N/A	N/A	N/A
5/25/2009	Malibu	A	8am	19		N/A	N/A	N/A	N/A
5/25/2009	Malibu	B	8am	20	1	N/A	N/A	N/A	N/A
5/25/2009	Malibu	C	8am	21	1	N/A	N/A	N/A	N/A
5/25/2009	Malibu	D	8am	22		N/A	N/A	N/A	N/A
5/25/2009	Malibu	E	8am	23		N/A	N/A	N/A	N/A
5/29/2009	Malibu	A	8am	28	1	N/A	N/A	N/A	N/A
5/29/2009	Malibu	B	8am	29	1	N/A	N/A	N/A	N/A
5/29/2009	Malibu	C	8am	30		N/A	N/A	N/A	N/A
5/29/2009	Malibu	D	8am	31		N/A	N/A	N/A	N/A
5/29/2009	Malibu	E	8am	32		N/A	N/A	N/A	N/A
5/30/2009	Malibu	A	8am	37	1	N/A	N/A	N/A	N/A
5/30/2009	Malibu	B	8am	38	1	N/A	N/A	N/A	N/A
5/30/2009	Malibu	C	8am	39		N/A	N/A	N/A	N/A
5/30/2009	Malibu	D	8am	40		N/A	N/A	N/A	N/A
5/30/2009	Malibu	E	8am	41		N/A	N/A	N/A	N/A
5/31/2009	Malibu	A	8am	46	1	N/A	N/A	N/A	N/A
5/31/2009	Malibu	B	8am	47		N/A	N/A	N/A	N/A
5/31/2009	Malibu	C	8am	48	1	N/A	N/A	N/A	N/A
5/31/2009	Malibu	D	8am	49		N/A	N/A	N/A	N/A
5/31/2009	Malibu	E	8am	50		N/A	N/A	N/A	N/A
6/6/2009	Malibu	A	8am	64	1	N/A	N/A	N/A	N/A
6/6/2009	Malibu	B	8am	65	1	N/A	N/A	N/A	N/A
6/6/2009	Malibu	C	8am	66	1	N/A	N/A	N/A	N/A
6/6/2009	Malibu	D	8am	67		N/A	N/A	N/A	N/A
6/6/2009	Malibu	E	8am	68		N/A	N/A	N/A	N/A
6/7/2009	Malibu	A	8am	73	1	N/A	N/A	N/A	N/A
6/7/2009	Malibu	B	8am	74	1	N/A	N/A	N/A	N/A
6/7/2009	Malibu	C	8am	75	1	N/A	N/A	N/A	N/A
6/7/2009	Malibu	D	8am	76		N/A	N/A	N/A	N/A
6/7/2009	Malibu	E	8am	77		N/A	N/A	N/A	N/A
6/13/2009	Malibu	A	8am	91	1	N/A	N/A	N/A	N/A
6/13/2009	Malibu	B	8am	92	1	N/A	N/A	N/A	N/A
6/13/2009	Malibu	C	8am	93	1	N/A	N/A	N/A	N/A
6/13/2009	Malibu	D	8am	94	1	N/A	N/A	N/A	N/A
6/13/2009	Malibu	E	8am	95	1	N/A	N/A	N/A	N/A
6/14/2009	Malibu	A	8am	100		N/A	N/A	N/A	N/A

6/14/2009	Malibu	B	8am	101	1	N/A	N/A	N/A	N/A
6/14/2009	Malibu	C	8am	102		N/A	N/A	N/A	N/A
6/14/2009	Malibu	D	8am	103		N/A	N/A	N/A	N/A
6/14/2009	Malibu	E	8am	104		N/A	N/A	N/A	N/A
6/19/2009	Malibu	A	8am	109	1	N/A	N/A	N/A	N/A
6/19/2009	Malibu	B	8am	110	1	N/A	N/A	N/A	N/A
6/19/2009	Malibu	C	8am	111	1	N/A	N/A	N/A	N/A
6/19/2009	Malibu	D	8am	112		N/A	N/A	N/A	N/A
6/19/2009	Malibu	E	8am	113	1	N/A	N/A	N/A	N/A
6/20/2009	Malibu	A	8am	118		N/A	N/A	N/A	N/A
6/20/2009	Malibu	B	8am	119	1	N/A	N/A	N/A	N/A
6/20/2009	Malibu	C	8am	120		N/A	N/A	N/A	N/A
6/20/2009	Malibu	D	8am	121		N/A	N/A	N/A	N/A
6/20/2009	Malibu	E	8am	122		N/A	N/A	N/A	N/A
6/21/2009	Malibu	A	8am	127		N/A	N/A	N/A	N/A
6/21/2009	Malibu	B	8am	128		N/A	N/A	N/A	N/A
6/21/2009	Malibu	C	8am	129		N/A	N/A	N/A	N/A
6/21/2009	Malibu	D	8am	130		N/A	N/A	N/A	N/A
6/21/2009	Malibu	E	8am	131		N/A	N/A	N/A	N/A
6/27/2009	Malibu	A	8am	145		N/A	N/A	N/A	N/A
6/27/2009	Malibu	B	8am	146		N/A	N/A	N/A	N/A
6/27/2009	Malibu	C	8am	147		N/A	N/A	N/A	N/A
6/27/2009	Malibu	D	8am	148		N/A	N/A	N/A	N/A
6/27/2009	Malibu	E	8am	149		N/A	N/A	N/A	N/A
6/28/2009	Malibu	A	8am	154		N/A	N/A	N/A	N/A
6/28/2009	Malibu	B	8am	155		N/A	N/A	N/A	N/A
6/28/2009	Malibu	C	8am	156		N/A	N/A	N/A	N/A
6/28/2009	Malibu	D	8am	157		N/A	N/A	N/A	N/A
6/28/2009	Malibu	E	8am	158		N/A	N/A	N/A	N/A
7/3/2009	Malibu	A	8am	172		N/A	N/A	N/A	N/A
7/3/2009	Malibu	B	8am	173		N/A	N/A	N/A	N/A
7/3/2009	Malibu	C	8am	174		N/A	N/A	N/A	N/A
7/3/2009	Malibu	D	8am	175		N/A	N/A	N/A	N/A
7/3/2009	Malibu	E	8am	176		N/A	N/A	N/A	N/A
7/4/2009	Malibu	A	8am	181		N/A	N/A	N/A	N/A
7/4/2009	Malibu	B	8am	182		N/A	N/A	N/A	N/A
7/4/2009	Malibu	C	8am	183		N/A	N/A	N/A	N/A
7/4/2009	Malibu	D	8am	184		N/A	N/A	N/A	N/A
7/4/2009	Malibu	E	8am	185		N/A	N/A	N/A	N/A
7/5/2009	Malibu	A	8am	190		N/A	N/A	N/A	N/A
7/5/2009	Malibu	B	8am	191		N/A	N/A	N/A	N/A
7/5/2009	Malibu	C	8am	192		N/A	N/A	N/A	N/A
7/5/2009	Malibu	D	8am	193		N/A	N/A	N/A	N/A

