ENTEROVIRUSES IN PROHIBITED OYSTERS AND MARINE SEDIMENTS

Final Report

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Administrative Summary

MASCG Project No. R/MT-3; Ellender, USM; Cook, GCRL.
Duration: January 1980 to December 1981.
Extension: to August 1982.
USM Project Numbers: 0221713409; 0221713509.
Funds: 1980, $26,943; 1981, $29,665.
No. Field Exercises:13
No. Man Months Expended: 9.1
Percent Completion of Objectives:98
Published Reports or Manuscripts in Preparation:

1. Tsai, S.A. and R.D. Ellender. 1981. Development and evaluation of methods for the elution of enteric viruses from Mississippi estuarine sediments. Miss.-Ala. Sea Grant Consortium Publication, MASGP-80-021, 70pp.
2. Tsai, S.C. and R.D. Ellender. 1982. Elution of enteroviruses from estuarine sediment. Appl. Environ. Microbiol. (in preparation).
3. Johnson, R.A. and R.D. Ellender. 1982. The effect of lecithin on the elution of enteroviruses from marine sediments.

Student Theses/Disserations:

1. V.L. Sheladia, Microbiology, Ph.D., Jan. 1981.
2. S.C. Tsai, Microbiology, M.S., May, 1981.
3. Y.J. Brady, Microbiology, M.S., May 1982
4. R.A. Johnson, Microbiology, M.S., Dec. 1982

Titles:

1. Sheladia: Extraction and Analysis of Enteroviruses for Oxidation Ponds, Shellfish and Sediment Samples.
2. Tsai: Development and evaluation of methods for the elution of enteric viruses from Mississippi estuarine sediments.
3. Brady: The role of sediment in the transmission of Channel Catfish Virus Disease.
4. Johnson: Lecithin as a component of eluents to extract enteric viruses from estuarine sediments.

Overall Objectives

1980: Field Investigations

examine contaminated oysters for enteroviruses and indicator bacteria and interface these data with the levels of enteric viruses and bacteria found in marine sediments.

1981: Laboratory Investigations

explore methods to improve the elution of viruses from estuarine sediment; consider the role of environmental factors in the viability of sediment-associated viruses and bacteria; evaluate the role of oyster feces as a potential source of sediment contamination.

Abstract

Methods were developed to recover sewage derived viruses from estuarine sediments collected along the Mississippi coast. This research was conducted to better understand the potential role of sediment as a reservoir of viral contamination of shellfish. The organic eluent mixtures commonly used by other investigators were found to be unsatisfactory for the analysis of these sediment types. Of the organic eluent solutions which were compared in seeded virus experiments, isoelectric casein produced the most promising results over the spectrum of sediments types examined. Additional experiments using isoelectric casein solutions containing lecithin as supplement were promising as a general eluent applicable to various sediment compositions. Studies conducted to determine the protective effect of sediment demonstrated that long term protection was temperature dependent and not directly related to sediment composition.

Fecal coliforms were found in high numbers in sediments collected in a prohibited oyster harvesting area. High sediment counts were associated with the colder months of the year and their release into the water column was viewed as a potential source of contamination for filter feeding shellfish. Additional laboratory studies to evaluate the viablity of coliforms in sediment suggested

that the standard response of this organism is to decline in number, at rates directly related to temperature. Sediments found to contain fecal coliforms over a long term sampling period must be considered to be contaminated on a regular basis. Separate trials using sediments seeded with Salmonella showed similar trends and documented the more rapid die-off of these pathogens as compared to the indicator bacteria.

Oyster feces, artificially contaminated through ingested water containing coliforms and virus, were found to be able to contaminate surrounding sediments.

Bacteriologically, the health control procedures applied to shellfish and their growing waters appears to apply to sediments as well. The standard times of relaying and depuration practices are adequate and sediments do not appear to significantly influence these considerations. Additional research must be forthcoming in regard to virus contamination, but dramatic modifications in the current shellfish guidelines are not expected.

Opening Remarks

It was the original intent of this investigation to evaluate the potential role of estuarine sediments as a reservoir for natural viral and bacterial contamination of eatible shellfish. Virologically, the accomplishment of this objective was impaired by toxic factors present in the sediments examined. A summary of the first year of virus isolations is outlined below. The bacteriological results were not hampered by such problem and are presented and analyzed in this report.

		Oysters*					Sediments				
Month		A	B	C	D	E	1	2	3	4A	4B
0	12	3	8	21	0	-	Jan.	0	0	0	
0^{C}	0^{C***}	0^{C}	3	0	0	0	Feb.	0	0	0^{C}	
-**	-	0^{C}	0^{C}	2^{C}	0^{C}	6	Mar.	0^{C}	0^{C}	0	
-	-	0^{C}	1	3^{C}	0^{C}	0	Apr.	0	0	0^{C}	
0^{C}	0^{C}	0^{C}	1	0	0^{C}	0^{C}	May	0^{C}	2	0^{C}	
1	-	0^{C}	0	0^{C}	0^{C}	0^{C}	June	0^{C}	1	1	
0	5	0	0^{C}	0	0^{C}	0^{C}	July	0	0	0	
-	-	0	0	1	3	-	Aug.	1	0	5	
-	-	0^{C}	0^{C}	0	-	0	Sept.	0	0	3	
-	-	0^{C}	0	0	2	-	Oct.	0	2	5	
3	-	-	0^{C}	-	0^{C}	-	Nov.	1	0	4	
-	-	-	0^{C}	0	1	0^{C}	Dec.	1	1	2	

ns/150 gr

ne

ntrate plaqued but cytotoxicity encountered.

* No. isolatio

** [-] = not do

*** No.^{C} = conce

The major problem associated with the proposed research involved the isolation of virus from sediment. Such field experiments had not been previously conducted using Mississippi estuarine sediments and as it later became apparent, elution methods had to be developed specifically for these types of samples. Therefore, a major thrust of the research effort was designed to evaluate various eluent mixtures capable of reducing sample cytotoxicity while allowing maximum virus recovery. These studies involved investigation of the effect of lecithin upon virus elution and a separate group of experiments which considered virus longevity in sediment in regard to time and temperature. Overall, the study raised additional questions concerning sediment virology and may prove, in the long term, that the quantitation of virus in sediment is a very appropriate indicator of the extent to which an estuarine area is contaminated by sewage.

The bacteriological portion of the study was just as valuable and has shed new light on several aspects of estuarine microbiology that were not previously understood.

The investigators thank the entire staff of the Mississippi-Alabama Sea Grant Consortium for their financial support, wherewithal and encouragement during 1980 and 1981.

VIROLOGY

The isolation of human enteric viruses from estuarine sediments (1,2,3) adds a new dimension to the overall complexity of sewage contamination of coastal waters. Enteric viruses appear to survive longer than indicator bacteria when attached to sediment particles rather than as free virions in seawater (4,5,10,11,17) and they exhibit type and strain dependence (6).

If the large number of enteric viruses entering estuarine waters form sewage outfalls (14,15,16) readily attach to particulate matter, accumulate in sediments (10), persist for extended periods (2,11,33) and remain viable when attatched to particulate matter (35),the potential for hydrotransportation to recreational waters or shellfish beds is increased. This type of contamination, as demonstrated by the isolation of indigenous sediment-associated virus (7,14,16,36), could explain the continuity of low level,natural viral isolations from oysters (12,13,18,19,20) observed when the animals are collected from approved growing waters.

The composition of estuarine sediment is an important factor in any study which relates to viral elution, inactivation or persistence (10,11,23,27). Clays appear to play a major role in virus removal from seawater. The adsorption of viruses to sand is less efficient while the role of silt in virus adsorption has yet to be examined.

The adsorption of viruses to clays is well documented (28-31,34) and the mechanisms by which viruses adhere to

colloidal surfaces has been investigated (32,33). Stotzky (33) has remarked that adsorption appears to be dependent on surface charge interactions wherein the charge on viruses are influenced by the pI or pKa of the environment (ionic changes in virus surface components); the charge on chrystalline clay minerals results primarily from isomorphous substitution with the clay and the net electrical charge is negative (33).The cation exchange capacities of certain clays have been shown to effect their ability to adsorb reovirus , but no conclusive evidence was found for T-1,T-3 phage or Herpesvirus hominis type 1 (32). Pretreatment of the clay with negatively charge d compounds reduced the adsorption of phage to clay but did not reduce the amount of reovirus or HSV-1 adsorbed. These results indicate that reovirus binds to the negatively charged sites on the clays, and that other sites (or ty pes of binding) are responsible for adsorption of thes e viruses (32,33).

The demonstration of sediment-bound virus is dependent upon elution-concentration procedures (7,9,10,2 1) which are yet to be tested in collaborative efforts or co mpared using sediments of varying composition or from differ ent geographical locations. Recent extraction metho ds rely on alkaline buffered eluents to desorb sediment-bo und virus; virus concentration from the suspending fluid i s usually accomplished by acid precipitation of soluble p roteins.

In these first studies which examine the e lution of

viruses from Mississippi coastal sediments, previously reported eluent mixtures are compared. The ultimate intent of this study, and those which follow, is to examine the degree to which estuarine sediments play a role in the natural viral contamination of shellfish-growing and recreational waters.

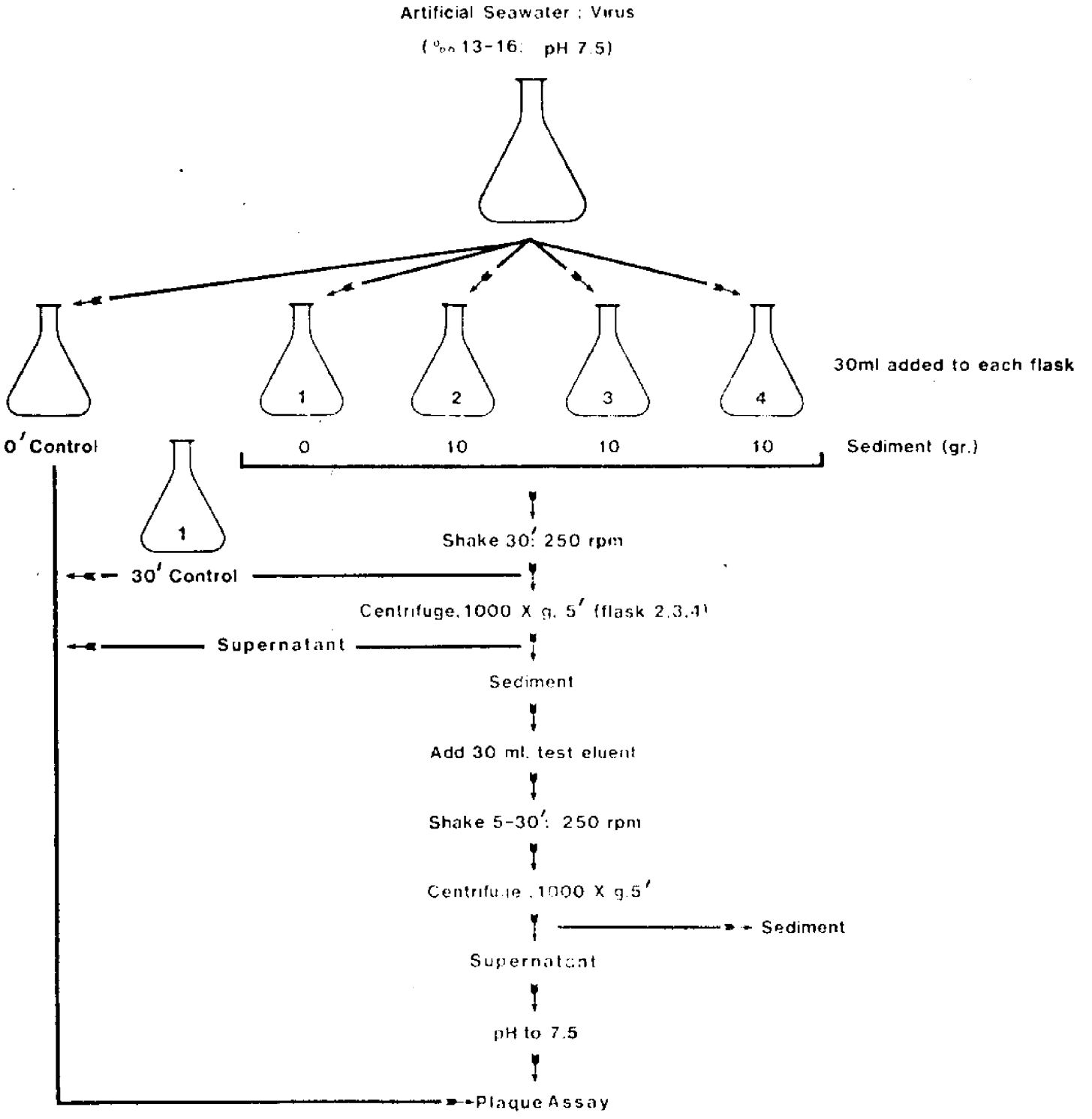
Materials and Methods:

1. Sediment collection: Sediments were collected in an area (Bayou Graveline) south of Pascagoula, Ms. which was known to be contaminated with sewage. This location was selected because previous studies (12,13) had demonstrated the presence of enteroviruses in oysters examined during a 24 month investigation. In this study, a procedure to evaluate the degree of sediment viral contamination was sought.

Sediments L2 and L3 were collected at the previous oyster harvesting site and the mouth of Bayou Graveline; the average composition (percent sand,silt,clay) for each sediment type was 10.1, 48.2, 41.7 for sediment L2 and 79.2, 11.8, 9.1 for sediment L3, respectively. Samples were collected using a hand held Ekman dredge (Wildco); the upper 2 to 3 cm of sediment were removed and placed into sterile plastic containers.

2. Sediment extraction: The procedure outlined in Figure 1 was used as the model for all experiments. Artificial seawater was prepared using Instant Ocean (R) (Eastlake, Ohio); seawater with a salinity range of 13-16‰ and a pH of 7.5 was used. Thirty ml of the seawater virus mixture,containing 100,000 to 1,000,000 plaque forming units (pfu) per ml, was mixed with 10 gr of sterile sediment. A flask containing no sediment was used as input

Figure 1. Sediment Extraction Procedure



control. Dry sediment was prepared as previously described (3); sterile, wet sediment was prepared by autoclaving. Supernatant and control samples were collected as indicated in the figure and either plaqued immediately or frozen at -70 C.

3. Cell cultures and viruses: The Buffalo African green monkey kidney cell line (BGM) (37) (passages 120-200) was used in all viral experiments. Briefly, cells were grown in MEM-L15 (1:1) medium supplemented with 10% newborn calf serum, 1% antibiotic-antimycotic mixture (100X), 1% L-glutamine and 1% sodium bicarbonate (7.5%) all of which were purchased from Grand Island Biological Company (Grand Island, New York). Viruses (poliovirus type 1, Sabin, VR-192 and coxsackievirus B3, Nancy, VR-30) were purchased from the American Type Culture Collection (Rockville, Md.) and used to produce large, monodispersed pools whose volumes were sufficient to serve as inocula in all seeded experiments. The titers of viral pools and the fluids associated with extraction studies were determined by a double agar overlay plaque method. Cell cultures (a minimum of 3 replicates) were inoculated with 0.2 to 0.5 ml of sample and placed on a rocker platform for 1 hr at 37 C. Following the addition of overlay medium (12), flasks were inverted and incubated at 37 C. Plaques were counted for 10 days or until no new plaques appeared after 2 consecutive days.

