Abstract

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Evaluation of different methods for detecting Male Specific Coliphages from marine water at Doheny Beach, CA

Male-specific (F+) coliphages, primarily F+ RNA coliphages, have been proposed as candidate indicators of enteric viruses in water. The purpose of the this study was to evaluate the use of F+ coliphages as viral indicators of fecal contamination in marine waters using: (1) the Coliphage Latex Agglutination and Typing (CLAT) immunoassay, a rapid and novel coliphage detection and typing method, (2) a rapid (5-hr) liquid culture enrichment, and (3) a standard overnight liquid culture enrichment method, EPA Method 1601, at Doheny Beach, CA. This study analyzed 75 1 L marine water samples following rapid enrichment and 101 samples following overnight enrichment. For both methods, samples were collected in 2007 and 2008. The study was continued for the summer of 2008 to better document the performance of modifications of the rapid coliphages methods that were intended to overcome deficiencies of the method identified in 2007. For samples collected in 2007 using the rapid-CLAT and the overnight-CLAT assay 3/75(4.00%) and 4/101(3.96%) samples were positive for F+ coliphages respectively. Higher detection rates were found by using rapid and overnight enrichment spot plating with 16/75 (21.3%) and 27/101 (26.7%), respectively. We tested the lysis zones of the spot plates for the presence of F+ coliphage with the CLAT and found that 16/101 (15.8%) were positive for rapid and 23/101 (22.7%) for overnight enrichments, respectively, both of which were significant increases in positivity ($p<0.05$). We analyzed the effect salinity has on the enrichment and found that by reducing the salt

concentration of the seawater samples by adding 1-liter of salt-free water, the growth of *E. coli* and the propagation of coliphages was improved. *E coli* growth during the rapid (5-hr) enrichment improved from 9.0×10^8 to 1.0×10^9 cfu/mL and coliphage propagation improved from 2.3×10^5 to 1.0×10^6 pfu/mL. This dilution of seawater was used for the analysis of 75 samples in 2008. Using the rapid-CLAT and the overnight-CLAT assay 16/75 (21.3%) and 24/101(23.7%) samples were positive for F+ coliphages respectively in 2008. Following rapid and overnight enrichment spot plating we found 37/75 (49.3%) and 76/101 (75.2%) F+ coliphage-positive samples, respectively, which was significantly different by Fisher Exact Test analysis ($p<0.05$). The results of this study suggest that the CLAT assay needs further improvement to (1) increase coliphage virus detection in seawater enrichments or (2) reduce to a lower coliphage concentration the lower detection limit of the agglutination immunoassay as approaches to achieving maximum F+ coliphage detection.

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List of Abbreviations

Amp	ampicillin
ATCC	America Type Culture Collection
BSA	Bovine serum albumin
C	Celsius
cDNA	complementary deoxyribonucleic acid
CLAT	coliphage latex agglutination and typing assay
CFU	colony forming units
DAL	double agar layer
DI	de-ionized water
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EC	Enterococci
EPA	United States Environmental Protection Agency
FC	fecal coliform
FIB	fecal indicator bacteria
g	gram
GuSCN	guanidinium thiocyanate

hr hour

IO instant ocean

L Liter

$MgCl_2$ magnesium chloride

ml mililiter

MPN most probable number

NaCl Sodium Chloride

nm nanometer

OD optical density

PCR polymerase chain reaction

PFU plaque forming unit

PPT parts per thousand

RLB reverse line blot hybridization

RNA ribonucleic acid

rpm revolutions per minute

RT-PCR reverse transcriptase polymerase chain reaction

SAL single agar layer

sec	second
TC	total coliform
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
µl	microliter
WHO	World Health Organization

Introduction

Access to safe and reliable water is important to human health. Every year over 1 million people die worldwide from consumption of unsafe and unreliable water sources (WHO, 2004). Microbial pathogens with the potential to cause respiratory, dermal, and gastrointestinal illness can be spread though water, and the common source for their presence is fecal pollution. In developed countries, pollution control measures have been taken and both wastewater effluent discharge and ambient water quality standards set to monitor water and maintain a level of quality that ensures safety. Despite these regulations and treatment, outbreaks of waterborne illness still occur.

Fecal indicator bacteria are commonly used as indicators of fecal contamination in recreational waters. A meta-analysis of the US EPA water quality monitoring standards showed that *E. coli* and Enterococci proved to be adequate indicators of GI illness in recreational waters (Wade et al., 2003). However, their usefulness in determining the level of viral contamination has been questioned (Weisberg et al, 2007). Human enteric viruses are transmitted by water and their presence in recreational water can pose serious risk to swimmers. Compared to fecal indicator bacteria, enteric viruses are more resistant to drinking water and wastewater treatments and can persist longer in both fresh and marine waters (Goyal et al, 2003).Bacterial viruses such as coliphages have been suggested as alternatives indicators of fecal contamination. They are present in the GI tract of both humans and animals and are generally the same size and shape as many of the pathogenic enteric viruses.

There is also a need for more rapid water quality monitoring tests because current tests take more then 24 hours to complete. It is possible for human exposure to occur by the time the results are available. It is also possible that the microbes of interest may return to levels below the threshold, resulting beaches remaining closed even when the threat from fecal contamination has subsided. A modified (5-hr) rapid enrichment has been described for determining the presence of coliphages from 1 L of water and when combined with a 1-minute Coliphage Latex Agglutination Typing assay (CLAT), which uses antibody coated latex beads to detect male specific coliphages. This new test system has the potential to provide rapid results and also give information on the possible source of contamination because it simultaneously serotypes the F+ coliphages present with the use of a antibody based typing assay (Love and Sobsey, 2007).This rapid coliphage detection will allow for a near real-time detection of fecal contamination, reducing swimmer exposure to human viruses and reducing unnecessary beach closures. The goal of this study is to evaluate the use of this method as a same day, reliable, and inexpensive method for detecting and typing indicator viruses for the monitoring of marine recreational waters.

Literature Review

Bacteriophages

Biology

Bacteriophages are viruses that infect bacteria. Coliphages are a type of bacteriophage that infects *Escherichia coli*. Bacteriophages have been suggested as candidates for use as an indicator of enteric viruses due to fecal contamination (Havelaar A.H., 1987). Coliphages have a number of characteristics that make them good candidate indicators that will be discussed later. They are often found in the intestinal tract of both humans and animals. Three groups of bacteriophages with potential to serve as indicators for enteric viruses are: male-specific coliphages (F+ Coliphages), somatic coliphages, and the bacteriophages that infect *Bacteroides fragilis*.

Bacteriophages usually undergo a lytic life cycle in which the bacteriophages open and destroy (lyse) the host bacterial cell releasing new viral particles capable of infecting new cells. Bacteriophage replication is a complex process involving several steps including adsorption, separation of the nucleic acid from the protein coat, expression and replication of the nucleic acid, assembly of new viral particles, and finally the release of new viruses (Goyal et al., 1987). Adsorption is attachment of the bacteriophage to specific host receptors on the surface of the bacterial cells including the pili or surface proteins. The bacteriophage injects its viral nucleic acid into the cell with a motion similar to that of a syringe. Once inside the cells bacterial ribosome's begin translating the viral nucleic acid into protein. As viral proteins are synthesized they are assembled by other proteins and packaged into a capsid. The new viral progeny are released by lysis of the cell and the new virus released to attack other cells.

F+ Coliphages

Male Specific Coliphages can be either DNA or RNA and they infect their host cells by attaching to the F-pilus of *E. coli* bacterium containing the F plasmid, also known as the F-factor or fertility factor. When this plasmid is present the bacterium is able to grow a pilus through which nucleic acids can be transferred from one bacterium to another. These bacteriophages range in size from 30nm to over 200nm. Male specific coliphages are grouped into three families, male specific RNA coliphages (F+RNA) families Leviviridae and Alloviridae, and male specific DNA coliphage (F+DNA) family Inoviridae.

The Leviviridae group of F+ coliphages is comprised of two sub groups. These coliphages are small ranging in size from 20-30nm, and contain a single strand of RNA. Their subgrouping was based on specific serological properties. Group I include MS-2-like and group II GA-like coliphages. The allolevividae family is comprised of group III Qß like coliphages and group IV containing Fi-like coliphages. Groups I and IV are commonly associated with animals and groups II and III are most commonly associated with humans although there are exceptions for both. Inoviridae is the second group of F+ coliphages made up of a single strand of DNA. This group is comprised of large filamentous phages that range in size from 760-1950 nm.

Somatic Coliphages

Somatic Coliphages are DNA coliphage that infect their host cells by attaching to their cell walls. They are a heterogeneous group of bacteriophages which have been mostly uncharacterized because of lack of interest, and focus on F+ coliphages. Somatic coliphages are not specific to *E. coli*. Some strains can replicate with other species of Enterobacteriaceaa

including *Klebsiella* and *Salmonella*. These coliphages are very persistent in the environment and have been found in waste and surface waters and in sand longer than the pathogenic viruses of interest (Kott et al., 1978 and Bonilla et al., 2007). There is also concern about potential propagation in the environment (Borrego et al., 1990).

Bacteroides fragilis phages

Bacteroides fragilis is an obligate anaerobic gram-negative bacterium present in high numbers in the intestinal tract of both humans and animals. Their phages are very host specific unlike somatic coliphages. They have been extensively detected in human sewage (Cornax et al., 1990). One of the characteristics that make the bacteroides phages good indicators of fecal contaminations is that it is thought that they do not replicate in the environment because of growth restrictions imposed by the host. Additionally, they are as resistant to natural inactivation processes and waste water treatment as F+ coliphages. *B. fragilis* phages are detected at lower levels than other phages therefore determining the precise ratio of these phages with enteric viruses is difficult. Their detection relies primarily on the use of PCR methods. Culture methods of these phages are time consuming and complex because their host is an anaerobic bacterium which is more difficult to cultivate than other enteric bacteria.

Fecal Indicator Bacteria and Recreational Water Quality Monitoring

Fecal contamination continues to be a problem in our coastal and recreational waters despite government regulations regarding water quality and treatment. The microbial pathogens that contaminate these water sources are capable of causing gastrointestinal, dermal, and respiratory illness. Current recreational water monitoring standards routinely

measure fecal bacterial contamination using fecal bacterial indicators (FIB) such as total coliforms (TC), *Escherichia coli*, and *Enterococcus spp* (EC). It is believed that when these organisms are present in high numbers, that pathogenic organisms are likely to be present as well.

Coliforms

Coliforms have been used as indicators of water quality (surface, recreational, drinking, and shellfish waters) for over one hundred years. Much has been discovered about coliforms since the first use of them as an indicator. Coliforms are rod-shaped, gram-negative, and non-spore forming bacteria. They are capable of fermenting lactose at temperatures within the range of 35-37°C. These bacteria are found in abundance in the intestinal tracts of animals and humans. Coliforms can also be found elsewhere in the environment. Coliforms are non pathogenic and simple to culture making them suitable for use as bacterial indicators of fecal contamination of water. Their behavior in the environment including but not limited to their biology, occurrence, and reaction to stress can differ greatly in comparison to the pathogenic organisms they serve as an indicator for, potentially limiting their usefulness as indicators of fecal pollution. Because of their ubiquitous presence in the environment, further tests are needed to conclude the source of their origin (Fecal or soil, animal or human).

Escherichia coli

E. coli is a member of the coliform group and is distinguished from other members by its ability to ferment the sugar lactose at 44.5°C.This organism serves as a good indicator of fecal contamination because it is non-pathogenic to humans except in a few cases, it is easily

detected, and it is generally present in concentrations higher than pathogens (Scott et al., 2002). Unlike coliforms as a group, *E. coli* are more exclusively of fecal origin. However, these bacteria have been reported to replicate in tropical and sub-tropical warm waters and in soils (Lasalde et al., 2005).

