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BENTONITE CLAY ADSORPTION PROCEDURE FOR CONCENTRATING ENTEROVIRUSES FROM WATER

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A method of adsorbing enteroviruses to bentonite clay was developed for use as a concentration technique designed to sample low levels of virus in environmental and tap waters. A divalentcation such as calcium was required to enhance the adsorption of large quantities of poliovirus onto bentonite within a 20 minute contact period. A minimum bentonite level of 50 mg/L was necessary to adsorb the virus and to still allow efficient recovery and reasonable sample size collection onto Milipore™ AP25 prefilters. Virus elution from the clay concentrated on the AP25 prefilters was most efficient with a solution mixture containing glycine (0.1 M), EDTA (0.01 M), and 1% bovine serum at pH 11, although tryptose phosphate broth at pH 7.2 was favored for field sampling with long field storage or shipping requirements. A water pH range of 4-10 did not significantly alter the efficiency of virus recovery. Although best recovery was obtained from highly polished water, good recoveries were also found for tap and wastewater with virus recoveries of 45-85%. The technique was found to be very versatile and could be easily scaled up for larger sample volumes without a loss in recovery efficiency. (continued)

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NOTICE

Note

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19. (Abstract continued)

The method could effectively recover virus concentrations of 10 PFU/L. Further tests should be done to determine if efficiency remains high at very low virus levels.

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Bentonite Clay Adsorption Procedure for Concentrating Enteroviruses from Water

SUMMARY

A method for adsorbing virus to bentonite clay was developed for use as a concentration technique designed to sample low virus levels in environmental waters. A divalent cation such as Ca^{++} was needed for the adsorption of large quantities of poliovirus within a 20 minute contact period. A minimum clay concentration of 50 mg/L was necessary to effectively adsorb the virus and to allow efficient recovery during the elution phase. Virus elution was most efficient with a solution containing a combination of glycine (0.1 M), EDTA (0.01 M), and 1% bovine serum at pH 11, (abreviated GES in the text) although tryptose phosphate broth at pH 7.2 was favored for field samples with long storage requirements.

A major advantage of the bentonite technique was that a pH range of 4 to 10 did not significantly alter the efficiency of virus recovery. Although recovery from highly polished water was greatest, good recoveries were also obtained from tap water and wastewater.

The technique was found to be very versatile and could easily be scaled up or down, depending on the sample volume. The technique can be used without modification for a variety of waters and can detect virus at concentrations less than 10 PFU/L. For all test waters, overall virus recovery ranged from approximately 45 to 85%.

INTRODUCTION

Since water was first implicated in the transmission of paralytic poliomyelitis and infectious hepatitis, health professionals have been interested in measuring viruses in environmental, potable, and wastewaters. Many of the early methods were qualitative in nature and relied upon inoculation of the contaminated water sample into nonhuman primates or rodents and then scoring for symptomatic illness or death. Gauze pads were used to concentrate viruses, which were then eluted and processed for animal inoculation. The advent of tissue culture methods in the late 1950's allowed direct virus analysis[1] by cell cytopathology or plaque assay.

Numerous attempts have been made during the past fifteen years to efficiently concentrate enteric viruses from water in a quantitative manner.[2-5] This was necessary because virus concentrations in environmental waters are generally too low (except in raw sewage) to make direct virus assays practical. Therefore, many methodologies that have evolved utilize the technology of concentrating viruses from large volumes of water into small volumes for subsequent assays. Many environmental virologists and public health experts agree that, even if the efficiency of the virus concentrating technique is high (yielding good recoveries), a practical target sample volume of potable water for the detection of viral pathogens would be between 100 and 1000 liters. At the present time, the precise number of viable enterovirus particles constituting an infectious dose by oral or respiratory routes in humans is not known. Until this question is resolved, a conservative approach has been taken to estimate what constitutes a "safe" level of virus. Further delineation of virus standards for drinking and recreational water and for wastewater effluent discharge cannot be made at this time.

A number of virus concentration techniques have been developed for large volumes of water.[6-13] Since most of these techniques concentrate viruses by membrane filtration and are used to sample highly polished drinking water, they may not be suitable for the assay of other types of water, such as surface water or wastewater. High turbidity and pH, as well as the presence of soluble organics, may interfere with the concentration and recovery of viruses. In addition, most concentration techniques do not measure virions adsorbed to suspended particles (natural turbidity), which may constitute a significant proportion of the virus population.[14]

This report presents the development of a unique method of concentrating viruses from water which relies on the adsorption of the virion to particles of bentonite (montmorillonite) clay, separation of the clay-adsorbed virions, and the elution of virus

with a small volume of medium. The basic concept was developed by the senior author and others[15-17] in their efforts to determine the nature and fate of viruses in the environment. Earlier studies by Schaub and Sagik[14] demonstrated that virus adsorption to clay, detritus, and other materials was very common in the water environment. The basic phenomenon obeyed the Freundlich adsorption isotherm equations and depended on pH, ionic strength, the size and quantity of particulates, and other colloid stability factors.[18]

The objective of the present study was to determine the feasibility of using a bentonite clay adsorption method for the quantitative recovery of enteric viruses from various types of water. Our primary concern was to develop a technique that was versatile, easy to use, inexpensive, flexible in sampling scale, and effective for a broad spectrum of enteric viruses.

MATERIALS AND METHODS

Virus Preparation and Assay

Poliovirus I (L-Sc-1 strain), echovirus 7, and coxsackievirus A9 and B3 were grown and assayed on the Buffalo Green Monkey Kidney (BGM) continuous cell line[19] kindly supplied by G. Berg, U.S. Environmental Protection Agency (USEPA), Cincinnati, Ohio. Stock virus was prepared in monolayer cultures incubated in an atmosphere of 5% CO_2 in air at 35° C for 15 hours using Medium 199 supplemented with 10% fetal bovine serum and 50 μg/mL Gentamycin. The culture fluids were freeze-thawed three times and centrifuged at 12,100 x g for 10 minutes. The supernatant fluid was filtered through a 0.22 μm membrane filter, and aliquots were frozen at -80° C. Columbia SK (Col SK) virus was grown in mouse L cell monolayers with Eagle's Minimal Essential Medium (MEM) supplemented with 5% fetal bovine serum and 50 μg/mL Gentamycin.

Plaque assays were used to measure viruses in all samples. A confluent monolayer of BGM cells was infected with the sample for 90 minutes and was overlayed with Ion Agar (1%) with the appropriate culture medium containing 5% fetal bovine serum, protamine sulfate (0.2 mg/mL), Gentamycin (100 μg/mL), and Kanamycin (100 μg/mL). Poliovirus and Columbia SK (COL SK) virus plaques were counted after 48 nours of incubation, whereas echovirus 7 and Coxsackievirus A9 and B3 plaques were counted after three days of incubation. Neutral red solution (1:10,000) was added to the solid overlay for several hours and was removed prior to plaque counting. Mycostatin, at a concentration of 100 units/mL, was added to all plaque media used for the determination of viruses in wastewater. Indigenous virus samples were assayed on BGM monolayers, using the same media constituents as required for poliovirus, and plaques were counted after three days.

General Procedures for Virus Concentration

Water

Distilled, deionized water was prepared by steam distillation and mixed-bed deionization followed by sterilization at 121° C for 30 minutes. The chlorine in tap water was neutralized with sodium thiosulfate (0.05 M) until no residual available chlorine was detected by the DPD test or by amperometric titration. Domestic primary or secondary (trickling filter) unchlorinated wastewater was obtained from the Pusey treatment plant at Edgewood Arsenal (Maryland) or the U.S. Army Medical Bioengineering Research and Development Laboratory (Fort Detrick, Maryland) pilot treatment plant.

Clay Adsorption

Batches of various test waters (100 mL) were prepared in screw-capped milk dilution bottles, with the pH adjusted between 6 and 7 with 0.01 M HCl or NaOH. Virus was added from stock solutions to the required concentration, followed by emulsified USP grande bentonite clay (Fisher Scientific) and calcium chloride solution prepared from $CaCl_2.2H_2O$ at the standard or experimental concentrations. The addition of divalent cation evidently serves as a "cross complexing agent" between the negatively charged bentonite and virions (Kessick and Wagner 1978).[20] The standard bentonite and $CaCl_2$ concentrations for the initial studies were 100 mg/L and 0.01 M, respectively. Virus was allowed to adsorb for the experimental or standard time period (20 min) with intermittent slow stirring or shaking.

The bentonite clay used in this study is a three-layer expanding montomorillonite clay with the general formula of $Al_2O_3SiO_2.H_2O$. It has an average particle size of less than 2 μm, in a platelet-like configuration which provides a very large surface area. It has an isoelectric point near pH 3.

