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TECHNIQUES FOR VIRUS DETECTION IN THE MARINE ENVIRONMENT

By

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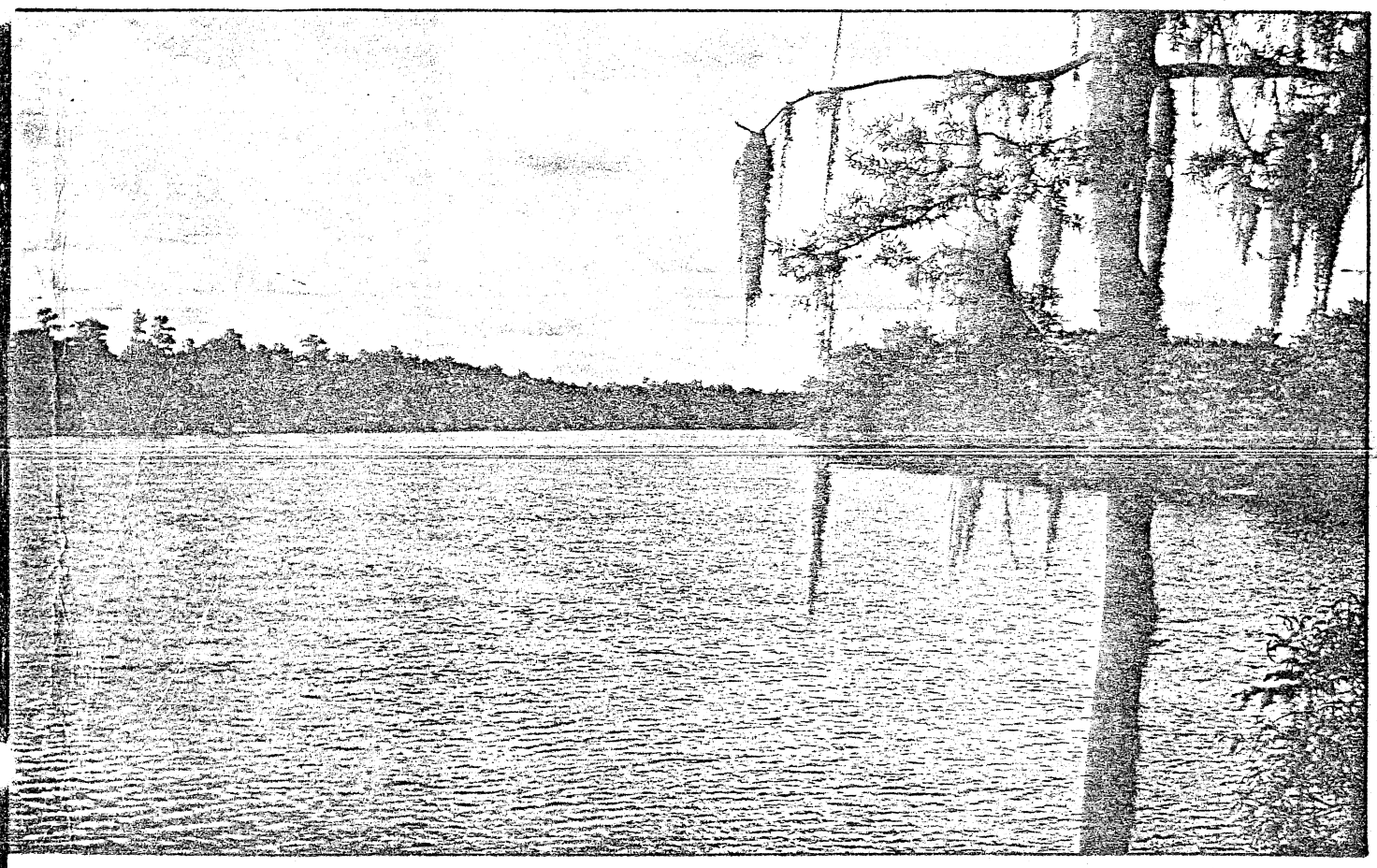

UNIVERSITY OF FLORIDA

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ABSTRACT

This study dealt with the developement of methodology for virus recovery from the marine environment.

We have explored a variety of methods for the recovery of enteroviruses from seawater which has been inoculated with high liters of poliovirus 1, 2, and 3, coxsackie B3 and echovirus 1 and 4. We have developed a technique based on the adsorption of viruses to Filterite filters, elution with 1% non-fat dry milk (NFDM), and organic flocculation of the eluates. This method resulted in the efficient recovery of most enteroviruses tested except for echovirus type 1. The method was also suitable for virus recovery from tap water.

Various eluents were tested for their ability to desorb viruses from marine sediments. In laboratory experiments 4M urea-0.05M lysine was the most efficient in virus recovery from sediment. Although the method is time consuming, it results in a small volume of concentrate.

The methods developed in the course of this study were used to recover indigenous viruses from estuarine water and sediments. Enteroviruses were found in both water and sediment in a section of Matanzas River which was closed with regard to shellfish harvesting.

PUBLICATIONS

G. Bitton, B.N. Feldberg and S.R. Farrah. 1979. Concentration of enteroviruses from seawater and tapwater by organic flocculation using non-fat dry milk and casein. Water Air and Soil Pollution. 12: 187-195.

G. Bitton, P. Chou and S.R. Farrah. Virus recovery from marine sediments. (In Preparation).

THESIS

Feldberg, B.N. 1979. The use of non-fat dry milk for the concentration of enteroviruses from seawater, tapwater, and sewage treatment plant effluents. M.S. thesis, 75 pp. Department of Environmental Engineering Sciences. University of Florida, Gainesville, Florida. 32611.

INTRODUCTION

Wastes resulting from man's activities have been traditionally (and are still) discharged into the ocean. These include liquid wastewater, sludge, oil, industrial and thermal wastes. With ever increasing populations along the coastlines around the world, it is by now realized that the ocean can no more be considered as the "infinite sink" for the disposal of fecal and other wastes. The coastal waters of the USA receive more than 8 billion gallons of municipal sewage in a single day. Approximately half of this domestic sewage receives secondary treatment (McNulty, 1977). Ocean outfalls are commonly used for the disposal of non treated or partially treated sewage. Their main advantages are low cost as compared to tertiary treatment of wastewater. Their design is based on location, water depth, distance from shore, and mixing regime.

Over the past 25 years much information has been gathered concerning the fate of enteric bacteria in marine waters. These pathogens are generally eliminated by physical dilution and by die-off mechanisms, which include temperature, predation, nutrient deficiency, algal and bacterial antibiotics or toxins, solar radiation, heavy metals and salinity. (Mitchell, 1968; Mitchell and Chamberlin, 1975). Other factors, such as sedimentation, lead to the accumulation of the bacterial pathogens in marine sediments. (Gerba et al., 1978; Rittenberg et al., 1958). With regard to viruses more information is being gathered about their occurrence and fate in marine and estuarine waters (Edmond et al., 1978; Goyal et al., 1978; 1979; LaBelle et al. 1980). However, more efforts must be spent on the improvement or development of methodology for virus detection in marine and estuarine waters as well as sediments.

This report explores the methodological aspects of virus occurrence in the marine environment.

LITERATURE REVIEW

We will briefly review the occurrence of viruses in the marine/estuarine environment, the potential public health hazards resulting from their entry in ocean waters, and the existing methodology for their detection in that particular environment.

Occurrence of Viruses in Marine Waters

The contamination of seawater by viruses mainly occurs through the disposal of sewage or sewage effluents into estuarine waters or offshore disposal via sewage outfalls.