A. Eluent mixtures: Four basic eluents solutions were used in this investigation: a) glycine buffer (0.25%) (Eastman Kodak), b) milk proteins, represented by 0.5% skim milk (SM) and 0.5% isoelectric casein (IC) (Difco), c) beef extract (BE) represented by paste (Difco) and powdered (Inolex) (IBE) preparations and d) nutrient broth (NB) (Difco). The basic eluent solutions were modified by the addition of one or more of the following reagent grade substances: a) ethylaminediaminetetraacetic acid (EDTA) (Sigma), b) citric acid (Sigma), c) sodium oxalate (Sigma), d) fetal (FCS) and newborn (NCS) calf sera (Gibco), e) sodium dodecyl sulfate (MCB) and f) disodium phosphate (Fisher). Unless otherwise stated, all eluents were prepared in tris (hydroxymethyl) aminomethane (Bio-Rad). Corrections in the pH of each eluent were performed using sterile 1N NaOH or 1N HCl solutions.

5. Expanded sediment investigation: The study was expanded during the second year of the profect to include four additional sediment sites whose samples varied in the content of sand,silt and clay (Table 12). All subsequent experiments were performed using these materials. Site selection was based on percent composition of the sediment, especially the content of clay. Eight kilogrms of each sediment type were collected, homogenized, analyzed and stored in plastic bags in 500 gr lots at -20C.

Eluents used in these experiments were selected on the basis of efficience of recovery in the studies previously

described (Inolex beef extract, isoelectric casein and nutrient broth) and were conducted using the procedure outlined in Figure 1. Although many eluent supplements were used in an attempt to increase virus recovery, one particular substance, lecithin (phosphatidylcholine), was promising and its examination was expanded into a major group of experiments. All lecithin preparations used were purchased from Sigma Chemical Co. with the exception of soybean lecithin (technical grade)(MCB). In addition to poliovirus type 1 and coxsackievirus type B-1 experiments, studies were also conducted using Echovirus type 11.

6. Sediments and Infectivity: Experiments were also conducted to determine if enteric viruses remain infective when adsorbed to sediment. Experimental variables included the time of adsorption, the sediment types described in Part 5 and the temperature of incubation of the virus-sediment mixture. Tests were also performed to determine the die-off rate of enteric virus in artificial seawater. The general procedure described in Figure 1 was also used in this part of the investigation.

7. Studies on the concentration and survival of enteric viruses in oyster feces: This supplemental investigation was performed to complement previous studies (12 , 13) of oyster virology and to investigate a potential source of concentrated sediment contamination. In these experiments, different levels of poliovirus type 1 were suspended in seawater and removed from suspension by oysters held at 15

and 25 C. In one group of experiments, 0.1ml of stock virus was diluted to 5 gallons and pumped into the tank system at a rate of 6ml/min. A second group of experiments employed 10 ml of stock virus with the other conditions held constant. Oysters (25 per experiment) were placed into the tank system (Figure 2) and allowed to accumulate the virus suspension for 14 hours. The system was turned off and the fecal material collected with sterile pipettes, being careful to maintain the ribbon-like texture of the fecal material. An effort was made to collect from as many different oysters as possible after which the fecal material was discharged into pro-weighed vials and stored at 15 or 25 C until removed at the appropriate time and transfered to the -70C freezer. On the sample day, the vials were removed, centrifuged, and the supernatant carefully poured off. The pellets were weighed and stored at -70C. In each type experiment, ampules were collected on day 0,7,14,21 and 28 from each of the 2 incubation temperatures. For virological analysis, vials were thawed at 37 C and mixed with 5 ml of 3% IBE in Tris-saline buffer (pH 9.0). The solution was mixed for one minute and centrifuged to remove the particulate matter. Supernatant fluid was filtered (0.45um) and analyzed by plaque assay (12, 13).

Results and Discussion:

Of the initial methods which we used to elute enteroviruses from estuarine sediments (3,7,14) or similar methods which have been applied to soils or other solids (23,24,39), none were found to be suitable for the extraction of virus from estuarine sediments examined during this study. Each of these procedures did recover sediment-bound virus but the efficiency of recovery was low (0.1 to 1%).

Nevertheless, enteroviruses do adsorb to Mississippi estuarine sediments and the epidemiological and ecological concerns expressed earlier (27) probably apply to this geographic location. The ability of sediments to bind virus was not influenced by the sampling location or the method of sterilization of the sediment prior to viral addition and elution. Poliovirus and coxsackievirus removal from seawater by each sediment type was consistent and averaged a three log decrease in the seawater-virus titer. Virus adsorption to sediment was rapid with an average of 92-95% of these viruses being adsorbed during the first minute of shaking. When the average viral titers of the zero and 30 minute seawater controls (no sediment) were compared there was no significant difference between virus levels.

Since the greater percentage of reports which deal with elution of virus from estuarine sediments have

utilized the glycine-EDTA procedure (1,3,5,7,16,27), this method was analyzed for its ability to recover virus from L2 and L3 sediments. The results of Table 1 demonstrate that recovery by this eluent is low but consistent (less than 12%).In Group A experiments, glycine buffer (0.25M) alone produced the lowest recovery from dry sediments (recovery range 0.4 to 1.0%). Glycine-EDTA (0.05M), glycine-FCS (10%) and glycine-EDTA-FCS mixtures gave recovery ranges of 0.9 to 1.7, 4.1 to 4.3 and and 2.4 to 2.6, respectively). The elution of poliovirus from autoclaved L3 sediments (Table 1,B) by glycine based eluents was higher than from dry sediment with glycine buffer containing 10% serum producing an average recovery of 10.9%. Glycine alone and glycine mixtures containing 0.05 and 0.1M EDTA and 0.05M sodium oxylate gave average recoveries of 5.2, 7.0, 4.2 and 4.0, respectively. When glycine eluents were used to extract coxsackievirus from autoclaved L3 sediment (Table 1,C), the efficiencies of recovery were approximately those observed during polio experiments. Similar trends were noted when each virus was eluted from type L2 sediment (Table 1,D,E). The highest percentage of recovery (11.1%) using glycine-EDTA as eluent was observed when coxsackievirus was eluted from L2 sediment. These experiments confirm the previous observations of Bitton and co-workers (36) concerning the use of glycine-EDTA as eluent and point to the need for eluent selection in regard to each sediment type under

Table 1. Summary of experiments designed to evaluate the elution of virus from sediment by glycine base eluents.

	Virus Sediment/Type	Titer (PFU/ml) Average Input	Average Recovery	Recovery Range (%)
A)*	L3/P	1.2×10^6	1.6×10^4	0.4 - 4.3 (1.3)
B)**	L3/P	1.9×10^6	1.1×10^5	2.3 - 10.9 (5.7)
C)	L3/C	6.5×10^5	2.8×10^4	3.4 - 4.8 (4.3)
D)	L2/P	1.0×10^6	2.2×10^4	0.1 - 4.2 (2.2)
E)	L2/C	3.6×10^5	2.8×10^4	4.2 - 11.1 (7.7)

* Type A experiments - sediments dried at 60° C.

** Types B-E experiments - sediments autoclaved.

consideration.

Eluents containing milk proteins have been used to recover viruses from soils (23) and marine sandy sediments (36). Table 2 summarizes the experiments designed to test the efficiency of elution of viruses from coastal sediments using skim milk eluents in this locale. A mixture of 0.5% skim milk (pH 9.0) did not elute a significant portion of virus adsorbed to dried sediments (Table 2,A) (range 0.1 to 0.5%); however, the recovery of poliovirus in this same group of experiments by 0.5% skim milk containing 10% FCS was 4.3%. The recoveries of virus from autoclaved L3 sediment (Table 2,B) using 0.5% SM (pH 9.0) was also low (0.2%) but recovery increased slightly when supplements were added to the basic mixture (10% FCS plus 0.25% SDS, 2.2%; 10% NCS plus 0.05M EDTA, 5.0%; 10% NCS plus 0.25% SDS, 3.3%). The best recovery (11%) was obtained using 0.5% SM plus 10% NCS. SDS and EDTA did not appear to positively affect the elution of virus from L3 sediment by SM mixtures. Recovery of poliovirus from autoclaved L2 sediment (Table 2,B) by SM mixtures was poor (range 0.1 to

Table 2. Summary of experiments to evaluate the elution of virus from sediments by solutions containing mild proteins.

Sediment	Virus/Type	Titer (PFU/ml) Average Input	Average Recovery	Recovery Range
Skim Milk				
A)*	L3/P	8.8×10^5	9.7×10^3	0.1 - 4.3
B)**	L3/P	2.0×10^6	8.9×10^4	0.2 - 11.0
C)	L3/C	6.5×10^5	5.4×10^4	6.0 - 10.7
D)	L2/P	1.2×10^6	5.6×10^3	0.1 - 0.8
E)	L2/C	3.0×10^5	2.3×10^4	6.7 - 11.7
Isoelectric Casein				
F)	L3/P	3.4×10^5	2.0×10^5	58.8
G)	L3/C	2.8×10^5	5.3×10^4	18.9

* Sediment dried @ 60° C.

** Experiments B-G, sediment autoclaved.

Table 3. Elution of poliovirus from autoclaved sediment using beef extract paste.

Sediment Type	% BEP	Titer (PFU/ml) Average Input	Average Recovery	Recovery Range (%)
L3	3	1.1×10^6	4.6×10^4	2.5 - 6.6
L3	10	2.5×10^6	7.5×10^4	2.8 - 3.2
L3	10*	8.0×10^5	9.5×10^4	11.8
L2	3	1.0×10^6	1.3×10^5	0.8 - 25.0

*Prepared in McIlvaine's buffer (pH 7.0)

The use of beef extract to recover virus from soils (23,25), river water solids (22), sludge (24,26), wastewater (38) and sediments (9,36) is well documented. In this investigation, two types of beef extract (paste and powdered) were tested; the results of experiments using Difco beef extract (paste) are presented in Table 3. No significant difference was noted when virus was eluted from L3 sediment by 3 or 10% beef extract solutions (pH 9.0). Ten percent beef extract did not produce higher recoveries when supplemented with either 10% NCS or 0.25% SDS (3.0 versus 3.2%); however, when 0.05M Na2HPO4 and 1.2 g/l citric acid (McIlvaine buffer, pH 7.0) were added to 10% BE, 11.8% of seeded virus was recovered. Berg and Dahling

iver water
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to 46%;
lower (L3,
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n L3 sediment

(22) reported recoveries of poliovirus from r
solids by this method which ranged from 17.4
L2 sediment was eluted with similar mixtures,
recovery (25%) was associated with the use of
containing 10%NCS.

The recovery of virus from sediment by p
extract (Inolex) was generally higher than th
using beef extract paste (Table 4). Poliovirus
3% IBE from L2 and L3 sediment ranged from 11
coxsackievirus recoveries by this eluent were
13%; L2, 29%). The highest eluent pH used, 11
decrease the efficiency of virus recovery fro
and may inactivate virus during the 30 minute
period. The ranges of poliovirus recovery fro

Table 4. Elution of poliovirus (P) and coxsackievirus (C) 3% beef extract powder.

Sediment/Virus Type	Eluent pH	Titer (PFU/ml) Average Input	Titer (PFU/ml) Average Recovery	Recovery Range (%)
L3/P	9	1.3×10^6	5.3×10^5	12.1 - 15.0 (41)
	10	1.1×10^6	5.1×10^5	37.1 - 65.0 (46)
	11	1.0×10^6	1.1×10^5	10.1 - 12.0 (11)
L2/P	9	9.7×10^5	3.9×10^5	25.0 - 42.1 (40)
L3/C	9	4.4×10^5	6.0×10^4	13.0 - 13.1 (13)
L2/C	9	3.2×10^5	9.3×10^4	- (29)

at pH values of 9,10 and 11 and from L2 sediment (pH 9) were: 12-15%, 37-65%, 10-12% and 25-42%. Coxsackievirus recovery from L3 and L2 sediments by 3% IBE was 13 and 29%, respectively. When 3% IBE mixtures were prepared in other buffers, no major differences of elution of poliovirus from L3 sediment were observed (Table 5). Tris and glycine buffers containing 3% IBE produced an increase in virus recovery as the pH was increased to 9.0 with Tris-IBE giving the highest recovery (42%). McIlvaine's buffer was as efficient as the tris and glycine mixtures.

The addition of various supplenents to IBE eluents did not produce a dramatic increase in the recovery of either poliovirus or coxsackievirus from L2 or L3 sediment (Table 6). Three percent IBE containing 0.025M SDS recovered 38% of the seeded virus as compared to the same eluent (and pH level) containing 10% NCS (18%). Overall, the presence of eluent supplements seemed to detract from the recovery of poliovirus from either sediment type; coxsackievirus recovery remained at the same level for L3 sediment and gave higher recoveries (26% versus 19%) from L2 sediment when IBE was used alone.

Many past investigations have utilized 3% BE but no specific concentration is characterized as a standard amount. Landry et al. (38) reported that powdered BE concentrations of less than 3% appeared to be as effective as 3% BE for virus reconcentration from wastewater effluent samples. Studies to determine the effect of IBE

Table 5. Effect of buffer, concentration and pH on the elution of poliovirus (P) and coxsackievirus (C) from sediment by powdered beef extract mixtures.

Virus Sediment/Type	Buffer	Eluting pH	Percent IBE	PFU/ml Average Input	PFU/ml Average Recovery:	%
L3/P	Tris (0.2m)	7	3	4.8×10^4	1.2×10^4:	25
		8	3	4.8×10^4	1.5×10^4:	31
		9	3	4.8×10^4	2.0×10^4:	42
		11	3	4.8×10^4	1.7×10^3:	
	Glycine (0.25m)	7	3	4.8×10^4	1.2×10^4:	25
		9	3	4.8×10^4	1.6×10^4:	33
	McIlvaine	7	3	6.2×10^4	2.0×10^4:	32
		7	10	8.0×10^5	2.5×10^5:	31
L3/C	Tris	9	3	3.3×10^5	4.3×10^4:	13
L2/C	Tris	9	3	3.2×10^5	8.3×10^4:	26

Table 6. Effect of supplements on the elution of virus from sediment by powdered beef extract mixtures.

