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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 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, eg. 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 (3, 17, 23). High densities of viruses have been found in marine waters that meet fecal coliform and total coliform standards (3, 10). This is of particular importance to marine coastal areas where discharge of sewage is commonplace almost irrespective of the sewage components and its impact on public health (8). 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 (11, 34). Predictably, the shellfish that are cultured in wastewater rich environments have been associated with hepatitis and gastroenteritis, sometimes with high human morbidity (8).

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 (21) reported two studies, one conducted in Ghana, and the other in Thailand, that suggests 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 compared to temperate aquatic systems. Bigger (5) first reported the growth of coliforms in tropical waters in 1937, while in 1939 Ragavachari and Iyer (29) showed that coliforms can survive for several months in natural tropical river waters. Recent studies in Puerto Rico (14, 31, 36) 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 (14, 31). 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.

(This study was part of the M.S. thesis of I. López de Cardona at the University of Puerto Rico, Río Piedras, Puerto Rico, 1984.)

MATERIALS AND METHODS

Study Site. Boquerón Bay is located at the southwest corner of Puerto Rico (18° 15" N and 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 (22) 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 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 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 (1). Water samples for chlorophyll *a* analysis were taken in 500 ml Nalgene amber bottles and analyzed by the trichromatic extraction method (1).

Total and fecal coliform counts were determined by the membrane filtration technique (1). 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 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 and developed by Richards et al. (30) and by Sobsey et al. (33), were used with minor modifications in the first procedure. The procedure by Richards et al. (30) consists of weighing 50 g of the whole oyster meat which was the homogenized for 2 min in a 1,000 ml blender with 450 ml of a glycine-NaCl buffer at pH 9.5. The homogenate was adjusted to pH 9.5 with 6N NaOH, this provides virus elution from the oyster meat. A 1% Cat-Floc solution (Calgon Co., Ponce, PR) was added to the homogenate at a rate of 10 ml per 50 g of sample. 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 (34). The homogenate was stirred for 5 min and transferred to 50 ml centrifuge tubes where it was let stand for 15 min at 4°C. After centrifugation the pellet was discarded. Equal volumes of 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 6N HCl, and was stirred slowly for 10 minutes to allow precipitation. The acid precipitation was followed by a centrifugation at 10,440 x g for 15 min at 4°C. Afterwards, the supernatant was discarded. The pellet was resuspended in no more than 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 1N NaOH. Contaminants were removed by treating the concentrate with antibiotics, 0.2 ml of penicillin-streptomycin 10,000 µg/ml-10,000 mcg ml^{-1} (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 1,000 ml blender with cold sterile distilled water (1:7 wt/vol), for 1-2 min. The homogenate pH is adjusted to 5.0 with 0.05 M glycine-HCl (pH 1.5) and to a salt concentration greater or equal to 2,000 mg NaCl $liter^{-1}$. The homogenate was centrifuged at 1,500 x 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 8,000 mg $liter^{-1}$ NaCl in a 1:7 wt/vol. The resuspension was centrifuged at 2,000 x 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 1,500 x 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 is adjusted to pH 7.2-7.4 with 0.05 M glycine-NaOH (pH 11.5). Thereafter 10X antibiotics (penicillin-streptomycin 10,000-10,000 mg ml^{-1}) 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 to 5 days (30). BGM cells were grown in Eagle's minimum essential medium with Hank's salts (HMEN) supplemented with 5% fetal bovine serum (FBS), antibiotics (5 ml penicillin-streptomycin 10,000 µg/ml-10,000 mcg ml^{-1} and 0.5 ml Gentamycin 50 mg/ml (GIBCO, Grand Island, NY), or 1 ml kanamycin 10,000 mcg ml^{-1} (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 fungizone 250 mcg ml^{-1} (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 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 to 2.5 ml penicillin-streptomycin 10,000-10,000 mcg ml^{-1} 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 (26). The cells were overlayed with 8 ml agar overlay medium that contained (per 100 ml) 50 ml 2X 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^{-1} -10,000 mcg ml^{-1}, and 1:300 neutral red (GIBCO, Grand Island, NY) mixed with 50 ml 3% agar (Difco, Detroit, MI). After overlaying, the flasks were incubated at 37°C in the dark. Plaques were enumerated every 24 h for 3 to 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 (16). 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 a 10^{-3} dilution 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 flasks contents were combined and centrifuged for 10 min at 2,000 rpm to rid the suspension of cell debris. The supernatant fluid was filtered pretreated with 3% heat-inactivated FBS to disperse the viruses. Viral aliquots of 10 ml were dispensed to sterile freezing tubes and frozen at -70°C until the virus titer was determined by the plaque assay previously described (26).

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 5,000 rpm 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^{-1}.

