Influence of Estuarine Sediment on Virus Survival Under Field Conditions

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The survival of poliovirus 1 (LSc) and echovirus 1 (Farouk) in estuarine water and sediment was studied in Galveston Bay, Texas. Viruses were suspended in estuarine water and sediment both in dialysis tubing and in chambers constructed with polycarbonate membrane walls. Virus inactivation rates in seawater were similar in both types of chambers. Virus adsorption to sediment greatly increased survival time. The time required to inactivate 99% (T-99) of poliovirus increased from 1.4 days in seawater alone to 6.0 days for virus adsorbed to sediment at a relatively nonpolluted site. At a more polluted site, poliovirus T-99 was increased from approximately 1 h to 4.25 days by virus adsorption to sediment. This study demonstrates that under field conditions virus association with estuarine sediment acts to prolong its survival in the marine environment.

The presence of viruses in estuarine recreational and shellfish-harvesting water is recognized as a potential public health problem (5, 20). Methodology to isolate viruses from polluted freshwater (10) and seawater (4, 18, 21, 23) has progressed to such an extent that standards for permissible concentrations of viruses have been proposed (1, 15, 20). However, water may not be the only source of viruses in the estuarine environment. Recent studies (6, 12a) have shown that viruses are present in polluted estuarine sediment at higher concentration than in the overlying seawater. We have previously shown (12) that enteric viruses readily adsorb to sediment, and in laboratory studies we have demonstrated (22) that enteroviruses survive longer in the presence of sediment than in seawater alone. However, laboratory studies may not reflect field conditions where environmental conditions are in a constant state of flux. In addition, phenomena controlling virus survival in the field are not necessarily identical to those observed in the laboratory. In this study we have attempted to determine whether sediment exhibits a protective effect on virus survival under circumstances more closely approaching field conditions.

MATERIALS AND METHODS

Virus and viral assays. Enterovirus assays were performed with the buffalo green monkey kidney cell line, which was passaged, grown, and maintained by previously described methods (16). Plaque-purified stocks of poliovirus 1 (strain LSc) and echovirus 1 (strain Farouk) were grown in buffalo green monkey cells, concentrated 10-fold and purified by membrane chromatography (8), and then stored at −20°C. Virus

samples were diluted in tris(hydroxymethyl)-aminomethane (Tris)-buffered saline which contained 20 mM Tris, 140 mM NaCl, 5 mM KCl, 0.4 mM Na₂HPO₄, 6 mM dextrose, 0.5 mM MgCl₂, 0.7 mM CaCl₂, 2% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). Virus assays were performed by the plaque-forming unit method as used in this laboratory (16).

Bacterial assays and physical measurements. Coliforms, fecal coliforms, salinity, temperature, pH, turbidity, and absorbance at 254 nm were determined as previously described (7, 22). The physical, chemical, and biological characteristics measured during the experiments at each site are listed in Table 1.

Source of sterile sediment. Sediment used in the sterile environment studies was obtained at site 3, washed 2 times with distilled water, and dried at 350°F overnight. Clumped, dried sediment was disaggregated in a mortar and pestle. Samples (5 g each) were autoclaved before use in the field studies.

Artificial seawater. Artificial seawater (34-g/kg salinity) was prepared by the formula of Dittmar, as given by Matossian and Garabedian (13).

Description of field sites. The sites of the virus survival study were located in two canal communities near Galveston, Texas. Site 1 was near a private residence in a canal community on the western end of Galveston Island. Sites 2 and 3 were located in a community north of Galveston on the mainland. Site 2 was 45 m upstream of a sewage outfall serving this community. Site 3 was halfway down a canal located two canals downstream of the sewage treatment plant, approximately 150 m away.

