CONCENTRATION OF ADENOVIRUSES AND NOROVIRUSES FROM SEAWATER WITH ARGONIDE NANOCERAM CARTRIDGE FILTERS: METHOD EFFECTIVENESS AND OCCURRENCE IN SOUTHERN CALIFORNIA RECREATIONAL WATERS

Christopher Gibbons

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Approved by:

Dr. Mark D. Sobsey

Dr. Otto D. Simmons, III

Dr. Louise M. Ball

ABSTRACT

Christopher Gibbons

Concentration of Adenoviruses and Noroviruses from Seawater with Argonide Nanoceram Cartridge Filters: Method Effectiveness and Occurrence in Southern California Recreational Waters
(Under the direction of Dr. Mark D. Sobsey)

The goals of this study were to evaluate Argonide Nanoceram cartridge filters for concentrating adenoviruses and noroviruses from seawater and then apply them to Southern California recreational water samples. The filters were challenged with 20-40 L of seawater seeded with test viruses; the recoveries of adenoviruses and noroviruses were 3.2% (± 2.3%) and 111% (± 28%), respectively. PEG precipitation was used as a secondary concentration step, and recovery rates with optimized conditions were 34% (± 12%) for adenovirus, and 59% (± 4.8%) for norovirus. These methods were applied to seawater samples from two Southern California beaches for the detection of both viruses. For Doheny Beach, 24 and 21 of 93 samples were positive for adenovirus and norovirus, respectively. For Avalon Beach, 26 and 2 of 286 samples were positive for adenovirus and norovirus, respectively. The presence of enteric viruses was not associated with the presence of coliphages in this study.

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LIST OF ABBREVIATIONS

µl	microliters
µm	micrometers
BE	beef extract
bp	base pairs
CPE	cytopathogenic effects
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
F+	male-specific
g	grams
GII.4	genogroup 2, strain 4
GuSCN	guanidinium thiocyanate
HAV	hepatitis A virus
HCl	hydrochloric acid
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
in.	inches
KCl	potassium chloride
KH_2PO_4	potassium phosphate
L	liters
M	molar

m^2	square meters
MEM	minimal essential media
mg	milligrams
$MgCl_2$	magnesium chloride
ml	milliliters
mM	millimolar
MPN	most probable number
mV	millivolts
Na_2HPO_4	sodium phosphate
NaCl	sodium chloride
NaOH	sodium hydroxide
nm	nanometers
NTU	nephelometric turbidity units
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PPT	parts per thousand
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
sec	seconds
st dev	standard deviation
TAE	Tris-acetate-EDTA

UV	ultraviolet
V	volts
VP	estimated viral particles

INTRODUCTION

The presence of human enteric viruses in recreational waters is currently a major public health concern. Viruses such as adenovirus, norovirus, rotavirus and enteroviruses are transmitted via the fecal-oral route, and may be capable of persisting in waters contaminated with fecal material for many days or longer, depending on the initial virus concentration (Fong & Lipp, 2005). These viruses are known to cause acute gastroenteritis in infected individuals, as well as other diseases (Henrickson et al., 2001). Transmission of these viruses can occur when contaminated water is accidentally ingested, or when contaminated shellfish are consumed (Griffin et al., 2003).

In order to reduce the risk of exposure in recreational water users, detection of human enteric viruses in water is crucial. Current water quality assessments are based on bacterial indicators of fecal contamination, which may not accurately predict the presence and concentration of human pathogenic viruses in water (Fong & Lipp, 2005). Therefore, the direct detection of enteric viruses by polymerase chain reaction (PCR) may be a valuable tool for determining the presence of these specific pathogens as well as others, such as bacteria and protozoan parasites.

Adenoviruses are non-enveloped double-stranded DNA viruses that range in size from 90 to 100 nm. Fifty-one human serotypes have been identified, some of which cause acute respiratory disease (types 1-7, 14, and 21), conjunctivitis (types 3, 7, 8, 11, 14, 19, and

37), and gastroenteritis (types 31, 40, and 41) (Rusin et al., 2000). Infected individuals spread adenoviruses via the respiratory or fecal-oral routes, because the viruses are shed in the respiratory secretions and feces of infected individuals for up to weeks after the onset of infection (Centers for Disease Control and Prevention, 2005). The serotypes that cause gastroenteritis are often found in wastewater and natural waters impacted by wastewater and urban runoff (Griffin et al., 2003; Jiang et al., 2001). Their physical structure makes adenoviruses exceptionally resistant to disinfection and other water and waste purification procedures. Their resistance is considered to be primarily due to DNA repair, such that a damaged strand of DNA may be repaired by the host cell using the undamaged complementary strand as a template (Fong & Lipp, 2005). In 1998, adenoviruses were added to the Environmental Protection Agency's "Candidate Contaminant List" as part of the Safe Drinking Water Act, and are currently one of only four viruses or virus groups included in the list (the others being caliciviruses, coxsackieviruses, and echoviruses).

Noroviruses, previously known as "Norwalk-like viruses," are small (25-40 nm), non-enveloped, single-stranded RNA viruses. They are members of the *Caliciviridae* family, and are genetically classified into five different genogroups (GI, GII, GIII, GIV, and GV), which are further divided into genotypes. Of these, only genogroups I, II, and IV are known to contain human strains (Atmar & Estes, 2006). Human noroviruses have traditionally been difficult to study because they cannot easily be propagated in cell cultures or in experimental animals (Estes et al., 2000; Haramoto et al., 2004; Straub et al., 2007). Symptoms of norovirus infection include vomiting, nausea, abdominal cramping, and diarrhea, and usually last 1-3 days (Centers for Disease Control and Prevention, 2006). Noroviruses are spread via the fecal-oral route by infected individuals, with fecal shedding for up to three weeks after

the onset of infection (Rockx et al., 2002). Noroviruses have frequently been found in treated wastewater and natural waters contaminated with fecal material (Beller et al., 1997; da Silva et al., 2007; Griffin et al., 2003; Lodder et al., 1999). This may be due to the fact that these viruses are resistant to some forms of chlorine disinfection, and can withstand freezing, as well as temperatures as high as 60˚ C (Griffin et al., 2003; Thornton et al., 2004).

In order to detect human enteric viruses in water using PCR analysis or other assay methods, the viruses must first be concentrated. This is due to the fact that enteric viruses are present only in low concentrations in recreational waters, and detection is limited by the volume that can be analyzed by PCR or other assay methods. Primary virus concentration from water is often achieved using filter adsorption-elution methods. Under the appropriate conditions, viruses in water will adsorb to positively-charged filters, and the adsorbed viruses may then be eluted using an alkaline solution of organic matter such as beef extract at pH 9.5 (Schwab et al., 1995). The effectiveness of this method depends on the efficiency of adsorption of the virus particles to the filter, and the efficiency of adsorbed viral particle recovery during elution.

Secondary concentration of the viruses is typically achieved by using a method such as polyethylene glycol (PEG) precipitation or organic flocculation. PEG precipitation creates saturated conditions in which proteins as well as viral particles precipitate from solution (Lewis & Metcalf, 1988). Centrifugation allows separation of the PEG-containing supernatant from the sediment or pellet, which contains the majority of the viral particles. Organic flocculation relies on the tendency of proteins to precipitate out of solution under acidic conditions. At a pH of approximately 3.5, virus particles will naturally bond to proteins in the eluent due to electrostatic interactions, and centrifugation allows the

separation of viruses and precipitable proteins from the supernatant (Katzenelson et al., 1976).

In this study, positively-charged Argonide Nanoceram alumina cartridge filters were evaluated for the recovery of adenoviruses and noroviruses from seawater. PEG precipitation was then evaluated as a secondary concentration step, followed by direct detection of the viral nucleic acids by PCR assay for adenoviruses and RT-PCR assay for noroviruses. The performance of the newly developed method was evaluated by applying it to waters considered to be high risk in causing waterborne illness due to fecal contamination and its attendant viral pathogens. The development of a reliable and effective method for the concentration of these enteric viruses from seawater is essential for determining recreational water quality and thereby reducing the risks of human exposure to waterborne pathogens.

STATEMENT OF OBJECTIVES

- Evaluate the use of Argonide Nanoceram positively-charged cartridge filters as a means of primary concentration of adenoviruses and noroviruses in seeded water samples
- Determine the effectiveness of beef extract as an eluent for recovery of viruses adsorbed to Argonide Nanoceram filters under various conditions
- Evaluate the use of PEG as a secondary concentration method for viruses in eluates from Argonide Nanoceram filters
- Determine the presence or absence of adenovirus and norovirus in seawater samples from two fecally contaminated Southern California beaches, Doheny Beach and Avalon Beach, using these methods in conjunction with (RT-)PCR analysis
- Statistically analyze for a correlation or lack thereof between the presence of adenovirus and norovirus, and between these two groups of human enteric viruses and coliphages in the seawater of the two beaches sampled

LITERATURE REVIEW

Introduction

The presence of human enteric viruses in coastal waters represents a serious health risk to swimmers and other recreational water users. By monitoring these areas for the presence of human pathogens, preventative actions can be taken to reduce the risk of human exposure. The studies summarized in this review are related to the concentration and detection of enteric viruses in water, and the practical application of each method under the environmental conditions associated with seawater.

Enteric Viruses in Seawater

Throughout the world, the eventual fate of wastewater is often to discharge it into ambient waters, many of which drain into the ocean. This can occur directly in the case of coastal communities, or indirectly if wastewater is discharged into a river and then carried to the ocean. In many cases wastewater is treated before it is released into the environment, but wastewater treatment processes may be ineffective at removing all human pathogens (da Silva et al., 2007). In fact, current U.S. regulations for treating wastewater are based on bacterial indicators, and the discharge of viral pathogens in treated sewage is not regulated (Griffin et al., 2003). This lack of attention to viruses in wastewater discharges is a concern

because enteric viruses generally tend to be more resistant to disinfection procedures than bacteria (Fong & Lipp, 2005).