Concentration

Bentonite-adsorbed virus complex was concentrated by centrifugation at 12,100 x g for 10 minutes in a Sorvall SS34 (angle head rotor) or by filtration through 47mm, 142mm, or 293mm Millipore AP25 fiberglass prefilters which are designed as bulk prefilters for use with 0.8 to 8.0 μm membrane filters and do not have a nominal pore size rating. In earlier studies, Gelman GN-6 membranes with 0.45 μm pore size were used. The amount of unadsorbed virus was determined by plaque assay of supernatant fluids from centrifugation or filtration.

Virus Elution and Efficiency of Recovery

Tryptose phosphate broth (Difco) at pH 7.2 was prepared in deionized water. The glycine-EDTA eluent, with or without serum, was prepared in deionized-distilled water at a concentration of 0.1 M glycine and 0.01 M EDTA; the solution was adjusted to pH 11 with 1 M NaOH. Initially, the elution volume used for virus recovery for 47 mm filters was 5 mL, and the contact time was 5 minutes. The volume was later reduced to 2 mL. The eluted virus suspension was collected under vacuum in graduated, conical centrifuge tubes placed under the filter funnel inside the filter flask. The pH of the eluent was immediately adjusted to 8 before plaque assay or storage. For virus elution from larger AP25 filters (142 mm or 293 mm), the top pressure plate of the filter housing was removed, and the eluent was pipetted directly onto

the filter and allowed to stand for 5 minutes. The eluent was then recovered by vacuum as described above.

Effect of Suspended Solids on Virus Recovery

Poliovirus, to provide 10^4 plaque forming units/mL (PFU/mL), was added to 1 liter of secondary treated wastewater from a trickling filter system, and the solution was stirred slowly at 20° C. At 0, 5, 15, 30, 60, and 90 minutes, 100-mL aliquots were taken and centrifuged in polypropylene bottles in a Sorvall RC-2B centrifuge at 4000 rpm for 5 minutes. The procedure is outlined in a flow diagram (Figure 1). Fluid was decanted, and 1 mL of supernatant was assayed for virus (centrifuge supernatant). The remaining fluid was mixed with 8 mg of bentonite and $CaCl_2$ was added to a concentration of 0.01 M. After 20 minutes the suspension was filtered through a 47 mm AP25 prefilter. The bentonite-virus complex was treated with an eluent mixture containing 2 mL of glycine (0.1 M), EDTA (0.01 M), fetal bovine serum (1%) (GES), at pH 11 for 5 minutes, and the eluate was drawn through the filter by vacuum. The pH was adjusted to 8, and the fluid was assayed for virus (bentonite supernatant).

The centrifuged pellet (above) was resuspended by titration in 99 mL of filtered (0.45 μm) wastewater from the same source. One mL of this suspension was assayed for virus (centrifuge pellet), and the remainder was divided into two equal portions. One-half of the material was concentrated by the bentonite-$CaCl_2$ standard procedure (bentonite pellet), and the second half was simply passed through an AP25 prefilter and eluted with GES (control pellet). All samples were assayed for virus by the plaque method, and the efficiency of concentration was determined as a percentage of the original virus titer which was taken from the large batch prior to the centrifugation of each sample.

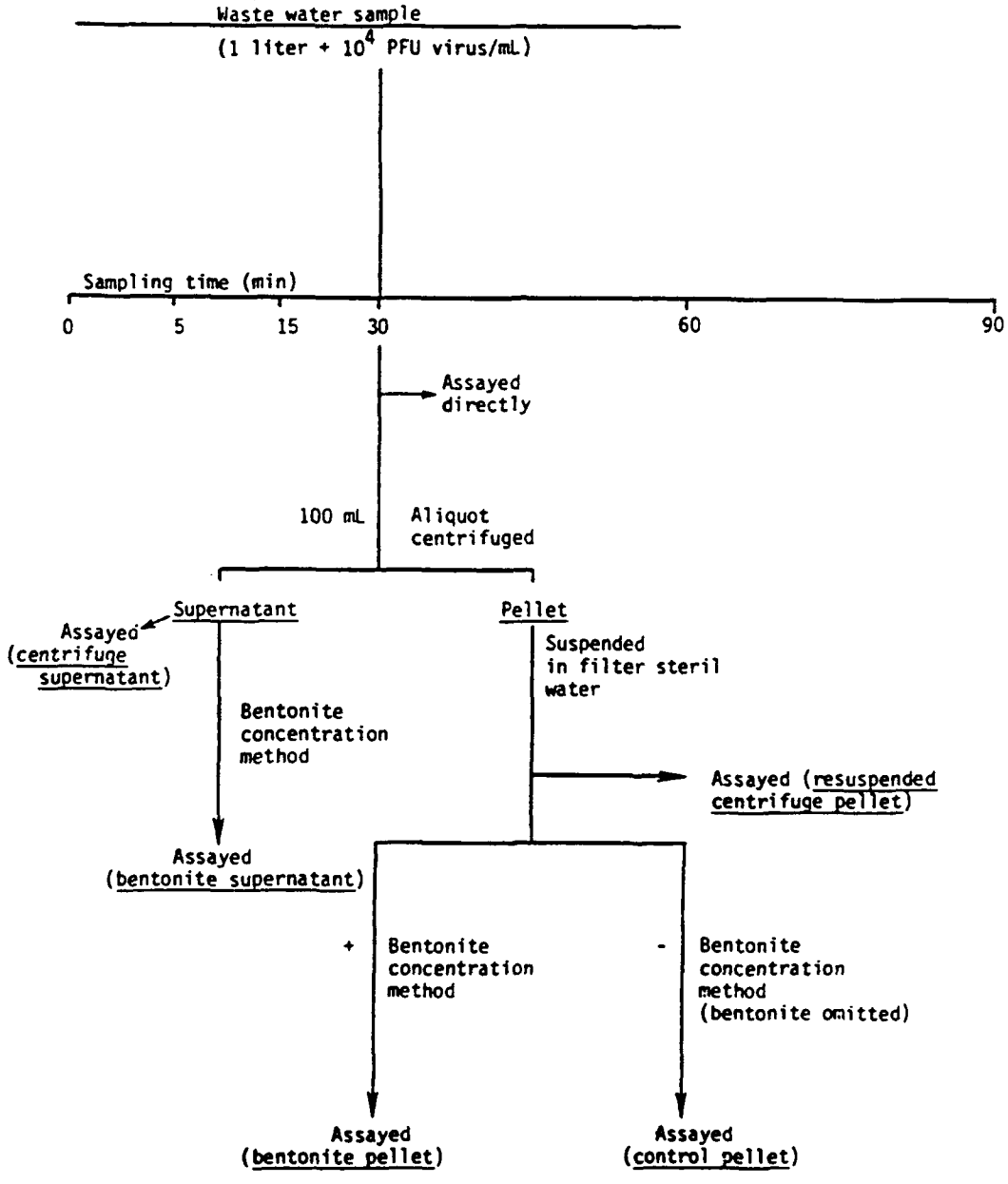

Figure 1. Flow diagram showing the procedure to study the effect of suspended solids on bentonite virus concentration method

RESULTS

The Effect of Cation Concentration on Adsorption

In these tests Col SK virus and poliovirus were added to various types of test water samples, followed by bentonite and monovalent (NaCl) or bivalent ($CaCl_2$) cations over a range of concentrations. Preliminary tests were performed to establish the general range of cation concentration required to obtain the maximum possible virus association with bentonite. After a 30 minute contact time (20 minutes adsorption period and 10 minutes centrifugation) the bentonite-associated virions were separated, and the supernatant fluids were assayed for residual virus. In the presence of Ca^{++} ions the adsorption of virus to bentonite became independent of cation concentrations above 0.001 M; the adsorption efficiency was similar with deionized water, tap water, or primary wastewater effluents (Figure 2). When Na^{+} was used as the cation, the adsorption of Col SK virus was less efficient at 0.001 M and 0.01 M than at 0.1 M. The virus adsorption to bentonite in the presence of NaCl required a nearly 100-fold higher molar concentration than with $CaCl_2$ to achieve the same level of virus adsorption. Maximum adsorption of viruses from deionized, tap, and primary effluent waters occured at a bivalent cation concentration of 0.01 M. Based on these studies, 0.01 M $CaCl_2$ was selected as the standard cation concentration, because it was well within the maximum range of the adsorption curve for Col SK (> 0.001 M), and because that level allowed the maximum adsorption of poliovirus from sewage.

Virus Adsorption Kinetics

The data shown in Figure 3 illustrate the kinetics of adsorption of poliovirus to USP grade bentonite (100 mg/L) in various types of water to which 0.01 M $CaCl_2$ had been added. After 5 minutes, only 50% of the virus from sewage effluents was adsorbed to bentonite compared to 81 - 85% adsorption to bentonite from tap or deionized water samples. After 20 minutes, however, more than 90% of the virus had adsorbed from sewage effluents and other water samples. There was no substantial increase in adsorption in these three types of water between 20 and 30 minutes of the study. Flocculation and settling of bentonite began occurring after 20 minutes in all of the samples when left undisturbed. Because very little additional virus adsorption occurred after 20 minutes, this time period was adopted as the standard time of adsorption for the remaining experiments.