7/5/2009	Malibu	E	8am	194	N/A	N/A	N/A	N/A
7/23/2009	Malibu	A	8am	271	N/A	N/A	N/A	N/A
7/23/2009	Malibu	B	8am	272	N/A	N/A	N/A	N/A
7/23/2009	Malibu	C	8am	273	N/A	N/A	N/A	N/A
7/23/2009	Malibu	D	8am	274	N/A	N/A	N/A	N/A
7/23/2009	Malibu	E	8am	275	N/A	N/A	N/A	N/A
7/24/2009	Malibu	A	8am	280	N/A	N/A	N/A	N/A
7/24/2009	Malibu	B	8am	281	N/A	N/A	N/A	N/A
7/24/2009	Malibu	C	8am	282	N/A	N/A	N/A	N/A
7/24/2009	Malibu	D	8am	283	N/A	N/A	N/A	N/A
7/24/2009	Malibu	E	8am	284	N/A	N/A	N/A	N/A
8/20/2009	Malibu	A	8am	415	N/A	N/A	N/A	N/A
8/20/2009	Malibu	B	8am	416	N/A	N/A	N/A	N/A
8/20/2009	Malibu	C	8am	417	N/A	N/A	N/A	N/A
8/20/2009	Malibu	D	8am	418	N/A	N/A	N/A	N/A
8/20/2009	Malibu	E	8am	419	N/A	N/A	N/A	N/A
8/22/2009	Malibu	A	8am	433	N/A	N/A	N/A	N/A
8/22/2009	Malibu	B	8am	434	N/A	N/A	N/A	N/A
8/22/2009	Malibu	C	8am	435	N/A	N/A	N/A	N/A
8/22/2009	Malibu	D	8am	436	N/A	N/A	N/A	N/A
8/22/2009	Malibu	E	8am	437	N/A	N/A	N/A	N/A
8/23/2009	Malibu	A	8am	442	N/A	N/A	N/A	N/A
8/23/2009	Malibu	B	8am	443	N/A	N/A	N/A	N/A
8/23/2009	Malibu	C	8am	444	N/A	N/A	N/A	N/A
8/23/2009	Malibu	D	8am	445	N/A	N/A	N/A	N/A
8/23/2009	Malibu	E	8am	446	N/A	N/A	N/A	N/A
8/29/2009	Malibu	A	8am	469	N/A	N/A	N/A	N/A
8/29/2009	Malibu	B	8am	470	N/A	N/A	N/A	N/A
8/29/2009	Malibu	C	8am	471	N/A	N/A	N/A	N/A
8/29/2009	Malibu	D	8am	472	N/A	N/A	N/A	N/A
8/29/2009	Malibu	E	8am	473	N/A	N/A	N/A	N/A
8/30/2009	Malibu	A	8am	478	N/A	N/A	N/A	N/A
8/30/2009	Malibu	B	8am	479	N/A	N/A	N/A	N/A
8/30/2009	Malibu	C	8am	480	N/A	N/A	N/A	N/A
8/30/2009	Malibu	D	8am	481	N/A	N/A	N/A	N/A
8/30/2009	Malibu	E	8am	482	N/A	N/A	N/A	N/A
9/5/2009	Malibu	A	8am	487	N/A	N/A	N/A	N/A
9/5/2009	Malibu	B	8am	488	N/A	N/A	N/A	N/A
9/5/2009	Malibu	C	8am	489	N/A	N/A	N/A	N/A
9/5/2009	Malibu	D	8am	490	N/A	N/A	N/A	N/A
9/5/2009	Malibu	E	8am	491	N/A	N/A	N/A	N/A
9/6/2009	Malibu	A	8am	496	N/A	N/A	N/A	N/A
9/6/2009	Malibu	B	8am	497	N/A	N/A	N/A	N/A