Survival Study. The chambers and their use is as described before (4, 13, 19, 20, 25, 35). The diffusion surface for viruses was created by two filters. A 10 nm internal filter (Nuclepore, Pleasanton,

Ca) (18) 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^{-1} and four with 10^7 bacterial cells ml^{-1} 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 (13).

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 (37).

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 210-200,000 CFU per 100 ml, while total coliforms ranged from 500-1,300,000 CFU per 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 per 100 ml) or total coliforms (1000 CFU per 100 ml) (6). Obviously site 1 was grossly contaminated with sewage of fecal origin. The effluent that was 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 a 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 virus 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^{-1}) 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 (12, 27, 32). Since both temperature and salinity 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 there rate of enterovirus inactivation in marine waters (2, 15, 16, 28). 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% with 24 h (Fig. 2). Fujioka et al. (9) reported a 90% reduction of poliovirus at 24°C in seawater samples, obtained from different 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. Aiken et al. (2) 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. (20), and Valdes-Collazo et al. (36) 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 having an antiviral effect in the absence of an antibacterial effect. Among the processes controlling virus inactivation, it has been suggested that microbial antagonism could be an important factor in marine environments (7, 35).

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 (9, 24). Akin et al. (2) and O'Brien and Newman (28) both reported that enteric viruses survived longer in filtered and/or autoclaved water than in raw water. Fujioka et al. (9) 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 also observed to have an antiviral effect in the absence of an antibacterial effect on **in situ** suspensions of poliovirus and E. coli, this suggests that microbial antagonism is also a major factor 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.

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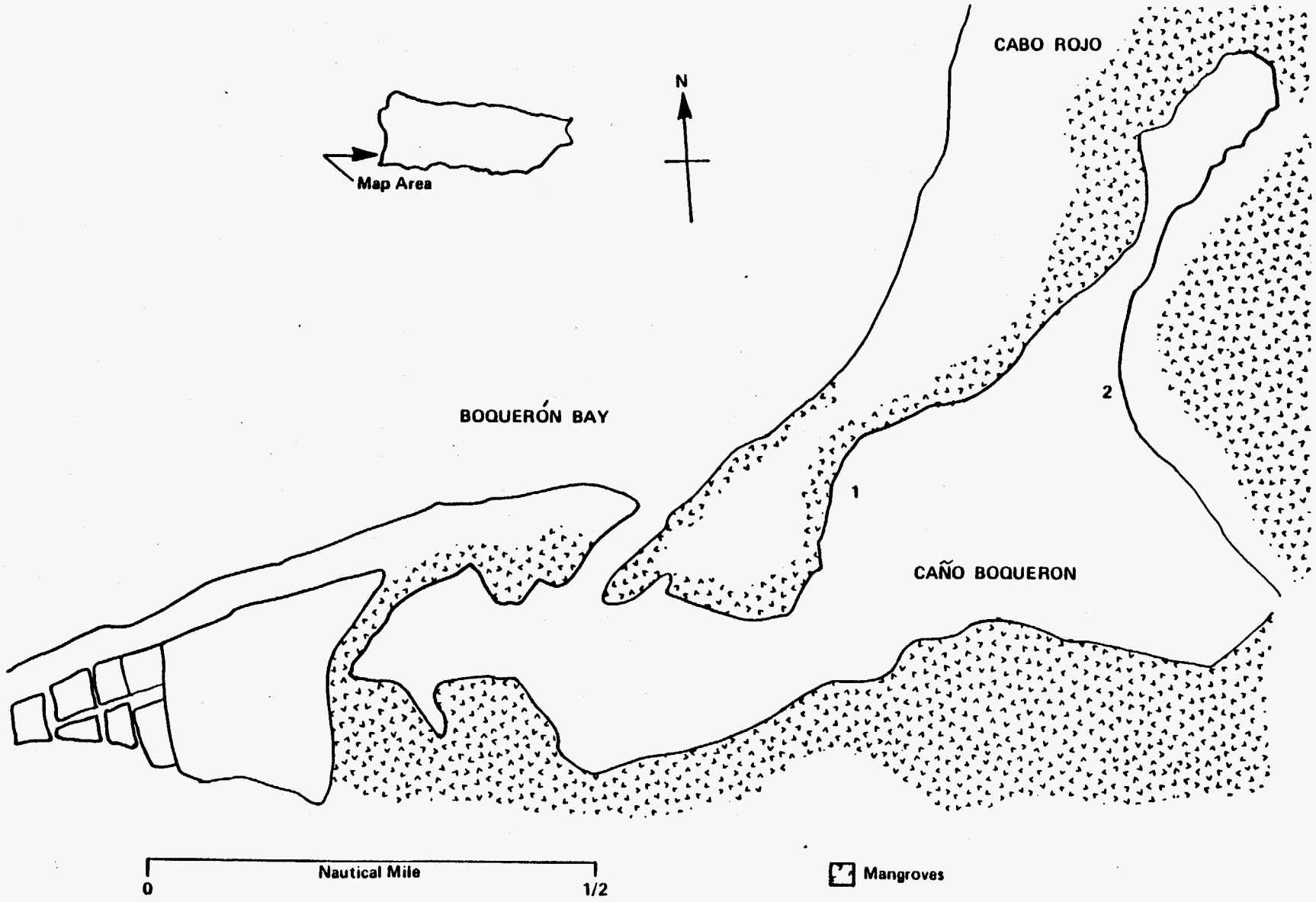
FIGURE LEGENDS