Some researchers have suggested using coliphages as indicators of fecal contamination in water. Coliphages are a type of bacteriophage that infects coliform bacteria present in the gut of warm-blooded animals, and are therefore frequently present in waters contaminated with fecal material. Because coliphages are viruses, they are generally more resistant to disinfection procedures than fecal indicator bacteria. Therefore, it has been suggested that their presence may more accurately indicate the presence of enteric viral pathogens, since enteric viruses are also resistant to disinfection. Two types of coliphages that have been considered as potential indicators of viral pathogens in water are somatic coliphages and male-specific (F+) coliphages. Somatic coliphages are a heterogenous group of bacteriophages that infect *E. coli* bacteria by attaching to a specific receptor site on the cell wall, whereas F+ coliphages are only infectious to "male-specific" bacteria possessing the F-plasmid, as they attach to a specific receptor site of the sex pilus (Leclerc et al., 2000). A number of studies have reported a positive association between human enteric viruses and somatic (Moce-Llivina et al., 2005; Skraber et al., 2004) or F+ coliphages (Colford et al., 2007; Dore et al., 2000; Jiang et al., 2001) in waters contaminated with fecal pollution. However, some uncertainty remains concerning the comparative survival of coliphages and human enteric viruses in natural aquatic environments due to factors such as removal of viruses by microbial predation and inactivation of viruses by solar radiation.

Another potential source of wastewater contamination stems from cities that use combined sewer systems that collect both wastewater and stormwater. In this case, during periods of high precipitation the treatment plants may become overloaded, and rainwater and

untreated sewage will be discharged directly into the receiving waters (Rodriguez, 2007). This phenomenon may also be observed in separate sewer systems if storm water leaks into the sewage system. Seawater may also become contaminated by urban, rural and agricultural runoff and subsurface seepage during storm events, which can carry pathogenic viruses from the land into streams and rivers, which eventually drain into the ocean (Fong & Lipp, 2005).

Once they reach the ocean, pathogenic human viruses may be able to persist for many days or weeks in a marine environment. For example, Enriquez et al. (1995) determined that poliovirus type 1 and adenoviruses 40 and 41 could survive in seawater at 15˚ C for 18, 77 and 85 days, respectively, before 99% reductions were observed. In addition to this survival study, numerous studies have demonstrated the presence of human enteric viruses in coastal waters impacted by wastewater or urban runoff (Goyal et al., 1979; Griffin et al., 1999; Jiang et al., 2001), and in estuaries that discharge directly into the ocean (Lodder & de Roda Husman, 2005; Noble et al., 2006).

The presence of human enteric viruses in seawater represents a serious health risk to recreational water users (Griffin et al., 1999; Griffin et al., 2003; Henrickson et al., 2001; Schaub & Oshiro, 2000). In addition, many studies have shown that certain human enteric viruses become concentrated in the tissues of bivalve mollusks such as oysters and mussels (Goyal et al., 1979; Myrmel et al., 2004; Pina et al., 1998). This phenomenon can lead to human disease following the consumption of contaminated shellfish (Griffin et al., 2003).

By carefully monitoring seawater quality or implementing more effective methods to reduce enteric virus contamination in seawater, the risk of disease from these pathogens may be significantly reduced. The monitoring of human viruses in water is often achieved by cell culture assays or direct detection of viral nucleic acid by polymerase chain reaction (PCR).

However, these methods are limited by the volume that can be analyzed, and most viruses are present in relatively low concentrations in environmental waters. Therefore, viruses must be concentrated before detection by these methods is feasible.

Concentrating Viruses from Tap, Fresh-, Sea-, and Wastewaters

The four basic steps often used for detecting viruses in water samples are concentration by filtration, elution or other recovery of viruses from the filter, reconcentration, and viral detection and assay (Pepper et al., 2000). Because virus particles are very small, filtration is usually dependent upon either ultrafiltration to physically retain viruses by exclusion, or adsorption to microporous filters by electrostatic or hydrophobic interactions between the viruses and the filter medium rather than physical entrapment or size exclusion. The initial volume which must be concentrated is usually dependent upon the anticipated number of virus particles present in the water, which usually depends on the type of water being processed. For example, treated wastewaters are likely to contain more viruses than natural waters, and far more viruses than treated tap water. Therefore, greater volumes of tap or natural waters must be filtered relative to wastewaters in order to concentrate a sufficient number of viruses for subsequent detection. In order to determine which type of filter to use, one must carefully consider the physical and chemical nature of the water media which is to be filtered. In general, wastewater and near-shore estuarine and seawater are more turbid than other water types, and seawater is the most saline. Natural waters may contain various amounts of natural and anthropogenic organic material, such as humic and fulvic acids, and the pH may also vary considerably among different water types.

Another important consideration when concentrating viruses based on electrostatic interactions is the isoelectric point and charge density or zeta potential of the viruses. Viral capsids contain numerous electrical charges as a result of the amino acid functional groups present in the surface proteins, most notably carboxyl, amino and sulfhydryl groups. Overall, these ionizable functional groups confer a net negative charge to viruses in solutions near neutral pH. However, if the pH of the solution is lowered, these surface proteins undergo protonation, and in some cases a conformational shift that results in a net positive charge (Reynolds & Pepper, 2000). The pH at which this transition between negative and positive charge occurs and there is a zero net charge is known as the isoelectric point. This surface charge-related ionic behavior may be utilized in order to capture viral particles on either negatively- or positively-charged filter materials when concentrating viruses from water.

Comparison of Electronegative and Electropositive Filters

Since the 1960s, many studies have supported the use of negatively-charged filters for concentrating viruses from different types of water. The electronegative filters examined in these studies included Millipore nitrocellulose filters (Millipore Corp., Bedford, MA), Cox epoxy fiberglass filters (Cox Instrument Corp., Detroit, MI), and Filterite epoxy fiberglass filters (Filterite Corp., Timonium, MD). Wallis & Melnick (1967) conducted one of the first such studies, in which poliovirus seeded into tap water was reported to adsorb to Millipore filter membranes in the presence of salts. Several years later, Sobsey et al. (1973) found that if the water is acidified prior to filtration, poliovirus may be adsorbed to Millipore or Cox filters without the addition of salts. Although neither study speculated on the underlying mechanisms, it was later revealed that adsorption was related to the isoelectric point of the

viruses and the membrane. At neutral pH, both the viruses and the filter possessed an overall negative charge and would repel one another, but lowering the pH caused the viruses to become positively-charged and therefore adsorb to the negative membrane (Sobsey & Jones, 1979). In addition, it was speculated that by adding multivalent cation salts to the water, the multivalent cations in solution served as a mediator between the negative surfaces and thereby allowed viral adsorption. The use of electronegative filters was expanded when Payment et al. (1976) applied this method to the concentration of polioviruses from seawater. In this study, poliovirus was seeded into 100-gallon samples of highly turbid estuarine water collected from the Gulf of Mexico, and the water was acidified to pH 3.5 and supplemented with 0.0015 M aluminum chloride to promote adsorption to Filterite filters. The adsorbed viruses were eluted with an alkaline solution, and virus recovery rates of 48-58% were reported. Numerous studies were conducted in subsequent years reporting the successful adsorption and recovery of enteric viruses from electronegative filters using large volumes of tap water (Farrah et al., 1976; Gerba et al., 1978), seawater (Farrah et al., 1977; Gerba et al., 1978; Goyal et al., 1979; Goyal & Gerba, 1983; Reynolds et al., 1995), and treated sewage (Gerba et al., 1978). Although most of these studies focused on the concentration of enteroviruses, such as poliovirus, echovirus, and coxsackievirus, Goyal & Gerba (1983) demonstrated that rotaviruses could also be concentrated from seawater using Filterite filters, with an overall recovery of 99% in seeded samples.

The electropositive filters evaluated in previous studies were Zeta Plus cellulose-diatomaceous earth-"charge-modified" resin filters (AMF Corp., Cuno Division, Meriden, CT) and Virosorb 1MDS charge-modified fiberglass filters (AMF Corp., Cuno Division, Meriden, CT). The use of positively-charged filters to concentrate viruses from water was

first suggested by Sobsey & Jones (1979). They hypothesized that viruses, which are naturally negatively-charged near neutral pH, would adsorb to positively-charged membranes without acidification of the water or the addition of multivalent cation salts. To support this hypothesis, they seeded poliovirus into tap water at ambient pH levels and succeeded in recovering the viruses from Zeta Plus filters with a 66% recovery rate. The concept of using electropositive filters for the recovery of viruses from water was quickly adopted by other researchers. Logan et al. (1981) used Zeta Plus filters to concentrate bacteriophages from seeded samples of natural river water with an estimated recovery of 75%, although the recovery was somewhat lower when the pH of the water was greater than 8. That same year, Chang et al. (1981) used Zeta Plus filters to successfully concentrate naturally-occurring enteroviruses from as much as 30 gallons of wastewater effluent collected from a wastewater treatment plant. Soon after, positively-charged filters were being used to concentrate rotaviruses from tap water using Zeta Plus and 1MDS filters (Guttman-Bass & Armon, 1983; Toranzos & Gerba, 1989). In addition, adenoviruses were detected by Pina et al. (1998) in 14 out of 15 (93%) raw sewage samples collected in Barcelona, Spain, in 2 out of 3 samples of primary treated wastewater effluent, and in 6 out of 20 river water samples collected near the point of wastewater discharge using Zeta Plus filters.

