Caribbean Journal of Science, Vol. 24, No. 3-4, pp. 102-111, 1988

Enteric Viruses in a Mangrove Lagoon, Survival and Shellfish Incidence

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ABSTRACT.—Mangrove oysters (*Crassostrea rhizophorae*) were screened for enteric viruses. For 18 months oysters were collected from Caño Boquerón, a tropical mangrove lagoon on the southwest coast of Puerto Rico. This popular tourist resort has two primary sewage treatment plants which service 158 single family cabañas. In spite of the heavy seasonal input of treated sewage to Caño Boquerón and high densities of fecal coliform bacteria, enteric viruses were not detected in shellfish meat. Because no viruses were detected in the oysters, a virus survival study was performed. Poliovirus type 1 was placed in diffusion chambers in situ at two sites in Caño Boquerón. More than 95% of the poliovirus inactivation occurred within 24 h. Virus inactivation was significantly different by site, indicating different inactivation rates within the lagoon. Chamber studies done simultaneously with *Escherichia coli* did not reveal differences between sites. It is suggested that the sewage effluent had an antiviral effect in the absence of an antibacterial effect. This study demonstrates the importance for establishing microbial contamination standards for shellfish growing waters in the tropics based upon in situ studies with tropical species, e.g., mangrove oyster.

INTRODUCTION

Coliform bacteria have been extensively used as indicators for determining the microbiological safety of water; however, current studies indicate that fecal coliform counts do not accurately reflect the degree of virological contamination of water (Berg et al., 1978; LaBelle et al., 1980; Marzouk et al., 1980). High densities of viruses have been found in marine waters that meet fecal coliform and total coliform standards (Berg et al., 1978; Goyal et al., 1979). This is of particular importance to marine coastal areas where discharge of sewage effluent is commonplace almost irrespective of the sewage components and its impact on public health (Cooper and MacCallum, 1984). Suspended virus-contaminated particulate matter can be easily ingested by filter-feeding organisms like oysters, clams, and mussels that inhabit coastal waters and are harvested for consumption as raw or partially cooked seafoods (Hamblet et al., 1969; Sobsey, 1982). Predictably, the shellfish that are cultured in wastewater rich environments have been associated with hepatitis and gastroenteritis, sometimes with high human morbidity (Cooper and MacCallum, 1984).

Very little information exists about the fate of enteric viruses in tropical waters. It is suspected that the period of viral inactivation is shorter in tropical waters, than it is in temperate waters, but nothing is known about how virus interaction with the physical-chemical conditions of tropical waters can affect virus survival. Lund (1978) reported two studies, one conducted in Ghana, and the other in Thailand, that suggest the enteric viruses are found throughout the year at similar concentrations in these tropical areas.

Environmental factors like temperature, solar radiation, seasonal variability, and concentration of nutrients, are quite different in tropical as contrasted to temperate aquatic systems. Bigger first reported the growth of coliforms in tropical waters

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in 1937, while in 1939 Ragavachari and Iver showed that coliforms can survive for several months in natural tropical river waters. Recent studies in Puerto Rico (Hazen et al., 1987; Santiago-Mercado and Hazen, 1987; Valdés-Collazo et al., 1987) showed that the survival of fecal coliforms increased in marine and freshwater systems possibly because of the presence of high nutrient concentrations. Thus high counts of total coliforms and fecal coliforms do not necessarily indicate recent fecal contamination. The reliability of coliforms and fecal coliforms as indicators of recent fecal contamination in tropical waters is questionable when no identifiable source of fecal contamination has been detected (Hazen et al., 1987; Santiago-Mercado and Hazen, 1987). The present study examines the extent of tropical oyster contamination in a lagoon known to receive human fecal contamination and the in situ survival of enteric viruses and *Escherichia coli* in this lagoon.

MATERIALS AND METHODS

Study Site.—Boquerón Bay is located at the southwest corner of Puerto Rico (18°15′N, 67°10′W) near Cabo Rojo (Fig. 1). This bay has an average depth of 3.28 m with a shallower inner lagoon, Caño Boquerón, which covers 0.687 km^2 with an average depth of 2.5 m. The lagoon is surrounded by a fringe mangrove forest (Martínez et al., 1979) where the majority of the oysters that are sold in the area are harvested. Caño Boquerón is adjacent to a public recreational resort (Centro Vacacional de Boquerón). This popular tourist resort has 158 single family cabañas that are fully occupied during summer months, December and during weekends all year around. The resort has two primary sewage treatment plants that pump effluents directly into the lagoon. Sampling site 1, was within 10 m of the outfall of primary water treatment plant 2. Site 2 was 600 m to the east of site 1.

