VIRAL EVALUATION OF PROHIBITED OYSTER GROWING WATERS

FINAL REPORT

January 1978 to December 1979

R. D. ELLENDER
DAVID W. COOK
UNIVERSITY OF SOUTHERN MISSISSIPPI
GULF COAST RESEARCH LABORATORY

Mississippi-Alabama Sea-Grant Consortium

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Administrative Summary

MASCG Project No. R/SP-1; Ellender, PI; Middlebrooks, Cook, Cake, AI

Duration: January 1, 1978 to December 31, 1979

USM Project Numbers: 0221713209, 0221713309

Funds: 1978, $26,859; 1979, $30,843

Project Objectives:

a. To evaluate the enterovirus concentration of oysters sampled from a prohibited reef versus the "natural viral background" of a reef bacteriologically approved for public shellfish collection. Samples for enterovirus detection will also be examined for hepatitis B surface antigen (1978) and hepatitis A antigen (1979).

b. Relate enterovirus contamination of oyster tissue with the level of fecal coliforms found in prohibited and approved waters.

c. Disseminate information to the public via oral and written presentations.

d. Determine if enterovirus contamination of shellfish corresponds to the categories now used for shellfish growing water classification.

e. Collect necessary methods to allow suitable evaluation of future depuration projects along the Gulf Coast.

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PART I. OVERVIEW OF THE PROJECT

The measurement of the concentration of fecal coliforms in estuarine water helps to determine whether a site containing shellfish is to be open or closed to public or private harvest. Bacteriological data of this type has been used with a great degree of certainty for 40-50 years and has been responsible for the phenomenal decrease in the extent of water borne disease worldwide. The concept of the indicator bacterium has been studied to the point that its applications are widespread and are usually standardized procedures. In the early 1960's efforts were begun to determine if indicator bacteria could determine the presence of viruses in water. It was rapidly obvious that direct extrapolation from bacteriological to virological consequences could only be rarely quantitated. The evidence gradually emerged that the same was true for other systems: sewage, soils, seawater and shellfish, etc. Only in the last five years have available standard methods for the determination of viruses in water become available, but they are not universally accepted.

The growth of shellfish virology has made most of its gains during the last decade. Still, virologists do not agree on a single procedure to quantitate viruses in shellfish. The problem is magnified by the type of microbe which is attempting to be isolated and identified and the assay system to be employed.

This investigation was designed to compare fecal coliform and virus isolations from approved and prohibited oysters collected along the Mississippi Gulf Coast. Other data were collected for comparative purposes or, as in the case of the quantitative measure of fecal coliforms in water, used because

of their classification as standard and well-recognized procedures. In Part II of this report, the bacterial studies verified the classification of the selected harvesting sites as approved and prohibited. However, no statistical relationships could be found to indicate that counts of fecal coliforms in estuarine waters were correlated to those found in oysters harvested at the same site. This was more obvious at the prohibited site where wide fluctuations in fecal coliform indicies in both types of samples were noted. In Part III, studies demonstrate that virus isolations from prohibited shellfish were more frequent than isolations from approved oysters. No obvious pattern of isolation was observed at either location; however, the finding of virus at the approved site was not expected. At the approved site, the number of isolations was consistent on an annual basis. At the prohibited site virus isolations during 1978 were higher than 1979, but none of the parameters used could have indicated this change. It became clear during this investigation that the virological examination of shellfish is now a routine procedure which should be used to supplement bacterial measurements.

Part IV of this report summarizes preliminary studies designed to quantitate hepatitis antigens in oyster tissue. As this section points out, the radioimmunological methods were not designed for this purpose and required modifications with some loss of sensitivity. The more difficult of the systems to modify was HAVAB, designed originally to detect hepatitis A antibody in human sera. We believe that these studies constitute an important first step toward an eventual method which is both economical and sensitive.

The conclusions of this study did lead to additional research which is attempting to examine the relationships estuarine sediments play in the epidemiology of viruses in the shellfish environment.

VIRAL EVALUATION OF PROHIBITED OYSTER GROWING WATERS

PART II. BACTERIOLOGICAL STUDIES

INTRODUCTION

This research was undertaken to gather quantitative data on fecal coliform bacteria in water samples from areas where oysters were being collected for viral examination. Further, data was collected on the levels of fecal coliforms in oysters taken from these areas.

These data provide documentation to verify the classification assigned to those areas by the Mississippi State Board of Health, Bureau of Environmental Health, Milk and Shellfish Division, and serve as a basis to correlate levels of fecal coliform bacteria in water and oyster samples to levels of enteroviruses in oysters.

MATERIALS AND METHODS

Sampling Areas:

The two areas in which sampling sites were established are shown in Map 1. Graveline Bayou is designated by the Mississippi State Board of Health as a "prohibited" shellfish harvesting area and the Pass Christian reef as an "approved" shellfish harvesting area.

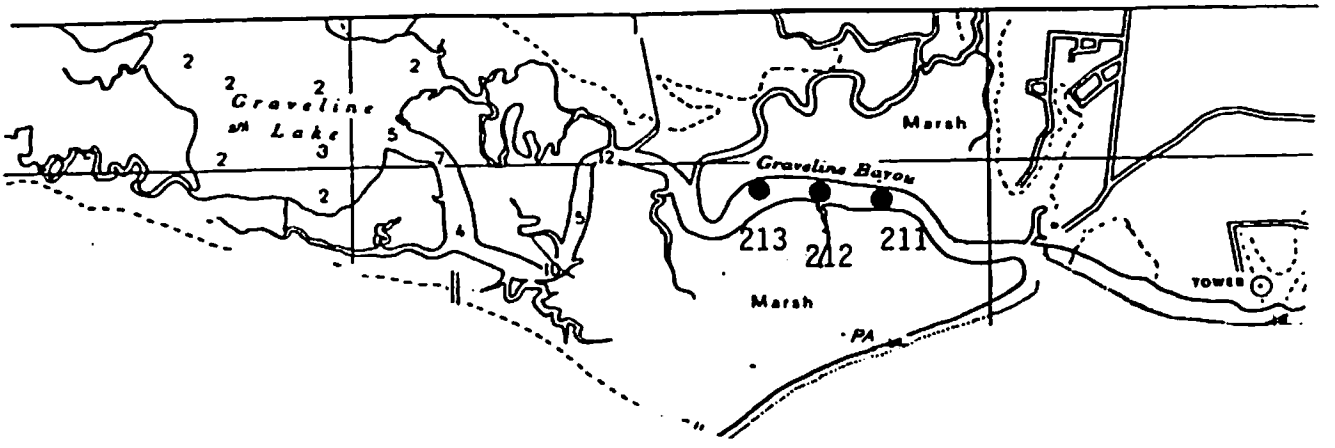
Sampling Frequency:

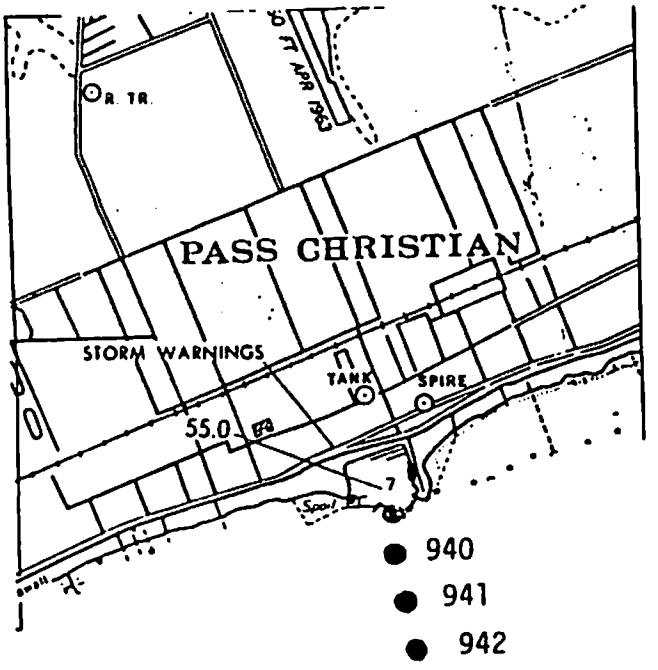
Previous studies (Cook, unpublished data) have shown that the water quality in Graveline Bayou is greatly influenced by local rainfall and tidal flushing and may change significantly within a short time period. The Pass Christian reef, on the other hand, is less subject to rapid changes because it does not lie directly in a drainage area or near a deep water channel. Therefore, the maximum sampling effort was expended in Graveline Bayou.

Three water sampling stations were established in each of the two areas. Oysters were taken in the area between the two outer stations. Sampling was conducted in both areas near the end of each calendar month for the calendar years of 1978 and 1979. Sampling was scheduled to coincide with the lowest tidal stage which occurred during the daylight hours on each sampling day.

In Graveline Bayou, water samples were collected at each station on three days prior to and on the same day as the oysters were collected. Both surface and bottom water samples were taken at each station on each of the four days. Therefore, a total of 24 water samples were collected from Graveline Bayou each month.

Map 1. Location of Water Sampling Stations. Oysters are found in the vicinity of the water sampling stations in both areas.





Sampling on the Pass Christian reef was limited to one day each month. Surface and bottom water samples were taken at all three stations on the same day the oysters were collected. A total of 6 water samples were collected from Pass Christian reef each month.

Sampling Technique:

Water samples labeled as surface were collected at a depth of 0.1 to 0.5m in sterile wide mouth bottles. Bottom water samples were collected approximately 0.3m above the sediment-water interface, in sterile bottles with the aid of a J-Z sampler. All samples were protected from heat and sunlight and returned to the laboratory for analysis within three hours of collection.

Measurements of surface water temperature were made with a mercury-in-glass thermometer at the time of sampling. Salinity measurements were made on a portion of the water sample collected for bacteriological analysis. A refractometer (American Optical, No. 10402) was used to measure the salinity.

Oysters were harvested with a dredge, culled and returned to the laboratory. A portion of these oysters were retained at GCRL for bacteriological analyses and the remainder were placed in an insulated box and shipped by bus to the University of Southern Mississippi at Hattiesburg, Mississippi. Oysters normally reached USM in less than 12 hours after they were harvested.

Bacteriological Analyses:

Bacteriological analyses were performed on both oyster and water samples using the 5-tube MPN procedure outlined in "Recommended Procedures for the Bacteriological Analysis of Seawater and Shellfish," 4th Edition, 1970, American Public Health Association.

RESULTS AND DISCUSSION

In the course of this study, 703 water samples and 96 oyster samples were analyzed for fecal coliform bacteria. Salinity and temperature measurements were also made on the water samples.

Pass Christian Reef:

The three sampling stations in this area were located close to one another and on each sampling date all three

stations had similar salinities. Similar numbers of fecal coliform bacteria were usually found in samples taken from these stations on the same day. However, there was a definite difference in these parameters as measured at the two levels, surface and bottom. Considering this, we chose to treat the three stations as replicates and pooled the data from the same level for all stations. Thus, all data presented here represents summation data.

Salinity and temperature data collected from water samples taken on the Pass Christian reef are displayed in Figures 1 and 2. The average of the salinities recorded during the two year period was 12.8 and 14.5 ppt for the surface and bottom waters, respectively.

Fecal coliform counts in the waters were usually very low during the summer and fall but increased during the winter and spring as shown in Figures 3 and 4. The median fecal coliform MPN value for all samples taken in that area was 2 per 100 ml for both the surface and bottom samples. Approximately 13% of both the surface and bottom samples had MPN values greater than 43 per 100 ml. This 13% represented 18 samples, 12 of which were collected during 2 months, 1/78 and 12/79.

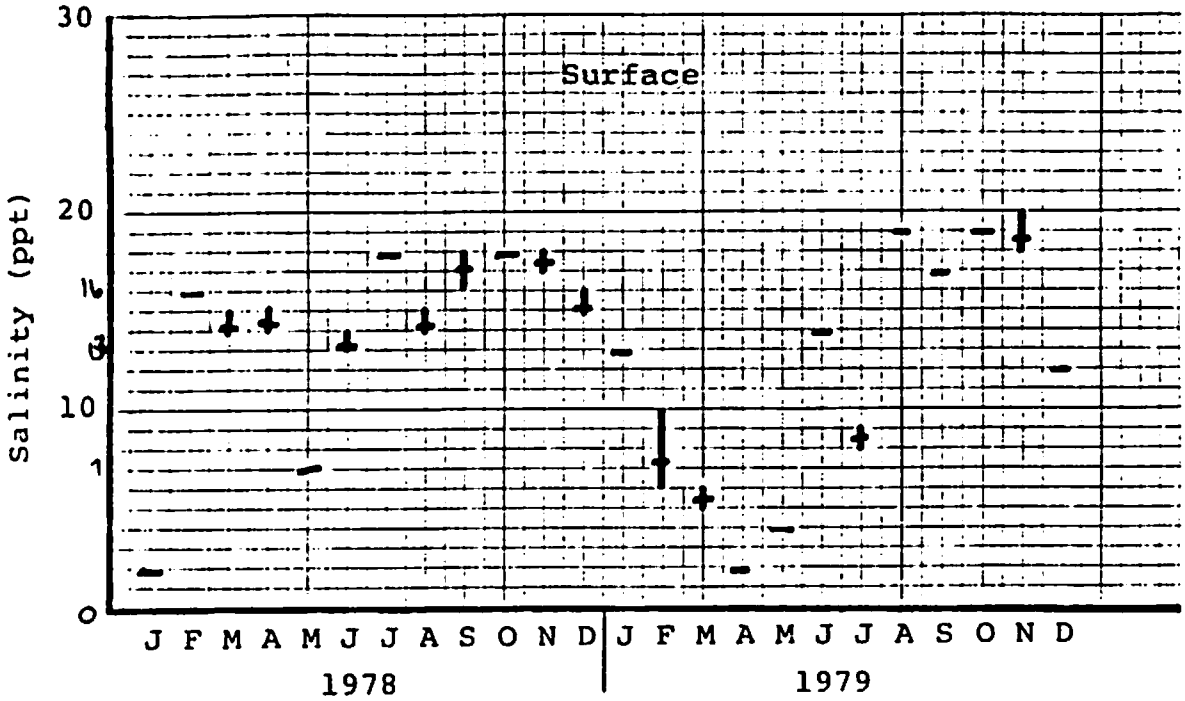
The fecal coliform data from the Pass Christian reef samples verifies the classification of that area as an "approved" shellfish harvesting area.

The fecal coliform counts in the oysters taken from the Pass Christian reef are recorded in Table 1. The median fecal coliform count in the oyster samples was 78/100g. Excessive fecal coliform counts (average count >500 per 100g) were recorded on three dates, 9/78, 5/79, 12/79. On only one of those dates, 12/79, was the fecal coliform count in the bottom water elevated above 2 per 100ml. There appears to be no correlation between fecal coliform counts in water and oyster samples collected at the same time in this area.

Graveline Bayou:

As with the Pass Christian reef samples, we found that in Graveline Bayou the data collected at all three stations on the same day were similar. However, data collected on each of the four consecutive sampling days may vary significantly depending on local meteorological and hydrographic conditions. Table 2 displays the average and median values for salinity and fecal coliform counts, respectively, for both the surface and bottom water samples collected on each sampling date. The three surface and three bottom water samples collected on the same day were treated as replicates to compute these figures. During some 4 day periods, average daily salinities varied over 10 ppt and median fecal coliform

Figure 1. Surface and bottom water salinities from the Pass Christian reef. Data for each month indicates the range and the average salinity of samples taken from three adjacent stations on the same day.



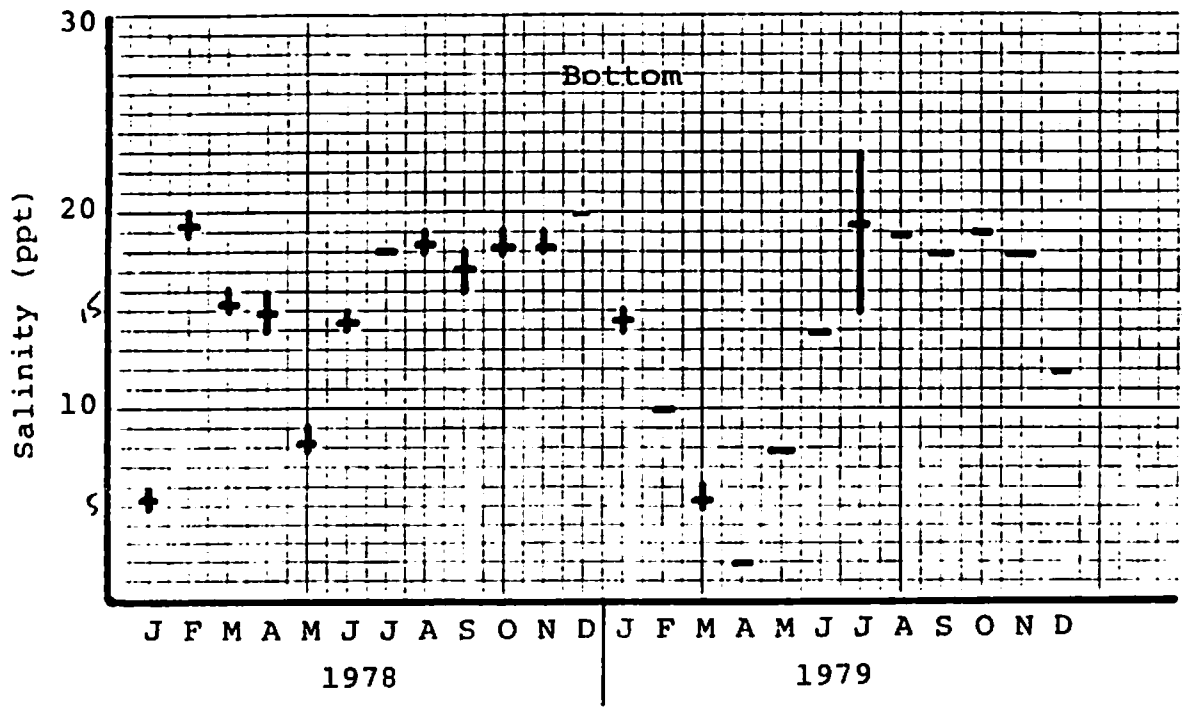


Figure 2. Surface water temperatures recorded on the Pass Christian reef. Data for each month indicates the range and the average temperature of samples taken from three adjacent stations on the same day.

