FINAL REPORT
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Collaborative National Study Using Molecular Techniques to Detect Hepatitis A Virus and Virulence Factor Genes in *E. coli*

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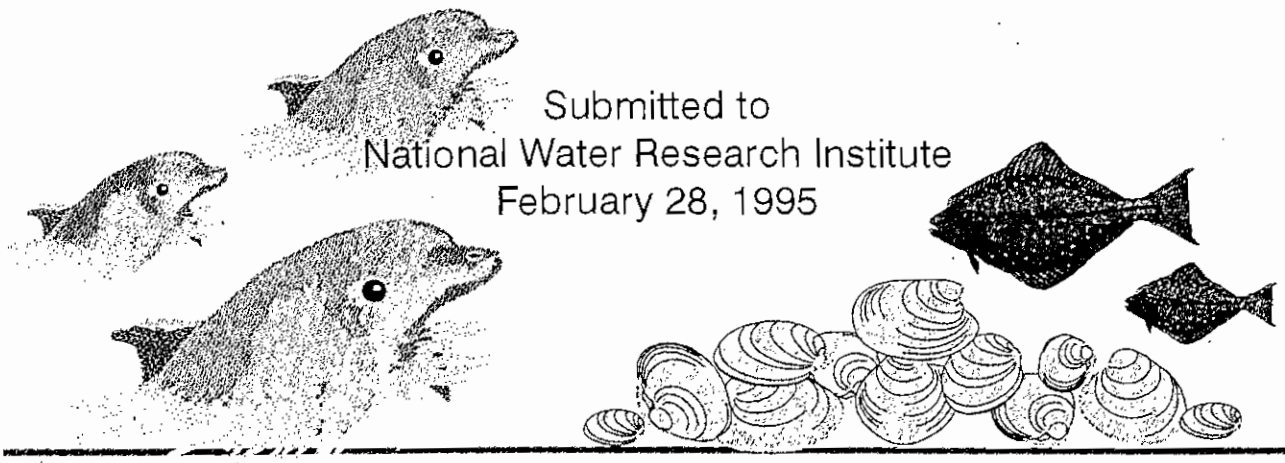

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INTRODUCTION

Infectious disease may be transmitted through the water route via direct contact with contaminated water or through consumption of aquatic organisms such as fish or shellfish, that have bioconcentrated pathogenic microorganisms from the water column in their tissues. It would be a formidable task to examine each water sample for the hundreds of different pathogenic microorganisms that may be present, thus, coliforms or enterococci are used as indicator or sentinel organisms to alert us to contamination events. Questions have been raised on the validity of the indicator system, particularly in marine waters. For example, enteric viruses are known to persist longer in marine water than do coliforms (Gerba and Goyal, 1988) and indicator bacteria do not correlate with the presence of indigenous marine pathogens (Rosas and Hazen, 1988) or predict the presence of other pathogens, such as *Staphylococcus aureus*, that survive well in estuarine environments and can become part of the normal microbial flora (Charoence and Fujioka, 1993; Palmer et al., 1991). In order to answer questions related to indicator validity, appropriate pathogen detection methods need to be developed, particularly for viruses such as hepatitis A virus (HAV) and Norwalk virus, which are difficult or impossible to detect in cell culture. In the past, this was a problem. Due to the development of molecular microbiology, however, this obstacle has been removed. Using the polymerase chain reaction (PCR) and gene probes, pathogens can be successfully detected in both clinical and (more recently) environmental samples even when they are present in very low numbers.

During the past few years there has been increased interest in the evaluation of water for the presence of specific pathogens. The research presented in this report represents an in-depth examination of coastal and other water sources for the presence of selected bacterial and viral pathogens. This project is one of the first major studies on the use of PCR and gene probes for water quality testing and the detection of pathogens in environmental samples. This study is unique not only in the use of genetic technology to determine water quality but because three distinctly different geographical coastal areas (representing tropical, sub-tropical, and temperate climates) were included in the survey. Of particular interest to this study was the fact that these three coastal areas use different bacterial indicators to determine ocean water quality. Hawaii uses enterococci (7/100ml), California uses total coliforms (1000/100ml) and North Carolina uses fecal coliforms (200/100ml). We report on the findings of our two year collaborative study using molecular methods to assess water quality.

STUDY SITES

The three coastal locations that were included in this study were: 1) Hawaii - representing a tropical ecosystem; 2) Southern California - representing a sub-tropical ecosystem; and 3) North Carolina - representing a temperate ecosystem. The Hawaii work was completed on the island of Oahu in the Waikiki and Kailua Bay areas (figure 1). The Waikiki beach site is a popular tourist destination while the Kailua Bay site is of recreational importance to local residents for both fishing and swimming. The California study was