Water Quality and Sample Collection.—Measurements in situ of pH, salinity, temperature, dissolved oxygen (DO), and conductivity were done using a Hydrolab surveyor (digital model 4041, Hydrolab Corp., Austin, TX). Water samples were collected in 500 ml Nalgene bottles for further analysis in the laboratory for: turbidity, ammonia, nitrate plus nitrite, phosphate, and total phosphorus. These water samples were preserved by fixation with sulfuric acid or mercuric chloride. Turbidity was measured by the spectrophotometric method (APHA, 1985). Water samples for chlorophyll *a* analysis were taken in 500 ml Nalgene amber bottles and analyzed by the trichromatic extraction method (APHA, 1985).

Total and fecal coliform counts were determined by the membrane filtration technique (APHA, 1985). HA type membrane filters (Millipore Corp., Bedford, MA) were placed on m-Endo media and incubated for 24 h at 37°C for total coliform determinations. Only green sheen colonies were enumerated. HC type membrane filters were placed on m-FC media and incubated at 44.5°C in a block type incubator (Millipore) for 24 h. Blue colonies were enumerated as fecal coliforms.

Oysters of the species *Crassostrea rhizophorae* were collected from the roots of the mangroves and placed in sterile 500 ml Whirl-Pak bags. The bags were placed on ice and transported to the laboratory for analysis. The oysters were washed and scrubbed with a bristle brush in running tap water, then rinsed with 70% alcohol and opened aseptically. The whole meat was removed, weighed, and stored in 150 ml Whirl-Pak bags at −70°C until assayed.

Enterovirus Extraction from Shellfish.—Two virus extraction procedures based on adsorption-elution precipitation developed by Richards et al. (1982) and by Sobsey et al. (1978) were used with minor modifications in the first procedure. The procedure by Richards et al. (1982) consists of weighing 50 g of the whole oyster meat and homogenizing it for 2 min in a 1000 ml blender with 450 ml of a glycine-NaCl buffer at pH 9.5, and when necessary adjustment to pH 9.5 with 6 N NaOH was done to allow virus elution from the oyster meat. A 1% Cat-Floc solution (Calgon Co., Ponce, PR) was added to the homogenate at a proportion of 10 ml/50 g of sample.