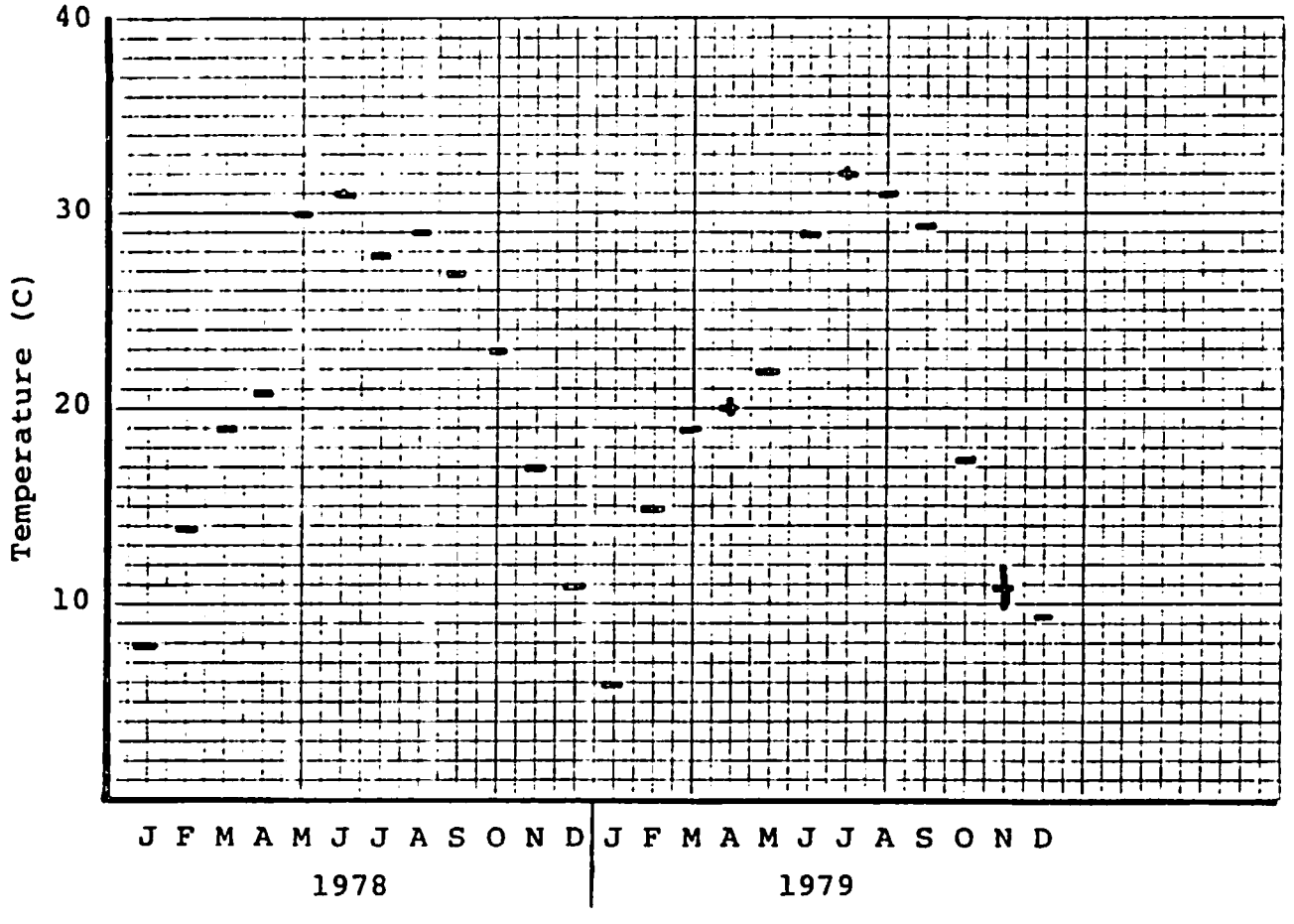


Figure 3. Fecal coliform bacteria in surface water samples from Pass Christian reef. Data for each month indicates the range and median count from samples taken at three adjacent stations on the same day.

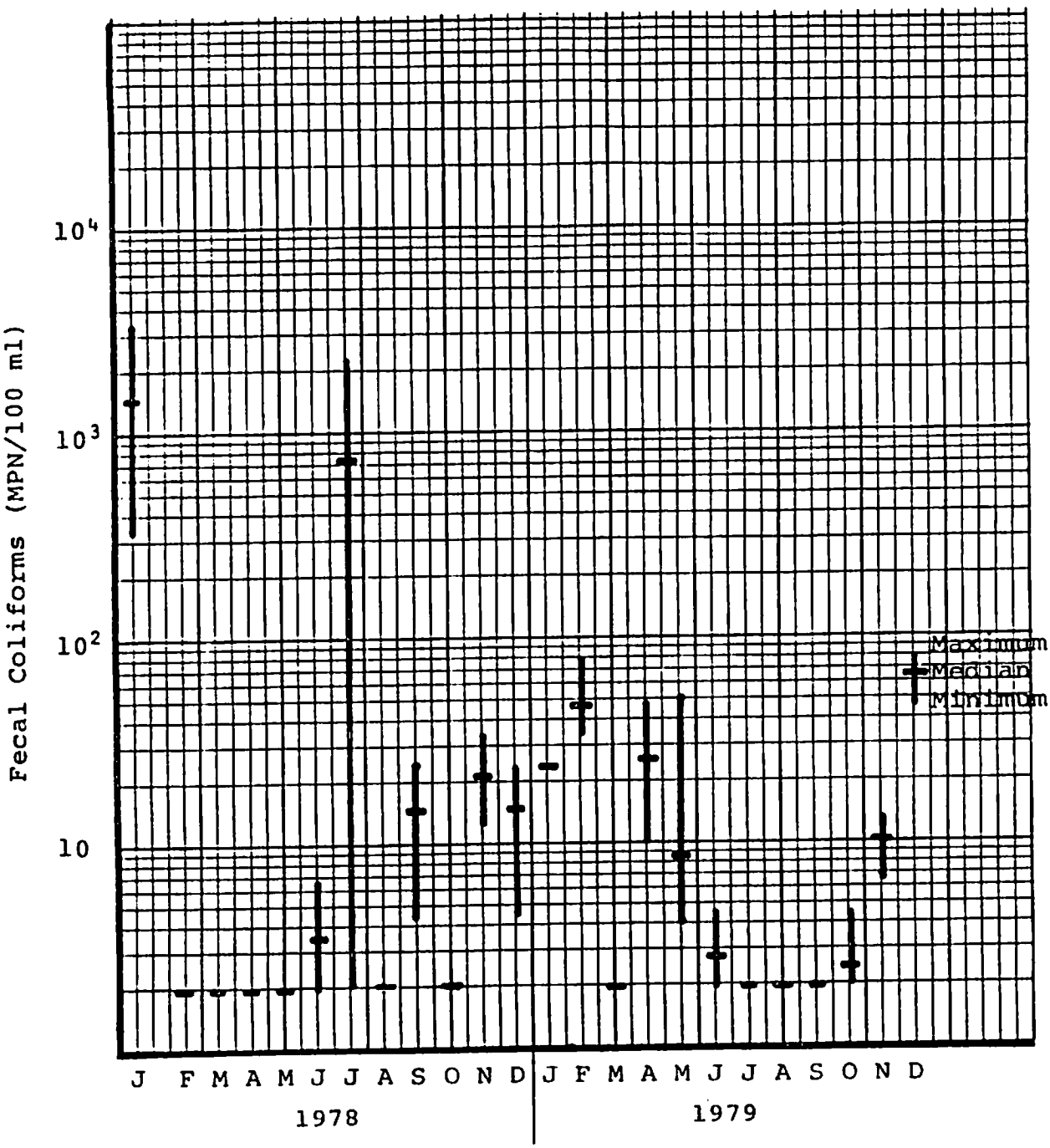


Figure 4. Fecal coliform bacteria in bottom water samples from the Pass Christian reef. Data for each month indicates the range and median count from samples taken at three adjacent stations on the same day.

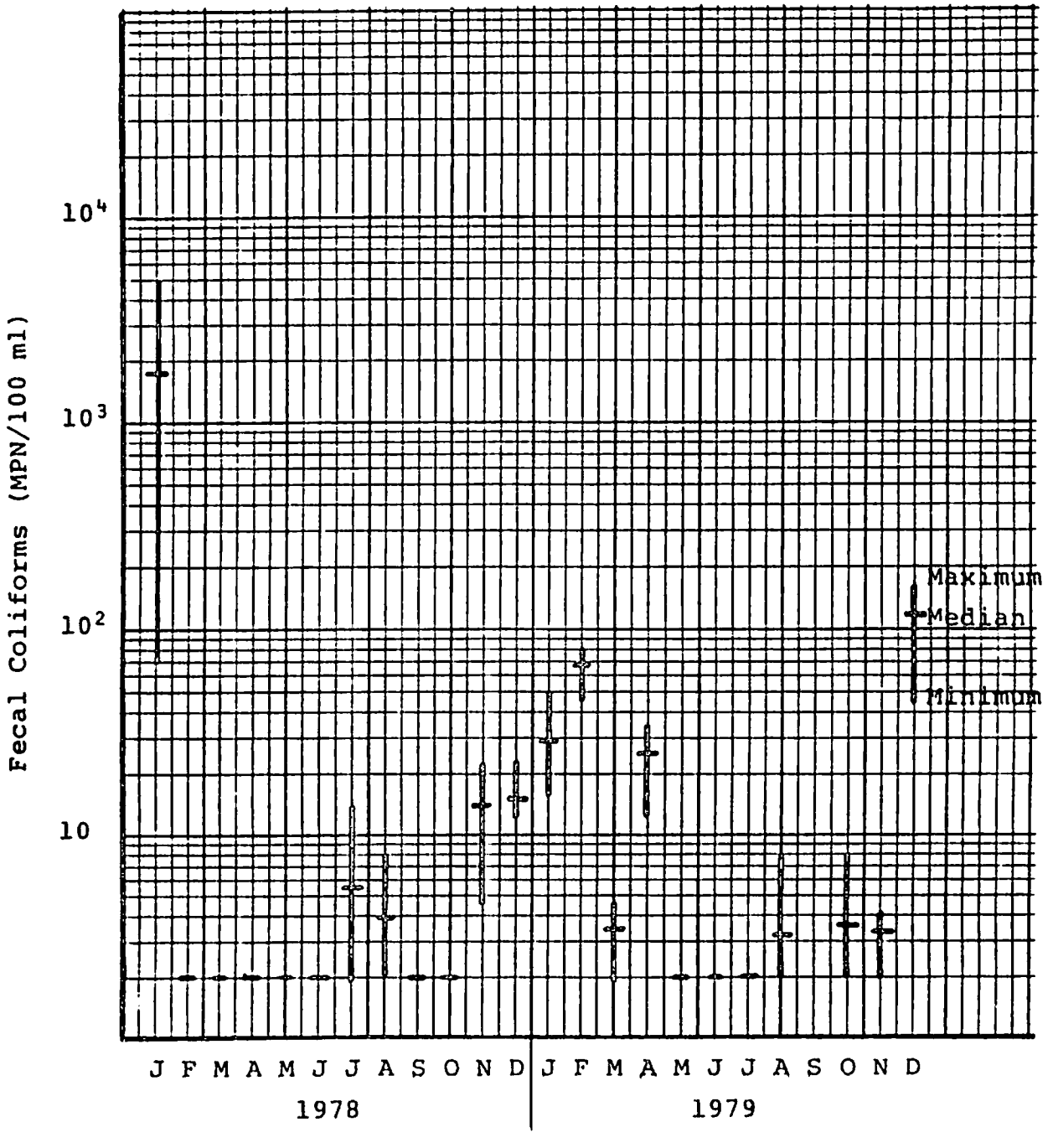


Table 1. Fecal Coliforms in Oysters from Pass Christian reef.

Month/Year	Fecal Coliforms per 100g		
	Sample A	Sample B	Average
1/78	68	78	73
2/78	45	45	45
3/78	110	140	130
4/78	<20	<20	<20
5/78	<20	<20	<20
6/78	45	78	62
7/78	310	490	400
8/78	230	490	360
9/78	7,900	17,000	12,000
10/78	<20	<20	<20
11/78	330	330	330
12/78	78	330	200
1/79	45	130	88
2/79	20	78	49
3/79	86	170	130
4/79	<20	230	<130
5/79	4,600	11,000	7,800
6/79	45	450	250
7/79	20	790	400
8/79	<20	20	<20
9/79	45	110	78
10/79	40	45	43
11/79	220	490	360
12/79	3,300	3,300	3,300

Table 2. Salinity and fecal coliform data from stations in Graveline Bayou. Data for each date represents combined data from three stations.

Date	Surface		Bottom	
	Average Salinity (ppt)	Median Fecal Coliform (MPN/100ml)	Average Salinity (ppt)	Median Fecal Coliform (MPN/100ml)
1/24/78	7.0	790	10.3	330
1/25/78	4.0	3,300	4.0	2,200
1/26/78	2.0	1,700	2.0	1,400
1/27/78	2.3	490	6.7	330
2/27/78	7.0	130	17.7	11
2/28/78	6.3	13	17.0	1.8
2/29/78	12.0	11	11.3	11
2/30/78	6.0	49	10.3	17
3/28/78	11.7	23	17.0	79
3/29/78	17.0	4.5	17.7	2.0
3/30/78	19.7	22	20.0	10
3/31/78	21.3	2.0	22.0	4.5
4/25/78	14.7	17	15.0	31
4/26/78	19.7	4.5	19.7	4.5
4/27/78	18.7	2.0	19.3	2.0
4/28/78	18.5	13	18.8	33
5/28/78	10.0	33	6.0	23
5/29/78	4.0	7.8	5.3	13
5/30/78	5.0	11	5.0	11
5/31/78	4.0	17	4.8	7.8
6/25/78	15.7	11	15.0	7.8
6/26/78	17.7	130	17.7	7.8
6/27/78	9.3	23	10.7	4.5
6/28/78	8.5	49	10.0	2.0
7/31/78	6.2	4,900	7.5	1,500
8/1/78	12.7	170	--	--
8/2/78	18.7	49	22.0	46
8/3/78	12.0	460	20.0	11
8/27/78	14.0	11	14.0	7.8
8/28/78	14.0	27	14.0	17
8/29/78	15.0	49	15.0	22
8/30/78	16.0	49	16.0	49
9/25/78	16.0	20	16.0	23
9/26/78	16.7	33	16.7	23
9/27/78	16.0	49	16.0	79
9/28/78	16.0	49	16.0	23

Table 2. (continued)

Date	Surface		Bottom	
	Average Salinity (ppt)	Median Fecal Coliform (MPN/100ml)	Average Salinity (ppt)	Median Fecal Coliform (MPN/100ml)
10/30/78	25.0	23	25.0	33
10/31/78	25.7	130	25.7	49
11/1/78	24.0	49	24.0	49
11/2/78	24.0	70	24.0	31
11/27/78	21.0	4,900	21.7	2,300
11/28/78	--	--	--	--
11/29/78	22.0	170	22.0	130
11/30/78	21.0	79	21.0	110
12/11/78	28.0	13	28.0	7.8
12/12/78	28.0	79	28.0	49
12/13/78	20.0	33	22.0	17
12/14/78	22.0	2.0	22.0	2.0
1/29/79	15.0	23	21.3	7.8
1/30/79	20.0	33	19.3	22
1/31/79	15.0	49	15.0	49
2/1/79	15.7	27	21.7	17
2/26/79	1.0	1,700	1.0	1,100
2/27/79	4.0	700	5.7	230
2/28/79	1.0	230	4.0	490
3/1/79	2.0	330	4.0	220
3/26/79	6.0	33	6.0	49
3/27/79	4.0	49	5.0	43
3/28/79	2.0	79	3.0	110
3/29/79	0.0	33	0.0	33
4/23/79	2.0	330	2.0	230
4/24/79	0.0	110	0.0	79
4/25/79	0.0	1,300	0.0	640
4/26/79	1.7	49	1.7	170
5/28/79	8.7	23	7.0	27
5/29/79	4.0	23	4.0	49
5/30/79	2.0	79	2.0	79
5/31/79	3.3	49	3.3	33
6/25/79	10.0	230	10.0	95
6/26/79	12.0	13	12.0	46
6/27/79	14.0	130	14.0	130
6/28/79	15.0	7.8	15.0	11

Table 2. (continued)

Date	Surface		Bottom	
	Average Salinity (ppt)	Median Fecal Coliform (MPN/100ml)	Average Salinity (ppt)	Median Fecal Coliform (MPN/100ml)
7/30/79	3.0	7.8	8.0	11
7/31/79	3.0	17	5.0	21
8/1/79	5.0	33	7.7	11
8/2/79	6.0	23	8.0	11
8/27/79	11.0	21	11.0	33
8/28/79	11.0	49	14.0	49
8/29/79	11.0	33	11.0	17
8/30/79	10.0	33	10.0	49
9/24/79	7.7	49	10.7	26
9/25/79	9.0	23	9.0	22
9/26/79	10.0	49	10.0	79
9/27/79	10.0	33	12.0	27
10/22/79	14.0	170	14.0	130
10/23/79	15.0	230	15.0	79
10/24/79	15.0	49	15.0	79
10/25/79	19.0	11	21.0	13
11/26/79	11.0	1,700	11.0	1,700
11/27/79	6.0	1,300	10.0	790
11/28/79	5.0	1,300	10.0	49
11/29/79	18.4	130	21.3	49
12/17/79	8.0	310	9.7	280
12/18/79	11.3	49	17.0	130
12/19/79	12.0	43	12.0	49
12/20/79	2.3	490	9.3	460

counts varied as much as 100 fold. This variation can best be seen in Figures 5, 6 and 7 in which all the data collected over the 4 day sampling period is displayed for each month. These data indicate the extreme variability in water quality encountered in Graveline Bayou.

In general, salinity extremes were greater in Graveline Bayou than on the Pass Christian reef. Average surface and bottom salinities in Graveline Bayou were 11.4 and 12.9, respectively. Temperatures recorded in Graveline Bayou are displayed in Figure 8.

Fecal coliform counts in surface samples from Graveline Bayou were frequently elevated. During only 2 sampling months did the median fecal coliform values fall below 14 per 100 ml. The median value for all surface samples was 49 and 51.9% of the samples exceeded an MPN of 43.

Fecal coliform counts in bottom water samples were consistently lower than those in surface samples. The median count obtained from all bottom water samples was 33 and the percent of samples with MPN values in excess of 43 was 46.0.

These data indicate that Graveline Bayou is correctly classified as a "prohibited" shellfish harvesting area since it exceeds both a median fecal coliform value of 14 and greater than 10% of the MPN values exceed 43.

As may be expected, the levels of fecal coliforms in oysters from Graveline Bayou as shown in Table 3 were higher than those from the Pass Christian reef. The median fecal coliform count in the oysters was 230 per 100g. No correlation was noted in the fecal coliform counts in the water and in the oysters.