Survival chambers. Two types of survival chambers were used to assess virus survival in the field: ¾-in. (ca. 1.9 cm) cellulose dialysis tubes (Arthur A. Thomas, Philadelphia, Pa.), and survival chambers constructed of plexiglass (14) designed to support diffusion membranes which then form a chamber. Polycarbonate membrane filters of 0.015-µm porosity (Nu-

clepore Corp., Pleasanton, Calif.) were used in this study. Cellulose dialysis tubes have been used in past survival studies (2, 9, 17) but have the disadvantage of being biodegradable (24). Polycarbonate membranes, which are non-biodegradable, have been used in bacterial studies (25), but this is the first report of their use in virus survival studies.

Since the polycarbonate membranes are only 10 μm in thickness, the survival chambers sometimes leaked if assembled as described by McFeters and Stuart (14). Therefore, it was necessary to make several modifications of the commercially available chambers. The assembly of the chamber as modified is illustrated in Fig. 1 and 2. A stainless steel 30-mesh screen was added to the exterior to protect the membrane from being punctured while in the environment (3). A 1.6-mm (1/16-in.) natural latex rubber gasket was used in the sandwich between the side piece and the center piece of Plexiglas to hold the membrane firmly in place against the center piece. The gasket was necessary to prevent leakage. Dow Corning silicone grease was used to provide a watertight seal between the membrane and the Plexiglas center piece. Diffusion tests and field tests were performed with the apparatus assembled as in Fig. 2.

Diffusion rates. In the laboratory determination of diffusion rates, a solution of artificial seawater diluted to 16-g/kg salinity with distilled water containing methylene blue was placed in dialysis tubing and in the polycarbonate membrane chamber. These chambers were suspended in artificial seawater (16 g/kg) for 24 h with periodic manual agitation. Samples were taken at regular intervals. When all samples were collected, the concentration of methylene blue was determined by measuring the absorption of each sample at 660 nm in a Gilford model 240 spectrophotometer. Laboratory diffusion rates of sucrose and phenol red were also determined. The concentration of sucrose was measured by index of refraction, and phenol red was measured by optical density at 260 nm. In the

TABLE 1. *Field survival site characteristics*

Characteristic	Site 1	Site 2	Site 3
Total coliforms in seawater	15–750/100 ml (68)[a]	93–1,100/100 ml (240)	43–240/100 ml (75)
Fecal coliforms in seawater	0–93/100 ml (12.5)	3–93/100 ml (4)	3–93/100 ml (9)
Total coliforms in sediment	46,000/100 ml	21,000/100 ml	15,000/100 ml
Fecal coliforms in sediment	24,000/100 ml	30/100 ml	90/100 ml
Optical density at 254 nm	0.055–0.101 (0.063)	0.285–0.393 (0.334)	0.188–0.268 (0.261)
Temperature	30–32°C (31)	30–33°C (32.5)	30–33°C (32.5)
Salinity (g/kg)	30–31 (30)	26–27 (26)	26.5–29 (27)
Turbidity (NTU)	5.4–7.1 (5.6)	6.5–8.8 (8.0)	7.1–11 (8.1)
pH, seawater	8.3–8.45 (8.35)	8.4–8.9 (8.76)	7.85–8.6 (8.5)
pH, sediment	8.0	ND[b]	ND

[a] Numbers in parentheses represent the median of the results obtained over the course of 6 to 7 days.
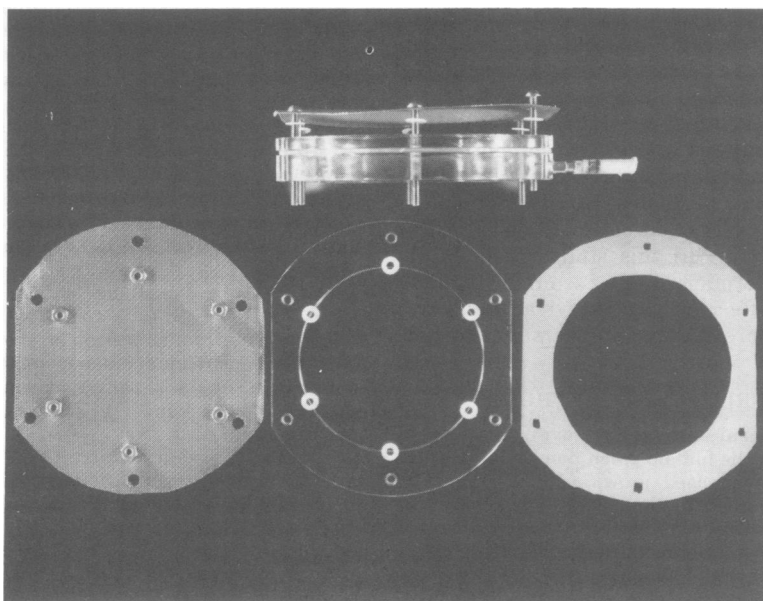[b] ND, Not done.

