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Relationships Between Environmental Factors, Bacterial Indicators, and the Occurrence of Enteric Viruses in Estuarine Sediments

RAYMOND L. LABELLE,[1] CHARLES P. GERBA,[1]* SAGAR M. GOYAL,[1] JOSEPH L. MELNICK,[1] IRINA CECH,[2] AND GREGORY F. BOGDAN[2]

Department of Virology and Epidemiology, Baylor College of Medicine,[1] and School of Public Health, University of Texas,[2] Houston, Texas 77030

Current standards for evaluation of the public health safety of recreational and shellfish-harvesting waters are based upon bacteriological analysis, but do not include an evaluation of the number of viruses. The objective of this study was to determine the occurrence of enteric viruses in estuarine sediments and to find a relationship, if any, between the presence of viruses in seawater or sediment or both and various biological and physicochemical characteristics of the environment. Viruses were found in greater numbers in sediment than in overlying seawater on a volume basis. Several types of enteroviruses were isolated: coxsackievirus types A16, B1, and B5, echovirus type 1, and poliovirus type 2. On several occasions, viruses were isolated from sediments when overlying seawaters met bacteriological water quality standards for recreational use. Statistical analysis of the relationship between viruses in seawater or in sediment and other variables measured yielded only one significant association: the number of viruses in sediment was found to be positively correlated with the number of fecal coliforms in sediment. No other physical, chemical, or biological characteristic of seawater or sediment that was measured showed statistically significant association with viral numbers. No correlation was found between bacterial indicators and virus in the overlying waters. The data indicated that evaluation of the presence of bacteria and viruses in sediment may provide additional insight into long-term water quality conditions and that indicator bacteria in water are not reflective of the concentration of enteric viruses in marine waters.

The construction of off-shore sewage outfalls and coastal canal communities with limited sewage treatment facilities has sparked an interest in the public health safety of the surrounding waters. The survival and persistence of viruses in the estuarine environment is of special interest since viruses appear to survive longer than coliform bacteria, which are presently used to judge water quality (35). Large numbers of viruses have been shown to be discharged into marine waters by offshore sewage outfalls (8, 10, 35), and they have also been detected in coastal canal water polluted by small-package sewage treatment plants and septic tanks (19). Using a recently developed technique to quantitate viruses in marine sediments (18), Gerba et al. found viruses in greater numbers in sediments near sewage outfalls than in the overlying seawater (15). Although viruses are inactivated more readily in seawater than in freshwater (27), they can survive much longer if adsorbed to sediments (36). Recent outbreaks of hepatitis in Houston, Tex., and in Calhoun, Ga. (26), caused by consumption of hepatitis A virus-containing oysters which were taken from water considered safe (by bacteriological standards) for shellfish harvesting, have caused concern about the present state of knowledge of virus survival and transport in the estuarine environment.

In 1905 Savage (34) concluded that samples of mud yielded more reliable bacteriological evidence of the degree of fecal pollution of a tidal river than samples of either water or oysters. More recent investigations (15, 23, 38) have found that coliforms, fecal coliforms, and salmonellae tend to concentrate in sediment and that they are almost totally located in the upper layers (top 2 in [ca. 5 cm]) of sediment (2, 38, 41). *Escherichia coli* has been shown to survive longer in sediment than in seawater (16). It appears that fecal bacteria concentrate and survive in sediments until they are resuspended by activities that disturb sediment, such as dredging and rapid rainwater runoff (17, 22, 29). It has been demonstrated that bottom-feeding fish have higher levels of coliforms, fecal coliforms, and fecal streptococci than do surface-feeding and predaceous fish (13). Also, oysters have been

shown to accumulate more virus if it is present as a crude suspension rather than as purified virus, indicating that virus associated with a small amount of particulate matter is taken up more efficiently by shellfish (24).

The current study was undertaken to determine whether the number of viruses in sediments could be correlated with various indicators used to judge water quality and to ascertain the number of viruses that could be expected to be present in sediments of areas into which domestic sewage was being discharged.