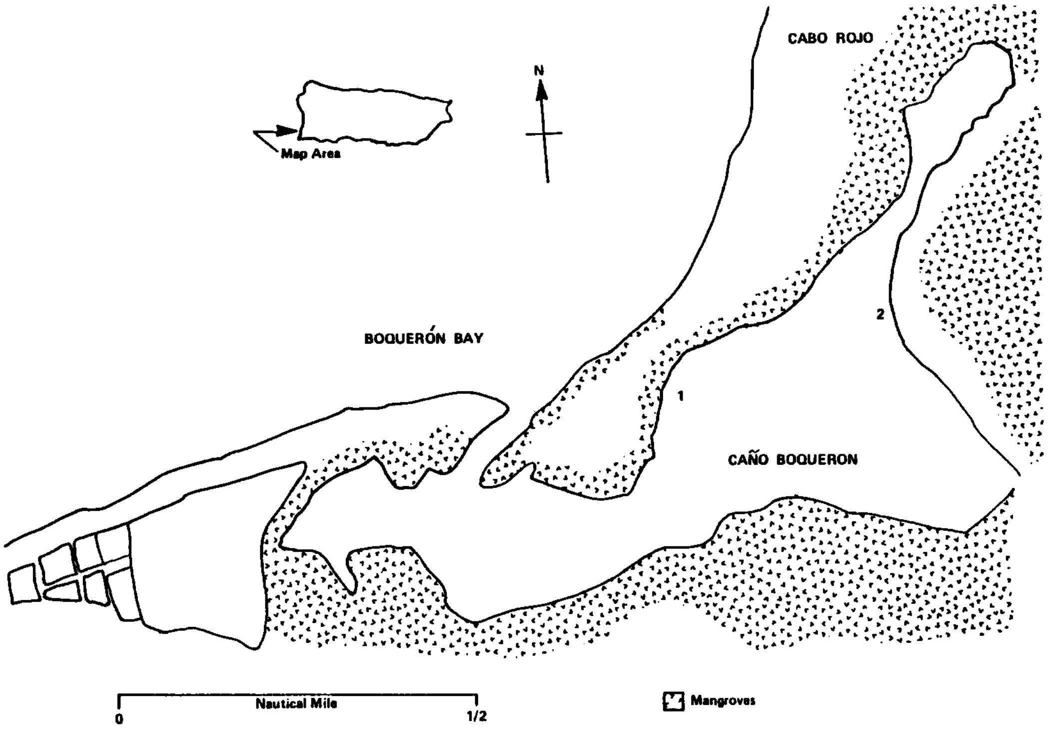

FIG. 1. Map of sampling sites in the Caño Boquerón Lagoon.

Cat-Floc is a cationic organic soluble polymer that precipitates shellfish-associated components that are toxic to the cell cultures used for virus assay of sample concentrate (Sobsey, 1982). The homogenate was stirred for 5 min and transferred to 50 ml centrifuge tubes where it was allowed to stand for 15 min at 4°C. After centrifugation the pellet was discarded. Equal volumes of a 3% beef extract solution at pH 9.5 was added to the supernatant. The mixture was acid precipitated by adjusting its pH to 3.5 with 6 N HCl, and was stirred slowly for 10 min to allow precipitation. Acid precipitation was followed by a centrifugation at 10,440 × *g* for 15 min at 4°C. Afterwards, the supernatant was discarded. The pellet was resuspended in 30 ml of 0.1 M Na_2HPO_4 at pH 9.5. The concentrate pH was then adjusted to 7.3–7.5 with 1 N NaOH. Contaminants were removed by treating the concentrate with antibiotics, 0.2 ml of penicillin-streptomycin 10,000 μg/ml-10,000 mcg/ml (Gibco, Grand Island, NY) followed by 1 h incubation at 37°C. After incubation the extract was frozen at −70°C until assayed on cell monolayer.

Sobsey's procedure consisted of homogenizing 50 g of shellfish meat in a 1000 ml blender with cold sterile distilled water (1:7 wt/vol), for 1–2 min. The pH of the homogenate was adjusted to 5.0 with 0.05 M glycine-HCl (pH 1.5) and to a salt concentration of 2000 mg NaCl/L. The homogenate was centrifuged at 1500 × *g* for 20 min. The supernatant fluid was discarded, and the sediment was resuspended in a glycine-NaCl buffer (0.05 M glycine and 0.15 M NaCl) at pH 7.5 and a conductivity of 8000 mg/L NaCl (1:7 wt/vol). The resuspension was centrifuged at 2000 × *g* for 15 min. The pellet was discarded, and the supernatant was filtered through a serum-treated 0.02 μm membrane filter. The filtrate was concentrated by precipitation at pH 3.5 by adding 0.05 M glycine-HCl (pH 1.5) and was slowly mixed for 10–15 min to allow precipitation. Then the sample was centrifuged at 1500 × *g* for 10 min. The supernatant fluid was discarded, and the pellet was resuspended in the least volume possible of 0.1 M Na_2HPO_4 buffer at pH 9.0. The resuspension was adjusted to pH 7.2–7.4 with 0.05 M glycine-NaOH (pH 11.5). Thereafter 10× antibiotics (penicillin–streptomycin 10,000–10,000 mg/ml) were added, and the sample was incubated for 1 h at 37°C. After incubation, the extraction was frozen at −70°C until assayed on cell monolayers.