Numerous studies have been designed to directly compare the performance of each type of filter for specific viruses and water conditions. In 1980, shortly after the introduction of the electropositive filter, Sobsey & Glass (1980) compared electronegative Filterite filters to electropositive Zeta Plus and 1MDS filters for the recovery of poliovirus seeded into tap water. They calculated similar recoveries for both filters, but noted that the electropositive filters were easier to use and did not subject the viruses to extreme pH levels. Several years

later, Sobsey & Glass (1984) performed another comparative experiment in which enteroviruses, echoviruses and adenoviruses were seeded into raw or treated lake water. They reported that overall, enterovirus recovery was slightly higher with 1MDS filters, but that adenovirus and echovirus recovery was slightly higher with Filterite filters. The major conclusion from this study was that neither filter is superior for all virus types and water conditions. That same year, Rose et al. (1984) tested the efficiency of Filterite, Zeta Plus and 1MDS filters for the recovery of naturally-occurring coliphages from primary (pH 7.4) and secondary (pH 6.8) sewage. They found that the positively-charged filters were superior in recovering these viruses from secondary sewage, and that there was no significant difference in recovery efficiency among different filters for viruses in primary sewage. The following year, Sobsey et al. (1985) conducted a study to determine recovery of hepatitis A virus (HAV) in drinking water at various pH levels. Under optimal conditions (pH 3.5 for Filterite filters, pH 4.5-6.5 for 1MDS filters), both filters adsorbed viruses efficiently (>97% for both). However, adsorption rates to 1MDS filters decreased significantly at higher pH levels (82% at pH 7.5, 43% at pH 8.5). In 1995, Enriquez & Gerba (1995) tested both types of filter for the recovery of human adenoviruses from tap water (pH 7.5), and obtained recoveries of 36.6% and 26.5% using Filterite and 1MDS filters, respectively. However, this observed difference in recovery was not statistically significant. The effect of salts on viral adsorption was tested by Lukasik et al. (2000). They found that the addition of sodium chloride or magnesium chloride to tap water significantly interfered with the adsorption of coliphages and poliovirus to positively-charged 1MDS filters, but had no effect on viral adsorption to Millipore or Filterite negatively-charged filters.

Based on these comparative studies, it seems that neither type of filter, positively-charged or negatively-charged, is ideal for all viruses, water types, and environmental conditions. Positively-charged filters are easier to use than negatively-charged filters, and do not require acidification of the water prior to filtration, which may promote inactivation of viral particles. However, electropositive filters may be inefficient when concentrating viral particles at elevated pH levels (>7.5) or in the presence of salts such as sodium chloride and magnesium chloride. Due to these limitations, the development of a new adsorbent filter capable of concentrating different viruses from a variety of water types and pH levels would be greatly advantageous.

Elution of Viruses from the Filter

Once viruses have adsorbed to a microporous filter, they must then be eluted with a solution that disrupts the electrostatic or hydrophobic interactions between the virus particles and the filter surface. The eluent may then be collected for further concentration or for detection of the viruses by cell culture or PCR. One of the most commonly used eluents is a beef extract (BE) solution at an elevated pH (usually 9-10) which contains a concentration of 1-3% beef extract. In many early studies, a glycine solution (usually 0.05 M) at elevated pH (10.5-11.5) was used to successfully elute viruses from negatively-charged membranes (Farrah et al., 1976; Gerba et al., 1978; Goyal et al., 1979; Payment et al., 1976; Sobsey et al., 1973). In both cases, the high pH increases the negative charge of the viruses and the filter, and the proteins and hydroxide ions in the solution compete with the viruses for adsorption sites on the filter, both of which lead to desorption of the virus (Pepper et al., 2000). Several studies have reported successful elution using an eluent with a combination

of these two properties, a beef extract solution and alkaline pH conditions (i.e. 0.3-3% beef extract solution containing 0.05 M glycine at pH 9.5) (Abbaszadegan et al., 1993; Enriquez & Gerba, 1995; Sobsey & Glass, 1980; Sobsey & Glass, 1984; Sobsey et al., 1985). Although this method has been proven effective in a number of studies, it is important to realize that successful elution is ultimately dependent on several factors, including the type of virus, the type of filter, and the presence of salts and organic materials which may affect the elution process. Therefore, it is always advisable to test the effectiveness of an eluent for each set of recovery conditions before proceeding with a field experiment.

Reconcentration

Because viruses are often present in the environment in very low numbers, it is frequently necessary to further concentrate the viruses in the filter eluent. Several reconcentration methods have been studied, including a second step of microfilter adsorption-elution, ultrafiltration, organic flocculation, and PEG precipitation.

A second step of microporous filter adsorption-elution and ultrafiltration are basically the same as initial filter adsorption-elution or initial ultrafiltration, except that they use a smaller filter and consequently a smaller eluent volume. Second step filtration processes have been used successfully in a number of studies (Logan et al., 1980; Logan et al., 1981; Sobsey et al., 1973). Disadvantages of these second step filtration methods are that most second step microfilters and ultrafilters can only process a small volume of liquid, so this method is ineffective or impractical when concentrating viruses from larger volumes. In addition, these filters can become clogged if a large amount of organic matter or particulates are present in the eluent.

Organic flocculation was first described by Katzenelson et al. (1976). They discovered that lowering the pH of a protein solution (such as beef extract) to 3.5 will produce flocculation of proteins, and the viruses in solution will naturally bond reversibly to these proteins. After lowering the pH, they centrifuged the beef extract solution in order to separate the proteins and viruses from the remaining liquid, and the pellet was resuspended in a sodium phosphate (Na_2HPO_4) solution. Using this method, they reported nearly a 100% recovery of poliovirus from 300 ml of a 3% beef extract solution. Since its discovery, this method has been used by many scientists to successfully reconcentrate enteroviruses (Abbaszadegan et al., 1993; Guttman-Bass & Nasser, 1984; Reynolds et al., 1995; Sobsey & Glass, 1980), rotaviruses (Goyal & Gerba, 1983; Guttman-Bass & Armon, 1983; Toranzos & Gerba, 1989), adenoviruses (Enriquez & Gerba, 1995), noroviruses (Griffin et al., 1999), and HAV (Sobsey et al., 1985) from beef extract solution eluates. Notable drawbacks to this method are that the process of lowering the pH to 3.5 may cause inactivation of acid-sensitive viruses and precipitate other material from solution that can interfere with subsequent steps for virus recovery and assay.

Polyethylene glycol (PEG) precipitation was carefully described as a method of reconcentrating human enteric viruses from eluates by Lewis & Metcalf (1988), but the method has been used for several decades to concentrate and purify a variety of viruses from various aqueous solutions (Adams, 1973; Friedmann and Haas, 1970; McSharry and Benzinger, 1970; Yamamoto et al., 1970). In the study by Lewis & Metcalf (1988), hepatitis A virus, human rotavirus, and poliovirus were seeded into natural bayou and estuary waters. The viruses were concentrated using an electronegative filter, and specified concentrations of PEG were added to the eluate. The solution was stirred for two hours at 4˚ C, centrifuged,

and the pellet was resuspended in a sodium phosphate (Na_2HPO_4) solution. For the freshwater samples, mean recoveries were 93%, 78% and 73% for rotavirus, hepatitis A virus and poliovirus, respectively. For the estuarine samples, mean recoveries were 61%, 95% and 64%, respectively. Optimum virus recovery was dependent on sample type in combination with PEG concentration and sodium chloride (NaCl) concentration. Since then, many other studies have used this method to effectively reconcentrate viruses (Atmar et al., 1993; da Silva et al., 2007; Schwab et al., 1995). PEG precipitation and organic flocculation were directly compared by Enriquez & Gerba (1995) for concentrating adenoviruses from tap water. They reported a 38.6% recovery using organic flocculation, and a 40% recovery using PEG precipitation, and concluded that these results were not significantly different. The combined results of these studies seem to indicate that PEG precipitation is an effective reconcentration step and that it may be performed at ambient pH levels, so inactivation of viruses is less likely to occur than with the organic flocculation method, which requires acidification to a pH of about 3.5.

Detection of Viruses

Cell culture assays have traditionally been used by many researchers to detect different enteric viruses in concentrated samples, including different enteroviruses (Chang et al., 1981; Farrah et al., 1976; Farrah et al., 1977; Gerba et al., 1978; Goyal et al., 1979; Payment et al., 1976; Sobsey et al., 1973; Sobsey & Jones, 1979; Sobsey & Glass, 1980; Sobsey & Glass, 1984), simian rotaviruses SA-11 (Goyal & Gerba, 1983; Guttman-Bass & Armon, 1983; Toranzos & Gerba, 1989), and hepatitis A virus HM-175 (Sobsey et al., 1985). The detection of viruses by cell culture is dependent on the ability of the viruses to infect the

cell line and propagate. Depending on the virus being analyzed, this may promote cell lysis, or it may produce visible changes in the cell morphology (Josephson et al., 2000). The limitations of this method are that it is costly and time-consuming, and some viruses, such as hepatitis A virus and human norovirus, are very difficult if not impossible to propagate in any cell lines currently available (Estes et al., 2000; Haramoto et al., 2004). In addition, cell lines are to varying degrees susceptible to toxic compounds present in environmental samples. Such cytotoxicity can potentially lead to false positive results if cytotoxic effects are interpreted as viral cytopathogenic effects (CPE) or false negative results if cell death occurs prior to virus replication.