FIG. 1. *Survival chamber for support of polycarbonate membranes shown disassembled.*
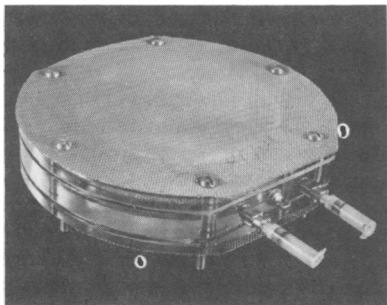
FIG. 2. *Survival chamber shown assembled.*

field studies, samples of sterile artificial seawater containing methylene blue were added to several dialysis tubes and one polycarbonate membrane chamber and were suspended in seawater at site 1. Periodic samples were taken, and the methylene blue concentration was again determined by absorption at 660 nm.

Determination of virus survival. Field survival studies were performed with natural seawater and sediment obtained at the site at the beginning of each experiment.

Virus survival in seawater alone and in seawater containing sediment was studied in dialysis tubes. To study viral inactivation in seawater alone, several dialysis tubes (sterilized by boiling) were each filled with 30 ml of seawater containing virus at a 1:100 to 1:1,000 dilution to yield a titer 10^5 to 10^6 per ml. To study virus survival in a mixture of sediment and seawater, 5 ml of natural sediment was mixed with 25 ml of natural seawater and inoculated with virus at the same concentration as seawater alone. One sample was assayed for virus before suspending the dialysis tubes in seawater. The samples were shaken vigorously in a sterile 50-ml polypropylene centrifuge tube periodically before sampling; they were then transferred to dialysis tubes. Survival of virus in seawater alone was also studied in chambers with polycarbonate membrane walls. Mixtures of seawater and sediment were not studied in this type of chamber because sediment samples could not be obtained through the narrow luer-lok syringe fitting.

Virus survival in sterile, sealed chambers was also studied at the field sites. Sealed polypropylene centrifuge tubes (Corning Glass Works, Corning, N.Y.; no. 25330, 50 ml) containing sterile-filtered (0.22 μm) artificial seawater or sterile-filtered artificial seawater plus autoclaved sediment were suspended in the same environment.

Samples in dialysis tubes and in sealed polypropylene centrifuge tubes were placed on the bottom of the canal (1 to 1.5 m) in a plastic bait bucket that had perforated sides and a stone for weight. The Plexiglas chambers were suspended independently of the bait bucket. All chambers were anchored to the shore with nylon string.

Survival data are expressed as the negative $\log_{10}$ of the survival ratio N_t/N_o, where N_o is the original number of viruses and N_t is the number of viruses surviving after a given period of time.

Elution of virus from survival chamber wall. Empty dialysis bags were returned from the field on

ice. After complete removal of the original fluid, 1 ml of 0.05 M glycine at pH 11.5 was added to each dialysis bag and was moved around the internal surface of the bag by external finger pressure. After approximately 5 min, the eluate was recovered with a sterile Pasteur pipette and diluted 1:10 in Tris buffer with 2% fetal calf serum containing 100 U of penicillin per ml and 100 μg of streptomycin per ml.