Enterovirus Enumeration Assays.—The environmental samples were assayed on low passage BGM and MA-104 cell lines, that were grown in 25 cm^2 flasks (Corning Glass Work, New York, NY) at 37°C, until a monolayer was achieved, usually 4–5 days (Richards et al., 1982). BGM cells were grown in Eagle's minimum essential medium with Hanks' salts (HMEN) supplemented with 5% fetal bovine serum (FBS), antibiotics (5 ml penicillin–streptomycin 10,000 μg/ml–10,000 mcg/ml and 0.5 ml gentamycin 50 mg/ml (GIBCO, Grand Island, NY), or 1 ml kanamycin 10,000 mcg/ml (GIBCO, Grand Island, NY)), 12 ml HEPES (Research Organics Inc., Cleveland, OH) at pH 7.4, 4 ml 200 mM L-glutamine (Flow Lab., McLean, VA) and 8 ml $NaHCO_3$ at 7.5%. When needed, 1 ml of fungizone 250 mcg/ml (Flow Lab., McLean, VA) was added. MA-104 cell line was grown under the same conditions except that 8% FBS was added to the culture media.

When confluent monolayers were observed, the spent medium was discarded and the cells were washed twice with 5 ml of Tris buffered saline (TBS) which contained 20 mM Tris, 140 mM NaCl, 5 mM KCl, 0.4 mM Na_2HPO_4, and 6 mM dextrose. Tenfold dilutions of the virus extraction were prepared in a diluting buffer which contained 100 ml of TBS plus 1–2.5 ml penicillin–streptomycin 10,000–10,000 mcg/ml and 0.25 ml gentamycin. The monolayers were inoculated with 0.5 ml of the virus dilution and incubated for 1 h at 37°C, rocking the flasks every 15 min to avoid monolayer desiccation. After incubation, the inoculum was discarded and the cells were washed once with 5 ml TBS prewarmed to 37°C. When CPE assays were run, 8 ml complete culture medium was added to each flask. The flasks were incubated at 37°C and observed for the presence of CPE every 24 h.

The technique used to overlay the cultures was as described by Melnick and Wenner (1969). The cells were overlayed with 8 ml agar overlay medium that contained (per 100 ml) 50 ml 2× MEM, 2% heat inactivated FBS, 3 ml $NaHCO_3$ at 7.5%, 2 ml 200 mM L-glutamine, 2.5 ml penicillin–streptomycin 10,000 μg/ml–10,000 mcg/ml, and 1:300 neutral red (GIBCO, Grand Island, NY) mixed with 50 ml 3% agar (Difco, Detroit, MI). After overlying, the flasks were incubated at 37°C in the dark. Plaques were enumerated every 24 h for 3–4 days. Only rounded single clear plaques were enumerated. Every plaque was assumed to represent one PFU.

Viral Inoculum Preparation.—Poliovirus assays were performed with the BGM cell line, which was passed, grown, and maintained by previously described methods (1980). BGM cell monolayers were grown in 75 cm^2 flasks (Corning Glass Work, New York, NY). The cell medium was drained, and the monolayers were rinsed twice with 5 ml TBS. The monolayers were inoculated with 0.5 ml of a 10^{-3} dilution of type 1 poliovirus (Sabin), provided by Dr. G. Kuno, CDC San Juan laboratories, PR. The flasks were incubated at 37°C and rocked every 15 min for 1 h. After incubation the inoculum was drained and the flasks were filled with 15 ml fresh media without serum. The flasks were incubated at 37°C for 48 h or until 4 plus CPE developed in which 99% of the cells were infected with the virus. The infected monolayers were then frozen at −70°C and thawed in a 37°C water bath three times. The contents of the flasks were combined and centrifuged for 10 min at 2000 × *g* to rid the suspension of cell debris. The supernatant fluid was filtered and pretreated with 3% heat-inactivated FBS to disperse the viruses. Viral aliquots of 10 ml were dispensed into sterile freezing tubes and frozen at −70°C until the virus titer was determined by the plaque assay previously described (Melnick and Wenner, 1969).

Bacterial Inoculum Preparation.—Pure cultures of *Escherichia coli* B was grown in nutrient broth (5% TSB) for 24 h at 37°C. The cells were harvested by centrifugation at 5000 × g for 10 min and washed in filter-sterilized PBS (pH 7.0). The number of cells per ml was estimated with a model ZF Coulter Counter (Coulter Electronics, Hialeah, FL) and adjusted to 10^7 cells/ml.