A survey carried out in the Mediterranean sea by the Environmental Health Laboratory of Hebrew University led to the detection of several types of enteric viruses (Poliovirus, Echovirus, Coxsackievirus) as far as 1500 m from the discharge point. Coliform monitoring showed that these bacteria were less stable than viruses in seawater. (Shuval et al., 1971). Another virus survey was carried out along the Houston ship channel and in Galveston Bay, Texas. (Metcalf et al., 1974). This study showed that sewage treatment plants may discharge from 70×10^6 to 1746×10^6 viruses per day. Examination of the Houston ship channel for viruses led to the detection of enteroviruses as far as 8 miles (≈13 kilometers) from the contamination source. Ruiter and Fujioka (1978) have shown that up to 8×10^{10} viruses are discharged daily into Mamala Bay via a sewage outfall. More recently human viruses were detected in coastal waters and sediments (Edmond et al. 1978; Goyal et al., 1978; 1979; LaBelle et al., 1980). These studies have essentially shown that marine/estuarine waters are frequently contaminated with viruses and that classical indicator bacteria are not suitable for demonstrating the presence or absence of viruses in the marine environment. These findings prompted world-wide research on the factors which control the fate of viruses in marine waters.

Virus Survival in Marine Waters

Laboratory studies have shown that virus survival is primarily controlled by physical, chemical, and biological factors (Bitton, 1978). Some of these laboratory experiments were confirmed by "in situ" studies. However, "the virucidal agent" remains yet to be identified. It was, however, repeatedly demonstrated that temperature is the most decisive factor controlling virus survival in seawater and other coastal waters as well.

Viruses display an affinity for suspended solids in the aquatic environment (Bitton, 1980a). These solids may be silts, clay minerals, cell debris and particulate organic matter. The solid-associated viruses tend to settle into bottom sediments where they reach higher concentrations than in the overlying waters (Gerba et al., 1978; Goyal et al., 1977). Therefore, sediments may serve as reservoirs for viruses as well as for enteric bacteria. This has a public health significance since resuspension of the upper layers of sediments may increase the virus concentration in the water column. Sediment resuspension is particularly significant in shallow waters and is due to motor boat activity, currents, swimming and changes in water quality.

Potential Health Hazards Due to the Presence of Enteric Viruses in Marine Waters

In the last decade, water has been recognized as a possible route for the transmission of enteric viral diseases. (Berg, 1967). We have now evidence that the infectious hepatitis virus may be transmitted via the water route. However there is a lack of epidemiological data concerning the association between swimming in water and viral diseases. Recently, it has been demonstrated that there was a statistically significant association between swimming and shigellosis. Shigella sonnei was isolated from the Mississippi River near Dubuque, Iowa and was shown to be responsible for disease in individuals who had water in their mouth while swimming (Rosenberg et al., 1976). These are interesting data since Shigella has a low infectious dose (10 to 100 bacterial cells) as compared to other pathogens. This infectious dose is not very far from the infectious dose of enteric viruses.

Viral diseases may also be contracted following the consumption of shellfish which have been harvested from beds contaminated with sewage effluents (Metcalf, 1976). Shellfish (oysters, clams, mussels) have the ability to concentrate viruses within their gastrointestinal tract. Viruses may later diffuse from the alimentary tract to other tissues. Although infectious hepatitis and gastroenteritis are the only diseases shown to be associated with shellfish consumption, it is possible that other viral diseases are transmitted via this route.

Methodology for virus detection in the marine environment

Microporous filters have been mostly used for virus concentration from marine and estuarine waters (Goyal and Gerba, 1981). The virus concentrator developed by Wallis et al., (1972) was used by Metcalf et al., (1974) to concentrate viruses from estuarine and surface waters. Clarifying filters (1-μm orlon filters) were used to remove large particles that tended to clog the Millipore filters that were used as virus adsorbents. It was noted that the clarifying filters tended to adsorb viruses as larger volumes of water were processed. Metcalf and co-workers (1974) used a modified version of the previous method to detect viruses in Houston ship channel and Galveston Bay. A procedure for concentration of enteroviruses from 15 to 100 gallons of turbid estuarine water using a filter aid (Celite) and Millipore membranes was described by Hill, et al., (1974). However, these procedures permitted recovery of less than 3% of added virus when low numbers of viruses were used to test the procedures.

Sobsey et al., (1977) used asbestos-fiberglass (Cox) filters in place of the nitrocellulose (Millipore) filters previously used. Since these filters were more resistant to clogging than Millipore filters, the clarifying filters were eliminated. The virus concentrator used by Sobsey et al., (1977) employed a single prefilter along with two flat sheets of membrane filters as the virus adsorbents. Virus in estuarine water at pH 3.5 and 0.0005 M aluminum chloride adsorbed to the filters and could be recovered by treating the filters with glycine buffer at pH=11.5. However, virus in this eluate could not be reconcentrated in a smaller volume using a second membrane

adsorption-elution step as was used when tapwater was sampled for virus (Sobsey et al., 1973). Adjusting eluates to pH 3.5 resulted in the formation of large amounts of floc that rapidly clogged filters used for reconcentration. However, the authors (Sobsey et al., 1977) observed that flocculation could be increased by addition of ferric chloride. The flocs that formed were efficient virus adsorbents and could be used to reconcentrate the virus to a final volume of 20 ml. The flat filters used by Sobsey et al., (1977) permitted relatively low initial flow rates and also clogged rapidly. Frequently, 1-2 hours were required to process 50 gallons of estuarine water. Since pleated fiber-glass filters were shown to permit processing of large volumes of tapwater at high flow rates (Farrah, et al., 1976), these filters were tested for their ability to concentrate virus from estuarine water by Payment et al., (1977). The use of pleated fiberglass filters permitted the concentration of virus from 400 to 1,000 liters of estuarine water. Virus was recovered by treating the filters with high pH glycine. A two-step flocculation procedure using aluminum chloride was used to concentrate virus to a final volume of 20-40 ml. This reconcentration procedure was found to give inconsistent recoveries when estuarine water with different amounts of organic compounds and from different sites were tested. Therefore, this reconcentration procedure was replaced by a procedure using aluminum hydroxide flocculation and hydroextraction. (Farrah et al., 1977). The completed procedure using pleated membrane filters as the virus adsorbent, glycine buffer to elute adsorbed virus, and aluminum hydroxide flocculation along with hydroextraction to reconcentrate eluted virus, was used in virus monitoring in Galveston Bay (Goyal et al., 1978) and in seawater near a deep sea sewage outfall off Miami, Florida (Edmond et al., 1978). It thus appears that the pleated Filterite microporous filters offer large surface areas for virus adsorption and are relatively resistant to clogging. The optimal virus adsorption to these filters requires an $AlCl_3$ concentration of 0.0005M to 0.0015M and a pH of 3.5. However, elution of adsorbed viruses with glycine buffer at high pH (pH=11.5) may be detrimental to some pH-sensitive viruses such as adenoviruses or rotaviruses. Therefore one needs to look for eluents which work efficiently at pH=9.0-9.5. In 1976, Katzenelson and his collaborators described a method which responded to this need. These investigators showed that beef extract could be used as an eluent in virus concentration from tapwater. After elution at pH=9.0, viruses could be concentrated via organic flocculation of the beef extract at pH=3.5. The sediment obtained from flocculation could be resolubilized with 0.15M Na_2HPO_4 at alkaline pH. It is therefore useful to explore virus detection methodology which uses eluents that work well at pH<10. The methods discussed above are summarized in Table 1.

Methodology for virus detection in sediments

Viruses as well as enteric bacteria may become associated with organic and inorganic particulates present in water. The solid-associated viruses tend to settle into the bottom sediments where they reach high concentrations (Edmond et al., 1978; Gerba et al., 1977; Goyal et. al., 1977; Matson et. al. 1978). It has been shown that sediment-associated viruses may survive longer than in the water column (DeFlora et al., 1975; Gerba and McLeod, 1976; Smith et al., 1978). The mechanism of the protective effect displayed by sediment is not clear (Bitton, 1980b). Sediments may therefore serve as reservoirs for enteric pathogens, particularly viruses. Resuspension of the upper layer of sediments through motor boat activity, swimming activity,

currents, dredging or changes in water quality, may lead to an increase of virus concentration in the water column.