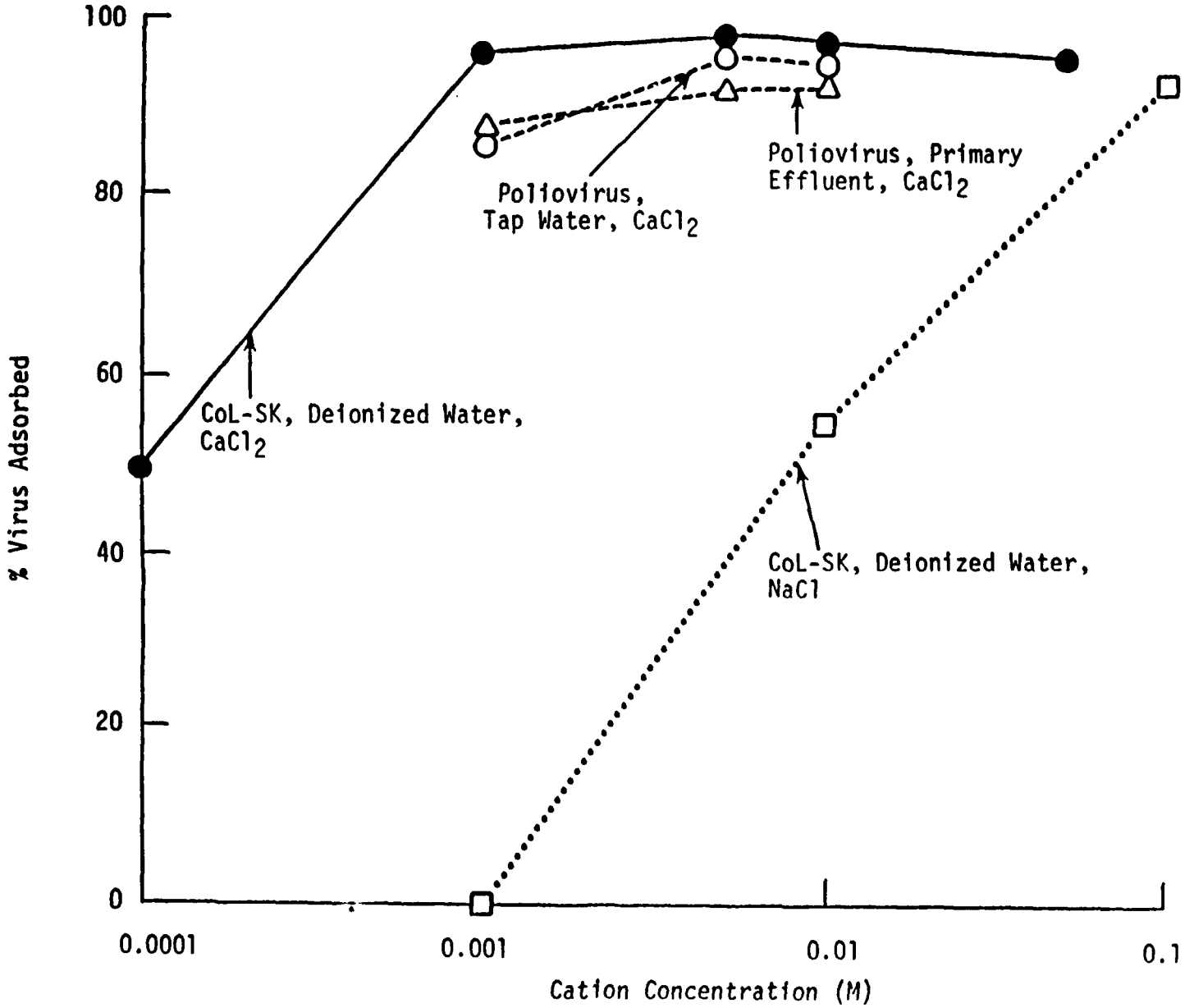

Figure 2. Enterovirus adsorption to bentonite at various cation concentrations

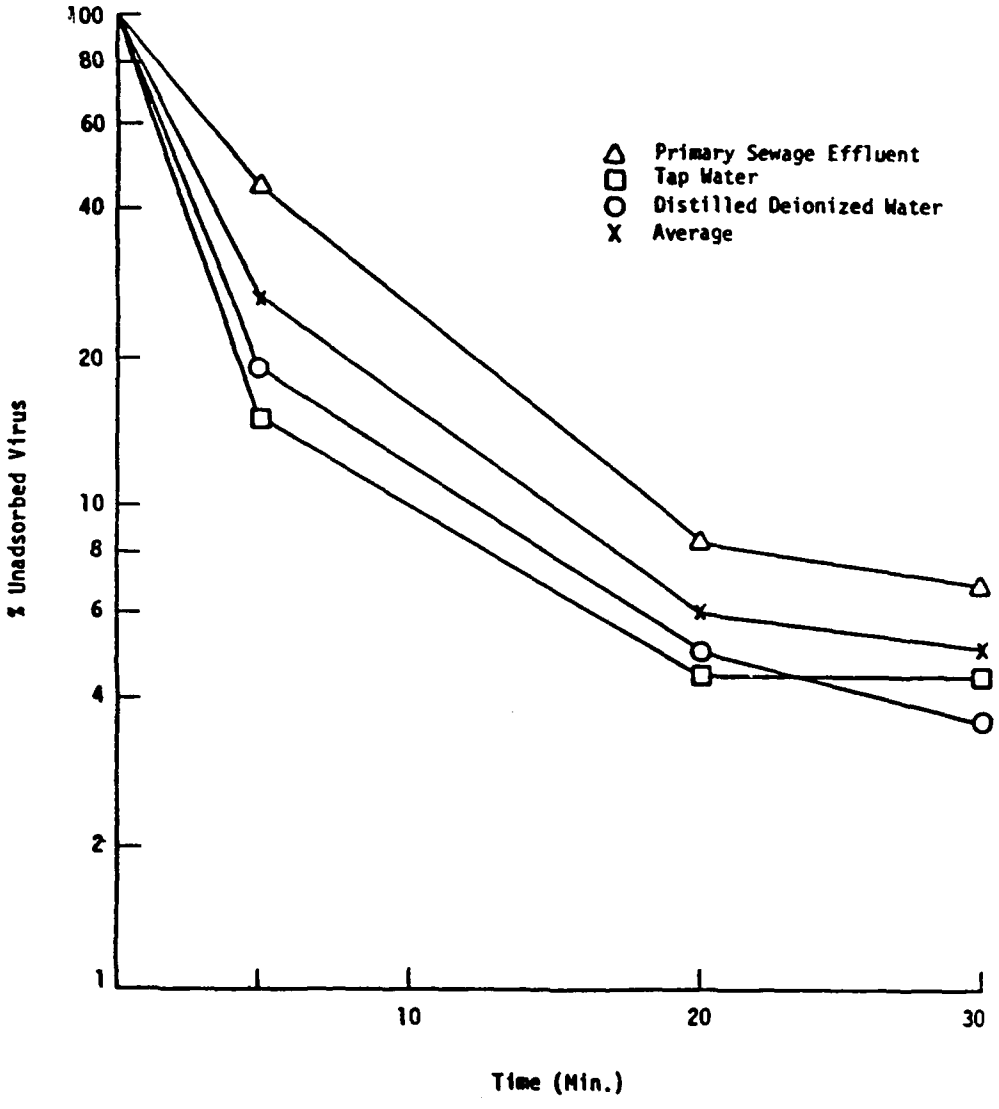

Figure 3. Kinetics of poliovirus adsorption to bentonite

Concentration of Bentonite-adsorbed Virus

Two methods were compared for their efficiencies of concentrating the bentonite-virus complex: filtration and low speed centrifugation. Both methods are simple and practical and would be generally available in most public health or field laboratories.

Our data (Table 1) show that filtration with AP25 fiberglass prefilters (samples 5-7) is more efficient for concentrating bentonite-associated poliovirus than centrifugation (samples 1-4). The percentage of the virus concentrated was calculated from the difference between virus titer in filtrate (or supernatant) and total original sample. The values shown in the table represent the amount of virus associated with the clay and filter (or centrifuged pellets). In the filtration method, 23.6% of the polio virus and 8.4% of Col SK were retained by the prefilter in the absence of cation and clay (Sample 8). The addition of 0.01 M Ca^{++} increased this value 4-fold for poliovirus in deionized and tap water and 2-fold for sewage effluents (samples 9-11). In the case of Col SK virus this increase from Ca^{++} was over 86% in distilled water. The addition of 100 mg/L of bentonite increased the efficiency of concentration to a mean value of nearly 99% (samples 5-7), and the type of water had no major effect.