Sampling Sites

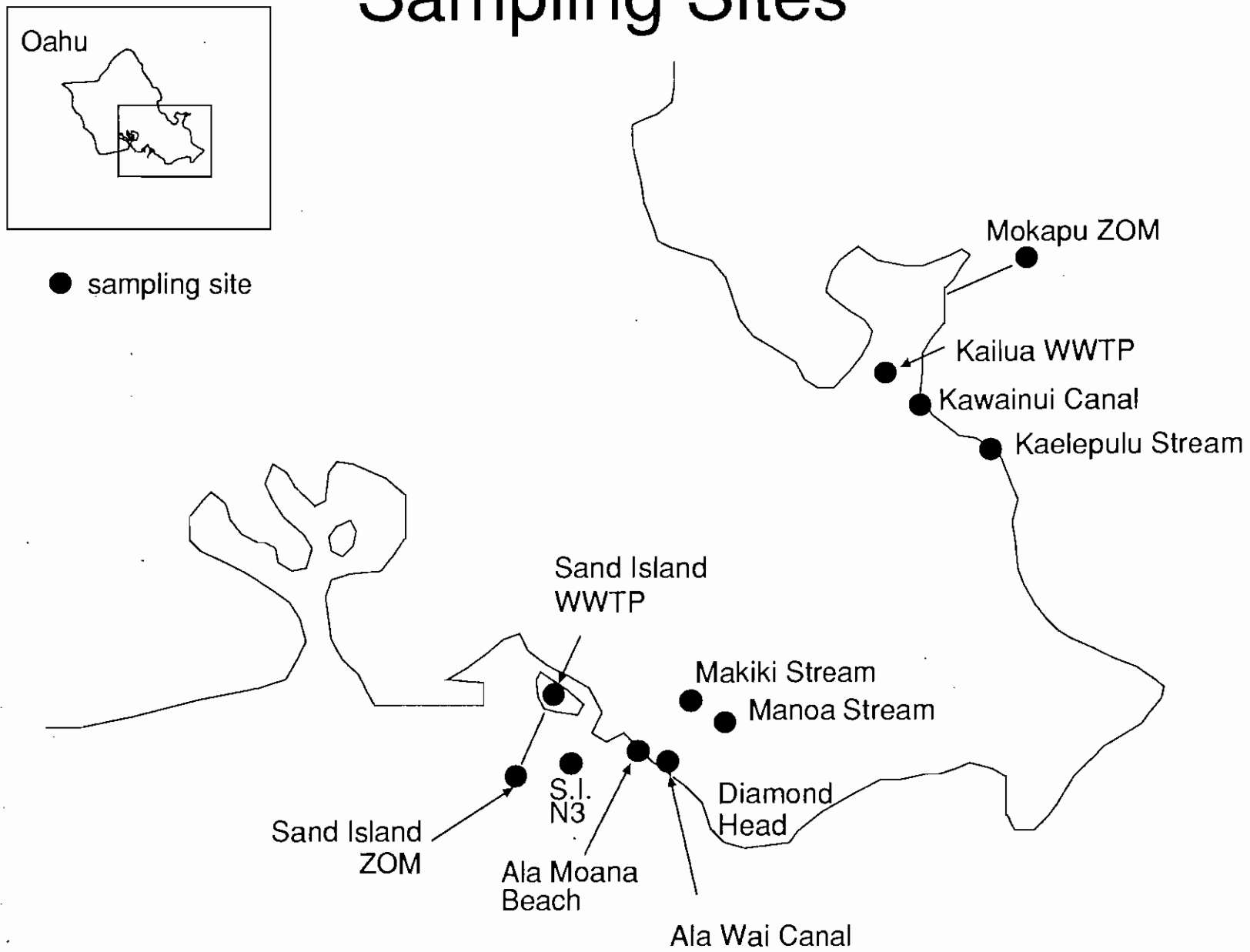

Figure 1. Hawaii Sampling Sites

Figure 2. Treated Sewage Ocean Outfall

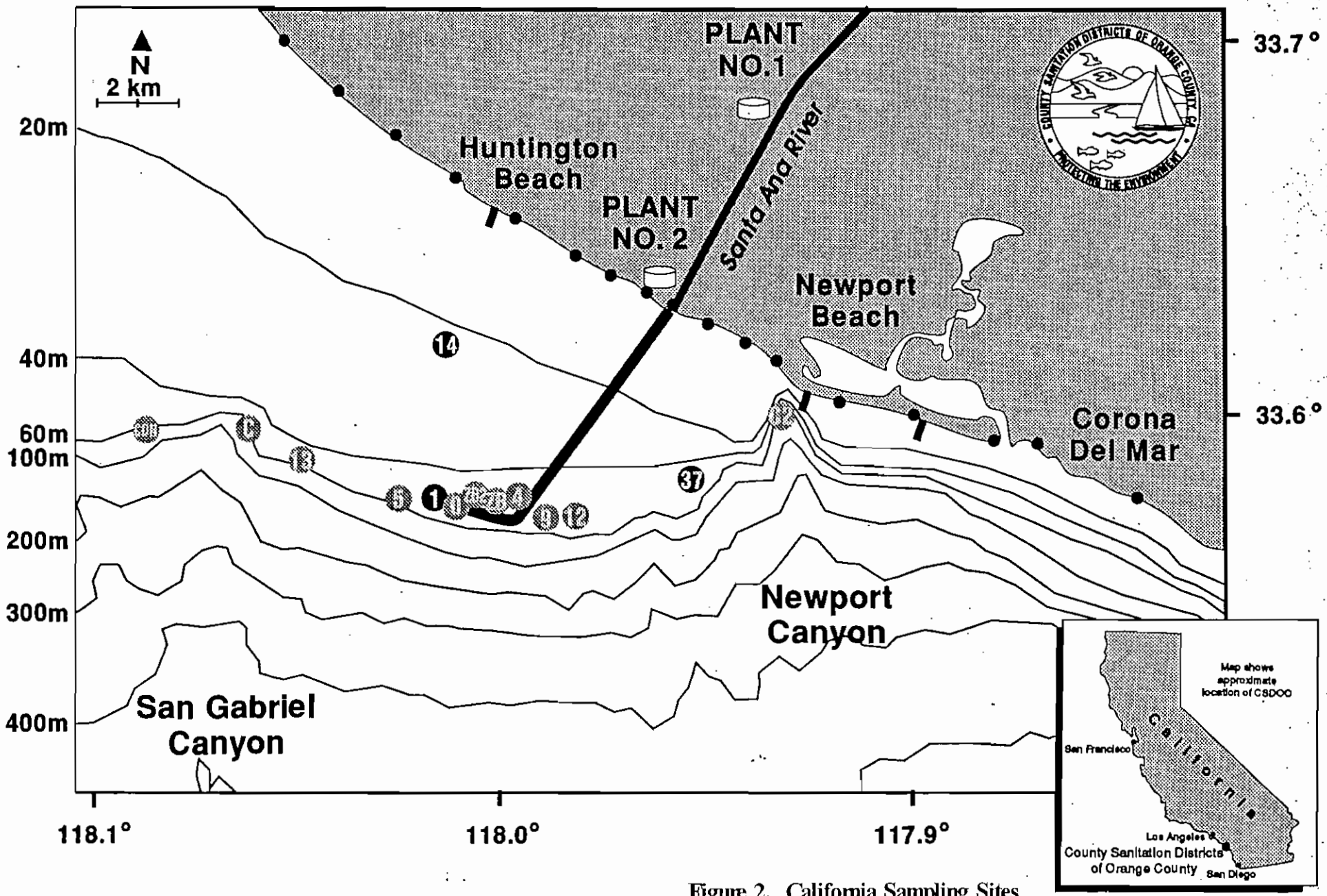

Figure 2. California Sampling Sites

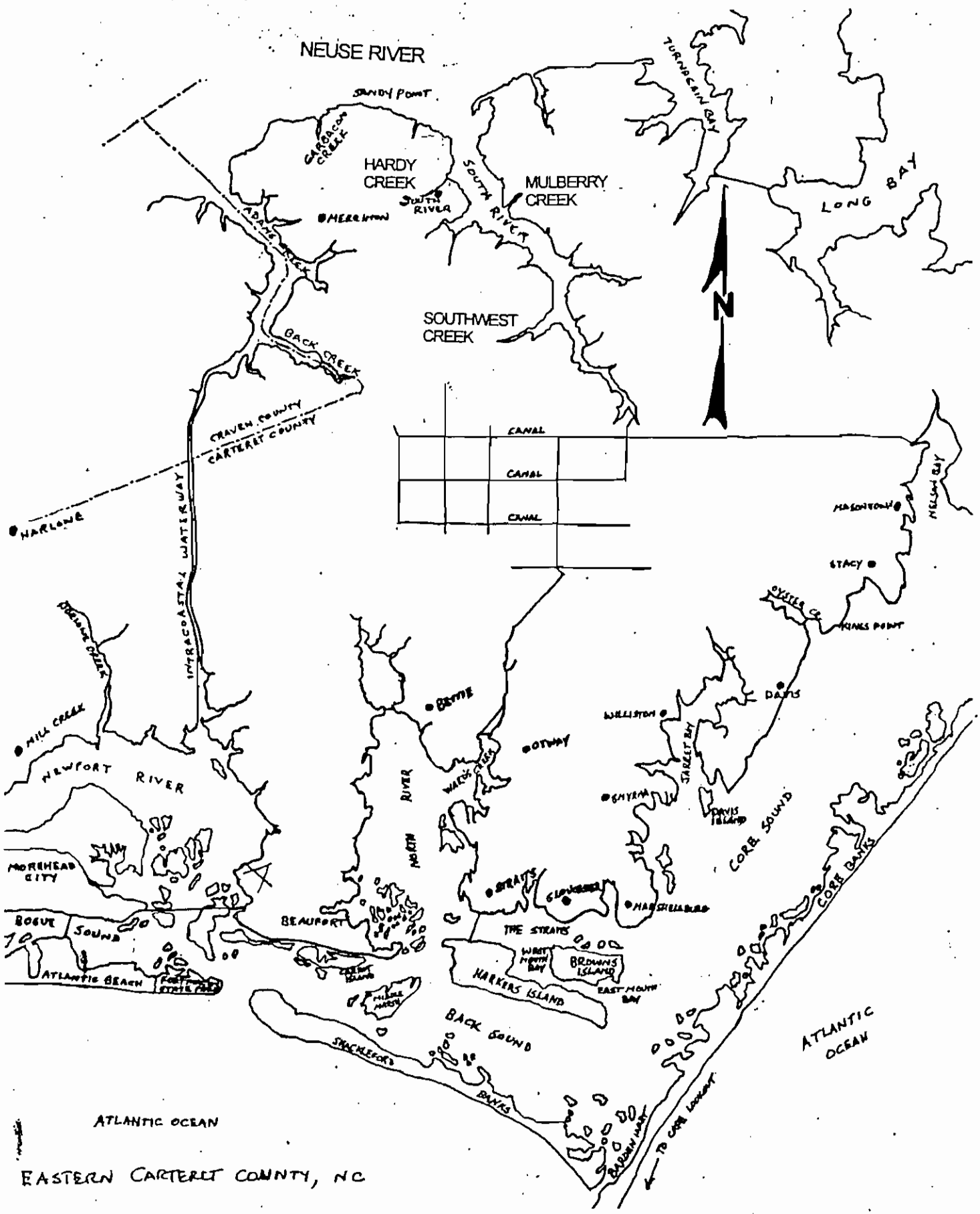

Figure 3. North Carolina Sampling Sites

completed in Orange County between Huntington and Newport beaches (figure 2), both of which are popular with local residents for boating, fishing, swimming and surfing. While Hawaii and California are highly urban areas, the North Carolina site (figure 3) included estuaries located in Beaufort which is a rural area with a relatively small population. The Beaufort estuaries contain valuable shellfish beds that are important to the local economy.

GOALS AND OBJECTIVES

The original goals and objectives of this research were to:

1. Determine the incidence of hepatitis A virus (HAV) in sewage-impacted coastal waters.
2. Define the survival time of HAV in three different marine waters from tropical, temperate, and sub-tropical areas.
3. Determine the percentage of pathogenic *E.coli* present in coastal waters.
4. Interconnect three laboratories from across the nation in a collaborative effort to study coastal waters using molecular technology.

As these goals were successfully accomplished during the course of this study, new objectives were added to the research. The studies above and beyond the original proposal included:

1. Expansion of bacteriological work to include *Legionella.*
2. A study on human immunodeficiency virus (HIV) in a sewage treatment plant.
3. The addition of polio, enterovirus, and rotavirus to the detection and survival studies.
4. Studies on the effects of proteolytic activity on viruses in seawater.
5. Sunlight inactivation studies on poliovirus.
6. Analysis of viral RNA persistence in ocean water.

RESULTS

Toxigenic *Escherichia coli*

Escherichia coli is a predominant specie of the normal flora of the intestine and plays an important role in maintaining intestinal physiology (Drasar and Hill, 1974). Although most *E. coli* strains are harmless, some strains are highly pathogenic causing distinct syndromes of diarrheal disease. Two groups that contain strains with the ability to cause disease are the enterotoxigenic *E. coli* (ETEC) and enterohemorrhagic *E. coli* (EHEC). The ETEC strains are most frequently associated with "travelers diarrhea" and produce a heat-labile (LT) enterotoxin and heat stable (ST) enterotoxin (Mosely et al., 1982). Clinical features of ETEC infections include profuse watery diarrhea, nausea, abdominal cramps, and low-grade fever. The EHEC strains produce potent cytotoxins similar to Shiga toxin and can cause bloody and copious diarrhea (Levin, 1987).

Both the ETEC and EHEC groups have been implicated in waterborne outbreaks. The EHEC group, in particular, has been linked to several recent outbreaks. In 1990, an outbreak occurred in Cabool, Missouri in which 243 people were infected and four died

(Swerdlow et al., 1992). This outbreak was traced to the town's drinking water supply which became contaminated with sewage when two water mains ruptured. A second recent outbreak was traced to a lake in Portland, Oregon. Twenty-one individuals who ingested contaminated water while swimming in a lake became ill with symptoms consistent with EHEC infection (Keene et al., 1994). The EHEC organisms were isolated from both the stools of ill individuals and lake water.

Because of this recent focus on water-borne transmission, we decided to examine coastal water in order to determine the prevalence of toxigenic *E. coli*. It is not possible to determine *E. coli* strain differences using the standard recovery techniques and growth media. The harmless *E. coli* grow and appear the same on culture media as the highly virulent strains. For this reason, we developed a multiplex PCR amplification method that could quickly detect pathogenic strains of ETEC or EHEC in water samples (Lang et al., 1994). We tested 377 *E. coli* isolated from both ocean water and shellfish to try to determine the relative rate of toxigenic coliforms in nearshore coastal areas. We found only one ETEC and one EHEC to be present. These strains (along with non-toxigenic strains) were sent to the California State health laboratory in a blind study to determine toxigenicity (or expression of the tox gene) and confirm the PCR findings. This was accomplished using the standard Y-1 adrenal cell (for LT toxin production in ETEC) and Vero cell bioassays (for shiga-toxin production in EHEC). The results from the State laboratory were an exact match to the PCR predicted toxigenic isolates that were sent. The toxigenic isolates identified using PCR were positive in bioassay and the isolates that were not positive by PCR were negative in the bioassay. Based on the low incidence of toxigenic *E. coli* found in nearshore coastal waters and shellfish, we concluded that there was a low public health risk from these organisms in coastal waters.

In Hawaii, a total of 135 *E. coli* isolates were obtained from raw (62), primary (62) and secondary sewage (11) and 45 additional isolates collected from coastal ocean water. The *E. coli* were isolated on mTEC agar and biochemically identified using the API 20E identification system. The PCR was performed to amplify and detect any EHEC organisms. None of the coastal isolates were positive for tox genes. One isolate was positive from the raw sewage, four were positive from primary treated sewage and none of the secondary sewage isolates were positive. The five positive isolates were tested in Vero cell toxin bioassays. Three of the five were positive in the bioassay. This indicates that two isolates that were positive for toxigenicity using PCR did not express the gene. This phenomena warrants further study which is beyond the scope of this grant.