9/6/2009	Malibu	C	8am	498	N/A	N/A	N/A	N/A
9/6/2009	Malibu	D	8am	499	N/A	N/A	N/A	N/A
9/6/2009	Malibu	E	8am	500	N/A	N/A	N/A	N/A
9/12/2009	Malibu	A	8am	532	N/A	N/A	N/A	N/A
9/12/2009	Malibu	B	8am	533	N/A	N/A	N/A	N/A
9/12/2009	Malibu	C	8am	534	N/A	N/A	N/A	N/A
9/12/2009	Malibu	D	8am	535	N/A	N/A	N/A	N/A
9/12/2009	Malibu	E	8am	536	N/A	N/A	N/A	N/A
9/13/2009	Malibu	A	8am	541	N/A	N/A	N/A	N/A
9/13/2009	Malibu	B	8am	542	N/A	N/A	N/A	N/A
9/13/2009	Malibu	C	8am	543	N/A	N/A	N/A	N/A
9/13/2009	Malibu	D	8am	544	N/A	N/A	N/A	N/A
9/13/2009	Malibu	E	8am	545	N/A	N/A	N/A	N/A
9/19/2009	Malibu	A	8am	550	N/A	N/A	N/A	N/A
9/19/2009	Malibu	B	8am	551	N/A	N/A	N/A	N/A
9/19/2009	Malibu	C	8am	552	N/A	N/A	N/A	N/A
9/19/2009	Malibu	D	8am	553	N/A	N/A	N/A	N/A
9/19/2009	Malibu	E	8am	554	N/A	N/A	N/A	N/A
9/20/2009	Malibu	A	8am	559	N/A	N/A	N/A	N/A
9/20/2009	Malibu	B	8am	560	N/A	N/A	N/A	N/A
9/20/2009	Malibu	C	8am	561	N/A	N/A	N/A	N/A
9/20/2009	Malibu	D	8am	562	N/A	N/A	N/A	N/A
9/20/2009	Malibu	E	8am	563	N/A	N/A	N/A	N/A

Figure A-1: Nested PCR Enterovirus Results for Avalon 6/26-8/10 and Malibu 9/13-9/20

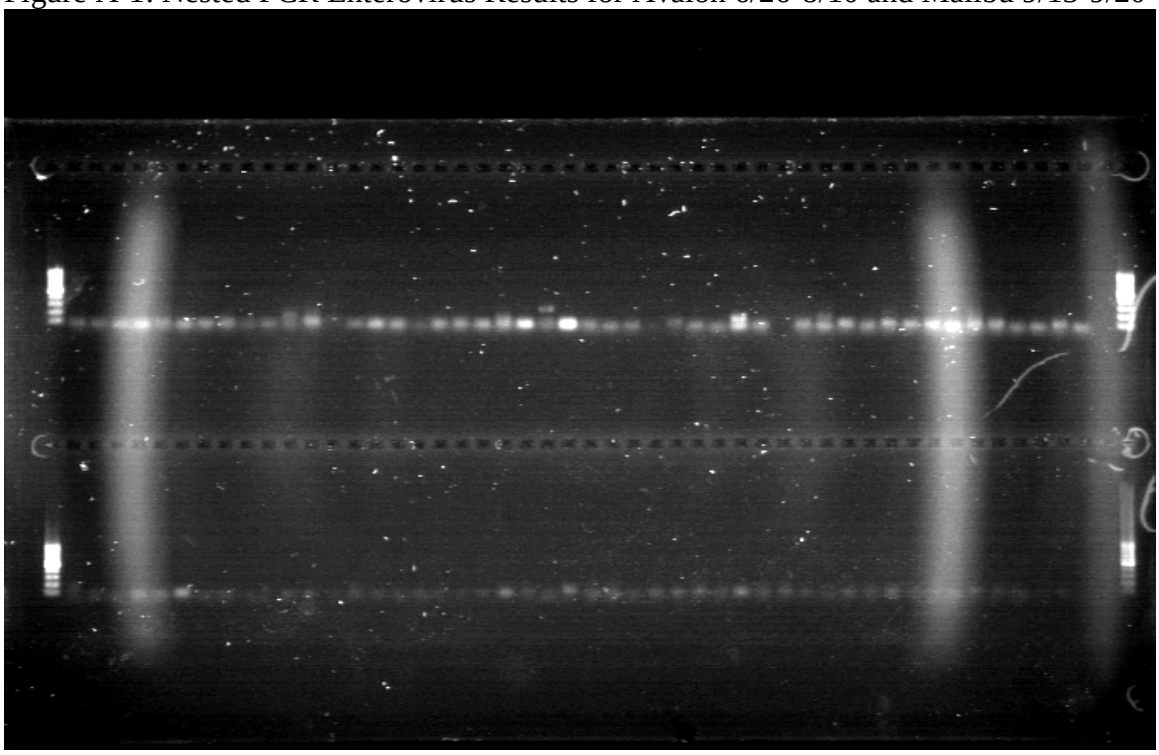


Figure A-2: Nested PCR Enterovirus Results for Doheny 8/10-8/14 and Avalon 7/13-7/20