Determination of Minimum Bentonite Concentration

Because the AP25 prefilters themselves adsorbed significant amounts of poliovirus in the absence of bentonite (Table 1, sample 8), experiments were conducted to determine the minimum bentonite concentration that would provide a high level of virus adsorption. The goal was to optimize virus concentration without unnecessarily overloading the filters. Poliovirus was adsorbed to various concentrations of clay under standard conditions. After centrifugation at 12,100 x g for 5 minutes, the supernatant fluids were assayed for virus, and the amount adsorbed was determined by difference from the initial titer. The mean values for virus adsorption from three types of water are shown in Table 2.

TABLE 1. CONCENTRATION OF VIRUSES FROM VARIOUS TYPES OF WATER USING BENTONITE ADSORPTION

Sample No.	Water Type	Treatment $CaCl_2$	Bentonite	Separation Process	% Concentration of Seeded Viruses* Columbia SK	Poliovirus A
1.	Distilled-Deionized	10^{-3}M	100 mg/L	Centrifugation @ 12,100 x g for 5 minutes	84.5	—
2.	Distilled-Deionized	10^{-2}M	100 mg/L	Centrifugation @ 12,100 x g for 5 minutes	90.9	93.5
3.	Tapwater	10^{-2}M	100 mg/L	Centrifugation @ 12,100 x g for 5 minutes	—	93.1
4.	Primary Sewage Effluent	10^{-2}M	100 mg/L	Centrifugation @ 12,100 x g for 5 minutes	—	80.1
5.	Distilled-Deionized	10^{-2}M	100 mg/L	Filtration on Millipore AP25 Fiberglass Prefilters	98.8	99.9
6.	Tapwater	10^{-2}M	100 mg/L	Filtration on Millipore AP25 Fiberglass Prefilters	—	99.8
7.	Primary Sewage Effluent	10^{-2}M	100 mg/L	Filtration on Millipore AP25 Fiberglass Prefilters	—	98.8

TABLE 1. (continued)

Sample No.	Water Type	Treatment $CaCl_2$	Bentonite	Separation Process	% Concentration of Seeded Viruses* Columbia SK	Poliovirus A
8.	Distilled-Deionized	None	None	Filtration on Millipore AP25 Fiberglass Prefilters	8.4	23.6
9.	Distilled-Deionized	10^{-2}M	None	Filtration on Millipore AP25 Fiberglass Prefilters	90.2	95.8
10.	Tapwater	10^{-2}M	None	Filtration on Millipore AP25 Fiberglass Prefilters	—	93.2
11.	Primary Sewage Effluent	10^{-2}M	None	Filtration on Millipore AP25 Fiberglass Prefilters	—	47.5

* All water samples were seeded with 10^4 PFU/ml viruses. Standard elution medium and assay procedures as outlined in the materials and methods were used.

TABLE 2. EFFECT OF BENTONITE CONCENTRATION ON THE ADSORPTION OF POLIOVIRUS[a]

Bentonite Concentration (mg/L)	% Poliovius Adsorbed to Bentonite[b]	Remained in Supernatant
0	34 (31-37)[c]	66
25	54 (50-57)	46
50	81 (74-87)	19
75	84 (82-87)	16
100	89 (85-91)	11

[a] Virus (10^4 PFU/mL) were exposed to various concentrations of bentonite in 100 mL water samples (distilled deionized, tap water or primary sewage-effluents) under standard conditions. After 20 minute contact time 10 mL aliquots were centrifuged (12,100 x g for 5 min) and supernatants were assayed.

[b] Calculated by difference between the virus titer in supernatant and total mixture

[c] Mean (range) of 3 water types.

Approximately 34% of the viruses were removed even without bentonite, probably due to adsorption to glass surfaces and natural turbidity components, especially in the sewage effluents. Bentonite at a concentration of 50 mg/L adsorbed 81% of the added virus, and concentrations up to 100 mg/L only provided an additional 8% adsorption. Thus it appeared that 50 mg/L was an optimum concentration of bentonite that could be used for the virus recovery from a broad range of unknown water samples.

Elution of Concentrated Virus

We had determined previously that depth filters such as the AP25 could not be backwashed easily to free virus or clay particles. Membrane filters could be backwashed but clogged too rapidly to be effective. Other filter media, such as 1 μm (nominal porosity) wool filter bags, and filter beds of sand, glass, or diatomaceous earth, did not retain clay-adsorbed virus as effectively as AP25 filters. Therefore, we investigated methods for eluting virus most efficiently from bentonite on depth filters.

Samples of distilled-deionized water, tap water, or primary sewage effluent (100 mL) containing 10^4 PFU/mL of poliovirus were adsorbed with bentonite under standard conditions. The suspensions were filtered through 47 mm AP25 prefilters, and 5 mL

of various eluents were added to the residue on the filter. After a 5 minute contact period, the eluents were collected by vacuum filtration and assayed for virus. The eluents used were distilled-deionized water containing fetal calf serum (pH 7); glycine (0.1 M)-EDTA (0.01 M), pH 11, with or without fetal calf serum (GES); and tryptose phosphate broth, pH 7.2 (Table 3).

Satisfactory recovery was obtained (68.1%) when glycine-EDTA was used as eluent, and the addition of 1% serum increased the recovery to 75.2% (Table 3). Addition of 10% serum, however, caused excessive foaming and gave lower recoveries. Virus elution was most efficient from the distilled water sample and least efficient with primary sewage effluent. The results with the tap water were intermediate (except for glycine-EDTA-1% serum eluent which gave the virus recovery of 90%).

GES was selected as the standard eluent for further experiments. However, tryptose phosphate broth was used (instead of GES) in field trials, because pH adjustments were not required. This broth was also preferred because it afforded long-term storage stability under field conditions, due to its protein content and good buffering capacity.

Effect of the Amount of Bentonite on Virus Recovery

The minimum amount of bentonite required in association with the AP25 filters for maximum poliovirus recovery from 3 types of water samples was determined using GES as eluent. Poliovirus was experimentally adsorbed to various amounts of bentonite for 20 minutes under standard conditions in 100 mL of each water type. They were filtered through AP25 prefilters, virus was eluted by GES, and the eluates were assayed.

Approximately 27% of the virus was recovered from the filters alone (Figure 2), and the inclusion of as little as 25 mg/L of bentonite increased recovery to 56%. The use of 50, 75, or 100 mg/L of bentonite increased virus recovery to a maximum of approximately 70%. The total virus recovery curve at various bentonite levels is similar in shape to the curve obtained for adsorption of virus to bentonite (Figure 4). Fifty mg/L of bentonite appeared to be the minimum amount needed for efficient virus recovery from a spectrum of waters; it was also the optimum concentration for virus adsorption to the clay.

TABLE 3. RECOVERY OF VIRUS FROM BENTONITE USING VARIOUS ELUENTS

Eluent	% Recovery of Viruses From Distilled-Deionized Water	Tap Water	Primary Effluent	Average Recovery All Samples
Distilled-deionized H_2O, 10% Fetal Calf Serum, pH 7.0	12.8	-	-	-
Glycine (0.1M), EDTA (0.01M), pH 11	82.3	64.3	57.7	68.1
Glycine (0.1M), EDTA (0.01M), 1% Fetal Calf Serum, pH 11	85.3	90.0	49.3	75.2
Glycine (0.1M), EDTA (0.01M), 10% Fetal Calf Serum, pH 11	52.6	--	--	--
Tryptose Phosphate Broth, pH 7.2	62.4	55.1	43.1	53.5