In recent years, many studies have advocated the use of PCR or RT-PCR for the detection of enteric viruses such as enteroviruses (Abbaszadegan et al., 1993; Noble et al., 2006; Reynolds et al., 1995), noroviruses (Atmar et al., 1993; Loisy et al., 2005; Myrmel et al., 2004), adenoviruses (Jiang et al., 2001; Pina et al., 1998), and hepatitis A virus (Griffin et al., 1999; Hafliger et al., 1997; Traore et al., 1998). The detection of viruses by PCR analysis relies on the use of primers and DNA polymerase to amplify specific regions of the viral genome. For conventional PCR, the target region of DNA is replicated exponentially, and may then be detected through the use of gel electrophoresis. In order to replicate an RNA strand, such as the genomes of many important enteric viruses including enteroviruses, noroviruses and hepatitis A and E viruses, reverse transcriptase PCR (RT-PCR) must be used instead. In this process, a reverse transcriptase enzyme is used to initially convert the RNA to its complementary double-stranded DNA prior to replication. Some researchers have suggested the use of nested PCR or RT-PCR in order to further amplify the target genetic sequence and thereby provide greater specificity and sensitivity (Hafliger et al., 1997; Vinje

et al., 2004). In this case, two PCR reactions are performed in succession to generate a more specific and highly amplified final product. Although all these forms of conventional PCR are highly sensitive and accurate, none of them provide quantitative data. The only way to quantify the number of viruses or amount of viral nucleic acid using these methods is by applying PCR or RT-PCR amplification to a dilution series of the sample in order to determine the last (greatest) dilution that yields a positive result. This approach allows for the estimation of virus or viral nucleic acid in the sample by endpoint dilution or Most Probable Number (MPN) quantification.

Another approach for determining the approximate number of virus particles present in the sample is quantitative PCR, otherwise known as real-time PCR. Real-time PCR is similar to conventional PCR, except an internal probe is added to the master mix that releases fluorescent light in proportion to the quantity of genetic material being replicated (Mackay et al., 2002). The real-time PCR instrument detects and quantifies the magnitude of the fluorescent light emitted by the amplified nucleic acid, and provides data that may be used to generate quantitative results.

One deficiency associated with PCR is its inability to distinguish between infectious and non-infectious viral particles. This is because PCR amplifies any nucleic acid target present, whether it came from an infectious virus or an inactivated virus. Another potential problem is that PCR is sensitive to inhibitors, which tend to be concentrated in environmental samples. In particular, beef extract is known to contain organic inhibitors, and compounds such as humic acids and certain salts may also interfere with PCR amplification (Arnal et al., 1999; Schwab et al., 1995). Commercial DNA and RNA extraction kits are designed to purify the genetic material by removing these inhibitory compounds, but sometimes they are

unable to completely remove them all, which can potentially lead to false negative results (Abbaszadegan et al., 1993).

Summary

No concentration method is ideal or optimal for all viruses, water types, and environmental conditions. The electropositive filters that have been previously tested, such as the 1MDS and Zeta Plus filters, appear to offer certain clear advantages over traditional negatively-charged membranes, but they have not been shown to effectively concentrate viruses from seawater due to the effects of high pH levels and salt concentrations typically found in this environment. The development of a new concentration method based on an improved electropositive adsorbent filter capable of efficiently recovering viruses from seawater could potentially overcome these limitations and more effectively detect enteric viruses in seawater.

EXPERIMENTAL DESIGN

Virus adsorption experiments were performed to test the effectiveness of Argonide Nanoceram cartridge filters for concentrating adenoviruses and noroviruses from seawater. Based on the concentrations of viruses in experimentally seeded test waters applied to filters and in the filtrates, the percent adsorption for each virus was estimated. Next, a series of experiments was conducted to determine the effectiveness of beef extract as an eluent for viruses adsorbed to the Argonide Nanoceram filters. The adsorbed viruses were eluted by continuously circulating a volume of beef extract solution at various pH levels through the filter using a peristaltic pump. For some experiments, a non-ionic detergent (Tween 80) was added to the eluent. PEG precipitation with different concentrations of PEG and sodium chloride (NaCl) was then evaluated as a secondary concentration step for viruses in the filter eluate. The percent recovery of initial viruses in seeded test seawater was determined for each virus under each set of experimental conditions using real-time PCR for adenoviruses and RT-PCR for noroviruses.

The virus elution and PEG precipitation conditions that yielded the highest recoveries from seeded samples were used to detect adenovirus and norovirus in seawater samples collected during the summer of 2007 from Doheny Beach, and Avalon Beach, California. These beaches are both impacted by non-point sources of fecal contamination. Viruses were concentrated from 40 L samples of seawater to approximately 6 ml by Argonide Nanoceram

filter adsorption-elution and then PEG precipitation. The viruses were then detected in the resulting concentrated samples using nested PCR and RT-PCR for adenoviruses and noroviruses, respectively. The presence of somatic and male-specific (F+) coliphages in the same seawater samples was determined for each sample by an enrichment-spot plate MPN method. Chi-squared analyses were then performed to determine the relationship between adenovirus and norovirus, and between these two groups of human enteric viruses and the coliphages. These analyses were used to determine if the presence of somatic or F+ coliphages was a reliable predictor of the presence of the two groups of human enteric viruses, adenoviruses and noroviruses, in the samples collected and analyzed.

MATERIALS AND METHODS FOR SEEDED EXPERIMENTS

Viruses

Adenovirus type 41 was propagated in G293 cells provided by the Centers for Disease Control and Prevention. The cells were grown in a minimal essential media (MEM) containing 1% glutamine and non-essential amino acids, 1% HEPES buffer, 1.5% sodium bicarbonate, and 10% fetal bovine serum. After propagating the virus, the virus suspension was purified by freezing and thawing, followed by chloroform extraction and centrifugation for 20 minutes at 4˚ C and 3000 x g. Norovirus genogroup II strain 4 (GII.4) was isolated from stool samples collected from a 2004 norovirus outbreak at a nursing home in Forsyth County, North Carolina. It was used as a 10-18% stool suspension in PBS that was then centrifuged for 20 minutes at room temperature and 3000 x g. The supernatant was then collected, aliquoted and frozen at -80˚ C.

Cartridge Filters

Positively-charged Nanoceram alumina cartridge filters (part number VS2.5-5, 2.5 in. x 5 in., Argonide Corporation, Sanford, FL) were used for all filtration experiments. Each filter was placed in an Argonide filter housing (part number H2.5-5, 2.5 in. x 5 in.) and challenged with 20-40 L of seawater containing 10^8 to 10^{10} PCR units adenovirus and 10^6 to 10^7 RT-PCR units norovirus. For the adsorption experiments, approximately 35 L of

unseeded seawater were passed through each cartridge filter, and the filtrate was discarded. Then, 500 ml of water seeded with adenovirus and norovirus were filtered, followed by an additional 5.5 L of unseeded water. Seawater was collected near Wilmington, North Carolina. The pH of the seawater was 7.95, the turbidity was 2.4 NTU, and the salinity was 27 PPT. Seawater was passed through the filter at a rate of approximately 25 L per minute using a 0.3 horsepower Emerson self-priming pump (ITT Jabsco, St. Louis, MO) with Nalgene 0.5 inch braided polypropylene tubing (Nalgene, Rochester, NY) and Swagelok stainless steel Quick-Connects (Swagelok Company, Solon, OH). Challenge water influent, filter effluent, and filter eluates were collected and assayed by real-time PCR for the quantification of adenovirus and norovirus.

Elution

A 3% beef extract solution in reagent water (BBL Beef Extract Powder, catalog number 212303, Becton, Dickinson and Company, Sparks, MD) containing 0.1 M glycine (pH 7.3) was used for all experiments. For some experiments, 0.1% or 0.01% Tween 80 (Fisher Scientific, Fair Lawn, NJ) was added to the beef extract solution. Using a Manostat variable-speed peristaltic pump (Barnant Company, Barrington, IL), 500 ml of the beef extract solution were passed through the cartridge filter at a flow rate of 1.25-2.75 L per minute for 10 minutes in the forward direction, and 5 minutes in the reverse direction. The pH of the beef extract was adjusted to 9.5 using a 5 M NaOH solution at the beginning of the elution process. At the end of the process, the pH of the eluent was adjusted to 7.3 using a 5 M HCl solution. For some experiments, beef extract solution at pH 7.3 was poured into the cartridge housing and the filter was left to soak at 4˚ C for three days prior to elution with the

peristaltic pump. This experiment was done to model the transport of filters that had been used to process California seawaters, which were supplemented with beef extract and shipped on freezer packs via air express to this University of North Carolina laboratory.

PEG Precipitation

Beef extract solution was prepared as described previously (pH 7.3) and seeded with 10^8 to 10^{10} PCR units adenovirus and 10^6 to 10^7 RT-PCR units norovirus. The seeded beef extract was sampled for real-time PCR analysis, and then split into 250 ml volumes and placed into Corning 250 ml polypropylene disposable conical bottles (Corning Inc., Corning, NY). Six percent or 9% PEG molecular weight 8000 (Fisher Scientific, Fair Lawn, NJ) and 0.1 M or 0.3 M sodium chloride (NaCl) were added to each bottle, and the bottles were shaken continuously overnight at 200 rpm and 4˚ C using a Lab-Line platform shaker (Barnstead International, Dubuque, IA). The bottles were then centrifuged for 1 hour at 4˚ C and 5100 x g. One ml of the supernatant was sampled and the rest was discarded, and the pellet was resuspended in 2 ml of phosphate buffered saline (PBS). One liter of PBS contains 8 g sodium chloride (NaCl), 0.2 g potassium chloride (KCl), 0.12 g potassium phosphate (KH_2PO_4), and 0.91 g anhydrous sodium phosphate (Na_2HPO_4). The resuspended PEG precipitate was sampled for real-time PCR analysis, and percent recoveries were determined for each set of experimental conditions by dividing the (RT-)PCR units in the resuspended PEG precipitate by the (RT-)PCR units in the spiked beef extract and multiplying by 100.