Few studies have been undertaken on the development of methods for virus detection in sediments. De Flora et al. (1975) showed an accumulation of viruses in marine sediments in the vicinity of sewage outfalls in Italy. They used sterilized seawater for eluting viruses from sediments. Glycine buffer (0.05M; pH=8.5) and nutrient broth (pH=8.5) have also been used for virus recovery from marine sediments (Metcalf et al. 1974; Vaughn and Metcalf, 1975). However, none of the above investigators determined the efficiency of the eluents used. Gerba et al. (1977b) described a method for virus recovery from marine sediments. The procedure consists of eluting the sediment with 0.25M glycine buffer, pH=11.5, containing 0.05M EDTA. Following a 10-minute contact time, the sediment was spun down and the supernatant was concentrated via membrane filtration. Virus was recovered from sediment with an efficiency of 50%. This method has two major drawbacks: The pH of the eluent is too high and may lead to inactivation of pH-sensitive viruses as was discussed previously. The second drawback is the clogging of microporous filters used in the reconcentration step. It is therefore necessary to evaluate a wide range of eluents for virus recovery from marine and estuarine sediments. These eluents should be used at a pH lower than 11.5. Moreover, some other means of eluate reconcentration (other than membrane filtration) should be explored. Since no study has dealt with methodology for virus recovery from freshwater sediments, some of the findings may be helpful in the development of methodology for virus recovery from these types of sediments.

Table 1 : Virus concentration from large volumes of seawater and sewage effluents.

	Concentration system	Adsorption conditions	Initial volume (l)	Final volume (ml)	Final percent recovery	Water type	Reference
Seawater	2 step adsorption elution	0.0005 M $AlCl_3$ pH = 3.5	95	10	63	Coastal	Metcalf et al. (1974)
	Adsorption, elution, inorganic flocculation	0.0015 M $AlCl_3$ pH = 3.5	378	20	53	Seawater	Gerba et al. (1978)
	Adsorption, elution, inorganic flocculation	0.0015 M $AlCl_3$ pH = 3.5	400	10-40	60-80	Estuarine	Farrah et al. (1977)
	Adsorption, elution, inorganic flocculation	0.0015 M $AlCl_3$ pH = 3.5	378	20-100	50	Seawater	Payment et al. (1976)
	Adsorption, elution, inorganic flocculation	0.0005 M $AlCl_3$ pH = 3.5	189	15-20	41	Seawater	Sobsey et al. (1977)

Table 1 ------continued.

	Concentration system	Adsorption conditions	Initial volume (l)	Final volume (ml)	Final percent recovery	Water type	Reference
Sewage effluents	Adsorption, elution, inorganic flocculation	0.0005 M $AlCl_3$ pH = 3.5	19-190	20	50	2° effluent	Gerba et al. (1978)
	Adsorption, beef extract elution, organic flocculation	0.0005 M $AlCl_3$ pH = 3.5	76-96	?	85	3° effluent	Landry et al. (1978)
	Adsorption, elution, inorganic flocculation	0.0005 M $AlCl_3$ pH = 3.5	76-96	?	36	3°	Landry et al. (1978)

MATERIALS AND METHODS

Viruses Used and Their Assay

Plaque-purified poliovirus type 1 (Sabin), poliovirus type 2 (natural isolate), poliovirus type 3 (natural isolate), ECHO 1 (Farouk), ECHO 4 (Pesacek), and Coxsackie B3 (Nancy) were used in these studies. Poliovirus types 2 and 3 were isolated from wastewater sludge.

Virus stocks were produced by infecting a monolayer of human amnion cells (AV3) or Rhesus monkey embryonic kidney cells (MA104) growing in 32 ounce bottles. Viruses were allowed to adsorb to the host cells for one hour at room temperature after which 40 ml of Eagle's Minimal Essential Medium (MEM) with 10% Fetal Calf Serum (FCS) was added. The monolayers were then incubated for 48 hours at 37°C. The virus was harvested by decanting the fluid, centrifuging at 1500 RPM for 15 minutes, and freezing the resultant supernatant at -70°C in small aliquots for later use.

Viruses used in seeded experiments were assayed on AV3, MA104, or BGM (Buffalo Green Monkey) host cells using a routine plaque procedure. For this assay monolayers were prepared by incubating host cells at 37°C in 32 ounce bottles containing 40 ml of Eagle's MEM with 10% FCS until confluent monolayers were obtained (approximately 60 hours). At confluence, the growth media was removed and the cells washed with 30 ml of a pre-trypsin solution. The monolayers were then treated with 10 ml of a standard trypsin-versene solution to separate the cells from the glass surface. After approximately 1 minute the latter solution was decanted and the monolayer maintained at room temperature until the cells were unbound (approximately 5 minutes). Then, 200 ml of Eagle's MEM with 10% FCS was added to suspend the cells and subsequently distributed to forty 2-ounce glass or plastic tissue culture bottles in 5 ml aliquots. The bottles were incubated at 37°C for 48 hours or until monolayer confluence was evident. At this stage the tissue cultures were ready for use in the standard plaque assay.

Mature tissue cultures were infected by decanting the growth medium and adding 0.2 ml of sample adjusted to isotonicity and pH=7.2-7.4. Samples were diluted using either Eagle's MEM with 10% FCS and antibiotics or phosphate-buffered saline (PBS) with 5% FCS and antibiotics. The tissue cultures were maintained at room temperature for one hour to allow for virus adsorption to the host cells. After the adsorption period the monolayers were overlayed with five ml of methyl cellulose and incubated at 37°C until the plaques were approximately 1-5 mm in diameter. At this stage the monolayers were stained using crystal violet or neutral red and the number of plaques determined.

Water Samples

Seawater was collected from Crescent Beach, Florida, and stored at 4°C prior to use. The seawater had a pH of 8.2 and a specific conductance

of 41,000 µmhos/cm (29.6 ppt salinity). Gainesville tap water used had a pH of 8.4 and a specific conductance of 250 µmhos/cm and was dechlorinated using sodium thiosulfate prior to viral seeding. Ortho-tolidine titrations (Standard Methods, 1976) were performed to determine the concentration of sodium thiosulfate necessary for complete dechlorination.

Concentration Step: Viral Adsorption and Elution

All experiments were performed in batch processes. The viruses used were suspended in three liters of seawater and adsorbed to a series (3.0 µm → 0.45 µm → 0.45 µm or 3.0 µm → 0.45 µm → 0.25 µm) of epoxy-fiberglass filters (Filterite Corp., Timonium, Md.) in the presence of 0.0005 M $AlCl_3$ at pH = 3.3-3.5. Viruses were eluted from the membrane filters using 0.5% (w/v) technical, purified, or isoelectric casein (Difco, Detroit, Mich.) in 0.05 M glycine buffer at pH = 9.0 or 10.0, or 1% (w/v) non-fat dry milk (NFDM) (Atlantic and Pacific Tea Co., Montvale, N.J.) in 0.05 M glycine buffer at pH = 9.0. Additional eluents used were 3% beef extract at pH = 9.0 (Katzenelson et al., (1976) and 0.05 M glycine, pH = 11.5 (Farrah et al., 1977). Both the filtrates and eluates were assayed to determine percent adsorption and elution, respectively.

In experiments using large volumes of water (20 to 100 gallons), suspended viruses were adsorbed to a 0.25 µm or 0.45 µm 10-inch epoxy-fiberglass cartridge filter (Filterite Corp., Timonium, Md.) in the presence of 0.0005 M $AlCl_3$ at pH = 3.3-3.5. This was done by pumping the prepared sample through the filter, using a gasoline (Sears Roebuck and Co., Chicago, Ill.) or electric (Sturdy Puppy, ITT Jabsco Prod., Costa Mesa, Calif.) powered centrifugal pump. Flow rates as high as 10 GPM were achieved using the gasoline powered pump. When 50 gallons of secondary effluent was processed a 3.0 µm 10-inch Filterite cartridge filter was used as a prefilter. Viruses were eluted by pumping 1 liter of 1% NFDM in 0.05 M glycine at pH 9.0 through the filters after a 1 minute contact period was allowed. In seeded experiments the filtrate and eluate were assayed to determine percent adsorption and elution, respectively.

For the concentration step the concentration factor ranged from 100 in the small volume trials to 400 in the large volume trials.

Reconcentration of filter eluates

Glycine eluates were reconcentrated according to Farrah et al. (1977).

Beef extract eluates were concentrated by organic flocculation as described by Katzenelson et al. (1976).