SUMMARY

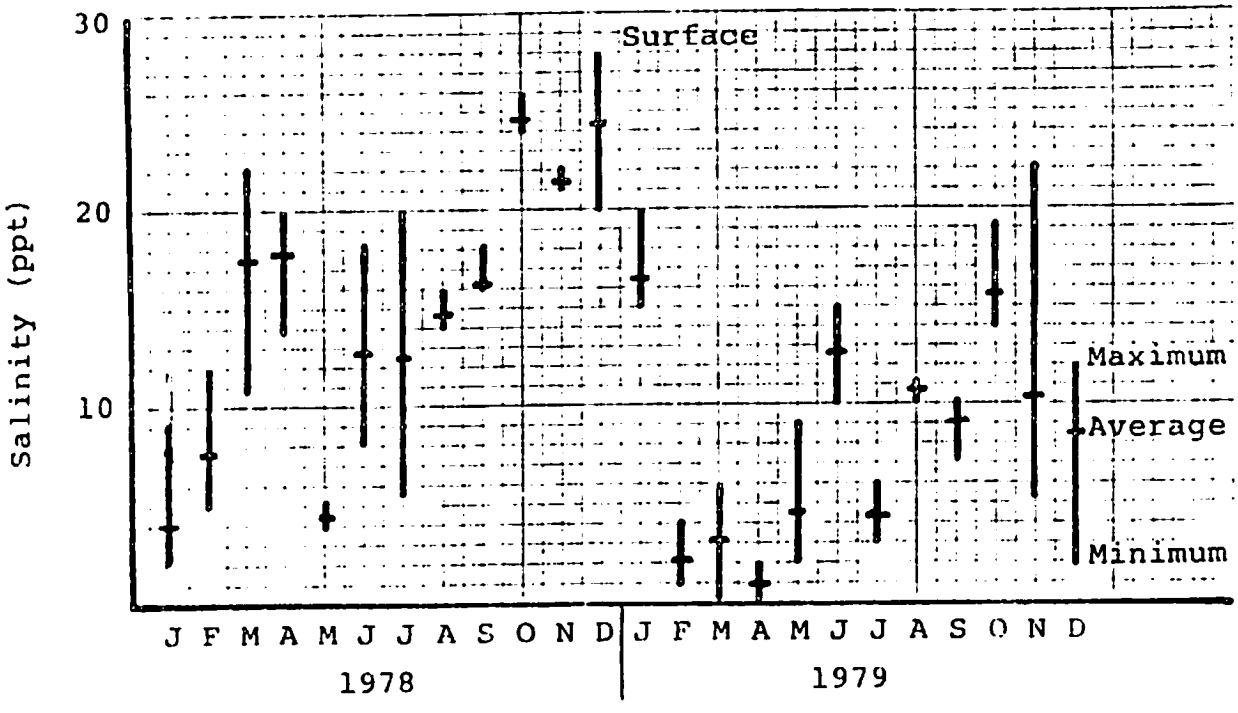
1. Bacteriological examination of water samples collected over the Pass Christian reef verified its classification as an "approved" shellfish harvesting area.

2. Bacteriological examination of water samples collected from Graveline Bayou verified its classification as a "prohibited" shellfish harvesting area.

3. Water quality in Graveline Bayou as measured by fecal coliform bacteria was extremely variable.

4. There appeared to be no correlation between the fecal coliform count in the water and the fecal coliform count in oyster samples collected from the Pass Christian reef at the same time. In the Graveline Bayou samples, a similar lack of correlation was noted but the relationship was less clear because of the high variability in fecal coliform count encountered during the four day sampling period prior to oyster harvest.

Figure 5. Surface and bottom water salinities in Graveline Bayou. Data for each month indicates the range and average salinity of all samples collected at three adjacent stations during four consecutive sampling days.



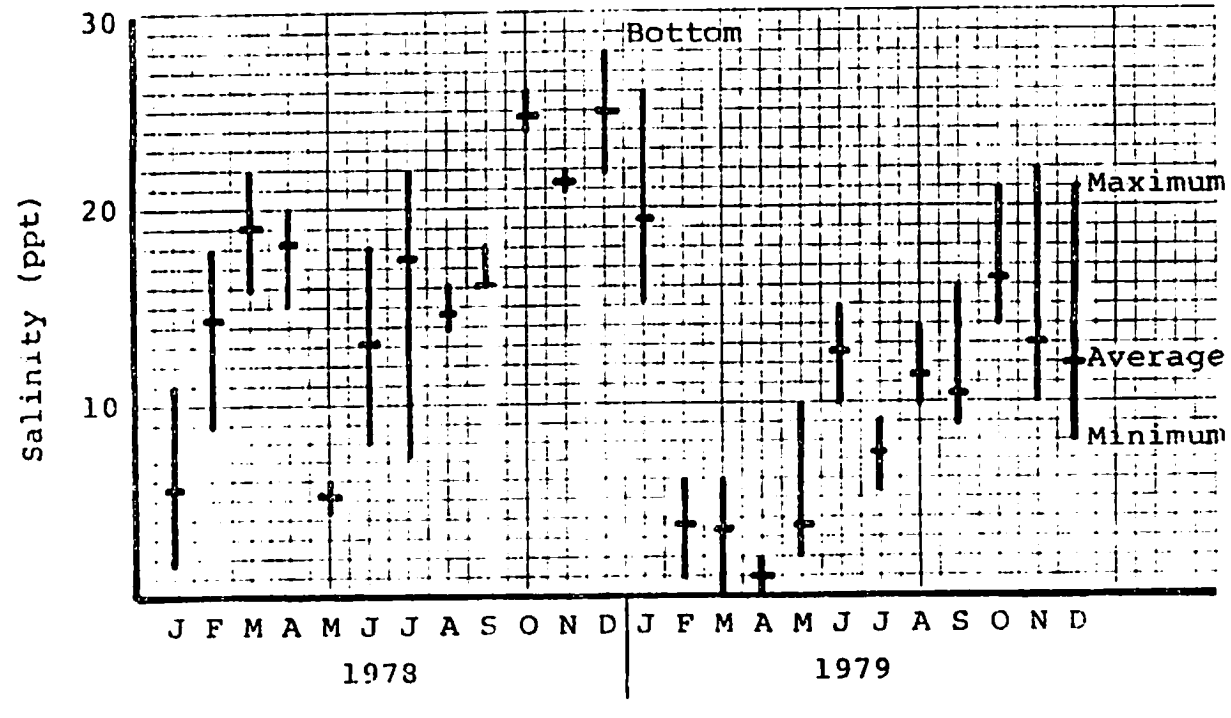


Figure 6. Fecal coliform bacteria in surface water samples from Graveline Bayou. Data for each month indicates the range and median count from all samples collected at three adjacent stations during four consecutive sampling days.

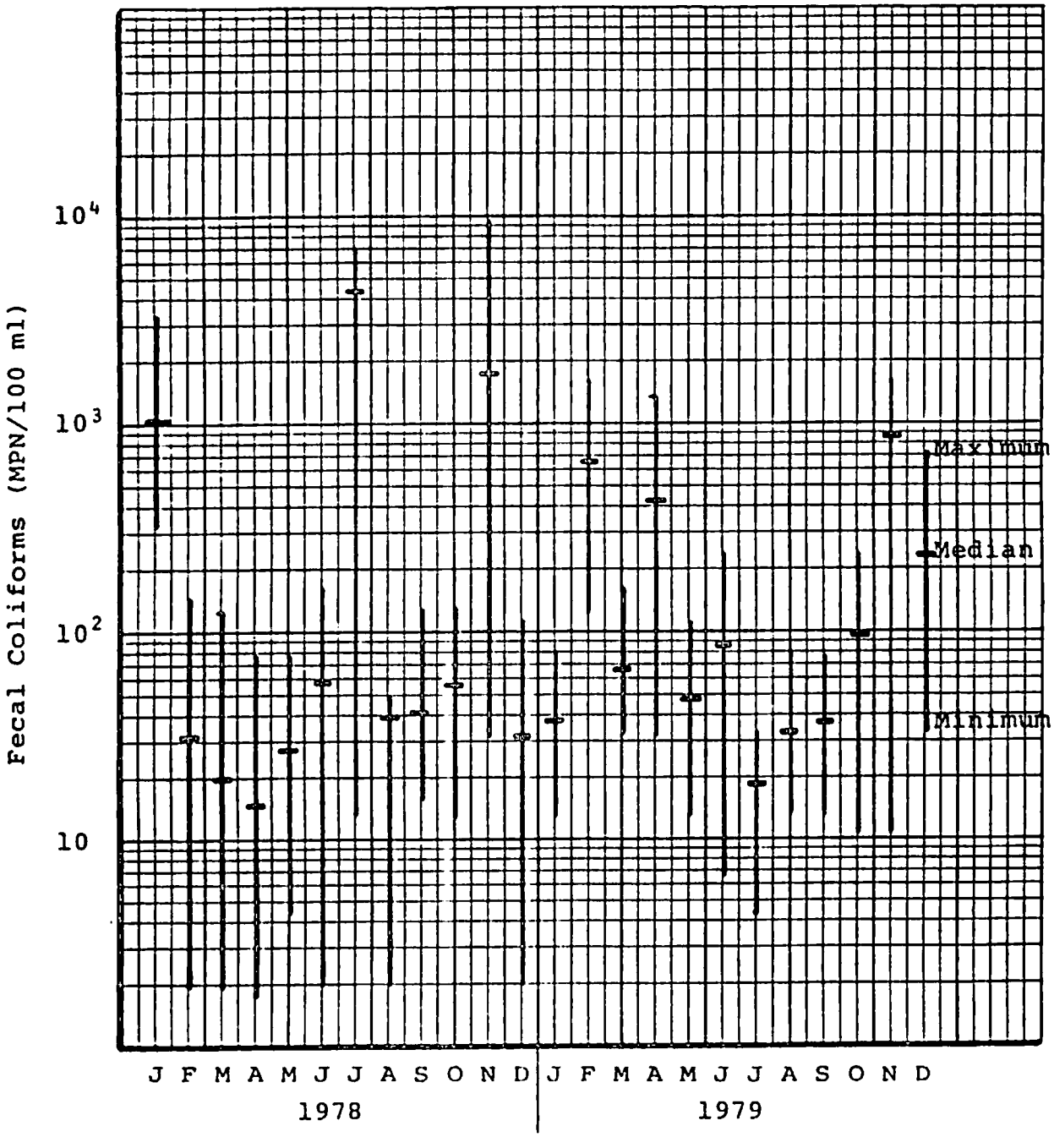


Figure 7. Fecal coliform bacteria in bottom water samples from Graveline Bayou. Data for each month indicates the range and median count from all samples collected at three adjacent stations during four consecutive sampling days.

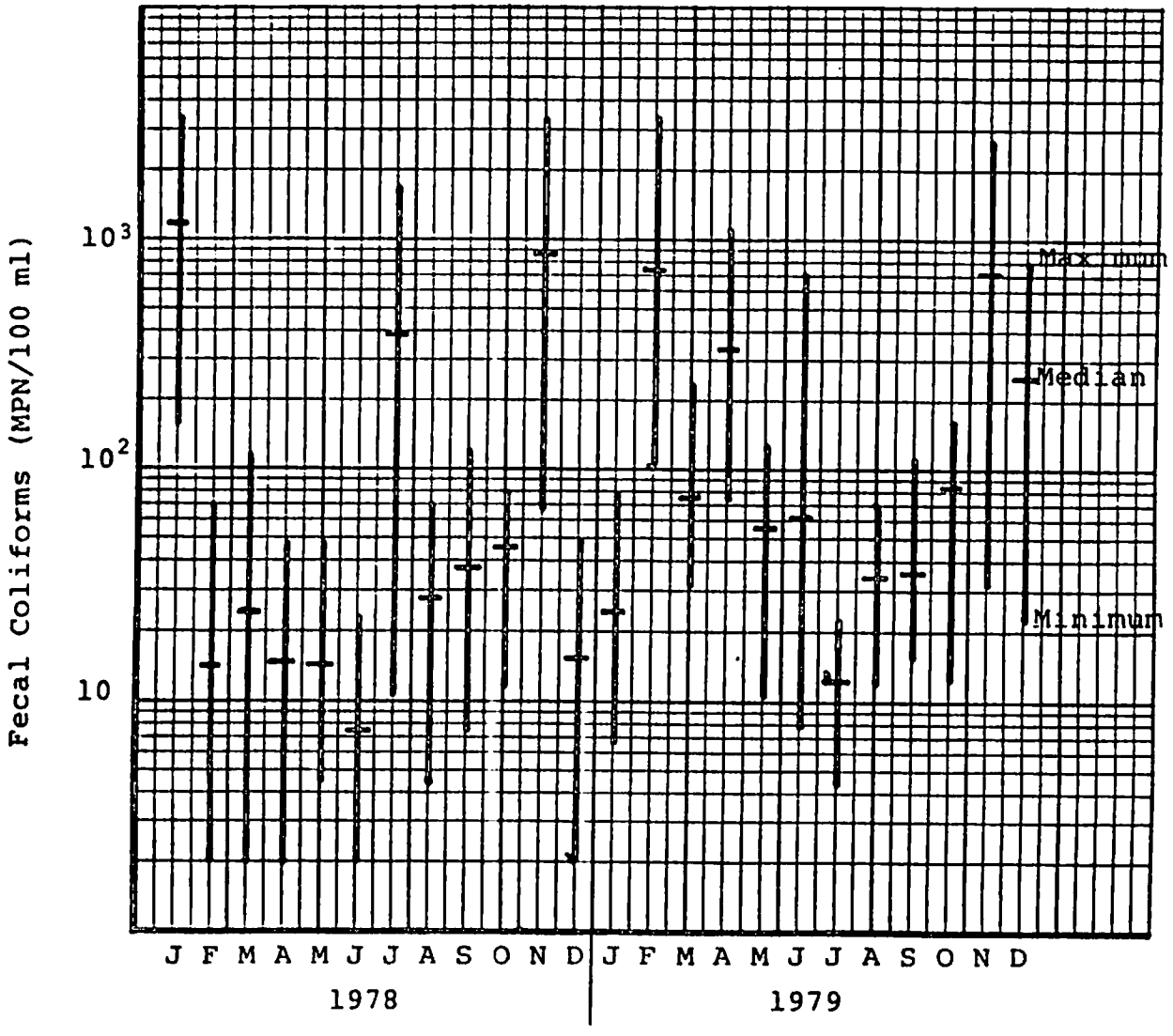
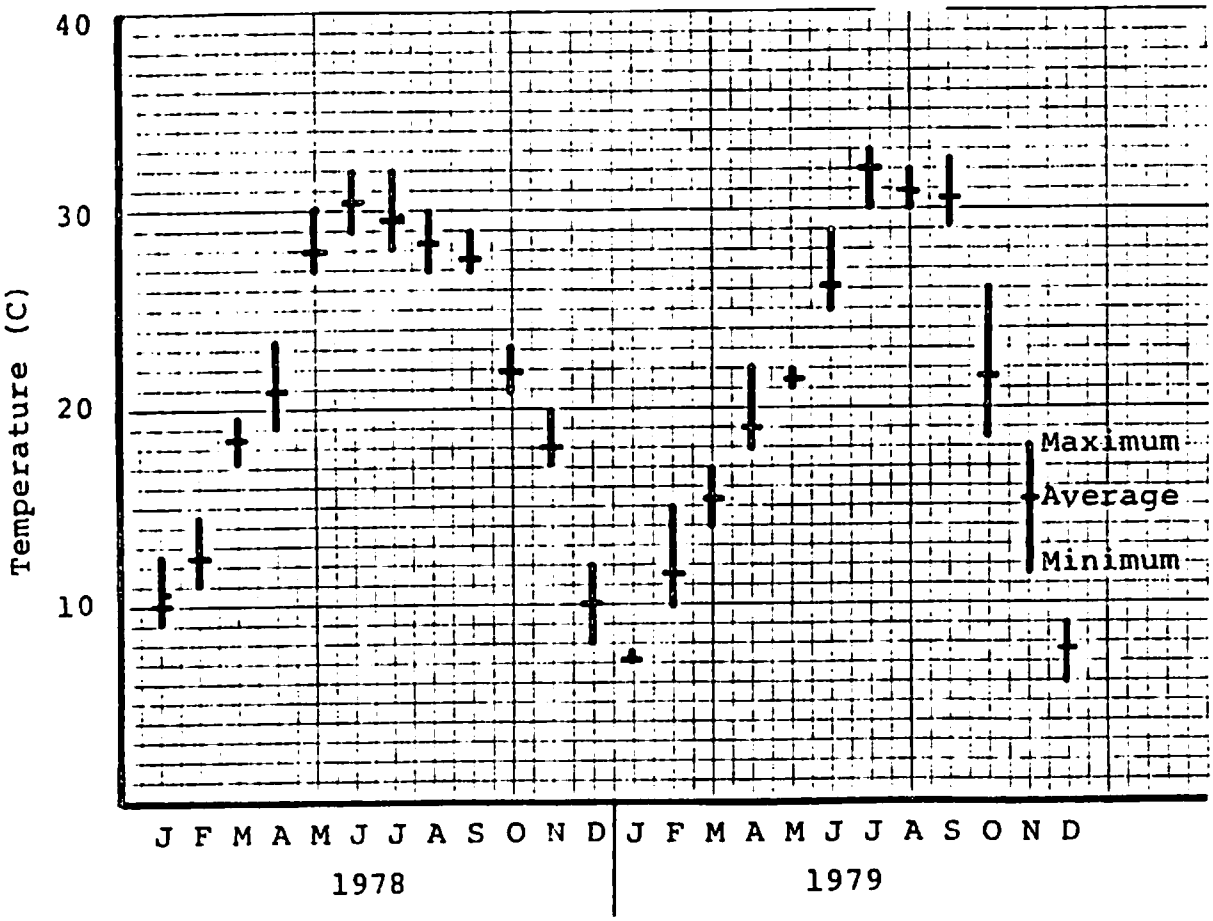


Figure 8. Surface water temperatures recorded in Graveline Bayou. Data for each month indicates the range and average temperatures of all samples collected at three adjacent stations during four consecutive sampling days.



VIRAL EVALUATION OF PROHIBITED OYSTER GROWING WATERS

PART III. VIROLOGICAL STUDIES

INTRODUCTION

These scientific data were collected (a) to determine the extent of natural viral contamination of oysters in Mississippi waters, (b) to virologically compare approved and prohibited oyster growing waters, (c) to examine the ability of fecal coliform indicies to predict viral contamination and as a supplementary study, (d) to evaluate methods to detect hepatitis B and hepatitis A antigens in shellfish tissues.

MATERIALS AND METHODS

A. Shellfish sampling:

Estuarine water and oysters (Crassostrea virginica) were collected from approved (Pass Christian) and prohibited (Graveline Bayou) shellfish growing areas (Figure 1) during 1978, 1979. Oysters were harvested with a hand dredge, culled and placed in an insulated box for shipment. The length of time between oyster harvesting and receipt in Hattiesburg or Ocean Springs was normally less than 12 hours. All samples were kept at 4°C until processed.