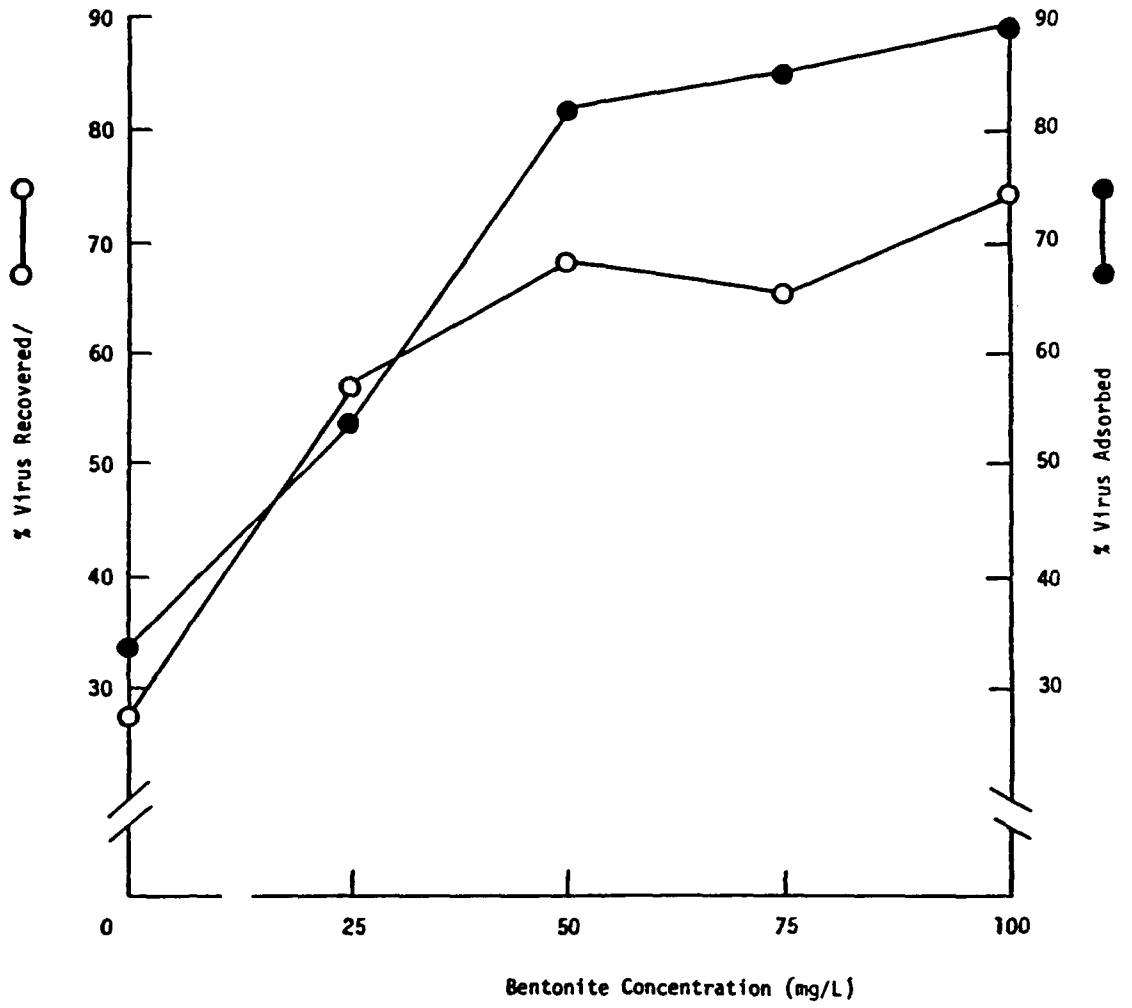

Figure 4. Effect of bentonite concentration on the adsorption and recovery of poliovirus

Effect of pH on Virus Recovery

Since the pH range of various types of natural water and wastewaters varies considerably, we determined the effect of pH during adsorption on the recovery of viruses from bentonite. Poliovirus was added to 100 mL samples of distilled-deionized water (10^4 PFU/mL) containing the standard bentonite and $CaCl_2$, but the pH was adjusted to 4, 6, 8, or 10. The suspensions were filtered and processed in the usual manner. The amount of virus in the filtrate and the amount recovered by elution with GES were both measured, and these data are shown in Table 4.

TABLE 4. POLIOVIRUS ADSORPTION TO BENTONITE AS A FUNCTION OF pH

	% of Virus at pH of Adsorption Mixture			
Sample	pH 4	6	8	10
Filtrate	0.06	1.1	1.3	2.0
Eluate (recovery)	49.00	64.0	57.0	55.0

The pH of the water samples appears to have little effect on the extent of adsorption (retention) of virions to bentonite and filters. Although the adsorption of virus at pH 4 was considerably more (99.94%) than at pH 10 (98%), the virus titers in the filtrate show (Table 4) that the leakage of unadsorbed viruses through AP25 filters was minimal and reached a maximum loss of only 2% at the higher pH. Virus recovery in these experiments was slightly higher at pH values near neutrality, but recovery varied little over the pH range of 4 to 10, indicating that precise pH monitoring and adjustment of typical environmental water samples is probably not necessary in this procedure.

Effect of Suspended Solids in Wastewater on Poliovirus Recovery

Wastewater obtained from a secondary treatment (trickling filter) plant was used as a typical "worst" example of environmental water to determine the effect of organic suspended solids on the bentonite concentration method. Poliovirus was added to 1 liter of wastewater at a concentration of 10^4 PFU/mL, and 100 mL aliquots were centrifuged at timed intervals from 5 to 90 minutes. A 1 mL aliquot of the supernatant fluid was used for a plaque assay (centrifuge supernatant) and the remainder was concentrated by the bentonite procedure. The virus level in the eluate from this procedure was also measured (bentonite supernatant). The pellet obtained from centrifugation was resuspended in 99 mL of wastewater, (sterilized by filtration with 0.45 μm Millipore filters) and assayed for virus (centrifuge

pellet). The remainder was divided into two equal fractions. One fraction was processed by the bentonite adsorption method (bentonite pellet) while the other fraction was processed without bentonite (control bentonite pellet). All samples and fractions were assayed for virus by the plaque method (Figure 1 and Table 5).

The results (Table 5) show that the added poliovirus did not adsorb appreciably to the solids in the wastewater over the 90 minute period. Approximately 96% of the input virus remained in the supernatant (sample 1). This unadsorbed virus from the centrifuge supernatant was recovered by the bentonite procedure with an efficiency of nearly 75% (sample 3). Only about 4% of the input virus was found in the centrifuged pellet (sample 2), and less than 25% of this virus in the sediment could be recovered by the bentonite method (sample 4). Recovery of virus from the resuspended pellet after AP25 filtration alone (without bentonite, sample 5) was variable, averaging only 0.16% of the input virus (<4% recovery of sedimented virus).

Concentration of Other Enteroviruses

The bentonite virus concentration method was examined for its efficiency to concentrate and recover other enteroviruses from water. ECHO 7, Coxsackie A9 and B3, encephalomyocarditis (EMC), and polio I (vaccine strain) viruses at 10^4 PFU/ml were concentrated from 100 mL distilled-deionized water samples using 70 mg/L bentonite and 0.01 M $CaCl_2$. The clay-virus complex was collected onto the 47 mm AP25 filters. The viruses were then eluted with 2 ml of the GES (glycine [0.1 M], EDTA [0.01 M], with 1% calf serum). All viruses in the eluent were assayed on BGM cells except for EMC virus whish was assayed on mouse L cell monolayers. In most cases, more than 95% of the irput virus was adsorbed by the bentonite (Table 6). Approximately 70% of each virus could be eluted with GES from bentonite. With the Coxsackie A9, however, recovery was 48%.

TABLE 5. PERCENT POLIOVIRUS RECOVERY FROM WASTEWATER CONTAINING SUSPENDED SOLIDS

No.	Sample[a]	Time in Minutes 0	5	15	30	60	90	Mean ± S.D.
1.	Centrifuge Supernatant	105.0*	96.0	74.0	102.0	101.0	98.0	96.0 ± 10.2
2.	Centrifuge Pellet	3.5	4.0	4.8	6.0	3.4	3.4	4.2 ± 0.9
3.	Bentonite Supernatant	84.0	82.0	80.0	65.0	72.0	66.0	74.8 ± 7.6
4.	Bentonite Pellet	0.84	0.84	0.84	0.65	0.75	0.82	0.79 ± 0.007
5.	Control Pellet (no bentonite)	0.11	0.26	0.11	0.066	0.15	0.27	0.16 ± 0.08

[a] See Flow-diagram (Figure 2) for the description of these sample fractions.

* These numbers indicate percent poliovirus recovery.

TABLE 6. CONCENTRATION AND RECOVERY OF VARIOUS ENTEROVIRUSES BY THE BENTONITE PROCEDURE

Virus Type	Concentration of Bentonite Adsorbed Virus (%)	Recovery of Virus Eluted From Bentonite (%)
Echovirus 7	97.3	75.2
Coxsackievirus A-9	99.5	48.4
Coxsackievirus B-3	93.8	69.1
EMC Virus	99.8	74.0
Poliovirus I (vaccine)	99.4	72.0

Concentration of Virus from Larger Sample Volumes

In most environmental studies, samples larger than 100 mL may be needed to meet virus detection requirements. Therefore the bentonite concentration procedure was scaled up from 47 mm prefilters for 100 mL of water sample to 142 mm and 293 mm AP25 prefilters for larger sample volumes. To test filter capacity and to determine the maximum sample volume that could be effectively processed, water samples containing 100 mg/L of bentonite were drawn through the three sizes of prefilters by vacuum, until the flow of filtrate was reduced to a separated stream (fast drip). For each increase in prefilter size, the sample volume that could be filtered also increased approximately 10-fold (Table 7).

TABLE 7. CONCENTRATION OF BENTONITE-ADSORBED POLIOVIRUS ON MILLIPORE AP25 FLAT FIBERGLASS PREFILTERS* FROM DISTILLED-DEIONIZED WATER

Filter Diameter (mm)	Effective Surface Area (cm^2)	Maximum Sample Vol. (L)	Minimum Required Eluent Vol. (mL)	Sample Concentration
47	9.6	0.36	2	180 fold
142	138.9	3.80	20	190 fold
293	576.8	40.10	100	400 fold

* Millipore AP25 used in appropriate funnel or pressure holders, filtered by negative pressure (laboratory vacuum).