Survival Study.—The chambers and their use have been described before (McFeters and Stuart, 1972; Biamón and Hazen, 1981; Hazen and Esch, 1983; Toranzo and Barja, 1983; López-Torres et al., 1987, 1988). The diffusion surface for viruses was created by two filters. A 10 nm internal filter (Nuclepore, Pleasanton, CA) (Landry et al., 1982) and a 0.45 μm, 142 mm diameter nylon reinforced Versapore membrane filter (Gelman Instrument Co., Ann Arbor, MI) were used to avoid viral diffusion, while allowing fluid exchange with the surrounding environment. The diffusion surface for bacteria was created by a 0.45 μm, 142 mm diameter nylon reinforced Versapore membrane filter.

Eight sterile diffusion chambers, four adjusted to contain 10^3 viral particles/ml and four with 10^7 bacterial cells/ml were placed 0.5 m below the surface at the study sites, site 1 and site 2. Site 1 chambers were placed in the shadows of the mangrove trees while site 2 chambers were placed in an exposed area.

Samples (1 ml) were taken from the chambers with sterile tuberculin syringes at different time intervals for 130 h. Bacterial samples were fixed with 1.5 ml 10% phosphate buffered formalin for further counting in the laboratory with the Coulter Counter (Hazen and Esch, 1983).

Virus samples were frozen at 4°C and then transported to the laboratory where they were placed at −70°C until assayed on BGM and/or MA-104 cell monolayers.

Data Analysis.—Statistical analysis was conducted with the programs developed for Apple II and IBM 370/148 computers. The two factor analysis of variance (FANOVA) was used to test the differences between sites and time of collection. The data were made more homoscedastic by transformation with $\log(x + 1)$. Any statistical probability equal to or less than 0.05 was considered significant (Zar, 1984).

Results and Discussion

Caño Boquerón is a saline, hypereutrophic, tropical mangrove lagoon, receiving seasonally moderate contamination from two primary sewage treatment plants. Representative water quality is given in Table 1. Site 1 had significantly higher phosphates, total phosphorus, ammonia, and nitrates plus nitrites than site 2. Fecal coliforms at site 1 ranged from 210 to 200,000 CFU/100 ml, while total coliforms ranged from 500 to 1,300,000 CFU/100 ml. However, site 2 which was approximately 600 m away from the outfall never exceeded recommended recreational water maximum contaminant levels (MCL) for fecal coliforms (200 CFU/100 ml) or total coliforms (1000 CFU/100 ml) (Cabelli, 1978). Site 1 was thus highly contaminated with sewage of fecal origin. The effluent adjacent to site 1 was receiving only primary treatment at best. Site 2 was minimally effected by this effluent.

Despite repeated attempts to demonstrate enteric viruses in mangrove oysters from site 1, none could be found (Table 2). Two different cell lines (BGM and MA-104), two different extraction techniques, and two different viability assays on samples taken at 15 different times over an 18 month period, failed to detect any enteric viruses. Because this study represents the first report of screening of mangrove oysters (*Crassostrea rhizophorae*), parts of two samples were also sent Dr. Charles P. Gerba, University of Arizona and Dr. Mark B. Sobsey, University of North Carolina for enteric virus analysis. Both laboratories independently confirmed that no enteric viruses were detectable in the shellfish tissue from site 1. Since fecal coliform and coliform densities were always high in the shellfish meats (>1000 CFU/ml) and since the surrounding waters were obviously contaminated with recent fecal contamination it was surprising that no enteric virus could be detected.

The rate of viral uptake and depuration in shellfish is normally a function of temperature and salinity (Seraichekas et al., 1968; Haven and Morales, 1970; Metcalf et al., 1979). Since both temperature and sa-

TABLE 1. Water physicochemical parameters by sampling site.

	Parameter					
	ATEMP	WTEMP	DO	pH	NO_{2+3}	TP
Site 1	26.4 ± 1.6	26.2 ± 0.9	5.3 ± 1.2	7.5 ± 0.1	0.26 ± 0.11	8.5 ± 1.9
Site 2	28.8 ± 2.4	26.8 ± 0.8	4.0 ± 0.9	7.3 ± 0.1	0.10 ± 0.02	6.9 ± 1.4

	Parameter					
	Chl *a*	SAL	NH_4	TURB	TC	FC
Site 1	3.08 ± 1.17	33.7 ± 1.3	0.31 ± 0.12	94 ± 2	5,160 ± 4,146	2,264 ± 1,643
Site 2	4.18 ± 1.03	32.7 ± 1.2	0.24 ± 0.08	97 ± 1	307 ± 173	105 ± 54