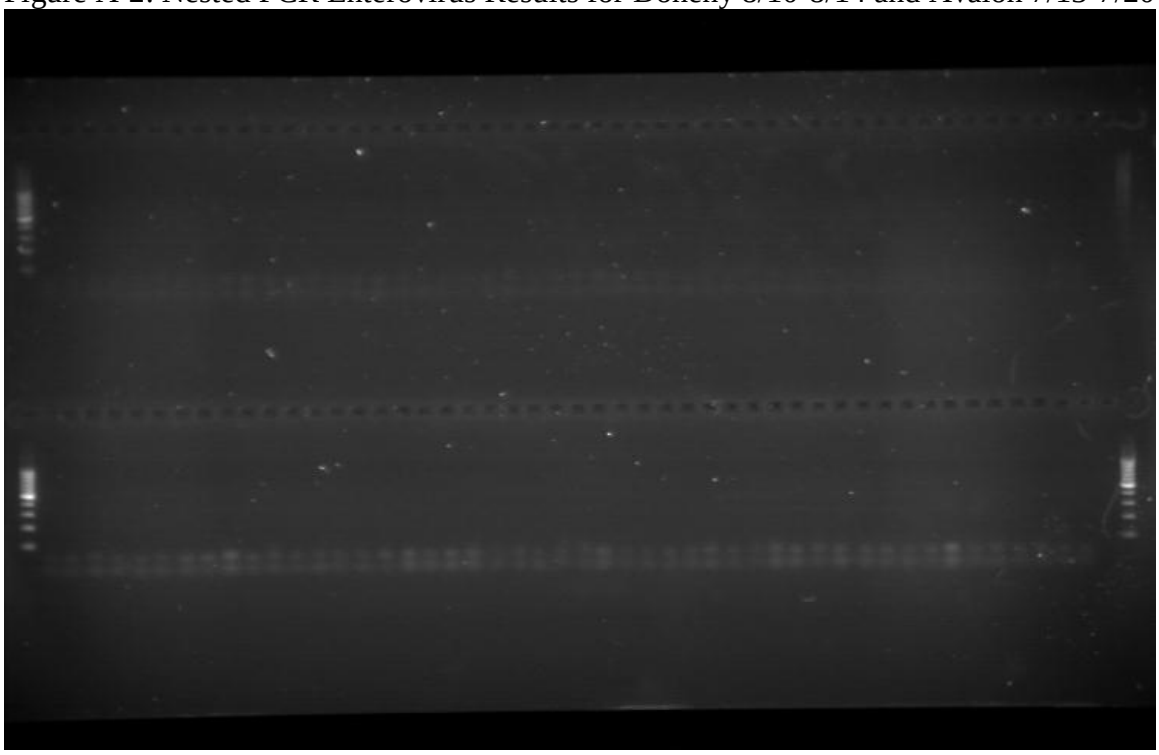


Figure A-3: Nested PCR Enterovirus Results for Malibu 5/23-5/29 and 6/21-7/24

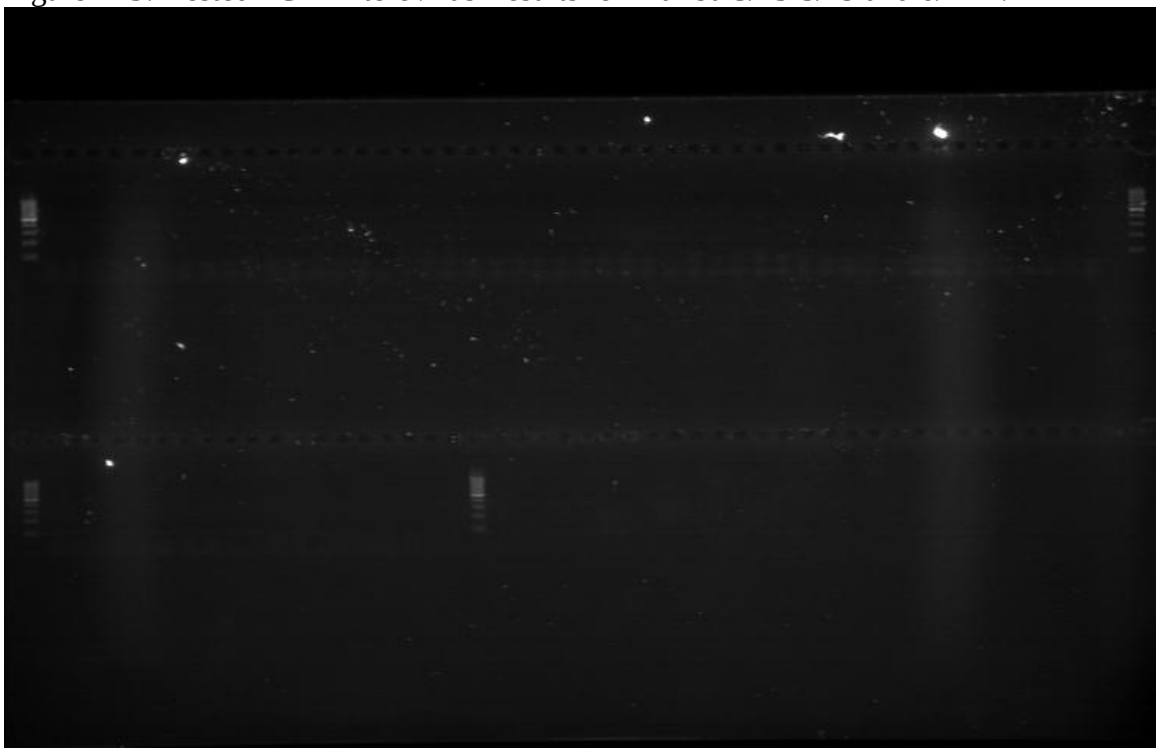


Figure A-4: Nested PCR Enterovirus Results for Malibu 5/23-6/20 and 8/20-9/21

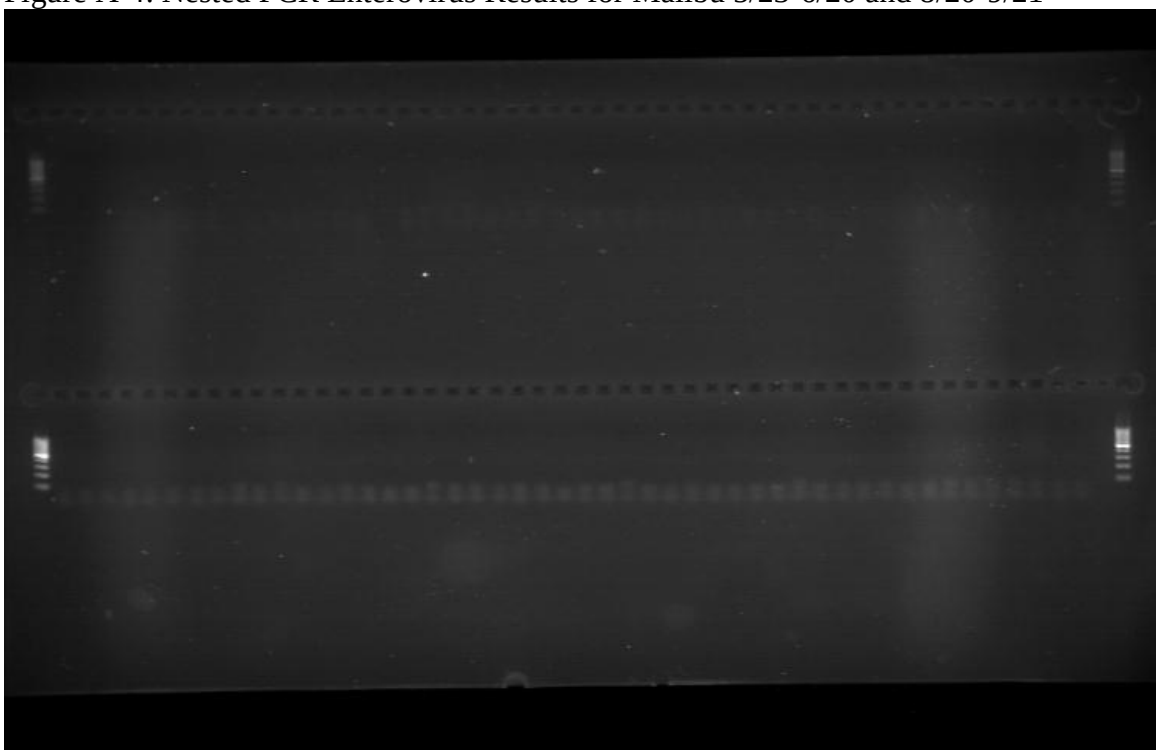