Solutions of casein and non-fat dry milk flocculate at pH 4.5-4.6 which is the isoelectric point of casein (Jenness and Patton, 1959). Eluates obtained by the above concentration step were flocculated at pH = 4.5-4.6 by the addition of 1 M glycine (pH = 2.0) and then centrifuged at 4500 RPM for 4 minutes. Eluates may also be allowed to settle and only the lower portion centrifuged. This was ascertained by seeding 30 ml of a 1% NFDM solution in 0.05 M glycine, adjusting the pH to 4.5, and allowing the flocs to settle undisturbed. The supernatant was withdrawn and the loose sediment adjusted to pH = 7 with 1 M glycine (pH = 11.0). Upon assay, an average of 117% of the added poliovirus was recovered in a final volume of 7 ml. This data suggests that the centrifugation step may also be carried out at a lower speed with no effect on virus recovery. Pellets obtained from centrifugation were redissolved in 0.15 M Na_2HPO_4 (pH = 9.0). The concentration factor for this step ranged from 10 (small volume) to 40 (large volume). The final sample was sonicated at 35 watt power output for 10 to 15 seconds with a Sonifier Cell Disrupter (model WI85D, Heat Systems Ultrasonics, Inc., Plainview, N.Y.).

When using casein or NFDM it was found to be advantageous to add a small quantity (one squirt) of an antifoam agent (Dow Corning Corp., Midland, Mich.) to inhibit foam production during elution and pellet resuspension. The use of an antifoam agent did not reduce poliovirus numbers or cause cytotoxicity to AV3 or MA104 host cells (Table 2 and 3).

When large volumes of water were processed using NFDM the final concentrates were dialyzed against PBS for 18 to 24 hours at 4°C prior to virus assay. This step was necessary to prevent cytotoxity to host cells caused by factors present in the undialyzed fluid.

Virus Recovery From Sediments

Marine sediments used in this study were a sandy sediment collected at Crescent Beach, Fla. and an organic muck sediment sampled at the Intercoastal Waterway near St. Augustine, Fla. The sediments were retrieved with the aid of a Ponar dredge and were stored at 4°C until use.

10g-sediment samples were mixed with 20 ml of seawater which has been seeded with poliovirus type 1. The mixtures were mechanically shaken for 30 minutes and then centrifuged at 4000 RPM for 4 minutes. The supernatants were poured off and assayed for viruses to determine the percent virus adsorption to the sediment. A blank, which consisted of seawater without sediment, was run simultaneously with the sediment samples.

Ten eluents were investigated for their ability to desorb viruses from marine sediments. These eluents were 4M urea + 0.05M lysine, 0.6M Na trichloroacetate + 0.02M glycine, 1.0% isoelectric casein, 1% non-fat dry milk, humic substances (color units = 5,700), 0.3% Na pyrophosphate, 10% fetal calf serum (FCS) in phosphate buffered saline (PBS), distilled water, 3% beef extract and 0.25 M glycine + 0.05M EDTA. All the eluents

were buffered to pH = 9.0 except for distilled water (pH = 7.0) and glycine-EDTA (pH = 11.5). Thirty ml of a given eluent were added to 10g of wet sediment. The mixtures were vortexed for 30s and then shaken for 30 min. However the shaking time was only 4.5 min and 1 min for glycine-EDTA and urea-lysine, respectively. The samples were centrifuged at 4000 RPM for 4 min and the supernatants were assayed for viruses to determine the % of virus eluted from the sediment.

Table 2: The effect of Antifoam A Spray on the recovery of poliovirus type 1 using AV3 host cells.

Treatment	Total PFU before treatment ($\times 10^3$)	Total PFU after treatment ($\times 10^3$)
With Antifoam	6.4	6.6
	5.5	6.8
	Mean: 6.0	Mean: 6.6
Without Antifoam	6.6	7.1
	6.5	6.0
	Mean: 6.6	Mean: 6.6

-Note: 50 ml of a 0.05 M glycine buffer solution (pH = 8.5) were seeded with poliovirus type 1 (Sabin), stirred with a magnetic bar for 1 minute and then assayed for virus. Two squirts of Antifoam A Spray (Dow Corning Corp., Midland, Mich.) were added to two test tubes while two other tubes received no treatment. All tubes were then strongly mixed for 5 minutes with a magnetic stirring bar and then assayed for virus on AV3 host cells.

Table 3: The effect of Antifoam A Spray on the recovery of poliovirus type 1 using MA104 host cells.

Treatment[a]	PFU/ml			
	Run 1[b]	Run 2	Run 3	Mean
MEM + Antifoam	440	440	420	430
MEM (control)	450	390	370	400

(a) 14.9 ml of Eagle's MEM were added to each of 6 test tubes and 0.1 ml of poliovirus was added to give a final virus titer of approximately 400 PFU/ml. Three tubes were treated (one squirt) with Antifoam A Spray (Dow Corning Corp., Midland, Mich.) while the three other tubes did not receive Antifoam. All 6 tubes were vortexed for 10 sec and the fluids assayed on MA104 host cells.

(b) Each run represents the mean number of 4 to 5 tissue culture bottles.

Concentration of Indigenous Virus from Field Samples

City of Tampa Sewage Treatment Plant Effluents and Tampa Bay Water

Table 4 shows conductivity, salinity, and pH values of sewage treatment plant effluents and Tampa Bay water which were examined for the presence of naturally occurring enteroviruses. Unchlorinated secondary effluent was collected immediately prior to tertiary treatment. Tertiary effluent was collected after approximately 10 minutes of chlorination (≈3 ppm available chlorine). This effluent was dechlorinated prior to processing as described for Gainesville tap water. Tertiary effluent from the City of Tampa sewage treatment plant is discharged to Tampa Bay by an underwater outfall. Sampling of the outfall was accomplished by anchoring a boat directly over the "boil" created by the discharge and pumping water from a five (5) foot depth into a holding tank on board the boat. Tampa Bay water was collected from a site 30 yards seawater of the outfall from a five foot depth. It is evident from conductivity and salinity values for effluents and bay water (Table 4) that the sample collected from the outfall site was quite dilute with effluent.

The virus concentration method, using 1% non-fat dry milk, is described in details in the "Results and Discussion" section.

Recovery of indigenous viruses from estuarine water and sediment in St. Augustine, Florida

The presence of indigenous viruses was monitored in two stations in St. Augustine, Fla (Figure 1). These are shellfish beds and shellfishing is prohibited. The first sampling point is on the Matanzas River just south of the Bridge of Lions in St. Augustine. The second sampling station is in the Salt Run at a location north of the public boat ramp. The station on the Matanzas River is in an area which is subject to fecal pollution from wastewater treatment plants (see Figure 1).

Forty to fifty gallons of estuarine water were sampled and processed following the non-fat dry milk method described previously.

Fifty to one hundred grams of sediment were sampled at both stations. They were immediately stored in ice and brought back to the laboratory where they were processed. The time between sampling and processing did not exceed 5 hours. The sediment samples were eluted with urea-lysine as described previously. The urea-lysine eluent must be prepared a few hours before use since older solutions may lead to lower recovery from sediment. The sediment eluates were concentrated according to the following procedure: $AlCl_3$ was added to give a final concentration of 0.005M. The eluate was then adjusted to pH 7 by the addition of 1M sodium carbonate and mixed for 5-10 minutes. The flocs formed were collected by centrifugation at 5000 RPM for 5 min. The supernatant was discarded and the floc was mixed with 5 volumes 0.1M EDTA 3% beef extract, pH=9.0. Most of the floc could be dissolved in this solution. The sample was then centrifuged at 5000 RPM for 5 min. and the supernatant was adjusted to pH7 and dialyzed against phosphate buffered saline at pH=7.0 overnight at 4°C. After dialysis the sample was reconcentrated by the organic flocculation method of Katzenelson et al. (1976). Our method resulted in a 3 ml concentrate from 50 to 100g sediment sample.

Concentrates from water and sediment were assayed on BGM cells and examined for cytopathic effects (CPE) for up to three weeks. The 50% tissue culture infective dose ($TCID_{50}$) was determined according to Reed and Muench (1938). Virus isolates were identified by using pools of neutralizing antiserum (Lim and Benyesh-Melnick, 1960).