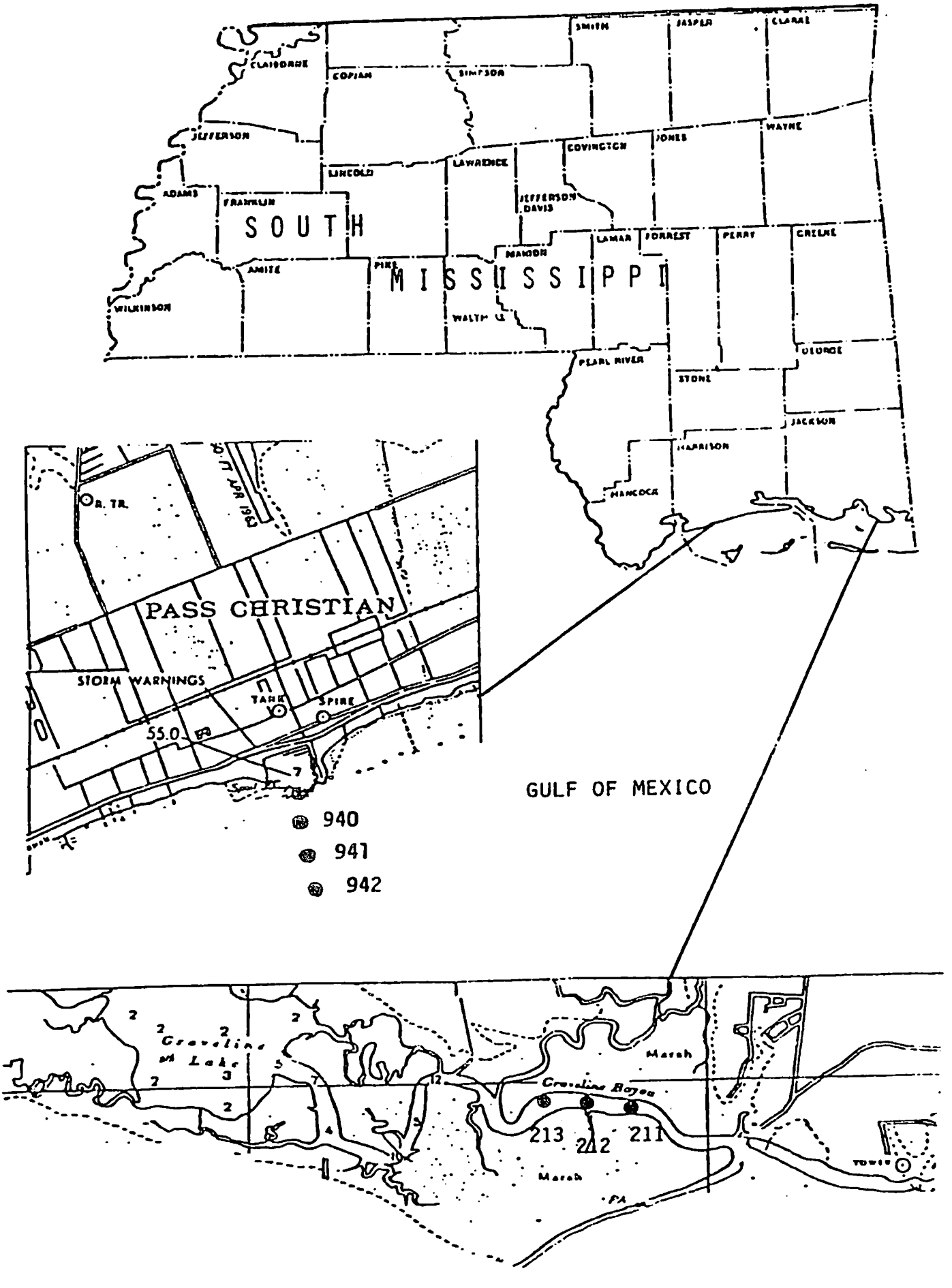
B. Sample analysis:

Oysters (130-150 gr lots) were extracted to determine virus concentration using a modification of the Sobsey procedure (Figure 2).

C. Tissue culture assay:

The African green monkey kidney cell line (BGM) was used to analyze all oyster concentrates. Virus samples and/or dilutions

Figure 1. Sample Collection Sites



(0.2 to 0.5 ml per 25 cm^2 plastic flask; 1-2 ml per 75 cm^2 plastic flask) were distributed over BGM monolayers (passages 100 to 120) for 1 hour at 37°C using a rocking apparatus (Bellco) at five rotations per minute. Growth medium for BGM cells consisted of MEM:L15 (1:1), 10% fetal calf serum and 1% L-glutamine (all purchased from Grand Island Biological Company).

Samples were quantitatively assayed by a modification of the plaque method. Plaque counts were made on a daily basis for five days or until no new plaques appeared after two consecutive days.

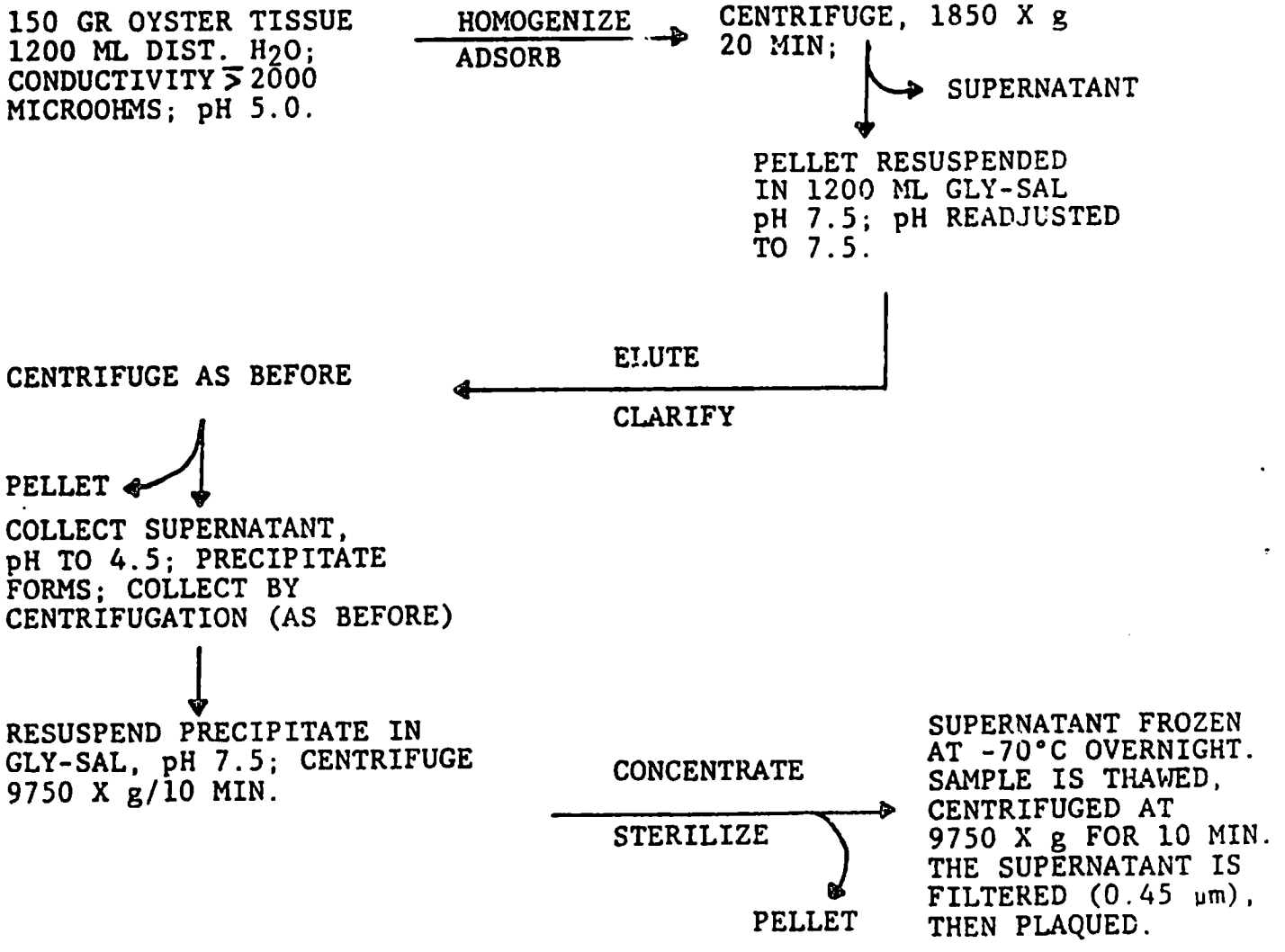
D. Plaque identification:

Individual plaques were picked when they were $\geq$ 1 mm in diameter. A Pasteur pipette, bent at the capillary end, was used to transfer an agar plug (area of plaque) and a small volume (0.05 ml) of wash fluid to a holding medium (1 ml MEM per tube). Samples were passaged three times in BGM cells with a minimum of one filtration step (0.45 µm). Two blind passages were made of all samples not producing observable cytopathic effect. Plaques identified as viruses were titered and identified serologically using LIM Benyesh-Melnick pools (A-H; J-P) or immunoperoxidase testing with known antisera.

E. Statistics:

Bacterial and viral counts in water and oyster samples were subjected to a square root transformation prior to calculation of linear correlation coefficients.

Figure 2. Oyster extraction procedure for virus isolation.



RESULTS AND DISCUSSION

During the 24-month study period, 3134 oysters in composite samples were examined for viruses. At the approved site (Pass Christian) a total of 93 plaques were examined of which 23 were confirmed as viruses. The prohibited location (Graveline Bayou) yielded a total of 1309 plaques of which 207 were confirmed as viruses. When a small number of plaques (1-20) were found in a sample, all plaques were virologically tested. On occasion, extremely high levels of plaques (100-500) were observed; when this occurred, no more than 50 random isolates were processed due to the length of time (2 weeks) required for identification. At the approved site during 1978, 1979, 3.4×10^{-2} and 3.3×10^{-2} confirmed virus isolations/oysters were determined. During 1978, 1.1×10^{-1} virus/oyster were found at Graveline Bayou. This figure dropped to 5.3×10^{-2} viruses/oyster during 1979. These data demonstrate that the number of viruses found in approved oysters remained constant over the two-year period. In contrast, a significant fluctuation in the virus numbers was observed in oysters collected at the prohibited site. The same general trend is evident in the bacteriological analyses of the two sites. The greater amount of fecal coliform fluctuation was observed at Graveline even though we have found no statistically significant correlations between these two parameters when compared for a single collection site. Actually, the number of virus isolations during 1978 were 2.4 times higher than those found in 1979. It is not presently understood whether this change was a result of physical alterations of the environment or whether less fecal

Table 1

Natural Viral Analysis of Approved Oysters
PASS CHRISTIAN (1978)

Month	# Oysters/ # Samples	*	# Plaque-like Isolates	# Plaques Identified As Viruses	
Jan.	24/2	0	2	0	
Feb.	32/2	0	4	0	
Mar.	24/2	0	6	0	
Apr.	30/2	2	19	11	
May	21/1	0	0	0	
June	38/2	0	0	0	
July	32/2	0	0	0	
Aug.	36/2	1	2	0	
Sept.	33/2	2	7	5	
Oct.	26/2	2	3	1	
Nov.	20/2	1	1	0	
Dec.	23/2	1	3	0	
	339/23	9	47	17	36%

Table 2

Natural Viral Analysis of Approved Oysters, Pass Christian Reef (1979)

Month	No. Oysters/No. Samples	*	No. Plaque-like Isolates Total: Per 100 g	No. Plaques Identified As Viruses Total: Per 100 g
January	30/3	0	20.0:4.4	0.0:0.0
February	31/4	2	17.0:2.8	8.0:1.3
March	29/3	0	1.0:0.2	0.0:0.0
April	23/3	0	3.0:0.7	0.0:0.0
May	31/3	0	0.0:0.0	0.0:0.0
June	28/3	1	3.0:0.7	1.0:0.2
July	36/3	1	1.0:0.2	1.0:0.2
August	29/2	0	6.0:2.0	0.0:0.0
September	36/2	0	0.0:0.0	0.0:0.0
October	23/2	0	0.0:0.0	0.0:0.0
November	28/2	0	1.0:0.3	0.0:0.0
December	18/2	0	1.0:0.3	0.0:0.0
TOTAL	343/33	4	53	10

*Number of samples containing confirmed virus isolates.

Figure 3

Viral Analysis of Pass Christian Reef Oysters: 1979

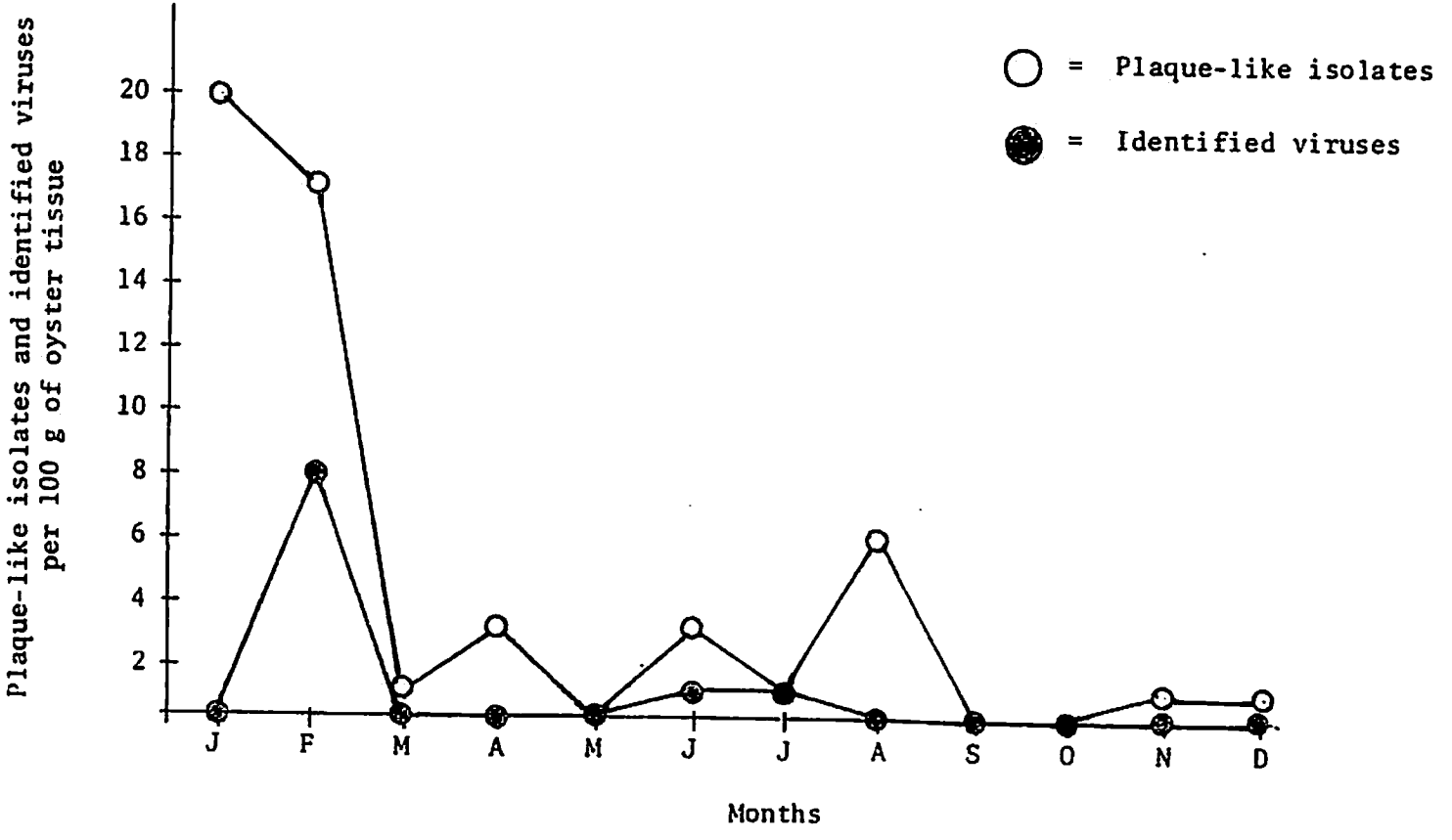


Table 3

PASS CHRISTIAN

Extractions and Isolations, January, 1978 to December, 1979

Collection Date	Sample	# of Oysters Processed/Wt.	Final Sample Volume*	Volume Plaqued	# of Plaques Isolated	# of Confirmed Isolates
1-27-78	145	13/150	53	20	0	0
	146	11/150	59	20	2	0
2-28-78	159	20/150	70	50	2	0
	160	12/100	40	25	2	0
3-31-78	164	13/100	38	38	0	0
	165	11/103	40	20	6	0
4-28-78	173	15/150	45	20	0	0
	174	15/150	50	30	19	11
6- 2-78	179	21/150	50	25	0	0
6-28-78	205	20/150	50	50	0	0
	206	18/150	50	35	0	0
8- 3-78	220	15/120	20	20	0	0
	221	17/150	42	22	0	0
8-30-78	232	13/86	45	20	1	0
	233	23/150	60	25	1	0
9-30-78	249	21/150	45	25	7	5
	250	12/150	15	15	0	0
11- 2-78	264	10/150	20	20	3	1
	265	16/150	30	20	0	0

Pass Christian (continued)

Collection Date	Sample	# of Oysters Processed/Wt.	Final Sample Volume*	Volume Plaqued	# of Plaques Isolated	# of Confirmed Isolates
11-30-78	272	13/150	25	20	1	0
	273	7/82	20	20	0	0
12-16-78	285	12/150	30	30	3	0
	286	11/150	30	20	0	0
2- 1-79	299	10/150	35	20	13	0
	300	10/150	40	27	7	0
	301	10/150	40	25	0	0
3- 2-79	317	7/150	35	30	2	0
	318	9/150	40	30	4	1
	319	7/150	40	40	8	7
	320	8/150	40	31	3	0
3-30-79	338	10/150	50	25	0	0
	339	9/150	51	25	0	0
	340	10/150	45	33	1	0
4-26-79	356	8/150	45	22	0	0
	357	7/150	70	51	3	0
	358	8/150	42	27	0	0
6- 1-79	368	13/150	60	32	0	0
	369	9/150	60	38	0	0
	370	9/130	50	22	0	0
7- 2-79	389	9/150	80	46	3	1
	390	12/150	62	37	0	0
	391	7/150	80	43	0	0
8- 3-79	402	12/150	70	46	1	1
	403	13/150	95	50	0	0
	404	11/110	60	31	0	0

Pass Christian (continued)

Collection Date	Sample	# of Oysters Processed/Wt.	Final Sample Volume*	Volume Plaqued	# of Plaques Isolated	# of Confirmed Isolates
8-31-79	415	15/150	55	42	5	0
	416	14/150	50	40	1	0
9-28-79	428	17/150	60	38	0	0
	429	19/150	50	40	0	0
10-26-79	440	10/150	60	30	0	0
	441	13/150	50	30	0	0
11-29-79	445	12/150	65	46	1	0
	446	16/150	58	50	0	0
12-20-79	450	10/161.2	60	55	0	0
	451	8/162.7	60	60	1	0
					53	10

*A minimum of one half the final sample volume was plaqued.