Viral Nucleic Acid Extraction

The viral nucleic acid was chemically extracted from 100 µl of each sample using a guanidinium thiocyanate (GuSCN) extraction method adapted from Boom et al. (1990). DNA and RNA were both extracted simultaneously using this same method. One-hundred microliters of the sample were mixed with 100 µl 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 for a total volume of 240 ml. Each sample was vortexed for 15 seconds and incubated at room temperature for 10 minutes. Then, 0.2 ml of 100% ethanol were added and the mixture was vortexed for 15 seconds. The solution was transferred to a HiBind RNA minicolumn (E.Z.N.A. RNA isolation system, OMEGA Bio-Tek, Doraville, GA) and centrifuged at 16,000 x g for 1 minute. The filtrate was discarded, and the column containing the nucleic acid 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 wash steps. The column was centrifuged for 2 minutes at 16,000 x g to dry the remaining ethanol, and then the nucleic acids were eluted from the column with 50 µl of nuclease free water and stored at -80˚ C until analyzed.

Quantification of Adenovirus by Real-time PCR

The primers and real-time PCR conditions used to detect adenovirus 41 were obtained from Jothikumar et al. (2005a). The primers and probe used for adenovirus are summarized in Table 1. The viral DNA was incubated at 95˚ C for 15 minutes to activate the polymerase.

Thermocycling conditions for the reaction consisted of 45 cycles of 10 sec at 95˚ C, 30 sec at 58˚ C, and 15 sec at 72˚ C. A series of 10-fold dilutions of the virus stock was analyzed by PCR to generate a series of Ct values and thereby create a dilution curve relating virus concentrations and Ct values. The equation of the curve was $\log_{10}$ VP = -0.2814 * Ct + 12.256, where VP is the estimated viral particles and Ct is the value generated by the real-time PCR machine. The correlation was $R^2 = 0.9986$. For the real-time PCR, the Qiagen Quantitech probe PCR kit (Qiagen, Valencia, CA) was used as described by the manufacturer for a total volume of 25 µl, including 2 µl of the extracted viral nucleic acid. A Smart Cycler (v. 2.0c, Cepheid, Sunnyvale, CA) was used for running the real-time PCR analysis.

Quantification of Norovirus by Real-time RT-PCR

The primers and real-time RT-PCR conditions used to detect norovirus genogroup II strain 4 (GII.4) were obtained from Jothikumar et al. (2005b). The primers and probe used for norovirus are summarized in Table 1. The viral RNA was held at 50˚ C for 30 minutes to initiate reverse transcription, and then incubated at 95˚ C for 15 minutes to activate the polymerase. Thermocycling conditions for the reaction consisted of 45 cycles of 10 sec at 95˚ C, 20 sec at 55˚ C, and 15 sec at 72˚ C. A series of 10-fold dilutions of the 10-18% norovirus stool suspension was analyzed by RT-PCR to generate a series of Ct values and thereby create a dilution curve relating virus concentrations and Ct values. The equation of the curve was $\log_{10}$ VP = -0.2726 * Ct + 10.362, where VP is the estimated viral particles and Ct is the value generated by the real-time PCR machine. The correlation was $R^2 = 0.9988$. For the real-time RT-PCR, the Qiagen Quantitech probe RT-PCR kit (Qiagen, Valencia, CA)

was used as described by the manufacturer for a total volume of 25 µl, including 2 µl of the extracted viral nucleic acid.

Table 1. Primers and probes used for the detection of adenovirus and norovirus by real-time PCR

Virus	Primer/Probe	Working Conc.	Name	Sequence (5′ - 3′)	Position
Adenovirus 41	Forward Primer	1 µM	JTVXF	GGA CGC CTC GGA GTA CCT GAG	271-291
	Reverse Primer	1 µM	JTVXR	ACI GTG GGG TTT CTG AAC TTG TT	344-366
	Probe	0.1 µM	JTVXP	CTG GTG CAG TTC GCC CGT GCC A	299-320
Norovirus II.4	Forward Primer	1 µM	JJGII	CAA GAG TCA ATG TTT AGG TGG ATG AG	5003-5028
	Reverse Primer	1 µM	COG2R	TCG ACG CCA TCT TCA TTC ACA	5080-5100
	Probe	0.1 µM	Ring2-TP	TGG GAG GGC GAT CGC AAT CT	5048-5067

MATERIALS AND METHODS FOR FIELD SAMPLES

Sample Collection

Forty-liter volumes of seawater were collected from the waters of Doheny Beach, and Avalon Beach, California. Samples were labeled according to the collection site (5 sites for Doheny Beach, 3 sites for Avalon Beach), collection date, and collection time (7 AM, 1 PM or 3 PM for Doheny Beach, 8 AM, 12 PM or 3 PM for Avalon Beach). For Doheny Beach, 93 samples were collected from June through July, 2007. For Avalon Beach, 286 samples were collected from July through September, 2007.

Cartridge Filters

Positively-charged Nanoceram alumina cartridge filters (part number VS2.5-5, 2.5 in. x 5 in., Argonide Corporation, Sanford, FL) were placed in Argonide filter housings (part number H2.5-5, 2.5 in. x 5 in.) and challenged with 40 L of seawater. Seawater was passed through the filters at a rate of approximately 25 L per minute using a 0.3 horsepower Emerson self-priming pump (ITT Jabsco, St. Louis, MO) with Nalgene 0.5 inch braided polypropylene tubing (Nalgene, Rochester, NY) and Swagelok stainless steel Quick-Connects (Swagelok Company, Solon, OH). Three-hundred-fifty milliliters of beef extract were poured into each cartridge housing, and the cartridge housings were sealed and shipped

on ice overnight to the University of North Carolina laboratory (Chapel Hill, North Carolina).

Elution

A 3% beef extract solution in reagent water (BBL Beef Extract Powder, catalog number 212303, Becton, Dickinson and Company, Sparks, MD) containing 0.1 M glycine (pH 7.3) was used for all field samples. Viruses were eluted using a peristaltic pump as described for the seeded water samples. Five hundred milliliters of beef extract were used to elute each filter, including the 350 ml shipped in each cartridge housing, and 150 ml added at the North Carolina laboratory. The pH of the beef extract was adjusted to approximately 9.7 using a 5 M NaOH solution at the beginning of the elution, and declined naturally to approximately 9.5 by the end of the elution period. At the end of the elution period, the pH of the eluent was adjusted to 7.3 using a 5 M HCl solution. The pH meter probe was sterilized between samples by submerging the probe in 0.1 M HCl for 2 minutes. The eluent was then stored at -20˚ C for further processing.

PEG Precipitation

Each 500 ml volume of beef extract eluent was split into two Corning 250 ml polypropylene disposable conical bottles (Corning Inc., Corning, NY). PEG molecular weight 8000 (Fisher Scientific, Fair Lawn, NJ) and sodium chloride (NaCl) were added to each bottle to final concentrations of 9% and 0.3 M, respectively, and the bottles were shaken continuously overnight at 200 rpm and 4˚ C using a Lab-Line platform shaker (Barnstead International, Dubuque, IA). The bottles were then centrifuged for 1 hour at 4˚ C and 5100 x

g. The supernatant was discarded, and the pellet was resuspended in 2 ml of PBS per bottle. The resuspended pellet was collected and stored at -80˚ C.

Viral Nucleic Acid Extraction

The viral nucleic acid was chemically extracted from 1.2 ml of the concentrate from the Doheny Beach, California samples and from 0.2 ml of the concentrate from the Avalon Beach, California samples. In both cases the nucleic acid (both DNA and RNA) was extracted using the guanidinium thiocyanate (GuSCN) extraction method previously described, except that two or three HiBind RNA minicolumns (E.Z.N.A. RNA isolation system, OMEGA Bio-Tek, Doraville, GA) were used for each sample due to the larger volumes. For the Doheny Beach samples, 1.2 ml of the sample were mixed with 1.2 ml of the GuSCN extraction buffer, and incubated at room temperature for 10 minutes. Then, 2.4 ml of 100% ethanol were added and the mixture was vortexed for 15 seconds, and then divided evenly into 3 minicolumns. For the Avalon Beach samples, 200 µl of the sample were mixed with 200 µl of the GuSCN extraction buffer, and incubated at room temperature for 10 minutes. Then, 400 µl of 100% ethanol were added and the mixture was vortexed for 15 seconds, and then divided evenly into 2 minicolumns.

Detection of Adenovirus by Nested PCR

The primers and nested PCR conditions used to detect adenovirus (groups A-F) were obtained from Ko et al. (2003). The primers used for both reactions are summarized in Table 2. A Qiagen One-Step PCR Kit and a Qiagen Hot Star Taq DNA Polymerase kit (Qiagen, Valencia, CA) were used as described by the manufacturer for both reactions. For the first

reaction, 15 µl of the extracted viral nucleic acid were mixed with 5 µl 10X PCR buffer with 15 mM $MgCl_2$, 10 µl 5X Q solution, 0.5 µl 25 mM $MgCl_2$, 1 µl of 10 mM each dNTP, 1 µl each of 50 µM Hex1 and Hex2, 0.2 µl of 5 units/µL Hot Star Taq, and 16.3 µl water, for a total volume of 50 µl. For the second reaction, 2 µl of the product from the first reaction were mixed with 5 µl 10X PCR buffer with 15 mM $MgCl_2$, 0.5 µl 25 mM $MgCl_2$, 1 µl of 10 mM each dNTP, 1 µl each of 50 µM Hex1 and Hex3, 0.2 µl of 5 units/µl Hot Star Taq, and 39.3 µl water, for a total volume of 50 µl. The PCR conditions for adenovirus were identical for the first and second reactions. The reaction mixture was incubated at 94˚ C for 10 minutes to activate the Taq polymerase. Thermocycling conditions for the reaction consisted of 40 cycles of 15 sec at 94˚ C, 15 sec at 50˚ C, and 30 sec at 72˚ C, with a final extension step of 72˚ C for 7 minutes. A Peltier Thermal Cycler (MJ Research, Waltham, MA) was used for performing the nested PCR analysis.