In a laboratory experiment designed to determine the amount of virus adsorbed to the internal surface of the survival chamber, 3 ml of artificial seawater (34-g/kg salinity) containing 5×10^4 plaque-forming units of virus per milliliter was enclosed in each of several dialysis tubes suspended in artificial seawater for 48 h. Periodically, a dialysis tube was collected and sampled for virus, and the internal wall was eluted with 1 ml of fetal bovine serum for 5 min. The eluate was diluted 1:10 in Tris containing 2% fetal calf serum plus antibiotics and frozen at −20°C until assayed. A similar laboratory experiment was conducted with the survival chamber with a polycarbonate membrane wall. Artificial seawater (60 ml) containing virus was added to a chamber that was then suspended in artificial seawater. Two chambers were used. One was sampled and eluted at 2 h, and the other was sampled and eluted at 24 h with 5 ml of fetal bovine serum diluted 1:10 in Tris containing 2% fetal calf serum plus antibiotics.

The polypropylene centrifuge tube (Corning Glass Works, Corning, N.Y.; 50 ml) was also tested for virus adsorption to its wall. Artificial seawater (10 ml) was added to several centrifuge tubes which were harvested at several time points over 48 h. The seawater was sampled for assay, and the seawater solution was then removed. Viruses were eluted from the wall with 1 ml of fetal bovine serum by swirling the serum around the wall for a few minutes; the serum was then diluted in Tris buffer containing 2% fetal calf serum plus antibiotics.

Determination of membrane integrity. A suspension of bacteria was grown by inoculating several 100-ml bottles of tryptose phosphate broth with a sample of natural seawater. After 48 h the bacterial suspension was autoclaved for 20 min, cooled, centrifuged at $1,400 \times g$ for 5 min, suspended in Tris buffer (without serum), and centrifuged and suspended two more times. This suspension was sonicated and then diluted to an approximate turbidity of 40 nephelometric turbidity units (NTU) measured in a Hach model 2100A turbidimeter. The suspension was placed in dialysis bags which were then suspended in the environment at sites 2 and 3 for 48 h, and the turbidity was again measured in the laboratory. The turbidity readings before and after suspension of the bacterial solution in the environment were compared to determine whether there was any leakage of material from the dialysis tubing in the field due to breaks in the membrane.

RESULTS

Characterization of survival chambers. The diffusion rates of sucrose, phenol red, and methylene blue from each of the chambers under laboratory and field conditions are shown in

Table 2. Diffusion of these substances was found to be several times slower from the chamber with a polycarbonate membrane than from the dialysis tube. However, since natural seawater and virus were placed in the chambers, the initial concentrations of virus-inactivating factors would be similar in both types of chambers.

The amount of virus adsorbed to the sides of the chambers during use was also investigated. The empty dialysis tubes were transported to the laboratory on ice and were eluted with either 0.5 M glycine at pH 11.5 or 100% fetal calf serum (no pH adjustment). The number of viruses eluted from the tube varied from 0.0095 to 2.2% of the total virus present in the tubing at that time in the survival test. In a separate laboratory test of the dialysis tubing, the polycarbonate membrane, and the polypropylene centrifuge tube for virus adsorption, only 0.6 to 1.9, 0.6 to 0.8, and 0.4 to 1.6% of the total virus present could be eluted off the chamber wall of the dialysis tube, the polycarbonate membrane, and the polypropylene tube, respectively, with fetal calf serum. Since the chamber wall did not appear to adsorb a significant amount of virus, it was not considered to have any effect on virus survival in the field.

Dialysis bags were checked for durability in the field by suspending dialysis tubes filled with a turbulent suspension of autoclaved and sonicated bacteria. At site 2, a suspension diluted to 23 NTU read 22 NTU after 2 days. At site 3, the contents of a tube containing a suspension originally diluted to 43 NTU read 33 NTU after 2 days' suspension in the environment. This indicates that there were no breaks in the dialysis tubes during the experiment, although there

TABLE 2. *Diffusion times of sucrose, phenol red, and methylene blue from virus survival chambers in seawater*

Reagent	Dialysis tubing		Polycarbonate membrane	
	T-50[a]	T-90[a]	T-50	T-90
Laboratory[b]				
Sucrose	1.25	7.5	39.0	85[c]
Phenol red	6.25	11.0	25.8	75[c]
Methylene blue	0.75	4.2	3.6	27
Field (site 1)				
Methylene blue	1.60	3.1	8.5	22

[a] T-50 and T-90 are the time in hours for 50% and 90%, respectively, of the reagent to diffuse out of the chamber.