Table 4

Natural Viral Analysis of Prohibited Oysters
BAYOU GRAVELINE (1978)

Month	# Oysters/ # Samples	# Plaque-Like Isolates	# Plaques Identified As Viruses	
Jan.	139/10	88	31	
Feb.	65/5	38	5	
Mar.	53/4	18	3	
Apr.	56/3	5	3	
May	102/6	51	18	
June	200/13	38	12	
July	180/10	44	23	
Aug.	128/7	39	11	
Sept.	191/11	46	27	
Oct.	129/9	15	10	
Nov.	87/8	18	4	
Dec.	35/3	25	1	
		425	148	35%

Table 5

Natural Viral Analysis of Prohibited Oysters, Graveline Bayou (1979)

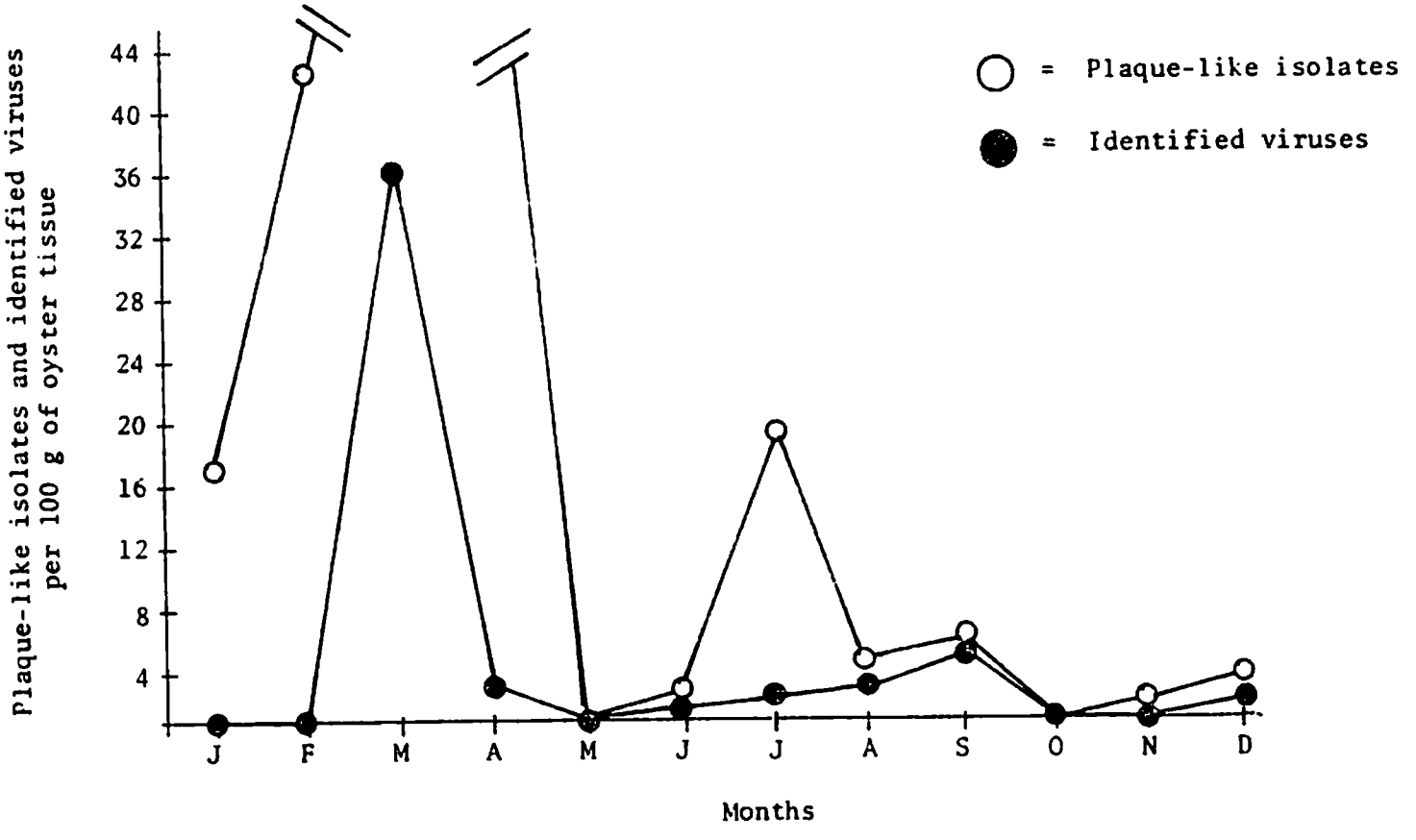
Month	No. Oysters/No. Samples	*	No. Plaque-like Isolates Total: Per 100 g	No. Plaques Identified As Viruses Total: Per 100 g	
January	39/3	0	17.0:3.8	0.0:0.0	
February	91/9	0	42.0:3.1	0.0:0.0	
March	75/10	3	503.0:33.5	**36.0:2.4	
April	89/10	2	70.0:4.7	3.0:0.2	
May	88/10	0	0.0:0.0	0.0:0.0	
June	104/10	1	2.0:0.1	1.0:0.1	
July	125/10	2	19.0:1.3	2.0:0.1	
August	117/9	6	5.0:0.4	3.0:0.2	
September	114/8	3	6.0:0.5	5.0:0.4	
October	88/7	0	0.0:0.0	0.0:0.0	
November	74/6	0	1.0:0.1	0.0:0.0	
December	21/2	1	4.0:1.3	1.0:0.3	
TOTAL	1043/94	18	673	51	8%

*Number of samples containing confirmed virus isolates.

**Forty plaques purified and typed.

Figure 4

Viral Analysis of Graveline Bayou Oysters: 1979



pollution was placed in the oysters' environment during the second year. The fecal coliform data does not indicate that less fecal material entered the system.

The virological analyses of oysters collected at the Pass Christian site during the two-year period are summarized in Tables 1-3 and Figure 3. Other data which point out the trends observed at this approved site during 1978 are presented in the enclosed publication. Summary Tables 1 and 2 show the fluctuation observed in approved systems and the sporatic isolations at certain periods (April, 1978; January and February, 1979). Although the number of plaque-like isolates observed during 1979 was higher than that of 1978, the percentage of confirmed isolates was lower (36% versus 19%). The fact that this is an area approved for public oyster harvest allows one to question the validity of the fecal coliform standard (this area met the bacteriological requirements for approved status during the 24-month period).

Virological data of oysters collected from the Graveline Bayou harvesting site are contained in Tables 4-6 and Figure 4. Other plotted data related to the first year of the project are contained in the enclosed publication (Figure 3). A much larger number of plaque-like isolates were observed at the prohibited site during the two-year periods. However, the number of confirmed virus isolations was not significantly different at the two collection sites. Since Graveline is heavily polluted, it may contain additional natural or fecal related adventitious agents which could produce cell damage and resemble a plaque. In addition, Graveline sample concentrates were often cytotoxic to

Table 6

BAYOU GRAVELINE

Extractions and Isolations, January 1978 to December, 1979

Collection Date	Sample	# of Oysters Processed/Wt.	Final Sample Volume	Volume Plaqued	# of Plaques Isolated	# of Confirmed Isolates
1-19-78	124	11/150	54	54	22	9
	125	8/150	68	20	0	0
	126	11/150	35	25	11	0
	127	13/150	50	50	0	0
	129	11/150	20	20	4	0
1-27-78	140	19/150	64	48	46	22
	141	16/150	50	0	0	0
	142	17/150	67	15	2	0
	143	17/150	62	34	1	0
	144	16/150	56	20	2	0
2-16-78	152	11/150	45	30	0	0
	153	13/130	49	31	18	0
	154	13/150	49	10	2	0
2-28-78	157	17/150	40	37	11	0
	158	11/100	40	25	7	5
3-15-78	161	13/150	48	48	12	3
	162	11/150	35	32	0	0
	165	11/103	40	20	6	0
3-31-78	168	18/150	42	12	0	0
4-13-78	170	16/150	40	40	0	0
4-28-78	175	22/145	14	12	2	0
	176	18/150	40	33	3	3

Bayou Graveline (continued)

Collection Date	Sample	# of Oysters Processed/Wt.	Final Sample Volume	Volume Plaqued	# of Plaques Isolated	# of Confirmed Isolates
5-17-78	177	19/150	45	20	1	0
6- 2-78	182	18/150	45	6	0	0
	183	15/150	55	15	2	0
	184	15/150	45	43	17	1
	185	18/150	42	42	26	11
	186	17/150	50	15	6	6
6-15-78	190	19/150	45	45	2	2
	191	19/150	43	43	0	0
	192	18/150	50	20	0	0
	193	21/150	45	10	0	0
	194	18/150	50	50	25	9
6-28-78	196	13/150	50	10	0	0
	198	14/150	55	17	0	0
	199	15/150	50	40	4	0
	200	15/150	55	50	0	0
	202	15/150	40	28	0	0
	203	16/150	50	15	0	0
	204	19/150	50	30	7	1
	206	18/150	50	15	0	0
7-14-78	207	18/150	50	15	0	0
	208	16/150	50	20	2	0
	209	16/150	50	35	4	0
	210	16/150	40	15	0	0
	211	15/150	40	25	3	0
	213	17/150	45	45	3	0
	214	14/150	55	35	0	0

Bayou Graveline (continued)

Collection Date	Sample	# of Oysters Processed/Wt.	Final Sample Volume	Volume Plaqued	# of Plaques Isolated	# of Confirmed Isolates
8- 3-78	216	25/150	55	55	27	23
	217	23/150	55	20	0	0
	218	20/150	45	40	5	0
8-15-78	222	17/150	60	60	21	2
	223	18/150	40	35	13	6
8-30-78	226	19/150	28	20	0	0
	227	19/150	50	30	2	2
	228	18/150	45	30	0	0
	229	17/150	30	20	0	0
	230	20/150	20	15	3	1
9-17-78	234	16/150	50	40	15	11
	236	14/150	55	30	3	0
	237	17/150	50	40	5	1
	238	19/150	60	45	0	0
	240	19/150	50	40	9	5
9-30-78	241	15/150	40	22	4	4
	244	19/150	45	35	0	0
	245	19/115	45	23	9	6
	247	21/150	49	18	0	0
	248	17/150	45	20	1	0
	251	15/150	45	25	0	0
10-16-78	255	18/150	45	25	0	0
	256	14/150	50	20	2	1
	257	16/150	50	27	5	4
11- 2-78	259	11/150	30	20	0	0
	260	14/150	30	21	0	0
	261	14/150	30	20	0	0

Bayou Graveline (continued)

Collection Date	Sample	# of Oysters Processed/Wt.	Final Sample Volume	Volume Plaqued	# of Plaques Isolated	# of Confirmed Isolates
11- 2-78	262	16/150	15	10	2	2
	263	10/150	30	28	6	3
	265	16/120	30	22	0	0
11-15-78	266	16/150	20	20	0	0
	267	16/150	30	24	0	0
	268	15/150	35	20	0	0
	269	11/150	30	18	0	0
11-30-78	274	8/150	35	35	15	
	275	12/150	40	26	3	
	276	9/150	30	20	0	0
	279	11/150	30	20	0	0
12-16-78	280	10/150	30	20	6	0
	281	12/150	30	30	10	1
	283	13/150	30	30	9	0
1-15-79	288	12/150	35	30	8	0
	289	13/150	40	32	4	0
	290	14/150	40	28	5	0
2- 1-79	293	11/150	30	25	4	0
	294	11/150	40	30	3	0
	295	11/150	40	30	7	0
	297	10/150	35	32	8	0
	298	11/150	40	20	7	0
2-15-79	305	11/150	30	18	6	0
	307	9/150	35	30	1	0
	308	9/150	40	22	1	0
	309	8/150	35	28	5	0

Bayou Graveline (continued)

Collection Date	Sample	# of Oysters Processed/Wt.	Final Sample Volume	Volume Plaqued	# of Plaques Isolated	# of Confirmed Isolates
3- 1-79	312	7/150	30	23	452	31
	313	8/150	35	20	10	0
	314	8/150	40	19	3	0
	315	7/150	30	22	5	0
	316	9/150	40	29	5	0
3-15-79	322	6/150	20	16	1	0
	323	8/150	30	21	8	4
	324	8/150	25	25	7	0
	325	7/150	35	18	0	0
	326	8/150	35	30	12	1
3-30-79	331	8/150	35	32	1	0
	332	5/150	40	30	0	0
	333	9/150	55	45	29	1
	334	8/150	42	42	22	2
	335	11/150	53	50	18	0
4-18-79	342	8/150	45	40	0	0
	343	10/150	52	40	0	0
	344	11/150	45	39	13	0
	345	8/150	55	20	0	0
	346	9/150	45	28	0	0
4-26-79	349	8/150	53	40	0	0
	350	9/150	50	25	0	0
	351	9/150	53	28	0	0
	352	6/150	45	35	0	0
	353	7/150	58	28	0	0
5-18-79	361	9/150	60	38	0	0
	362	10/150	55	43	0	0
	363	10/150	68	39	0	0

Bayou Graveline (continued)

Collection Date	Sample	# of Oysters Processed/Wt.	Final Sample Volume	Volume Plaqued	# of Plaques Isolated	# of Confirmed Isolates
5-18-79	364	10/150	58	27	0	0
	365	10/150	60	35	0	0
5- 1-79	371	10/150	60	30	0	0
	372	10/150	52	46	0	0
	373	11/150	50	25	0	0
	374	13/150	50	25	0	0
	375	11/150	50	47	2	1
7- 2-79	392	14/150	55	26	0	0
	393	13/150	55	30	0	0
	394	14/150	70	43	1	0
	395	10/150	75	62	2	1
	396	13/150	60	55	1	0
7-16-79	397	11/150	50	48	10	0
	398	12/150	57	35	2	0
	399	11/150	60	38	1	0
	400	13/150	53	22	1	1
	401	15/150	80	35	0	0
8- 3-79	405	12/150	65	37	0	0
	406	15/150	65	40	1	0
	407	16/150	60	40	1	0
	408	15/150	80	20	0	0
8-16-79	410	13/150	43	35	1	1
	411	11/150	50	23	1	1
	412	9/150	50	37	1	1
	413	14/150	45	40	0	0
	414	12/150	60	40	0	0

Bayou Graveline (continued)

Collection Date	Sample	# of Oysters Processed/Wt.	Final Sample Volume	Volume Plaqued	# of Plaques Isolated	# of Confirmed Isolates
8-31-79	418	12/150	50	27	0	0
	419	18/150	75	70	3	3
	420	17/150	65	32	0	0
	421	15/150	70	39	1	0
	422	15/150	55	43	2	2
9-28-79	423	13/150	45	40	0	0
	424	12/150	50	20	0	0
	425	12/150	50	20	0	0
10-15-79	431	13/150	70	30	0	0
	432	12/150	58	50	0	0
	434	12/150	60	32	0	0
10-26-79	435	13/150	62	22	0	0
	436	12/150	45	20	0	0
	437	13/150	60	20	0	0
	438	13/150	50	20	0	0
11-19-79	442	13/150	78	43	1	0
	443	12/150	55	25	0	0
	444	12/150	50	20	0	0
11-29-79	447	15/150	65	20	0	0
	448	13/150	75	20	0	0
	449	9/150	35	28	0	0
12-20-79	452	8/151.9	55	18	0	0
	453	13/152	60	47	4	1

Table 8

GRAVELINE BAYOU

	Month	S °/oo Surface	S °/oo Bottom	T°C	Median (10^1) FC Water Surface	Median (10^1) FC Water Bottom	FC Oysters Per 100 g	PLI Per 100 g	CV Per 100 g
1978	Jan.	4.0	5.6	10.0	100.0	103.0	310	5.8	2.2
	Feb.	8.0	14.3	12.5	3.2	1.6	104	5.0	0.7
	Mar.	17.5	19.0	18.5	2.0	2.5	3,250	3.0	0.5
	Apr.	18.0	18.2	21.0	1.6	1.6	765	1.1	0.7
	May	4.5	5.1	28.0	2.8	1.5	170	5.6	2.0
	June	12.8	13.0	30.5	5.8	0.6	44	1.9	0.6
	July	12.5	17.5	29.5	430.0	39.0	815	2.9	1.5
	Aug.	14.7	14.8	28.3	3.9	2.8	17,650	5.0	1.4
	Sept.	16.0	16.0	27.6	4.0	3.7	745	2.7	1.5
	Oct.	24.5	24.8	22.0	5.5	4.6	230	1.1	0.7
	Nov.	21.3	21.3	18.0	108.0	88.0	945	0.8	0.3
	Dec.	24.3	25.0	10.0	3.2	1.6	715	5.5	0.2
1979	Jan.	16.5	19.4	7.2	3.8	2.5	73	3.8	0.0
	Feb.	2.0	3.8	11.6	65.0	73.0	120	3.1	0.0
	Mar.	3.0	3.3	15.3	6.8	7.5	30	33.5	2.4
	Apr.	1.0	1.0	19.0	42.0	34.0	49	4.7	0.2
	May	4.5	3.7	21.4	4.8	5.6	745	0.0	0.0
	June	12.7	12.7	26.1	8.7	6.0	320	0.1	0.1
	July	4.3	7.3	32.0	1.8	1.3	124	1.3	0.1
	Aug.	10.8	11.3	30.9	3.3	3.4	94	0.4	0.2
	Sept.	9.0	10.3	30.5	3.7	3.6	194	0.5	0.4
	Oct.	15.7	16.3	21.6	9.7	8.3	135	0.0	0.0
	Nov.	10.3	13.0	15.2	88.0	70.0	1,170	0.1	0.0
	Dec.	8.5	12.0	7.8	23.0	26.0	1,095	1.3	0.3

Table 7

PASS CHRISTIAN

	Month	S °/oo Surface	S °/oo Bottom	T°C	Median (10^1) FC Water Surface	Median (10^1) FC Water Bottom	FC Oysters Per 100 g	PLI Per 100 g	CV Per 100 g
1978	Jan.	2	5	8	150	180	73	0.6	0.0
	Feb.	16	19	14	0.2	0.2	45	1.3	0.0
	Mar.	14	15	19	0.2	0.2	125	2.0	0.0
	Apr.	14	15	21	0.2	0.2	<20	6.3	3.6
	May	7	8	30	0.2	0.2	<20	0.0	0.0
	June	13	14	31	0.4	0.2	61	0.0	0.0
	July	18	18	28	70	0.5	400	0.0	0.0
	Aug.	14	18	29	0.2	0.4	360	0.6	0.0
	Sept.	17	17	27	1.5	0.2	12,450	0.0	0.0
	Oct.	18	18	23	0.2	0.2	<20	0.6	0.3
	Nov.	17	18	17	2.0	1.5	330	0.0	0.0
	Dec.	15	20	11	1.5	1.6	204	1.0	0.0
1979	Jan.	13	14	6	2.3	2.0	88	4.4	0.0
	Feb.	7	10	15	4.7	7.0	49	2.8	1.3
	Mar.	5	5	19	0.2	0.3	130	0.2	0.0
	Apr.	2	2	20	2.7	2.6	130	0.7	0.0
	May	4	8	22	0.8	0.2	7,800	0.0	0.0
	June	14	14	29	0.3	0.2	250	0.7	0.2
	July	8	19	32	0.2	0.2	400	0.2	0.2
	Aug.	19	19	31	0.2	0.3	<20	2.0	0.0
	Sept.	17	18	29	0.2	---	78	0.0	0.0
	Oct.	19	19	17	0.2	0.3	43	0.0	0.0
	Nov.	18	18	11	1.0	0.3	360	0.3	0.0
	Dec.	12	12	9	6.0	120	3,300	0.3	0.0

Table 9

Correlation Coefficients: Pass Christian

January, 1978 to December, 1979

	MON	SS	SB	T	FCS	FCB	FCO	PLI	CV
MON	1.00000								
SS	.41101	1.00000							
SB	.32886	.92442	1.00000						
T	.36257	.67603	.88680	1.00000					
FCS	.28354	.13814	.15305	.13364	1.00000				
FCB	.11745	.15036	.16546	.15509	.75231	1.00000			
FCO	.01410	.03685	.07147	.08420	.10549	.00211	1.00000		
PLI	.22307	.15595	.20168	.23140	.08989	.07562	.24259	1.0000	
CV	.18918	.09406	.11528	.11215	.08654	.08488	.13768	.78575	1.0000

Underlined numbers are significant at $P \leq 0.05$ level of significance.