***Legionella* species**

Legionella bacteria have been well documented as the cause of Legionnaires' disease and Pontiac fever. While it is not known why two clinically different diseases can result from exposure to the same organism, it has been suggested that nonviable legionella may cause the less severe Pontiac fever (Miller et al., 1993). *Legionella* are known to be ubiquitous in environmental waters and are transmitted to humans through aerosolization of legionellae contaminated water droplets. People have become infected through aerosolization

of water from cooling towers (Adiss, et al, 1989), air conditioners (Dondero et al, 1980), hot tubs (Mangione, et al., 1982), showerhead water (Tobin et al., 1980), public fountains (Fenstersheib, et al, 1990) and even a supermarket misting machine (LaMaire, et al, 1990).

Detection of legionellae in environmental water samples is difficult. These organisms grow very slowly and can take upwards of a week to appear on the recommended recovery media. Environmental samples typically contain a plethora of other organisms that can grow rapidly and appear overnight and thus, the legionellae can be easily missed. The use of genetic technology allows for rapid detection of these elusive bacteria and a Legionella PCR kit is now available commercially (EnviroAmp, Perkin Elmer, Foster City, CA). The PCR proved its effectiveness during a recent outbreak, where 13 of 34 guests at a resort became ill with symptoms of Pontiac fever (Miller et al, 1993). While *Legionella* was suspected, it could not be cultured. *Legionella pneumophila* was detected from the resort hot tub using PCR and DFA and the serogroup matched results from serologic tests performed on the stricken guests. Without the PCR, the environmental reservoir of this organism would not have been located and additional illness could have occurred.

In a previous study, we reported that *Legionella* species survived through both the primary and secondary treatment processes of sewage treatment (Palmer et al., 1993) and concluded that sewage treatment plants (STP) are an environmental reservoir for the legionellae. In this current research we expanded the sewage study by comparing legionellae presence in STPs in both California and Hawaii to determine the level of agreement in the results. We found that both treatment plants, although geographically isolated from each other, contained *Legionella* species in all phases of the treatment process. Using PCR, *Legionella* was consistently detected in high concentrations following all treatments although treatment, especially chlorination, resulted in reduced ability to recover *Legionella* by culture methods (Palmer et al., 1993; Roll and Fujioka, 1994). Since the legionellae are known to survive inside protozoan or algae cells, we can conclude that biofilms in STP pipes may play an important role in protecting and maintaining *Legionella* population numbers and could explain why we did not see a decrease in their numbers through the sewage treatment process. Thus, our study added a newly defined habitat for *Legionella* species and our results suggest that the legionellae may be a member of the indigenous biofilm population in all STPs.

Interest in water reuse is growing rapidly, particularly in drought prone areas such as Southern California. Since we were able to show that the legionellae clearly survived through primary and secondary treatment in both California and Hawaii treatment plants, and because of the increased interest in using reclaimed water for irrigation, landscaping, and watering greenbelts, we wanted to evaluate highly treated reclaimed water for the presence of *Legionella* species. We also evaluated air collected over a secondary treatment basin to determine if *Legionella* could be captured from aerosols at a treatment plant. Four different sites that used reclaimed water were chosen for evaluation. Using PCR and DFA, we found that reclaimed water in both California and Hawaii contained *Legionella* species even after chlorination (Palmer et al., 1995). The *Legionella*, however, could not be cultured on plating media which indicates that they may be either dead, injured, or in a viable but non-culturable

state. We were, however, able to culture *Legionella* species (not including *L. pneumophila*) from the air samples above the secondary sewage treatment basin in addition to detection using PCR. This finding suggests that viable legionellae are aerosoled during the sewage treatment process.

Infectivity and RT-PCR to evaluate HIV in sewage

The human immunodeficiency virus (HIV) is known to be transmitted through sexual contact, intravenous drug use, blood transfusions, and in-utero or perinatal transmission from mother to baby during pregnancy. It has even been suggested that HIV may be transmitted via exposure during dental care (Ciesielski et al., 1992) but this appears to be a remote possibility. Studies on HIV-infected individuals have shown that HIV can be detected in various body fluids, including saliva (Ho et al., 1985), urine (Li et al., 1992), semen (Ho et al., 1984), feces (Yolken et al., 1991), tears (Fujikawa, et al, 1985), cerebrospinal fluid (Ho et al, 1985), breast milk (Thiry et al., 1985), and cervical secretions (Vogt et al., 1987). These findings have caused concerns related to other possible modes of transmission of HIV.

Environmental transmission of HIV via the water route has been a topic of interest for the past several years, and several studies have been completed (Ansari, et al, 1992; Casson et al, 1992; Enriquez, et al., 1993; Moore, 1993; Preston, et al., 1991; Slade et al., 1989). These studies have been in response to questions raised on the potential of acquiring HIV through contact with waters receiving treated wastewater effluent and sharing hot-tubs and swimming pools with HIV-infected individuals (Zuckerman, 1986). The potential for HIV transmission to sewage treatment plant workers through contact with wastewater containing infectious HIV is also of interest since they are often in close physical contact with sewage. Although it is known that HIV is a labile virus and it is thought to have limited potential for survival in the environment outside of the host, it is important to investigate the possibilities for waterborne transmission in order to answer public concerns.

Two previous studies have shown that HIV nucleic acid can be detected from sewage (Ansari, et al., 1992; Preston, et al., 1991) but it was not determined if any infectious virus was present. Our study differed from previous work since we tested the sewage for both HIV RNA and infectious virus. Sewage samples were tested for HIV-1 genomic RNA by reverse transcriptase polymerase chain reaction (RT-PCR) using primers SK38/SK39 and M667/AA55. Infectivity assays employed peripheral blood mononuclear cells (PBMC) stimulated with phytohemagglutinin. Coxsackievirus B4, echovirus 7, and poliovirus 1, enteroviruses normally present in sewage, were tested for replication in PBMCs. A p24 antigen capture ELISA test was performed to determine the results from tissue culture infectivity assays. Sewage was also seeded with HIV-1 to ensure that sewage does not contain substances that would hinder HIV infectivity in the PBMC coculture assay and to show that if HIV-1 was present, it could be detected with the methods used in this study. In order to eliminate other enteroviruses that may also infect the PBMCs and interfere with HIV-1 testing, concentrated sewage was treated with human immunoglobulin (free of HIV antibodies) and poliovirus antisera before performing infectivity assays.

All sewage samples tested negative for HIV-1 by all methods used. The HIV-1 seeded into sewage, however, remained infectious in the assay, indicating the sewage water sample did not interfere with HIV infectivity nor was it toxic to the PBMCs. Poliovirus 1 was found to infect the PBMCs which was an unexpected result and may play a role in the pathogenesis of poliovirus. A manuscript detailing this work has been submitted for publication consideration. [The HIV research was an extension of our collaborative efforts and we would like to acknowledge the other researchers and coauthors on the paper - Dr. Moon Lee of Harbor UCLA Medical Center, Los Angeles, CA and Dr. Edward Siwak of Baylor School of Medicine, Houston, TX.]

Molecular Detection of Enterovirus and Hepatitis A Virus in Water and Wastewater.

Enteric viruses have caused numerous outbreaks of waterborne gastroenteritis (Hedberg and Osterholm, 1993; Wanke and Guerrarnt, 1987; Richards, 1985) and the consumption of raw shellfish (oysters and mussels) has been strongly implicated in outbreaks of hepatitis A (Desenclos et al., 1991; Enriquez et al., 1992; Xu et al., 1992). Clearly, waterborne transmission of viruses is a major health problem. The numbers of outbreaks and incidence of disease are probably grossly underreported since, historically, viruses have been difficult to detect from water and shellfish samples. Outbreaks are usually identified by symptoms and serological tests of ill patients but these results can not positively identify the source of the virus. Waterborne viruses are difficult to recover due to: 1) a lack of susceptible cell lines for many important waterborne viruses; 2) low or poor sensitivity in existing cell lines; 3) low numbers of viruses in environmental waters which make it necessary to concentrate large volumes for detection and; 4) the inefficiency of existing concentration methods. Moreover, once the water sample is concentrated, virus detection using traditional tissue culture methods is labor intensive and it can take up to six weeks to obtain the results.

Molecular techniques offer a way to solve virus detection problems since sensitivity and specificity are increased and analysis time is substantially decreased. However, reduced detection sensitivity may be observed when RT-PCR and oligonucleotide probing (OP) are used directly to detect enteric viruses present in complex environmental samples such as sewage, environmental waters and sludge. The suspected causes are inhibitory substances of RT-PCR that are present in environmental samples, such as humic acid, polysaccharides and metal ions (e.g., iron and aluminum). In addition, the abundant RNAses in environmental samples can cause additional problems when using RT-PCR technology. Thus, it was important to develop a method to purify and concentrate the intact virions from environmental samples in order to minimize RNA exposure to inhibitory and degradative agents until an adequate degree of purification has been achieved.

Our collaborative study had three major foci that addressed environmental virus detection; 1) improvement of water concentration methods, 2) removal of inhibitory substances and, 3) development of a rapid RT-PCR protocol. After development of improved methodologies to concentrate water, remove PCR inhibitors, and rapidly detect viruses, we performed virus field testing in all three coastal areas. We also evaluated seeded

water for virus survival times and conditions that could impact virus persistence in coastal waters.

Water concentration for virus detection. A simple method of water concentration was developed and tested for its ability to isolate viruses from environmental water samples (Tsai et al., 1993). This method used vortex flow filtration (VFF) to concentrate 15 liters of ocean water into 100 ml. Further concentration was achieved by using an ultrafiltration method consisting of Centriprep and Centricon microconcentrators (Amicon, Beverly, MA). Ultrafiltration concentrated the sample from 100 ml into 100 µl. Since only 2 µl is required to perform RT-PCR, more than enough sample was available for testing after these concentration steps. For sewage samples, only 100 ml was needed for starting volume. Thus, the VFF step was not necessary for sewage and samples were placed immediately into the microconcentrators. Both enteroviruses and HAV were detected in raw sewage (primary influent) and treated sewage effluent (primary and secondary effluent). The ocean virus results are presented in the field recovery portion of this report.