MATERIALS AND METHODS

Samples and sampling sites. Seawater samples to be used for viral analysis were obtained as grab samples in 20-liter stainless-steel pressure vessels (Millipore Corp., Bedford, Mass.). Sediment samples were obtained with the aid of an Ekman dredge and were stored in plastic bags on ice until they were processed in the laboratory for viruses and bacteria. The sample obtained by the Ekman dredge covered a square area, 15 by 15 cm, and was dug to an average depth of 2 cm. Samples of approximately 100 to 400 g could be obtained, depending upon the compaction of the surface of the sediment. Sediment was divided into 100-ml samples for bacterial analysis and 400-ml samples for viral analysis. Samples were processed immediately after returning to the laboratory. Water and sediment samples were obtained from shore stations.

Seven coastal canals and four oyster beds situated in the Galveston Bay area, located within 80 km (50 miles) of Houston, Tex., on the Gulf of Mexico, were studied. Detailed maps of their locations have been published (15; R. LaBelle, Ph.D. dissertation, Baylor College of Medicine, Houston, Tex., 1979). Samples were obtained on two to four occasions from the polluted sites (H-86, H-86a, H-86b, H-81, and K-27), whereas only one or two samples were obtained from the less-polluted sites (T-50 and 323 and oyster beds no. 1, 2, 3, and 4). A total of 30 seawater and 42 sediment samples were analyzed. Seawater temperature varied from 9 to 16°C through the course of this study, which was conducted from November 1977 to early April 1978.

Virus concentration from water. Attempts to isolate viruses from 20-liter samples of estuarine water were performed according to the procedure of Payment et al. (33). The following is a brief summary of the procedure. A 20-liter seawater sample was adjusted to pH 3.5 using 1 N HCl, and $AlCl_3$ was added to a final concentration of 0.0015 M. The solution was then passed through 3.0- and 0.45-μm-pore size Filterite filters (Filterite Corp., Timonium, Md.; Duo-Fine filters) in a flat 142-mm-diameter filter holder. Viruses were eluted with 50 ml of 0.05 M glycine (pH 11.5). The eluate was adjusted to neutrality using 1 M glycine (pH 2.0). The efficiency of this method was approximately 50% (14).

Virus concentration from sediment. Attempts to isolate viruses from sediment were performed according to a previously developed procedure (18), with one modification: tryptose phosphate broth containing 10% fetal calf serum adjusted to pH 11.5 was used as the eluent in the reconcentration step. The modified procedure for virus isolation is as follows: (i) addition of 400 ml (approximately 500 g [wet weight]) of sediment to 1,500 ml of 0.25 M glycine buffer containing 0.05 M ethylenediaminetetraacetic acid with a final pH of 11.0; (ii) shaking of the sediment-eluent mixture for 5 min; (iii) removal of sediment by centrifugation; (iv) adjustment of the supernatant to pH 3.5 by addition of 1 M glycine buffer (pH 2.0) (neutralization of the eluate should be performed as quickly as possible [within 15 min] to prevent inactivation of the virus due to the high pH [11]); (v) addition of 1 M $AlCl_3$ to a final concentration of 0.06 M; (vi) passage of the eluate through a series of adsorbent membrane filters (3.0- and 0.45-μm Filterite filters); (vii) elution of the virus from the filters with tryptose phosphate broth containing 10% fetal calf serum (pH 11.5); and (viii) neutralization of the filter eluate with 1 M glycine buffer (pH 2.0). The average efficiency was approximately 50% for the sediments examined in this study (18).

Virus isolation. Virus assays were performed by the plaque-forming unit method (39), using passages 140 to 150 of the buffalo green monkey kidney (BGM) cell line which was passaged, grown, and maintained by previously described methods (31). Samples of 2 ml were inoculated directly onto a monolayer of BGM cells in a 75-cm^2 flask. Virus was allowed to adsorb for 90 to 120 min at 37°C before the cells were overlaid with agar. Since the samples were obtained from polluted areas, 10 μg of neomycin sulfate and 100 U of mycostatin per ml were added to the overlay agar, in addition to 500 U of penicillin and 500 μg of streptomycin per ml. The overlaid cell cultures were incubated at 37°C and were examined daily for 10 to 14 days for the presence of plaques. All plaques were picked and passed once in BGM cells under liquid medium, and progeny from some samples were identified using pools of enterovirus antisera (30). From 50 to 100% of the total eluate volume of each sample was assayed, depending upon the concentration of virus in the final concentrate.