Enterococcus

Enterococcus spp are a sub-group of fecal streptococci. These bacteria are differentiated based on their ability to grow under different environmental conditions including high pH and temperature. Their ability to survive in these types of conditions mimics many of the pathogens for which they serve as an indicator. *E. faecalis* and *E. faecium* are the species most commonly associated with humans and have been successfully used as indicators of fecal pollution in recreational waters. Problems associated with using *Enterococci* as indicators of fecal pollution are very similar to those of *E.coli* including re-growth in the environment as well as the presence of *Enterococci* in the environment without any fecal contamination (Hartz et al., 2008). In addition to concerns with pH and temperature, predators in the environment contribute to die off of FC and EC (Davies et al., 1995 and Anderson et al., 2005).

Human Viruses (HV) in Water and their Relationship with Fecal Indicator Bacteria (FIB) and Coliphages

There are more then 100 enteric viruses associated with fecal matter such as Norovirus, Adenovirus, Hepatitis A Virus, Rotavirus, and Enterovirus, as well as many others that can be transmitted in water and cause serious disease. Viruses are a large concern

because they are resistant to many drinking water and wastewater treatment processes. FIB and viruses do not behave in the same manner under the same environmental conditions. In a comparison with F+ coliphages, bacteria were found to be more sensitive to sunlight and this higher rate of inactivation is increased in seawater (Sinton et al., 2002). The monitoring of enteric viruses in water is not commonly performed because it is time consuming and costly, giving preference to the use of fecal indicators. However, it has been reported that in some cases, human viruses are present in the absence of FIBs (Allwood et al., 2003). EC was found to be inactivated at a significantly higher rate than F+ coliphages (Schiff et al., 2004). For example, infectious enterovirus was detected off the coast of Florida where standard microbial indicators like FC and EC were found at levels under the recommended levels (Wetz et al., 2004).

It was found that the presence of adenoviruses was significantly correlated to the concentration of F+ coliphages in coastal waters (Jiang et al., 2001). In a cohort study of beachgoers in California, it was found that only F+ coliphages from a study group that included FIBs had an association with illness (Colford Jr. et al., 2007). High correlations have been observed between FRNA coliphages and enteroviruses in a variety of environments(Havelaar et al., 1993). Another study found that coliphages provide information in addition to that provided by bacteria regarding the fate of indicators both bacterial and viral in river waters (Lucena, F. et al., 2003). There was an increase in the number of positive river water samples for pathogenic viral genome with increasing concentration of somatic coliphages, with TC concentrations having no relation to the samples positive with viral genome (Skraber et al., 2004). It has been suggested that using a suite of indicator organisms might be more predictive of the presence of bacterial as well as

viral pathogens in water monitoring (Harwood et al., 2005). It appears that bacterial indicators are inadequate and unreliable in determining the level of viral contamination due to fecal pollution, so alternative indicators of water quality are needed (Weisberg et al., 2007).

Methods for Coliphage detection

Plaque Enumeration

Currently there are several methods used to detect coliphages from environmental samples. The single agar layer method (SAL) is a plaque method used to enumerate coliphages in volumes up to 100 ml. It is performed by assaying 100-ml of sample with Magnesium Chloride ($MgCl_2$), log-phase host bacteria specific for the coliphage of interest, and 100 ml of double strength molten Tryptic Soy Agar (TSA). The sample is mixed thoroughly and the total volume is poured into 5-10 plates. Following overnight incubation, lysis zones (plaques) are counted and summed for all plates from a single sample and the quantity expressed as plaque forming units (PFU)/100 ml. (EPA Method 1602, USEPA 2001). The double agar layer (DAL) method is similar to the SAL, with the addition of a underlying agar monolayer to increase plaque enumeration. DAL is generally used to enumerate stock suspension of coliphage used in spiking experiments. Higher coliphage counts have been observed than with the SAL (Havelaar, A.H. and Hogeboom, W.M., 1983).

Two-step enrichment spot-plate

Method 1601 is a presence /absence test that can be modified as a most probable number (MPN) quantification test for coliphages. One liter samples of water are supplemented with $MgCl_2$, log-phase host bacteria specific for the coliphage of interest, and Tryptic Soy Broth (TSB). After overnight incubation, samples are spotted onto Tryptic Soy

Agar (TSA) with specific host bacteria for each type of coliphage and incubated overnight and examined for plaques (Method 1601, USEPA 2001). The two step enrichment method can be used to analyze larger volumes of water, with a detection limit as low as 1 PFU/1L. It was found that enrichment methods were preferred over filter methods because of their ease and ability to quantify low levels of coliphages in large samples (Sobsey et al., 2004).

Membrane Filtration

Filter absorption methods have also been employed to detect coliphages from the environment. In this method the sample is passed through a filter usually 0.45um in size. The filter is then removed and placed face down onto an agar plate with TSA and incubated overnight. These filters usually require or have special characteristics that help to retain different organisms; coliphages are attached to the filter by isoelectric charge. Following overnight incubation the plaques are enumerated for a PFU count per 100 ml (Sobsey et al., 1990). This method has been modified over the years with the addition of different elulents to remove the coliphages attached to the filters. A method combining membrane filtration (MF) and swirling elution (SE) showed that recovery rates of coliphages could be improved from the 12-49% described by Sobsey et al. to 75% for FRNA coliphages (Sinton et al.,1995). A problem associated with membrane filtration is that this method can be cumbersome because of the amount of materials and equipment needed to perform the analysis.

Molecular Methods

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) is a method used to enzymatically amplify nucleic acid. Before PCR can begin, a reverse transcription step is

needed to convert the RNA to cDNA. The PCR reaction consists of three steps: denaturation, anneling, and elongation. In the denaturation step the temperature of the reaction is brought up to an elevated temperature where the two strands of DNA are separated. The annealing step occurs when the specific primers align and binds to the now single strand of DNA on its complementary sequence. An enzyme called Taq polymerase use the 3' OH terminal of the primer to start the polymerization of the complementary DNA strain at a temperature ranging between 50 to 60 °C. The final step is elongation where the polymerase completes synthesis of the target molecule essentially doubling the target, the temperature of this step is the optimum temperature for the polymerase usually 72 °C. These three steps continue for 30 to 40 cycles creating an exponential amount of the target molecule. Molecular methods using PCR can be time consuming, expensive, and complex requiring extensive training. Results from a study that compared the results of plaque assay to RT-RCR from environmental samples showed that there were differences in levels of two types of coliphages (cultureable and unculturable), and that the non-viable organisms detected by these methods do not provide useful information regarding human health risks (Rose et al., 1997).Detection limits are also variable, so the inability to detect a virus doesn't necessarily mean there is no virus present. The study found that RT-PCR methods detected F+ coliphages in samples where the plaque assay yielded negative results (Rose et al., 1997). RT-PCR was positive for all F RNA plaques and was able to distinguish FRNA coliphages in mixed populations of coliphages (Yee et al., 2006). In addition to the variable detection limits associated with this method another disadvantage includes problems associated with inhibition of the PCR assay.

F+ RNA Coliphage Typing and Source Tracking

Improving standard detection of coliphages is essential to assisting water quality mangers as they work to address issues related to water bodies. Current detection methods can provide both quantitative and qualitative information regarding the presence and concentration of particular coliphages; however they do not provide information about the potential source of contamination. Microbial source tracking is an important piece of the water quality monitoring puzzle, aiding in water quality assessment (Stewart-Pullaro et al., 2006).

The typing of F+ coliphages have the potential application for source tracking purposes because it provides information about the distribution of different sub-groups of F+ coliphages from different sources (Schaper et al., 2002 and Vantarkis et al., 2006). Other source tracking approaches include the generation of large libraries. Libraries of different microbial characteristics from different pollution sources are collected for each study area. Isolates from water samples are then compared to the information stored in these large libraries. A major problem with these libraries is that they vary from site to site and season to season. And furthermore for them to be representative of an area hundreds of thousands of isolates may be necessary (Wiggins et al., 2003). Source tracking using F+ coliphage typing can be accomplished by serotyping or genotyping isolates (Hsu et al., 1995). The typing of F+ coliphages on a large scale has also been demonstrated with the use of RT-PCR and reverse line blot hybridization (RLB) (Vinje et al., 2004).

The Importance of Rapid Detection

There is also a need for rapid water quality monitoring tests, because current test take more then 24 hours to complete. Often by the time the results are available, human exposure has already occurred or the microbes of interest may have already returned to levels below the action threshold, leaving beaches closed even when the threat from fecal contamination has subsided. Recently, a rapid typing assay known as the Coliphage Latex Agglutination Typing assay (CLAT) with the capacity to distinguish between coliphages belonging to male-specific) F+ RNA Serogroups I, II, III, and IV and the F+DNA coliphage group was developed. Distinguishing between the groups or types of male specific F+ RNA coliphages can aid in the identification of the source of the fecal contamination as being human or animal (Hsu et al., 1995; Schaper et al., 2002).The CLAT uses antibody-coated latex beads to detect male-specific coliphages so that they may be identified and the information used to take actions intended to control both human and animal sources of fecal pollution (Love and Sobsey, 2007).The goal for this test is to have a same day, reliable, and inexpensive method for detecting and typing indicator viruses for the monitoring of recreational waters. However, the effectiveness of its use in real samples has not yet been determined.

Objectives

The overall goal of this project was to compare the detection of male-specific (F+) coliphages in marine waters by the overnight enrichment-spot plate method (EPA Method 1601) with a modified version of the enrichment method for rapid (5hr) detection by the CLAT assay. Specific objectives were to:

- Evaluate the use of the Coliphage Latex Agglutination Typing (CLAT) assay for detection of Male-Specific Coliphages in samples of recreational seawater.
- Evaluate a rapid coliphage enrichment method and modifications of it for rapid detection of male-specific coliphages in marine recreational seawaters.
- Evaluate the application of coliphage typing with the Coliphage Latex Agglutination and Typing (CLAT) assay to enriched marine recreational seawater samples for the purpose of rapid microbial source tracking.

Materials and Methods

Sample Collection.

During the summers of 2007 and 2008, 101 and 328 water samples were collected from five sampling sites at Doheny State Beach in Dana Point, CA respectively. This beach has experienced relatively large densities of fecal indicator bacteria in its waters and has been subject to frequent posted warning to swimmers about excess fecal contamination levels in previous years. All Doheny Beach sampling sites were located linearly along the beach except one (site "C"), which was located in a lagoon of San Juan Creek, located across a sand beam from the Pacific Ocean.

Two-liter water samples were collected in sterile containers and at a depth of approximately 0.5m (ankle to knee depth) defined by the California County Health Departments. Water sample collection times were 8 AM, 1PM, and 3 PM for Doheny Beach. Water samples were then sent overnight on blue ice (freezer packs) by commercial air carrier to UNC-Chapel Hill for analysis.

Modified F + Coliphage Culture and Enumeration.

Two-Step Enrichment (Method 1601, USEPA 2001).