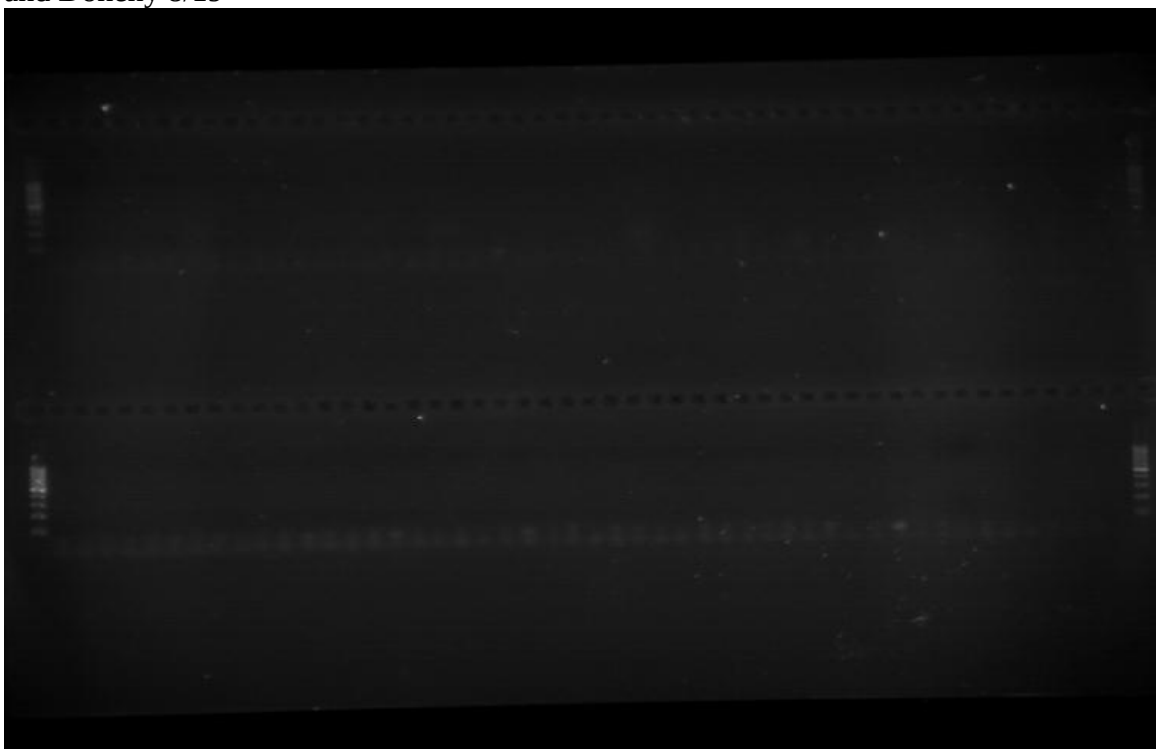


Figure A-5: Nested PCR Enterovirus Results for Malibu 9/13-9/20, Avalon 8/15-8/31, and Doheny 8/19



Literature Cited

Atmar RL, Estes MK. Diagnosis of noncultivatable gastroenteritis viruses, the human caliciviruses. Clin Microbiol Rev. 2001;14:15-37.