In another phase of this work we determined if HAV could be efficiently concentrated from seawater by adsorption to and elution from fiberglass microporous filters. The concentration and recovery of HAV and polio virus 1 (PV1) was compared from seeded samples of North Carolina seawater by adsorption to and elution from electronegative (Filterite) and electropositive (Virosorb 1MDS) filters. Both HAV and PV1 were adsorbed efficiently by Filterite filters at pH 3.5 in the presence of added $AlCl_3$. They were adsorbed less efficiently by Filterite filters at pH 6-8 in the presence of added $MgCl_2$ or by 1MDS filters at pH 6-8 with no added multivalent cation salts. Overall, adsorption efficiency was higher for poliovirus than for HAV. Both viruses were eluted with moderate but variable efficiency (30-60%) from both filters by 3% beef extract, pH 9.5. Recovery efficiencies using Filterite filters were ~40% for poliovirus, ~70% for HAV using $AlCl_3$, ~70% for PV1 and ~15% for HAV using $MgCl_2$. For 1MDS filters, recoveries were ~25% for PV1 and ~10% for HAV. In general, electronegative Filterite filters gave better recoveries than electropositive 1MDS filters. Overall, the results of these experiments show that HAV, like PV1 and other enteroviruses, can be recovered from seawater by adsorption to and elution from fiberglass microporous filters, with the highest recoveries from electronegative Filterite filters at pH 3.5 and 0.0005-0.005 M $AlCl_3$.

Removal of inhibitory substances in environmental samples. In this study we developed a simple and inexpensive method to purify and concentrate intact virions from up to several liters of sewage by a process that involves (1) polyethylene glycol (PEG) precipitation (8% PEG and 0.3M NaCl), (2) chloroform or Freon extraction, and (3) centrifugal ultrafiltration with Amicon Centricon 100 units (Tai, 1994). This method has the advantage of being able to process large volumes of sewage so that even low concentrations of relatively rare enteric viruses will be detected. When this method was used to concentrate PV1 and HAV from 1/4th liter volumes of virus-seeded sewage to a volume of <1 ml, virus infectivity recoveries averaged 18% for PV1 and 10% for HAV, and the viruses were detectable in 10µl volumes by direct RT-PCR and OP (RT-PCR-OP) after heating to 99°C to release virion RNA. For

PV1, the detection endpoint by RT-PCR-OP ranged from 0.5-0.0005 infectious units of viruses, and for HAV, it was 4-0.0001 infectious units of viruses. These results indicate that RT-PCR-OP is more sensitive than cell culture for enteric virus detection. Using this method, enteroviruses were readily detected in unseeded raw sewage samples, even when they were not detectable by infectivity in cell cultures. As expected, the levels of indigenous enteroviruses in raw sewage varied seasonally, with the highest levels in the late summer and early fall and lower levels at other times of the year.

Guanidinium Extraction. Poor detection sensitivity was sometimes observed when we applied direct RT-PCR-OP methods to some sewage samples, apparently due to residual PCR inhibitors. However, we could effectively eliminate this RT-PCR inhibition from processed sewage and other excreta samples by extracting viral RNAs and separating them from virion capsids and other materials present in the samples. This was done using a one-step guanidinium extraction procedure immediately prior to RT-PCR (Shieh et al., 1995). The detection sensitivity and specificity of guanidinium extraction-RT-PCR were established initially for enteroviruses in infected cell extracts. For 9 different enteroviruses, the endpoint of detection after guanidinium extraction was 0.6-0.003 infectious units of virus infectivity. Because guanidinium solution protects RNA from degradation and further purifies viral RNA from sample constituents, there was improved viral detection sensitivity by RT-PCR in processed and concentrated samples of sewage and other excreta. When sewage, concentrated up to 385,000-fold and seeded with 1 to 3 PFU of poliovirus, was extracted with guanidinium solution, viruses were detectable in samples originally judged negative by direct RT-PCR without guanidinium extraction. Eleven field samples processed by a series of steps that included PEG concentration, solvent extraction, and Sephadex G200 spin column chromatography were examined for enteroviruses by RT-PCR, both with and without guanidinium extraction. Guanidinium extraction eliminated sample inhibitory substances and increased the proportion of enterovirus positive samples from 3 of 11 to 7 of 11. These results show that guanidinium extraction in conjunction with virion concentration improved RT-PCR detection of viruses in sewage.

Detection in Beef Extract Eluates. In another phase of this study we developed a concentration and purification procedure for RT-PCR-OP detection of enteric viruses in water sample concentrates obtained by conventional filter adsorption-elution methods (Schwab et al., 1995). One liter beef extract (BE)-glycine mock eluates with or without humic acid and seeded with PV1 and HAV were used as a model system and the eluent was further processed for RT-PCR compatibility. The concentration and purification procedures for 1-liter BE samples consisted of: (1) polyethylene glycol (PEG) precipitation, (2) Pro-Cipitate precipitation, (3) a second PEG precipitation, (4) Sephadex G200 spin-column chromatography, and Centricon 100 centrifugal ultrafiltration. Sample volumes were reduced from 1 l to 20-50 µl and purified sufficiently for viral detection by RT-PCR-OP. The ability to detect low numbers of enteric viruses by these molecular techniques was compared directly to enteric virus recovery by cell culture infectivity. As little as 3 PFU of poliovirus 1 in an initial 1 liter volume of BE could be recovered and detected by RT-PCR-OP.

When this method was tested in a series of experiments on 1-liter beef extract mock eluates seeded with different levels of PV1, the percentage of positive samples using this method averaged 50% and did not decrease significantly at low input virus levels (P=0.84, Fisher Exact Test) (Table 1).

Table 1: RT-PCR Detection of PV1 in Seeded 1L BE Samples

Input PFU Per Sample	No. Trials	RT-PCR Detection No. Neg	No. Pos	% Positive Samples
10^2	7	3	4	57%
10^1	33	16	17	52%
$<10^1$	10	6	4	40%
Total	50	25	25	50%

In further experiments on PV1 recovery from seeded 1-liter samples of BE we compared the two virus detection methods of RT-PCR-OP and cell culture infectivity for samples seeded with about 10 infectious units of PV1. About 1/3rd of the final sample concentrate was assayed by each of the two methods (the last third was saved as a contingency). As shown by the results of 22 experiments summarized in Table 2, both detection methods gave positive results: 6 by infectivity and 10 by RT-PCR, with 4 samples positive by both methods. There was no difference in virus detection by the two assay methods ($p = 0.34$, Fisher exact test, two tailed). The mean recovery of virus infectivity was about 16% overall.

Table 2: PV1 Detection by RT-PCR and Quantal Infectivity Assay in Processed 1L BE Samples

	Infectivity Positive	Infectivity Negative	Totals
RT-PCR Positive	4	6	10
RT-PCR Negative	2	10	12
Totals	6	16	22

Development of a Triplex RT-PCR Method. The reverse-transcriptase polymerase chain reaction (RT-PCR) is of great value for the detection of RNA viruses. The RT-PCRs that have been performed, however, are monoplex. This means that only one set of primers are included in the reaction mixture and only one virus or one target RNA sequence can be detected at a time. We decided to enhance this procedure through streamlining the time required to analyze water samples using RT-PCR. A triplex RT-PCR was developed that could simultaneously detect three different viruses, HAV, poliovirus, and rotavirus, in one reaction (Tsai et al., 1994). This method was developed by carefully combining three different primer pairs, each unique to a different specific target virus, in one reaction mixture and adjusting the primer concentrations and PCR cycling times so that all three viruses would be amplified at once. This technique has both environmental and clinical applications since it can considerably reduce analysis time and costs and provide a rapid turn-around time for results.

Virus Field Studies - North Carolina

In North Carolina we chose to study the occurrence of human enteric viruses in the South River (a tributary of the Neuse River), an estuarine river in central coastal North Carolina that is closed to shellfishing due to excessive levels on fecal coliforms in water and shellfish. The impacts of different sources of fecal contamination in the South River are unclear, because there are multiple sources. These include: (1) household septic waste discharges from the small coastal community of South River (located on Hardy Creek, a tributary discharging into the South River in its northeast section), and (2) agricultural waste (surface runoff and ditch/canal drainage) from a large commercial farm with about 3,000 head of beef cattle that is located on the south and southwest southerly shores of the South River. Mullberry Creek, which drains into the northwest section of the South River also contains high levels of fecal coliforms, but there are no appreciable sources of human or animal fecal wastes on this tributary.

We attempted to determine the presence of human enteric viruses in regions of the South River impacted by the different pollution sources: Hardy Creek (human waste sources), Southwest fork (cattle waste sources) and Mulberry Creek (no major waste sources). Samples of these waters were collected monthly over a 6-month period and processed through Filterite filters by the methods described in previous sections of this report. Most of the final sample concentrate volume was subjected to culturable enteric virus infectivity assay by inoculating it into cell cultures (Buffalo Green Monkey Kidney and 2° African Green monkey kidney cells). Cultures were scored for the development of viral cytopathogenic effects (CPE). In addition, CPE-negative cultures were RNA-extracted, and the extracted RNA was further analyzed for enteroviruses by RT-PCR-OP. Also, a portion of each final sample concentrate was further processed by the methods described above and directly analyzed for enteroviruses and HAV by RT-PCR-OP. As shown by the infectivity data summarized in table 3, culturable enteric virus-positive samples were obtained from each of the three areas of the South River, regardless of their status with respect to major pollution sources. Of the 18 samples analyzed, 5 were positive for culturable enteric viruses: 1 from Hardy Creek, and 2 each from Mulberry and Southwest Creeks. These results suggest that culturable enteric viruses are widely distributed in the South River and are not confined to only limited sections of the river.

Table 3. Enteric Virus Analysis of South River Water: Hardy Creek, Mulberry Creek and Southwest Creek*

(Infectivity Results, Positive/Negative (Sample Volume, L)

Date	Hardy Creek	Mulberry Creek	Southwest Creek
7/93	Negative (114)	Negative (114)	Negative (114)
8/93	Negative (136)	Negative (151)	Negative (189)
9/93	Negative (114)	Negative (114)	Negative (189)
10/93	Negative (163)	Negative (159)	Negative (151)
11/93	Negative (151)	Positive (189)	Positive (189)
12/93	Positive (189)	Positive (151)	Positive (151)

*Adsorb to Filterite fiberglass filters, 0.45 μM pore size, at pH 3.5 and 0.0005 M $AlCl_3$; elute with 1.5% beef extract, pH 9.5; reconcentrate by PEG precipitation; inoculate into flasks of BGMK AND AGMK cell cultures.