Two-liter marine water samples were split into two 1-liter bottles, one for male specific (F+) coliphage culture and the other for somatic coliphage culture. An enrichment medium diluted to a final concentration of 0.5X tryptic soy broth (TSB), 0.125X magnesium chloride ($MgCl_2$), 0.1X antibiotic and 50ml of *E. coli* log phase host was added to each one liter bottle. For male specific coliphages, the host was *E. coli* F_{amp} (ATCC # 70089) and the

antibiotics added included Streptomycin and Ampicillin (15µg/ml). Somatic coliphages were cultured using *E. coli* CN13 as host in a medium with Naldixic Acid antibiotic at a final concentration of 0.1X. Within five minutes of the addition of *E. coli* host, each 1-liter sample was split into triplicate volumes each of 300, 30, and 3 ml and placed in a 36°C incubator. After five hr of enrichment or after overnight enrichment, 0.75 ml sub-samples were taken in 1.5 ml microcentrifuge tubes for subsequent coliphage detection by "spot plating."

Spot Plating

Enriched sub-samples were centrifuged at 14,000 rpm (Eppendorf Centrifuge 5415C; Brinkman Instruments; Westbury, NY) for 1 min at room temperature in a microcentrifuge to pellet (physically remove by sedimentation) bacterial cells. Ten microliters (µl) of enrichment supernatants were spotted onto Tryptic Soy Agar (TSA) plates made of Bacto Agar and TSB media containing antibiotics and log-phase *E. coli* host. After the applied spots dried, the plates were inverted and incubated overnight at 36°C. Spot plates were prepared by autoclaving TSA for 15 minutes. Following autoclaving, the agar was allowed to cool to a temperature between 45 to 47°C before adding 1% by volume of 100X Streptomycin/Ampicillin stock solution and 2% (2ml/100ml) of log phase *E. coli* Famp host for detection of male specific coliphages to achieve final concentrations of 0.1Xfor streptomycin and Ampicillin, respectively. For somatic coliphages, the agar was again cooled to 45 to 47°C before adding 1% by volume of 100X Naladixic Acid Solution solution and 2% (2ml/100ml) of log phase *E. coli* CN-13.The agar-host mixture was then poured into a 150mm diameter plate and allowed to cool. Spot plate lysis zones (clear zones produced by coliphage lysis of host) were enumerated and results calculated as most probable number

(MPN) of coliphage/100 ml using a MPN calculator (MPN Calculator, Build 23), based on which of the enrichment volumes and their corresponding spots were positive and negative.

Rapid F+ coliphage detection by enrichment and CLAT.

The CLAT assay utilizes a panel of five different serotyping solutions composed of polyclonal anti-coliphage rabbit sera absorbed onto 0.3 um diameter polystyrene beads in a solution of PBS with 0.01% bovine serum albumin (BSA) (pH 7.2). These serotyping solutions were prepared using coliphage antisera raised individually in rabbits for F+ RNA coliphages of groups I, II, III, IV, and F+ DNA coliphages. The CLAT assay was performed as previously described (Love and Sobsey., 2007) by mixing five µl of an enriched sample and five µl of a CLAT solution on an agglutination card for ~60 sec with a sterile toothpick. Agglutination was scored positive if the formation of clumps was visible on the agglutination card after 60 sec and negative if clumping was absent. CLAT was also performed on re-enrichments and on recovered virus lysis zone material of spot plates (suspended in 1ml of 0.5X TSB, 0.1X Streptomycin and Ampicilin, and 0.125X MgCl2)to better characterize method performance, accuracy and precision.

Serotyping Improvement Methods.

For Doheny Beach samples during 2007, spots of samples that had positive lysis zones following standard overnight and rapid enrichment but were F+ coliphage negative by direct CLAT serotyping from enrichments, had their lysis zones picked for further analysis. The zones were picked, inoculated into 25 µl of 1X TSB and stored at -80°C. These frozen lysis zones were subjected to F+ coliphage re-enrichment and CLAT serotyping as follows. One ml volumes of TSB enrichment media containing log phase *E. coli* host were prepared in

1.5 ml microcentrifuge tubes. Using sterilized wood sticks, scrapings of each frozen isolate were inoculated into a separate tube of TSB-host, incubated for 18 hours at 37°C and serotyped using the CLAT procedure described above.

E. coli Growth Curve.

To observe the growth kinetics of *E. coil* F_{amp} in seawater, the culture methods for rapid coliphage detection were used. An initial overnight culture of *E. coli* F_{amp} was grown in 1X TSB at 37°C with shaking for 18 hours. Following overnight growth, an aliquot of the culture was grown to a log phase optical density of between 0.25-0.6 .Then a 12.5-ml volume of the log phase culture was added to a bottle containing 250 ml broth culture consisting of 0.5X TSB, 0.125X $MgCl_2$ stock solution (4M; final concentration 0.05 M) and 0.1X Streptomycin/Ampicillin stock solution. A volume of 25 ml of log phase culture was added to a second bottle containing 250 ml of the same ingredients plus an equal (250 ml) volume of Instant Ocean (IO) at a salinity of 32 parts per thousand (ppt) to make the final concentration of NaCl 16 ppt. The culture was then grown at 37°C with shaking for 5 hours. Samples were taken every 30 minutes and measured for optical density at 520 nm (OD) in a spectrophotometer (Spectronic 1201; Milton Roy Company) to determine the effects of salt concentration on *E. coli* growth rate.

Coliphage and *E. Coli* Growth Studies.

Rapid coliphage culture methods were used to determine the concentration of both *E. coli* F_{amp} in CFU/100ml and F+ Coliphage group I (MS2) and group III (Qß) under dilute and undiluted conditions. Undilute conditions consisted of IO in dionized water at a concentration of 32 ppt, 0.5X TSB, 0.125X $MgCl_2$, 0.1X Streptomycin/Ampicillin, 50 ml of

log phase *E. coli* F_{amp} host, and 1PFU of both coliphage MS2 and Qß coliphage, in a total volume of 1L. Dilute conditions consisted of the same conditions as the undilute sample adjusted to volumes to account for the addition of 1 L of DI water (0.5X TSB, 0.125X $MgCl_2$, 0.1X Streptomycin/Ampicillin, 100 ml of log phase *E. coli* Famp host, and 1PFU of both coliphage MS2 and Qß coliphage) for a final IO concentration of 16 ppt in a total volume of 2 L. Every hour for 5 hours 100 µl were removed to quantify coliphage concentration using real-time PCR. And every hour, 1 ml and 10 ml of the sample was removed to determine *E. coli* concentration as CFU and coliphage infectivity concentration as PFU, respectively.

Viral Nucleic Acid Extraction.

In order to obtain purified viral nucleic acid (RNA), 100 µl volumes of each sample were chemically extracted using a guanidinium thocynanate (GuSCN) extraction method adapted by Boom et al. (1990). One-hundred microliters of the sample were mixed with 100µl of the GuSCN extraction buffer. The GuSCN extraction buffer contains 120 g of guanidinium thiocyanate, 100 ml of TE Buffer pH 8.0 (Ambion, Austin, TX), 55 mM sodium chloride, 33 mM sodium acetate, and 4.4 mg of polyadenylic acid (5') potassium salt in a total volume of 240 ml. Each sample was vortex mixed for 15 seconds and incubated at room temperature for 10 minutes. Then, 0.2 ml of 100% ethanol were added and the mixture vortex mixed for another 15 seconds. The solution was transferred to a HiBind RNA minicolumn (E.Z.N.A. RNA isolation system, OMEGA BioTek, Doraville, GA) and centrifuged at 16,000 xg for 1 minute. The filtrate was discarded, and the column containing the RNA was washed by loading 0.5 ml of 75% ethanol into the column, centrifuging for 1 minute at 16,000 x g, and then discarding the filtrate. This ethanol wash step was repeated twice more,

for a total of three ethanol washes. The column was centrifuged for 2 minutes at 16,000 x g to dry the remaining ethanol. Then, the nucleic acids were eluted from the column with 50 µl of nuclease free water and the eluted nucleic acid was stored at -80°C until analyzed.

Quantification of F+ RNA Coliphages by Real-Time PCR.

The Quantitech RT-PCR kit (Qiagen, Valencia, CA) was used for real-time PCR quantification of MS2 and Qß RNA. The RT-PCR reaction was performed in volumes of 25µL, with 0.2 µM of each primers and 0.2µM of probe and 2 µL of sample RNA extract added to the reaction mixture. The real time PCR primers and probe for the quantification of MS2 were previously described by Bae and Schwab (2008), and the primers and probe for the quantification of the coliphage Q b were described by Kirs and Smith (2007). The RT-PCR amplification parameters were as follow: for MS2, 50°C for 30 minutes, 94°C for 15 minutes, and 45 cycles of 94 °C for 15 sec, 55° for 30 sec, and 72°C for 15 sec.; for Qß, 50°C for 30 minutes, 94°C for 15 minutes, and 45 cycles of 94°C for 15 sec, 60 °C for 30 sec, and 72°C for 15 sec. The primers specific for the detection of replicase gene of MS2 were MS2KS1: 5' CTCTCTGGCTACCGATCGTC 3' and MS2KS2: 5'ACACTCCGTTCCCTCAACG 3'. The dual label probe was MS2KS3 probe: 5'ACACGCGGTCCGCTATAACGAGT 3', labeled with FAM in the 5' end and with black quencher in the 3' end. For Qß coat proteins genes the primers were: Group 3 Forward: 5'CCGCGTGGGGTAAATCC'3, Group 3 Reverse: 5'-TTACGATTGCGAGAAGGCTG-3', and the dual label probe:

5' ACGGCAAGCGGGTGCAGTTCCTGCCGT 3', labeled in the 5' end with FAM and in the 3' end with black quencher.

A calibration curve was generated by diluting the stock of each coliphage (MS2 and Qß) in ten fold dilutions. A Smart Cycler (v. 2.0c, Cepheid, Sunnyvale, CA) was used for performing the real-time analysis. For Qß we used the equation $y=10^{-0.306x + 13.66}$ to convert CT values to PCR units. The equation $y=-0.2627x + 10.152$ was used to convert CT values for MS2 into PCR units/µl. PCR units/µl were converted to PCR units/ml by multiplying PCR unit/µl X volume fraction.

Statistical Analysis.

Means and standard deviations for the concentrations (MPN/100ml) of F+ coliphage were calculated using Microsoft Office Excel 2007 (Microsoft Corp., Redmond, WA). Fisher's Exact Tests and McNemar's Tests were done to determine differences between the different coliphage methods and were calculated using QuickCalcs (GraphPad Software Inc., La Jolla, CA). ANOVAs were performed on two or more variables to determine differences between sampling times and sites using Minitab v 14. (Minitab, Inc., State College, PA). The Wilcoxon-Ranked Sign Test was performed to compare coliphage methods using Minitab v 14 as well.

Results

Method Performance

Dohney Beach Coliphage Results, 2007

During the summer of 2007, 101 marine water samples were analyzed for F+ coliphage at Doheny Beach, CA. Using the standard EPA method 1601, consisting of two steps: overnight (OV) enrichment and spot plating, F+ coliphages were detected in 27 (26.7%) of the 101 samples (Table 1). However, detection of F+ coliphage by the OV enrichment-CLAT assay resulted in only 4 (3.96%) positive samples. From the positive samples by OV enrichment and spot-plating, lysis zones picked from spot plates and assayed with the CLAT resulted in 23 (22.7%) positive samples. There was no significant difference in positivity between OV enrichment and spot plate assay (26.7% positive) and CLAT assay applied to OV-enrichment and spot plates (22.7% positive) ($p>0.05$, Fisher's Exact Test). Using a modified EPA method 1601, in which samples were rapidly (5 hr) enriched 19 (18.8%) were positive by spot plating lower than after overnight enrichment and spot plating (26.7%) but this reduction was not significant (Fisher's Exact Test ($p > 0.05$). When assaying using the 5hr-enrichment-CLAT only 5 (4.95%) of the 101 were positive, compared to the 5 hr enrichment-spot plate assay, in which 12 of 101 (11%) were positive but was not a significant difference in positivity (Fisher's Exact Test ($p>0.05$).