All values are mean ± one standard error ($n = 7$). ATEMP = air temperature (°C), WTEMP = water temperature (°C), DO = dissolved oxygen (mg/L), TURB = turbidity (% transmittance), SAL = salinity (ppt), NH_4 = ammonium (mg/L), NO_{2+3} = nitrites plus nitrates (mg/L), TP = total phosphorus (mg/L), Chl *a* = chlorophyll *a* (mg/L), TC = total coliforms (CFU/100 ml), FC = fecal coliforms (CFU/100 ml).

linity are higher in Caño Boquerón than previous studies in temperate and subtropical areas, lower rates of uptake and/or faster depuration may be occurring in this lagoon. Other studies in temperate marine waters have suggested that temperature is the principal environmental factor that influences the rate of enterovirus inactivation in marine waters (Akin et al., 1976; O'Brien and Newman, 1977; LaBelle and Gerba, 1979, 1980). Thus higher rates of inactivation would be predicted in the higher temperature water of Caño Boquerón.

Poliovirus inactivation in the in situ diffusion chambers was complete within 72 h at both sites and had reached 98% within 24 h (Fig. 2). Fujioka et al. (1980) reported a 90% reduction of poliovirus at 24°C in seawater samples, obtained from different

TABLE 2. Virus extraction assays.

Date	Cell line	Extraction	Assay	Dilutions	Results
08-17-83	BGM	S1-06/83	PFU	10^{-1}-10^{-11}	0 PFU
09-24-83	MA-104	S1-06/83	CPE	10^{-2}-10^{-10}	0 CPE
09-25-83	MA-104	S1-06/83	PFU	10^{-2}-10^{-10}	0 PFU
09-25-84	MA-104	S2-06/83	PFU	10^{-2}-10^{-10}	0 PFU
09-29-83	BGM	S1-06/83	CPE	10^{0}-10^{-3}	0 CPE
10-01-83	BGM	S2-06/83	CPE	10^{0}-10^{-6}	0 CPE
10-04-83	BGM	S1-06/83	PFU	10^{0}-10^{-6}	0 PFU
	BGM	S2-06/83	PFU	10^{0}-10^{-6}	0 PFU
11-15-83	MA-104	S1-09/83	CPE	10^{-2}-10^{-10}	0 CPE
	MA-104	S2-09/83	CPE	10^{-2}-10^{-1}	0 CPE
11-16-83	MA-104	S1-09/83	PFU	10^{-2}-10^{-10}	0 PFU
	MA-104	S2-09/83	PFU	10^{-2}-10^{-10}	0 PFU
11-29-83	MA-104	S1-11/83	CPE	10^{0}-10^{-10}	0 CPE
12-05-83	MA-104	S2-11/83	CPE	10^{0}-10^{-10}	0 CPE
12-14-83	MA-104	S1-12/83	CPE	10^{0}-10^{-10}	0 CPE
12-15-83	MA-104	S2-12/83	CPE	10^{0}-10^{-10}	0 CPE
02-15-84	BGM	S1-09/83	PFU	10^{0}-10^{-8}	0 PFU
	BGM	S2-09/83	PFU	10^{0}-10^{-8}	0 PFU
02-21-84	BGM	S1-11/83	PFU	10^{0}-10^{-8}	0 PFU
	BGM	S2-11/83	PFU	10^{0}-10^{-8}	0 PFU
04-17-84	BGM	S1-12/83	PFU	10^{0}-10^{-8}	0 PFU
	BGM	S2-12/83	PFU	10^{0}-10^{-8}	0 PFU

PFU = plaque forming unit, CPE = cytopathological effect.