As the prefilter diameter was increased, a larger volume of GES eluent was needed to elute adsorbed virus (Table 7). In order to determine this eluent volume more precisely, one-liter samples of distilled-deionized water containing 10^2 PFU/mL of poliovirus were adsorbed with 70 mg/L of bentonite under standard conditions and filtered through a 142 mm AP25 prefilter. Different volumes of eluent were added to the filter surfaces and recovered by vacuum filtration after 5 minutes. Assay of the filtrates showed that virus recovery increased linearly as eluent volume increased (Table 8). A recovery of 81% was obtained with an eluent volume of 20 mL, the maximum volume that could be retained on the surface of this filter without spilling over the sides. Although a second eluent volume could have been used, virus concentration would have been decreased significantly with only minor improvement in total recovery. Linear regression analysis of the data summarized in Table 8 indicated that the theoretical eluent volume needed to recover 100% of the virus was 24.08 mL ($r = 0.98$); these data are significant at the 0.05 level. The maximum sample volume for a 293 mm filter was determined to be 40.1 liters, and the maximum eluent volume for elution was 100 mL. The virus concentration factors resulting from these procedures ranged from 180- to 400-fold (Table 7).

TABLE 8. EFFECT OF ELUENT VOLUME ON RECOVERY OF BENTONITE ADSORBED POLIOVIRUS FROM 142 MM AP25 PREFILTERS

Eluent Volume (mL)	Virus Recovered (% of Total)
7.5	22
10.0	46
15.0	60
20.0	81

Effect of the Amount of Poliovirus on Concentration and Recovery

Because the amount of virus found in surface and ground waters is generally very low, we determined the effectiveness of the bentonite procedure on different types of water containing poliovirus at concentrations of 10^2 to 10^{-1} PFU/mL. The standard procedure was used with 100 mL water samples which were processed through 47 mm prefilters. The results (Table 9) suggest that poliovirus was recovered more efficiently from water than from sewage, but the differences may not be significant when comparison is made between tap water and sewage. The initial virus concentration had no appreciable effect on the efficiency of recovery. The variations observed are inherent in the experimental procedure itself (extrapolation of virus

TABLE 9. LOW LEVEL POLIOVIRUS RECOVERY*

Test Water	Sample Concentrations (PFU/mL) 10^2	10^1	10^0	10^{-1}	Mean ± S.D. (range)
Distilled-deionized					
Test # 1	107**	138	74	***	
Test # 2	86	78	46	63	84.6 ± 28 (46 - 138)
Tap Water					
Test # 1	34	29	50	154	
Test # 2	83	58	48	**	65.1 ± 40 (29 - 154)
Secondary Treated Wastewater					
Test # 1	70	42	41	71	
Test # 2	35	28	30	54	46.4 ± 16 (28 - 71)
Mean ± S.D.	69.2 ± 27	62.2 ± 38	48.2 ± 13	85.5 ± 40	

* Virus was adsorbed to 100 mg/L bentonite clay and eluted under standard conditions.

** These numbers indicate percent poliovirus recovery from 100 mL samples.

*** Not tested.

quantitation at lower serial dilutions) as well as the plaque assay, especially with eluates containing very few virus particles.

Recovery of Indigenous Viruses from Wastewater

The bentonite procedure was tested with wastewater effluents from several treatment plants to determine its capability for recovering virus under natural conditions. Bentonite was added to sewage samples to a level of 70 mg/L of water and $CaCl_2$ at a concentration of 0.01 M. After the 20-minute adsorption period, the suspension was filtered through a 142 mm AP25 prefilter, and the virus was eluted with tryptose phosphate broth (pH 7.2). This eluent was used by necessity for our field trials, because its final pH was not excessively high and it was thus better suited for refrigerated transportation and long-term frozen storage (~20° C) of eluted virus. Eluent volume was 20 mL for study 1 and 18 mL for studies 2 and 3. The amount of virus recovered, shown in Table 10, is in general agreement with published literature values.[21-23] Turk et al.[24] recovered 74% of poliovirus I from seeded primary sludge eluates using the bentonite procedure. The assay system used in that study is estimated to detect approximately 40% of the enteroviruses actually present, based on the results here reported using several laboratory enterovirus strains.

TABLE 10. USE OF BENTONITE ADSORPTION TECHNIQUE FOR RECOVERY OF INDIGENOUS VIRUSES FROM WASTEWATER*

Study No.	Wastewater Type	Number of Samples	Virus Detected (PFU/L) Mean	Range
3	Raw	3	138	60-201
3	Primary **	5	45	20- 93
3	Secondary	5	28	13- 47
2	Primary ***	3	118	41-195
2	Secondary	2	15	15
1	Primary	7	219	110-300

* Elution was performed with tryptose phosphate broth, pH 7.2, eluent volume was 20 mL for study 1 and 18 mL for studies 2 and 3.

** Trickling filter plant.

*** Extended aeration, activated sludge plant.

(Wastewater sample composites - sampling period was 6 hours.)

DISCUSSION

This study shows that high recovery of enteroviruses from various types of water by adsorption to bentonite is feasible and that the method provides good concentration, elution, and overall recovery potential. Moreover, this method is simple, rapid, and ideal for field use as well as for public health laboratories

The method was conceived as an outgrowth of results obtained from the studies of Drewry and Eliassen (1968)[16] and Schaub and Sagik (1975),[14] who demonstrated the adsorption of small RNA viruses to clays and organic particulates in the presence of mono-, bi-, or trivalent cations. Columbia SK virus adsorbed to crude bentonite in distilled water (36 mg/L) in the presence of Ca^{++} ions (Schaub and Sagik 1975).[14] In the present study, the adsorption of enteroviruses to USP grade bentonite (a 3-layer expanding montmorillonite clay), and elution, were studied in detail.

Initial studies in this laboratory provided further confirmation that cations enhance virus adsorption to clay particles and also conformed to the Schultz-Hardy rule for flocculation and virus adsorption originally determined for viruses by Carlson et al. (1968).[15] Maximum adsorption of poliovirus or Columbia SK virus to clay particles in various types of water occurred at Ca^{++} ion concentrations in the range of 5×10^{-3} M to 1×10^{-2} M. Numerous experiments in this laboratory confirmed that a Ca^{++} ion concentration of 10^{-2} M resulted in maximum adsorption of either poliovirus or Columbia SK to bentonite particles. When cation and clay concentrations are constant, the rates at which viruses adsorb from various types of water are similar. Initially, the rate is very high, and a maximum is reached after about 20 minutes of mixing (contact time).

In these studies, centrifugation and filtration of clay-adsorbed virions were compared for their efficiency to concentrate viruses from water. Centrifugation was satisfactory but was not effective for processing large sample volumes, often necessary for water analyses. In addition, centrifugation is not acceptable for some field applications. Filtration was more effective and appropriate for wider applications.

Several types of filter media including membranes, sand, diatomaceous earth, powdered glass, and charcoal were tested (unpublished data). The membrane filters were very effective in concentrating viruses but clogged rapidly with flat clay particles or natural turbidity, so that very little water could be processed. The other filter media tested neither retained sufficiently large amounts of the clay-virus complex nor appreciably enhanced the volume of the sample that could be

filtered. The millipore AP25 fiberglass prefilter provided excellent retention of clay and had significant virus adsorption capacity in the absence of clay (Table 1). The clay retention of these fiberglass prefilters was superior to that of many of the filter media tested, and a reasonable sample volume could be processed. We tested only Millipore^{tm} prefilters, but this does not imply or suggest that fiberglass prefilters produced by other manufacturers would not be suitable.

The minimum concentration of bentonite required to provide a high degree (over 90%) of adsorption of the viruses in a water sample containing 0.01 M $CaCl_2$ was determined. Use of a minimal concentration of clay in this procedure is important since clay concentration determines the amount of sample suspension that can be filtered on a given size filter. Because the technique was primarily developed as an all-purpose virus sampling procedure, several natural and processed waters, including wastewater from treatment plant, dechlorinated tap water, and pure, high resistivity water (18 megohm) were tested.

The bentonite adsorption studies (Figure 4) showed that 50 mg/L was the minimum level required for adsorbing high levels of the virus from all types of water. Increasing the level of bentonite to 100 mg/L did not improve the efficiency of adsorption significantly. A concentration of 50 mg/L was also the optimum amount of bentonite required for efficient recovery of virus from bentonite-virus complex on the prefilters (Figure 4). The effective sampling capacity is reduced when bentonite concentration is increased. Therefore, for water samples containing few virions, doubling the sample size may be more effective than increasing the bentonite concentration. The method was intended as a general purpose one designed for the field assay of all water types. However, the concentration and recovery of viruses could probably be enhanced significantly for repeated sampling at a specific water source, through fine tuning, such as optimizing clay and cation concentrations, adsorption time, and pH.