About 5% of the final concentrate volume of the same samples was further processed and analyzed for enteroviruses by RT-PCR. Only one (1) sample (Southwest Creek, 11/93) was positive by this analysis. While this approach yielded less positive samples than infectivity analysis, it is possible that more samples would have been virus positive by RT-PCR if larger sample volumes had been analyzed. In future studies, equal volumes of sample concentrates should be analyzed by infectivity and RT-PCR to obtain a better comparison of their relative sensitivities in virus detection. However, the fact that positive results were obtained by RT-PCR in at least one sample shows that enteric virus detection by molecular techniques is a potentially useful and effective method to detect enteric viruses in environmental waters and other environmental samples.

Virus Field Studies - Hawaii

A total of 24 water samples from various sources including sewage, ocean water and streams were analyzed for presence of human enteric viruses by culture method and by RT-PCR. These water samples were also analyzed for salinity, temperature, pH, turbidity and for indicator bacteria. The results of these analyses are summarized in Table 4. The standard membrane filtration method was utilized to analyze 100 ml of sample for the bacterial indicators (*E. coli*, fecal coliforms, enterococci, *C. perfringens)*. Larger volumes (20 liters) of environmental water samples were concentrated by the VFF method and analyzed for human enteric viruses by RT-PCR and tissue culture while only 100 ml of sewage effluent was concentrated and similarly assayed for virus. Samples analyzed include six ocean water, two sewage effluent and 16 from streams that serve as urban drainage for the cities of Honolulu and Kailua located on the island of Oahu.

Of 24 samples analyzed, eight samples were positive for pan enterovirus gene sequences while no hepatitis A gene sequences were detected. All 24 samples were negative for culturable human enteric viruses on BGM (Buffalo green monkey) cells. Ten of these samples were cultured for HAV on PMK (Primary monkey kidney) cells and were also negative. Twenty-two of the 24 samples were also analyzed for indicator bacteria and at least one indicator bacteria was recovered in 21 of 22 samples.

The detection of human enteric viruses from only 100 ml from both of the sewage effluent samples by RT-PCR and the absence of virus by culture indicates the sensitivity of the RT PCR method. Of the six ocean waters sampled, only the zone of mixing (ZOM) over the Mokapu sewage outfall was positive for human enterovirus using the RT-PCR method. This is not unexpected as the ZOM is the area where sewage from Kailua sewage outfall is initially mixed with ocean water in Kailua Bay. Three other ocean samples were collected from Ala Moana Beach Park and were negative for both culture and PCR. The two remaining ocean water samples were collected at near shore sites (Sand Island N3) located between the Sand Island sewage ocean outfall and the Ala Moana Beach Park (figure 1). These samples were virus negative using both PCR and tissue culture.

Sixteen samples were collected from streams that discharge into coastal ocean waters in the vicinity of recreational waters. Four of these samples were collected from the Ala Wai,

Table 4. Evaluation of Hawaii H_2O Samples for Virus and Indicator Bacteria.

						CFU/ 100ml				PCR and Hybridization Results				Cell culture
		Salinity	Temp.		Turbidity	mFC	mTEC	mE	mCP	Pan entero		Hepatitis A		BGM
Source	Date	ppt	°C	pH	NTU	Fecal Coliform	*E. coli*	Enterococci	*C. perfringens*	PCR	Hybrid	PCR	Hybrid	Pos/Neg
Kailua Eff	01/11/94	0	20	ND	ND	ND	ND	ND	ND	Pos	Pos	Neg	Neg	Neg
Kailua Eff	01/12/94	0	'24	ND	ND	ND	ND	ND	ND	Pos	Pos	Neg	Neg	Neg
Makapu ZOM	01/11/94	34	20	8.22	.12	128	86	13	0	Pos	Pos	Neg	Neg	Neg
Sand Island N3	01/12/94	34	20	8.17	.12	4	2	9	0	Neg	Neg	Neg	Neg	Neg
Sand Island N3	02/05/94	34	18	8.1	4.8	1	0	0	0	Neg	Neg	Neg	Neg	Neg
Ala Moana Beach	12/29/93	34	22	8.1	3.5	6.2×10^2	6.6×10^2	4	29	Neg	Neg	Neg	Neg	Neg
Manoa Stream UH	01/05/94	0	21	7.9	3.9	1.4×10^3	1.5×10^3	2.5×10^2	1.3×10^2	Neg	Neg	Neg	Neg	Neg
Manoa Stream UH	01/18/94	0	18	7.2	1.4	1.3×10^4	8.8×10^3	8.6×10^2	6.5×10^2	Neg	Neg	Neg	Neg	Neg
Ala Wai Canal	01/31/94	16	22	8.1	1.2	6	2	0	0	Pos	Pos	Neg	Neg	Neg
Ala Wai Canal	02/07/94	28	24	8.2	3.8	1	0	0	5	Pos	Pos	Neg	Neg	Neg
Kawainui Canal	03/12/94	15	20	7.3	4.9	5.2×10^2	4.1×10^2	48	28	Pos	Pos	Neg	Neg	Neg
Kaelepulu Stream	03/12/94	23	21	7.3	2.4	4.6 x 102	3.1 x 102	94	6	Neg	Neg	Neg	Neg	Neg
Kaelepulu Stream	03/19/94	29	21	8.0	1.5	3.0×10^2	2.1×10^2	3.6×10^2	0	Pos	Pos	Neg	Neg	Neg
Kawainui Canal	03/19/94	16	22	7.3	4.0	1.4×10^2	1.2×10^2	86	0	Pos	Pos	Neg	Neg	Neg
Manoa Stream	03/26/94	0	20	8.0	4.0	6.3×10^2	5.5×10^2	8.2×10^2	24	Neg	Neg	Neg	Neg	Neg
Ala Wai Canal	03/26/94	18	21.2	8.1	10	3.6×10^3	3.6×10^3	2.0×10^3	118	Neg	Neg	Neg	Neg	Neg
Manoa Stream	04/02/94	0	21	8.1	2.0	1.6×10^3	1.6×10^3	80	16	Neg	Neg	Neg	Neg	Neg
Ala Wai Canal	04/02/94	13	23	7.6	2.5	1.1×10^3	2.7×10^2	6.2×10^2	26	Neg	Neg	Neg	Neg	Neg
Manoa Stream	04/09/94	0	19	7.0	4.0	3.4×10^2	3.4×10^2	3.9×10^2	7	Neg	Neg	Neg	Neg	Neg
Makiki Stream	04/09/94	0	20	7.5	1.0	2.3×10^2	2.1×10^2	44	0	Neg	Neg	Neg	Neg	Neg
Maona Stream	04/16/94	0	19	7.5	.65	80	80	9	0	Neg	Neg	Neg	Neg	Neg
Makiki Stream	04/16/94	0	19	7.7	.65	389	288	84	0	Neg	Neg	Neg	Neg	Neg
Ala Moana Beach	04/23/94	33	24	8.1	.55	0	0	0	4	Neg	Neg	Neg	Neg	Neg
Ala Moana Beach	04/23/94	33	24	8.0	2.0	0	0	0	0	Neg	Neg	Neg	Neg	Neg

an urban stormwater canal, which discharges in the vicinity of Waikiki and Ala Moana beach parks. Two of the four samples collected from this site were positive for human enterovirus by the RT PCR method. It is significant to note that on both occasions when samples from these sites were positive for pan enterovirus sequences, the bacterial indicator, enterococci, was not recovered. Four samples were collected from the Kawainui Canal and Kaelepulu stream which serve as urban drainage for the city of Kailua, located on the windward side of Oahu. These drainage systems discharge into Kailua Bay and impact windward Oahu's most popular bathing beach. Of the four samples collected from these sites, three were positive for pan enterovirus PCR products. The remaining eight samples were collected from Manoa and Makiki Streams which serve as urban drainage for the city of Honolulu. Of the eight samples collected from these sites no pan enterovirus or HAV PCR products were detected.

The PCR results for virus did not consistently correlate with the absence or presence of fecal indicator bacteria. Samples negative for virus by PCR contained high concentrations of indicator bacteria. However, two samples collected from the Ala Wai canal were positive for pan enterovirus gene sequences while no enterococci were detected in these samples. This may be related to differences in the methods used, volumes of samples analyzed and the fact that the PCR method detects virus gene products while the bacterial indicator assay measures live bacteria. A significant finding was the detection of human enterovirus by RT PCR in urban drainage systems with no known sources of sewage contamination. These drainage systems have previously demonstrated high levels of indicator bacteria resulting from storm water discharges and animal feces. Further research should be conducted in order to determine the sources of these enteric viruses as detected by PCR.

Virus Field Studies - California

California water samples were collected from nearshore coastal areas between Newport and Huntington Beaches, an ocean sewage outfall area located five miles offshore in 200 ft of water, and from the Santa Ana River and flood control channels that drain into the coastal waters. Water samples were concentrated using the VFF method described earlier in this report. Water samples were treated with a chloroform:isoamyl alcohol solutions to provide better virus separation from particulates and then the sample was further concentrated using ultrafiltration. The RT-PCR was performed to detect both enterovirus and HAV. Results from sewage and nearshore coastal areas indicate that both enterovirus and HAV can be detected in these waters (Table 5) Enteroviruses were detected in greater than 50% of the sewage samples tested but in less than 25% of the coastal water and river samples. The HAV was detected in approximately 25% of both sewage and coastal/river samples.

Water was collected monthly for ten months from the ocean outfall area and was tested for enterovirus, HAV, and coliforms. The sample sites included the outfall surface and 36 meter depth (station 1), and surface water from 4 km east (station 14) and northwest (station 37) of the outfall (figure 2). Viruses were detected using both tissue culture and RT-PCR and coliform numbers were determined using Colilert-MW (Idexx, Westport, MA). Results using RT-PCR showed that enterovirus were recovered from 30% (3/10) of the outfall depth stations but only 10% (1/10) of outfall surface water samples (Table 6). Enterovirus

were detected in the surface samples from the east and northwest stations in 20% of the samples. Results from the outfall deep station showed HAV in 70% (7/10) of the samples and the outfall surface was positive only 10% (1/10) of the time. Stations 14 and 37 were positive in 20% (2/10) and 10% (1/10) of the samples respectively. Tissue culture results did not correlate with the RT-PCR results since in most cases, tissue culture was negative. Total coliform counts were less than 20 in most samples which would indicate that no pathogens were present. Our results clearly showed that the coliforms were not accurate predictors of the presence of viruses in ocean water samples.