Table 1. Comparison of the rapid 5hr enrichment CLAT assay with the two step enrichment-spot plate USEPA method 1601 for the F+ Coliphage Detection From seawater of Doheny Beach (n= 101 Samples)

Beach	Five-hr enrichment			Overnight enrichment		
Detection Methods	Spot Plate	CLAT[a] using broth	CLAT[b] using Lysis Zones	Spot Plate	CLAT using broth	CLAT using Lysis Zones
No. positive	19	5	12	27	4	19
(% positive)[c]	(18.8%)	(5.00%)	(11.1%)	(26.7%)	(4.00%)	(18.6%)

[a] CLAT = coliphage latex agglutination test applied to broth enrichment cultures

[b] CLAT applied to coliphages collected from lysis zones of spot plates.

[c] Percent positive was determined from 101 samples

The different methods for the detection of coliphages were compared using an Wilcoxon's Ranks Test (Table 2). Overnight enrichment-spot plate results were significantly different from rapid enrichment spot plate (P= 0.02). However the coliphage detection by rapid enrichment-CLAT was not significantly different from the overnight enrichment CLAT assay (Table 2, p> 0.1)

Table 2. Pair-wise comparison of the different coliphage methods by Wilcoxon' Rank Test

		5 hour enrichment			OV Enrichment		
		SPOT	CLAT	Lysis-CLAT	SPOT	CLAT	Lysis-CLAT
5 hour enrichment	Spot Plate	1.000					
	CLAT	0.000	1.000				
	Lysis-CLAT	0.008	0.035	1.000			
OV enrichment	Spot-Plate	0.033	0.000	0.000	1.000		
	CLAT	0.000	0.655	0.046	0.000	1.000	
	Lysis-CLAT	1.000	0.001	0.020	0.005	0.002	1.00

A comparison of the results from the standard overnight enrichment-spot plate assay with the CLAT assay after overnight enrichment (Table 3) revealed that the CLAT assay has a sensitivity of 22.2% and a specificity of 100%. Of the 101 samples analyzed, only 6 were positive by both the enrichment spot plate method and the CLAT assay. Twenty-one samples were positive by the enrichment-spot plate method and negative when assayed with the CLAT. Seventy-four of 101 samples (73.2%) were negative by both methods. The difference between the standard enrichment-spot plate method and the enrichment-CLAT assay is statistically significant ($p<0.0001$, McNemar's Test, a non-parametric method used to compare paired proportions).

Table 3. Cross tabulation of the F+ coliphage results detected by CLAT and by Spot plate from Doheny Beach, CA Samples Enriched Overnight.

		Spot Plate Pos.	Spot Plate Neg.	Total
CLAT	Pos.	6	0	6
	Neg.	21	74	95
	Total	27	74	101

The average concentration of F+ coliphage in Doheny Beach samples negative by using rapid 5hr enrichment CLAT assay, was 0.66 MPN/100ml as determined using an MPN version of the USEPA method 1601 (Table 4). In samples positive by the 5hr enrichment-CLAT assay, the average F+ coliphage concentration was 13.4 MPN/100ml. There were no instances in which the CLAT assay applied to overnight enrichments detected a positive sample and the standard overnight-spot plate method did not.

Table 4. Comparison of F+ coliphage concentrations in Doheny Beach samples positive and negative by the rapid CLAT test when assayed by the standard overnight enrichment-spot plate method.

Test Result	Mean (MPN/100ml)	Maximum (MPN/100ml)
Neg.	0.66 ± 2.7	15
Pos.	13.4 ± 15.6	33

Doheny Beach Coliphage Results, 2008

Comparison of F+ coliphage detection by spot plating after overnight enrichment and after rapid (5hr) enrichment is shown in Table 5. At Doheny Beach 261 (80.0%) of 326 samples were positive for F+ coliphage by the standard overnight enrichment-spot plate method. Following rapid (5-hour) enrichment-spot plating, only 117 (35.9%) of 326 samples were positive for F+ coliphage. The difference in coliphage positivity between the overnight and rapid enrichment is statistically significant ($p<0.001$, Fisher's Exact Test).

Table 5 F+ coliphage detection in seawater by two step enrichment-spot plate (USEPA method 1601) after rapid (5-hour) vs. overnight enrichment incubation.

Beach	5-hr Incub. Spot Plate	O/N Incub. Spot Plate
Doheny	117 (35.9%)[a]	261 (80.0%)

[a] Percent positive was determined from 326 samples

Using the CLAT to assay for F+ coliphages following rapid (5-hr) enrichment, 16 (21.3%) of 75 samples were positive. For the same samples using spot plate following rapid enrichment 38 (50.6%) of 75 samples were positive (Table 6). A significant difference in positivity was found between the rapid enrichment spot plate and the rapid CLAT assay (Table 6 $p<0.0003$, Fisher's Exact Test). Fourty two percent of F+ coliphage enrichments positive by spot plate were positive with the CLAT assay after rapid enrichment. Overnight enrichment of the 102 samples analyzed by CLAT assay, 24 (23.5%) were positive for F+ coliphage. Of the 102 samples analyzed following OV-spot plate, 77 (75.4%) were positive for F+ coliphage. The difference of positivity between the OV-enrichment spot plate and the OV-CLAT assay plus spot plate after 5-hr enrichment is also significant (Table 6 $p<0.0001$, Fisher's Exact Test) After overnight enrichment, 31.1% of F+ coliphages enrichments detected by spot plate were detectable using the CLAT assay.

Table 6 Detection of F+ Coliphage using the CLAT assay and the spot plate assay after Rapid and Overnight Enrichment at Doheny Beach, CA 2008.

F+ coliphage detection	Five-hr enrichment (n=75)		Overnight enrichment (n=102)	
	Spot Plate	CLAT[a] using broth	Spot Plate	CLAT using broth
No. positive	38	16	77	24
(% positive)	(50.6%)	(21.3%)	(75.4%)	(23.5%)
(% Spot Plate)	(NA)[b]	(42.1%)	(NA)	(31.1%)

[a] CLAT = coliphage latex agglutination test

[b] NA = Not applicable

Based on a standard overnight enrichment-spot plate assay (USEPA Method 1601) in an MPN format, the average concentration of F+ coliphage in the original Doheny Beach samples that were negative using rapid 5hr enrichment CLAT assay, was 2.84 MPN/100ml (Table 7). In samples positive by the 5hr enrichment-CLAT assay, the F+ coliphage concentration was 10.8 MPN/100ml by overnight enrichment-spot plate MPN assay. There is a significant difference between the mean concentrations of coliphages in the broths of samples negative and positive using the 5-hr enrichment-CLAT assay ($p<0.0001$, Un-paired T-test).

Table 7. Comparison of F+ coliphage concentrations in Doheny Beach 2008 samples positive and negative by the rapid CLAT test when assayed by the standard overnight enrichment-spot plate method.

Test Result	Mean (MPN/100ml)	Maximum (MPN/100ml)
Neg.	2.8 ± 8.3	37
Pos.	10.8 ± 15.7	37

In a comparison of F+ coliphage detection results by spot plating and CLAT assay after rapid (5-hr) enrichment, the CLAT assay had a sensitivity of 25.8% and a specificity of 94.1% (Table 8). Of the 75 samples analyzed with the CLAT assay and the rapid enrichment, 15 (30.0%) were positive for both methods and 16 (21.3%) were negative by both methods. One (1.3%) sample was positive by the CLAT assay alone while forty-three samples (57.3%) were positive by the spot plate method alone. Hence when only one of the two assays was positive for F+ coliphages, the spot plate was about 3.6 times more likely to be positive than was the CLAT. There is a significant difference in coliphage positivity between the standard enrichment method and the CLAT assay ($p<0.0001$, McNemar's Test).

Table 8 Cross tabulation of F+ coliphage results detected by CLAT and spot-plate from summer 2008, Doheny Beach CA. samples after rapid enrichment.

		Spot Plate		
		Pos.	Neg.	Total
CLAT	Pos.	15	1	16
	Neg.	43	16	59
	Total	58	17	75

In a comparison of F+ coliphage detection results by spot plating and CLAT assay after overnight enrichment (Table 9), the CLAT assay has a sensitivity of 24.0% and a specificity of 85.0%. Of the 102 samples analyzed by both the CLAT assay and spot plating, 18 (17.6%) were positive for both methods and 23 (22.5%) were negative by both methods. Four samples (3.92%) were positive by the CLAT assay alone and 57 samples (55.8%) were positive by the spot plate method alone. Hence when only one of the two assays were positive for F+ coliphages the spot plating was 14 times more likely to be positive than was the CLAT. There is a significant difference in positivity between the standard method and the CLAT assay ($p<0.0001$, McNemar's Test).

Table 9 Cross tabulation comparison of F+ coliphage results detected by CLAT and spot-plating from summer, 2008 Doheny Beach, CA samples following Overnight Enrichment.

		Spot Plate		
		Pos.	Neg.	Total
CLAT	Pos.	18	4	22
	Neg.	57	23	80
	Total	75	27	102

Comparisons of Coliphage Results and Detection Methods in 2007 and 2008

To determine how performance of both the standard overnight enrichment and rapid-enrichment differed from 2007 to 2008, we examined the percent of samples positive for F+ coliphage and the average F+ coliphage concentrations over similar sample days for both years (Table 9). A key comparison was the extent to which the percentage of enriched samples positive CLAT was higher relative to the percentage of enriched samples positive by spot plate + positive CLAT/+positive spot-plate). This would indicate improved detection of coliphages by CLAT relative to spot plate, which is the standard coliphage detection procedure. As shown in Table 10, the percentage of samples positive by enrichment-CLAT relative to those positive by enrichment-spot plate was higher after enrichment for both 5 hours (43.2% versus 18.8%) and overnight (33.3% versus 15%). These findings document greater coliphage detectability by enrichment-CLAT, relative to enrichment-spot plate.

For both of the enrichment periods (5-hr and overnight) for the enrichment spot plate method there was greater coliphage detection in 2008, when 1-L of DI water was used to dilute to the NaCl of samples by half. Of the 101 samples analyzed over those two years 31 (30.7%) and 19 (18.8%) were positive by spot plating for 2008 and 2007, respectively following overnight enrichment. For the standard overnight enrichment the number of positive samples by spot plate increased from 27 (26.7%) to 76 (75.2%) from 2007 to 2008. For the rapid enrichment there was an increase in coliphage concentration as MPN/100 ml from 0.948 to 1.86 from 2007 to 2008. For overnight enrichment the average concentration increased from 1.46 MPN/100ml in 2007 to 2.62 MPN/100ml in 2008. There was a significant increase ($P<0.0049$, un-paired T-test) in F+ concentration for both rapid and overnight enrichment from 2007 to 2008.

Table 10 Comparison of spot plate positives and average MPN/100ml over the same sample incubation time period for both 2007 and 2008 samples.