For each sample, 10 µl of the PCR product from the second reaction were mixed with 2 µl of 6X Blue/Orange Loading Dye (Promega, Madison, WI). Each sample and a 100 base pair DNA ladder (Promega, Madison, WI) were loaded into a 2% agarose gel in TAE buffer. Each 100 ml gel contains 2 g of agarose (Acros Organics, Morris Plains, NJ), 100 ml of 1X TAE buffer, and 5 µl of ethidium bromide solution (10 mg/ml, Promega, Madison, WI). One liter of 1X TAE buffer contains 0.484 g Tris-Base (Fisher Scientific, Fair Lawn, NJ), 1.142 ml acetic acid, and 2 ml 0.5 M EDTA (pH 8) in reagent water. The gel was placed into a Fotodyne wide format horizontal electrophoresis chamber (Fotodyne Inc., Hartland, WI) and electrophoresed at 150 V for 45 minutes. At the end of electrophoresis, the gel was analyzed by taking a picture in the presence of ultraviolet radiation. If a visible band appeared at 443 bp, the sample was designated as positive for the presence of adenovirus.

Detection of Norovirus by Nested RT-PCR

The primers and nested PCR conditions used to detect norovirus were obtained from Vinje et al. (2004) for the first reaction and from Maloney (2006) for the second reaction. The primers used for both reactions are summarized in Table 2. A Qiagen One-Step RT-PCR Kit and a Qiagen Hot Star Taq DNA Polymerase kit (Qiagen, Valencia, CA) were used as described by the manufacturer. For the first reaction, 5 µl of the extracted viral nucleic acid were mixed with 5 µl 5X Qiagen One-Step RT-PCR buffer, 5 µl 5X Q solution, 1 µl of 10 mM each dNTP, 1 µl each of 50 µM MJV12 and RegA, 1 µl Qiagen enzyme mix (provided in the kit), 0.25 µl Rnasin (RNase inhibitor), and 5.75 µl water, for a total volume of 25 µl. The viral RNA was held at 50˚ C for 30 minutes to initiate reverse transcription, and then incubated at 94˚ C for 15 minutes to activate the polymerase. Thermocycling conditions for the reaction consisted of 40 cycles of 15 sec at 94˚ C, 15 sec at 50˚ C, and 30 sec at 72˚ C, with a final extension step of 72˚ C for 7 minutes. For the second reaction, 2 µl of the product from the first reaction were mixed with 5 µl 10X PCR buffer with 15 mM $MgCl_2$, 1 µl of 25 mM magnesium chloride ($MgCl_2$), 1 µl of 10 mM each dNTP, 1 µl each of 50 µM RegA and MP290, 0.2 µl of 5 units/µl Hot Star Taq, and 38.8 µl water, for a total volume of 50 µl. The viral DNA was incubated at 94˚ C for 10 minutes to activate the Taq polymerase. Thermocycling conditions for this reaction consisted of 40 cycles of 30 sec at 94˚ C, 30 sec at 49˚ C, and 1 minute at 72˚ C, with a final extension step of 72˚ C for 7 minutes. The final PCR product was analyzed using the same agarose gel electrophoresis method described for adenovirus detection. If a visible band appeared at 317 bp, the sample was designated as positive for the presence of norovirus.

Statistical Analysis

Chi-squared tests of independence were performed using Minitab Statistical Software (Minitab Inc., State College, PA) to determine whether or not the presence of adenovirus was associated with the presence of norovirus in the samples, and whether the presence of these human enteric viruses was associated with the presence of somatic or male-specific (F+) coliphages. The presence of these coliphages was determined in a 1 L volume by overnight enrichment in a multiple volume format of 3 x 300 ml, 3 x 30 ml and 3 x 3 ml (EPA method 1601). These data were then used to determine whether or not somatic or F+ coliphages were predictors of human enteric viruses in the samples collected, and the likelihood that the enteric viruses found in the samples originated from the same source of contamination.

Table 2. Primers used for the detection of adenovirus and norovirus by nested PCR

Virus	PCR Reaction	Working Conc.	Primer	Sequence (5′ - 3′)	Product Size
Adenovirus (A-F)	First	1 µM	Hex1	TTC CCC ATG GCI CAC TAA CAC	482 bp
	First	1 µM	Hex2	CCC TGG TAG TCC AGA TAG TTG TA	
	Nested	1 µM	Hex1	TTC CCC ATG GCI CAC TAA CAC	443 bp
	Nested	1 µM	Hex3	AGG AAC CAA GTC CTT TAG GTC AT	
Norovirus (GI-GII)	First	1 µM	MJV12	TAY CAY TAT GAT GCH GAY TA	327 bp
	First	1 µM	RegA	CTC RTC ATC ICC ATA RAA IGA	
	Nested	1 µM	RegA	CTC RTC ATC ICC ATA RAA IGA	317 bp
	Nested	1 µM	MP290	GAY TAC TCY CSI TGG GAY TC	

RESULTS FOR SEEDED EXPERIMENTS

Adsorption Experiments

Virus adsorption was determined by passing approximately 35 L of unseeded seawater through the filter, followed by 500 ml of seawater seeded with adenovirus and norovirus, and then 5.5 L of unseeded water to push any remaining unadsorbed viruses through the filter. Samples of the 500 ml of seeded influent and the 6 L of effluent (corresponding to the 500 ml of seeded seawater filtrate and the subsequent 5.5 L of unseeded seawater filtrate) were collected for real-time PCR or RT-PCR analysis. The results of these experiments are displayed in Table 3 for adenovirus 41. The average percent adsorption for adenovirus 41 in seawater was 97.6% (± 0.64%). In all three experiments, the concentration of norovirus in the seawater filtrate was below the detection limit of the real-time RT-PCR assay. The minimum RT-PCR units needed for detection was calculated to be 1.5×10^6. Using this detection limit and comparing it to the number of RT-PCR units detected in the spiked seawater, the percent adsorption of norovirus was calculated to be greater than or equal to 43%.

Table 3. Percent adsorption of adenovirus 41 in seawater to Argonide Nanoceram cartridge filters

Trial	Virus in Influent (500 ml)	Virus in Filtrate (6 L)	Percent Adsorption
1	$2.3x10^9$	$3.8x10^7$	98.3%
2	$2.2x10^9$	$6.5x10^7$	97.0%
3	$2.6x10^9$	$6.5x10^7$	97.5%
Average			97.6% (± 0.64%)

Elution Experiments

The results of the elution experiments are shown in Table 4 and Table 5 for adenovirus 41, and in Table 6 for norovirus GII.4. The concentration of each virus in PCR or RT-PCR units in the seeded seawater was estimated using the Ct value of the viral stock solution, and the eluent was sampled for quantitative analysis by real-time PCR or RT-PCR. The concentration of viruses in the 20-40 L of spiked seawater was below the detection limit of the PCR assay. Average percent recoveries for adenovirus with immediate elution were 3.2% (± 2.3%), 1.0% (± 0.30%) and 0.69% (± 0.40%) using beef extract, beef extract with 0.1% Tween 80 and beef extract with 0.01% Tween 80, respectively. The average percent recovery for adenovirus with a 3-day soak in beef extract prior to elution was 0.69% (± 0.17%). Average norovirus percent recoveries with immediate elution were 111% (± 29%), 119% (± 26%) and 88% (± 24%) using beef extract, beef extract with 0.1% Tween 80 and beef extract with 0.01% Tween 80, respectively. Norovirus recovery could not be determined for the 3-day soak experiments because the virus stock used in previous experiments was exhausted and the titer of the new norovirus stock solution was too low for detection in the eluate by real-time RT-PCR. The addition of Tween 80 did not improve elution of either virus from the filter.

Table 4. Elution of adenovirus 41 with 3% beef extract using a peristaltic pump

Eluent [a]	Estimated Adenovirus input (PCR units)	Elution replicates - % recovered				Average adenovirus % recovered (± st dev)
		1	2	3	4	
3% BE	$1.0x10^9$	4.6%	1.9%	5.7%	0.65%	3.2% (± 2.3%)
3% BE, 0.1% Tween 80	$1.0x10^9$	1.0%	0.61%	1.2%	1.3%	1.0% (± 0.30%)
3% BE, 0.01% Tween 80	$1.0x10^9$	1.3%	0.46%	0.58%	0.43%	0.69% (± 0.40%)

[a] 3% BE = 3% beef extract – 0.1M glycine eluent at pH 9.5 with and without 0.1% - 0.01% Tween 80

Table 5. Percent recovery of adenovirus 41 with a 3-day soak in 3% beef extract followed by elution with a peristaltic pump

Trial	Virus in Influent (40 L)	Virus in Eluate[a] (500 ml)	Percent Recovery
1	$8.3x10^8$	$5.3x10^6$	0.64%
2	$1.3x10^9$	$7.4x10^6$	0.55%
3	$1.0x10^9$	$8.8x10^6$	0.88%
Average			0.69% (± 0.17%)

[a] Eluate = 3% beef extract with 0.1M glycine

Table 6. Elution of norovirus GII.4 with 3% beef extract using a peristaltic pump

Eluent [a]	Estimated Norovirus input (PCR units)	Elution replicates - % recovered				Average norovirus % recovered (± st dev)
		1	2	3	4	
3% BE	$3.5x10^6$	86%	88%	133%	139%	111% (± 29%)
3% BE, 0.1% Tween 80	$3.5x10^6$	95%	140%	99%	141%	119% (± 26%)
3% BE, 0.01% Tween 80	$3.5x10^6$	99%	53%	103%	98%	88% (± 24%)

[a] 3% BE = 3% beef extract – 0.1M glycine eluent at pH 9.5 with and without 0.1% - 0.01% Tween 80

PEG Precipitation Experiments

The results of the PEG precipitation experiments are shown in Table 7. For adenovirus, average percent recoveries were 10% (± 3.6%), 36% (± 14%), 42% (± 13%), and 89% (± 20%) using 6% PEG - 0.1 M NaCl, 6% PEG - 0.3 M NaCl, 9% PEG - 0.1 M NaCl,

and 9% PEG - 0.3 M NaCl, respectively. For norovirus, average percent recoveries were 38% (± 3.7%), 43% (± 7.3%), 51%, and 69% (± 4.0%) using 6% PEG - 0.1 M NaCl, 6% PEG - 0.3 M NaCl, 9% PEG - 0.1 M NaCl, and 9% PEG - 0.3 M NaCl, respectively. The standard deviation is not given for one experiment because a bottle broke in the centrifuge, resulting in only two experimental replicates. The highest recoveries for both viruses were observed using 9% PEG and 0.3 M NaCl (unpaired *t*-test, $p < 0.05$).