Figure 1. Map of sites in the Caño Boquerón Lagoon.

Figure 2. Survival of poliovirus serotype 1 at Caño Boquerón Lagoon.

Figure 3. Survival of Escherichia coli at Caño Boquerón Lagoon (Mean ± one standard error, n=4).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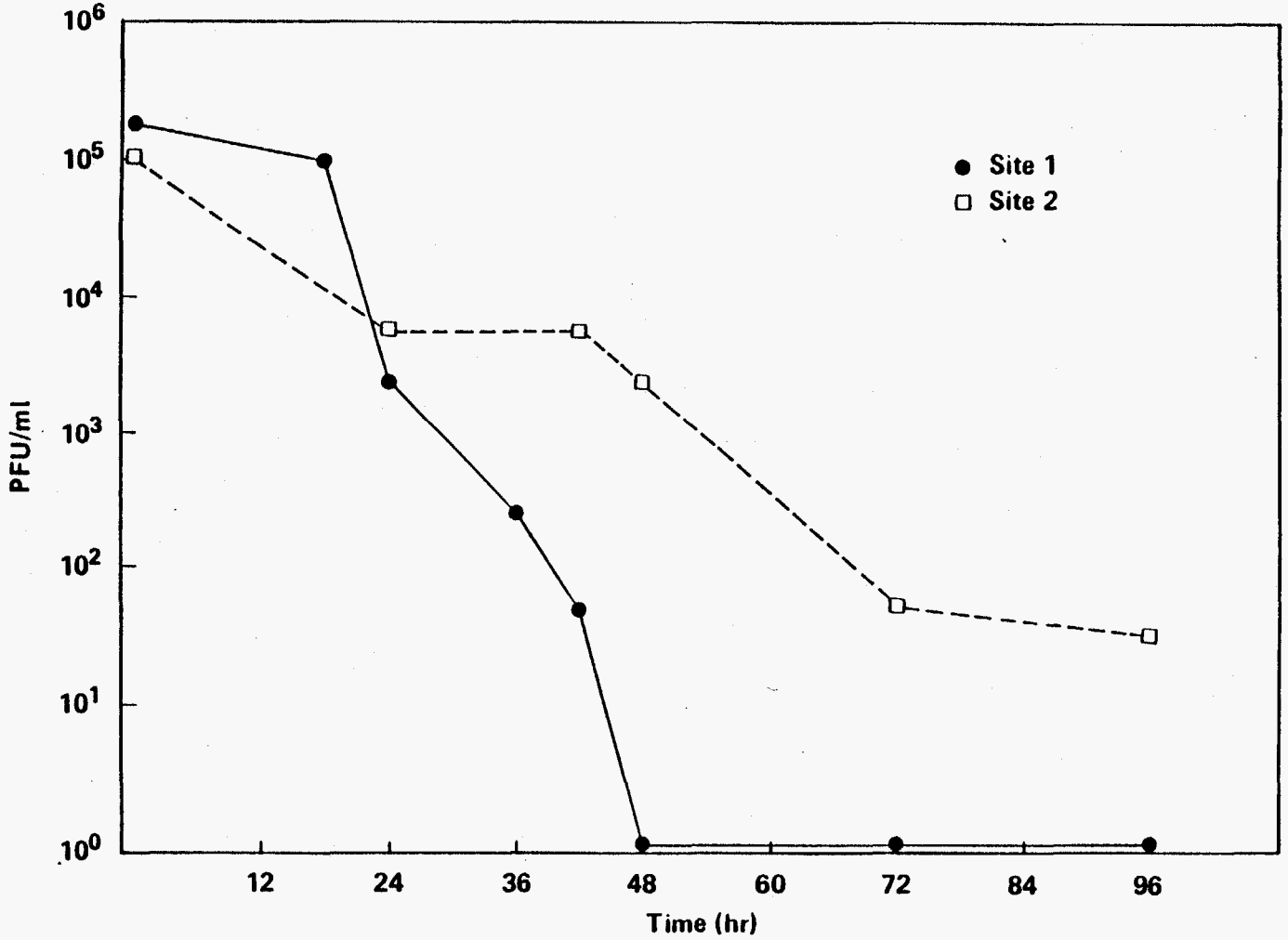
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96
Time (hr)