Afonina I, Ankoudinova I, Mills A, Lokhov S, Huynh P, Mahoney W. Primers with 5' flaps improve real-time PCR. BioTechniques 2007;43:770-774.

Batigelli DA, Sobsey MD, Libe DC. The inactivation of hepatits A virus and other model viruses by UV irradiation. Water Sci Technol. 1993;27:339-42.

Beversdorf LJ, Bornstein-Forst SM, McLellan SL. The potential for beach sand to serve as a reservoir for *escherichia coli* and the physical influences on cell die-off. Journal of Applied Microbiology. 2006;102:1372.

Boehm AB, Ashbolt NJ, Colford JM, Dunbar LE, Fleming LE, Gold MA, et al. A sea change ahead for recreational water quality criteria. Journal of Water and Health. 2009;07(1):9.

Bordalo AA. Microbiological water quality in urban coastal beaches: The influence of water dynamics and optimization of the sampling strategy. Water Res. 2003 JUL;37(13):3233-41.

Cabelli VJ, Dufour AP, McCabe LJ, Levin MA. Swimming-associated gastroenteritis and water quality. American Journal of Epidemiology. 1982;115(4):606.

Castor ML, Beach MJ. Reducing illness transmission from disinfected recreational water venues: Swimming, diarrhea and the emergence of a new public health concern. The Pediatric Infectious Disease Journal. 2004;23(9).

Chigbu P, Gordon S, Strange T. Influence of inter-annual variations in climatic factors on fecal coliform levels in mississippi sound. Water Research. 2004;38:4341.

Colford JM, Wade TJ, Schiff KC, Wright CC, Griffith JF, Sandhu SK, et al. Water quality indicators and the risk of illness at beaches with nonpoint sources of fecal contamination. Epidemiology. 2007;18(1):27.

Corbett SJ, Rubin GL, Curry GK, Kleinbaum DG, Syndey Beach Users Study Advisory Group, . The health effects of swimming at sydney beaches. American Journal of Public Health. 1993;83(12):1701.

Drago L, Lombardi A, Vecchi ED, Giuliani G, Bartolone R, Gismondo MR. Comparison of nested PCR and real time PCR of herpesviurs infections of central nervous system in HIV patients. BMC Infectious Diseases. 2004;4(55).

Dwight RH, Baker DB, Semenza JC, Olson BH. Health effects associated with recreational coastal water use: Urban versus rural california. American Journal of Public Health. 2004;94(4):565.

Efstratiou MA. Managing coastal bathing water quality: The contribution of microbiology and epidemiology. Marine pollution bulletin. 2001;42(6):425.

Efstratiou MA. Managing coastal bathing water quality: The contribution of microbiology and epidemiology. Mar Pollut Bull. 2001 JUN;42(6):425-32.

Elmir SM, Wright ME, Abdelzaher A, Solo-Gabriele HM, Fleming LE, Miller G, et al. Quantitative evaluation of bacteria released by bathers in a marine water. Water research. 2007;41:3.

Fang Y, Wu W, Pepper IL, Larsen JL, Marras SAE, Nelson EA, et al. Comparison of real-time, quantitative PCR with molecular beacons to nested PCR and culture methods for detection of *mycobacterium avium* subsp. *paratuberculosis* in bovine fecal samples. Journal of Clinical Microbiology. 2002;40(1):287-91.

Fankhause RL, Monroe SS, Noel JS, Humphrey CD, Bresee JS, Parashar UD, et al. Epidemiologic and molecular trends of "norwalk-like viruses" associated with outbreaks of gastroenteritis in the united states. J Infect Dis. 2002;186:1-7.

Fleisher JM, Kay D, Salmon RL, Jones F, Wyer MD, Godfree AF. Marine waters contaminated with domestic sewage: Nonenteric illnesses associated with bather exposure in the united kingdom. American Journal of Pulbic Health. 1996;9:1228.

Fong TT, Griffin DW, Lipp EK. Molecular assays for targeting human and bovine enteric viruses in coastal waters and their application for library-independent source tracking. Appl Environ Microbiol. 2005 APR;71(4):2070-8.

Geary PM, Davies CM. Bacterial source tracking and shellfish contamination in a coastal catchment. Water Science and Technology. 2003;47(7-8):95-100.

Grant SB, Kim JH, Jones BH, Jenkins SA, Wasyl J, Cudaback C. Surf zone entrainment, along-shore transport, and human health implications of pollution from tidal outlets. Journal of Geophysical Research. 2005;110:C10025.

Green KY, Chanock RM, Kapikian Z. Human caliciviruses. In: Knipe DM, Howley PM, editors. Fields Virology. Philadelphia, PA.: Lippincott Williams and Wilkins; 2001.

Gregory JB, Litaker W, Noble RT. Rapid one-step quantitative reverse transcriptase PCR assay with competitive internal positive control for detection of enteroviruses in environmental samples. Applied and Environmental Microbiology. 2006;72(6):3960-7.