	Rapid		**Overnight**	
Year	2008	2007	2008	2007
Spot Plate (+)	37	16	76	27
CLAT (+)	16	3	23	4
Total Samples (n)	75	75	101	101
Percent (+) SP	49.3%	21.3%	75.2%	26.7%
Percent (+) CLAT	21.3%	4.00%	22.8%	4.00%
Percent + CLAT/Percent + SP	43.2%	18.8	30.3	15.0
AVG MPN	4.12	1.21	4.45	1.46

Frequency of F+ Coliphage Occurrence by Sampling Site and Time at Doheny Beach by Standard Overnight Enrichment and 5-hr enrichment-Spot Plate Method in 2007

At Doheny Beach in 2007, the occurrence of F+ coliphage-positive samples declined throughout the day for both overnight and rapid enrichment followed by spot plating (Figure 1). The data collected at Pond sampling site were excluded from the calculations because only data for morning samples were available. The frequency of F+ coliphage positive samples was highest in the morning and decreased later in the day. Mean F+ coliphage concentrations also declined over the course of the day, from 0.675±2.5 MPN/100 ml at 7 AM , to 0.549±2.5 MPN/100 ml at 12 PM, to 0.1±0.0 MPN/100 ml at 4 PM . Maximum F+ coliphage concentrations also declined by the end of the day, from 14 MPN/10 ml at 7 AM and 1PM to 0.11 MPN/100ml at 3PM. It is noteworthy that the majority of 3PM samples were F+ coliphage-negative. The observed decrease in F+ coliphage concentration with increasing time of day at Doheny Beach by overnight enrichment and spot plating assay was statistically significant (ANOVA, $P<0.019$).

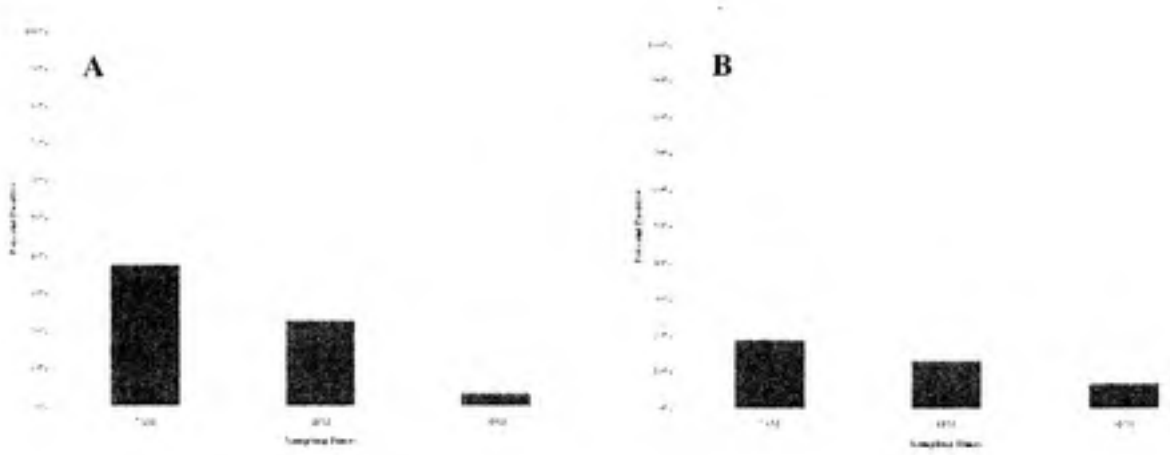

Figure 1 Percent of samples positive for F+ coliphages at Doheny Beach in 2007 at different daily sampling times for overnight (A) and rapid (5hr) enrichment (B) and spot-plate assay.

Sampling days were primarily Friday (June 9th,16th, 23rd, 30th), but other sampling days included Monday (May 26th, June 3rd, and July 4th) and Sunday (July 1st) in summer 2007. The highest coliphage concentrations were detected on May 26th at 33 MPN/100ml and on July 1st at 31 MPN/100ml. Those specific sampling days fell on holiday weekends, specifically Memorial Day and the fourth of July, in which beach goer populations are increased. It could be assumed that the increase in the population contributed to increased viral loading and thus higher coliphage concentrations. Despite inconsistent sampling times by day of the week we were able to recognize trends in F+ coliphage frequencies of occurrence and concentrations at Doheny Beach during the summer of 2007 on the basis of day of the week or summer period (early or late summer season).

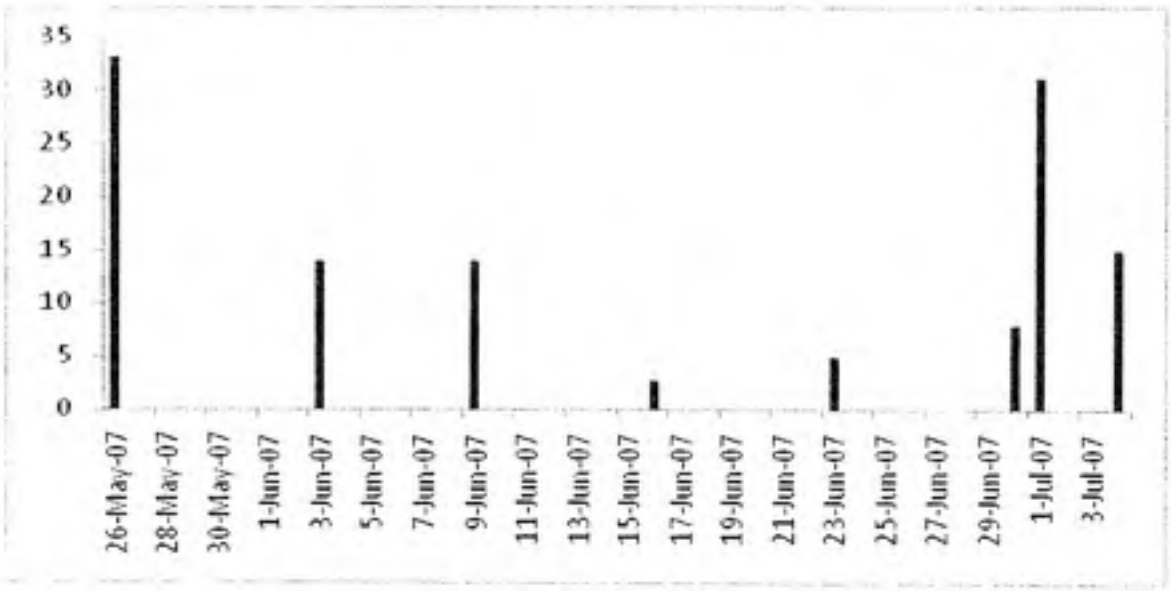

Figure 2 F+ Coliphage concentrations in morning (8:00 AM) samples over the duration of the 2007 study by overnight enrichment spot-plate method.

As shown in Table 11, F+ coliphage concentrations differed significantly among beach sites ($p<0.000$, ANOVA) in 2007. The highest mean concentrations were detected at the Pond with an MPN of 13.2 ± 12.8; these samples were only collected in the morning. There were not significant differences in the concentrations of F+ coliphages between all the beach stations ($P> 0.1$, Turkey pair wise comparisons). However, the concentration of coliphages in samples collected at the station located in the pond (Pond") was significantly different from the beach station ($P < 0.001$ Turkey pair wise comparison)

Table 11. Mean F+ Coliphage concentration at Sampling Sites of Doheny Beach in 2007

Sample Site	MPN/100ml ± STD Dev	Sample Size (n)	Min-Max
Jeddy	0.3±0.6	23	2.7
North Beach	0.7±2.8	24	13.9
South Beach 1	0.7±2.8	24	13.9
South Beach 2	0.1±0.02	22	0.13
Pond	13.2±12.8	8	32.9

Frequency of F+ Coliphage Occurrence by Sampling Site and Time at Doheny Beach in 2008 by Standard Overnight Spot Plate and Enrichment

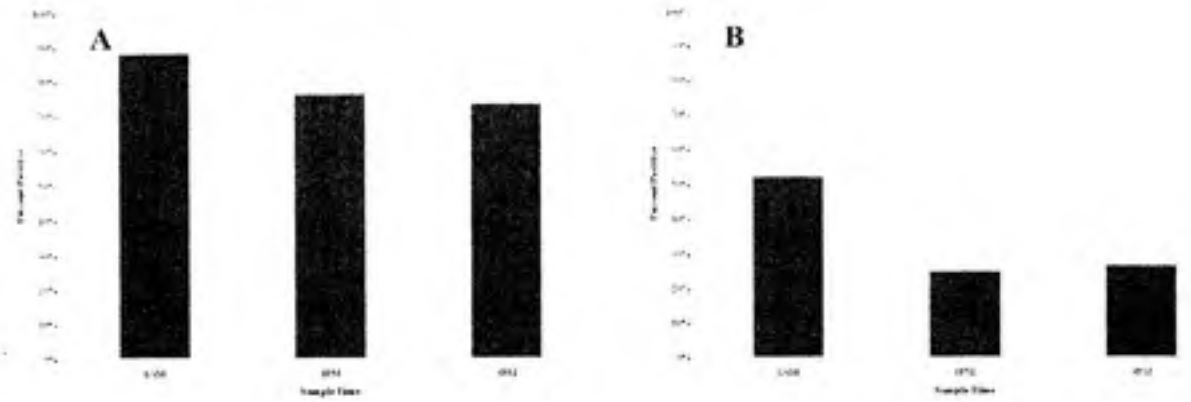

Figure 3 Percent of samples positive for F+ coliphages at Doheny Beach in 2008 at different daily sampling times for overnight (A) and rapid enrichment (B).

As shown in Figure 3, the occurrence of F+ coliphage-positive samples in 2008 at Doheny Beach, declined throughout the day for both overnight (Figure 3A) and rapid (Figure 3B) enrichment followed by spot plating. The data collected at Pond sampling site were excluded from the calculations because only data for morning samples were available. The frequency of F+ coliphage positive samples was highest in the morning and was lower later in the day. The average concentration for F+ coliphages detected at 8AM was 2.8±8.2 with the maximum concentration detected at 37 MPN/100ml. At 1 PM, the average concentration was 1.4±4.4 with the maximum concentration detected at 37 MPN/100ml. At the end of the sampling day at 3PM, the concentration was lowest at 0.7±1.60 with the maximum concentration at 8 MPN/100ml.

In the summer of 2008, weekly sampling days were primarily Saturday and Sunday, with exceptions of Monday May 26thand August 1st and Friday July 4th and August 1st. Samples were not taken on July 12th, 26th, August 9th, 16th, 23rdst, or September 6th, which is why no value is recorded for these dates on Figure 4. Throughout the summer, the F+

coliphage concentration sometimes reached the maximum detection limit of the assay at 37 MPN/100ml. Through the summer including the final day of sampling, the lower detection limit of the assay of 0.1 MPN/100ml also was routinely reached.

Figure 4 Average F+ Coliphages concentration detected in the morning (8:00 AM) samples by MPN Overnight Spot-Plate for the 2008 Summer Sampling Period.

As shown in Table 12, the highest concentrations of F+ coliphage were detected in 2008 at the Pond with an MPN of 19.4 ± 12.8. These Pond samples were only collected in the morning. Samples collected in the station located at the beach waters had concentrations of coliphages significantly lower from the samples collected at the pond ($p < 0.001$ Tukey pair wise comparisons). However, there were not significant differences between any of the samples collected at the beach ($p > 0.1$ Tukey pair wise comparisons).

Table 12 Mean F+ coliphages concentration per each sampling site at Doheny Beach.