A major advantage of the bentonite (adsorption) procedure is the ability to elute adsorbed virus into a small volume in a single-stage procedure, resulting in several hundred-fold concentration of virions. The principal conditions required for efficient elution are a maintenance of high pH, reduction of available cation concentration, and addition of soluble protein. The high pH increases the net negative charges on the clay and virions, thus they repel each other by disrupting the short range attractive van der Waals force. Reducing cation concentration, by complexing with EDTA, weakens its neutralization of the net negative surface charges of virus and clay. The soluble proteins probably exert their effect by competing with virus for adsorption sites on the clay particles.

Our data (Table 3) show that the GES (glycine [0.1M]), EDTA [0.01M], 1% calf serum solution at pH 11) was the most efficient of the eluents tested and that the absence of serum appeared to reduce recovery efficiency slightly. Within the pH range (4 to 10) of the water tested, the pH of the medium appeared to have little effect on adsorption, concentration, or recovery (elution) of virus (Table 4), a feature that is especially important in field studies. It is generally known that the presence of soluble protein enhances virus survival; thus, the tryptose phosphate broth eluent, at neutral pH, was also useful for field-specific applications.

Similarly, the presence of organic particulates in wastewater appeared not to interfere with recovery, because over a 90 minute period only about 5% of the added virus adsorbed to the wastewater borne particulates. The remainder could be recovered efficiently with the bentonite procedure. Recovery of the 5% virus that adsorbed to the sewage solids, using the bentonite adsorption method was not as effective, perhaps due to irreversible virus adsorption, incorporation into the floc, or inactivation.

Although poliovirus was used extensively in these studies, the bentonite method was highly effective with other enteroviruses (Table 6). The lower recovery efficiency of Coxsackie A9 virus, in spite of an excellent adsorption efficiency, suggests that recovery may not be complete with some specific enteroviruses. The Coxsackie A viruses apparently have an unusually high pK value of 8.6,[25] which may partly explain the reduced recovery.

We have shown that the bentonite concentration method is rather effective for small to moderate size samples (up to 40 liters) without modification other than increased filter size and the adjustment of eluent volume required for efficient virus recovery. This results in a 400-fold concentration of the original virus (Table 7). We are currently investigating the use of much larger filters, capable of sampling 100 to 1,000 liter samples, with possible concentration factors of 50,000, using a second stage concentration technique (organic flocculation with beef extract eluent).

The effect of additional volumes of an eluent solution would probably have little effect on virus recovery. This is indicated by our experiments with the 47 mm prefilter, where we found no significant difference in virus recovery with 2 mL vs 5 mL of eluent. Also, the regression analysis applied to the data shown in Table 8 for 142 mm filters indicated that slightly more than 24 mL would have been required to elute 100% of the adsorbed virus, assuming, of course, that the elution process was 100% effective. Earlier work[14] indicated that virus desorption is essentially instantaneous upon contact of clay and eluent, thus

it seems that if the layer of clay-virus complex is saturated with eluent then elution would be complete, unless mechanical trapping on the filter matrix occurs. Thus, the primary effect of the eluent is for virus desorption by reversing electrostatic attraction forces, while the secondary effect is washing the released virions from the filter bed to a collection vessel.

No appreciable differences were observed in virus recovery when virus concentration from specific categories of water was reduced to 10^{-1} PFU/mL. However, recovery efficiency was higher from clean waters (deionized) than from wastewater. Additional work is needed to determine recovery efficiency with initial virus concentrations in the 0.1 PFU/L range or less.

The bentonite procedure was used to concentrate and subsequently assay viruses in wastewater from three different treatment plants. The number of enteroviruses recovered (Table 10) was within the typical range of accepted values from such plants, even though our recovery efficiency was estimated to be only about 40% (most of these experiments were performed before optimum conditions were established). Shaffer et al.[26] have used the bentonite method in direct comparison with the carborundum virus concentrator in field studies. The bentonite method was generally more efficient in virus recovery in that study. However, the carborundum concentrator was a scaled-up model capable of sampling several hundred liters of water.

A most important aspect of existing procedures for detecting virus in water is the efficiency with which they are retained on and recovered from the adsorbent. A wide range of virus recoveries using various media have been reported in literature. Farrah et al.[6] obtained 40-50% recovery of the input virus from tap water using pleated membrane filters. Rao and Labzoffsky[11] recovered about 50% of the input poliovirus from 10 to 500 mL raw samples of river water using a combination of prefilter pad and membrane filters. Hill et al.[3] obtained inconsistant recoveries, ranging from less than 1 to 74% of the seeded poliovirus from estuarine water, with the combined use of microporous filters and celite. In later studies[8] they recovered several types of enteric viruses from large volumes of drinking water with an overall efficiency of 28 to 42%. In these studies the investigators compared 4 microporous type filter media: nitrocellulose, epoxy-fiberglass-asbestos, yarn-wound glass-fiber depth filter, and epoxy-fiberglass filter tubes.

Most of these microporous filters employed in concentrating viruses are negatively charged at pHs above neutrality. Because most of the viruses are also negatively charged in the pH range of natural waters (pH 5 to 9), acidification or addition of multivalent cations was necessary to facilitate virus adsorption to these filters. With the advent of the positively charged Zeta Plus filters, the necessity for the addition of acid (or salt)

was considerably reduced or even eliminated. However, the use of the positively charged filters is effective only for entrapment of the virus when the pH of the sample water is below 7.5.[13]

Jones and Sobsey[10] used Zeta Plus filters (50s and 60s) and reported recovery of 64% of the seeded poliovirus from 12 liters of tap water. However, when the method was used to demonstrate the applicability of Zeta Plus 50s filters to large volumes of water, the virus recovery was 22.5%. This large scale virus concentration procedure necessitated a two stage filter adsorption-elution procedure to reduce a 380 liter water sample to a final volume of 25 mL. Recently Chang et al. (1981)[27] obtained an overall recovery of 46.5% while concentrating enteroviruses from 19 liters of wastewater, using Zeta Plus 30s filters. Although the final volume of eluate was reduced to 1 to 2 mL, the procedure involved the initial elution with large volumes of urea-lysine buffer followed by 2 flocculation steps (inorganic and organic), two centrifugations at 4000 x g, and an 8 hour dialysis in phosphate-saline buffer at 4 C. This method, as such, is very time consuming, cumbersome, and not suitable for field studies.

Virus recovery with the bentonite method for all experiments, including those in which optimal conditions were being established, had a mean value of 71 $\pm$ 12%. the mean value for recovery from deionized water was 83.8%; from tap water, 79%; and from sewage, 55%. It is to be noted that these recoveries were obtained using a rapid, simple, single-stage method that does not require elaborate equipment. The complete processing of a 10 L sample of tap water requires approximately one half hour. As such, it is ideally suited for field use in monitoring viruses in water and at the same time has broad applications for general analytical uses in public health laboratories.

The general procedure for batch virus concentration from waste or environmental water, using the bentonite method, is shown in Figure 5, and a summary of the bentonite procedure is provided in Table 11.