Table 4: Water quality parameters of City of Tampa sewage treatment plant effluents and Tampa Bay water.

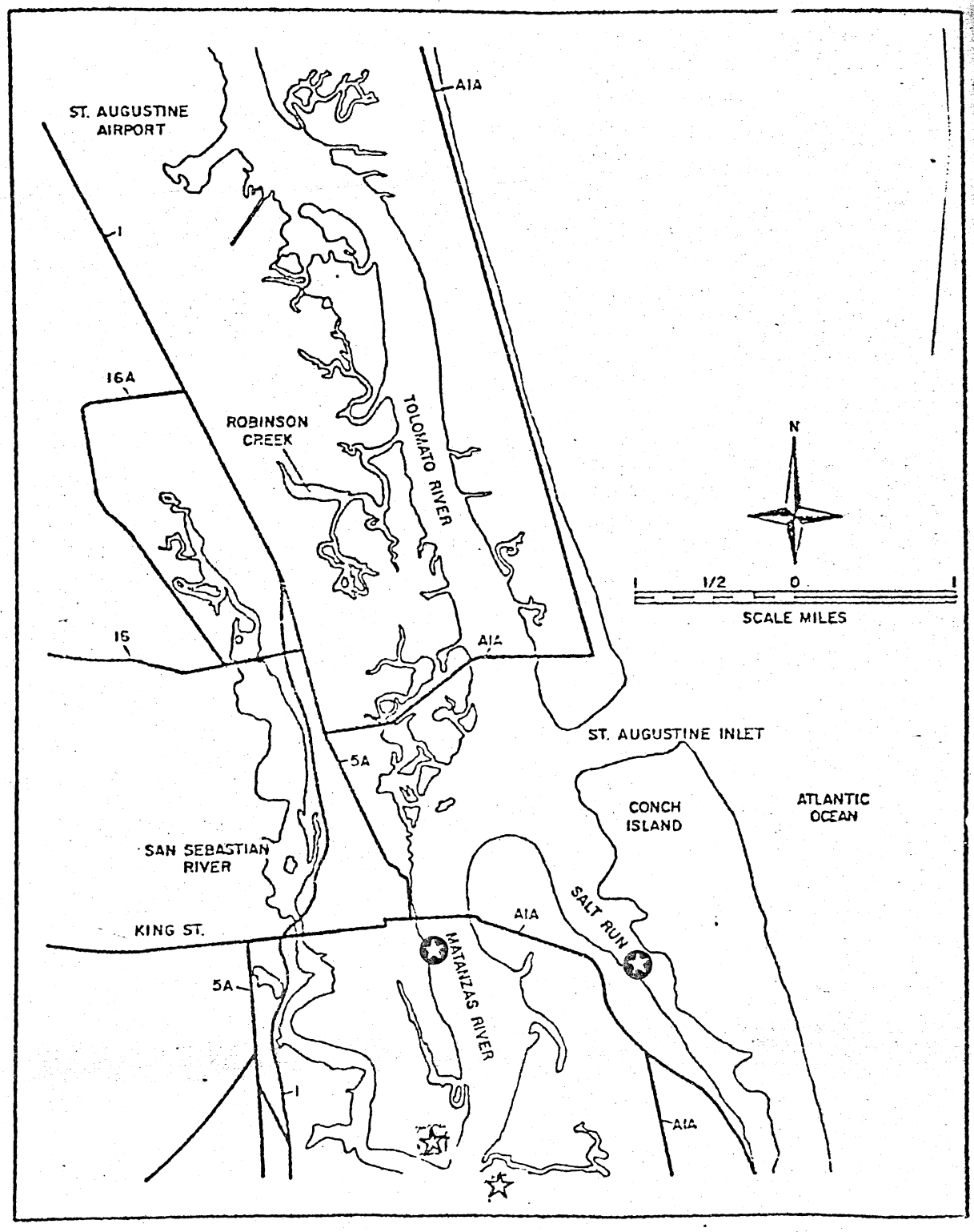
Water sample	Conductivity (μmhos/cm)	Salinity[(a)] (ppt)	pH
Secondary effluent (unchlorinated)	960	0.7	6.5
Tertiary effluent (chlorinated)	884	0.6	6.8
Tertiary effluent outfall, Tampa Bay	14,820	10.7	7.7
Tampa Bay, 30 yards from outfall	15,150	10.9	7.7

(a) Salinity values were computed from conductivity measurements.

Figure 1: Location of wastewater treatment plants and virus sampling stations in St. Augustine, Fla.

☆ Wastewater treatment plants

Virus sampling (Water and sediments)


ST. AUGUSTINE AIRPORT
A1A
I
16A
ROBINSON CREEK
TOLOMATO RIVER
N
1
1/2
0
1
SCALE MILES
15
A1A
ST. AUGUSTINE INLET
5A
CONCH ISLAND
ATLANTIC OCEAN
SAN SEBASTIAN RIVER
KING ST.
A1A
SALT RUN
5A
MATANZAS RIVER
I
A1A

RESULTS AND DISCUSSION

Comparison of Existing Methods for the Recovery of Poliovirus from Seawater

Two existing methods of concentrating viruses were examined with respect to recovery efficiency, using samples of seawater. Table 5 compares poliovirus recoveries using methods involving: 1) Elution with glycine (pH = 11.5) followed by inorganic flocculation using $AlCl_3$ (Farrah et al., 1977), and 2) Elution with beef extract (pH = 9.0) followed by organic flocculation (Katzenelson et al., 1976).

All methods examined shared a common first concentration step, that is the adsorption of viruses to epoxy-fiberglass (Filterite) filters in the presence of 0.0005 M $AlCl_3$ at pH = 3.3-3.5. The modified method of Farrah et al. (1977) used in our study involved elution with 0.05 M glycine buffer (pH = 11.5) and reconcentration using Al $(OH)_3$ flocs. Use of this method yielded mean recoveries of 47% elution., 25% from the reconcentration step, and 12% overall recovery. Farrah et al. (1977) reported that 85% to 95% of poliovirus adsorbed to Al $(OH)_3$ flocs were recovered by elution with the glycine-FCS mixture. The particular problem in the recovery of poliovirus in these trials (Table 5) was the elution of viruses adsorbed to the flocs. Greater than 90% of viruses present in the filter eluates were adsorbed to the inorganic flocs.

The second existing method of concentrating viruses is based upon elution of viruses adsorbed to membrane filters with 3% beef extract (pH = 9.0) followed by organic flocculation (Katzenelson et al., 1976). Use of beef extract method yielded higher poliovirus recovery than the glycine method and resulted in an overall recovery of 56% (Table 5). Using this method, Katzenelson 1977 was able to recover 63% of added poliovirus from 35 liters of seawater.

The Use of Technical, Purified, and Isoelectric Casein in the Concentration of Poliovirus from Seawater

A set of preliminary experiments were performed to learn whether dilute casein solutions could be used to elute poliovirus adsorbed to membrane filters and further concentrate the viruses by organic flocculation. Table 6 shows percent viral recovery using 0.2% technical casein in 0.05 M glycine (pH = 11.0-11.5), 0.5% technical casein in 0.05 M glycine (pH = 11.5). All trials yielded greater than 75% recovery from elution and greater than 65% recovery from organic flocculation (reconcentration). Overall recoveries for the three tials were greater than 50%.

The main advantage of the beef extract method is that elution is carried out at pH = 9.0, thus not affecting the survival of pH-sensitive viruses (reoviruses, rotaviruses, and adenoviruses) which are inactivated by exposure to pH = 11.5 for even short times (Fields and Metcalf, 1975, Sobsey et al., 1980). Furthermore, even the exposure of poliovirus type 1 to pH = 11.5 glycine buffer for more then 10 minutes may be virucidal (Gerba et al., 1977b). For this reason, one must work with great speed when using pH = 11.5 glycine buffer as an eluent for enteric viruses.

Table 5: Comparison of two existing methods for the concentration of poliovirus type 1 from seawater.