Griffin DW, Gibson CJ3, Lipp EK, Riley K, Paul 3rd JH, Rose JB. Detection of viral pathogens by reverse transcriptase PCR and of microbial indicators by standard methods in the canals of the florida keys. Appl Environ Microbiol. 1999;65:4118-25.

Griffith JF, Schiff KC, Lyon GS, Fuhrman JA. Microbiological water quality at non-human influenced reference beaches in southern california during wet weather. Mar Pollut Bull. 2010 APR;60(4):500-8.

Haile RW, Witte JS, Gold M, Cressey R, McGee C, Millikan RC, et al. The health effects of swimming in ocean water contaminated by storm drain runoff. Epidemiology. 1999;10(4).

Hartz A, Cuvelier M, Noowosielski K, Bonilla TD, Green M, Esiobu N, et al. Survival potential of *escherichia coli* and enterococci in subtropical beach sand: Implications for water quality managers. J Environ Qual. 2008;37:898.

Herrera A, Suarez P. Bacterial markers as tools for coastal water environmental quality measurement. Interciencia. 2005 MAR;30(3):171-6.

Hoebe CJ, Vennema H, de Roda Husman, A. M., van Duynhoven YT. Norovirus outbreak among primary schoolchildren who had played in a recreational water fountain. J Infect Dis. 2004.;189:699-705.

Ibekwe AM, Lyon SR. Microbiological evaluation of fecal bacterial composition from surface water through aquifer sand material. Journal of Water and Health. 2008 SEP;6(3):411-21.

Jiang SC, Chu W. PCR detection of pathogenic viruses in southern california urban rivers. J Applied Microbiology. 2004;97:17-28.

Jothikumar, N., J. A. Lowther, K. Henshilwood, D. N. Lees, V. R. Hill, and J. Vinje. 2005. Rapid and sensitive detection of noroviruses by using TaqMan-based one-step reverse transcription-PCR assays and application to naturally contaminated shellfish samples. Appl. Environ. Microbiol. 71:1870-1875.

Kageyama T, Shinohara M, Uchida K, Fukushi S, Hoshino FB, Kojima S, et al. Coexistence of multiple genotypes, including newly identified genotypes, in outbreaks of gastroenteritis due to norovirus in japan. J Clin Microbiol. 2004;42:2988-95.

Kamizoulis G, Saliba L. Development of coastal recreational water quality standards in the mediterranean. Environment International. 2004;30:841.

Kawada J, Klmura H, Ito Y, Hoshlno Y, Tanaka-Kitajlma N, Ando Y, et al. Comparison of real-time and nested PCR assays for detection of herpes simplex virus DNA. Microbiol Immunol. 2004;48(5):411-5.

Kay D, Fleisher JM, Salmon RL, Jones F, Wyer MD, Godfree AF, et al. Predicting likelihood of gastroenteritis from sea bathing: Results from randomised exposure. Lancet. 1994;344:905.

Kay D, Kershaw S, Lee R, Wyer MD, Watkins J, Francis C. Results of field investigations into the impact of intermittent sewage discharges on the microbiological quality of wild mussels (mytilus edulis) in a tidal estuary. Water Res. 2008 JUN;42(12):3033-46.

Keswick BH, Gerba CP, DuPont HL, Rose JB. Detection of enteric viruses in treated drinking water. Appl Environ Microbiol. 1984;47:1290-4.

Ki SJ, Ensari S, Kim JH. Solar and tidal modulations of fecal indicator bacteria in coastal waters at huntington beach, california. . Environ Manage. 2007;39:867.

Kite-Powell HL, Fleming LE, Backer LC, Faustman EM, Hoagland P, Tsuchiya A, et al. Linking the oceans to public health: Current efforts and future directions. Environmental Health. 2008;7(Suppl 2):S6.

Kopecka H, Dubrou S, Prevot J, Marechal J, Lopez-Pila JM. Detection of naturally occurring enteroviruses in waters by reverse transcription, polymerase chain reaction, and hybridization. Appl Environ Microbiol. 1993;59:1213-9.

Lee RJ, Morgan OC. Environmental factors influencing the microbiological contamination of commercially harvested shellfish. Water Science and Technology. 2003;47(3):65-70.

Levy K, Hubbard AE, Nelson KL, Eisenberg JNS. Drivers of water quality variability in northern coastal ecuador. Environ Sci Technol. 2009 MAR 15;43(6):1788-97.

Lindesmith LC, Donaldson EF, Lobue AD, Cannon JL, Zheng DP, Vinje J, et al. Mechanisms of GII.4 norovirus persistence in human populations. PLoS Med. 2008;5:e31.

Mansilha CR, Coelho CA, Heitor AM, Amado J, Martins JP, Gameiro P. Bathing waters: New directive, new standards, new quality approach. Mar Pollut Bull. 2009 OCT;58(10):1562-5.

Melnick JL, Rennick V. Infectivity titers of enterovirus as found in human stools. J Med Virol. 1980;5:205-20.

Mieszkin S, Furet J, Corthier G, Gourmelon M. Estimation of pig fecal contamination in a river catchment by real-time PCR using two pig-specific bacteroidales 16S rRNA genetic markers. Appl Environ Microbiol. 2009 MAY 15;75(10):3045-54.

National Resources Defense Council (NRDC), . Testing the waters 2005. A guide to water quality at vacation beaches. Washington, DC.: NRDC; 2005.