Virus Sediment/Type	Eluent pH	% IBE	Supplement	PFU/ml Average Input	PFU/ml Average Recovery:	%
L3/P	9	3	10% NCS	8.5×10^5	1.5×10^5:	18
	10	3	10% NCS	8.5×10^5	2.3×10^5:	29
	11	3	10% NCS	8.5×10^5	2.0×10^5:	25
	9	3	0.25% SDS	2.5×10^6	3.3×10^5:	13
	9	3	0.025% SDS	9.0×10^5	3.5×10^5:	38
	9	3	10% NCS + 0.025% SDS	9.0×10^5	3.0×10^5:	33
	9	10	0.25% SDS	2.5×10^5	3.8×10^5:	15
L2/P	9	3	10% NCS	1.0×10^6	3.0×10^5:	30
L3/C	9	3	10% NCS	3.3×10^5	4.2×10^4:	13
L2/C	9	3	10% NCS	3.2×10^5	6.0×10^4:	19

concentration on poliovirus elution are presented in Table 7. As the quanity of IBE increased from 1 to 15%, a gradual increase in the efficiency of recovery occurred. Concentrations of IBE greater than 15% were viscous and the resultant lower percentages of virus recovery could be due to interference with virus desorption from sediment or attachment of virus to BGM cells. A similar pattern of recovery was reported by Berg and Dahling (22) when 10-20% beef extract eluents were used.

The effect of time and the use of different lots of IBE on the elution of poliovirus from L3 sediment are presented in Table 8. Hurst (24) reported that 30 second and 1 minute mixing times could yield similar or higher results that longer periods, for example, 15 minutes when conducting studies on activated sludge. In this study, 3% IBE recovered the greatest amount of virus following the 30 minute elution (43.8%). When the time of elution was increased to 45 minutes, recovery decreased (34.2%). Berg and Dahling (22) reported that different lots of beef extract could have different eluting capacities, but the six lots tested in this study recovered approximately the same quantities of poliovirus (average of all lots, 30%; range, 22.7-39.1%).

Studies of elution of viruses using nutrient broth (Table 9) favorved the recovery of poliovirus. The average recovery of polioviurs from L3 sediment was 53% (range, 25-63.2%). The lower recoveries of poliovirus from L2

Table 7. Effect of concentration of inolex beef extract at pH 9.0 on the elution of poliovirus from autoclaved L3 sediment.

Concentration of Beef extract (%)	Virus Input (PFU/ml)	Virus Recovery (PFU/ml: %)
1	1.8×10^6	3.5×10^5: 19.4
2	1.8×10^6	5.0×10^5: 27.8
3	1.3×10^6	5.7×10^5: 43.8
5	4.8×10^4	2.0×10^4: 42.0
8	4.6×10^4	2.1×10^4: 46.0
10	4.5×10^4	2.2×10^4: 48.0
15	9.2×10^5	4.4×10^5: 47.8
20	9.2×10^5	1.4×10^5: 15.2
25	1.8×10^6	3.0×10^5: 16.6
30	1.2×10^6	1.5×10^5: 12.5
40	4.6×10^4	1.0×10^4: 21.7

Table 8. Effect of time and lot of beef extract on the elution of poliovirus from L3 sediment by 3% IBE, pH 9.0.

Variable	Time of Elution (min)	Average % Recovery
	5	31.5
	15	34.2
	30	43.8
	45	34.2
Lot No.		
	5485	36.2
	5667	32.4
	3924	24.2
	011961	31.6
	014507	22.7
	012036	30.3

Table 9. Elution of virus from autoclaved sediments by 4% nutrient broth (pH 7.5).

Sediment/Virus Type	PFU/ml Average Input	Average Recovery: %
L3/P	1.7×10^6	9.0×10^5: 53
L2/P	1.0×10^6	4.0×10^5: 40
L3/C	3.3×10^5	3.5×10^4: 15.4
L2/C	3.2×10^5	8.3×10^4: 25.9

sediment (40.0%) was higher that the recovery of coxsackievirus from either of the sediment types (L3, 15.4%; L2, 25.9%).

A comparison of all of the experimental eluents used to desorb virus from L2 and L3 sediments demonstrated that higher recoveries were achieved using organic eluents. The highest recoveries obtained in individual experiments were 63.2% (recovery of poliovirus from L3 sediment by 4% NB at pH 7.5) and 65% (recovery of poliovirus from L3 sediment by 10 [illegible] pH 10.5 glycine and SM solutions removed higher percentages of coxsackieviruses than poliovirus from both types of sediment but in an overall sense were not found to be effective for virus removal.

As demonstrated in Table 10, IC, NB and IBE eluents allowed the highest recoveries of poliovirus from L3 sediment. Beef extract paste, glycine and SM mixtures were consistently low. The same trend was evident with L2 sediment and coxsackievirus experiments. Overall, the elution pattern of coxsackievirus from either sediment type was lower that the recovery of poliovirus. This ranking was not found to be consistent with the data accumulated to establish the effect of eluent composition on virus viability (Table 11). Here, the lowest percentage loss of poliovirus (7%) was realized with 4% NB (pH 7.5); the highest losses of virus occurred in 0.25M glycine (pH 11.0) and 0.5% IC (pH 9.0). The titer of coxsackievirus suspended in 4% NB (pH 9.0) dropped 10% but no virus loss occured in

Table 10. Eluent comparisons based on virus and sediment types.

Poliovirus			
L3		L2	
IC	58.8*	IBE	40.0
NB	53.0	NB	40.0
IBE	32.7	DBE	9.3
DBE	4.0	GLY	2.2
GLY	3.5	SM	0.5
SM	2.7		

Coxsackievirus			
L3		L2	
IC	18.9	IBE	29.0
NB	15.4	NB	25.9
IBE	13.0	SM	9.2
SM	8.3	GLY	7.7
GLY	4.3		

* Average % recovery for each eluent

Table 11. Effect of eluent composition on virus titer during 30 minute shaking period.

Virus	Eluent	Average Recovery (PFU/ml)	Average % Reduction in Titer
Polio*			
	0.25 M GLY, pH 11.0	2.5×10^5	42
	3.0% IBE, pH 9.0	3.6×10^5	17
	4% NB, pH 7.5	4.0×10^5	7
	4% NB, pH 9.0	3.8×10^5	12
	0.5% IC, pH 9.0	2.7×10^5	37
Coxsackievirus**			
	3% IBE, pH 9.0	6.2×10^5	0
	4% NB, pH 7.5	6.8×10^5	+9
	4% NB, pH 9.0	5.6×10^5	10

* Poliovirus inoculum, 4.3×10^5 PFU/ml.

** Coxsackievirus inoculum, 6.2×10^5 PFU/ml.

either 3% IBE (pH 9.0) or 4% NB (pH 7.5).

The results of this investigation again point to the need to understand the nature of the virus-sediment complex. No doubt differences of virus adsorption and elution (6,40) are associated with differences in virus composition, but in the study of soil (42) and sediment virology, we will need a more complete understanding of the makeup of the sediment matrix and not just the percentages of sand, silt and clay. For example, the type of clay (32,33,34,43), the presence of organic matter (44,45,10) and inorganic salts (27,41) and the physio-chemical conditions of adsorption and elution all affect the recovery of virus. These factors will gain a greater degree of importance as future investigations attempt to determine the level and persistence of viruses in contaminated soils and sediments.

As noted in section five of Materials and Methods, additional sediment types (Table 12) were collected and used in experiments designed to test the recovery by elution of polio, coxsackie and echoviruses. The results of the initial elutions using IBE, NB and IC solutions are presented in Table 13. These eluents had been found during the previous phase of this investigation to remove virus from Mississippi estuarine sediments; however, they had not been used with these sediment types. The best results were obtained when poliovirus was eluted at an alkaline pH with

Table 12. Sediment Profiles

	Site	GRVL	Sediment (%) Sand	Silt	Clay	Size Term
I.	Off Marsh Point	0	99.0	1.0	0.0	Medium sand
II.	N.W. VA Admin. Center	0	52.3	30.3	17.4	Medium silty, fine sand
III.	East of Point Cadet	0.1	89.3	6.0	4.6	Slightly granular muddy fine sand
IV.	Magnolia Bend	0	37.3	39.2	23.5	Very fine, sandy mud

Table 13. Elution of poliovirus from four sediment types by NB, IC and IBE mixtures.

Eluent	Sediment No. 1*	2	3	4
4% NB (pH 7.5)	6.2	10.4	7.3	5.3
4% NB (pH 9.0)	32.5	2.0	3.3	2.0
5% IBE (pH 9.0)	48.9	3.1	0.6	1.8
0.5% IC (pH 9.0)	65.3	0.3	29.4	1.2

* Each value is an average of two experiments.

isoelectric casein (65.3% recovery). Isoelectric casein was also able to remove approximately 30% of the virus from sediment 3.

Since IC appeared to best favor the removal of poliovirus from the four estuarine sediments, it was used in all subsequent studies designed to evaluate the use of lecithin as an aid to virus desorption. The results of 156 sediment elutions are shown in Table 14. Although IC-lecithin mixtures appear to reduce the recovery efficiencies for certain viruses, the overall effect of elution for all virus types is a trend toward higher recoveries from all sediments and a stability of the level of recovery achieved. For example, 0.5% IC, pH 9.0) alone was able to recover 65.3% of polio from sediment type 1 and 29.4% from sediment type 2 but the recoveries from sediments 2 and 4 were extremely low (0.3 and 1.2%, respectively). Three percent semi-purified lecithin was able to increase dramatically the recovery of polio from all sediments but especially sediments 2 and 3. The effect was more explicit when one compares the overall recoveries of coxsackie and echoviruses. The results demonstrate recovery consistency for these viruses, an effect that was not achieved in the first series of experiments with coxsackievirus.

The various mixtures appear to favor coxsackie and echovirus recovery and not poliovirus; this again underscores the reality of the possible use of multiple

Table 14. Recovery of 3 enteric viruses by 0.5% IC containing lecithin mixtures.

Eluent Mixture	Virus**	Sediment No. 1	2	3	4
A. 1% Soybean***	P	24.4*	16.1	39.1	6.0
	C	22.5	37.8	12.6	13.9
	E	17.0	42.4	6.7	5.5
B. 3% Soybean***	P	14.3	6.8	25.4	1.0
	C	45.6	80.9	40.2	21.8
	E	46.7	94.7	43.5	33.2
C. 1% Semi-purified soybean****	P	47.9	0.5	29.3	0.2
	C	55.6	55.1	61.6	48.4
	E	81.9	66.3	68.6	31.6
D. 3% Semi-purified soybean	P	75.1	9.7	61.4	2.6
	C	54.4	91.5	78.4	55.7
	E	58.9	78.5	69.0	56.8
E. 1% Egg*****	P	56.4	1.0	36.3	0.2
	C	64.9	54.5	69.0	38.6
	E	138.4	90.1	80.6	66.7
F. 3% Egg	P	102.6	1.1	65.5	0.6
	C	41.9	67.6	73.4	73.1
	E	44.6	50.4	48.8	43.3

* Each value is an average of at least two replicate trials. The basal medium consisted of 0.5% IC in 0.2 M tris buffer (pH 9.0) with 0.85% NaCl. Specific lecithin types and concentrations were added to this medium.

** P (Poliovirus type I), C (Coxsackievirus B-1), E (Echovirus type II).

*** Mathesm, Coleman and Bell, Soybean lecithin, technical grade (LX 210).

**** Sigma Chemical Co.; (P. 5638) Type II-S, from soybean, with 50% lecithin.

***** Sigma Chemical Co.; (P. 5394) Type X-E, from dried egg yolk, app. 60% lecithin.

eluents to achieve multiple virus isolations. This could be performed on the same sediment by repeated extractions for different virus types but this would require some degree of eluent compatability, for example, pH consistency.

If we rate the various lecithin mixtures on the basis of existing experimental results (Table 15), the value of the various eluents (highest to lowest) is:D:(F or A or E):(B or C). For poliovirus, the most promising eluent mixture is A; for coxsackie and echoviruses, mixtures D and E, respectively. For the various sediments compositions, only sediment 3 is clearly defined as to the eluent of choice. For sediment 4, mixture F is more appropriate for coxsackie and echovirus. The data of Table 14 was analysed statistically to determine the one eluent mixture suitable for the elution of all enteric viruses investigated. These results are presented in Table 16 and the pattern of overall best eluent was:D>C>F>E>B>A.

The initial studies presented in Table 17 demonstrate that virus survival in seawater containing no sediment decreases during 7 days of incubation, but this loss of virus is greater at the higher temperature (25 C). With the exception of sediment 3, virus in suspension (experiment 1) is protected by suspended sediment, though only to a small degree. Although the percentage of virus recovery from each sediment type in experiment 1 is low, virus does persist after 7 days in sediment and the low recovery could be due

Table 15. Comparison of IC-lecithin mixtures for all viruses and sediment types.

		Sediment No. 1	2	3	4
Virus	P	F*	A	D	A
	C	C	D	D	F
	E	E	B	D	F

* Eluent mixtures of Table 14.

Table 16. Statistical analysis of lecithin eluent recoveries of 3 viruses from 4 sediment types.

Eluent	Mean.	Min.	Max.	Std. deviation
A	20.3	5.5	42.4	12.6
B	37.8	1.0	94.7	26.7
C	45.6	0.2	81.9	24.7
D	54.5	0.6	91.5	28.1
E	39.3	0.2	138.4	43.1
F	43.0	0.6	73.4	22.9

Table 17. Recovery of poliovirus in PFU/ml from supernatant fluid and sediment after 7 days incubation at 25 and 5° C.

Experiment	Sample	Sediment No. 1	2	3	4
1 (25°C)	Su^7	3.2×10^3 (96.1)$^{++}$	9.6×10^2 (98.8)	1.7×10^2 (99.8)	2.9×10^3 (96.5)
	Se^{7*}	1.0×10^3 (1.2)$^{+++}$	2.7×10^3 (3.3)	6.6×10^2 (0.8)	4.5×10^2 (0.6)
	**(30 min. control = 8.2×10^4 PFU/ml; 7 day control, 3.6×10^2 PFU/ml)				
2 (5°C)	Su^0	4.8×10^2 (99.5)$^{+}$	3.8×10^1 (99.9)	5.6×10^1 (99.9)	3.7×10^1 (99.9)
	Su^7	1.5×10^2 (99.8)$^{++}$	3.2×10^1 (99.9)	9.5×10^1 (99.9)	3.1×10^1 (99.9)
	Se^7	2.3×10^3 (2.3)$^{+++}$	3.4×10^3 (3.5)	7.9×10^2 (0.8)	1.9×10^2 (0.2)
	(30 min. control, 9.8×10^4; 7 day control, 1.2×10^3 PFU/ml)				

* Eluent 4% IBE in 0.2 M tris (pH 9.0)

** Controls represent seawater-virus mixture (No sediment)

$^{+}$ % loss of virus from suspension after mixture with sediment

$^{++}$ % loss of virus after 7 days incubation

$^{+++}$ % recovery of virus from sediment after 7 days incubation

to a gradual increase in the bonding between virus and sediment particles. Note that in all cases, the amount of virus recovered from the sediment is greater that the 7 day seawater control. Experiment 2 controls were not as different as those of the first experiment and this effect is attributed to the low temperature of incubation. A high percentage (98.8%) of free virus is lost when virus is suspended in seawater but the loss is less than that observed at 25 C. The amount of virus remaining in suspension after removal of sediment by centrifugation is slightly greater on day 0 as compared to day 7 indicating that some loss of suspended virus does occur even during incubation at low temperature. As expected, virus is able to be retrieved from sediments maintained at 5 C for 7 days and the percentages of virus recovery are approximately the same for each sediment; a similar result occurred in experiment 1. This experiment pointed to a need to know the concentration of virus able to be recovered from each type sediment on day 0.