Method[a]	Percent elution	Percent reconcentration	Percent overall recovery
Elution with 0.05 M glycine (pH = 11.5) followed by inorganic flocculation using $AlCl_3$[b]	47	25	12
Elution using 3% beef extract (pH = 9.0) followed by organic flocculation[c]	86	66	56

(a) Poliovirus type 1 (Sabin) suspended in 3 liters of seawater, was adsorbed to a series (3.0 μm → 0.45 μm → 0.25 μm) of Filterite filters in the presence of 0.0005 M $AlCl_3$ at pH = 3.3-3.5. Viruses were eluted with 30 ml of a designated eluent (see references below). Final concentrates obtained were 2 to 4 milliliters. Numbers given are mean values for 2 to 4 trials.

(b) Trials performed according to Farrah et al. (1977).

(c) Trials performed according to Katzenelson et al. (1976).

Table 6: Use of technical and purified casein in the concentration of poliovirus type 1 from seawater.

Treatment[(a)]	Total PFU adsorbed to filters	Percent elution	Organic flocculation percent recovery	Percent overall recovery
0.2% technical casein in 0.05 M glycine (pH = 11-11.5)	4.06×10^6	77	79	56
0.5% technical casein in 0.05 M glycine (pH = 11.5)	5.47×10^6	76	67	51
0.5% purified casein in 0.05 M glycine (pH = 11.5)	4.64×10^6	81	72	58

(a) Poliovirus type 1 (Sabin), suspended in 3 liters of seawater, was adsorbed to a series (3.0 μm → 0.45 μm → 0.25 μm) of Filterite filters in the presence of 0.0005 M $AlCl_3$, pH = 3.5. Viruses were eluted using 30 ml of a designated eluent. Organic flocculation was induced by adding 0.5 M glycine (pH = 2) to the casein eluates until a pH of 4.5 was reached. The floc formed was pelleted by centrifugation at 4500 RPM for 4 minutes and re-dissolved in 3 ml 0.15M Na_2HPO_4, pH = 9.0. Values given are mean values from duplicate or triplicate trials.

It then became of interest to examine the effect of pH upon the elution of poliovirus type 1 from membrane filters. Table 7 shows that elution was most efficient at pH = 10.0 (100%) and least efficient at pH = 11.5 (64%). It is worth noting that viral elution was relatively high (76%) even at pH = 8.0.

Use of 0.5% isoelectric casein in 0.05 M glycine (pH = 10.0) permitted the highest recovery efficieny for elution (>100%) of the three types of casein examined so far (Table 8). Overall viral recovery from 3 liters of seawater using isoelectric casein was 83%.

Use of Non-Fat Dry Milk for the Concentration of Poliovirus from Seawater

While contemplating the potential use of casein for viral detection and concentration from seawater, the thought arose that since caseins and lipids (fats) are the major components of milk, then perhaps non-fat dry milk (NFDM) could be used as an inexpensive alternative for casein. Preliminary unseeded experiments showed that a 1% (w/v) solution of NFDM in 0.05 M glycine behaved like purified or isoelectric caseins in that flocculation occurred at pH = 4.5 and centrifugation produced a pellet and a clear supernatant.

Using 1% NFDM in 0.05 M glycine, a study was undertaken to investigate the influence of pH on the efficiency of elution of poliovirus type 1 from membrane filters. As shown in Table 9 the elution was maximum (116%) at pH = 9.0 and decreased to 35% and 43% at pH = 8.0 and pH = 7.0, respectively.

Since the ultimate goal of a concentration technique is to achieve a high concentration factor, it was decided to examine the effect of NFDM concentration on the efficiency of poliovirus recovery from seawater, the rationale being that the more dilute the NFDM solution used, the smaller the centrifuged pellet and hence the smaller the volume of final concentrate. As shown in Table 10 efficiency decreased with decreasing NFDM concentration (from 71% with 1% NFDM to 35% with 0.25% NFDM). However, recovery from organic flocculation was always high and ranged from 81% to 100%. Use of NFDM concentrations of 1%, 0.75%, 0.50% and 0.25% yielded overall poliovirus recoveries of 71%, 48%, 33%, and 28%, respectively.

Concentration of Poliovirus from Large Volumes of Seawater and Tap Water Using the NFDM Technique

As the NFDM technique proved efficient for the concentration of poliovirus from small volumes of seawater (3 liters), its application to the processing of large volumes of seawater and other types of water became of interest.

Twenty gallons (≈76 liter) samples of seawater were adjusted to pH = 3.3-3.5 and 0.0005 M $AlCl_3$, seeded with poliovirus, and processed by the NFDM technique (Table 11). Recoveries obtained for two trials were 54% and 87% elution recovery, 87% and 115% reconcentration recovery, and 74% and 100% overall recovery; the mean overall recovery for the two trials being 74%.

Next, fifty gallon (189 liters)-samples of dechlorinated tap water were seeded with high (≈7 x 10^7 PFU) and low (≈600 PFU) inputs of poliovirus type 1 and processed by the NFDM technique. As shown in Table 12 high virus input trials resulted in a mean overall recovery of 80% while low input trials recovered an average of 75% of the initial virus titer. Concentration factors ranging from 2363 to 6750 were obtained in these trials.

Table 7: Effect of pH on elution of poliovirus type 1 from membrane filters using 0.5% purified casein.

pH of casein	Percent elution[(a)]
8	76
9	74
10	100
11.5	64

(a) Poliovirus 1 (Sabin), suspended in 1 liter of seawater, was adsorbed to a series (3.0 µm → 0.45 µm → 0.25 µm) of Filterite filters in the presence of 0.0005 M $AlCl_3$ at pH = 3.5. The adsorbed viruses were eluted with 0.5% purified casein in 0.5 M glycine (30 ml) adjusted to various pHs with 0.5 M glycine. Each number represents the mean for 2 to 4 trials.

Table 8: Use of isoelectric casein in the concentration of poliovirus type 1 from seawater.

Total number of viruses adsorbed to membrane filters PFU	Percent elution	Organic flocculation percent recovery	Percent overall recovery efficiency
2.06×10^6	136	80	110
2.51×10^6	104	54	56
Mean:	120	67	83

Note: Poliovirus type 1 (Sabin), suspended in 3 liters of seawater, was adsorbed to a series (3.0 µm → 0.45 µm → 0.45 µm) of Filterite filters in the presence of 0.0005 M $AlCl_3$ at pH = 3.5. Viruses were eluted from the filters with 30 ml of a 0.5% isoelectric casein solution in 0.05 M glycine (pH = 10.0). Organic flocculation was undertaken by adding 0.5 M glycine (pH = 2) to the eluate until a pH of 4.5 was reached. The floc formed was pelleted by centrifugation and redissolved in 2.5 ml of 0.15 M Na_2HPO_4, pH = 9.0.

Table 9: Effect of pH on elution of poliovirus type 1 from Filterite filters using 1% (w/v) non-fat dry milk (NFDM).

pH of NFDM eluent	Percent elution[a]
7.0	43
8.0	35
9.0	116
10.0	86
11.5	69

(a) Poliovirus 1 (Sabin), suspended in 1 liter of seawater, was adsorbed to a series (3.0 μm → 0.45 → 0.45 μm) of Filterite filters in the presence of 0.0005 M $AlCl_3$ at pH = 3.5. Viruses were eluted with 10 ml 1% NFDM in 0.05 M glycine. Each number represents the mean of 2 to 4 trials.

Table 10: Concentration of poliovirus type 1 from seawater using various concentrations of non-fat dry milk (NFDM).

Treatment[a]	Total PFU adsorbed ($\times 10^6$)	Percent elution	Organic flocculation percent recovery	Percent overall recovery
1.00% NFDM[b]	51	71	101	71
0.75% NFDM	1.86	58	84	48
0.50% NFDM	1.36	35	94	33
0.25% NFDM	1.22	35	82	28

(a) Poliovirus type 1, suspended in seawater, was adsorbed to a series (3.0 µm → 0.45 µm → 0.45 µm) of Filterite filters in the presence of 0.0005 M $AlCl_3$ at pH = 3.5. Viruses were eluted with the various concentrations of NFDM in 0.05 M glycine, pH = 9.0. Organic flocculation was undertaken by adding 0.5 M glycine to the filter eluate until a pH of 4.5-4.6 was reached. The floc formed was pelleted by centrifugation at 4500 RPM for 4 minutes and the pellet redissolved in 0.15 M Na_2HPO_4, pH = 9.0 Each number represents the mean value obtained from duplicate trials.