Sample Site	MPN/100ml ±SD	Sample Size (n)	Range
Jeddy	1.8±5.2	75	36.9
N Beach	1.8±6.2	76	36.9
S Beach 1	2.1±6.3	74	36.9
S Beach 2	1.0±4.3	75	36.9
Pond	19.4±17.9	26	36.9

The Effect of Salinity in the Growth Kinetics of the *E. coli* Host and the Propagation of F+ Coliphages

Growth of *E. coli* was followed over the course of 5 hours under two salinity conditions: (1) a high salt concentration of 32 ppt and (2) a moderate salt concentration of 16 ppt, following 1:1 dilution of the seawater with DI water. Two trials were undertaken for each concentration. Two hundred and fifty ml of DI water with an IO concentration of 32 ppt with the appropriate amount of nutrients from the media 0.5X TSB, 0.125X $MgCl_2$, 0.1X antibiotics (Streptomycin/Ampicillin), and 12.5 ml of log phase host were analyzed in one bottle. A volume of 25 ml of log phase culture was added to a second bottle containing 250 ml of DI water with IO at a salinity of 32 parts per thousand (ppt) and the same ingredients doubled to account for the addition of an equal (250 ml) volume of DI water to make the final concentration of NaCl 16 ppt along with log phase host. Enrichments were sampled every 30 minutes and the OD 520nm recorded. The initial growth of *E. coli* at both salinity concentrations remained about the same until 120 minutes into the growth period. From there the rate of growth of 16 ppt sample during exponential growth period was more rapid compared to the 32 ppt sample, and at the end of the incubation period the OD 520 of the 16 ppt sample was almost 2.0, while that of the 32 ppt sample was only between 1 and 1.2. The

increased *E. coli* OD in the 16 ppt sample compared to the 32 ppt sample was statistically significant ($p<0.004$, ANOVA).

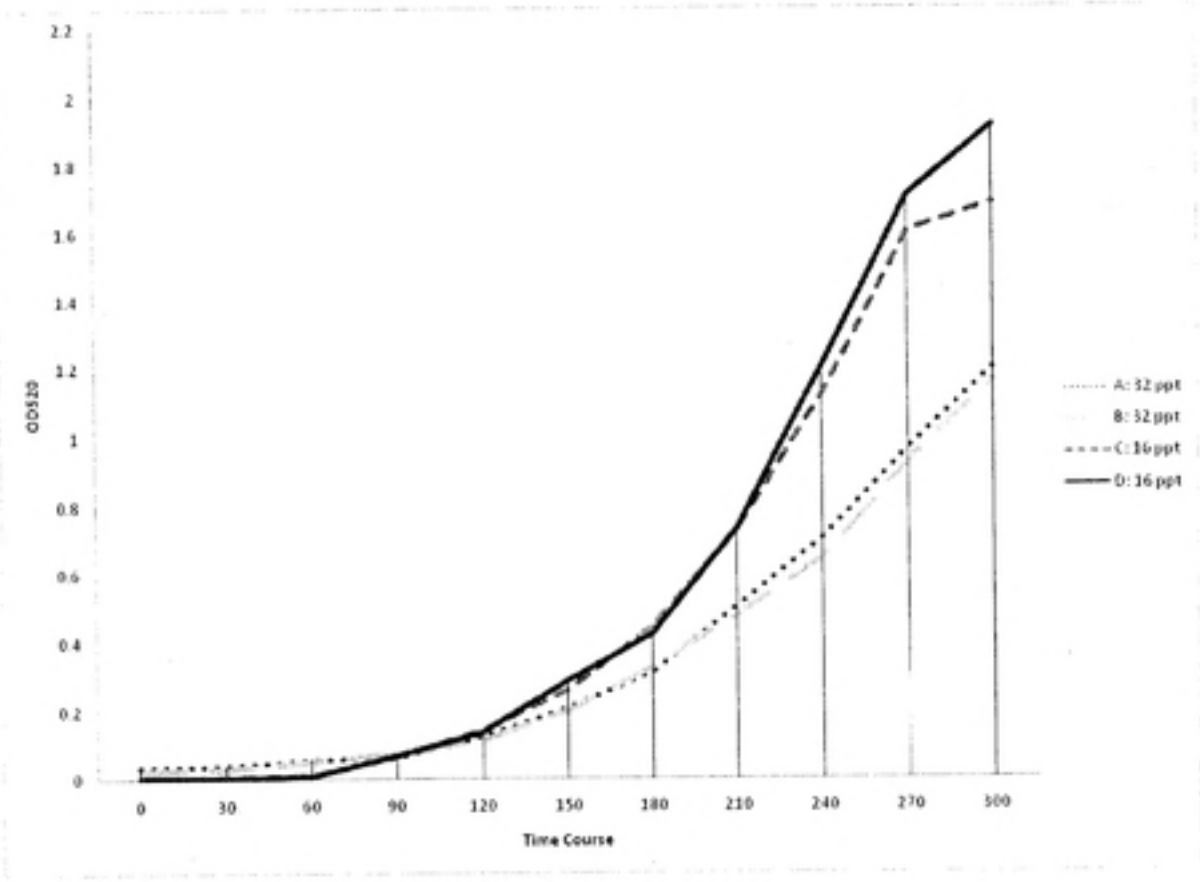

Figure 5. Growth Curve of *Escherichia coli* (*E. coli* F_{amp} (ATCC # 700891) at two different salinities, 32 and 16 PPT of Instant Ocean with 0.5X`TSB over a five hour time course at 37 °C.

***E. coli* and Coliphage Growth Studies.**

Another growth study was undertaken to quantify the concentrations of *E. coli* and F+ coliphage during rapid enrichment, in order to gain a better understanding of *E. coli* growth under high salt (32 ppt) and moderate salt (16 ppt) conditions. One liter of 32 ppt (undilute) NaCl and two-liter of 16 ppt (dilute) NaCl with the appropriate amounts (All media doubled to account for added volume) of TSB, $MgCl_2$, antibiotics, and log phase host were sampled every hour and 10 ml of sample were removed to determine the CFU/ml (Figure B) using spread plating for *E. coli* and PFU/ml (Figure A) for F+ coliphages using SAL method. Additionally, 100 microliters were sampled every hour to quantify coliphage concentration using real-time PCR. As shown in Figure 6B, the dilution of the sample to 16 ppt slightly increased the final yield of CFU/ml of *E. coli* after 5 hours of growth from 9.0×10^8 to 1.0×10^9 and the PFU/ml of F+ coliphage from 2.3×10^5 to 1.0×10^6. However, these concentrations of *E. coli* or F+ coliphages in 32 ppt and 16 ppt samples were not significantly different ($P>0.05$). After five hours the PCR units/ml in the 32 ppt samples for QB were 8.85×10^6 /mL and for the 16 ppt 5.77×10^7 mL. For MS2 the PCR units/ml were 4.44×10^6 /mL in 32 ppt samples and they were not detected in the 16 ppt enrichments. The lower detection limit of the assay is 10^3 PCR units/mL

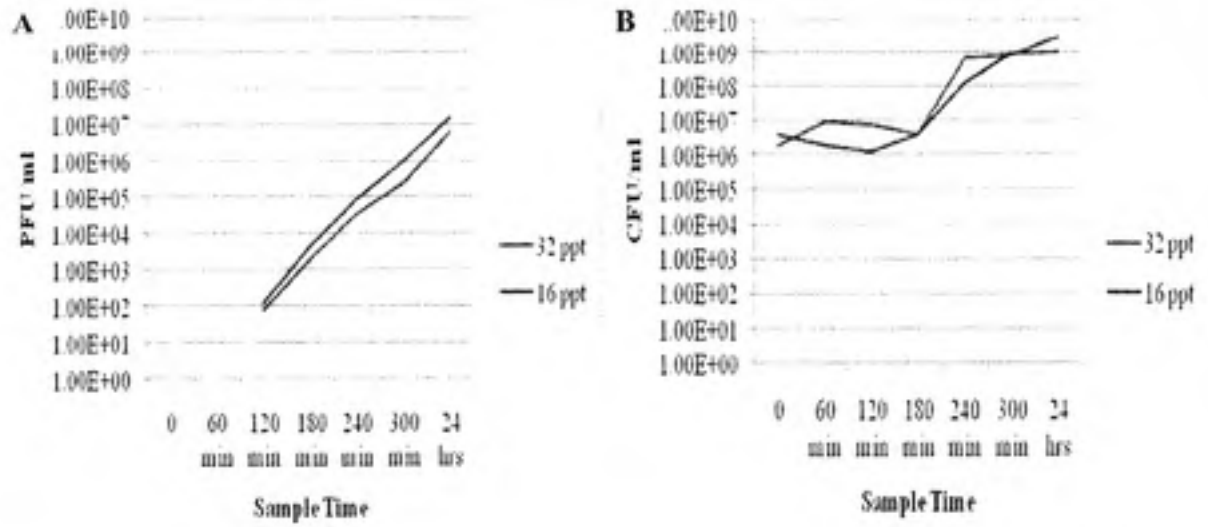

Figure 6 *E. coli* and Coliphage Growth Kinetics in 32 and 16 PPT NaCl waters over a 5-hr Incubation Period at 37 °C

Serotypes of F+ Coliphages

F+ Coliphage Serotypes Detected by CLAT after Enrichment Assay or Enrichment-Spot Plate Assay for Summer, 2007 Samples

Of the F+ coliphages that were serotyped with the CLAT assay, following rapid enrichment that yielded 27 positive samples by overnight enrichment-spot plate assay, 19%, 4%, and 4% were F+ DNA, F+ RNA Group I, and F+ RNA Group II, respectively (Figure 7A). Using plaques picked from the lysis zones of spot-plates for these samples, 33%, 26%, and 4% were F+ DNA, F+ RNA Group I, and F+ RNA Group II, respectively (Figure 7C). Hence, the method of applying the CLAT assay to spot lysis zones after enrichment resulted in an increased detection of F+ RNA serogroup 1 coliphages. By direct CLAT assay

following overnight enrichment, F+ DNA and Group I F+ RNA coliphages were detected in 11% and 4% of the samples, respectively, for total percent F+ coliphage-positive of 15% (Figure 7B). When lysis zones were picked and typed, the percent F+ coliphage positivity rate of samples increased to 59%, 41%, 4%, 4%, and 4% for F+ DNA, F+ RNA Group I, F+ RNA Group II, F+ RNA Group III, and F+ RNA Group IV respectively, for an overall percent positive rate of 74% (Figure 7D).

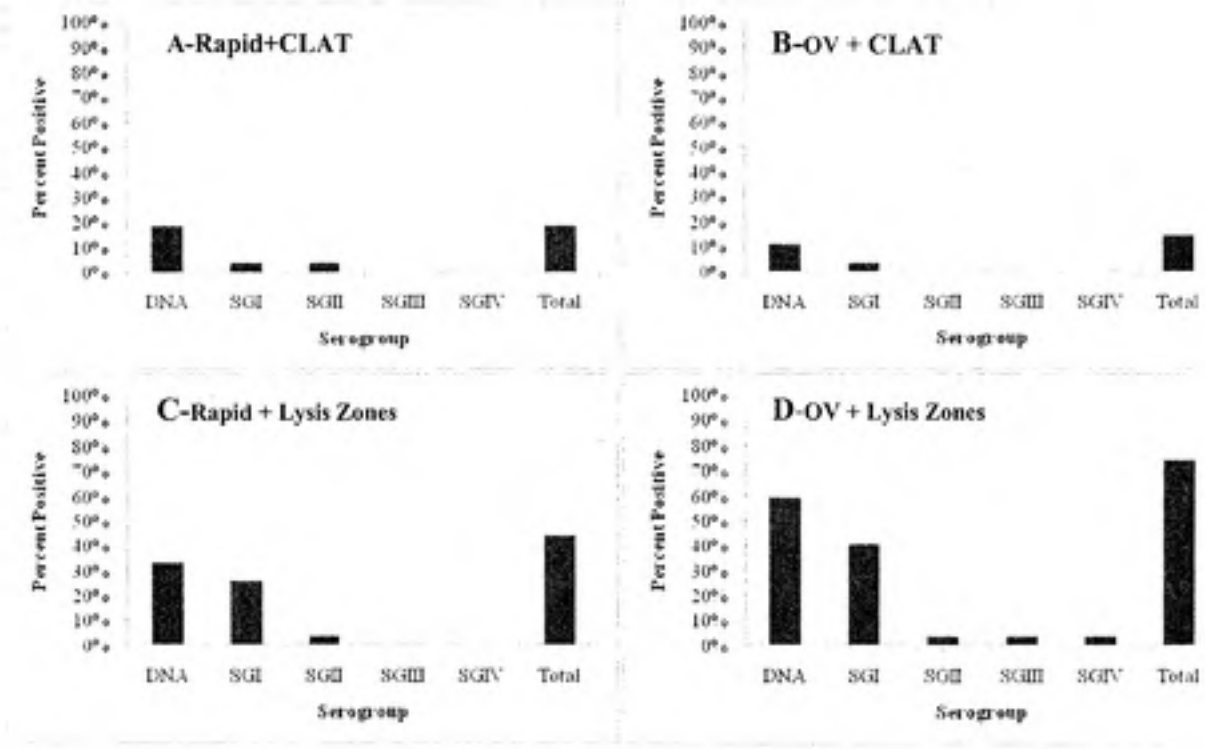

Figure 7. F+ Coliphage Serotypes at Doheny Beach by CLAT Methods Applied to Enrichments or Enrichment Spot Plate Lysis Zones, following Rapid and Overnight Enrichment