[b] Sterile-filtered artificial seawater at 16-g/kg salinity was used.

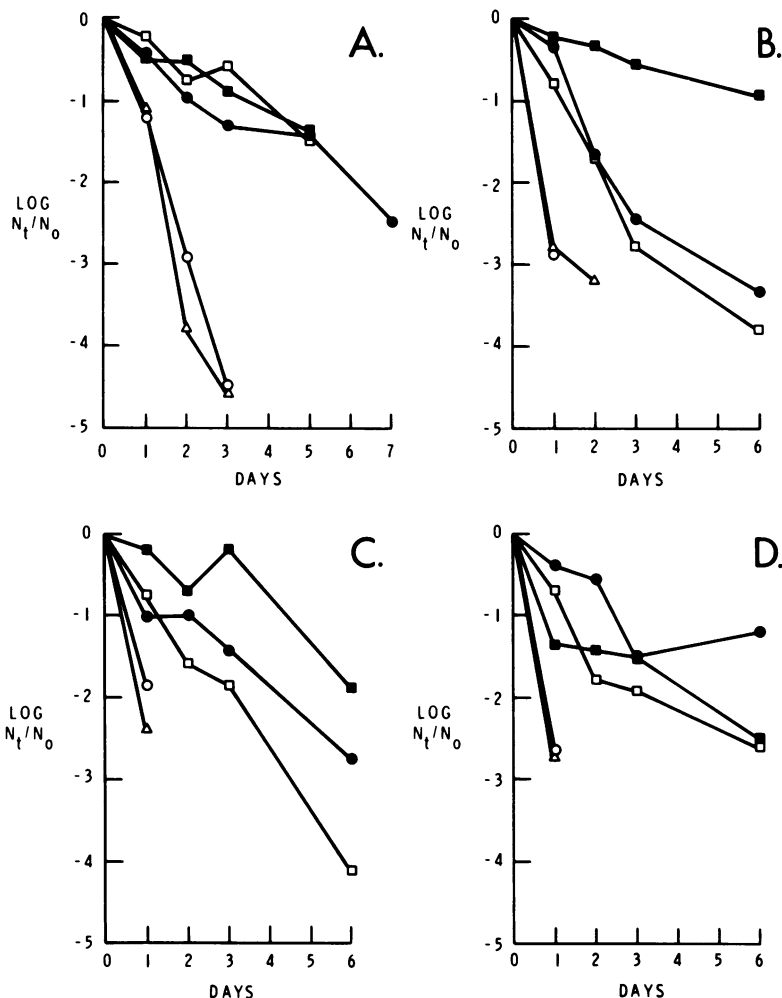[c] Estimated by extending the slope of the diffusion observed to the T-90 value.

may have been some aggregation of the suspension at site 3, since it did demonstrate a slightly lower reading after 2 days.

Virus survival. The survival of poliovirus 1 (strain LSc) at site 1 is illustrated in Fig. 3A. Poliovirus exposed to seawater alone was inactivated in both dialysis tubing and polycarbonate membrane chambers much faster than in the presence of sediment. The inactivation rates for virus in the sterile containers and for virus adsorbed to natural sediment appeared to be about equal. In this experiment, the addition of sediment under sterile conditions did not enhance virus survival.

Figure 3B illustrates inactivation of poliovirus 1 in a polluted area that was about 45 m from a sewage outfall discharging secondary treated sewage (site 2). Virus was again inactivated more rapidly in seawater alone than in the presence of sediment. The inactivation rate of virus in the presence of natural seawater and sediment paralleled virus inactivation in sterile artificial seawater alone. However, virus survived longer in the presence of sterile seawater containing sediment than in sterile seawater alone, in contrast to the results at site 1. Apparently, sediment protected poliovirus against the effects of temperature or salinity or both to a greater extent in this experiment than it did in the previous one (Fig. 3A).