MON - Month
SS - Salinity:Surface Water
SB - Salinity:Bottom Water
T - Temperature°C
FCS - Fecal Coliforms Surface Water: 100 ml
FCB - Fecal Coliforms: Bottom Water: 100 ml
FCO - Fecal Coliforms: Oyster Tissue: 100 g
PLI - Plaque-like Isolates: 100 g
CV - Confirmed Virus Isolates: 100 g

Table 10

Correlation Coefficients: Graveline Bayou

January, 1978 to December, 1979

	MON	SS	SB	T	FCS	FCB	FCO	PLI	CV
MON	1.00000								
SS	.18698	1.00000							
SB	.21181	.96757	1.00000						
T	.02834	.00204	.07350	1.00000					
FCS	.14853	.02910	.08070	.07254	1.00000				
FCB	.03053	.21243	.18425	.40043	.48042	1.00000			
FCO	.31583	.22894	.21780	.03852	.10075	.15296	1.00000		
PLI	.27614	.10952	.11902	.21291	.09917	.12708	.55320	1.00000	
CV	.55797	.19078	.19876	.10390	.22032	.07284	.05285	.45160	1.00000

Underlined numbers are significant at $P \leq 0.05$ level of significance.

MON - Month
SS - Salinity:Surface Water
SB - Salinity:Bottom Water
T - Temperature°C
FCS - Fecal Coliforms: Surface Water: 100 ml
FCB - Fecal Coliforms: Bottom Water: 100 ml
FCO - Fecal Coliforms: Oyster Tissue: 100 g
PLI - Plaque-like Isolates: 100 g
CV - Confirmed Virus Isolates: 100 g

the cell cultures. If this effect was localized during the assay, it could appear as a plaque. The procedure used to confirm the nature of a viral isolate was very specific. If the PLI could not be carried through three subcultivations in BGM cells, it was discarded. Perhaps the procedure was too stringent and could not compensate for viruses which replicated weakly in BGM cells. Tables 4 and 5 which summarize two years of Graveline isolations demonstrate the wide variability observed at this one site. The number of PLI during 1979 was 1.6 x greater than 1978 although the number of confirmed isolates was lower (35 versus 8%). Part of the discrepancy results from the isolates of March, 1979. Here, 503 PLI were observed, but only 40 plaques were confirmed. The problem was time; two weeks are required to confirm an isolate. As the results show, 36 of the 40 plaques were viruses. If more could have been done, the final percentages would have changed drastically. It is obvious that virological isolates at Graveline are more frequent than observed at the approved area. It would take additional data of this type, perhaps 3-5 additional years, to firmly determine if virological data can be used to classify oyster growing waters.

Tables 7 and 8 are combinations of the bacteriological and virological studies and include the physical parameters collected during the grant period. Fecal coliform and virus isolations are compared on the basis of 100 gr quantities. These data were analyzed using an SPSS program on the Sigma 9/CP-V computer system at USM. The results of the coefficients of correlation are shown in Tables 9 and 10. At the approved sampling location, found in open waters of the Mississippi Sound, positive correlations

were found to exist between (a) month of the year and surface water salinity, (b) surface and bottom water salinity, (c) temperature versus surface and bottom water salinity measurements, (d) fecal coliforms in surface and bottom waters and (e) the number of plaque-like isolates and confirmed virus isolates per 100 gr oyster tissue. Correlations were not observed between fecal coliform and virological indicies although both locations are classified using fecal coliform counts in addition to the other measurements of sanitary surveys. Of the positive microbial correlations, the strongest value was observed between plaque-like isolates and confirmed viruses. Fecal coliforms in surface and bottom water also correlated strongly indicating that the mixing of water in the Sound contributes to an even distribution of microorganisms. It is not clear how mixing relates to the correlation between plaque-like isolates and confirmed viruses.

At the prohibited oyster harvesting site, Graveline Bayou, different positive correlations or different correlation strengths were observed (Table 10). For example, the positive relationship between surface and bottom water salinity measurements was greater at the prohibited site. However, temperature did not relate to the salinity measurements at Graveline as it did at the Pass Christian location. Only a weak positive correlation was observed in water samples (surface and bottom) collected at the Graveline location indicating that less mixing occurs at this site than noted at the approved study area. As expected, plaque-like isolates from Graveline oysters correlated with confirmed virus isolations; however, the strength of the correlation was very weak as compared to the figure observed in approved oysters. A

moderate correlation was also observed between the months of the year and the confirmed virus number but the observed rise and fall during the two-year period did not follow the usual epidemiological patterns (more shedding of virus during the warmer months). It is possible that virus isolations do not relate to the fluctuations of sewage entering the system. At present, we have no way to gauge the length of time that an isolate has been oyster associated.

The types of confirmed virus isolates followed the general pattern observed by other investigators. At the approved area during 1978, 67% were polioviruses; four could not be identified by the procedure employed. These findings were repeated during 1979 with seven of the ten confirmed isolates identified as poliovirus type 1 or type 2 and the remainder as unidentifiable.

In comparison, 148 confirmed isolates (35% of the number of plaque-like isolates) were purified from Graveline samples in 1978. Of 55 random isolates identified, 50 were polio type 1, one was polio type 2, one was echovirus 24 and three were unidentifiable. The 51 confirmed isolates purified during 1979 were identified and again the largest percentage were identified as polio types 1 and 2. Echovirus type 24 was identified on three occasions, and coxsackievirus types A9 and B3 were each isolated once. Five isolates could not be identified by the immunological methods used.

VIRAL EVALUATION OF PROHIBITED OYSTER GROWING WATERS

PART IV. RADIOIMMUNOLOGICAL STUDIES

INTRODUCTION

Hepatitis A (HAV) and hepatitis B (HBV) viruses have long been considered distinct etiologic agents of viral hepatitis. One distinguishing feature of each virus has been its mode of transmission: HAV by the fecal/oral route; HBV by parenteral transfer. However, the classical mechanisms of transmission are today being cautiously reexamined. HBV contamination of shellfish or their growing water was not considered likely until Mahoney and co-workers identified HBV in clams collected along the Maine coast near a hospital sewage outfall.

PROCEDURES AND DISCUSSION: HEPATITIS B

Standard procedures of hepatitis B surface antigen (HBsAg) analysis by radioimmunoassay (RIA) are available for human serum but have not been applied in studies of oyster contamination. Similar methods for HAV analysis are not routinely available. This study was performed to determine: (a) if it is practical to use RIA to detect HBsAg in oyster tissue and (b) the sensitivity of the assay system when applied to oyster tissue. Ausria II test systems and an autologic 101 gamma counter were purchased from Abbott Laboratories, North Chicago. Abbott investigators supplied two milliliters of HBsAg concentrate (100 µg/ml) which we diluted with fetal bovine serum to produce a pool containing 1000 ng/ml. This pool was used in all seeded oyster experiments.

Oyster homogenates (1 gr tissue:1 ml distilled water) were prepared by blending for one minute and seeded with HBsAg

(1 to 500 ng/ml) while control homogenates contained no antigen. Results demonstrated: (a) that non-specific absorption of oyster proteins to system components inflated the gamma counts by a factor of 2-4 X when compared to serum controls, (b) that when homogenates were centrifuged prior to analysis (500 X g; 20 minutes), 90% of the antigen was found in the supernatant, (c) that 20 ng/ml HBsAg in a homogenate could be statistically detected (as compared to a minimal level of 2 ng/ml of HBsAg in serum), and (d) that the test system became saturated when antigen levels exceeded 250 ng/ml. Various methods including treatment of seeded homogenate with acid, base, lipid solvents, proteolytic enzymes or digestants such as trisodium phosphate, failed to reduce non-specific binding or destroyed some component of the RIA test system.

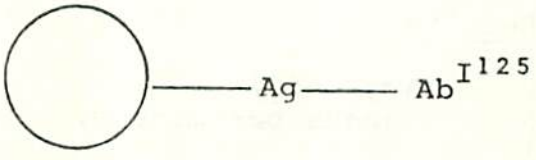
Further experimentation demonstrated that the detectable level of HBsAg in oyster supernatants could be lowered to 5 ng/ml if the mixture was centrifuged at 12000 X g for 30 minutes prior to analysis. The quality of the oyster tissue was a determinant, i.e. the sensitivity of the system varied from 5-20 ng/ml supernatant when polluted oysters were analyzed. Washed, retail oysters allowed the greatest sensitivity.

Studies to determine if a current method to extract entero-viruses from oysters could concentrate HBsAg to a detectable RIA level were conducted. Results demonstrated that polluted oysters would have to contain at least 50 ng HBsAg in each milliliter of original homogenate to yield a positive, final concentrate. It is highly unlikely that this HBsAg level would be found in natural oyster tissue requiring other, more efficient methods of antigen concentration.

Field trials of the above procedure were tested using oysters collected from the Graveline site during 1978. A total of 37 150 gr oyster samples were tested (a minimum of two samples per month) during the twelve month study, none of which were positive for HBsAg. Testing of this procedure was terminated in lieu of experiments (1979) to detect hepatitis A virus antigen in oyster tissue.

PROCEDURES AND DISCUSSION: HEPATITIS A

The initial series of tests in this experiment were designed to detect the presence of varying concentrations of Hep A Ag in phosphate buffered saline. The detection procedure is based upon a competition established between Hep A Ag in solution, anti Hep $A^{I^{125}}$ in solution, and Hep A Ag in solid phase conjugation to the beads supplied with the Abbott kits. In theory, the greater the concentration of Ag present in solution, the more $Ab^{I^{125}}$ will be bound and made unreactive in respect to the solid phase Ag, thus reducing the count on the beads.



$Ab^{I^{125}}$ (can react with bead)

$Ab^{I^{125}}$---- Ag (cannot react with bead)

A low count on the bead is indicative of a higher concentration of Ag in solution as opposed to a high count which is indicative of a lower concentration of Ag in solution.

The first experiment involved testing decimal dilutions of Ag against undiluted I^{125} Ab. The dilution we prepared used PBS and was carried to a 10^{-10} value. Fifty ml of Ag dilution and 200 ml of I^{125} anti-HAV were added to the wells. This mixture was allowed to react for 2 hours at 45°C. After 2 hours the Ag labeled beads were added to the wells and incubated for 2 hours at 45°C. The beads were then washed (10 ml of acetic acid and formaldehyde solution) and counted for 1 minute.

Results

10^{-1}	8978	(Avg. of 6 wells)
10^{-2}	10269	(counts per minute)
10^{-3}	10246	
10^{-4}	9949	
10^{-5}	10004	
10^{-6}	10564	
10^{-7}	9938	
10^{-8}	10618	
10^{-9}	10696	
10^{-10}	10751	
Neg. Cont. (kit)	11,790	(Avg. of 10)
Pos. Cont. (kit)	618	(Avg. of 10)

The first test indicated a possible early extinction of the antigen. A check was run using two-fold dilutions and following the same procedure.

Results

Neg. Cont.	9102	(Avg. of 2)
1/2	5915	(counts per minute)
1/4	5911	
1/8	6209	
1/16	8029	
1/32	8006	
1/64	7762	
1/128	8498	
1/256	7835	

The first test and the check indicate that a count difference was evident, thought to be due to the change in the Ag concentration.

The second test determined if dilutions of the $Ab^{I^{125}}$ would increase the sensitivity of the test. The procedure followed was identical to the first test. A PBS control was included. $Ab^{I^{125}}$ dilutions tested were 1/2, 1/4, 1/8, and 1/16. Ag dilutions used were 1/4, 1/16, 1/64, and 1/256.

Results of Second Test

Pos. Cont. 433 (Avg. of 5)
Neg. Cont. 10565

1/2 $Ab^{I^{125}}$ Dilution	
1/4 Ag	3674 CPM
1/16 Ag	6057 CPM
1/64 Ag	7922 CPM
1/256 Ag	7728 CPM
PBS	8649 CPM

1/4 $Ab^{I^{125}}$ Dilution	
1/4 Ag	2780 CPM
1/16 Ag	2941 CPM
1/64 Ag	3693 CPM
1/256 Ag	4432 CPM
PBS	4698 CPM

1/8 $Ab^{I^{125}}$ Dilution	
1/4 Ag	977 CPM
1/16 Ag	1391 CPM
1/64 AG	3380 CPM
1/256	2848 CPM
PBS	2488 CPM

1/16 $Ab^{I^{125}}$ Dilution	
1/4 Ag	1148 CPM
1/64 Ag	1190 CPM
PBS	1578 CPM

The third test was performed in the same manner as the second. The incubation period was changed to the effect that the $Ab^{I^{125}}$, Hep A Ag, and beads were all incubated together.

Results

Pos. Cont.	541
Neg. Cont.	18809

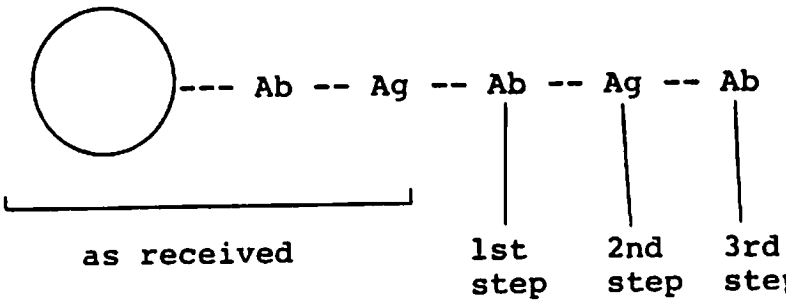
1/2 $Ab^{I^{125}}$ Dilution	
1/8 Ag	10559 CPM
1/16 Ag	10790 CPM
1/64 Ag	11678 CPM
1/256 Ag	11523 CPM
PBS	12079 CPM

1/4 $Ab^{I^{125}}$ Dilution	
1/8 Ag	6208 CPM
1/16 Ag	7013 CPM
1/64 Ag	7149 CPM
1/256 Ag	7351 CPM
PBS	6848 CPM

1/8 $Ab^{I^{125}}$ Dilution	
1/8 Ag	3729 CPM
1/16 Ag	3832 CPM
1/64 Ag	3748 CPM
1/256 Ag	3977 CPM
PBS	3849 CPM

1/1 Ab (No Dilution)	
1/8 Ag	17207 CPM
1/64 Ag	15486 CPM
PBS	18126 CPM

The above tests indicate that it is possible to detect the presence of Hep A Ag in dilution prepared using PBS diluent. The sensitivity of the competitive procedure does not seem to be optimum. The next series of tests were designed using a sandwich method. The beads were received from Abbott Labs coated with inactivated Hep A Ag, attached to the bead via unlabeled conjugated anti-Hep A antibody. Ab is added to the bead resulting in Ab on the outside of the bead. Ag is then added, followed by labeled Ab resulting in a sandwich.



The fourth test procedure was considerably different than that of the first three tests. The beads were incubated overnight with 1/1 labeled antibody (I^{125}). They were washed with the acetic-acid formaldehyde solution and incubated for 4 hours with the Ag dilution. They were then washed with the acetic-acid formaldehyde and incubated overnight with the 1/1 I^{125} labeled Ab, washed and counted. The quantity of Ag is read as an increase in counts over the PBS control.

Results

Antigen Dilution	Counts Per Minute
1/8 Ag	75665 (7 test avg.)
1/16 Ag	58364
1/32 Ag	46006
1/64 Ag	33766
1/128 Ag	30629
1/256 Ag	25522
PBS	24911

Note that in the sandwich method, the count is directly proportional to the amount of antigen present.

The fifth test procedure was identical to that of the fourth except that each incubation period was for a 24-hour period. This test was to test the sensitivity of dilutions of the $Ab^{I^{125}}$.

Results

1/2 Ab Test	CPM	
1/10 Ag	29332	(Avg. of 7 tests)
1/20 Ag	22478	
1/40 Ag	20947	
1/80 Ag	18964	
1/160 Ag	17203	
1/320 Ag	16882	
PBS	16794	

1/4 Ab Test	CPM
1/10 Ag	19359
1/20 Ag	17397
1/40 Ag	15555
1/80 Ag	12903
1/160 Ag	14722
1/320 Ag	13480
PBS	13760

The sixth test followed the same protocol as the fifth with the following exception. The initial incubation was for 2 overnight periods and the first coating antibody was not labeled. All washing steps used PBS, not the acetic acid-formaldehyde solution.

1/1 Ab Test	CPM	
1/20 Ag	6146	(5 tests)
1/40 Ag	3649	
1/80 Ag	2148	
PBS	873	

1/2 Ab Test	CPM
1/20 Ag	5279
1/40 Ag	3520
1/80 Ag	2140
PBS	754

The sandwich technique appears to be superior to the competitive procedure in detecting the presence of the Hep A Ag.

The seventh test was designed to detect the presence of Hep A Ag in oyster tissue. The test was prepared as follows:

1. Oyster tissue blended and 1.0 ml of Ag added.
2. Osmolarity adjusted to 850.
3. pH lowered to 4.3.

4. Spun at 1850 g.
5. Pellet resuspended in Gly-saline buffer pH 7.5.
6. Blended and adjusted to 7.5 pH.
7. Spun at 1850 g supernate saved.
8. pH adjusted to 4.5.
9. Spun down, pellet collected.
10. Pellet resuspended and pH adjusted to 7.5.
11. Spun at 10,000 g for 10 minutes. Supernate should contain virus.

A control was prepared and processed as above without antigen. In the RIA procedure, since 7 ml of test oyster material were recovered, a 1/7 dilution of Ag was the initial Ag standard.

Results

1/1 Ab Test	CPM
1/7	11708
1/10 of 1/7	1948
1/20 of 1/7	1235
1/40 of 1/7	930
PBS	520

1/2 Ab Test	CPM
1/7	7144
1/10 of 1/7	1536
1/20 of 1/7	1057
1/40 of 1/7	792
PBS	475

1/1 Ab +	Oyster Control	544
+	1/7 Oyster Test	2132
	1/10 Oyster Test	688
	1/20 Oyster Test	736
	1/40 Oyster Test	552
1/2 Ab +	Oyster Control	458
	1/7 Oyster Test	1710
	1/10 Oyster Test	604
	1/20 Oyster Test	539
	1/40 Oyster Test	491

These results indicate that it is possible to detect the presence of Hep A Ag in oyster tissue. The primary problem to overcome are to increase the sensitivity of the tests, shorten the time required, and improve on the extraction procedure.

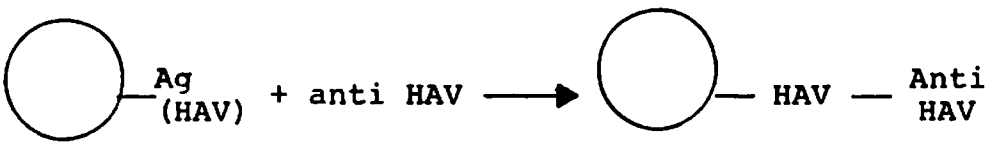
Our initial studies during 1979 to detect hepatitis A antigen in oyster tissue were successful but the sensitivity of the test modification described herein needs to be increased. The following summary statement points to certain possible changes for future experimentation.