Table 7. Percent recovery of adenovirus 41 and norovirus GII.4 from seeded beef extract using PEG precipitation

	6% PEG		**9% PEG**	
	0.1 M NaCl	0.3 M NaCl	0.1 M NaCl	0.3 M NaCl
Adenovirus 41 (n=4)	10% (± 3.6%)*	36% (± 14%)	42% (± 13%)**	89% (± 20%)
Norovirus GII.4 (n=3)	38% (± 3.7%)	43% (± 7.3%)	51%***	69% (± 4.0%)

* percent recovery (± st dev)
** n=3
*** n=2

Next, the efficiency of PEG precipitation was determined for actual seawater eluents. This experiment was performed in order to determine what effects the salts and anything else concentrated from seawater might have on PEG precipitation. The results of this experiment are shown in Table 8. Adenovirus average percent recoveries were 1.9% (± 0.48%), 3.7% (± 1.7%), 29% (± 14%), and 34% (± 12%) using 6% PEG - 0.1 M NaCl, 6% PEG - 0.3 M NaCl, 9% PEG - 0.1 M NaCl, and 9% PEG - 0.3 M NaCl, respectively. Norovirus average percent recoveries were 5.6% (± 1.1%), 5.4% (± 0.46%), 52% (± 7.8%), and 59% (± 4.8%) using 6% PEG - 0.1 M NaCl, 6% PEG - 0.3 M NaCl, 9% PEG - 0.1 M NaCl, and 9% PEG - 0.3 M NaCl, respectively. Overall, percent recoveries were considerably lower using actual seawater eluents. Higher average percent recoveries were observed for both viruses using

9% PEG rather than 6% PEG (unpaired *t*-test, $p < 0.05$). However, no significant difference in average percent recoveries was observed between 0.1 M NaCl and 0.3 M NaCl when combined with 9% PEG for either adenovirus (unpaired *t*-test, $p = 0.325$) or norovirus (unpaired *t*-test, $p = 0.122$).

Table 8. Percent recovery of adenovirus 41 and norovirus GII.4 from actual eluates using PEG precipitation

	6% PEG		**9% PEG**	
	0.1 M NaCl	0.3 M NaCl	0.1 M NaCl	0.3 M NaCl
Adenovirus 41 (n=4)	1.9% (± 0.48%)*	3.7% (± 1.7%)	29% (± 14%)	34% (± 12%)
Norovirus GII.4 (n=3)	5.6% (± 1.1%)	5.4% (± 0.46%)	52% (± 7.8%)	59% (± 4.8%)

* percent recovery (± st dev)

RESULTS FOR FIELD SAMPLES

Doheny Beach

Of the 93 samples collected from the waters of Doheny Beach, California, 24 samples (26%) were positive for the presence of adenovirus, and 21 samples (23%) were positive for the presence of norovirus. The equivalent volume analyzed for adenovirus was 2.1 L, and the equivalent volume analyzed for norovirus was 0.7 L. Assuming that 1 PCR or RT-PCR unit is detectable in the assayed volume of the extracted viral nucleic acid, optimal detection limits were calculated as 18 PCR units per 40 L of seawater for adenovirus, and 55 RT-PCR units per 40 L of seawater for norovirus. Actual detection limits were then calculated using the average percent recoveries of the viruses from the seeded experiments. The actual detection limits were estimated at 1472 PCR units per 40 L (37 PCR units per L) for adenovirus, and 93 RT-PCR units per 40 L (2.3 RT-PCR units per L) for norovirus. Somatic coliphages were detected in 78 (84%) of the samples, and male-specific (F+) coliphages were detected in 24 (26%) of the samples. These data were analyzed using Chi-squared tests of independence, and the results are displayed in Table 9. The presence of adenovirus was correlated with the presence of norovirus (X^2, $p < 0.05$, $r = 0.210$). However, the presence of the enteric viruses was not correlated with the presence of the coliphages, with the exception of adenovirus and F+ coliphages, which were negatively correlated (X^2, $p = 0.042$, $r = -0.236$).

The data were then stratified into subsets in order to test for potential associations between viruses at specific collection times or sampling sites. Separate Chi-squared tests of independence were used to test for associations between virus groups in the samples collected at 7 AM, followed by the samples collected at 1 PM, and then the samples collected at 3 PM. The same procedure was used to test for virus associations in samples collected at each of the five different sampling sites. No association was found between adenovirus and norovirus, or between the enteric viruses and the somatic or F+ coliphages in any of these data subsets. However, the percentage of samples positive for both F+ coliphages and somatic coliphages was highest in the morning samples, and was lower in samples collected progressively later in the day. F+ coliphages were detected in 16 (46%) of the samples collected at 7 AM, 7 (23%) of the samples collected at 1 PM, and 1 (4%) of the samples collected at 3 PM. Somatic coliphages were detected in 33 (94%) of the samples collected at 7 AM, 25 (81%) of the samples collected at 1 PM, and 20 (74%) of the samples collected at 3 PM. The frequency of adenovirus and norovirus presence in samples did not appear to be affected by the sampling time. Adenovirus was detected in 9 (26%) of the samples collected at 7 AM, 6 (19%) of the samples collected at 1 PM, and 9 (33%) of the samples collected at 3 PM. Norovirus was detected in 4 (11%) of the samples collected at 7 AM, 9 (29%) of the samples collected at 1 PM, and 8 (30%) of the samples collected at 3 PM.

Avalon Beach

Of the 286 samples collected from the waters of Avalon Beach, California, 26 samples (9%) were positive for the presence of adenovirus, and 2 samples (<1%) were positive for the presence of norovirus. The equivalent volume analyzed for adenovirus was

240 ml, and the equivalent volume analyzed for norovirus was 80 ml. The equivalent volumes analyzed for Avalon Beach were smaller than the equivalent volumes analyzed for Doheny Beach because at the higher equivalent volumes, most of the Avalon Beach samples analyzed were negative for both viruses. Samples were re-analyzed at the smaller equivalent volumes in order to reduce any effects of sample-related inhibition. The optimal detection limits were calculated as 100 PCR units per 40 L of seawater for adenovirus, and 300 RT-PCR units per 40 L of seawater for norovirus. The actual detection limits were estimated at 8029 PCR units per 40 L (200 PCR units per L) for adenovirus, and 507 RT-PCR units per 40 L (13 RT-PCR units per L) for norovirus. Somatic and F+ coliphages were detected in 127 (44%) and 159 (56%) of the samples, respectively. These data were analyzed using Chi-squared tests of independence, but no statistically significant association was found between adenovirus and norovirus, or between the enteric viruses and the coliphages ($p > 0.05$).

The data were then stratified into subsets in order to test for potential associations between viruses at specific collection times or sampling sites. Separate Chi-squared tests of independence were used to test for associations between virus groups in the samples collected at 8 AM, followed by the samples collected at 12 PM, and then the samples collected at 3 PM. The same procedure was used to test for virus associations in samples collected at each of the three different sampling sites. No association was found between adenovirus and norovirus, or between the enteric viruses and the somatic or F+ coliphages in any of these data subsets. However, the percentage of samples positive for each coliphage was highest in the morning and was lower for samples collected progressively later in the day. F+ coliphages were detected in 54 (68%) of the samples collected at 8 AM, 74 (52%) of the samples collected at 12 PM, and 31 (48%) of the samples collected at 3 PM. Somatic

coliphages were detected in 69 (86%) of the samples collected at 8 AM, 45 (32%) of the samples collected at 12 PM, and 13 (20%) of the samples collected at 3 PM. The frequency of adenovirus and norovirus presence in samples did not appear to be affected by the sampling time. Adenovirus was detected in 8 (10%) of the samples collected at 8 AM, 14 (10%) of the samples collected at 12 PM, and 4 (6%) of the samples collected at 3 PM. Norovirus was detected in 1 (1%) of the samples collected at 8 AM, 1 (<1%) of the samples collected at 12 PM, and was not detected in any of the samples collected at 3 PM.