(b) Trials using 1% NFDM used seawater samples of 20 gallons (76 liters) processed through 0.45 µm Filterite cartridge filters. Other trials used 1-3 liters seawater samples and were processed as described above.

Table 11: Concentration of poliovirus from 20 gallons (76 liters) of seawater using the NFDM technique.

PFU adsorbed to membrane filters	Percent elution	Percent reconcentration	Percent overall recovery
6.98×10^7	54	87	47
3.16×10^7	87	115	100

Note: Poliovirus type 1 (Sabin), suspended in 20 gallons (76 liters) of seawater was adsorbed to a 0.45 µm Filterite cartridge (10") in the presence of 0.0005 M $AlCl_3$, pH = 3.3-3.5. Viruses were eluted with 1 liter of 1% NFDM in 0.05 M glycine (pH = 9.0). Filter eluates were brought to pH 4.5 with 1 M glycine (pH = 2.0) and the resultant flocs centrifuged at 4500 RPM for 4 minutes. The pellets were then resuspended in 0.15 M Na_2HPO_4, pH = 9.0.

Table 12: Concentration of poliovirus type 1 from 50 gallons (189 liters) of tapwater by the non-fat dry milk (NFDM) technique.

Total virus input PFU	Percent elution	Organic flocculation percent recovery	Final volume (ml)	Percent overall recovery
High virus input[a]				
7.12×10^7	72	110	80	79
7.17×10^7	118	68	55	81
Low virus input[a,b]				
641	-	-	28	72
569	-	-	30	78

(a) Poliovirus type 1, suspended in 50 gallons (189 liters) of dechlorinated tapwater, was adsorbed to a 0.25 µm or 0.45 µm Filterite cartridge filter in the presence of 0.0005 M $AlCl_3$ at pH = 3.5. Viruses were eluted from the filters using 1% NFDM in 0.05 M glycine, pH = 9.0. Filter eluates were brought to pH = 4.5-4.6 with 1 M glycine (pH = 2.0) and the resultant floc centrifuged at 4500 RPM for 4 minutes. The pellets were then resuspended in 10-63 ml Na_2HPO_4, pH = 9.0.

(b) Concentrates from low virus input trials were dialyzed for 18-24 hours against PBS at 4°C prior to direct assay on host cells.

These results agree with those reported by other researchers using large volumes of water but different methods. Wallis et al. (1972a, 1972b, 1972c) and Sobsey et al. (1973) reported poliovirus recoveries ranging from 61% to 92% from large volumes of tap water using two step filter-adsorption elution. Using filter-adsorption elution and aluminum hydroxide flocculation reconcentration, Farrah et al. (1977) were able to recover 40% to 50% of poliovirus added to 500 gallons of tap water. Katzenelson et al. (1976) recovered 74% of added poliovirus from 500 liters of tap water using beef extract elution and organic flocculation. Using low numbers (16 to 50 PFU) of added poliovirus per 100 gallons of tap water, Hill et al. (1974) were able to recover 25% to 50% of the initial virus titers using two step filter-adsorption elution. However, Sobsey (1979) reported that the above method recovered only <1% to 25% of low numbers of nine enteroviruses, each added to 100 gallons of tap water.

Concentration of Other Enteroviruses from Seawater Using the NFDM Technique

The concentration of six enterovirus (poliovirus type 1, 2, and 3, coxsackie B3, ECHO 1, and ECHO 4) by the NFDM technique is shown in Table 13. It is evident that this method is efficient for the recovery of the three types of poliovirus as well as coxsackie B3; overall recoveries for the above four viruses ranged from 59% to 75%. Using NFDM, 31% overall recovery of ECHO 4 was obtained, but only 8% overall recovery was obtained using ECHO 1. The poor performance of the NFDM technique with regard to ECHO 1 recovery efficiency is mainly due to the inability to concentrate this virus on membrane filters and casein flocs. Other researchers working in this area have also encountered recovery problems regarding ECHO 1. Charles (1979) reported that ECHO 1 was poorly adsorbed to a sandy soil and to isoelectric casein and NFDM flocs as compared to poliovirus type 1, coxsackie B3, and ECHO 4. ECHO 1 was found to adsorb poorly to Filterite filters and lagoon sludge solids as compared to poliovirus type 1 (Pancorbo et al., unpublished observation). Goyal and Melnick (1978) observed that ECHO 1 was adsorbed significantly less to soils than poliovirus types 1, 2, and 3, coxsackie B3, and other ECHO virus types and suggest the presence of intratypic differences in adsorptive potential between ECHO virus isolated strains.

Table 13: Concentration of six enteroviruses from seawater by the non-fat dry milk (NFDM) technique.

Virus[a]	Virus input PFU ($\times 10^6$)	Percent elution	Organic flocculation percent recovery	Percent overall recovery
Polio 1[b]	31.6-69.8	71	100	71
Polio 2	29.9-30.9	76	78	59
Polio 3	61.1-69.6	111	72	75
Coxsackie B3	0.46	87	75	65
ECHO 1	17.0	78	10	8
ECHO 4	0.8-1.6	72	43	31

(a) The various viruses, suspended in 1-3 liters of seawater, were adsorbed to a series (3.0 µm → 0.45 µm → 0.45 µm) of Filterite filters in the presence of 0.0005 M $AlCl_3$ at pH = 3.5. The filters were eluted with 1% NFDM in 0.05 M glycine, pH = 9.0. Filter eluates were brought to pH = 4.5-4.6 with 1 M glycine (pH = 2.0) and the resultant floc centrifuged at 4500 RPM for 4 minutes. The pellets were then resuspended in 0.15 M Na_2HPO_4, pH = 9.0. Each number represents the mean of duplicate trials.

(b) The polio 1 trials used seawater samples of 20 gallons (76 liters) processed through 0.45 µm Filterite cartridge filters. Other trials used 1-3 liters seawater samples and were processed as described above.

VIRUS RECOVERY FROM MARINE SEDIMENTS

Prior to evaluating the ability of various eluents to desorb virus from marine sediments, experiments were undertaken to study the sorption ability of the sediments toward viruses. Table 14 shows that the sandy sediment sampled at Crescent Beach, Fla. retained 99% of added viruses. The Intercoastal Waterway sediment adsorbed 100% of added virus (only two samples were run for this sediment). Gerba et al., (1977) found similar results with estuarine sediments from Galveston Bay, Texas.

Ten eluents were investigated for their ability to desorb viruses from the Crescent Beach sediment. Three to six replicates were run for each eluent.

It was observed that virus elution from the sediment surface was generally relatively low and ranged from <1% to 43.9% (Table 15). Urea-lysine and TCA-glycine were found to be the most efficient among all the eluents tested. A surprising finding was the low recovery (<1%) obtained with 0.25 M glycine + 0.05 M EDTA pH = 11.0. Similar results were obtained with the Intercoastal Waterway sediment (Table 16). These findings do not agree with those of Gerba et al. (1977) who reported that glycine-EDTA eluted more than 50% of adsorbed viruses. In our study we made sure that the viruses were exposed to the high pH for no more than 15 minutes. Urea-lysine, which eluted 43.9% of poliovirus, has been successfully used for virus elution from sludge (Farrah et al., submitted for publication), and from positively charged microporous filters (Chang et al., unpublished). We have (Chang et al., unpublished; Farrah et al., submitted for publication). a two-step concentration scheme for urea-lysine eluates. This method led to a 50% virus recovery from these eluates.

Addition of distilled water (Table 15) to the marine sediment resulted in a poor virus recovery (1.6%) and this agrees with the results reported by Gerba et al. (1977). The use of 2% isoelectric casein resulted in a 35% recovery while less than 10% recovery was achieved with 3% beef extract (Table 15).

It was therefore decided to use 4M urea + 0.05M lysine (pH 9.0) for the detection of indigenous viruses in estuarine sediments.