Bacteriological analysis. The determination of most probable numbers of total coliform and fecal coliform bacteria was carried out according to *Standard Methods for the Examination of Water and Wastewater* (3) and as detailed elsewhere (19). Clostridia were enumerated using differential reinforced clostridial medium as a presumptive medium in a most-probable-number test (9). The inoculated tubes were incubated anaerobically in a GasPak system (BBL Microbiology Systems, Cockeysville, Md.) at 37°C for 48 h. Blackening of the medium indicated the presence of clostridia. Cultures from positive tubes were transferred to litmus broth tubes, which were incubated at 37°C for 48 h. Acidification, coagulation, and gas production were considered positive for *Clostridium perfringens* (9).

Statistical analysis. The statistical evaluation of the relationships between the number of viruses in the sediments and the environmental variables was performed through multivariate regression analysis. Before this analysis, the scatter plots of the viral counts against each independent variable were examined.

This step proved to be very helpful in the search for a mathematical approximation of the complex process studied. For all variables (except for rain index, which was a discrete variable), the bivariate relationships with the viral count as a dependent variable were first approximated by a least-squares fit of a linear model. Further, the possibility was also considered that an exponential curve may provide better approximation of the biological relationships under study, and therefore the least-squares fit of the log-transformed data was tried.

The number of viruses in the sediments, denoted by Y, was studied as a function of various characteristics of water quality and sediments, denoted by $X_1, X_2, \cdots X_{13}$. This hypothesized functional dependence was evaluated by stepwise multiple regression using the SPSS computer package version 7 (32).

RESULTS

Water quality information obtained at the collection sites is presented in Table 1.

Since we were primarily interested in determining whether there was a relationship between the presence of viruses in sediment and the presence of viruses in seawater, most of the sites were chosen because of their known pollution (20). The most-probable-number values for coliforms at the sampling sites at the time of virus isolation are shown in Table 2. In all but one case of virus isolation from water, the total and fecal coliform counts exceeded the values recommended for recreational waters (1,000 total coliforms per 100 ml, 70 fecal coliforms per 100 ml) (12). On one occasion, when water met the standards for both recreational and shellfish-harvesting waters (70 coliforms per 100 ml), viruses were isolated from the water overlying an oyster bed. In four cases when the overlying seawater met the recommended standards for recreational water, viruses were isolated from sediment. At one of these sites (H-86), two of these cases occurred at the same approximate area on the same day and yielded virus isolates. This site is a sewage outfall area of a canal community. The total and fecal coliform counts in the sediment were also the highest observed at this site. The two other sites had relatively low numbers of total and fecal coliforms in the sediment. This indicates that, even though indicator organisms were present in low numbers in sediment, it was possible for virus to be present.

Of the 440 natural isolates obtained, 83 plaques were plucked, and attempts were made to grow virus in one or two passages in BGM cell culture. Twenty-three isolates were successfully passaged, and serotyping was attempted using enterovirus typing pools (30). Of the viruses grown in cell culture, eight were typed as coxsackievirus type B5 (coxsackie B5), seven as echovirus (echo) 1, and one each as coxsackie B1, coxsackie A16, and poliovirus (polio) 2. Five isolates were not typable by the enterovirus typing sera. We have isolated viruses from these waters in a previous study (20) and obtained several different types of enteroviruses. The two studies have only one virus in common: echo 1.

As stated above, the first step in the statistical evaluation of sampling data was the analysis of bivariate scatter plots for viral counts versus other individual characteristics of water or sediment. Such analysis indicated that out of 12 independent variables, only 1, the number of fecal coliforms found in the sediments, had a significant regression coefficient ($P < 0.0001$) with the number of viruses isolated from sediments. The association of the number of fecal coliforms and viruses in sediments was direct, meaning that the increase in fecal coliform number was associated with higher viral numbers, with the coefficient of linear correlation R equal to 0.76 (Fig. 1). The regression remained statistically significant when the semi-log transformation was applied (Fig. 2), although log-log transform worsened the fit.

Other variables were not found to be of statistical significance when studied on a one-to-one basis with the dependent variable (which was numbers of virus, either in water or sediment), and the exponential curve approximations were not substantially better than the linear. It is important that neither the number of viruses nor the number of fecal coliforms in water appeared to be a good predictor of the number of viruses found in the sediments, and vice versa.