Table 5. Detection of enteroviruses and hepatitis A virus from sewage and ocean water using RT-PCR.

Sample Site	EV[a]			HAV[b]		
	Total	Positive	%	Total	Positive	%
Raw Influent	5	4	80	4	1	25
1° Eeffluent[c]	5	3	60	4	1	25
2° Effluent[d]	5	2	40	4	1	25
HP[e]	6	1	17	4	1	25
SAR[f]	7	1	14	7	2	28
D2[g]	10	2	20	10	1	10
OS[h]	8	1	12	8	1	12
OD[i]	8	5	62	8	2	25

[a] EV, enteroviruses
[b] HAV, hepatitis A virus
[c] 1° Eff., primary effluent
[d] 2° Eff., secondary effluent
[e] HP, surfzone water
[f] SAR, a river brackish water
[g] D2, brackish water from a flood control channel
[h] OS, surface ocean water above a sewage discharge pipe
[i] OD, deep ocean water (30 m depth) around sewage discharge pipe

Table 6. Hepatitis A virus, total coliform and *E. coli* per 100 mL in ocean water around treated sewage outfall

Distance from discharge pipe: Station 1 = 0.5 km West, Station 14 = 4.0 km East, Station 37 = 4.0 km Northwest

	Hepatitis A Virus																			
	Station 1 (surface)					Station 1 (depth)					Station 14 (surface)					Station 37 (surface)				
Date	PCR	PFU	CPE	Total Coliform	*E. coli*	PCR	PFU	CPE	Total Coliform	*E. coli*	PCR	PFU	CPE	Total Coliform	*E. coli*	PCR	PFU	CPE	Total Coliform	*E. coli*
June 93	-	ND	ND	ND	ND	+	ND	ND	ND	ND	+	ND	ND	ND	ND	-	ND	ND	ND	ND
July 93	+	ND	ND	ND	ND	+	ND	ND	ND	ND	+	ND	ND	ND	ND	-	ND	ND	ND	ND
August 93	-	ND	ND	ND	ND	+	ND	ND	ND	<20	-	ND	ND	ND	ND	-	ND	ND	ND	ND
September 93	-	-	-	<20	<20	+	-	-	<20	<20	-	-	-	<20	<20	-	-	-	<20	<20
October 93	-	-	-	<20	<20	+/-	-	-	<20	<20	-	-	-	<20	<20	-	-	-	<20	<20
November 93	-	-	-	<20	<20	+	-	-	16000	3000	-	-	-	<20	<20	+	-	-	<20	<20
December 93	-	-	-	<20	<20	-	-	-	600	40	-	-	-	<20	<20	-	-	-	<20	<20
January 94	-	-	-	<20	<20	-	-	+	40	<20	-	-	-	<20	<20	-	-	-	<20	<20
February 94	-	-	-	<20	<20	-	-	+/-	170	20	-	-	-	<20	<20	-	-	-	<20	<20
March 94	-	-	-	<20	<20	+	-	-	20	<20	-	-	-	<20	<20	-	-	-	<20	<20
% POSITIVE	10	0	0	0	0	60-70	0	14-28	71	43	20	0	0	0	0	10	0	0	14	0

Enteroviruses in ocean water around treated sewage outfall

	Enteroviruses																			
	Station 1 (surface)					Station 1 (depth)					Station 14 (surface)					Station 37 (surface)				
Date	PCR	PFU	CPE	Total Coliform	*E. coli*	PCR	PFU	CPE	Total Coliform	*E. coli*	PCR	PFU	CPE	Total Coliform	*E. coli*	PCR	PFU	CPE	Total Coliform	*E. coli*
June 93	-	ND	ND	ND	ND	-	ND	ND	ND	ND	-	ND	ND	ND	ND	-	ND	ND	ND	ND
July 93	-	-	-	ND	ND	+	50/15 L	+	ND	ND	-	-	-	ND	ND	-	38/15 L	+	ND	ND
August 93	-	-	-	ND	ND	-	-	-	ND	<20	-	-	-	ND	ND	-	-	-	ND	ND
September 93	-	-	-	<20	<20	-	-	-	<20	<20	+	-	-	<20	<20	+	-	-	<20	<20
October 93	+	-	-	<20	<20	+	-	-	<20	<20	+	-	-	<20	<20	+/-	-	-	<20	<20
November 93	-	-	-	<20	<20	-	-	+/-	16000	3000	-	-	-	<20	<20	-	-	-	<20	<20
December 93	-	-	-	<20	<20	+	-	-	800	40	-	-	-	<20	<20	+	-	-	<20	<20
January 94	-	-	-	<20	<20	-	-	-	40	<20	-	-	-	<20	<20	-	-	-	<20	<20
February 94	-	-	-	<20	<20	-	-	-	170	20	-	-	-	<20	<20	-	-	-	<20	<20
March 94	-	-	-	<20	<20	-	-	-	20	<20	-	-	-	<20	<20	-	-	-	<20	<20
% POSITIVE	10	0	0	0	0	30	11	11-22	71	43	20	0	0	0	0	20-30	11	11	14	0

Survival of HAV and Other Viruses in Geographically Diverse Seawater.

The survival of pathogenic enteric viruses such as HAV in seawater is important in the extent to which seawater receiving sewage and other human wastes poses a human health risk from bathing, other primary contact recreation and eating shellfish from such water. Previous studies on virus survival in seawater gave different survival times for the same virus, indicating geographic differences in water quality contributing to virus survival differences. No studies have simultaneously compared the survival of viruses in geographically different seawaters in the same experiments. Because monitoring for human enteric viral pathogens in seawater is not yet practical, monitoring for good indicators of enteric viruses is of interest. Therefore, the survival of candidate viral indicators in seawater is also important. Enteric bacteriophages (viruses infecting enteric bacteria) may serve as indicators of human enteric viruses if they survive as well as viruses in seawater and have the other necessary attributes to be reliable virus indicators.

Comparative Virus Inactivation in Seawaters of North Carolina, California and Hawaii.

We compared the survival of two groups of candidate indicator viruses, F-specific coliphages (FRNA phages) and somatic *Salmonella* bacteriophages (SS phages), to the survival of two human enteric viruses, HAV and PV1, in coastal seawater from three different sources: North Carolina, Orange County, and Hawaii at 20°C. Seawater samples were obtained on the same day, shipped to the laboratory in North Carolina, and within 1 day, they were seeded with test viruses, assayed for initial virus concentration after seeding and incubated with slow mixing. Samples were taken periodically and assayed for surviving viruses over time.

For each virus, inactivation was similar among the three seawaters, indicating no geographic difference, despite measurable differences in chemical quality (salinity = 28-40 ppt, pH = 7.6-8.8, turbidity = .02-9.1 NTU, suspended solids = 10-600 mg/L and volatile solids = 3.5-60 mg/L). By 30 days, in all three seawaters, SS phages were inactivated by ~2 $\log_{10}$, HAV was inactivated by ~4 $\log_{10}$, and PV-1 and FRNA phages were inactivated $\geq$ 6 $\log_{10}$ (detection limits). The virus survival data as a composite for all three seawaters at 20°C are summarized in Table 7 as mean 2 and 4 $\log_{10}$ (99% and 99.99%) reduction times. The T_{99} and $T_{99.99}$ values in Table 7 indicate that SS phage survival is considerably longer than the other viruses in this study. On average, both T_{99} and $T_{99.99}$ values were beyond the length of the experimental time period and had to be extrapolated. A notable aspect of the results for all viruses is that they exhibit retardant "die-off" kinetics, particularly SS phages and HA. HA had a T_{99} value of 6.0 days but a $T_{99.99}$ value of 49.8 days, which was beyond the 30-day experimental period. In contrast, PV-1 and FRNA phages reach T_{99} relatively rapidly (3.1 and 5.0 days, respectively), and both reach $T_{99.99}$ in ~12 days.

Table 7. Mean values for 2 and 4 $\log_{10}$ (99 and 99.99%) reduction

Virus Type	T_{99} (days)	$T_{99.99}$ (days)
FRNA phage	5.0	12.7
PV1	3.1	12.1
HAV	6.0	49.8*
SS phage	35.5	>100*

*values extrapolated beyond the experimental time period

Using repeated measures ANOVA tests to determine which variables influenced virus survival (virus type and seawater source) the type of viruses was significant ($p<0.001$). The p-values for significance testing of mean difference in $\log_{10}$ virus reductions over a 30-day time period are shown in table 8. The inactivation of PV1 and FRNA phages are not significantly different ($p=.475$), but for all the other virus pairs, the inactivation rates are significantly different ($p<0.001$).

Table 8. P-Values for Tests of Differences in Mean Virus Reduction

Virus Type	SS phage	FRNA phage	HAV	PV1
PV1	<0.001	0.475	<0.0001	-
HAV	0.0005	0.0007	-	
FRNA phage	0.00002	-		

The results of this study suggest that the reported difference in the inactivation of the same virus types in seawater from different studies are probably a consequence of methodological differences and not true differences in the virucidal activity of the seawaters. Geographically diverse seawaters were not very different with respect to the rate of and extent of virus inactivation when studied simultaneously under identical conditions. The results of this study indicate SS phages survive significantly longer than HAV, PV1 and FRNA phages in 20°C seawater. The long survival of SS phages in seawater warrants further investigation into their possible use as indicators of fecal contamination. In particular, the various possible sources of SS phages in fecally contaminated waters need to be better documented. The longer survival of SS phages compared to the survival of HAV and PV1 suggests that these phages may be reliable and conservative indicators of enteric virus survival in seawater. In

this study the inactivation of FRNA phages is similar to that of PV1 in seawater. However, the inactivation of FRNA phages was more rapid than that of HAV in seawater. Hence, while FRNA phages may be a reliable indicator of human enteroviruses like PV1, they may not be a good indicator of more persistent enteric viruses like HAV.

Role of marine microbial protease activity in virus inactivation.