To determine if higher temperatures would influence the recovery of virus adsorbed to sediment for 7 days, the results of Table 18 were accumulated. At the higher temperature of 37 C, poliovirus in seawater dies at a faster rate that the same suspension held at 30 C. Virus was able to be recovered after 7 days in greater quantities at the lower temperature but it is significant that at 37 C virus is still able to be isolated after a week of

Table 18. Recovery of poliovirus (PFU/m
incubation at 30 and 37° C.

Experiment	Sample	1
3 (30°C)	Su^0	9.8×10^2 (98.8)+
	Su^7	6.9×10^3 (91.8)++
	Se^7	3.1×10^3 (3.7)+++
(30 min. control, 8.2×10^4; 7 day		
4 (37°C)	Su^0	9.8×10^2 (98.8)
	Su^7	3.7×10^1 (99.9)
	Se^7	7.1×10^1 (.09)
(30 min. control, 8.2×10^4; 7-day		

\+ % loss of virus from suspension

++ % loss of virus from suspension

+++ % recovery of virus from sedimer

incubation. At the lower temperature, the titer of the supernatant increased after 7 days incubation, possibly indicating that virus desorption was reversed at 30 C. In comparison to the percent recovery of virus from each sediment type held at 5, 25 and 30 C, 37 C appeared to produce a decrease in titer.

In all of the experiments described above, some portion of virus remained viable after a week of incubation. The following experiments are again intended to test virus viability as a function of time and temperature; however, a 30 day trial period is included for comparison. Here, the amount of virus able to be extracted from sediment on day 0 is also summarized.

Table 19 experiments were conducted at 25 C. It is clear from the control samples that poliovirus suspended in seawater declines in titer during the 30 day period (41,000 to 5800 to 43 PFU/ml). When sediment is added to the system, a significant portion of the suspended virus is adsorbed but the change in supernatant virus levels is not as drastic: sediment 1, 260 to 97 to 5.4; sediment 2, 17 to 17 to 10; sediment 3, 24 to 19 to 1.3; sediment 4, 36 to 33 to 11. The most dramatic change occurs with the supernatant fluid of sediment 1 which contains the lowest percentage of clay. It appears that once clay is added to the system and while a higher portion of the original virus suspension is removed by adsorption, the virus that remains suspended does retain its infectivity for longer periods.

Table 19. Recovery of poliovirus in PFU/ml from supernatant fluid and sediment following incubations of 7 and 30 days at 25° C.

Experiment	Sample	Sediment No. 1	2	3	4
5	Su^0	2.6×10^2	1.7×10^1	2.4×10^1	3.6×10^1
	Se^0	3.5×10^4	4.0×10^4	3.9×10^4	3.8×10^4
	Su^7	9.7×10^1	1.7×10^1	1.9×10^1	3.3×10^1
	Se^7	7.8×10^2	9.1×10^3	6.3×10^2	4.6×10^3
	Su^{30}	5.4×10^0	1.0×10^1	1.3×10^0	1.1×10^1
	Se^{30}	3.5×10^0	4.8×10^2	7.8×10^1	3.6×10^2

(30 min. control, 4.1×10^4; 7 day control, 5.8×10^3; 30 day control, 4.3×10^1)

This experimental procedure was repeated at 37 C and the results are presented in Table 20. The decrease in seawater control samples approximate the results obtained at 25 C. Although a general decline in supernatant and sediment titers is present in the results, the data demonstrate that virus is more likely to persist in association with particulate matter.

To determine whether cold temperatures could retard viral inactivation in the presence of sediment during a 30 day period, the same basic procedure was performed at 5 C (Table 21). At this temperature, viral infectivity is decreased but it is not as rapid as was observed at the higher temperatures. This same trend also applies to supernatant and sediment titers for all of the sediments tested and implies that virus contamination of estuarine sediments during the colder months is likely to persist until the warmer months increase the rate of inactivation.

The greatest change in the titer of virus suspended in seawater occurs between day 0 and day 7 (Table 22). The percent decrease from 7 to 30 days is sizeable for the 5 and 25 C incubations but is not as obvious at 37 C because little virus remains after 7 days. The greater recovery of virus in sediment held for 7 days at 25 C as compared to the recovery at 5 C has not been explained. Overall, less virus adsorbs to the four sediment types at the 5 C incubation temperature; the degree of adsorption at 25 and 37 C is higher and more consistent at these temperatures.

Table 20. Recovery of poliovirus (PFU/ml) from supernatant fluid and sediment following incubations of 7 and 30 days at 37° C.

Experiment	Sample	Sediment No. 1	2	3	4
6	Su^0	7.4			
	Se^0	3.5			
	Su^7	1.0 x 10^2	9.2×10^1	7.4×10^1	6.9×10^1
	Se^7	1.1 x 10^4	3.8×10^4	3.7×10^4	3.8×10^4
	Su^{30}	4.3 x 10^2	5.8×10^0	1.9×10^0	2.9×10^0
	Se^{30}	3.9 x 10^2	1.4×10^3	1.5×10^2	8.8×10^2
(30 min. control; 3.3×10^0 control; 9.9×10^1)			0.4×10^0	0.2×10^0	0.2×10^0
			3.8×10^2	4.3×10^1	3.4×10^2
			$.9 \times 10^4$; 7 day control 4.3×10^2; 30 day		

Table 21. Recovery of poliovirus (PFU/ml) from supernatant fluid and sediment following incubations of 7 and 30 days at 5° C.

Experiment	Sample	Sediment No. 1	2	3	4
7	Su^0	8.7×10^2	4.1×10^1	9.7×10^1	5.2×10^1
	Se^0	4.0×10^4	3.7×10^4	3.1×10^4	3.6×10^4
	Su^7	5.5×10^2	3.7×10^1	6.6×10^1	4.0×10^1
	Se^7	2.4×10^3	9.3×10^3	5.1×10^3	7.2×10^3
	Su^{30}	1.2×10^2	1.1×10^1	2.3×10^1	2.9×10^1
	Su^{30}	3.7×10^2	2.6×10^3	7.9×10^2	1.1×10^3

(30 min. control, $4.5_2 \times 10^4$; 7 day control, 3.9×10^3; 30 day control, 2.2×10^2)

Table 22. Percent virus recovery after 7 and 30 days of incubation of seawater held at 5, 25, and 37° C and adsorption to sediment on day 0.

°C	% Recovery 7	30
5°C	8.7%	0.5%
25°C	14%	0.1%
37°C	1.1%	0.3%

°C	% Adsorption to Sediment No. 1	2	3	4
5°	89.3	82.2	69.8	80.1
25°	85.3	97.8	95.1	92.6
37°	90.2	98.2	95.1	98.4

As a rule it can be stated that suspended virus does not remain free in the water column for long periods and the degree of adsorption and protection is rapid and long lasting.

The fact that sediment-associated virus does persist for extended periods led to experiments designed to evaluate the role of shellfish in this type of microenvironment. Viruses which are taken up during the shellfish feeding process are likely to be engulfed by cells of the digestive tract and can enter normally sterile tissue in phagocytes. Viruses may possibly be excreted in shellfish feces and this topic was considered in this investigation (Table 23). These resluts demonstrate that virus persistence in oyster castings are dependent on the initial concentration of virus in the growing waters and the environmental temperature. Virus, in low seeded experiments, was recovered after 7 days at 15 C but was not detected when the incubation temperature was 25 C. In comparison, a higher level of seeded virus, as indicated in type B experiments, was able to be demonstrated for 28 days at 15 C and 14 days at 25 C. Overall, virus would remain infective in sediments, including those contaminated by oyster feces, to a greater degree during the colder months of the year.

Table 23. Effect of time, temperature and level of viral inoculum on the maintenance of poliovirus in oyster feces.

Experiment Type		PFU/gr. Days of Treatment 0*	7	14	21	28
A		17				
	15°C		3.0	0.0	0.0	0.0
	25°C		0.0	0.0	0.0	0.0
B		169				
	15°C		361	92	58	105
	25°C		73	16	0.0	0.0

* Average of a minimum of 2 ampules.

BACTERIOLOGY

Introduction

To be approved for oyster harvesting an estuarine area must be free from dangerous concentrations of pathogenic microorganisms. This determination is based on a sanitary survey of the area and verified by bacteriological studies on water samples. However, most approved oyster growing areas do receive pathogenic microorganisms during catestrophic events, such as floods, and relaying areas receive pathogenic microorganisms when oysters from polluted areas are brought in (relaid) for cleansing.

A multitude of chemical, physical and biological factors are involved in the decline of non-marine bacteria in seawater. Of these, adsorption and sedimentation can cause a decline in populations in the water without killing the bacteria which ultimately end up in the sediments. Once there, these bacteria are still subject to biological, chemical and physical pressures which lead to their demise. However, recent research (8, 48) has demonstrated that coliforms survive longer in seawater to which sediment has been added than in seawater alone.

In the overall effort to protect the health of raw oyster consumers, it is of equal importance to understand the fate of indicator bacteria and human pathogens in sediments and in water. The investigations reported here consider the fate of those bacteria in sediments and the potential for oyster contamination by pathogens from sediments and oyster feces. Studies with viruses were not addressed here but are being conducted in an adjunct project.

Review of Literature

Numerous papers have explored the fate of enteric bacteria that enter the marine environment through sewage disposal pipes or in contaminated river water. Many of these studies have been reviewed by Greenberg (54), Orlob (59), Carlucci and Parmer (47) and Mitchell (57).

Enteric bacteria are removed from seawater by death or by adsorption and sedimentation. Factors found to be of the greatest significance in the death of bacteria include sunlight (52, 53) and the natural microflora (49, 51, 58).

Adsorption and sedimentation have long been recognized as a non-destructive mechanism for removal of enteric bacteria from contaminated waters. Weiss (62) observed that natural waters usually contain sufficient particulate matter to adsorb the *E. coli* present and that adsorption to particles increased the rate of sedimentation of bacterial cells. Further, diluted seawater increases the rate of flocculation of silt and thus the sedimentation rate. Studies of sediments around three marine sewage outfalls led Rittenberg et al. (60) to conclude that coliforms reached the sediments by sedimentation from the overlying waters.

Once in the sediments, physical and biological pressures still exist which destroy the bacteria, but this process is slowed as has been noted by some researchers. Gerba and McLeod (8) concluded that the greater organic content of sediments was responsible for the increased survival of *E. coli* in sediments over survival in seawater. Similar findings were reported by Chang et al, (48) who noted a direct

relationship among sediment particle size, organic content and survival of E. aerogenes.

The above cited literature documents one pathway by which enteric bacteria may gain entry into sediment. A second mechanism is the deposition of oyster feces, which contains enteric bacteria, directly onto the sediment. T would be the case when oysters are relaid from contaminat areas to approved areas for cleansing. Fecal material deposited by oysters during the first few hours of cleans may contain thousands of indicator bacteria per gram (56) These indicator bacteria have not been observed to multip in those deposits (56), but there is no information on th survival rate. Such biodeposits are high in organic matt (61) which may protect and extend the life of the enteric bacteria.

Oysters are filter feeders. Water is pumped through shell by the ciliary action of the gills. Particles in t water are passed along the gills to the labial palps, a p of fleshy folds surrounding the mouth. The palps sort th particles according to size, rejecting those that are too large and delivering the appropriate sized ones to the mo for ingestion. Though large sand particles are excluded, small silt and clay particles to which bacteria would be adsorbed are ingested. This thus provides a mechanism by which adsorbed enteric bacteria find their way into oyste

It should be remembered that the water-sediment inte where the sediments first collect and where oyster feces deposited is not a static system (8). Sediments are easi

resuspended by fast running tides or storm surges, thus promoting desorption of bacteria from the sediment. The absence of indicator bacteria in the water column may not necessarily indicate that an area is free of pathogens since abrupt water movements may suspend sediment and introduce into the water column bacteria which were attached to the sediments. An understanding of the sedimentation and survival of enteric bacteria in estuarine areas is therefore essential to the proper management of the oyster industry and protection of the public health.

Materials and Methods

Sampling Sites: Field Studies

Figure 1 is a map of the study area with the location of the sampling stations marked. Station 1 is located in about 4 feet of water just inside Graveline Lake at its connection with Graveline Bayou. Station 2 is located in about 3 feet of water at the junction of Graveline Bayou and an unnamed branch running to the north. Station 3 is on the north side of Graveline Bayou at the junction with the unnamed tributary running to the north. Because of the coarse nature of the materials in the bottom of the channel, it was necessary to take samples very close to the shore at this station. Station 4A is located in the Sound approximately 50 yards offshore in about 2 feet of water. Station 4B is located at the upper end of a bayou which receives discharge from a sewage treatment plant.

Oysters were harvested in Graveline Bayou between Stations 2 and 3.

The sampling site was visited at approximately 30 day intervals over a one year period and all samples collected during each calendar month were taken on the same day.

Sediment Sampling:

Sediment samples were retrieved with a hand-held Ekman dredge (Wildco, No. 196). The upper 2 to 3 cm of sediment were removed from the dredge with a clean spoon and placed in a sterile plastic container. Sampling was repeated until a total of 1000 ml of sediment was collected at each station. These samples were placed in an insulated chest and returned

to the laboratory. In all cases, bacteriological analysis of the samples were begun within 4 hours after collection.

Oyster Sampling:

An oyster dredge was used to harvest approximately 100 market size oysters on the same day the sediment samples were taken. The oysters were culled and protected from the sun while being returned to the laboratory. In all cases, oyster samples were processed within 4 hours after harvest.

Processing of Oyster Samples:

The shells of the oysters were scrubbed under cold tap water to remove mud and fouling organisms prior to being disinfected by dipping in 70% alcohol.

The oysters planned for use in virological analyses were shucked into a sterile pan which had a perforated bottom. After a 30-minute draining period, the oysters were packed in 150 g amounts into each of five plastic bags, labeled and frozen at -70 C. Oysters were held at that temperature until they could be transported to the Microbiology Department at the University of Southern Mississippi.

The oysters used in bacteriological analysis were shucked into sterile plastic beakers. Two 200 g samples were analyzed each month as described below.

Processing of Sediment Samples:

Sediment samples from each station were handled separately. Each sample was placed in a sterile blender jar and thoroughly mixed. 500 g of the sediment was weighed into a sterile plastic bag, labeled and frozen at -70 C. This sample was used for viral analysis. Two 100 ml samples were set aside

for bacteriological analysis. Once each quarter, sediment samples were set aside for grain size analysis.

Bacteriological Analysis of Oyster Samples:

The 5-tube MPN procedure as recommended by The American Public Health Association (46) was used to enumerate fecal coliforms in each oyster sample.

Bacteriological Analysis of Sediment Samples:

The procedure for the analysis of the blended sediment samples was essentially that of Babinchak et al. (63). All dilutions were made on a volume-to-volume basis using 0.5% sterile peptone water as the diluent. The initial dilution was made by volume displacement of the diluent by the sample in a bottle which had been calibrated at 200 ml and containing 100 ml of diluent. The weight of the sediment sample displacing the 100 ml volume was recorded.