SUMMARY: HEPATITIS A

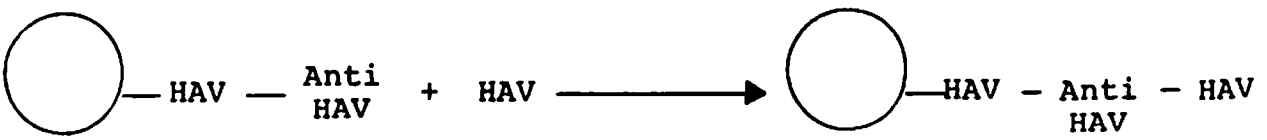
The Abbott HĀVAB kit has been modified to detect HA virus in solution. The procedure is a sandwich method that detects the viral particles via an attachment to an antibody-coated bead.

SANDWICH PROCEDURE

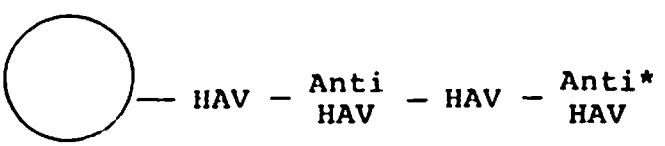
1. Incubate bead with anti hepatitis A virus (HAV).



2. Incubate bead with HAV Ag.

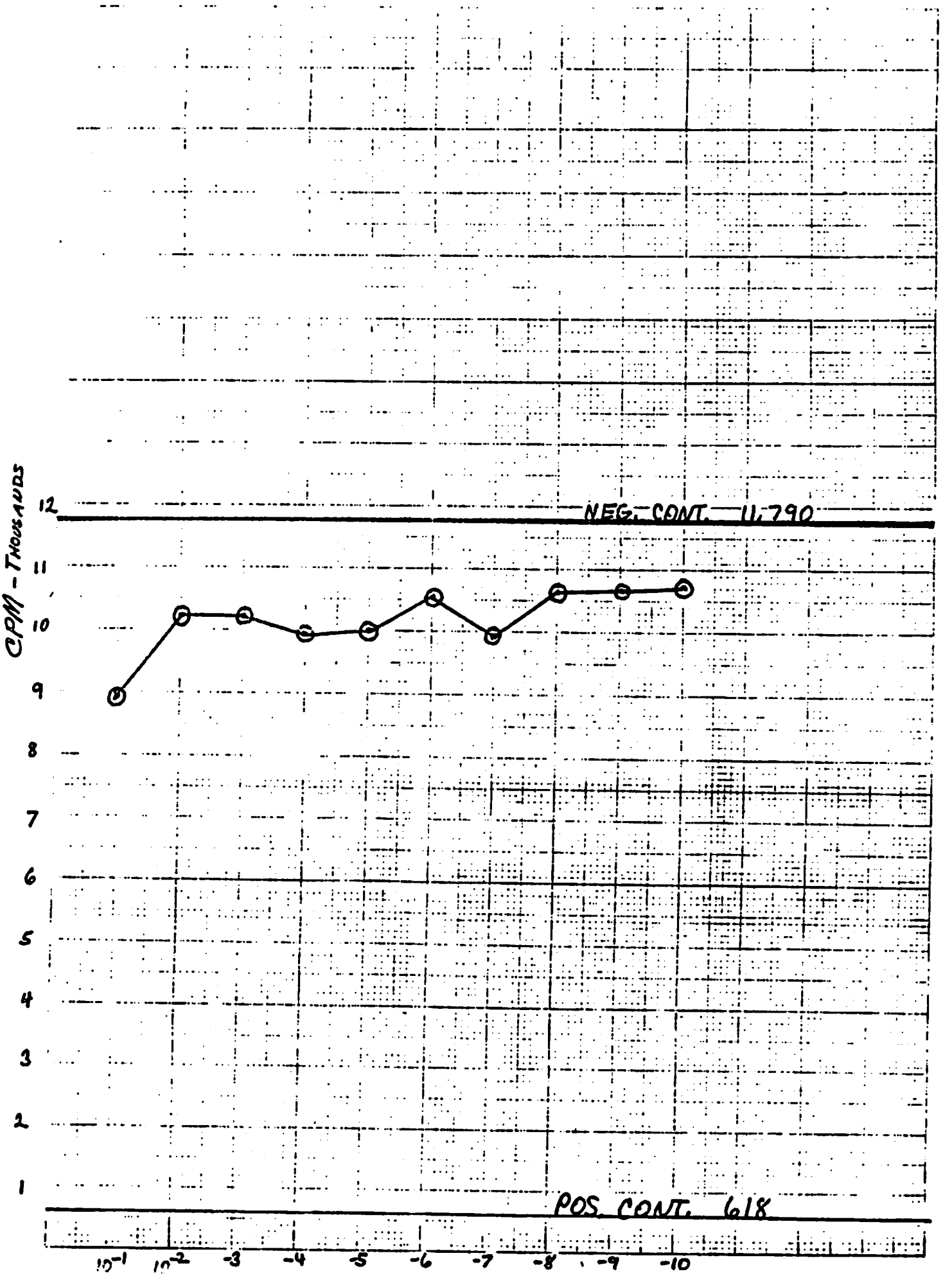


3. Incubate beads with anti HAV$^{I^{125}}$.

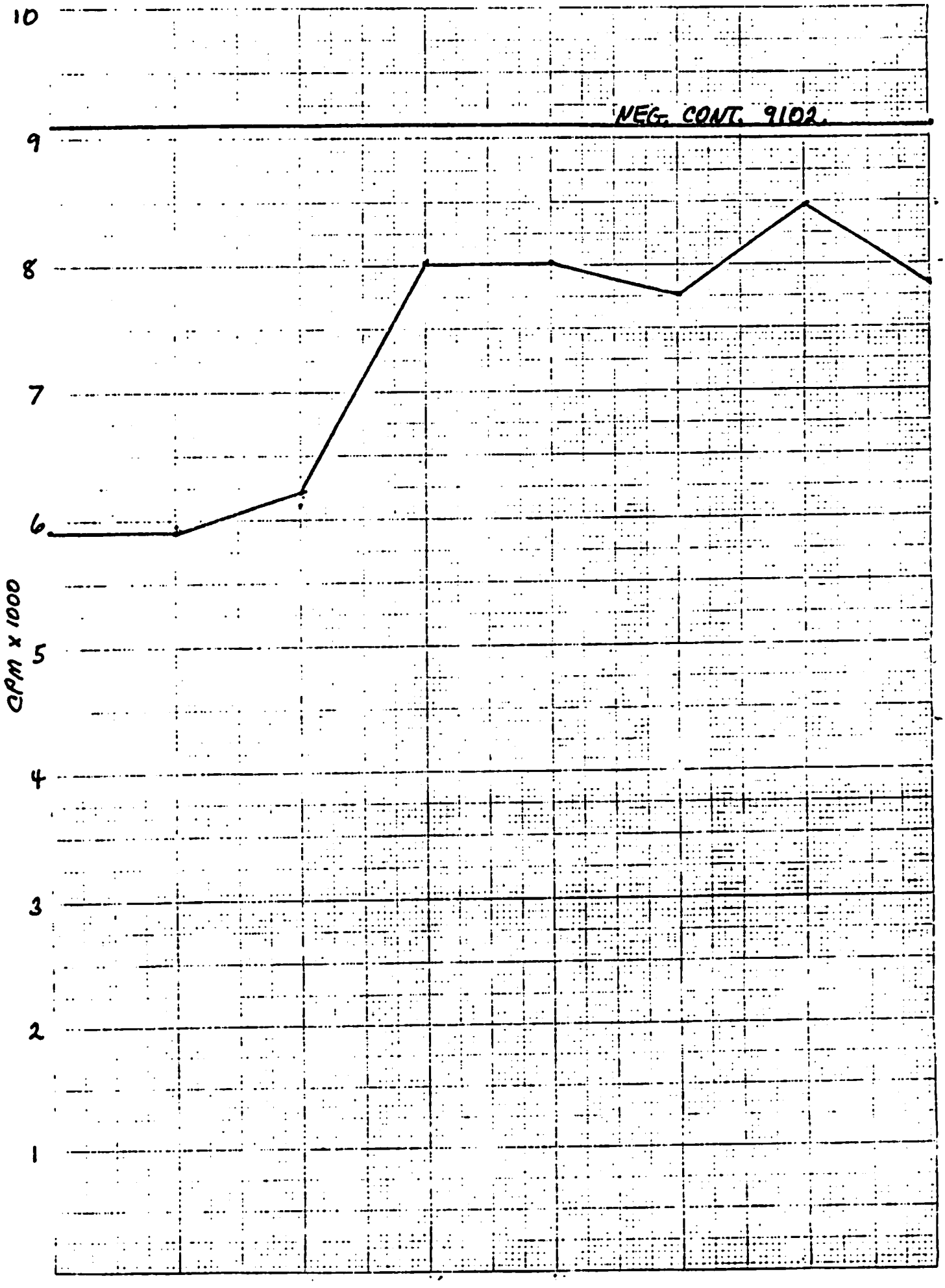


4. Count beads. High count = high concentration of virus.

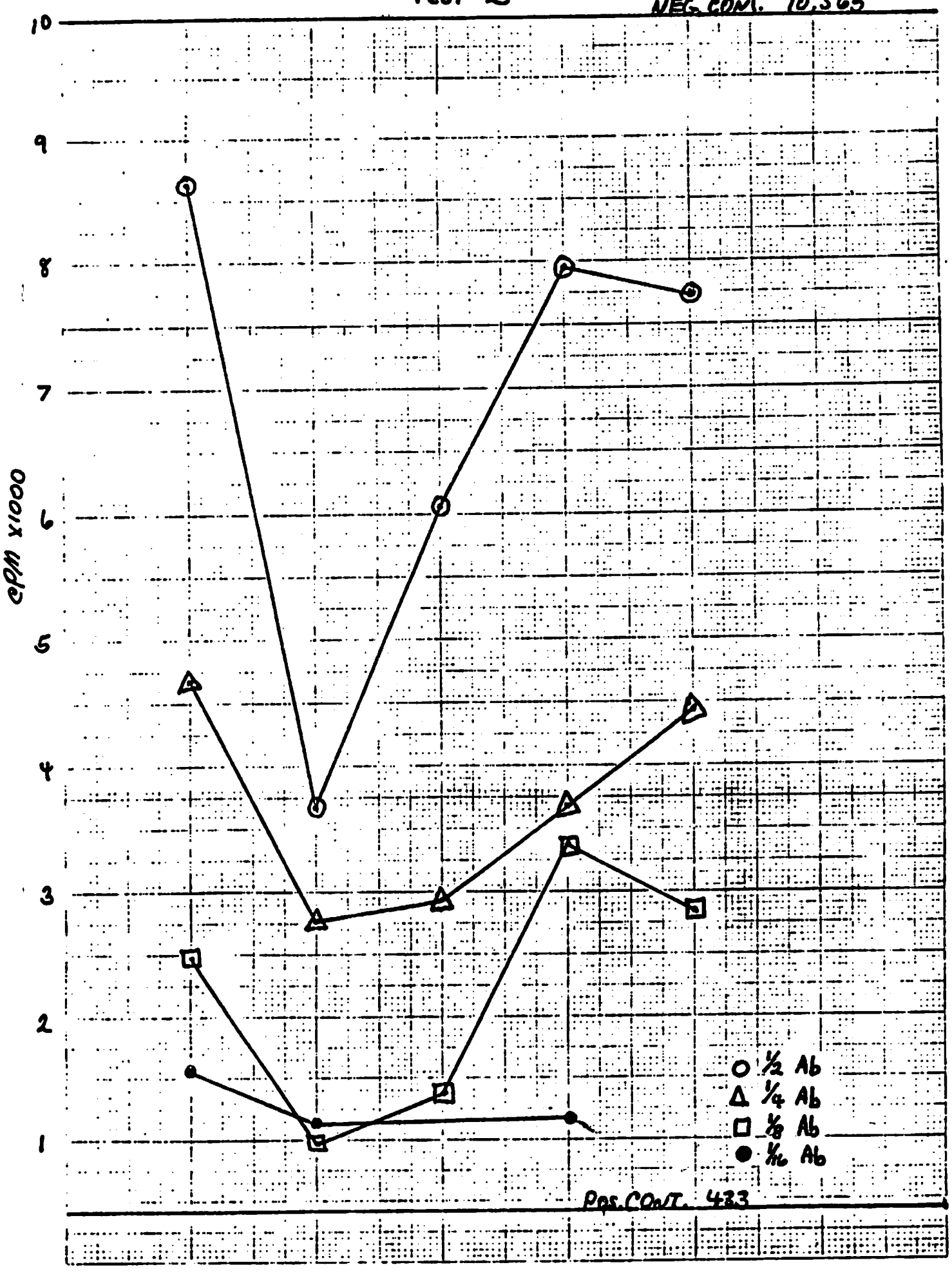
Test 1


CPM - Thousands
12
11
10
9
8
7
6
5
4
3
2
1
NEG. CONT. 4,790
POS. CONT. 618
10⁻¹
10⁻²
-3
-4
-5
-6
-7
-8
-9
-10
DILUTION