The scattergram of the number of viruses versus pH (Fig. 3) may be noteworthy. The examination of the scattergram suggested that a pattern might exist here, i.e., the number of viruses appeared to increase only in a certain range of pH, from about 7.8 to 8.4; it dropped at both higher and lower ends of the pH scale. It is not possible to judge presently whether this pattern is real or artificial, but this should be studied further when more data become available.

The effect of each factor in the presence of others on the number of viruses in the sediments was evaluated using the stepwise multiple regression analysis, as explained in Materials and Methods. This showed essentially that a single variable, the fecal coliform count, explained as much as 57% of the variation in the number of viruses found in the sediment. The variance contribution due to other variables was insubstantial. With the addition of each new variable, the changes in R^2 were small. A sum-

TABLE 1. *Water quality at the collecting stations*

Water quality criteria[a]	Collecting stations[b]							
	H-86	H-86a	H-86b	H-81	K-27	323	T-50	Oyster beds no. 1-4
pH	7.5–8.3 (8.0)	7.5–8.3 (7.92)	7.6–8.0 (7.97)	7.6–7.9 (7.83)	8.0–8.7 (8.35)	8.06–8.13 (8.10)	8.11	8.1–8.4 (8.35)
Salinity (g/kg)	4–24 (19.0)	4–25 (14.5)	5.5–24 (22.0)	5–20 (12)	16–17 (16.5)	25.5–29 (27.3)	23	14–28 (17.5)
Turbidity (NTU)	3.5–22.0 (7.5)	2.7–19.0 (7.0)	4.0–5.0 (4.0)	4.0–18.0 (10)	5.5–19 (12.25)	2.9–3.5 (3.2)	2.5	11–33 (24)
TCW	200–24,000 (11,000)	460–24,000 (13,950)	240–24,000 (2,300)	7,500–24,000 (24,000)	4,600–24,000 (14,300)	240–7,500 (3,870)	23	4–460 (233)
FCW	90–24,000 (11,000)	400–24,000 (5,730)	93–24,000 (300)	2,300–11,000 (11,000)	210–4,600 (2,405)	240–390 (315)	<3	4–460 (233)
Cl.W	<3–2,400 (75)	<3–2,400 (240)	<3–93 (30)	<3–2,400 (1,500)	43–430 (237)	43–460 (252)	43	<3–9 (3.5)
Cl.p.W	<3–2,400 (75)	<3–2,400 (240)	<3–43 (30)	<3–2,400 (1,500)	43–430 (237)	7–460 (234)	4	<3–9 (3.5)
TCS	43,000–460,000 (210,000)	11,000–2,400,000 (240,000)	430–240,000 (7,500)	46,000–240,000 (110,000)	240,000–1,100,000 (670,000)	2,400–9,300 (5,850)	930	400–15,000 (1,450)
FCS	4,000–460,000 (46,000)	2,400–240,000 (22,000)	90–24,000 (7,500)	43,000–110,000 (46,000)	2,100–46,000 (24,050)	2,100–2,400 (2,350)	90	<300–15,000 (1,450)
Cl.S	11,000–240,000 (93,000)	110,000–460,000 (240,000)	900–110,000 (46,000)	43,000–240,000 (75,000)	2,400,000–2,400,000 (2,400,000)	400–2,800 (1,600)	3,900	400–240,000 (18,000)
Cl.p.S	11,000–240,000 (46,000)	110,000–460,000 (225,000)	900–46,000 (24,000)	20,000–240,000 (43,000)	1,100,000–2,400,000 (1,750,000)	400–1,500 (950)	2,400	400–15,000 (9,300)
Virus/20 l	0–528 (10.5)	0–30 (2.5)	0–1 (0.5)	0–8 (0)	1–2 (1.5)	0–0 (0)	0	0–7 (0)
Virus/20 l sediment	0–350 (70)	0–480 (50)	0–0 (0)	0–0 (0)	0–0 (0)	0–0 (0)	170	0–0 (0)

[a] TCW, FCW, TCS, FCS, Most probable number per 100 ml of total coliforms in water, fecal coliforms in water, total coliforms in sediment, and fecal coliforms in sediment, respectively. Cl.W, Cl.p.W, Cl.S, Cl.p.S, Most probable number per 100 ml of *Clostridium* spp. in water, *C. perfringens* in water, *Clostridium* spp. in sediment, and *C. perfringens* in sediment, respectively. Virus/20 l, Number of viruses isolated per 20 liters of seawater. Virus/20 l sediment, Number of viruses isolated per 20 liters of sediment. NTU, Nephelometric turbidity units.