Samples were thoroughly mixed by shaking and transferred rapidly into diluent or media. Figure 2 diagrams the dilution and inoculation procedure. Fecal coliforms were enumerated by the 5-tube MPN method using lauryl sulfate tryptose broth (LST) in the presumptive test and confirmed in EC broth incubated at 44.5 C according to Recommended Procedures (46).

Sediment Grain Size Analysis:

Once each quarter a portion of the blended sediment sample was provided to the Gulf Coast Research Laboratory Geology Section for grain size analysis. Results provided the percent silt, sand and clay in each sample.

Temperature and Salinity Measurements:

These measurements were made on surface water samples at the site where the oysters were harvested. Temperature was measured in situ with a hand-held thermometer and salinity was determined with a refractometer (American Optical Co.).

Sediment Collection: Laboratory Studies

Sediments (8 kg) were collected with an Ekman Sampler, screened to remove large particulate matter and analyzed for grain size (same sediments as described in Table 12).

Bacteriological Procedures

All dilutions of bacteria and sediment samples were made in phosphate buffered dilution water (46).

E. coli in sediment samples which had been sterilized prior to inoculation were enumerated using the spread plate technique on plate count agar (Difco). In non-sterile samples the E. coli were enumerated as fecal coliforms.

Coliforms and fecal coliforms were enumerated by accepted techniques (46).

Salmonella were measured by the following semiquantitative technique. One part sediment was added to 9 parts buffered peptone water, (BPW) (50). This mixture was vortexed and deciaml dilutions made as required in tubes of BPW. Following a 4 hour resuscitation period at 25 C, the tubes were incubated at 35 C for 20 hr. One ml from each tube was then inoculated into 10 ml of Brilliant Green Broth (BGB) containing 1% mannitol instead of lactose. This medium was incubated at 43 C for 24 hours. All tubes showing growth were streaked onto plates of Hektoen Enteric Agar (Difco), incubated

35 C and examined after 24 and 48 hours for glossy black colonies with or without a blue-green edge.

Adsorption Studies

Sediment samples were sterilized by autoclaving and mixed with sterile diluted seawater (15 ppt) in a 1 to 3 ratio by weight. Following inoculation with a dilute culture of E. coli, samples were mixed for 30 minutes and then allowed to settle for a one hour period. The supernatant was taken for analysis. Counts were compared with a duplicate inoculated sediment-seawater mixture which had been agitated for the full 90 minute period. Inoculated seawater, to which sediment had not been added, acted as the control.

Survival Studies with Sediments

The sediment was placed in a blender with sufficient seawater (15 ppt) to permit mixing. The organisms to be added were suspended in a few milliliters of water and added to the sediment while vortexing. Following a 30 second mixing period, the slurry was poured into wide mouth 250 ml jars. Jars were covered with aluminum foil to prevent evaporation during the extended incubation period.

Sediments were sampled with a mini-corer fashioned from a 5 cc disposable plastic syringe which had the lower part of the barrel removed. Only sediment from the upper 2.5 cm was used for analysis.

Collection of Oyster Feces

A system diagrammed in Figure 3 was used to inoculate the water in which the oysters were feeding with a constant

level of the test organism. The fecal collection procedure followed the following pattern. Oysters were placed in the glass tray and inoculated seawater was allowed to flow through the system overnight. The following morning, the system was drained and all fecal material removed. Oysters were replaced in the clean tank and the water flow started again. If the fecal output was rapid, collection could be made later that day but if it was minimal, collection was made the following morning.

A dropper with rubber bulb was used to collect the fecal strands. These strands could generally be picked up whole with a dropper and deposited into a sterile tube which were numbered and weighed to the nearest one hundredth gram. From approximately twelve oysters, sufficient fecal material could be collected to prepare 24 tubes for testing with 0.1 to 0.2 g in each. Care was taken to randomize the placement of fecal material in tubes and each tube received a portion of the fecal material from several oysters. A small amount of water was allowed to remain in each tube and each tube was covered with foil to prevent drying during storage.

Analysis of Oyster Fecal Material

At the appropriate time for analysis, tubes were selected randomly and centrifuged to compact the fecal material. The overlying water was drained from the tube and any remaining droplets adsorbed with a tissue. The tube and fecal material were weighed and the weight of the tube subtracted to determine the weight of the feces. Sufficient buffered distilled water was pipetted into the tube to make a 1:10 dilution and with

the aid of a pipet, the sample was mixed. Appropriate dilutions were made and media inoculated.

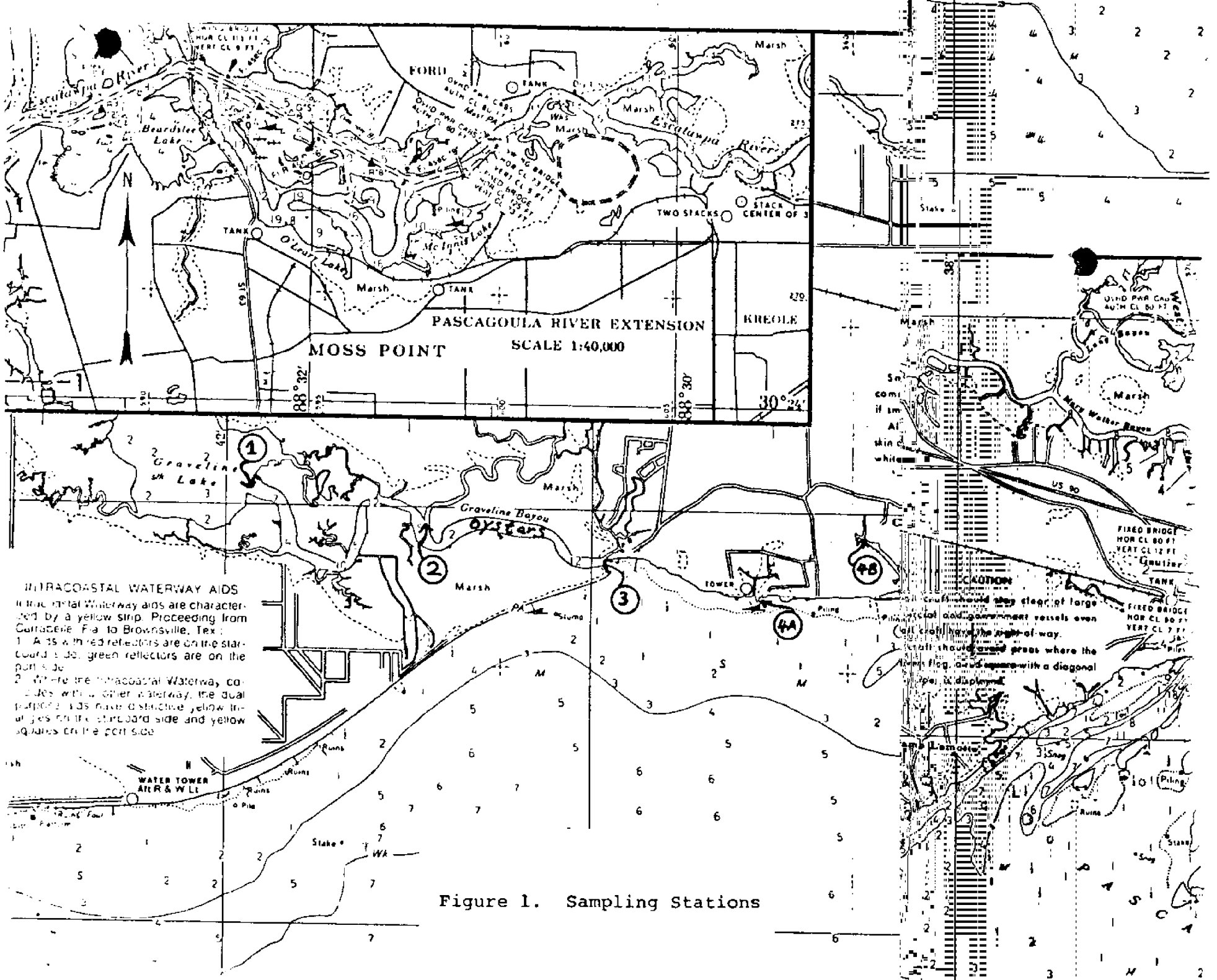


Figure 1. Sampling Stations

Figure 2. Sediment dilution and inoculation scheme.

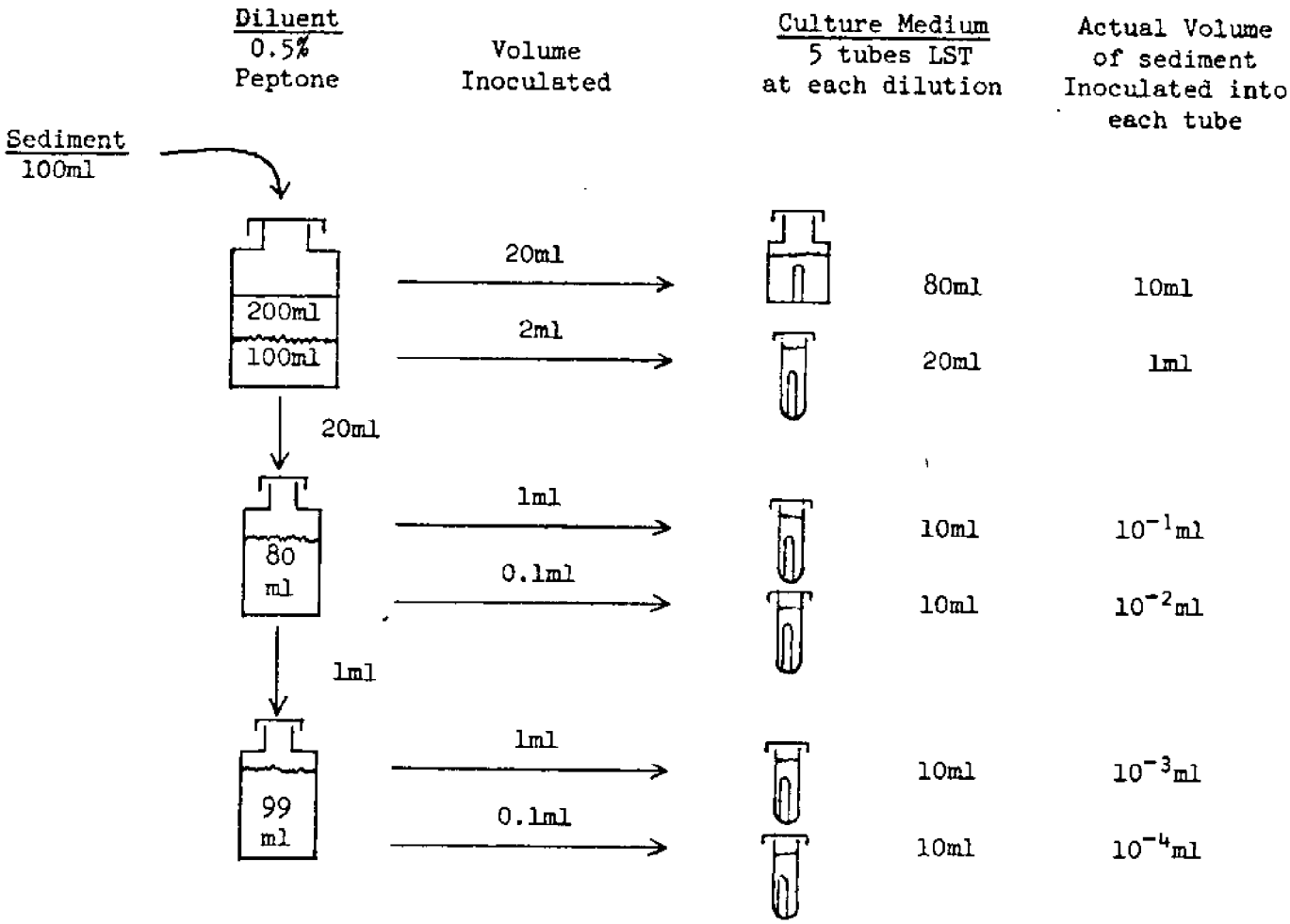
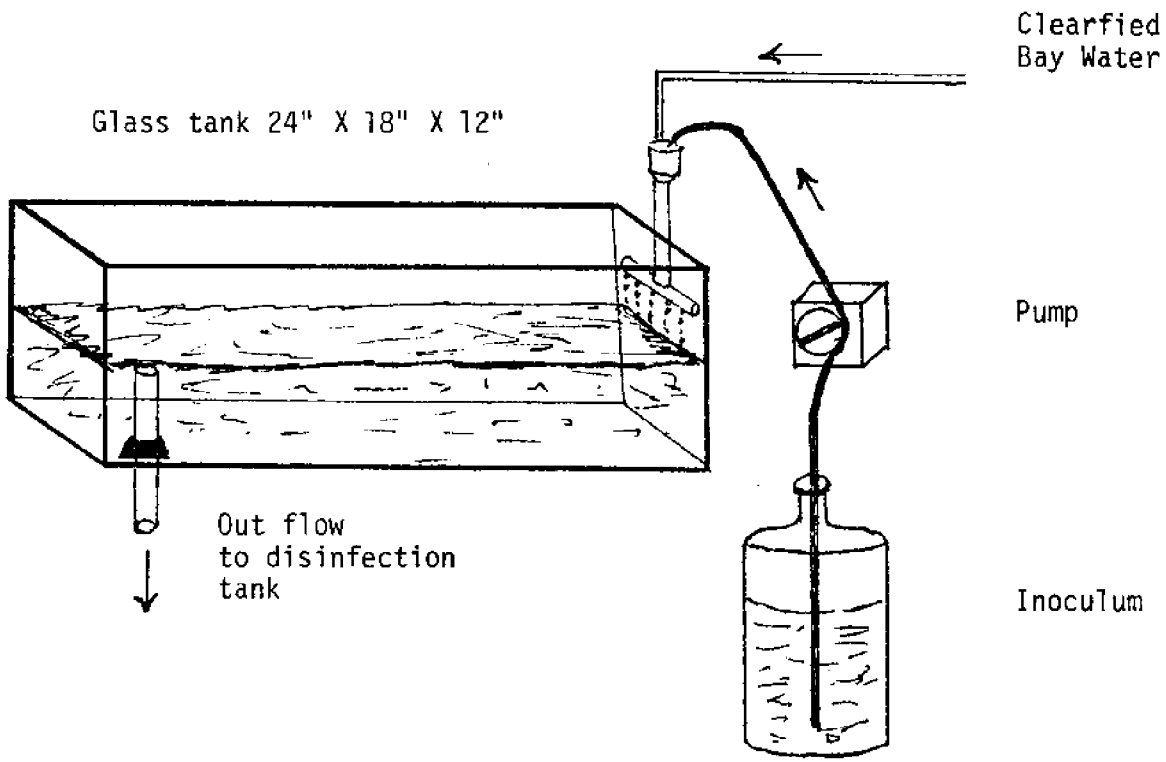


Figure 3. System used to hold oysters for fecal collection. Oysters placed in a single layer on bottom of tank with two inch spacing between oysters.