In further experiments we investigated the effects of marine microbial proteolytic activity on virus survival in seawater. In these experiments samples of North Carolina seawater were seeded with test viruses (HAV, PV1, FRNA coliphages and SS phages), and half the sample was supplemented with a protease inhibitor. Samples were incubated at 20°C for nearly two weeks and aliquots were taken for virus assays at intervals during this period. Both PV1 and FRNA coliphages were inactivated relatively rapidly in normal seawater, with about 4 $\log_{10}$ (99.99%) reduction in two weeks. In samples treated with protease inhibitor PV1 and FRNA coliphage inactivation was appreciably less, with only about 2 $\log_{10}$ (99%)in two weeks. However, the presence of protease inhibitor did not improve the survival of HAV and SS phages. These two viruses survived generally better than poliovirus and F-specific coliphages, with only 2 to 3 $\log_{10}$ (99-99.9%) inactivation in two weeks. Assay of the seawater samples for proteolytic bacteria showed that untreated seawater contained 10^6 to 10^7 proteolytic CFU/ml while treated samples contained <10 CFU/ml. Numbers of total (non-proteolytic) bacteria per ml were similar in both treated and untreated samples. The results of these experiments indicate that marine microbial protease activity contributes greatly to the inactivation of some enteric viruses, such as PV1 and FRNA coliphages, in seawater. However, other enteric viruses, such as HAV and SS phages are not very susceptible to marine microbial protease activity. Hence, viruses differ in the susceptibility to marine microbial protease activity.

Sunlight Inactivation Studies

A series of inactivation studies utilizing poliovirus were conducted in order to determine the effects of naturally occurring sunlight on the cultural viability and the persistence of viral genetic material. Ocean water was collected from a relatively pristine site located near Diamond Head. Poliovirus (Type II, Lsc2ab) was seeded into filtered ocean water, unfiltered ocean water and phosphate buffered saline and exposed to natural sunlight for two 6 hour periods. Samples were then placed in a dark cold room for an additional four days. Control samples were kept in the dark throughout the experiment. Samples were collected at regular intervals during this period and submitted to cultural isolation and detection using RT PCR.

We observed a four log reduction in culturally viable poliovirus in ocean water, filtered ocean water and PBS after 6 hours of exposure to sunlight. The type of water utilized in these experiments had little impact on the rate at which poliovirus was inactivated (figures 4, 5 and 6). No culturally viable poliovirus was detected after 10 hours of exposure to sunlight. For controls held in the dark, there was a two log reduction after 144 hours of exposure. These results indicated sunlight appears to be responsible for a dramatic decrease in

poliovirus cultural viability. As seen in tables 9 and 10, PCR products were detected throughout the exposure period. Although cultural viability was lost after 10 hour of exposure to sunlight, viral RNA appears to persist for longer than 144 hours.

In addition to the sunlight inactivation studies, other survival experiments were performed using ocean water from Pearl Harbor. The waters of Pearl Harbor are characterized by being high in turbidity and influenced by nonpoint source pollution. In these waters there was a four log reduction of culturally viable poliovirus after 72 hours of exposure. In filtered Pearl Harbor water there was a 2 log reduction of poliovirus after 7 days of exposure. The control sample using PBS showed approximately a 1 log reduction after 7 days of exposure. These results indicate filterable agents in Pearl Harbor water appears to influence the cultural viability of poliovirus. This would suggest that polluted waters like those found in Pearl Harbor may assist in inactivating poliovirus. In summary these results indicated that sunlight and the polluted water found in Pearl Harbor play a role in the inactivation of poliovirus in ocean waters. PCR detection of viral RNA indicated that target sequences can be detected for at least 5 days after a loss of cultural viability.

Table 9. Virus Survival Exposed to Sunlight

Time (Hour)	PFU/ml PBS	Filtered Ocean	Ocean	PCR Product
0	8.0×10^5	7.8×10^5	8.0×10^5	+
2	2.3×10^4	4.8×10^4	3.7×10^4	ND
4	2.0×10^2	6.0×10^2	4.0×10^2	ND
6	$<2 \times 10^2$	2.0×10^2	2.0×10^2	+
24	68	92	64	+
26	8	<2	4	ND
28	<2	<2	<2	ND
30	<2	<2	<2	ND
72	<2	<2	<2	+
96	<2	<2	10	+
120	<2	<2	<2	+
144	<2	<2	<2	+

Sunlight exposure
Absence of sunlight

Figure 4. Poliovirus Sunlight Inactivation In Ocean Water.

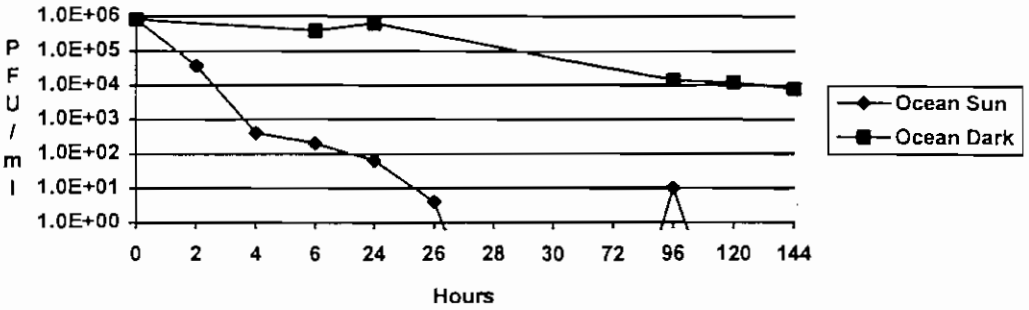

Figure 5. Polio Sunlight Inactivation In Filtered Ocean Water.

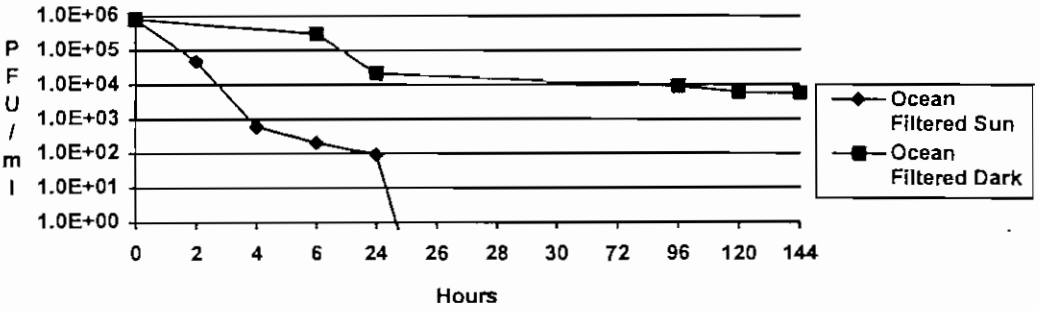

Figure 6. Polio Sunlight Inactivation In PBS.

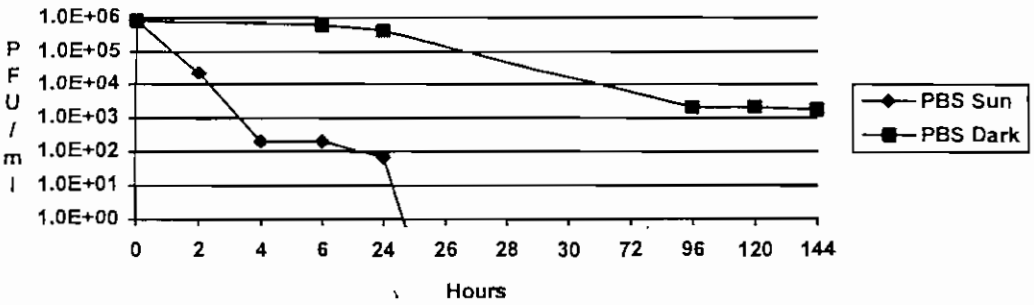

Table 10. Virus Survival in the Absence of Sunlight

Time (Hour)	PFU/ml PBS	Filtered Ocean	Ocean	PCR Product
0	8.0×10^5	7.8×10^5	8.0×10^5	+
6	6.0×10^5	3.0×10^5	3.8×10^5	ND
24	4.1×10^5	2.1×10^5	6.3×10^5	ND
48	2.0×10^5	1.3×10^5	2.7×10^4	+
96	2.0×10^3	9.0×10^3	1.4×10^4	+
120	2.1×10^3	6.0×10^3	1.2×10^4	+
144	1.8×10^3	5.8×10^3	8.0×10^3	+

Analysis of Viral RNA Persistence in Seawater. Throughout this study, we have used RT-PCR to detect viruses in aquatic environments. Therefore, we felt it was important to determine the stability of naked viral RNA in seawater since false positives could be obtained if the PCR amplified free RNA instead of RNA from intact viruses. The viral RNA in each virion is protected by a protein coat or an envelope. We wanted to determine how long the free RNA would survive in seawater if the outer protection is removed. Extracted RNA was seeded into both filtered and unfiltered seawater and the preparations were incubated at both 4°C and room temperature. The RNA seeded into the unfiltered seawater could not be detected after 2 days of incubation at either temperature while in the filtered seawater, RNA could still be detected after 21 days of incubation in both temperatures. Thus, in the natural environment (unfiltered seawater), the antiviral activity of seawater (including other organisms, RNAses, etc.) facilitate degradation of lysed viruses. When PCR is performed and positives obtained, the chances that the results indicate intact viruses are high due to the rapid degradation of free RNA from lysed virus. A manuscript has been published on these findings (Tsai et al., 1995) and is included in the appendix of this report.

CONCLUSIONS

This study provided an in-depth analysis of sewage, river, stream, estuarine and ocean water for the presence of a wide array of pathogenic microorganisms including toxigenic *E. coli, Legionella*, HIV, HAV, and the enteroviruses (including polio and rotavirus) using state-of-the-art molecular microbiology in tandem with traditional microbiology. The collaborative effort between the three laboratories in Hawaii, California, and North Carolina allowed for a remarkable exchange of technical advances, particularly in the area of environmental PCR, and for the comparison of water quality and results from these three different climatic environments. In addition, this project supported three graduate students and provided training via workshops that were attended by students, public health workers and water utility staff. Professional presentations, publications, graduate theses titles, and workshops are listed in appendix A. Publications are included in appendix B. A summary and highlights of our study are as follows:

1. Overall, molecular detection methods were more likely to yield positive results. In many cases, the target organisms would have been missed by traditional microbiological methods. While molecular detection affords increased sensitivity and a more thorough evaluation of the microbial quality of water, it also raises questions on acceptable population numbers of these organisms. We do not currently have enough information to understand the relative public health risk of our findings but our results certainly add significant information on the habitats and occurrence of certain pathogens in a wide array of water types.