[b] Numbers in parentheses represent the median values.

TABLE 2. *Isolation of virus from water and sediment and comparison to bacterial indicators*

Site	Date	PFU/20 l seawater[a]	PFU/20 l sediment[b]	MPN[c] of indicator organisms per 100 ml			
				Total coliforms		Fecal coliforms	
				Water	Sediment	Water	Sediment
H-86	11/15/77	6	200	24,000	46,000	24,000	46,000
	11/22/77	15	0	11,000	240,000	11,000	46,000
	4/12/78	374	0	24,000	240,000	11,000	110,000
	4/12/78	528	140	4,600	210,000	4,600	15,000
	2/27/78	0	350	200	460,000	90	460,000
	2/27/78	0	280	200	460,000	90	460,000
H-86a	11/15/77	0	50	460	11,000	460	2,400
	11/22/77	5	50	24,000	240,000	24,000	21,000
	12/01/77	30	480	24,000	240,000	11,000	240,000
T-50	11/15/77	0	170	23	930	<3	90
H-86b	11/22/77	1	0	24,000	7,500	24,000	7,500
H-81	11/22/77	8	0	24,000	110,000	11,000	110,000
K-27	12/09/77	2	0	24,000	240,000	4,600	46,000
	12/21/77	1	0	4,600	1,100,000	210	2,100
H-86[d]	2/27/78	0	50	50	1,100	6	200
Oyster bed no. 1	3/03/78	7	0	4	400	4	<300

[a] Number of viruses isolated per 20 liters of seawater.
[b] Number of viruses isolated per 20 liters of sediment.
[c] MPN, Most probable number.
[d] Sample taken 300 feet downstream.

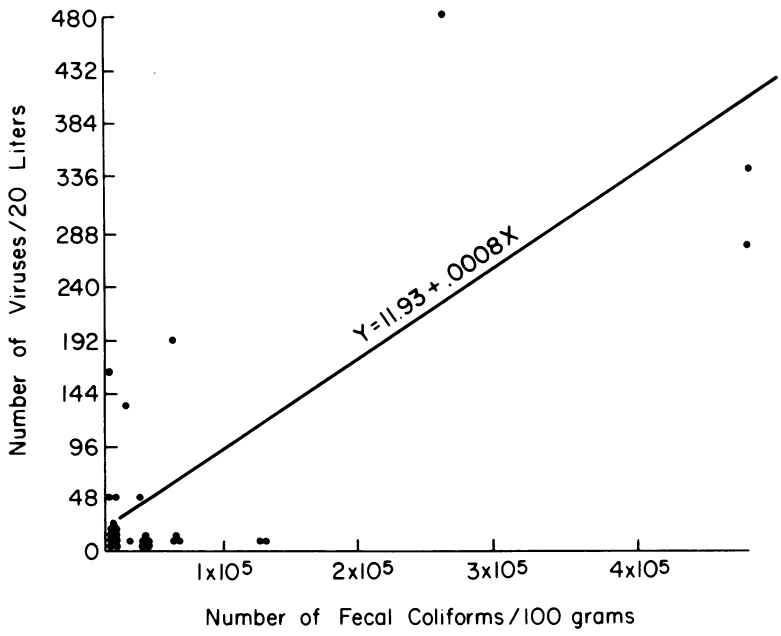

FIG. 1. *Scattergram of the number of fecal coliforms against the number of viruses found in sediment.*

mary of the stepwise multiple regression is given in Table 3. It can be seen that a model using 10 predictors, or independent variables, accounted for some 63% of the variability in the number of viruses, which is only 6% more than that accounted for by a single and easily measurable factor, fecal coliform count in the sediment. The logarithmic transformation of data for multiple regression was found to be disadvantageous.

An equation describing the relationship be-

tween the number of viruses in sediments (Y) and the number of fecal coliforms in sediments (X) is as follows: $Y = 11.93 + 0.0008X$.