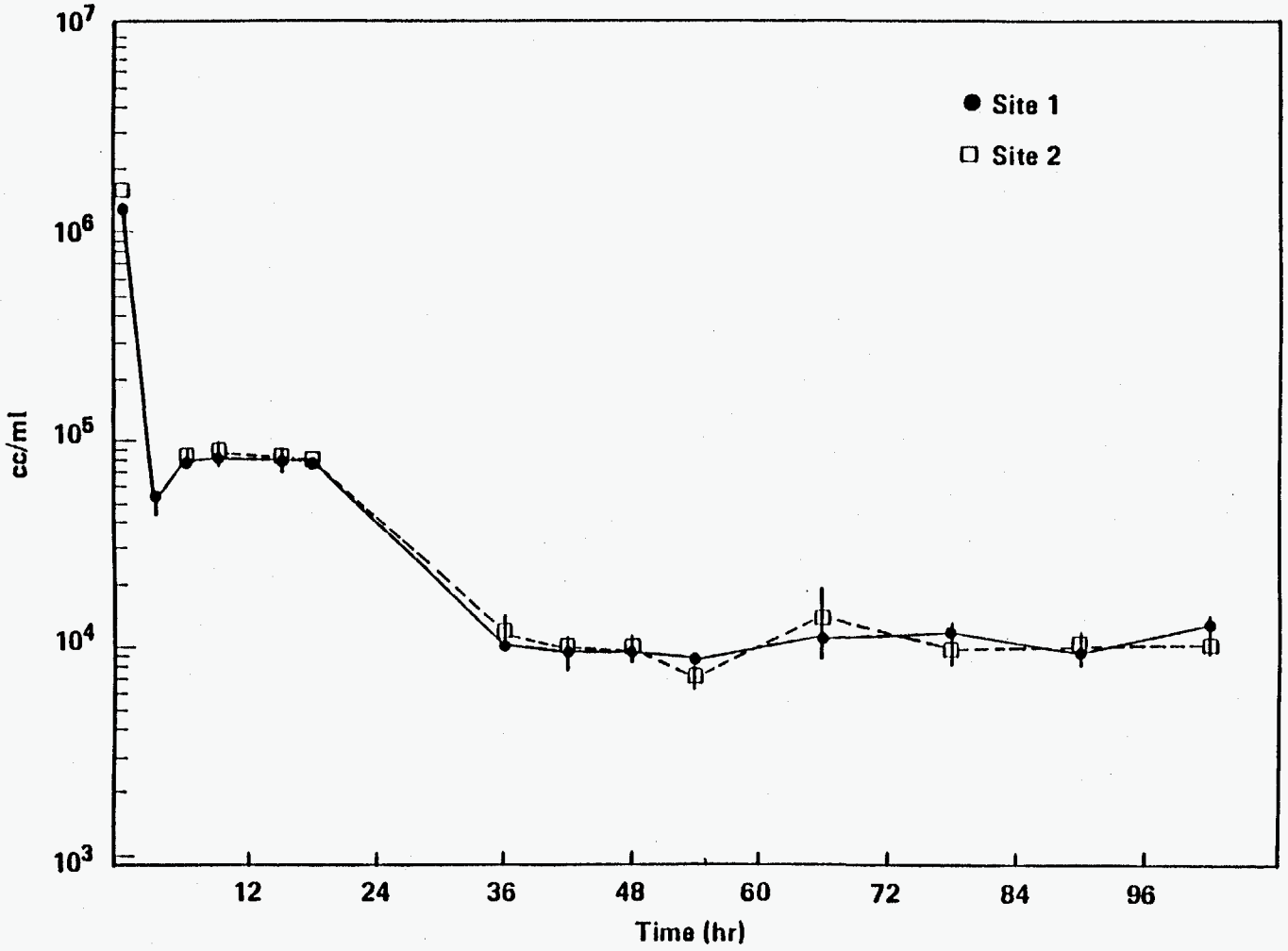
10^7
10^6
10^5
10^4
10^3
cc/ml
Site 1
Site 2
12
24
36
48
60
72
84
96
Time (hr)

Table 1. Water physicochemical parameters by sampling site.

Parameter	ATEMP	WTEMP	DO	pH	$NO_{2\pm3}$	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 ± 1643
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), T C = total coliforms (CFU/100 ml), F C = fecal coliforms (CFU/100 ml).

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