Figure 3C illustrates the inactivation of poliovirus 1 at site 3. The results are similar to those observed at the previous two sites. Under sterile conditions, sediment appeared to retard inactivation of poliovirus 1. The viral inactivation capacity of seawater at this site appeared to be much more rapid than at the other sites. The 0 h value of virus suspended in natural seawater alone was 1.69 logs (in a polycarbonate membrane chamber) to 2.2 logs (in dialysis tubing) lower than the 0 h standard of virus in sterile artificial seawater. However, in Fig. 3C the values obtained were used as the starting point at 0 h. Since all samples were seeded with the same amount of virus during their preparation, inactivation must have occurred between the time samples were inoculated and the time they were assayed. Approximately 15 min elapsed between inoculation and the initial sampling, indicating a very rapid rate of inactivation at this site. No assayable virus was present in the seawater samples at 24 h.

Figure 3D demonstrates the survival of echovirus 1 (strain Farouk) at site 2. The results are similar to those observed for poliovirus 1 in Fig. 3B. Echovirus 1 was also protected from inactivation by the presence of sediment. It was inactivated in seawater alone at the same rate as

FIG. 3. *Poliovirus 1 (strain LSc) and echovirus 1 (strain Farouk) survival at sites 1, 2, and 3. Symbols: ○, virus suspended in seawater in dialysis tubes; ●, virus suspended in seawater and sediment in dialysis tubes; △, virus suspended in seawater in the Plexiglas chamber with polycarbonate membrane (0.015-μm pore size) wall; □, sealed polypropylene centrifuge tube containing sterile artificial seawater; ■, sealed polypropylene centrifuge tube containing sterile artificial seawater and sterile sediment. Panels represent: poliovirus 1 survival at site 1 (A), site 2 (B), and site 3 (C); and echovirus 1 survival at site 2 (D).*

poliovirus, but appeared to survive longer when mixed with sediment. The presence of sediment under sterile conditions seemed to have only slightly increased virus survival.

It is apparent from the survival data that viruses adsorbed to sediment were protected from environmental inactivating factors under field conditions. Even under sterile conditions, sediment appeared to protect virus from inactivation, possibly by temperature, salinity, or other factors. Virus inactivation was more rapid in seawater at sites 2 and 3 than at site 1. However, virus inactivation in the presence of

sediment seemed to be more similar at all three sites.

Virus inactivation rates in seawater appeared to be the same in both types of survival chambers, in spite of the differences in their diffusion rates. Thus, we conclude that sufficient virus-inactivating factors were present in the initial sample of seawater to inactivate virus or the virus-inactivating factor(s) were more readily diffused than the compounds tested or both.

DISCUSSION

The results of the field survival study confirm

previous work on virus survival under laboratory conditions (22). It was shown that viruses survived longer in the presence of sediment in seawater. Virus inactivation occurred faster in natural seawater than it did in the laboratory study, but the temperature at which the present study was conducted was 5 to 7°C higher than the temperature of the previous study. It is also possible that other inactivating factors might have been present in the seawater. Sealed sterile chambers containing virus were exposed to the estuarine environment to determine whether salinity, temperature, or the absence of microorganisms had a major effect on virus survival. If virus inactivation in seawater is greatly influenced by salt concentration or temperature, then the virus inactivation rates for the sterile and nonsterile seawater environments would be similar. Figure 3A through D demonstrates that poliovirus and echovirus survival in sterile artificial seawater is much better than in natural seawater. This indicates that the elimination of microorganisms leads to a greatly increased survival rate, but indicates little regarding the effects of salinity and temperature. In two of three experiments, poliovirus survived longer in sterile sediment and seawater than in sterile seawater alone. This indicates that the sediment in the sterile environment may be protecting virus from inactivation by heat or salinity or both. Thus, all three factors appear to have some effect on virus survival in seawater. However, further studies are necessary to determine the mechanism of the protective effect of sediment.