Results and Discussion

Field Investigations:

Tables 1 and 2 present the data obtained during the field investigations.

If the level of fecal coliforms in sediments are related to the level of pollution in the water as the literature (60, 64) indicates, then we must assume the pollution enters Graveline Bayou at or near its mouth. Of the three stations in the bayou, Station 3 had higher average counts during every month except January and on an overall basis. The median fecal coliform count at Station 3 was 20 times higher than the count at Station 1, and 5 times higher than the count at Station 2 (See Table 3).

One possible source of pollution into Graveline Bayou is a sewage treatment plant which discharges its effluent into a bayou which connects with the Mississippi Sound at a point 1.7 miles east of the mouth of Graveline Bayou. The longshore currents in that area run east to west. Sediment from sampling station 4B which was located in that bayou contained large numbers of fecal coliforms (See Table 4).

If the fecal coliforms discharged at Station 4B were the source of fecal coliforms in the sediments at Station 3, high concentrations of fecal coliforms would be expected at Station 4A. However, Station 4A had the lowest average number of fecal coliforms of all five stations.

A knowledge of the sediments and hydrographic conditions of that area may explain this discrepancy in the data. Water depth at Station 4A is less than 2 feet at mean low water.

Table 1. Compilation of project data.

Date	Rep	Station 1		Station 2		Station 3		Station 4A		Station 4B		Fecal Coliforms per 100g Oysters	Surface Water over Oysters	
		Weight of 100ml Sediment	Fecal Coliform MPN/100ml	Weight of 100ml Sediment	Fecal Coliform MPN/100ml	Weight of 100ml Sediment	Fecal Coliform MPN/100ml	Weight of 100ml Sediment	Fecal Coliform MPN/100ml	Weight of 100ml Sediment	Fecal Coliform MPN/100ml		Temp (C)	Salinity (ppt)
1/23/80	A	125.0	7,900	124.7	7,900	153.7	7,900	189.2	13,000	---	---	130	--	—
	B	127.5	3,300	125.1	49,000	150.1	13,000	194.0	33,000	---	---	40		
2/22/80	A	118.5	4,900	113.9	3,300	143.3	7,900	---	--	147.6	360,000	<20	13.5	2
	B	114.6	3,300	115.8	3,100	150.1	4,900	---	--	153.6	360,000	40		
3/14/80	A	121.2	1,300	121.7	7,900	159.5	11,000	208.1	1,400	133.2	1.3×10^6	4,900	18	25
	B	126.3	790	112.4	4,900	168.8	17,000	201.9	2,400	140.3	1.6×10^6	1,700		
4/15/80	A	125.8	49,000	131.3	17,000	158.9	130,000	188.7	4,600	175.3	490,000	20	16	0
	B	124.0	33,000	135.3	11,000	160.4	110,000	190.3	4,900	175.3	490,000	20		
5/15/80	A	122.6	3,300	125.5	35,000	169.6	4,900	213.4	1,300	179.6	130,000	3,300	--	--
	B	125.4	4,900	125.8	3,300	175.3	49,000	208.5	1,300	185.7	$>1.6 \times 10^6$	210		
6/16/80	A	128.0	<200	125.6	<200	160.3	7,000	184.2	<200	168.7	130,000	40	28	5
	B	120.9	<200	126.6	200	164.1	7,000	183.9	<200	178.3	240,000	170		
7/15/80	A	120.6	13	125.4	13	174.9	170	187.4	2	154.8	7,900	<20	31	21
	B	122.8	33	120.4	7.8	171.9	330	195.0	4.5	154.9	11,000	40		
8/11/80	A	122.5	23	126.6	350	180.6	1,100	196.7	79	154.0	11,000	110	31	17
	B	121.1	46	126.9	220	191.7	170	200.5	79	143.6	3,300	40		
9/15/80	A	125.7	4.0	129.0	7.8	177.0	130	193.9	23	126.2	13,000	170	29	21
	B	125.8	13	128.2	2.0	175.2	240	190.9	17	131.0	130,000	460		
10/22/80	A	115.8	22	130.9	110	154.9	790	200.4	70	125.2	1.6×10^7	93	21	20
	B	123.3	79	126.9	350	159.6	790	204.9	170	133.3	1.6×10^7	<20		
11/17/80	A	133.2	330	125.9	1,300	165.4	33,000	193.6	490	120.6	22,000	2,200	19.5	19
	B	134.6	240	122.9	7,900	161.7	33,000	209.8	700	122.4	13,000	1,700		
12/15/80	A	127.4	140	122.9	490	168.4	1,300	192.4	170	139.3	79,000	65	15	18
	B	129.6	130	119.6	460	156.9	3,300	186.1	330	140.3	240,000	78		

Table 2. Composition of sediment samples.

Station	Date Collected	Sediment (%) Sand	Silt	Clay	Size Term
1	3/14/80	4.1	50.9	45.0	Mud
	6/16/80	12.0	54.8	33.2	Very fine sandy mud
	9/15/80	5.9	45.9	48.2	Mud
	12/15/80	29.6	29.6	40.8	Very fine sandy mud
2	3/14/80	10.8	39.7	49.5	Very fine sandy mud
	6/16/80	5.5	63.7	30.8	Silt
	9/15/80	18.0	50.3	31.7	Very fine sandy mud
	12/15/80	6.2	39.0	54.8	Mud
3	3/14/80	60.2	26.3	13.5	Muddy fine sand
	6/16/80	92.9	5.1	2.0	Fine sand
	9/15/80	89.4	3.0	7.6	Clayey fine sand
	12/15/80	74.2	12.6	13.2	Muddy fine sand
4A	3/14/80	94.2	2.5	3.3	Fine sand
	6/16/80	95.3	3.7	1.0	Fine sand
	9/15/80	95.8	2.4	1.8	Fine sand
	12/15/80	90.2	6.5	3.3	Fine sand
um silt		4B	3/14/80	63.7	21.3	15.0	Fine sand
			6/16/80	39.8	36.5	23.7	Fine sand
ne sand			9/15/80	82.7	12.5	4.8	Medium si
um silt			12/15/80	44.3	39.5	16.2	Fine sand

Table 3. Monthly average* of fecal coliforms per 100ml of sediments or 100g of oysters.

	Sediment Samples from Stations					
Month	1	2	3	4A	4B	Oysters
JAN	5,500	28,000	10,000	23,000	--	85
FEB	4,100	3,200	6,400	--	360,000	<30
MAR	1,000	6,400	14,000	19,000	$1.5X10^6$	3,300
APR	41,000	14,000	120,000	4,800	490,000	20
MAY	4,100	19,000	27,000	1,300	>870,000	1,700
JUN	<200	<200	7,000	<200	190,000	100
JUL	23	10	250	3.3	9,500	<20
AUG	35	290	640	79	7,200	78
SEP	8	5	190	20	72,000	320
OCT	51	230	790	120	$1.6X10^7$	<55
NOV	290	4,600	33,000	600	18,000	2,000
DEC	140	480	2,300	250	160,000	62
Overall**	220	900	4,900	<200	130,000	86

* Average of duplicate analyses. All stations sampled on the same day of each month.

** Median of all samples.

Table 4. Percent of samples from each station with fecal coliform counts in each range.

Fecal Coliforms per 100ml of sediment	Station Numbers				
	1	2	3	4A	4B
$>10^7$	0	0	0	0	9.1
$>10^6$ $<10^7$	0	0	0	0	13.6
$>10^5$ $<10^6$	0	0	8.3	4.5	[40.9]
$>10^4$ $<10^5$	8.3	16.6	25.0	9.1	27.3
$>10^3$ $<10^4$	29.2	33.3	[37.5]	22.7	9.1
$>10^2$ $<10^3$	[20.8]**	[33.3]	29.2	[31.8]	0
>10 $<10^2$	37.5	4.2	0	22.7	0
<10	4.2	12.5	0	9.1	0
N*	24	24	24	22	22

* N = Number of samples

** [ ] = Range in which the 50 percentile value is found

Wind stress out of the south causes wave action which constantly disturbs the bottom, resulting in a bottom type of greater than 90% sand (Table 2). Silts and clays, the two sediment fractions to which bacteria and viruses normally absorb or attach, are unable to settle out of the water column in the area around Station 4A due to the wave action. The currents transport these silt and clay laden waters into the calm bayou, where the suspended solids, with their attached microorganisms, settle out.

There, of course, may be other explanations for the high level of pollution at Station 3, such as the pollution source being the unnamed bayou which junctions Graveline Bayou at Station 3.

Fecal coliform levels in the sediments are not constant throughout the year. The trend indicated in Table 3 is for lower counts during the summer and fall, and higher counts in the winter and spring and was noted at all stations. A previous report (13) indicated a similar trend in the level of fecal coliforms in the waters of Graveline Bayou and an explanation for seasonal trends has been postulated by Cook and Hamilton (49).

Laboratory Investigations:

Adsorption of E. coli to estuarine sediments. Sediments found in different portions of a single estuary frequently differ in composition as measured by grain size analysis. To determine if sediment composition affects the adsorption of indicator bacteria, sediments of different composition (Table 5) were mixed with E. coli inoculated seawater. E. coli

was adsorbed to all sediments causing a reduction in the bacterial count of the water at the end of the settling period (Table 6). The least reduction occurred with sediment type 1 which was almost pure sand. With this exception, there was no obvious correlation between adsorption and the sediment composition which suggests that factors other than grain size may be involved in adsorption.

Survival of indicator bacteria in sediments. Sediments from the same four areas described in Table 5 were used in the survival studies. E. coli was adsorbed onto both fresh sediments and sediments which had been autoclaved. All sediments were stored at 25 C in the dark. Periodically samples were removed and the E. coli enumerated as fecal coliforms by the MPN technique.

Sediment type 1 adsorbed too few bacteria to be enumerated and no multiplication was noted in the autoclaved sample, probably reflecting the low organic content of that sediment. The other autoclaved sediments showed a large increase in the number of E. coli (Table 7). The E. coli in non-autoclaved sediments decreased in number with time and >94% were lost within 16 days. There was a difference in death rate of E. coli in the various sediment types, but it did not appear to relate to the sediment grain size.

Researchers (55) have been critical of survival studies which employed laboratory strains. Therefore, additional studies were made using indicator bacteria from sewage.

Sediments were obtained at three sites, each representing an area with a different level of pollution. Site 1 was the

Table 5. Source and composition of sediments used in adsorption and survival studies.

Sediment Type	Collected From	Composition Sand	Silt	Clay	Size Term
1	Off Marsh Point, Biloxi Bay	99	1.0	0	Medium Sand
2	East of Point Cadet Biloxi Bay	89.3	6.0	4.6	Muddy Fine Sand
3	North of Veterans Administration Center Back Bay of Biloxi	52.3	30.3	17.4	Medium Silty Fine Sand
4	Magnolia Bend Back Bay of Biloxi	37.3	39.3	23.5	Very Fine Sandy Mud

Table 6. Effect of different sediment types on the removal of E. coli from seawater by adsorption and settling.

Sediment Type	Trial	E. coli/ml Mixed	E. coli/ml Settled	% Reduction	Av. % Reduction
1	a	11,500	6,700	41.7	44.1
	b	1,830	1,200	34.4	
	c	1,900	830	56.3	
2	a	11,500	120	98.9	>98.7
	b	1,850	<30	>98.4	
	c	2,600	<30	>98.8	
3	a	11,700	2,100	82.1	82.7
	b	2,000	370	81.7	
	c	2,900	460	84.2	
4	a	11,000	2,000	81.8	81.6
	b	2,300	360	84.3	
	c	2,600	550	78.8	
Control No Sediment	a	1,800	1,900	-5.5	2.5
	b	1,900	1,700	10.5	

Table 7. Effect of sediment type and autoclaving on the survival of E. coli in sediments.

Sediment Type	Autoclaved Sediment Days 0	Autoclaved Sediment Days 7	Non-Autoclaved Sediment Days 0	Non-Autoclaved Sediment Days 7	Non-Autoclaved Sediment Days 16
1	<2*	<2	<2	<2	--
2	330	>16,000	100	6.9 (93.1)**	<2 (>98.0)
3	410	>16,000	390	120 (69.2)	21 (94.6)
4	280	>16,000	340	64 (81.1)	<4.5 (>98.5)

* - E. coli/g sediment. Average of two samples.

** - Percent reduction in E. coli.

Figure 4. Change in the number of indicator bacteria in sewage inoculated sediments during storage at two temperatures. Sediments obtained from an area which has been heavily contaminated by sewage.

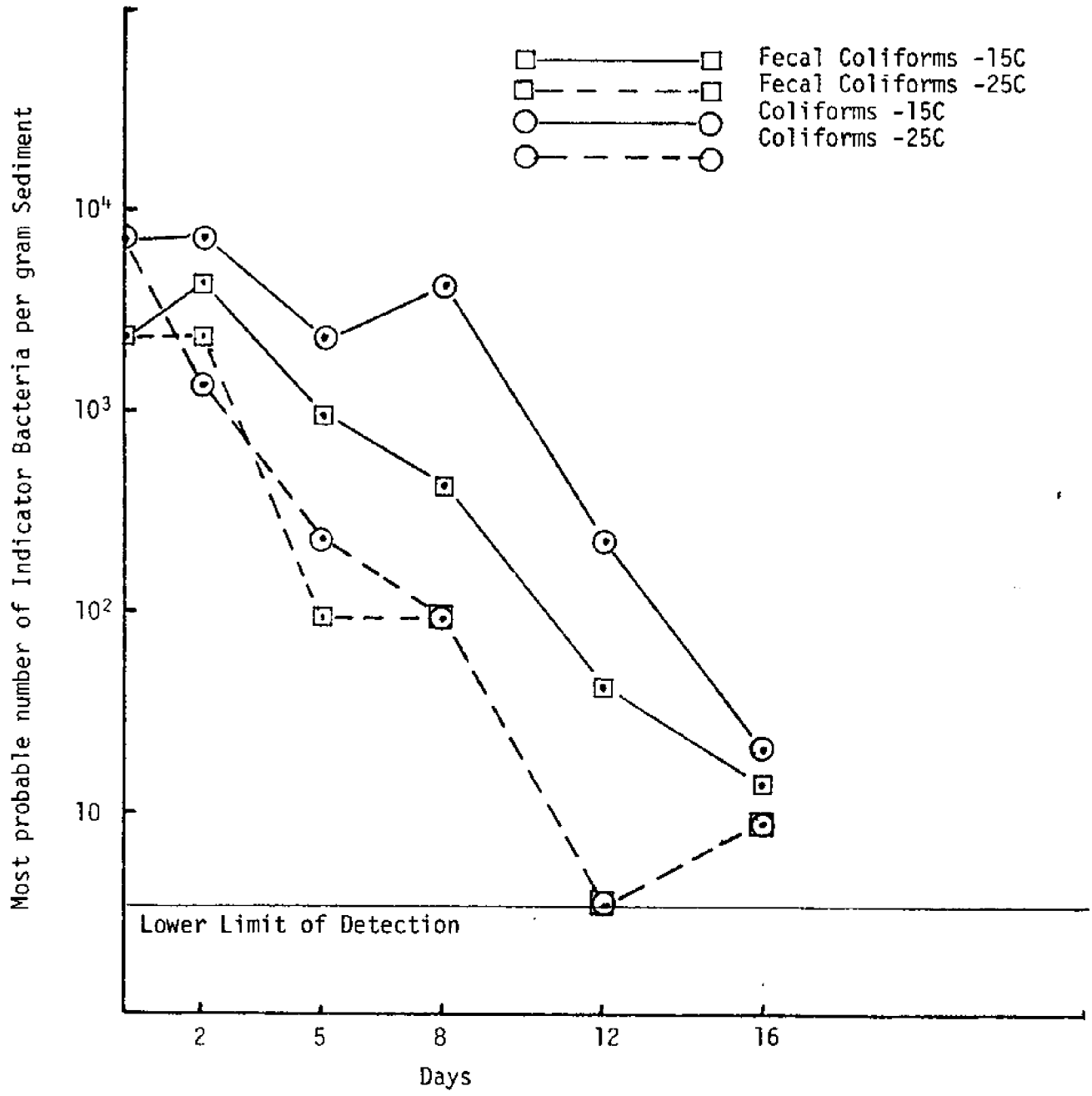


Figure 5. Change in the number of indicator bacteria in sewage inoculated sediments during storage at two temperatures. Sediments obtained from an area which receives intermittent sewage pollution.