F+ Coliphage Serotypes Detected by CLAT after Enrichment-Spot Plate Assay for Summer, 2008 Samples

Following rapid enrichment, 75 samples underwent enrichment-spot plate analysis and CLAT assay serotyping. Of the 75 samples analyzed, 38 were positive by rapid enrichment-spot plate assay. Sixteen of the 75 samples positive by rapid enrichment-CLAT had proportions of F+ coliphage serotype positivity of 21%, 18%, 3%, 16%, and 11% for F+ RNA Serogroups I, II, III, IV, and F+ DNA coliphages, respectively (Figure 8A). After overnight enrichment, 104 samples were analyzed by both overnight enrichment-spot plate and serotyping with the CLAT assay. Of the 101 samples analyzed, 77 were positive for F+ coliphage by spot plating. Twenty-two of the 101 samples were positive by CLAT, with 22 % ,13%, 1%, 4%, and 3% of the samples positive for F+ coliphages of RNA serogroups I, II, III, IV, and F+ DNA (Figure 8B).

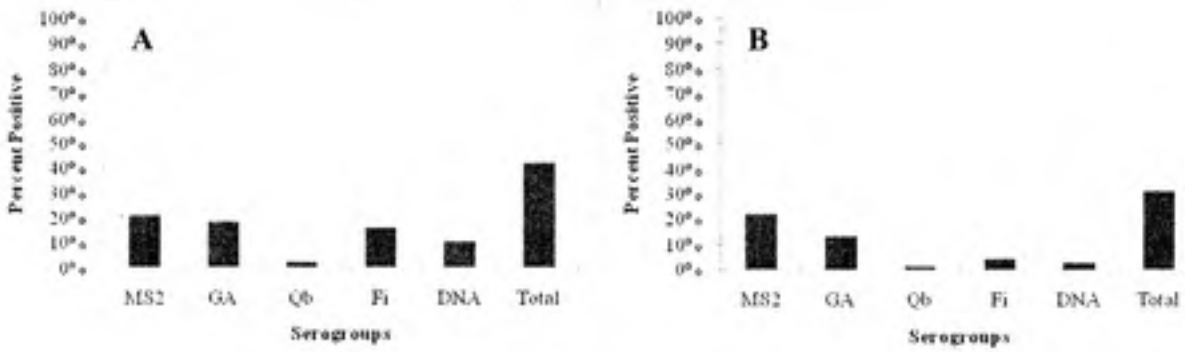

Figure 8 Methods for Serotyping at Doheny Beach following Rapid (A) Enrichment-Direct CLAT Assay and Overnight (B) Enrichment- Direct CLAT Assay

Discussion

Rapid Enrichment in Seawater

The overall goal of this study was to compare the performance of standard EPA Method 1601, an enrichment-spot plate assay, to a modified rapid (5-hour enrichment and CLAT assay) method for the detection of male-specific (F+) coliphages in marine recreational waters. A previous study demonstrated the potential of a rapid (180 minute) enrichment assay for detection of F+ coliphages, in which the F+ coliphages were enriched to detectable levels for a rapid (= 1 minute) particle immunoagglutination assay (CLAT) after 180 minutes (Love and Sobsey, 2007). However, this rapid method has never been tested in the field and never on seawater samples. For rapid F+ coliphage detection in environmental waters, there is a need for an *E. coli* host bacterium that can grow in sub-optimal conditions, such as high salinity, and a need for coliphages to replicate rapidly and to a high yield (burst size) on the bacterial host under such conditions. It has been reported that $>10^4$ CFU/ml of *E. coli* is needed for male specific coliphages to replicate (Wiggins and Alexander, 1985). It has been reported that an F+ coliphage concentration ranging from of 5×10^5 to 5×10^8 PFU/ml is needed for a positive CLAT result in low salinity water (Love and Sobsey, 2007). Therefore, in order for this assay to yield positive results in 5 hours of enrichment by CLAT assay detection, as little as 1 PFU in the enrichment culture volume needs to replicate to $5 \times 10^5$to 5×10^8 PFU/ml by 5 hours of enrichment.

A possible explanation for the poor performance of 5-hr rapid enrichment with coliphage detection by CLAT assay is the high salinity of the sample water. Several experiments on the effect of salt on the growth of the *E. coli* host and propagation of coliphages demonstrated that *E. coli* is adversely affected by the sub-optimal conditions of

the high salt concentration of 32 ppt, which is typical of seawater, when compared to growth at 16 ppt. This reduction in *E. coli* growth in water of high (32 ppt) salinity also affects the propagation of coliphages. In growth studies we found that F+ coliphage concentrations reached 2.36 X 10^5 PFU/ml at 5 hours and 6.00 x 10^6 PFU/ml at 24 hours in an enrichment environment of 32 ppt and they reached concentrations of 1.00 x 10^6 PFU/ml at 5 hours and 1.60 x 10^7 PFU/ml at 24 hours in an enrichment culture environment having a salt concentration of 16 ppt.

There have been several reports on the effect of salinity on the growth of *E. coli*. Sub-lethal stress in *E. coli* was observed at high salinities (30 ppt) in varying growth media, with the most pronounced decrease in survival in EC medium (Anderson et al. 1979). Another study demonstrated that *E. coli* cells starved in seawater or grown in seawater containing organic material can partially adapt to various marine conditions. However, the *E. coli* can undergo changes in metabolic functions and modifications to its structure, affecting other important properties like bacteriophage attachment (Munro et al., 1987). Hence, the stress experienced as a result of high salinities can affect cell growth, cell structure and susceptibility to phage infection, as documented in these previous studies.

In order to reduce stress to *E. coli* brought on by the high salt environment of the undiluted seawater of this study, 1-L of DI water was added to 1 L of sample water in a modified spot plate enrichment method during the summer of 2008 to effectively reduce the salinity of the sample water by half without affecting the concentration of the other enrichment materials (the concentration of all medium ingredients were doubled to account for added volume of DI water). With this dilution, the total volume of water doubled.

The recovery of F+ coliphages improved from 2007 to 2008 with the addition of the dilution step during the enrichment process. Besides to this improvement in F+ coliphage recovery by reducing seawater salinity through dilution, the recovery of somatic coliphages from 2007 to 2008 did not change significantly. The somatic coliphage enrichment methods from 2007 to 2008 were exactly the same, with no addition of DI water to reduce the concentration of salt in the sample. Hence, these F+ coliphage results for 2007 and 2008 suggest that the reduction in salt concentration and not other variables including uv-inactivation, temperature change, and differences in weather patterns possibly aided *E. coli* growth, which resulted in an increase of the percent of F+ coliphage-positive samples detected, as well as an increase in the average F+ coliphage concentration in samples of Doheny Beach during 2008.

Despite the increases in the concentration of F+ coliphage detected in seawater samples as well as increased percent positivity of seawater samples, the sensitivity of the modified method needs to be further improved. The lowest amount of an antigen that can be detected by a serological test is known as the test's sensitivity. Specificity is the ability of an antibody test to distinguish a single type of antigen. The sensitivity and specificity of the rapid CLAT assay was 22.2% and 100% respectively. Following, the addition of the DI water dilution step prior to enrichment culturing, the sensitivity of the rapid method improved to 25.8% and the specificity decreased to 94.1%. There is no significant difference in the sensitivities and specificities between 2007 and 2008 (Fisher's Exact Test, $p>0.05$). It is assumed that samples negative by the spot plate method would also be negative when using the CLAT assay for detection because the standard spot plate method has a lower detection limit than that of the CLAT assay. The two step enrichment spot plate method can

detect as little as 33 PFU/ml of coliphage in a 1-L sample, in contrast the lower detection limit of the CLAT assay was found to range between 5 x 10^3 to 1 X 10^5 PFU/ml. This assumption is supported by the results of this study, as all samples positive by the CLAT assay were also positive by spot plating techniques.

The CLAT assay is an antibody based agglutination test. Similar tests have been developed and used to assay for various other microorganisms in clinical settings. A rapid and sensitive latex agglutination test was developed for detection of Rotavirus A in clinical settings with a sensitivity and specificity of 98.5% and 100 %, respectively (Andrade de Goes et al., 2008). A similar test was developed for avian flu in specific fluids and tissues with detection rates comparable to other diagnostic tests (Chen et al., 2007). The high sensitivities and specificities found in the clinical setting are largely due to the high titers of the target viruses in the samples being analyzed. Because of the low levels of viruses in the environment, most tests used to identify F-specific RNA sub-groups emply methods that are of greater sensitivity but are also more technically intensive, time-consuming and expensive, such nucleic acid amplification-hybridization tests. The reverse line blot hybridization assay described by Vinje et al., 2004 is one such test. It was able to genotype 99% of enriched samples from various environmental sources.

Hybridization has been shown to be successful in distinguishing between F-specific coliphages for the purpose of source tracking (Stapleton et al., 2007 and Vantarkis et al., 2006). Because of the complexity and the time required to complete this type of analysis, an alternative method for simple and rapid typing coliphages is warranted. Although the CLAT assay in its current form does not appear to have a high sensitivity, it could still be used as a preliminary or screening test to rapidly determine the source of male specific coliphage in

recreational waters, until a more sensitive hybridization can be performed. Additionally, future modifications and improvements to the F-specific coliphage CLAT assay may make it more appropriate for standardized testing of recreational waters.

CLAT detection of F+ Coliphages for the years 2007 and 2008

Viruses were unable to be enriched consistently to levels that were detectable by the CLAT assay, resulting in lower detections than the standard 1601 method. At Doheny Beach during the summer of 2007, lysis zones were picked to address concerns regarding low virus titers in enriched samples. When plaques were picked from spot plates, the number of F+ coliphage positive samples for each F+ coliphage serogroup increased. Picking plaques ensured that there would be a sufficient concentration of viruses to react in the CLAT assay because they are so highly concentrated in the plaques themselves. This finding demonstrated that the coliphages detected in the lysis zones of spot plates can be readily detected by the CLAT assay although the concentrations of coliphages in the enrichment broth itself is not very high and often not high enough to be detected directly by CLAT assay.