2. We determined that indicator organisms, both coliforms and enterococci, are of limited (if any) value in predicting the presence of the pathogens detected in our study. Coliforms and enterococci did not correlate with virus detection in ocean water or with *Legionella* detection in reclaimed water.

3. We determined that toxigenic *E. coli* are probably not a public health risk in coastal waters and are present in negligible numbers in both sewage and ocean water.

4. We identified a new habitat for *Legionella* species since we found these organisms in all phases of the sewage treatment system in both California and Hawaii. We also identified reclaimed water as a habitat for legionellae. Further work on pipe biofilms, particularly on pipes utilized in reclaimed water is recommended. These studies would aid in understanding the microbial interactions in pipe biofilms and if *Legionella* would be a useful target organism in pipe disinfection tests.

5. It is unlikely that HIV is present in treated sewage effluent. HIV is a very labile virus and would not be expected to survive long in the environment outside of the host. We used both infectivity assays and RT-PCR to try to detect HIV in treated effluent. The fact that none was detected should help aleviate concerns from individuals who use waters receiving treated sewage for recreational pursuits. An exciting offshoot of this study was the fact that we discovered that polio infects PBMC cells. This could explain how polio enters the central nervous system and thus could shed more light on the method of polio pathogenesis..

6. According to seeding studies, HAV survives much longer than polio in ocean water and was the predominant virus detected in the California field study. Hepatitis A was not detected in Hawaii water in any of the water types sampled. Enteroviruses were not found in Hawaii ocean water but were recovered from streams entering coastal waters. Of interest in North Carolina was the finding that three different estuarine areas, one with human waste, one with cattle waste and one with no waste, all contained enteroviruses.

7. There were no differences between ocean water from the three study sites in virus seeding experiments. Although we suspected that the different physical components (salinity, pH, etc) would affect virus survival, it did not. Sunlight, however, was shown to impact the ability to culture viruses, since after two 6 hour periods of sunlight exposure, viruses could only be detected by PCR and they no longer infected cells lines. We also showed that naked viral RNA does not survive well in ocean water without its protective outer protein coat. This

information combined with the knowledge that our concentration methods eliminate cellular debris from burst organisms gives us confidence that PCR is detecting intact organisms and not artifacts or pieces of nucleic acid from dead or lysed organisms.

In conclusion, we obtained all major goals set forth in our grant proposal plus completed six additional goals. Our findings represent and portray some of the pioneering efforts in developing and applying molecular technology to the field of water quality. Because PCR is not currently able to differentiate live from dead organisms or viral infectivity, our findings can not be used as the basis for public health decisions. However, the fact that these organisms were detected indicates that additional research should be completed to better define the public health implications and to assist in further understanding the complexity of microbial systems.

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APPENDIX A

Publications, Presentations, Student Support, and Workshops

PUBLICATIONS

1. Palmer, C.J., Y.L Tsai, C.Paszko-Kolva, L.R. Sangermano, G.F. Bonilla, B.Roll, and R.S. Fujioka. 1993. Detection of *Legionella* species in sewage and ocean water in California and Hawaii. In Proceedings: Water Environment Federation Annual Conference, Anaheim, CA 123-131.

2. Palmer, C.J., Y.L. Tsai, C.McGee, L.R. Sangermano, R.S. Fujioka, and M.D. Sobsey. 1993. Collaborative national study using molecular techniques to detect pathogens in coastal waters. In Proceedings: Water Environment Federation Annual Conference, Anaheim, CA 257-266.

3. Tsai, Y.L., M.D. Sobsey, L.R.Sangermano, and C.J. Palmer. 1993. Simple method of concentrating enteroviruses and hepatitis A virus from sewage and ocean water for rapid detection by reverse transcriptase-polymerase chain reaction. Applied and Environmental Microbiology 59:3488-3491.

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5. Lang, A.L., Y.L. Tsai, C.L. Mayer, K.C. Patton, and C.J. Palmer. 1994. Multiplex PCR for detection of the heat-labile toxin gene and shiga-like toxin I and II genes in *Escherichia coli* isolated from natural waters. Applied and Environmental Microbiology 60:3145-3149.

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7. Palmer, C.J., G.F. Bonilla, B. Roll, C.Paszko-Kolva, L.R. Sangermano, and R.S. Fujioka. 1995. Detection of *Legionella* species in reclaimed water and air with the EnviroAmp Legionella PCR detection kit and direct fluorescent antibody staining. Applied and Environmental Microbiology. 61:407-412.

8. Palmer, C.J., M.Lee, G.F. Bonilla, B.J. Javier, E.B. Siwak, and Y.L. Tsai. Analysis of sewage effluent for human immunodeficiency virus (HIV) using infectivity assay and reverse-transcriptase polymerase chain reaction. Submitted 11/30/94.

9. Roll, B.M. and R.S. Fujioka. 1995. Detection of *Legionella* bacteria in sewage by polymerase chain reaction and standard culture method. Proceedings Water Quality International "94, International Association on Water Quality, London. In Press.

10. Schwab, K.J., R. DeLeon, and M.D. Sobsey. 1995. Concentration and purification of mock beef extract eluates from water samples for the detection of enteroviruses, hepatitis A virus, and Norwalk virus by reverse transcription-PCR. Applied and Environmental Microbiology. 61:531-537.

11. Callahan, K.M., D. Taylor and M.D. Sobsey. 1995. Comparative survival of hepatitis A virus, poliovirus and indicator viruses in geographically diverse seawaters. Water Science and Technology. Proceedings Water Quality International "94, International Association on Water Quality, London. In Press.

12. Shieh, Y.S, D. Wait, L. Tai, and M.D. Sobsey. 1995. Methods to remove inhibitors in sewage and other fecal wastes for enterovirus detection by polymerase chain reaction. In Press. J. Virological Methods.

PRESENTATIONS

1. Roll, B., R. Fujioka, and C.J. Palmer. 1993. DNA hybridization detection of pathogenic *Escherichia coli* in raw and treated sewage in Hawaii. American Society for Microbiology Annual Meeting, Atlanta, GA.

2. Callahan, K., M. Gray, and M.D. Sobsey. 1993 Survival of fecal indicator bacteriophages in geographically diverse seawaters. American Society for Microbiology Annual Meeting, Atlanta, GA

3. Palmer, C.J. 1993. Collaborative national study using molecular techniques to detect hepatitis A virus and virulence genes in *E. coli* in sewage and ocean receiving waters. Hawaii Water Pollution Control Association, Honolulu, HI Feb. 93

4. Palmer, C.J., M.H. Lee, G.F. Bonilla, B.J. Javier, Y.L. Tsai, and L.R. Sangermano. 1994. Analysis of sewage effluent for human immunodeficiency virus (HIV) using infectivity assay and reverse transcriptase PCR. American Society for Microbiology Annual Meeting, Las Vegas, NV

5 Palmer, C.J., G.F. Bonilla, C. Paszko-Kolva, B.Roll, R.S. Fujioka, and L.R. Sangermano. 1994. Detection of *Legionella* spp. in chlorinated effluent using the EnviroAmp Legionella PCR kit and direct fluorescent antibody staining. American Society for Microbiology Annual Meeting, Las Vegas, NV

6. Roll, B.M. and R.S. Fujioka. 1992. Development of new methods for the detection of waterborne pathogens in Hawaii's recreational waters. Environmental health seminar series, School of Public Health, University of Hawaii

7. Roll, B.M. and R.S. Fujioka. 1992. Detection of waterborne pathogens using gene probes and the polymerase chain reaction. Seminar series, Dept. of Microbiology, University of Hawaii.

8. Roll, B.M. and R.S. Fujioka. 1993. Detection of pathogenic *Escherichia coli* in sewage using gene probes and the polymerase chain reaction. Environmental health seminar series, School of Public Health, University of Hawaii.

9. Roll, B.M. and R.S. Fujioka. 1993. Alternative methods for the detection of enteric viruses and pathogenic bacteria in recreational waters. Environmental health seminar series, School of Public Health, University of Hawaii.

10. Roll, B.M. and R.S. Fujioka. 1993. Development of new methods for the detection of waterborne pathogens in sewage and recreational waters. Seminar series, Dept. of Microbiology, University of Hawaii.

11. Roll, B.M. and R.S. Fujioka. 1994. Detection of *Legionella* in sewage and air using the polymerase chain reaction and standard culture method. Seminar, Wahiawa Wastewater Treatment Facility, Wahiawa, Hawaii.

12. Roll, B.M., R.S. Fujioka, and C.J. Palmer. 1995. The theory, benefits and limitations of gene probes and the polymerase chain reaction for the detection of waterborne pathogens. Hawaii Water Environment Association, Honolulu, HI (Feb. 95)

GRADUATE STUDENT SUPPORT and THESIS TITLES

Masters level

1. Kathleen Callahan, M.S. University of North Carolina Chapel Hill, Dept. of Environmental Sciences and Engineering. Thesis: "Comparative survival of hepatitis A virus, poliovirus, and indicator organisms in geographically diverse seawater."

2. Ling Tai, M.S. University of North Carolina Chapel Hill, Dept. of Environmental Sciences and Engineering. Thesis: "Detection of norwalk virus, hepatitis A virus, and enterovirus in wastewater by RT-PCR and oligonucleotide hybridization."

3. Bruce Roll, MPH University of Hawaii at Manoa, Dept. of Public Health. Thesis: "Detection of *Legionella* bacteria in sewage by polymerase chain reaction and standard culture method"

Doctoral level

1. Bruce Roll, Ph.D. University of Hawaii at Manoa, Dept. of Microbiology Dissertation: "Development of polymerase chain reaction (PCR) techniques for the detection of waterborne pathogens in environmental waters."

WORKSHOPS

1. Palmer, C.J. and Y.L. Tsai. "Genetic Analysis DNA Methods Workshop", August 31- September 15, 1992 University of Hawaii at Manoa

2. Palmer, C.J. and Y.L. Tsai. "Environmental Virus Detection Workshop," August 1-7, 1993 University of Hawaii at Manoa