Table 14: Adsorption of Poliovirus type 1 to a marine sandy sediment[a]

Run	Total Virus Imput (PFU)	Total PFU retained by sediment	% Virus adsorbed to sediment
1	8.8×10^6	8.74×10^6	99.3
2	2.0×10^6	1.98×10^6	99.4
3	1.9×10^6	1.87×10^6	98.9
4	2.6×10^6	2.56×10^6	98.7
5	2.0×110^6	1.98×10^6	99.2

(a) 20 ml of seawater seeded with poliovirus type 1 were added to 10g of sediment. (From Crescent Beach, Fla.). The mixtures were shaken for 30 min and centrifuged at 4000 RPM for 10 min. The supernatants were assayed to determine the % virus adsorbed to the sediment.

Table 15: Elution of Poliovirus Type 1 from a Marine Sandy Sediment[a]

Eluent	% Elution Mean[b] ± S.D.
4M Urea + 0.05M Lysine (pH = 9.0)	43.9 ± 14.3
0.6M TCA + 0.02M Glycine (pH= 9.0)	41.3 ± 1.3
2% Isoelectric Casein (pH = 9.0)	35.0 ± 6.5
1% Isoelectric Casein (pH = 9.0)	23.4 ± 6.0
1% Non-Fat Dry Milk (pH = 9.0)	16.0 ± 3.8
Humic Substances (color units = 5,700; pH = 9.0)	11.3 ± 0.6
0.3% Na Pyrophosphate (pH = 9.0)	11.2 ± 2.7
3% Beef Extract (pH = 9.0)	9.3 ± 2.9
10% FCS in Phosphate Buffered Saline (pH = 9.0)	6.9 ± 1.1
Distilled Water	1.6 ± 0.2
0.25M Glycine + 0.05M EDTA (pH=9.0)	< 1%

(a) Thirty ml of eluent were added to 10g of sediment. The samples were vortexed for 30s and then shaken for 30 min (the shaking time was only 4.5 min and 1 min for glycine-EDTA and urea-lysine, respectively). The samples were centrifuged at 4000 RPM for 4 min and the supernatants were assayed for viruses.

(b) Mean of 3 to 6 replicates.

Table 16: Recovery of Poliovirus Type 1 from Intercoastal Waterway Sediment.

Eluent	% Elution	Reconcentration step % Recovery	% Overall Recovery
3% Beef Extract (pH = 9.0)	11	87	9.6
0.25M Glycine + 0.05M EDTA (pH = 11.5)	<1	<1	<1

RECOVERY OF INDIGENOUS ENTEROVIRUSES FROM SEAWATER AND MARINE SEDIMENTS

Virus Recovery from Tampa Bay.

In February, 1979, a trip was made to the City of Tampa Sewage Treatment Plant for the purpose of testing the efficacy of the NFDM technique in the recovery of naturally occurring enteroviruses (Table 17).

Fifty gallons (189 liters) of unchlorinated secondary treated effluent were processed by membrane filtration using a series (3.0 μm → 0.45 μm) of Filterite cartridge filters followed by NFDM elution and organic flocculation to yield a final concentrate of 22.7 ml and 27.8 ml for the two filters, respectively, as each filter was eluted separately. The final concentrates were assayed following chloroform treatment to remove bacterial and fungal contamination. Concentrates from both the prefilter and filter were positive with regard to enteroviruses (Table 17). No attempts were made to quantify viruses since the method was designed specifically for marine waters.

Chlorinated tertiary effluent was collected immediately prior to discharge into Tampa Bay after approximately 10 minutes contact time with about 3 ppm available chlorine. According to the sewage treatment plant operator the concentration of available chlorine should have only been 1 ppm (personal communication). This latter fact implies that the excess chlorine addition was due to either human error or a desire to produce high quality water with respect to the presence of viruses. No virus was detected in a sample of chlorinated tertiary effluent. Similarly no virus was detected in Tampa Bay water (Table 17).

Detection of indigenous enteroviruses in estuarine water and sediment in St. Augustine, Fla.

Indigenous enteroviruses were monitored at two stations (Salt Run and Matanzas River) in St. Augustine, Fla. (See Figure 1). Estuarine water was processed according to the non-fat dry milk method which was discussed previously. Virus detection in sediments was performed according to the urea-lysine method. The results are shown in Table 18. In June 1980 no virus was detected in Salt Run in both water and sediment. In Matanzas River (near the Bridge of Lions in St. Augustine, Fla.) viruses were detected in both estuarine water and sediments.

The virus concentrations found were 207 $TCID_{50}$/50 gallons of water and 41.4 $TCID_{50}$/100g of wet sediment. Poliovirus type 1 and type 3 were identified in concentrates from water and sediment, respectively. Salt Run sediment was sampled again on September 1980 and enteroviruses were detected at a concentration of 18 $TCID_{50}$/100g of sediments.

Virus concentration in Matanzas River is within the range found by other investigators. Gerba et al. (1977) reported virus concentrations of 2-16 PFU/10 liters in coastal canals in Texas (2-16 PFU/10 liters is equivalent to 38-304 PFU/50 gallons). However, Edmond et al. (1978) found lower virus concentrations (21-42/100 gallons) in Miami Beach marine outfall. Virus concentration in Matanzas River sediment is similar to that found by DeFlora et al. (1975) in Italy.

Table 17: Use of the non-fat dry milk (NFDM) technique for the recovery of naturally occurring enterovirus from sewage treatment plant effluents and Tampa Bay water.

Water sample	Volume processed (gallons)	Final volume (ml)	Volume assayed (ml)	Virus presence	Concentration factor
Secondary effluent (unchlorinated)	50	27.8[a]	13.10	+	3,743
		22.7[b]	15.20	+	
Tertiary effluent (chlorinated)	100	23.4	5.60	N.D[c]	16,154
Tertiary effluent outfall, Tampa Bay	80	30.2	14.65	N.D	10,000
Tampa Bay water, 30 yards from outfall	75	18.2	2.80	N.D	15,604

(a) Volume of final concentrate from the elution of the 0.45 µm filter.

(b) Volume of final concentrate obtained from the elution of the 3.0 µm pre-filter.

(c) Not detected.

Table 18: Detection of indigenous enteroviruses in estuarine water and sediment in St. Augustine, Fla. (June 1980).

Sample type	Salt Run	Matanzas River (Near Bridge of Lions)
Water ($TCID_{50}$/50 gallons)	N.D[a]	207 (P1)[b]
Sediment ($TCID_{50}$/100g)	N.D	41.4 (P3)[c]

(a) Not detected
(b) Poliovirus type 1
(c) Poliovirus type 3

CONCLUSIONS

The method of beef extract elution and organic flocculation has recently been used for the concentration of poliovirus in tap water, seawater, waste water effluent, and anaerobic sludge. This study has essentially shown that enteroviruses (poliovirus types 1, 2, and 3, coxsackie B3, and ECHO 1 and 4) can be recovered from seawater or tap water by elution and organic flocculation using 1% NFDM in 0.05 M glycine.

Elution with NFDM is most efficient at pH=9.0 and this is an advantage over methods which use glycine buffer at pH=11.5 due to the virucidal effect of such a pH.

Elution and organic flocculation with technical casein, purified casein, isoelectric casein, NFDM, or beef extract resulted in high overall recoveries (51% to 83%) of poliovirus type 1 from seawater. Results obtained here are in agreement with those reported by Katzenelson (1977), pertaining to the recovery of poliovirus from seawater using the beef extract method.

High overall recoveries (72% to 81%) of both high and low quantities of poliovirus type 1 were recovered from tap water using the NFDM technique.

The use of the NFDM technique resulted in the efficient elution of (71% to >100%) of six enterovirus from membrane filters. Problems were encountered during the organic flocculation step where low recoveries of ECHO 1 and 4 (10% and 43%, respectively) were observed.

Of the ten eluents investigated for desorption of viruses from marine sediments, 4M urea-0.05M lysine was found to be the most efficient.

The methods develop in the course of this study were successful in the recovery of indigenous enteroviruses in estuarine water and sediment.

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