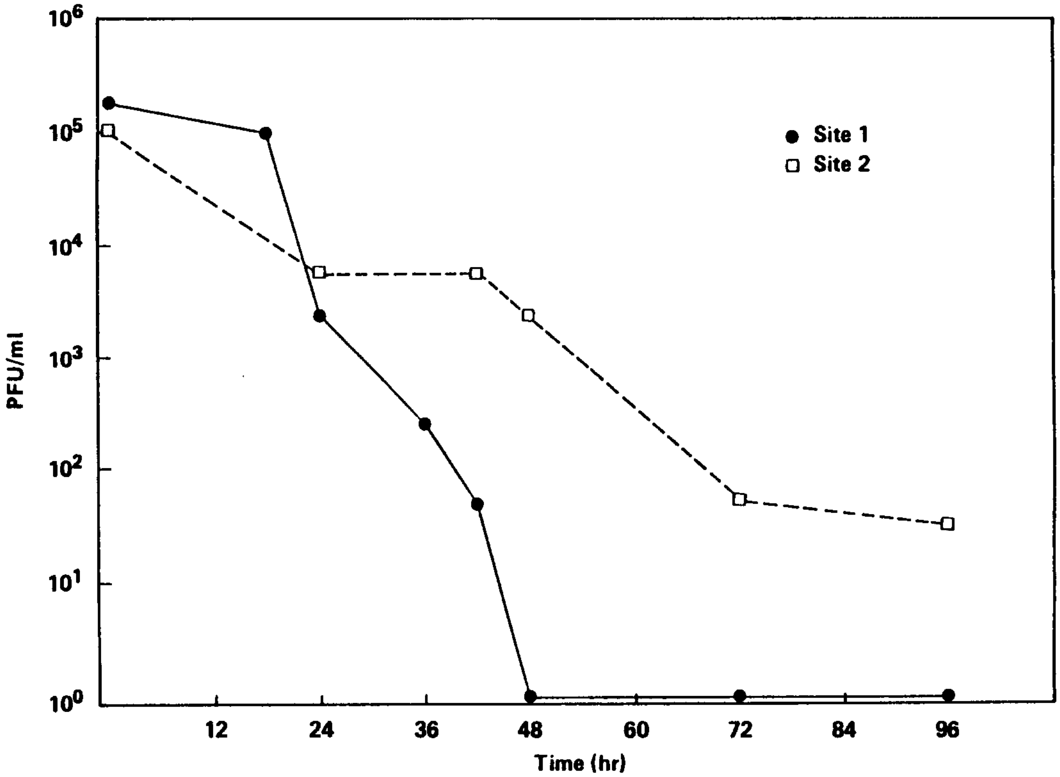

FIG. 2. Survival of poliovirus serotype 1 at Caño Boquerón Lagoon by site (mean density of poliovirus in plaque forming units/ml).

sites in Hawaii, within 48 h and complete inactivation within 96 h. The higher temperature (24.5–29°C) and in situ conditions of our study might explain the faster inactivation. Akin et al. (1976) using Gulf of Mexico water at 24°C observed complete inactivation of poliovirus only after 120 h. Significant differences in virus inactivation between sites was also observed in Caño Boquerón. Poliovirus inactivation was higher at the effluent point source (site 1) than at site 2 (Fig. 2). Thus the specific conditions at the sewage outfall caused faster than normal inactivation of poliovirus.

Densities of *E. coli* in the diffusion chambers declined very rapidly after the first 3 h, but stabilized and continued to decrease slowly until the end of the study (Fig. 3). Densities of *E. coli* were significantly different over time, but not by site. These results are similar to those obtained by López et al. (1988) and Valdés-Collazo et al. (1987) for *E. coli* in other coastal marine waters receiving high organic contamination in Puerto Rico. The differences between sites observed for poliovirus inactivation were not observed for *E. coli* survival. Thus the sewage outfall was associated with an antiviral effect but not an antibacterial effect. Among the processes controlling virus inactivation, it has been suggested that microbial antagonism could be an important factor in marine environments (Cliver and Herrmann, 1972; Toranzo and Barja, 1983).