DISCUSSION

A previous study (36) indicated that viruses could be found in higher numbers on a volume basis in estuarine sediment than in seawater. This study was undertaken to further investigate the presence of viruses in sediment and to attempt to correlate their presence in sediment with other indicators of fecal pollution. Research

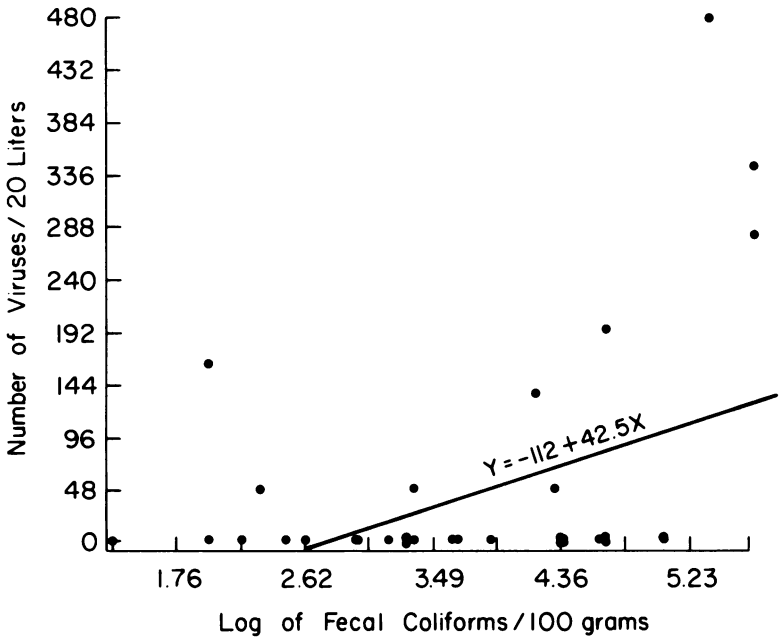

FIG. 2. *Scattergram of the logarithm of the number of fecal coliforms in sediment against the number of viruses in sediment.*

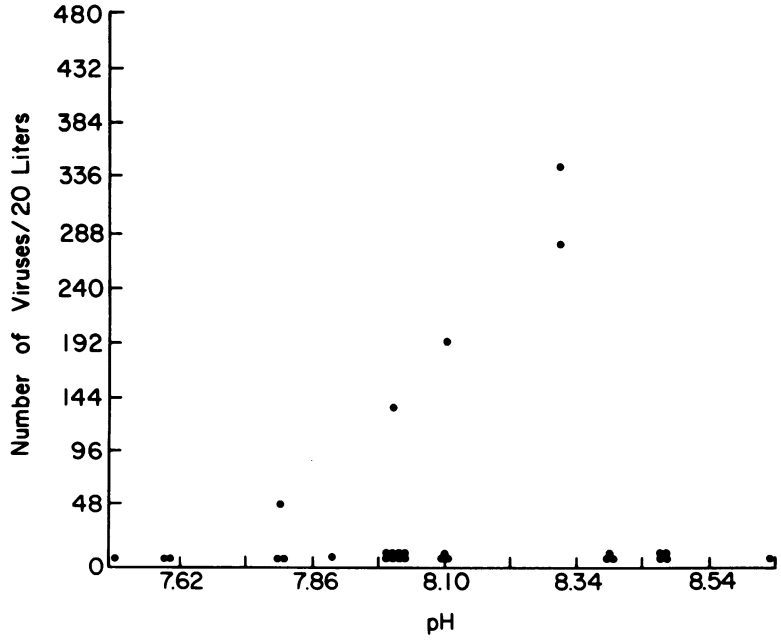

FIG. 3. *Scattergram of the pH of water against the number of viruses in sediment.*

TABLE 3. *Summary of the stepwise multiple regression of viruses in sediment with other environmental variables*

Variable[a]	Significance of individual variables	Multiple R	R^2	R^2 change	Simple R	Overall F	Significance of the regression
FCS	0.000	0.75672	0.57262	0.57262	0.75672	45.55436	0.000
Rainfall	0.180	0.77174	0.59558	0.02296	0.00429	24.29928	0.000
Cl.W	0.382	0.77800	0.60529	0.00970	0.18697	16.35707	0.000
Turbidity	0.387	0.78413	0.61487	0.00958	−0.00011	12.37284	0.000
pH	0.344	0.79145	0.62640	0.01153	−0.13200	10.05995	0.000
FCW	0.751	0.79229	0.62772	0.00132	0.09221	8.14962	0.000
TCW	0.567	0.79507	0.63214	0.00442	0.09426	6.87356	0.000
Salinity	0.727	0.79613	0.63382	0.00168	−0.25673	5.84174	0.000
Cl.p.S	0.771	0.79689	0.63503	0.00121	−0.10359	5.02649	0.001
TCS	0.887	0.79707	0.63533	0.00030	0.07235	4.35547	0.001

[a] Abbreviations as defined in Table 1.

conducted in this laboratory (20) indicated that the presence of enteric viruses in seawater was related to the number of total coliforms in sediment. However, the isolation of viruses from sediment was not attempted in that study.