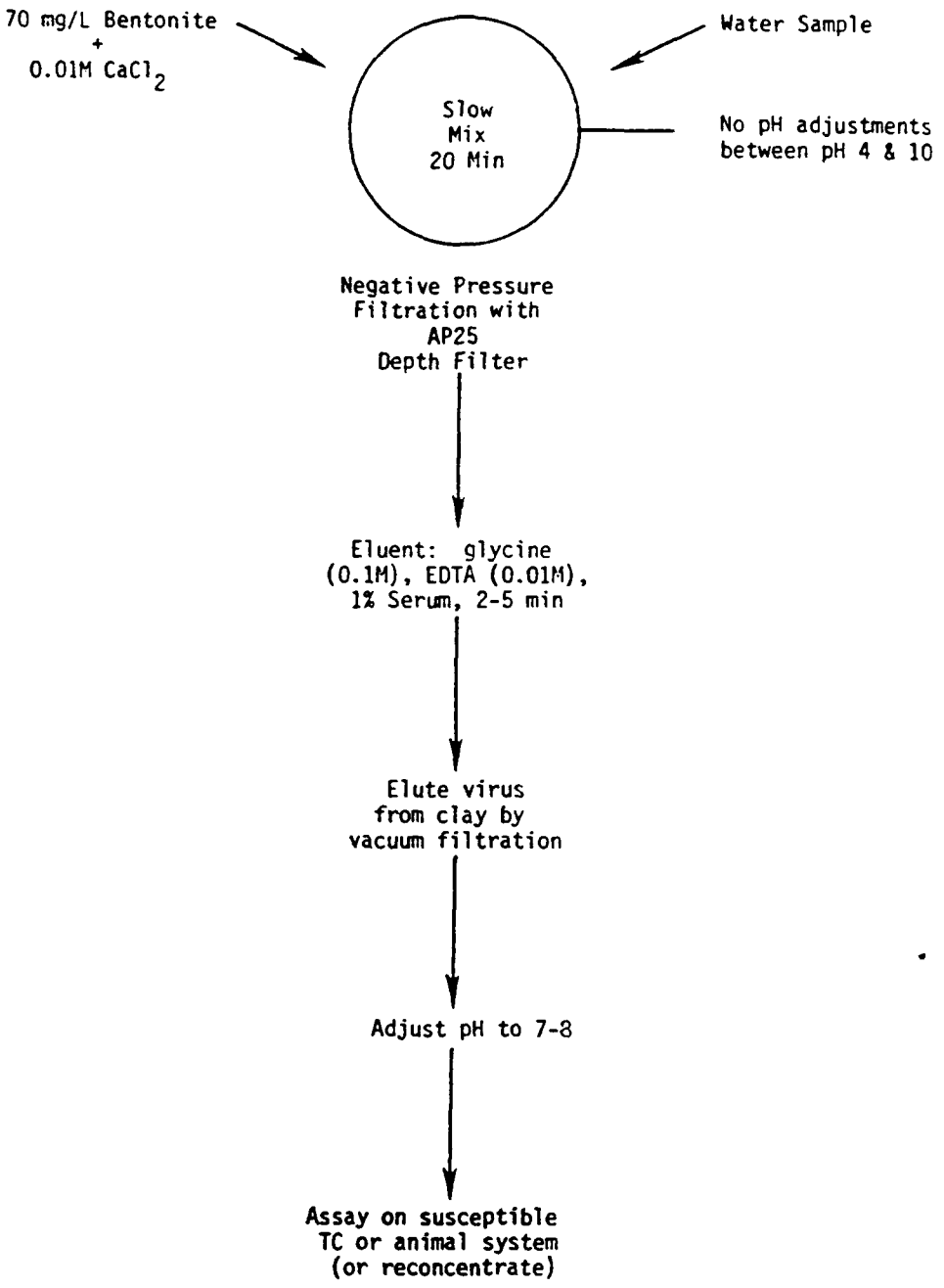

Figure 5. Flow diagram showing the batch virus concentration procedure

TABLE 11. STEPWISE SUMMARY OF THE TYPICAL BENTONITE VIRUS ADSORPTION PROCEDURE

A. Adsorption

1. 50-70 mg/L (or equivalent) USP grade powdered bentonite is added to water samples.

2. A 1 M solution of $CaCl_2$ is prepared and added to bentonite suspension to yield a 10^{-2} M final concentration.

3. The mixture is stirred slowly for 20 minutes.

B. Concentration

1. AP 25 Millipore prefilters are placed in an appropriate holder (high pressure holders are not necessary), which is selected on the basis of sample size (see Table 7).

2. The bentonite-virus complex is drawn through the filters by vacuum (positive pressure may also be used but at a reasonable flow rate to extend filter life).

3. After sample is filtered or flow rate slows to a slow drip, residual bentonite-virus complex is used for the elution of the viruses.

C. Elution

1. The proper amount (Table 7) of GES eluent (glycine-EDTA-bovine serum @ pH 11), or tryptose phosphate broth, pH 7.2) is added to soak the filter surface by pipette (or pouring) and allowed to soak for 5 minutes.

2. Eluent is drawn through the filter by vacuum (attempt to draw as much eluent through the filter as possible) and collected in a small flask or centrifuge tube inside the suction flask.

3. Virus is assayed on appropriate cells after adjusting the pH to 7-7.5 immediately to prevent loss of virus. The sample may be assayed directly or stored frozen below -20° C (preferably -80° C) for future assay.

LITERATURE CITED

1. Rawal, B.D. and S.H. Godbole. 1964. A simple tissue culture method for detection of viruses in drinking water; sample incorporation method. Environ. Health 6:234-236.

2. Cookson, J.T. 1969. Mechanism of virus adsorption on activated carbon. J. Am. Water Assn. 61:52-56.

3. Hill, W.F. Jr., E.W. Akin, W.H. Benton, C.J. Mayhew, and T.G. Metcalf. 1974. Pecovery of poliovirus from turbid estuarine water on microporous filters by the use of Celite. Appl. Microbiol. 27(3):506-512.

4. Rao, V.C., U. Chandorkar, N.U. Rao, P. Rumaran, and S.B. Lakhe. 1972. A simple method for concentrating and detecting viruses in wastewater. Water Research 6:1565-1576.

5. Wallis, C., M. Henderson, and J.L. Melnick. 1972. Enterovirus concentration on cellulose membranes. Appl. Microbiol. 23:476.

6. Farrah, S.R., C.P. Gerba, C. Wallis, and J.L. Melnick. 1976. Concentration of viruses from large volumes of tap water using pleated membrane filters. Appl. Environ. Microbiol. 31(2):221-226.

7. Gerba, C.P., S.R. Farrah, S.M. Goyal, C. Wallis, and J.L. Melnick. 1978. Concentration of enteroviruses from large volumes of tap water, treated sewage, and seawater. Appl. Environ. Microbiol. 35:540-548.

8. Hill, W.F., Jr., W. Jakubowski, E.W. Akin, and N.A. Clarke. 1976. Detection of virus in water: Sensitivity of the tentative standard method for drinking water. Appl. Environ. Microbiol. 31(2):254-261.

9. Jakubowski, W., S.L. Chang, T.H. Erickson, E.C. Lippy, and E.W. Akin. 1978. Large volume sampling of water supplies for microorganisms. Proc. Am. Water Assn. (Water Technology/Quality). December:702-706.

10. Jones, B.L. and M.D. Sobsey. 1978. Concentration of poliovirus from tap water using positively charged microporous filters. Proc. 98th Ann. Conf. Am. Water Works Assn., Atlantic City, NJ., June 25-30, 1978.

11. Rao, N.U. and N.A. Labzoffsky. 1969. A simple method for the detection of low concentrations of viruses in large volumes of water by the membrane filter technique. Can. J. Microbiol. 15:399-403.

12. Sobsey, M.D. and B.L. Jones. 1979. Concentration of poliovirus from tap water using positively charged microporous filters. Appl. Environ. Microbiol. 37:588-595

13. Sobsey, M.D. and J.S. Glass. 1980. Poliovirus concentration from tap water with electropositive adsorbent filters. Appl. Environ. Microbiol. 40:201-210.

14. Schaub, S.A. and B.P. Sagik. 1975. Association of enteroviruses with natural and artificially introduced colloidal solids in water and infectivity of solids-associated virions. Appl. Microbiol. 30:212-222.

15. Carlson, G.F., Jr., F.E. Woodard, D.F. Wentworth, and O.J. Sproul. 1968. Virus inactivation on clay particles in natural waters. J. Water Pollut. Control Fed. 40:R89-R106.

16. Drewry, W.A. and R.L. Eliassen. 1968. Virus movement in soil. J. Water Pollut. Control Fed. 40:R257-R271.

17. Moore, B.E., B.P. Sagik, and C.A. Sorber. 1979. Procedure for recovery of airborne human enteric viruses during spray irrigation of treated wastewater. Appl. Environ. Microbiol. 384:688-693.

18. Van Olphen, H. 1963. An Introduction to Clay Colloid Chemistry. Wiley-Interscience, New York.

19. Schmidt, N.J., H.H. Ho, J.L. Riggs, and E.H. Lennette. 1978. Comparative sensitivity of various cell culture systems for isolation of viruses from wastewater and fecal samples. Appl. Environ. Microbiol. 36(3):480-486.

20. Kessick, M.A. and R.A. Wagner. 1978. Electrophoretic mobilities of virus adsorbing filter materials. Water Research 12:263-268.

21. Kelly, S. and W.W. Sanderson. 1959. The effect of sewage treatment on viruses. Sewage Ind. Wastes 31:683-689.

22. Kelly, S., W.W. Sanderson, and C. Reidl. 1961. Removal of enteroviruses from sewage by activated sludge. J. Water pollut. Control Fed. 33:1056-1062.

23. Sproul, O.J. 1976. Removal of viruses by treatment processes. In G. Berg, et al. eds. Viruses in Water, pp. 167-179. Am. Public Health Assn., Washington, D.C.

24. Turk, C.A., B.E. Moore, B.G. Sagik, and C.A. Sober. 1980. Recovery of indigenous viruses from wastewater sludges, using a bentonite concentration procedure. Appl. Environ. Microbiol. 40:423-425.

25. Polson, A. and D. Deeks. 1962. Zone electrophoresis of enteroviruses. J. Hyg. (Cambridge) 60:217-236.

26. Shaffer, P.T.B., R.E. Meierer, and C.D. McGee. 1977. Virus recovery from natural water. J. Am. Water Works Assn. 69:528-531.

27. Chang, L.T., S.R. Farrah, and G.Bitton. 1981. Positively charged filters for virus recovery from waste water treatment plant effluents. Appl. Environ. Microbiol. 42(5):921924.

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