The higher rates of viral inactivation observed at site 1 could be due to extracellular bacterial antiviral activity. As discussed, densities of indicator bacteria were several orders of magnitude higher at site 1. Proteolytic or virolytic antimicrobial action of seawater has been reported by several investigators (Matossian and Garabedian, 1967; Fujioka et al., 1980). Akin et al. (1976) and O'Brien and Newman (1977) both re-

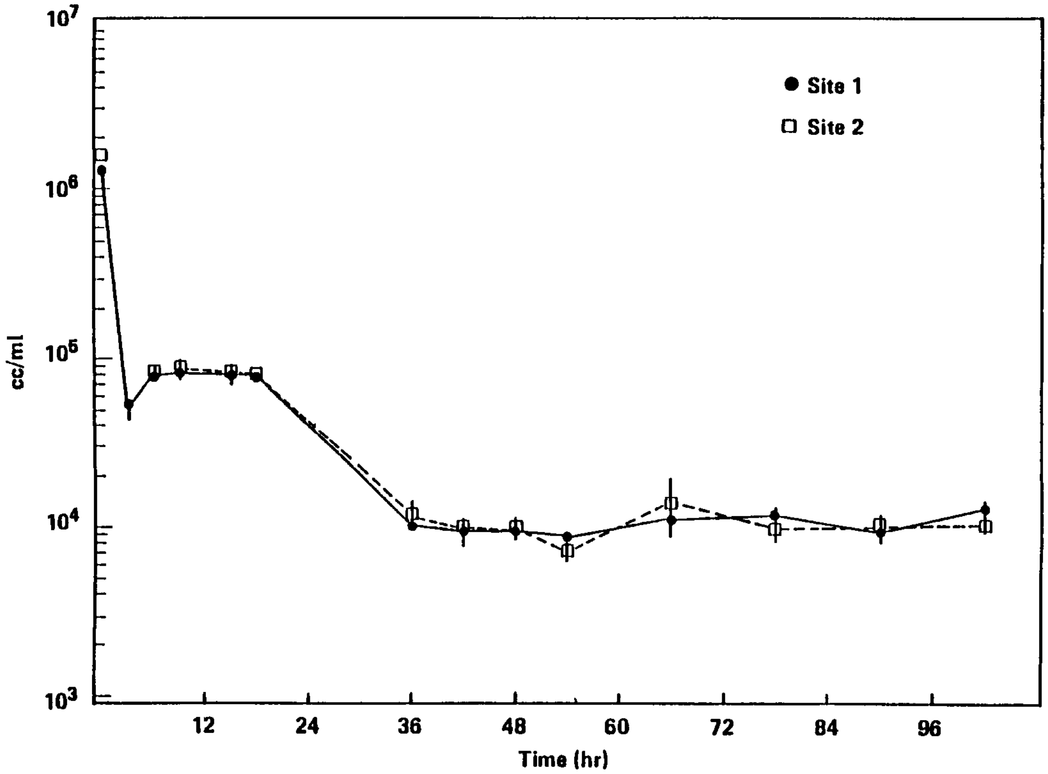

FIG. 3. Survival of *Escherichia coli* at Caño Boquerón Lagoon by site (mean ± one standard error, $n = 4$).

ported that enteric viruses survived longer in filtered and/or autoclaved water than in raw water. Fujioka et al. (1980) also showed that enteric viruses survive longer if antibacterial compounds are added to the water. Microbial antagonism is probably a major factor affecting virus inactivation in Caño Boquerón.

Undetectable levels of enteric viruses were observed in mangrove oysters from a sewage contaminated lagoon. Virus inactivation rates were significantly higher in this tropical lagoon than in temperate areas, probably due to higher temperature and salinity. The sewage outfall was associated with an antiviral effect but not an antibacterial effect on in situ suspensions of poliovirus and *E. coli*, this suggests that microbial antagonism also may be a major factor in increasing viral inactivation rates in the lagoon. The lack of correlation between densities of fecal coliforms and/or coliforms and virus incidence suggest that new standards for shellfish growing waters must be developed for the tropics using tropical environmental conditions and tropical species of shellfish.

Acknowledgments.—We thank Susan Rivera, Francisco Fuentes, Andrés Calvo, William Arias, Inés Muñiz, Jésus Santiago and Luz Rodríguez for their technical assistance. We are especially gratetul to Dr. Gary A. Toranzos who made many helpful suggestions about the manuscript. This work was supported by the Water Resources Research Institute of the University of Puerto Rico at Mayagüez and in part by Sea Grant R/LR-08-87-THA1 and Public Health Service grants RR-2657 and RR-8102 from the National Institutes of Health. In addition, portions of the information contained in this article were developed during the course of work under Contract No. DE-AC09-76SR00001 with the U.S. Department of Energy.

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Accepted: 23 November 1988.