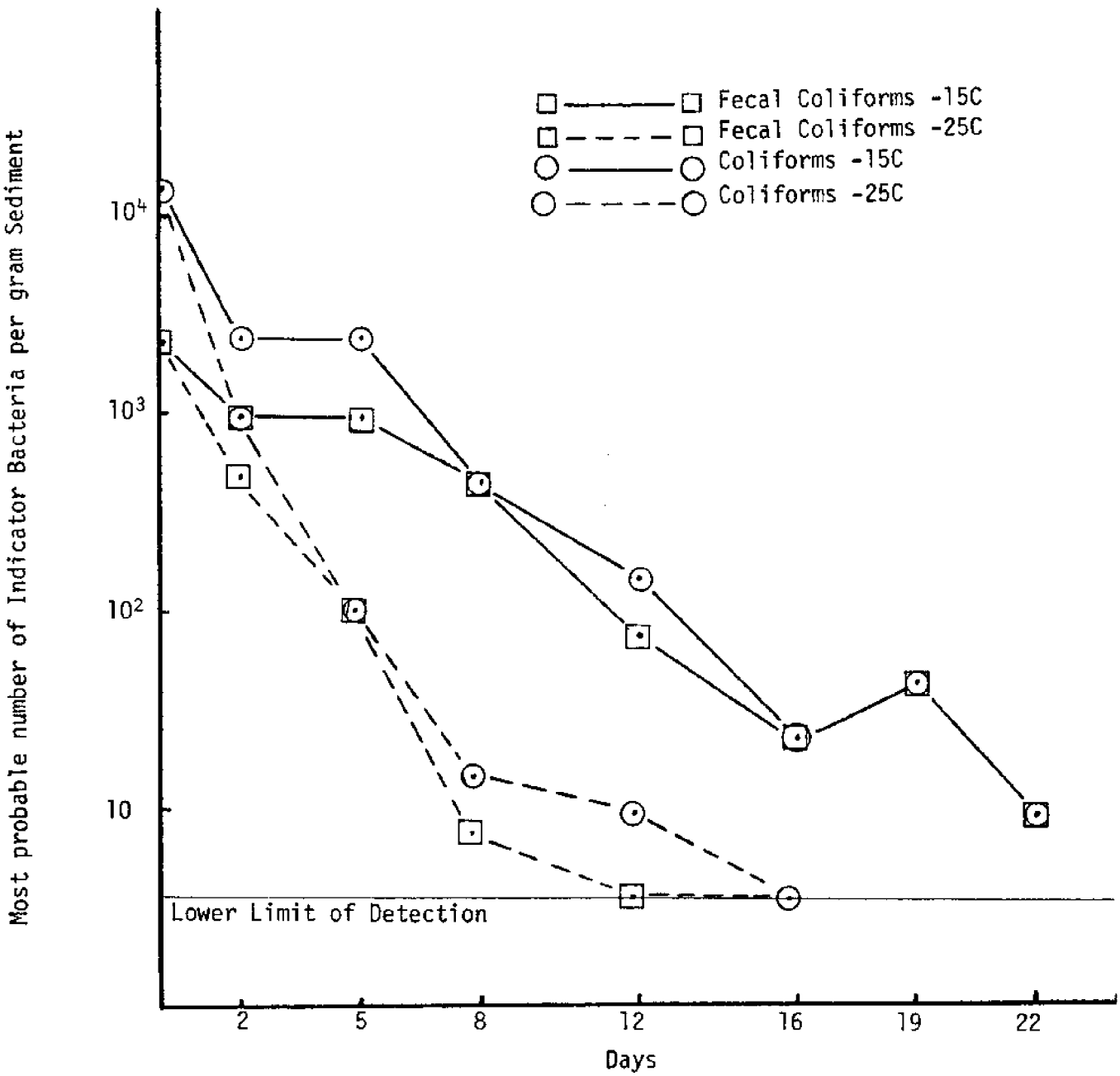
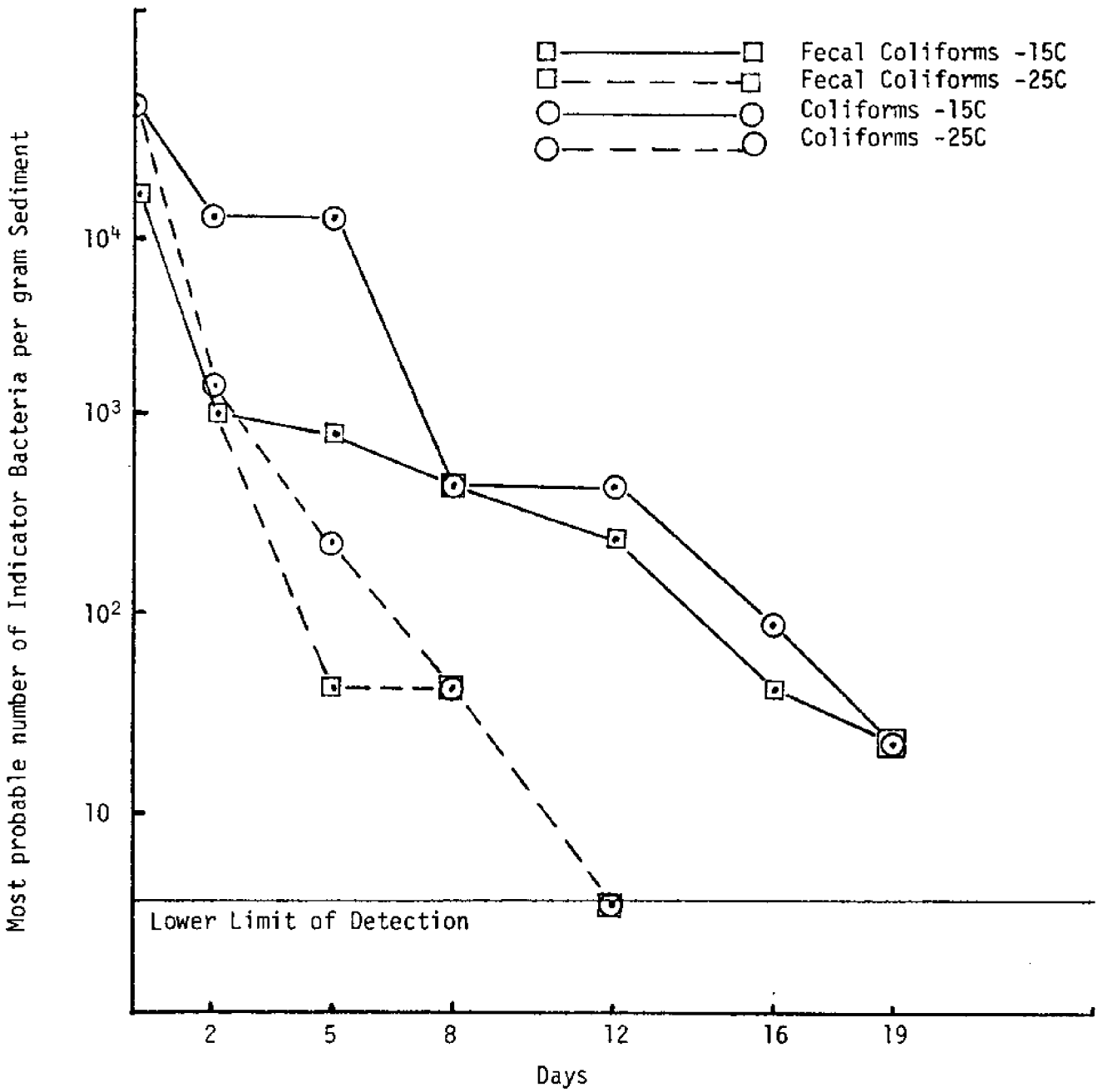


Figure 6. Change in the number of Indicator bacteria in sewage inoculated sediments during storage at two temperatures. Sediments obtained from an area free of sewage pollution.



heavily contaminated Ocean Springs Small Craft Harbor, Site 2 was an area of Biloxi Bay which is closed to oyster harvesting, but which receives only intermittent pollution, and Site 3 was an area essentially free of sewage pollution which has been used for relaying oysters. Each fresh sediment sample was inoculated with fresh sewage and thoroughly mixed. One half of each sample was placed in a glass jar and stored at 25 C which approximates the average warm weather sediment temperature and the other half was stored at 15 C which approximates the average cool weather sediment temperature. Each sample was analyzed for both total and fecal coliforms periodically. The results presented in Figures 4, 5, and 6 clearly indicate that cooler temperatures promote survival of both coliforms and fecal coliforms. In all sediments incubated at 25 C the coliforms and fecal coliforms reached the minimum level of detection in 10 to 15 days. At the lower temperatures, it is projected that 20 to 25 days would be required before the coliforms would reach a non-detectable level. The rate at which the indicator bacteria populations decreased was approximately equal in all sediments, despite the pollution history of the area from which the samples were taken.

A further study was made to determine how quickly the indicator bacteria eliminated from naturally contaminated sediments. Two sediment samples were taken inside the Ocean Springs Small Craft Harbor and two near, but outside, the harbor mouth. This harbor has received treated and untreated

sewage effluent for over 30 years. Each sample was divided into two portions, one for incubation at 25 C and one for incubation at 15 C.

The coliform and fecal coliform levels in the heavily contaminated sediments incubated at 25 C reached a low to non-detectable level within 22 days (Table 8). Samples held at 15 C retained high counts in excess of 28 days. Samples taken near the mouth of the harbor contained few indicator bacteria which were quickly reduced to non-detectable levels.

These studies have shown that estuarine sediments do not provide an environment conducive to the survival of indicator bacteria. Since the antibacterial activity is eliminated by sterilization of the sediments, and since its rate is keyed to temperature, biological agents in the sediments are probably responsible for the death of the bacteria. Further, these studies have shown that natural processes will eliminate both lab grown E. coli and indicator bacteria of sewage origin, from sediments. This self-cleansing process appears to be irrespective of the past pollution history of the sediment and the type of sediment. Contaminated sediments may be expected to cleanse of indicator bacteria in two to three weeks in warm weather, or require four to six weeks in cold weather.

Survival of Salmonella in Sediments. Techniques similar to those used to study indicator bacteria survival were employed to study Salmonella survival. However, the Salmonella detection techniques were only semi-quantitative and not as reliable as the enumeration techniques for indicator bacteria.

Table 8. Effect of temperature on the survival of indicator bacteria in sediments from contaminated areas. Sediments A and B were collected near the mouth of the Ocean Springs Small Craft Harbor and samples C and D were from within the inner part of that harbor.

	Total Coliform/gm							
	15 C				25 C			
Days	A	B	C	D	A	B	C	D
0	7.8	79	1300	1100	7.8	79	1300	1100
6	<2	13	1100	490	<2	2	220	240
13	<2	7.8	490	280	<2	<2	49	49
22	<2	<2	240	140	<2	<2	7.8	<2
28	<2	<2	170	140	<2	<2	23	<2

	Fecal Coliform/gm							
	15 C				25 C			
Days	A	B	C	D	A	B	C	D
0	2	12	1300	490	2	2	1300	490
6	<2	2	240	330	<2	<2	46	79
13	<2	<2	79	280	<2	<2	23	23
22	<2	<2	79	140	<2	<2	7.8	<2
28	<2	<2	49	79	<2	<2	<2	<2

Table 9. Survival of Salmonella typhimurium in sediments taken from the Mississippi Sound (Type I) and from the Gulf of Mexico south of Dog Keys Pass (Type II). Inoculated sediments incubated at 15 C and 25 C.

Days after inoculation	Sediment Type I		Sediment Type II	
	15 C	25 C	15 C	25 C
1	$\geq 10^4$*	$\geq 10^4$	$\geq 10^4$	$\geq 10^4$
4	10^2	10	10^3	10^2
10	ND**	ND	1	ND
14	ND	ND	ND	ND

* Salmonella per gram of sediment.
** Salmonella not detected.