Noble RT, Fuhrman JA. Enteroviruses detected by reverse transcriptase polymerase chain reaction from the coastal waters of santa monica bay, california: Low correlation to bacterial indicator levels. Hydrobiologia;460:175-84.

Noble RT, Fuhrman JA. Enteroviruses detected by reverse transcriptase polymerase chain reaction from the coastal waters of santa monica bay, california: Low correlation to bacterial indicator levels. Hydrobiologia. 2001 SEP 15;460:175-84.

Nygard K, Vold L, Halvorsen E, Bringeland E, Rottingen JA, Aavitsland P. Waterborne outbreak of gastroenteritis in a religious summer camp in norway, 2002. Epidemiol Infect. 2004;132:223-9.

Oberste MS, Maher K, Kilpatrick DR, Pallansch MA. Molecular evolution of the human enteroviruses: Correlation of serotype with VP1 sequence and application to picornavirus classification. J Virol. 1999;73:1941-8.

Payment P, Tremblay M, Trudel M. Relative resistance to chlorine of poliovirus and coxsackievirus isolates from environmental sources and drinking water. Appl Environ Microbiol. 1985;49:981-3.

Portnoy JW, Allen JR. Effects of tidal restrictions and potential benefits of tidal restoration on fecal coliform and shellfish-water quality. J Shellfish Res. 2006 AUG;25(2):609-17.

Prieto MD, Lopez B, Juanes JA, Revilla JA, Llorca J, Delgado-Rodriguez M. Recreation in coastal waters: Health risk associated with bathing in sea water. J Epidemiol Community Health. 2001;55:442.

Pruss A. Review Of epidemiological studies on health effects from exposure to recreational water. International Journal of Epidemiology. 1998;27(1).

Qaigen. AllPrep® 96 DNA/RNA handbook: For simultaneous purification of genomicDNA and total RNA from animal and humancells and tissues in 96-well format. ; 2008.

Renault T. Monitoring of marine environment and shellfish health protection. Bulletin De L Academie Veterinaire De France. 2009 JUL-SEP;162(3):247-51.

Reynolds KA, Roll K, Fujioka RS, Gerba CP, Pepper IL. Incidence of enteroviruses in mamala bay, hawaii using cell culture and direct polymerase chain reaction methodologies. Can J Microbiol. 1998;44:598-604.

Rodríguez RA, Thie L, Gibbons CD, Sobsey MD. Reducing the effects of environmental inhibition in quantitative real-time PCR detection of adenovirus and norovirus in recreational seawaters. J Virol Methods. 2012 Apr;181(1):43-50. Epub 2012 Feb 3.

Romero JR. Reverse-transcription polymerase chain reaction detection of the enteroviruses. Arch Pathol Lab Med. 1999;123:1161-9.

Rusin P, Enriquez C, Johnson D, Gerba CP. Enteric viruses. In: Maier R, Pepper IL, Gerba CP, editors. Environmental Microbiology, vol. 1. London: Academic Press; 2000. p. 472-84.

Seyfried PL, Tobin RS, Brown NE, Ness PF. A prospective study of swimming-related illness I. swimming-associated health risk. American Journal of Public Health. 1985;75(9).

Shuval H. Thalassogenic infectious diseases caused by wasterwater pollution of the marine environment: An estimate of the worldwide occurrence. In: Belkin , Colwell , editors. Oceans and Health: Pathogens in the Marine Environment. New York: Springer; 2005. p. 373.

Skraber S, Gassilloud B, Schwartzbrod L, Gantzer C. Survival of infectious poliovirus-1 in river water compared to the persistence of somatic coliphages, thermotolerant coliforms and poliovirus-1 genome. Water Res. 2004;38(2927):2933.

Soller JA, Bartrand T, Ashbolt NJ, Ravenscroft J, Wade TJ. Estimating the primary etiologic agents in recreational freshwaters impacted by human sources of faecal contamination. Water Research. 2010;44:4736-47.

Stevenson A. Studies of bathing water quality and health. American Journal of Public Health. 1953;43:529.

Stewart JR, Gast RJ, Fujioka RS, Solo-Gabriele HM, Meschke JS, Amaral-Zettler LA, et al. The coastal environment and human health: Microbial indicators, pathogens, sentinels and reservoirs. Environmental Health. 2008;7(Suppl 2):S3.

Turbow D. Addressing disease surveillance needs for marine recreational bathers. Journal of Water and Health. 2009;7(1):45.

Vinje J, Altena SA, Koopmans MP. The incidence and genetic variability of small round-structured viruses in outbreaks of gastroenteritis in the netherlands. J Infect Dis. 1997;176(1374):1378.

Watkins-Riedel T, Woegerbauer M, Hollemann D, Hufnagl P. Rapid diagnosis of enterovirus infections by real-time PCR on the LightCycler using the TaqMan format. Diagnostic Microbiology and Infectious Disease. 2002;42:99-105.

Wetz JJ, Lipp EK, Griffin DW, Lukasik J, Wait D, Sobsey MD, et al. Presence, infectivity, and stability of enteric viruses in seawater: Relationship to marine water quality in the florida keys. Mar Pollut Bull. 2004;48(698):704.

WHO. Guidelines for safe recreational water environments, vol 1 coastal and fresh waters. Geneva, Switzerland: World Health Organization; 2003.

Yoder J, Roberts V, Craun GF, Hill V, Hicks LA, Alexander NT, et al. Surveillance for waterborne disease and outbreaks associated with drinking water and water not intended for drinking--united states, 2005-2006. MMWR Surveill Summ. 2008(57):39-62.