This is the first reported instance in which polycarbonate membrane chambers have been used to study virus survival. Although the diffusion rate is much slower for the compounds tested from the polycarbonate chamber compared to dialysis tubing, viral inactivation rates were apparently the same in each type of chamber. This was probably due to the fact that virus-inactivating factors were not required to diffuse into the chamber since they would presumably be placed into the chamber with the natural seawater. An experiment that would require virus-inactivating factors to diffuse into the chamber may not yield results similar to those obtained in this study.

In the previous laboratory study (22), virus associated with polluted sediments survived longer than in less polluted sediments. This finding was not observed in the present study. Virus survival in the presence of sediment was similar from site to site, although virus associated with sediment from a less polluted site appeared to survive slightly longer. However, the difference may not be significant. Poliovirus survived longer in seawater from site 1 although it was inactivated quite rapidly at sites 2 and 3. Previous coliform counts of seawater and sediment at sites 2 and 3 indicated that these were moderately to heavily polluted sites, but the numbers of coliforms were low during the survival studies. However, the optical density at 254-nm wavelength of ultraviolet light was higher at sites 2 and 3 than at site 1. It has been shown that the reading at this wavelength has a general correlation with the presence of pollution (11). Most of the viruses at these sites were inactivated in 24 h or less. Echovirus showed a similarly rapid inactivation in seawater at site 2. Therefore, viruses may be inactivated more rapidly in polluted estuarine water. Poliovirus survival in sterile artificial seawater was greater than virus suspended in natural seawater in all cases. Survival in sterile seawater paralleled the survival of poliovirus in natural sediment for all three sites. Poliovirus survival in sterile seawater containing sediment was better than survival in sterile seawater alone at sites 2 and 3, but they were similar at site 1.

Echovirus also exhibited similar rates of inactivation in both sterile environments, although survival in sterile sediment was slightly better. This indicated that under sterile conditions, sediment is capable of protecting virus from inactivating factors other than microorganisms, such as temperature and salinity. In Fig. 3D, echovirus 1 survival in sediment under nonsterile conditions appears to be greater than in sterile sediment. However, the efficiency of plating of virus adsorbed to natural sediment in assay was less than the efficiency of plating of virus assayed from washed, dried, and autoclaved sediment. The titer of virus at 0 h in natural sediment was approximately 1 log lower than the other tests, and the virus titer appeared to decline gradually from this point. Therefore, virus survival in sterile and nonsterile sediment was comparable when one considers the relative inability to assay virus adsorbed to natural sediment. This may indicate that there is some factor that inactivates virus during adsorption or elution or both in natural sediment which is not present in the laboratory-processed sediment. The high heat used for drying the sediment and in autoclaving may destroy some substance that is antagonistic to echovirus survival.

The presence of natural sediment significantly increased virus survival under field conditions. For example, at site 3 the estimated time to inactivate 99% (T-99) of poliovirus 1 in seawater, about 1 h (extrapolated from the 15-min inacti-

vation rate), increased to approximately 4.25 days when poliovirus was adsorbed to natural sediment. This observed increase in survival rate is manyfold greater than the increase previously observed in the laboratory. At site 1, which was less polluted, the increase in virus survival was not as great, but was still significant. The T-99 (determined from Fig. 3A) was 1.4 days in seawater alone compared to a T-99 of 6.0 days for virus adsorbed to sediment at this site. Virus inactivation time in estuarine water was increased from a few hours to several days simply by virus adsorption to sediment.

Thus, an assay of water alone for virus could possibly lead to an underestimation of the number of viruses actually present in any estuarine area. It has been demonstrated previously that viruses and bacteria are present in higher numbers on a volume basis in sediment (6, 12a). It has also been demonstrated that viruses adsorbed to clay and sediment are infectious in the adsorbed state and can be assayed in tissue culture (12, 19). These data indicate that the microbiological quality of sediment may be an important indicator of the public health safety of recreational estuarine areas which is not currently being evaluated but which should be included in the evaluation of the microbiological hazards present in such areas.

ACKNOWLEDGMENTS

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