In summary, the standard overnight enrichment-spot plate method performed significantly better than the modified rapid enrichment method with direct CLAT assay. This work has shown that there may be potential directions for the development of improved or alternative methods that would lead to more efficient (same-day) F+ coliphage results to rapidly determine the quality of recreational waters. Future directions for work to improve the modified enrichment method may include but are not be limited to: (1) further dilution of the sample water, (2) membrane or other filtration to physically separate the F+ coliphages from the salt from the sample by capturing them on the filter, and different incubation

temperature from 37°C to identify temperatures at which the host can grow best and provide maximum coliphage growth. Development of a strain of *E. coli* that is halotolerant and expresses the F+ plasmid for improved growth in marine waters without any dilution or filtration needed could also serve to improve the rapid F+ coliphage detection method in seawater samples.

F+ Coliphage Occurrence Trends at Doheny Beach

At Doheny Beach, apparent die-off of F+ coliphages was observed throughout the day for both summers. This die-off is believed to occur primarily because of UV-inactivation. In a study to determine the effects of sunlight on the infectivity of *Cryptosporidium parvum*, salinity and sunlight were found to enhance die off of MS_2, *E. coli*, and *C. parvum* oocysts (Nasser et al., 2007). The highest concentrations of F+ coliphages were found at all locations at 8 am. As the day continued, radiation from the sun could have resulted in decreased concentrations of F+ coliphages. At the last sampling period for most days, F+ coliphages were rarely detected. Sinton et al. (1999) showed that F+ coliphages are susceptible to longer solar wavelengths and that the longer these coliphages were exposed to these wavelengths the faster they were inactivated. The results from this study provide water quality managers with information regarding times at which monitoring should begin to maximize coliphage detection, which is in the morning.

Shifts in tidal patterns from 2007 to 2008 occurred, meaning that on any given day the composition of the water is never the same. During the spring cycle, tides can either be higher than average tides or lower than average tides. The two stages of the tidal cycle are the ebb tide in which the water lowers over several hours and the flood tide when the water rises

over several hours. When changes in tide range (Spring/neap) and stage (ebb/flood) are considered together the Spring-ebb tide had the greatest chance of yielding Enterococci concentrations higher than that of the single-sample standard at a majority of southern California beaches(Boehm and Weisberg, 2005). The creek that flowed past the pond intermittingly overflowed and there was an increase in rainfall during the summer of 2008, spilling large amounts of bacteria from the runoff and fecal material from birds and other sources onto the beach. During the summer of 2007 there was a drought in the region so runoff from the creek was minimal. Perhaps as a result, only F+ DNA and group-I coliphages were detected in 2007. Differences in these variables can explain the average F+ coliphage concentrations detected in the morning (8AM) throughout the summer. Without rain to add enteric microbes and other fecal material to the water and inconsistent sampling days it is difficult to determine a predictable pattern of occurrence for coliphages. Nevertheless, F+ coliphages were detected, but primarily in the pond next to the creek. In the summer of 2008 the average F+ coliphage concentration detected was >37 MPN/100ml. This was the maximum detection limit of the overnight spot plate method. The increase in the number of days of rain added enteric microbes and other fecal material to the beach and as a result other groups of F+ RNA coliphages, associated with both animals and humans, were detected. In a study of marine beaches impacted by a continuous point source of Enterococci, it was determined that dilution was the primary cause of decline in bacterial concentrations, followed by UV- inactivation and grazing (Boehm, A.B. et al., 2005). Although bacteria and viruses behave differently in the environment, studies such as this give insight into how variables other than UV-inactivation and temperature affect the concentration of enteric microbes in the water.

In addition to UV-inactivation, tidal patterns, and temperature, population increases during specific times of the summer may have contributed to the F+ coliphage concentrations detected. In the analysis of the morning samples during both summers, there was an increase in F+ concentration on days where one might expect increased populations at the beach. During 2007, sampling days were inconsistent so a clear picture of what was occurring with F+ coliphage occurrence and concentrations is hard to determine. However, the highest concentrations were found on May 26th at 33 MPN/100ml and July 1st at 31 MPN/100ml, which corresponds to the weekends of Memorial Day and the Fourth of July, respectively. During 2008, concentrations of F+ coliphages were also high for the majority of sampling days including Memorial Day and the Fourth of July. The increase in population during the holidays and on weekends increased the number of potential swimmers and bathers who shed their own bacteria and fecal material into the beach.

Potential effects of rainfall and population on the results of this study are consistent with findings of previous studies. An increase in population and an increase in rain activity resulted in an increase in the concentration of indicator organisms in the ground water of Nanded City, India (Chitanand et al., 2008). An increase in upstream loading due to an increase in urbaninization resulted in higher levels of FIB in Turkey (Dere et al., 2006). And in Tokyo, Japan the Tama River was shown to receive significant bacterial concentration loading due to sewer overflow and stormwater effluents in a highly population dense area (Ham et al., 2008). Fecal indicator bacteria were highest on busy holiday weekends in coastal water of North Carolina when there were more boats in marinas (Sobsey et al., 2003).

Microbial Source Tracking and F+ Coliphages

Understanding the ecology of F+ coliphages in the marine environment is important to interpret their occurrence, concentrations, and possibly identify the origin of the contamination. Microbial source tracking (MST) aims to identify the origin of the indicator by analyzing the characteristics of the ones found in the samples analyzed (Scott et al., 2002). In this study, we applied a novel, simple, and rapid method for typing F+ coliphages. The CLAT assay is an antibody based immunoassay that can distinguish between F+ RNA and its four sub-groups (I,II,III, & IV) and F+ DNA in <60 seconds (Love and Sobsey, 2007). However, when combined with rapid enrichment culture and used as a means to detect F+ coliphages, the standard methods of enrichment-CLAT assay performed poorly compared to the standard enrichment spot plate method for coliphage detection. Nevertheless, the method was valuable when used for serotyping the enrichment broths and lysis zones of spot plates, providing rapid distinction between five serogroups for potential source tracking. The results for the enrichment-spot plate-CLAT assay are an improvement over other F+ coliphage detection and typing methods because they are faster and simpler than previously described membrane-based immunoassay methods or nucleic acid hybridization methods.

F+ DNA was the most prevalent coliphage group detected at Doheny beach during 2007. Tests on the initial culture broth following rapid enrichment were 19% DNA-like, 4% MS2-like, and 4% GA-like. Following overnight enrichment, detection was only 11% DNA-like and 4% MS2-like. There was an increase in F+ coliphage positivity when lysis zones were picked from spot plates following both rapid and overnight enrichments. For the rapid test, 33%, 26%, and 4% were F+ DNA-like, F+ RNA MS2-like (Group I), and F+ RNA GA-like (Group II), respectively. When the same was done for the overnight spot plate lysis zones subjected to CLAT assay, F+ DNA-like, F+ RNA MS2-like (I) , GA-like II), Qß-like

(III), and FI-like (IV) were detected in 59%, 41%, 4%, 4%, and 4% of samples respectively. This increase in positivity when spot plate lysis zones were picked indicates the need for higher virus titers. Material picked from lysis zones on spot plates provides far higher virus titers than does direct seawater enrichments for robust detection and typing by CLAT assay.

For the CLAT assay to become a rapid and simple method for typing coliphages it would seem advisable that plaques or lysis zones of samples be isolated for potential further analysis by CLAT. However, steps need to be taken to increase virus titers during the enrichment to lower (improve) the detection limit of the CLAT assay. A study conducted on the effects of freezing and storage temperature on MS2 viability found that viruses decayed faster at temperatures greater than or equal to 10°C and at -20°C. And that the better the quality of the water sampled, the faster the decay (Olson, M.R. et al, 2004). That study highlights the importance of being able to type a sample immediately to avoid coliphage degradation due to freezing and storage.

The dilution step added to the method to reduce salinity during the summer of 2008 aided the CLAT assay in detecting and distinguishing the five F+ coliphage groups of interest, increasing the percent positive for overnight samples from 11% to 22% for DNA-like coliphages. However, there was little increase (19% to 21%) in sample positivity for DNA-like coliphages following rapid enrichment. Despite lower than expected samples positive for specific coliphage groups, there was an increase in the percent positive of the Qß-like and FI-like F+ RNA coliphage groups after rapid enrichment. The dilution appears to have helped enrich coliphage types thought not to be present in the water according to results from 2007. In growth studies of *E. coli* and the propagation of two F+ RNA coliphages, MS2 and Qß, it was found that Qß was enriched to higher levels than that of MS2 over the course

of five hours using real-time RT-PCR. This demonstrates that MS2 in some cases may not be preferentially enriched over other coliphage types as suggested by other studies. Other variables like pH, temperature, and UV-inactivation may play a role in the types of coliphages present in the water at any given time. MS2 is more resistant than Qß to UV-inactivation with MS2 having a 2.3-log reduction as opposed to Qß 4-log reduction at the same UV dose (Simonet and Gantzer., 2006). In a study examining the effects of pH and temperature on the survival of MS2 and Qß coliphages, MS2 was found to generally be more stable over the range of pH (6-8) and temperature (5-35°C) studied (Feng, Y.Y., et al.,2003). Genogroups II (GA type) and III (Qß type) were more sensitive to environmental stresses and treatment, especially when compared to genogroup I (Schaper et al., 2002). These findings might explain the higher detection of MS2 at Doheny Beach over both summers. More specific knowledge about the beaches, especially related to animal fecal sources, such as water fowl, also could contribute to the understand of the higher occurrence of MS2 (Group I) over the other types of coliphages. F+ DNA coliphages were shown to primarily be associated with human related pollution (Long et al., 2005). Observation of F+ DNA coliphages higher than F+ RNA coliphages in samples at Doheny Beach suggest that human wastes are contributing to the levels of F+ coliphage detected.

The results of this study indicate that the dilution of seawater improved *E. coli* growth so that F+ coliphages could be enriched to titers high enough to be detected more frequently using the CLAT assay. However, more work needs to be done to improve the lower detection limit of the assay. The dilution of samples to a lower salinity also improved the ability of the CLAT assay to detect and distinguish between specific groups of coliphages previously thought to not exist and provided valuable information to water resource

managers who rely on data like this to make decisions regarding the water bodies they protect.

In addition to increasing our knowledge about the occurrence and behavior of these viruses in the environment, studies should be undertaken that attempt to directly correlate coliphage concentrations to human health effects, such as gastrointestinal illness. Previous attempts to correlate coliphage concentration to pathogen occurrence or concentrations have made it difficult to understand the impact of viruses in recreational waters on human health. A major contributor to this lack of understanding is the differences in methods used to detect both coliphages and human viral pathogens. Better information on coliphage and human viral pathogen occurrence and concentrations, their relationships to each other and their associations with human health risks are still needed. The findings of this study have at least contributed improved methods for rapid coliphage detection and typing, even though further improvements in the methods are still needed.

Conclusions

1. High salt concentrations of marine waters affect the growth of *E. coli* and thus affect the enumeration of F+ coliphages by standard overnight Method 1601 enrichment and spot plating.

2. Five hours is insufficient amount of time to enrich F+ coliphages to levels adequate for reliable detection using the CLAT assay or the modified (5hr) Method 1601 enrichment-spot plate assay.

3. Dilution of the marine water samples in DI water to lower salinity increases *E. coli* growth, coliphage growth and the ability to better detect and distinguish between F+ RNA sub-groups for source tracking.

4. The low virus titers in rapid (5-hr) coliphage enrichments that prevent reliable and sensitive serotyping by CLAT assay can be overcome by picking lysis zones from spot plate assays and subjecting the recovered coliphages to CLAT assay.

5. More work is needed before the rapid Method 1601 enrichment with CLAT assay can be used in place of the standard Method 1601 and other established F+ coliphage sero- and geno typing methods for sensitive detection and typing of F+ coliphages in recreational seawaters.

6. Based on F+ coliphages detected, it appears that both animal and human fecal sources are contributing to the F+ coliphage population detected at Doheny Beach.

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