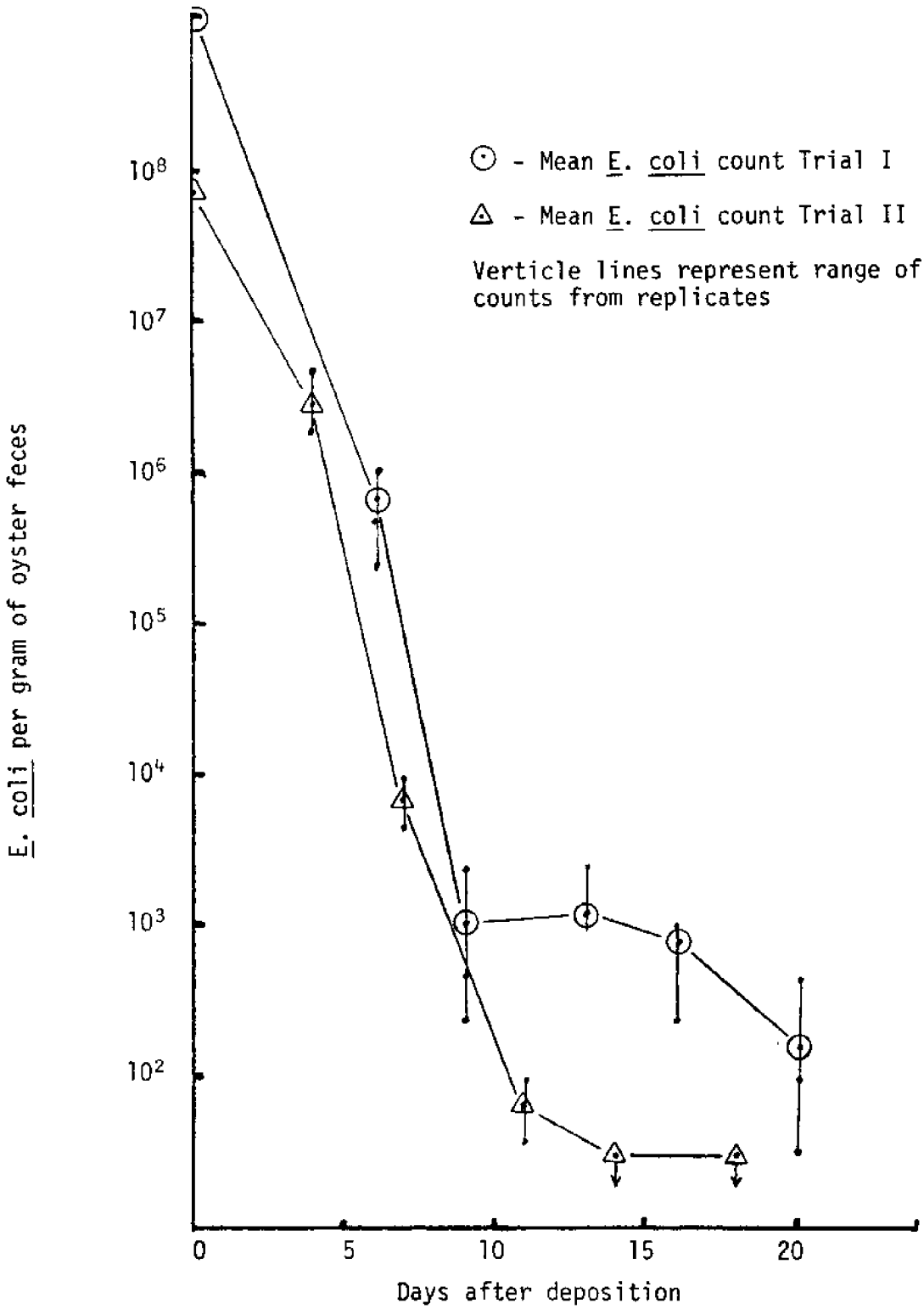
Sediments from both contaminated and non-contaminated areas were used in these studies. In general, Salmonella typhimurium decreased from initial concentration of $>10^4$ per gram to an undetectable level in less than 10 days at both incubation temperatures. However, in one sediment sample collected from offshore waters, the Salmonella remained detectable in low numbers for 34 days.

Table 9 presents typical data from a Salmonella survival study using sediments from two sources held at two temperatures. This data confirms that this pathogen dies faster at cool than at warm temperatures and would not be expected to survive two weeks in sediments.

Survival of Indicator Bacteria in Oyster Feces. Fecal material excreted by oysters which have been feeding in contaminated waters will contain large numbers of indicator bacteria. We have observed that oysters remaining in a tank of flowing bay water which contained an average of 750 E. coli per ml excreted feces which contained approximately 7.1×10^7 E. coli per gram. This represents a 100,000 X concentration factor. Since it is permissible to relay oysters from waters contaminated with large numbers of indicator bacteria, large numbers of fecal coliforms may be expected to be deposited directly onto the sediments by relaid oysters. The following studies were designated to gather information on the survival of indicator bacteria in oyster feces.

Freshly deposited fecal material from oysters was collected and held at 25 C in the laboratory. The death rate of E. coli under this condition is as shown in Figure 7.

Figure 7. Survival of E. coli in oyster feces after deposition. Feces held at 25 C.



In Trial I, the average count in the fecal material was reduced by greater than 5 logs in nine days with a slow decline thereafter. In Trial II, similar results were obtained and the E. coli population reduced below the detectable level within 14 days.

Oysters were then allowed to feed on sewage and the feces collected for study. The death rate of indicator bacteria were as shown in Figure 8. Both coliforms and fecal coliform populations decreased at approximately the same rate at both temperatures. However, both groups of bacteria decreased faster at 25 C than 15 C. At 25 C, a 4 log (99.99%) reduction was achieved in 14 days, whereas, 21 days was required to achieve a 2 log (99.0%) reduction when the oyster feces was held at 15 C.

Survival of Salmonella in Oyster Feces. As shown in Table 10, Salmonella died off in oyster feces at a rate approximately equal to the indicator bacteria, i.e., >4 logs (>99.99%) in 19 days. The Salmonella also died more rapidly at 25 C than they did at 15 C (Table 11).

Two routes have been postulated through which enteric bacteria can find their way into sediments in approved shellfish harvesting areas. These are: (1) adsorption of the bacteria introduced during catastrophic flooding to suspended sediments and settling out of the water column and (2) deposition of fecal material by oysters relaid from contaminated areas.

This research has demonstrated that enteric bacteria do not multiply in natural estuarine sediments or in deposited

Figure 8. Change in the number of indicator bacteria in oyster feces during storage at two temperatures.

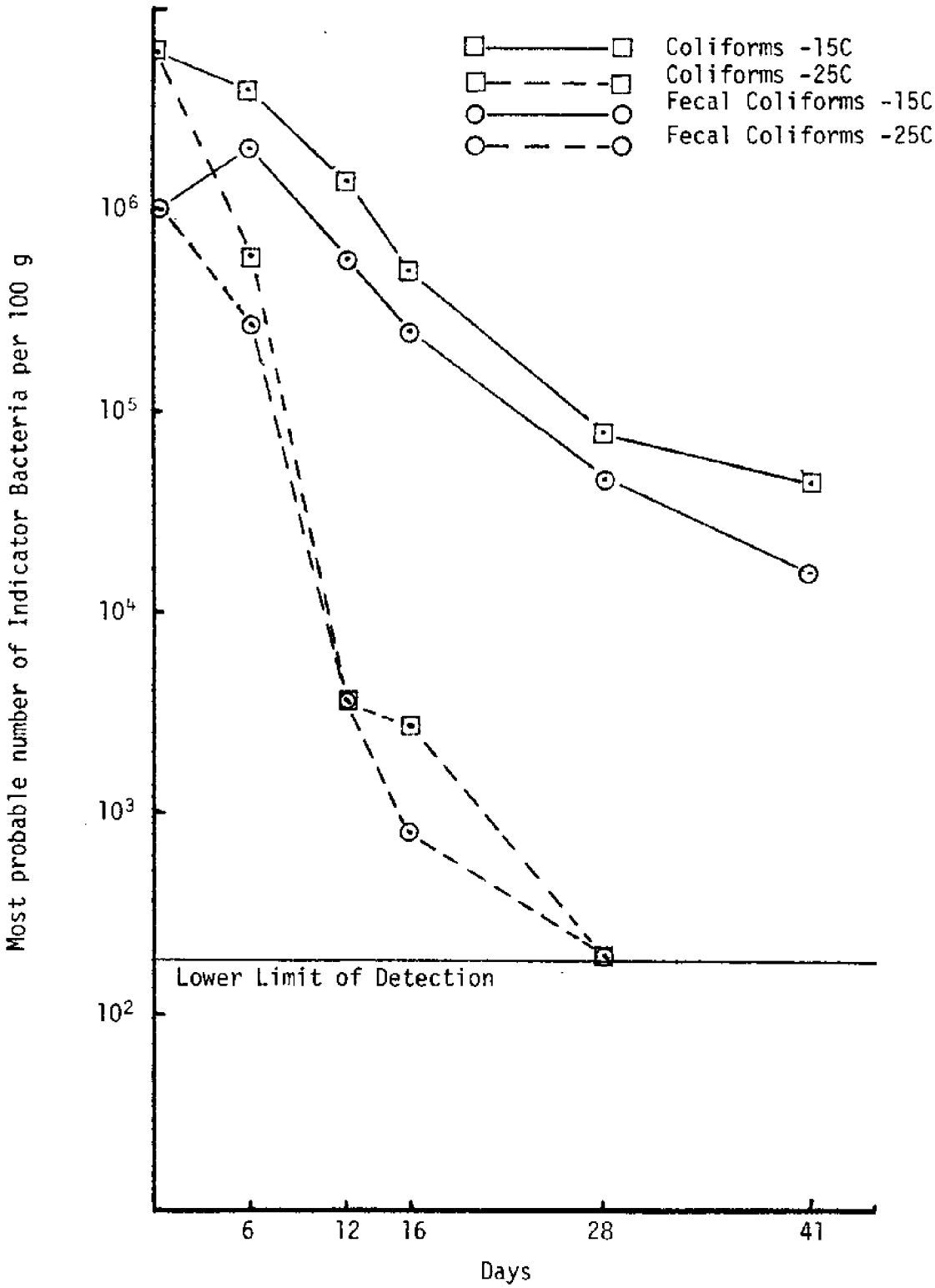


Table 10 *Salmonella* survival in oyster feces held at 25 C.

Day After Collection	Replicate A	B	C	D	AV	Reduction in Salmonella (%)
0	$>10^5$*	$>10^5$	$>10^5$	$>10^5$	$>10^5$	
6	10^2	10^2	ND**	10^2	10^2	>99.9
19	10	10	10	10	10	>99.99
25	10	10	10	ND	10	>99.99

* - *Salmonella* per gram of oyster feces

** - *Salmonella* not detected

Table 11. Effect of temperature on survival of Salmonella in oyster feces.

Day After Collection	Salmonella (No./gm) in oyster feces stored at	
	15C	25C
0	10^4	10^4
3	10^4	10^3
10	10^2	10
17	ND*	ND

* - Salmonella not detected

oyster feces. The death rate of enteric bacteria in sediments was directly related to the temperature and appeared to be independent of sediment type or past contamination history. Salmonella typhimurium died at a rate equal to or slightly greater than fecal coliforms.

In oyster feces, the death rates of the enteric bacteria was less than in sediments; probably, a response to the higher level of organic material in the oyster feces. Salmonella died at a faster rate than did fecal coliforms in the oyster feces.

If we assume that all enteric bacteria respond as did the indicator bacteria and Salmonella used in this study, we do not feel that intermittent contamination of sediments in approved shellfish harvesting areas poses a health hazard provided the following precautions are taken:

(1) Approved shellfishing areas that are contaminated by flooding should be closed to harvesting for a two week period after the water quality, as measured by the fecal coliform test, returns to an acceptable level.

(2) Oysters, relaid into an area for cleansing should not be harvested for a minimum of two weeks after the last oysters are moved into the area.

The time period suggested above is based on the following rational. It would be unlikely that sediments in approved shellfish areas would become heavily contaminated from a single event (flood) and if such event occurred frequently, the approved classification of the area would be questioned.

In nearly every case in this study, the number of enteric bacteria introduced into the sediments was reduced by greater than 99% within 14 days. Therefore, low numbers of indicator bacteria in naturally contaminated sediments should be reduced to background level within this period and Salmonella which would be present at a lower level than the indicator bacteria and which die at a faster rate should be eliminated within the quarantine period.

Although our studies showed a longer survival period for indicator bacteria and Salmonella in oyster feces than in sediments, under natural conditions this pattern may not hold true. In the laboratory studies, the fecal strands were held under conditions which maintained their physical integrity. In natural conditions, the fecal strands would probably be disrupted by currents or bottom feeding organisms. Disruption may lead to an increased death rate of the bacteria or suspension in the water column and removal from the area by tidal currents. Considering this and the information presented herein, 14 days appears adequate to significantly reduce or eliminate the enteric bacteria in excreted oyster feces.

Project Summary

Mechanisms to evaluate the microbiological status of contaminated estuarine areas were conducted. The main objective of the project was to evaluate the role of sediment in the continued contamination of shellfish tissues and growing waters. Initial studies undertaken during the first year were designed to evaluate oyster tissue and sediments collected at the same site or sediment from other local potential zones of contamination. These field investigations were successful in regard to the bacteriological data accumulated, but the virological phase of the study was hampered by sample cytotoxicity. During this period a total of 98 virus isolations were performed from oyster and sediment samples. The level of virus comatmination of oysters was consistent with the frequency of isolations previouslyreported (12,13); however, the number of sediment isolations was regared as an underestimation of the actual virus level.

The bacteriological field investigations conducted during the first year of the study demonstrated that sediments which are near to a source of sewage discharge are more likely to contain high levels of fecal coliforms. Although it could not be shown that a direct correlation existed between sediment associated fecal coliforms and the levels of fecal coliforms found in oyster tissues collected

at the same site, other possible interrelationships were noted and carried over as part of the second year investigations.

A major effort to deliniate the best method to elute virus from Mississippi estuarine sediment began midway through the first year and continued to the end of the grant period. Initial experiments discouraged the use of inorganic glycine eluent mixtures or the use of skim milk solutions in favor of eluents containing beef extract, isoelectric casein or nutrient broth. This research set a firm pattern for continued virus elution studies but was limited by the low number and variability of the sediments employed. The study continued in a more specific manner thereby, a) increasing the number of sediment types with regard to their composition of sand, silt and clay, b) expanding the number of virus types used (to include echovirus) and c) to analyze various lecithin mixtures in regard to their ability to desorb virus. In brief, the addition of lecithin to isoelectic casein solutions allowed high percentages of virus recovery and a recovery reliability not previously achieved. Other virological studies performed demonstrated that sediment-bound virus would remain infective (and able to be recovered from sediment) after 30 days incubation. In these experiments, temperature was observed to indirectly affect virus survival; virus recovery was higher when sediment-virus mixtures were stored at low temperatures. Virus was shown

to remain viable in oyster feces after 28 days at 15 C indicating the greater potential of contamination during the colder months of the year.

Laboratory studies performed to measure the fate of fecal coliforms in estuarine sediments generally demonstrated that a decline in indicator bacterial populations occurred as a direct result of nutritional competition or antagonism or both. As was found in the studies of virus adsorption, E. coli did adhere to sediment particles present in seawater. Sediments containing silt and clay particles were more efficient binders as compared to sediments composed mostly of sand. Bacterial numbers increased in autoclaved sediments indicating that sufficient nutrients were presnet in such samples to allow the replication of E. coli when there is no competition from normal estuarine flora. The levels of E. coli in non-sterilized sediments declined rapidly and appeared independent of sediment composition. Sewage inoculated sediment experiments did confirm this general finding in that fecal coliforms decline in sediment irregardless of the pollution history of the sediment collection site. Throughout these experiments, cooler temperatures favored the long term detection of E. coli in sediment; viruses, as discussed previously, followed a similar pattern.

Salmonella survival in sediments was investigated and the rate of decline of these bacteria was greater than that observed for E. coli. This pathogen was found to die more

rapidly at the cooler temperatures. When Salmonella were placed into oyster feces the rate of decline approximated that of the fecal coliform.

These studies demonstrate that indicator bacteria and bacterial pathogens do not survive for extended periods in estuarine sediments. Adsorption of bacteria to sediment particles is an active process mediated by the bacterial pili and the lack of a chemical stability in this interaction could account for bacterial suspectibility in this environment. Viruses, on the other hand, bind to sediment particles by covalent or electrostatic forces, and this relationship appears to increase the long term survival of virus in sediment.

As indicated in previous studies devoted to water or shellfish analysis, viruses in estuarine sediments may be reliable indicators of the pollutional status of an estuarine area.

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