Table 9. Chi-squared tests of independence for the correlation of enteric viruses and coliphages in samples from Doheny Beach, California

X^2, p = 0.023, R = -0.236		F+ Coliphages		
		Negative	Positive	Total
Adenovirus	Negative	47	22	69
	Positive	22	2	24
	Total	69	24	93

X^2, p = 0.228, r = 0.125		Somatic Coliphages		
		Negative	Positive	Total
Adenovirus	Negative	13	56	69
	Positive	2	22	24
	Total	15	78	93

X^2, p = 0.170, r = -0.142		F+ Coliphages		
		Negative	Positive	Total
Norovirus	Negative	51	21	72
	Positive	18	3	21
	Total	69	24	93

X^2, p = 0.277, r = -0.113		Somatic Coliphages		
		Negative	Positive	Total
Norovirus	Negative	10	62	72
	Positive	5	16	21
	Total	15	78	93

X^2, p = 0.042, r = 0.210		Norovirus		
		Negative	Positive	Total
Adenovirus	Negative	57	12	69
	Positive	15	9	24
	Total	72	21	93

DISCUSSION

According to the manufacturer, the nano alumina fibers that are used to create Argonide Nanoceram cartridge filters are 2 nm in diameter and 0.3 µm in length (Tepper & Kaledin, 2007). This corresponds to an external surface area of approximately 500 m^2 per gram of material, which provides a large area for the adsorption of electronegative particles. In addition, the zeta potential of the Nanoceram filters is approximately 50 mV at neutral pH, and the isoelectric point is approximately pH 9.4. Therefore, the filters are highly electropositive throughout the recommended pH range of 5 through 9. We demonstrated that the adsorption of adenoviruses to the filter in seeded seawater was very high (>96%). Norovirus adsorption was difficult to determine because of the low virus concentration achievable in seeded samples. Unfortunately, the titer of the virus stock suspension was not high enough to seed sufficient quantities of the virus into the influent to reliably quantify adsorption efficiency. Using the lower detection limit of the virus assay as a reference, norovirus adsorption was calculated to be at least 43%. The high norovirus recovery rates overall at least suggest that norovirus adsorption rates were also high.

Some studies have suggested that 1MDS electropositive filters may not be efficient at adsorbing certain viruses from water at pH levels greater than 7.5 or containing salts such as sodium chloride (NaCl) and magnesium chloride ($MgCl_2$). Sobsey and Jones (1979) reported that virus adsorption to filters is related to the isoelectric point of the viruses and the filter

membrane. Near neutral pH, 1MDS filters possess an overall net positive charge, and the viruses possess an overall net negative charge, which promotes adsorption of the viruses to the filter. However, at elevated pH levels the filter becomes less electropositive, and adsorption of the negatively-charged viruses is diminished. Sobsey et al. (1985) conducted an experiment in which adsorption rates of HAV in tap water to 1MDS filters were 97%, 82% and 43%, for pH levels of 6.5, 7.5 and 8.5, respectively. Lukasik et al. (2000) reported a 79% adsorption rate of poliovirus to 1MDS filters in de-ionized water at pH 7, but when 0.1 M NaCl or 0.1 M $MgCl_2$ was added, adsorption dropped to 7% or 9%, respectively. In the current study, adenovirus adsorption was not adversely affected by the pH conditions and salt concentrations found in natural seawater. This could be due to the relatively large surface area and strong electropositive charge of the Nanoceram filters. More work is needed to determine if these filters are capable of efficiently adsorbing other enteric viruses from seawater.

Circulating beef extract through the cartridge filter with a peristaltic pump resulted in poor adenovirus elution and recovery (<4% on average), but nearly 100% norovirus elution and recovery. Previous experiments have reported successful elution of enteroviruses (Sobsey & Glass, 1980), HAV (Sobsey et al., 1985), and adenoviruses (Enriquez & Gerba, 1995; Sobsey & Glass, 1984) in tap water from positively-charged filters using similar eluates. Specifically, Sobsey & Glass (1984) reported simian adenovirus SV-11 recoveries of 13-22%, and Enriquez & Gerba (1995) reported a 26.5% recovery of human adenovirus 40 by eluting 1MDS filters with beef extract. The percent recoveries for adenovirus in the current study are lower than the recoveries reported in these previous studies. One possible explanation is that the fibers which protrude from the adenovirus capsid may be capable of

physically interacting with the alumina fiber matrix. If this is true, adenovirus elution by interruption of electrostatic binding could still be hindered by physical entrapment within the filter matrix, while norovirus elution would be unaffected due to its smaller, nearly spherical size and lack of the long protruding surface fibers possessed by adenoviruses.

PEG precipitation yielded lower recoveries for both viruses when using actual eluates versus seeded beef extract solution. Lewis & Metcalf (1988) reported a similar reduction in performance when PEG precipitation was used to concentrate human rotaviruses from freshwater and estuarine samples. In their study, rotavirus recovery was 93% from freshwater eluates, but only 61% from estuarine water eluates. The reduced virus recovery with actual filter eluates was not specifically attributable to PCR inhibitors in their experiments. This is because infectivity assays, rather than nucleic acid assays, were used to detect the virus in these previous experiments. However, some substance(s) could have been concentrated during filtration and elution and subsequently interfered with PEG precipitation of the viruses.

A number of studies have suggested that somatic (Moce-Llivina et al., 2005) and F+ coliphages (Colford et al., 2007; Dore et al., 2000; Jiang et al., 2001) may be good indicators of human enteric viruses in water. In this present study, no positive association was found between the occurrence of human enteric viruses and coliphages in seawater for either sampling site. One reason for this lack of association could be related to the survival and persistence of human enteric viruses and coliphages in natural waters. Sinton et al. (1999) conducted an experiment to determine the inactivation rate of coliphages in sewage-polluted seawater, and determined that F+ coliphages are particularly susceptible to longer solar wavelengths that are frequently present in the marine environment. In addition, an

experiment by Kohn and Nelson (2007) showed that MS2 coliphages may be inactivated directly by sunlight, or indirectly by sunlight-mediated production of highly reactive singlet oxygen in natural waters.

It has also been suggested that human enteric viruses such as adenovirus may be able to persist for many days in seawater due to their relatively high resistance to inactivation by solar radiation and UV radiation in general (Jiang et al., 2001). The data of this present study documented a marked decline in the presence of both somatic and F+ coliphages throughout the day, which could be the result of inactivation by solar radiation. However, adenovirus and norovirus presence was relatively constant at different time periods throughout the day. If enteric viruses are able to survive for longer periods of time in natural waters than coliphages, this could explain why the two virus types were not positively associated. It is also possible that the lack of association of adenovirus and norovirus presence in samples with time of day is due to the unit of measure, which is either presence or absence. The actual concentrations of these viruses were not examined in samples collected at different times of the day. Therefore, it is possible that virus concentrations differ among samples collected at different times of the day, but human enteric virus concentrations were not measured in this study.

Another possible reason for the lack of positive association between coliphages and human enteric viruses could be related to the detection limit of the PCR assay for human enteric viruses. If human enteric viruses are present in low numbers they may not be detected, which can potentially generate false negative results. In this study, human enteric viruses were frequently detected in the absence of coliphages, so it is unlikely that the lower detection limit of the assay was responsible for the lack of positive association.

The lack of association could also be due to the different methods used to detect enteric viruses and coliphages in the field samples. Enteric viruses were detected by PCR assay, which is capable of detecting viral genomes of both infectious and non-infectious viruses, but coliphages were detected by cell infectivity assay, which only detects infectious virus particles. Gantzer et al. (1998) compared the two methods for the detection of coxsackievirus in PBS, and reported that viral genomes survived twice as long as infectious viruses. Choi & Jiang (2005) conducted a study in which adenoviruses were isolated from river water, and found that most of the viruses detected by PCR were non-infectious. Another comparative experiment was performed by Wetz et al. (2004), in which poliovirus was seeded into four different water types (de-ionized water, artificial seawater, filtered seawater, and unfiltered seawater). Their results indicated that viral genomes persisted longer than infectious viruses in all types of water except unfiltered seawater. They concluded that viral genomes probably do not persist for long periods of time in natural seawater due to factors such as microbial predation, nucleases, and solar radiation.

In the current study, it is uncertain how frequently non-infectious viral particles were detected by the PCR assay. However, it is likely that the viruses detected in these samples were associated with recent contamination, because published evidence suggests that viral genomes and damaged viral particles are quickly eliminated in natural seawater. Even so, in future studies it would be valuable to consider using PCR analysis to detect both the indicator organisms and the pathogenic viruses in order to generate more comparable results.

CONCLUSIONS

Overall, Argonide Nanoceram filters appear to offer certain distinct advantages over other electropositive filters for the recovery of adenoviruses and noroviruses from seawater. Specifically, the pH conditions and salt concentrations of the seawater did not seem to adversely affect virus adsorption to this filter. However, adsorbed adenoviruses could not be efficiently eluted using the beef extract solution described. Future research should be directed towards the improvement of adenovirus elution by disrupting both electrostatic interactions and potential physical adsorption or entrapment in the filter matrix.

PEG precipitation was shown to be an effective secondary concentration procedure, although further work is recommended to identify and remove or otherwise overcome the inhibitory compounds present in virus filter concentrates from seawater. When applied to seawater samples impacted by non-point sources of fecal contamination, Argonide filter adsorption followed by beef extract elution and PEG precipitation resulted in adenovirus and norovirus detection by PCR in 50/379 (13%) and 23/379 (6%) of the samples analyzed, respectively.

Finally, the results of the field studies suggest that somatic and F+ coliphages may not be reliable indicators of human adenovirus and norovirus in natural seawater contaminated with non-point sources of fecal contamination. It would be beneficial to further analyze these data in relation to reported swimmer illnesses in these waters in order to

determine whether or not the presence of adenovirus or norovirus (human enteric viruses), or somatic or F+ coliphages (fecal indicator viruses), was predictive of swimmer illnesses such as gastroenteritis, which may be caused by both noroviruses and adenoviruses, or respiratory syndromes, which may be caused by adenoviruses.

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