Table 2 of the present study shows that viruses were isolated from sediments when overlying water met recreational water standards. Virus was also isolated from seawater when bacterial counts were below the recommended standard, which is in agreement with findings in a previous study (20). Viruses that were isolated and grown in tissue culture were typed as coxsackie B5, echo 1, coxsackie B1, coxsackie A16, and polio 2 (in order of the frequency of isolation). Only one virus type, echo 1, was the same as isolates obtained in a study conducted 2 years previously in many of the same sites. This indicates a change in the enterovirus population in the area, possibly due to selection because of acquired immunity in the host population.

Statistical analysis indicated that the number of viruses isolated from sediment was associated with only 1 of the other 12 factors measured, i.e., fecal coliform numbers in sediment. None of the other factors studied was related in any significant way to the number of viruses in sediment. In this study, indicator bacteria could not be correlated with the concentration of virus in water or any other variable. In a previous study on the occurrence of viruses in oyster-harvesting areas of Galveston Bay (21), a moderate correlation between total coliforms and viruses was observed, but this was not seen in the present study. This finding is significant because it indicates that no characteristic of overlying waters examined in this study could predict the occurrence of viruses in sediments and that only one characteristic of sediment, the number of fecal coliforms, correlated to the number of viruses in sediment. Statistical analysis permitted the derivation of a linear model relating the number of viruses in sediment and the number of fecal coliforms in sediment (see Results). The observed correlation between a bacterial indicator and virus in sediment is stronger than any observed in two previous studies we have conducted in Galveston Bay (20, 21).

The data also confirm a previous finding (15) that viruses are present in sediment at much higher concentrations than in seawater. It has been shown that bacteria (40) and viruses (25) readily adsorb to estuarine sediments, and *E. coli* and enteroviruses (16, 36) have also been shown to survive longer in the presence of sediment than in seawater alone.

Since there is obviously no opportunity for growth of viruses in sediment, their continued presence appears to be due to some protective effect of sediment. Thus, sediment may act as a reservoir of pathogens which may be resuspended by any kind of turbulent activity, such as boating, storms, dredging, etc. In terms of public health significance, the number of fecal coliforms and enteroviruses in sediment at a particular location may be a better indication of water quality over a long period of time than the number of bacteria and viruses in the overlying water.

European workers advocate the use of *C. perfringens* in the examination of chlorinated waters and the detection of remote pollution. It has been suggested also that in terms of survival, distribution of *C. perfringens* spores in the aquatic environment may be more analogous to that of enteroviruses (6). Several investigators have demonstrated widespread presence of this organism in coastal waters and their underlying sediments (5, 7, 28, 37). Since we have previously demonstrated longer survival of enteroviruses in sediments (36) and since *C. perfringens* spores are expected to settle to the bottom, survive, and accumulate (6), it was intended to determine

whether a relationship existed between this organism and enteroviruses. No relationship was found to occur between clostridia and enteroviruses.

In summary, we have demonstrated that viruses do appear in contaminated sediments at higher concentrations than in the overlying seawaters, and the number of viruses present in the sediments could be correlated with the number of fecal coliforms in the sediments. However, the number of viruses in seawater showed no correlation with the number of bacteria in seawater. It was shown that virus could be isolated from sediment and seawater when seawater contained few coliform bacteria. The results and relationships observed in the present study may be subject to variation in local physical, chemical, and biological conditions, as well as the variation in the efficiency of the sampling procedure for virus and bacteria. Thus, further research in similar ecosystems at other localities is certainly indicated.

The data suggest that an evaluation of the presence of bacteria and viruses in sediment may provide additional insight into long-term water quality conditions, and that current bacteriological standards for recreational and shellfish-harvesting waters need further study.

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