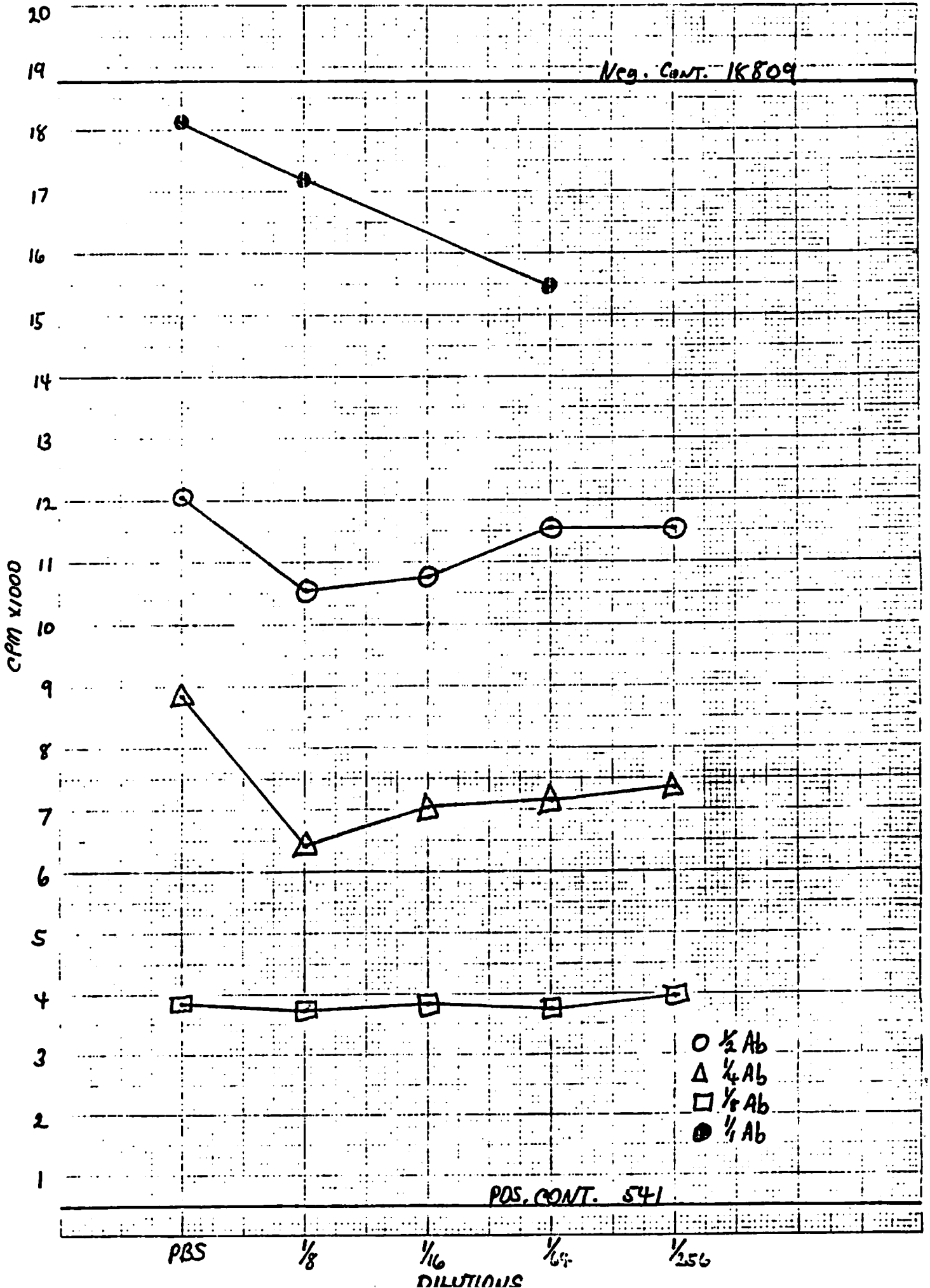
TEST 1 A



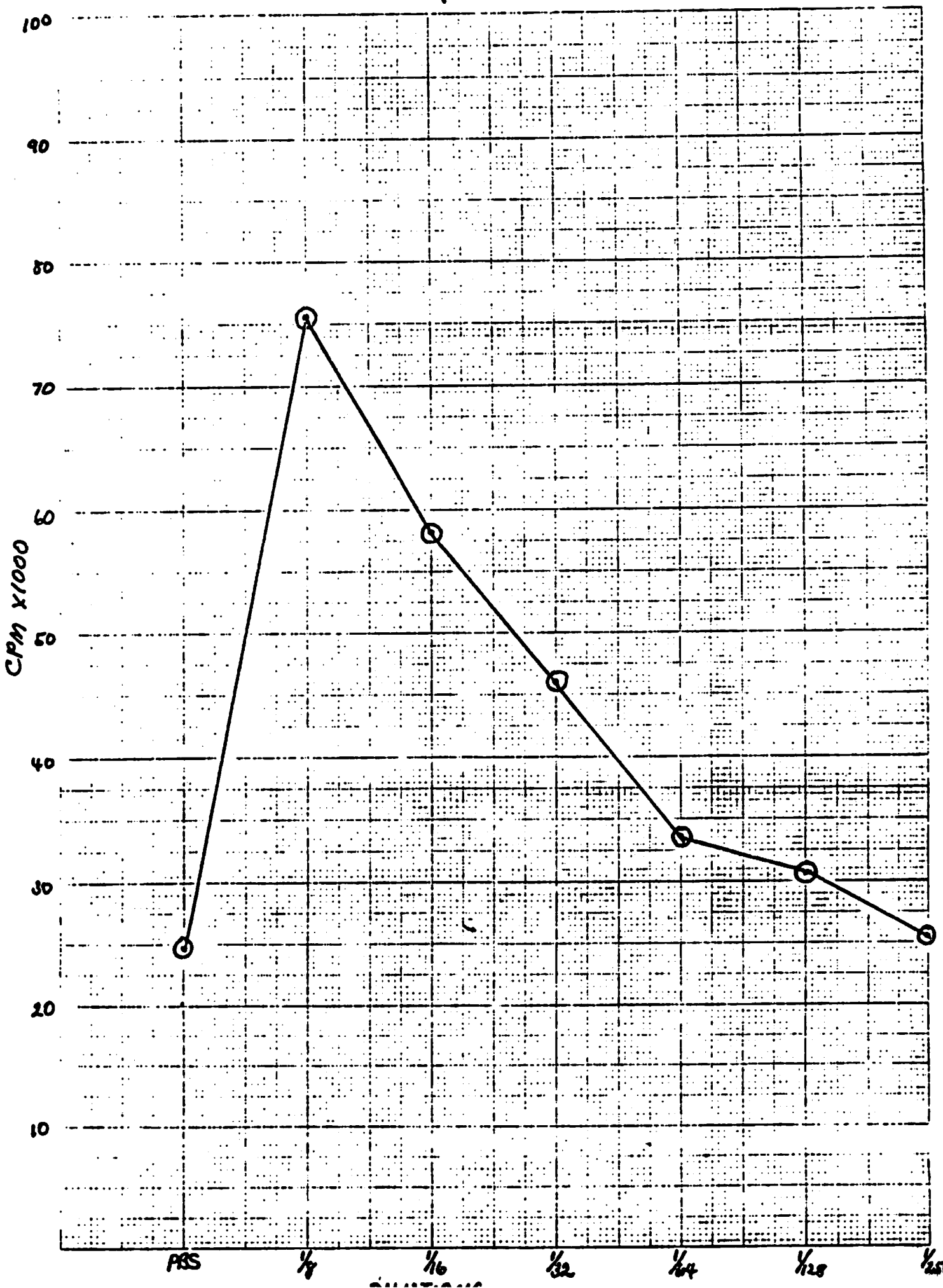
DILUTIONS

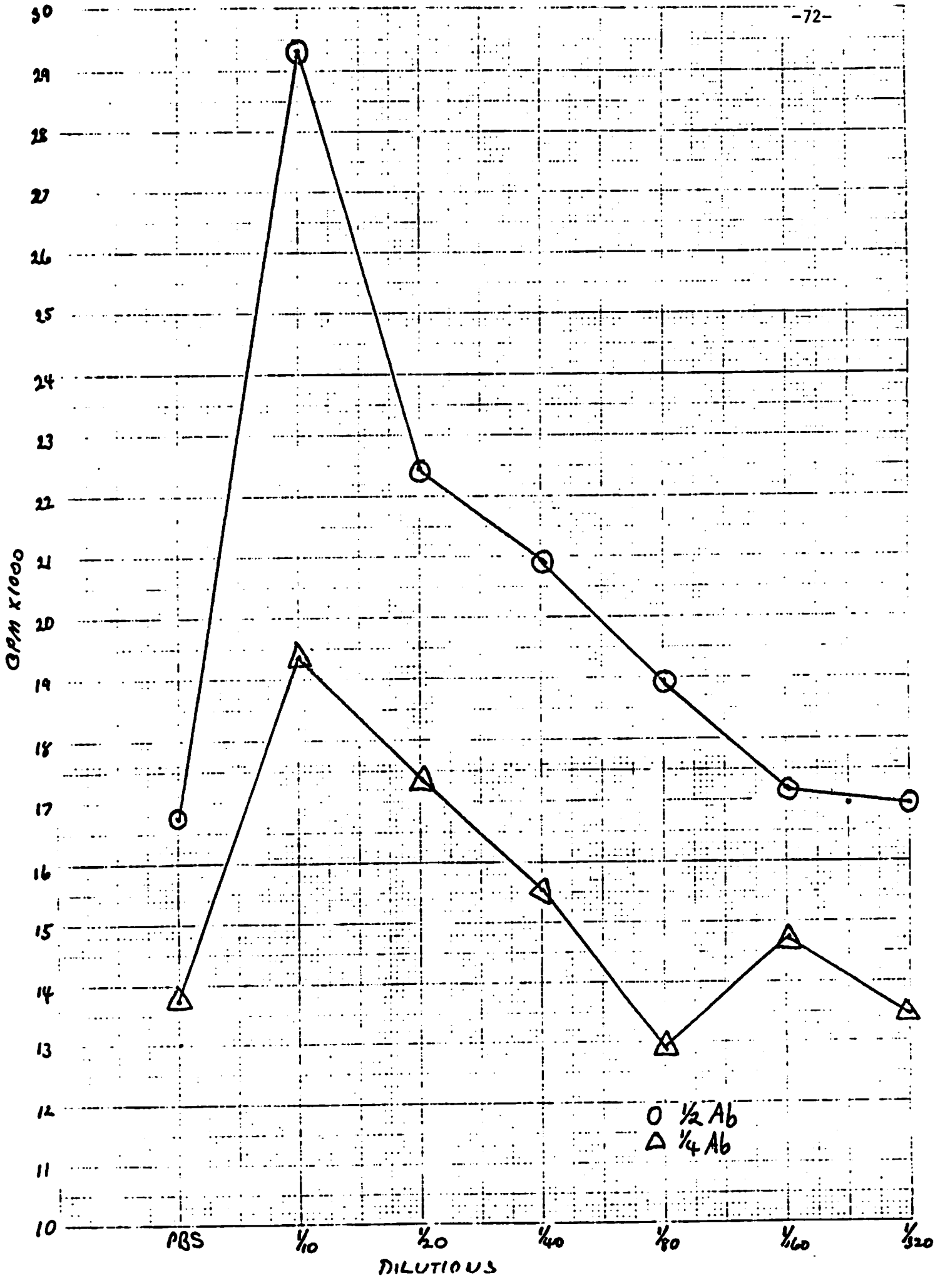

Test 2
NEG. CONT. 10,565
cpm x1000
10
9
8
7
6
5
4
3
2
1
½ Ab
¼ Ab
⅛ Ab
1/16 Ab
Pos. CONT. 433

Test 3

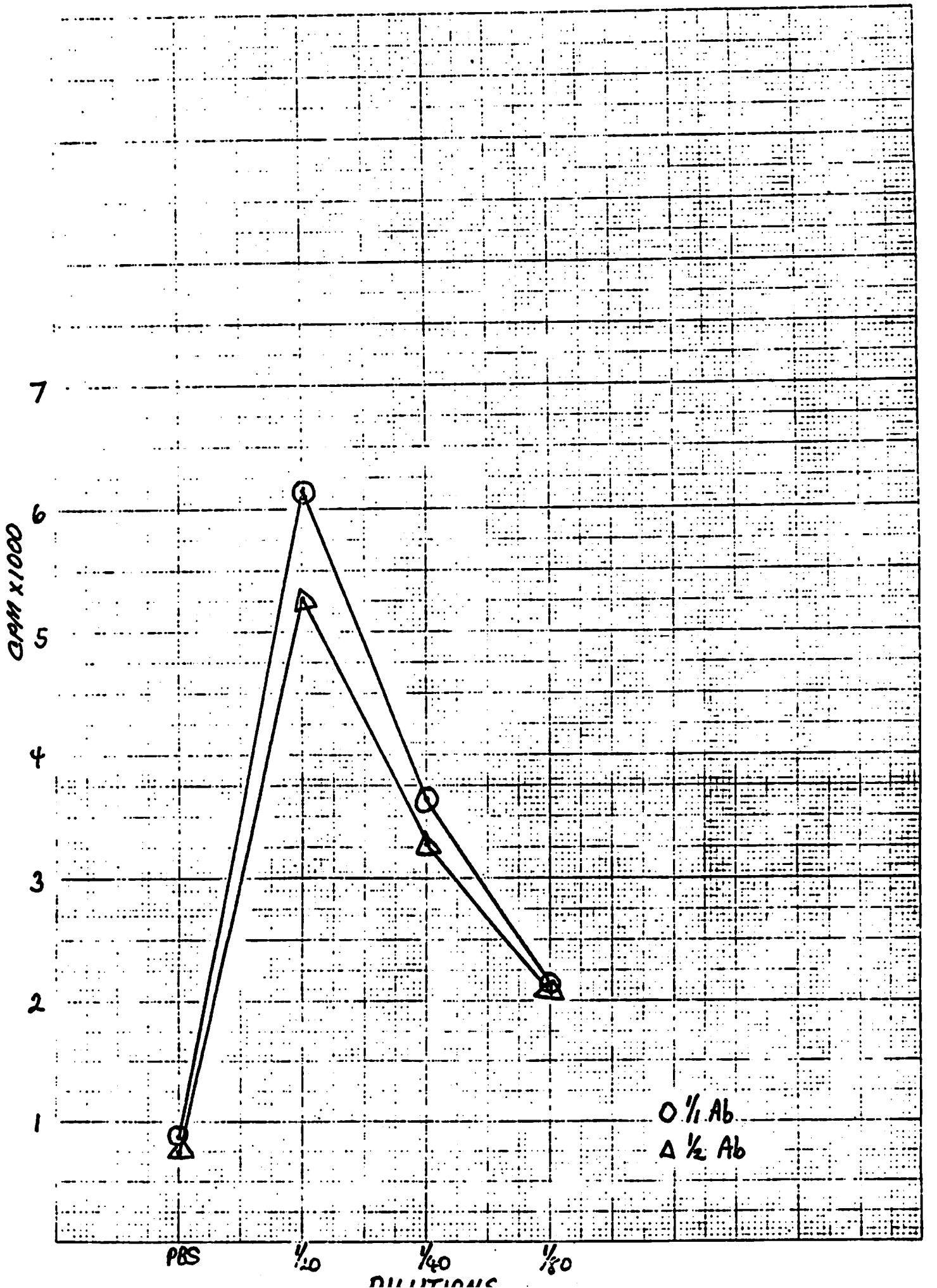

Neg. Cont. 1K809
Pos. Cont. 541
CPM x1000
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
PBS
1/8
1/16
1/64
1/256
DILUTIONS
○ 1/2 Ab
△ 1/4 Ab
□ 1/8 Ab
● 1/16 Ab

TEST 4 (1ST SANDWICH)

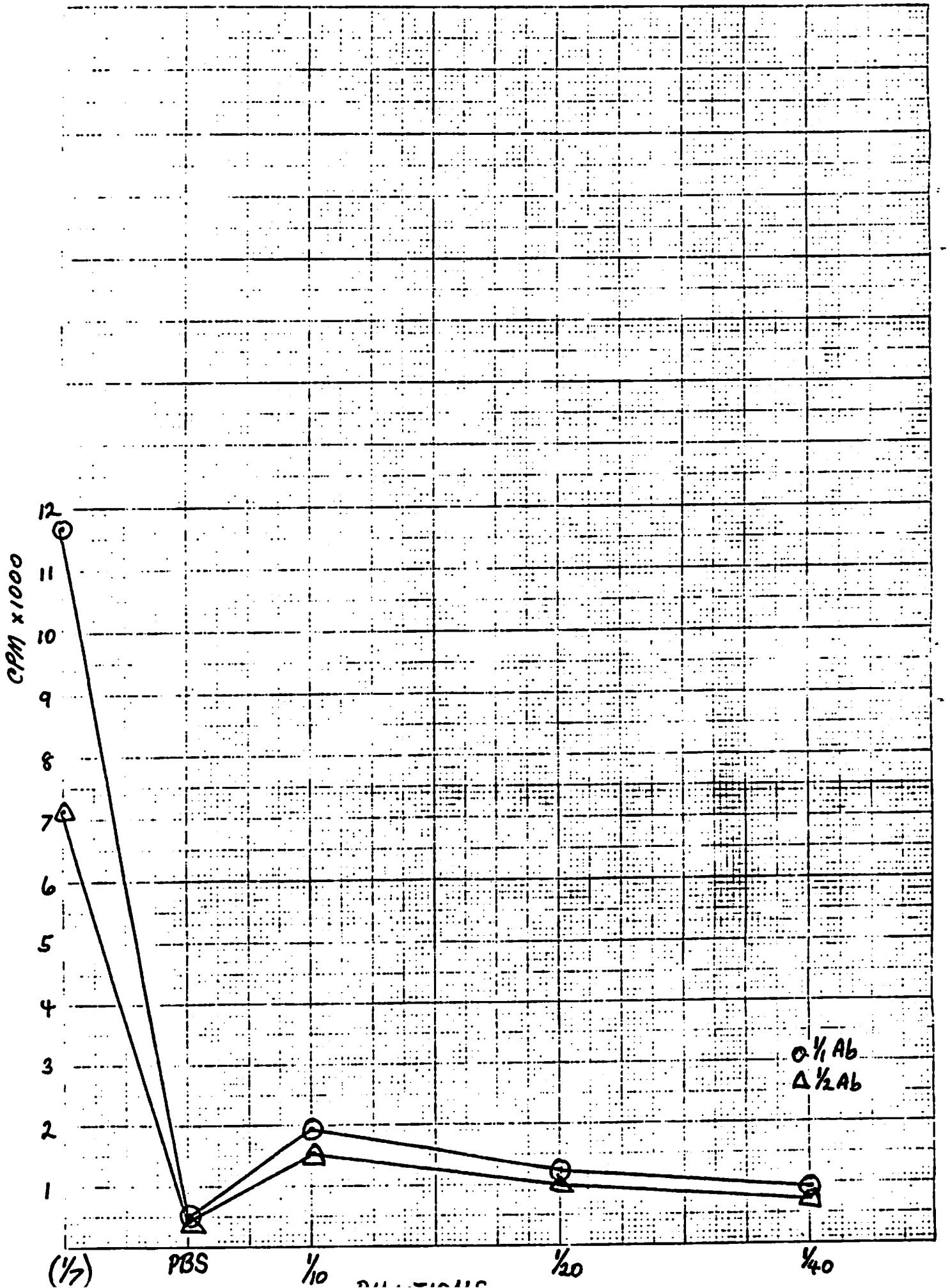



30
29
28
27
26
25
24
23
22
21
20
19
18
17
16
15
14
13
12
11
10
CPM X1000
PBS
1/10
1/20
1/40
1/80
1/160
1/320
DILUTIONS
○ 1/2 Ab
△ 1/4 Ab

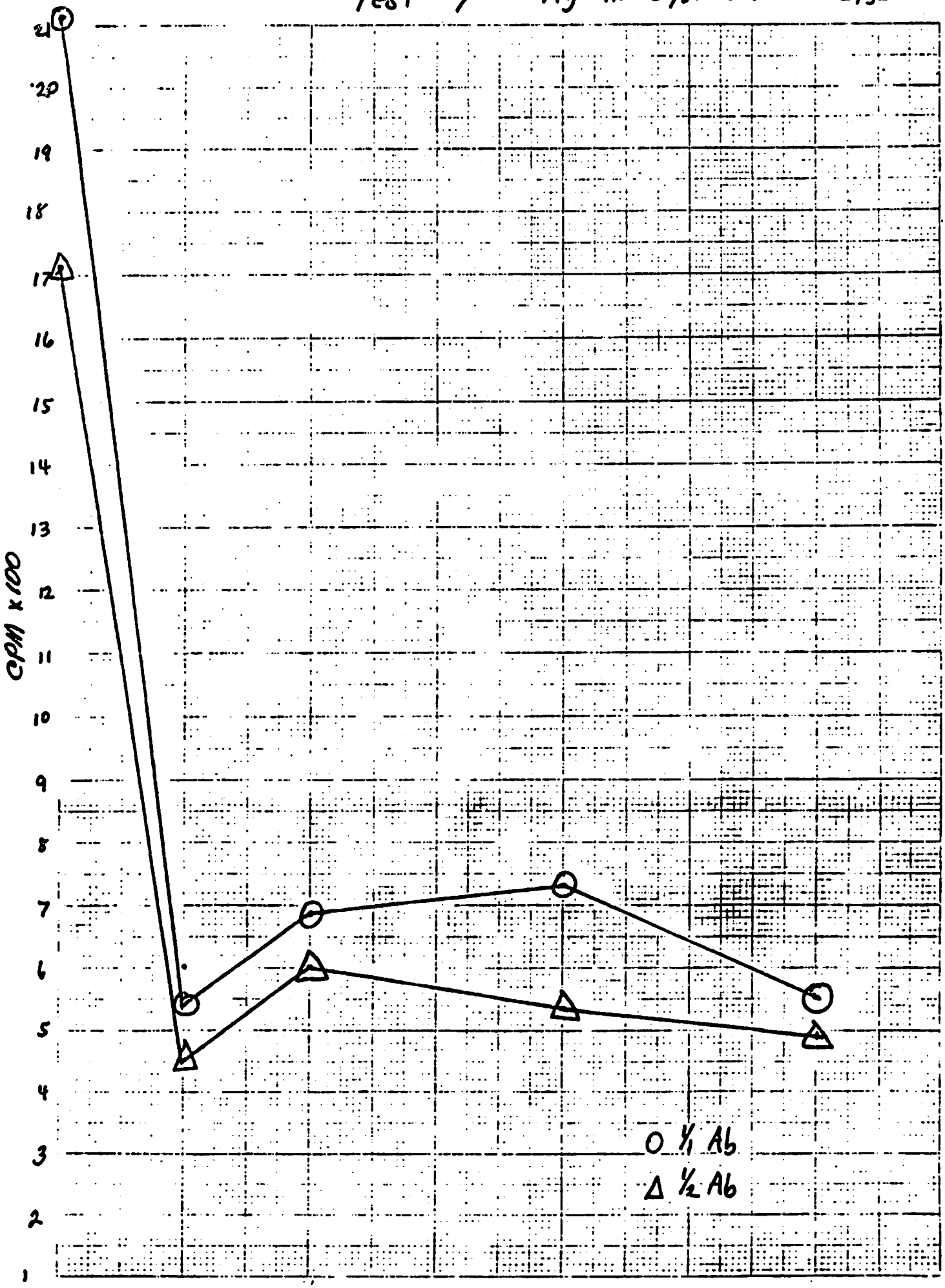
TEST ●



Test / Ag in PBS



Test 7 Ag in Oyster Tissue



DILUTIONS

This procedure works well with phosphate buffered saline (PBS) dilution of HAV. Results involving tests of seeded oyster extracts indicate limited virus recovery. The Gerba procedure for the extraction of enteroviruses was employed in an attempt to extract HAV from oyster tissue. At present we can detect only limited differences in the counts of control oysters and test oysters. We believe that the virus adsorbs to the oyster tissue and we are presently devising a method for its detection. This procedure is basically as follows:

1. Seed test oyster with HAV. Seed control oyster with PBS.
2. Incubate with unlabeled anti HAV.
3. Test the supernatant for its ability to inhibit the binding amount of anti HAV$^{I^{125}}$ in the Abbott procedure.

A high bead count indicates that the unlabeled antibody was bound to the virus in oyster tissue and was not present to compete with the labeled Ab for the bead. Thus, a high count indicates a large amount of virus in the sample. The converse is true.

VIRAL EVALUATION OF PROHIBITED OYSTER GROWING WATERS

PART V. CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

Relationship to project objectives:

1. Natural viral contamination occurs in oysters taken from both approved and prohibited shellfish growing waters when such waters are contaminated with human sewage. It is not correct to designate an approved reef as a "control", since its virological background is usually not known.

2. Virus isolations from oysters are consistent with the enteric virus group shed in feces. Polioviruses were by far the most frequently isolated group. This finding is not unusual since vaccination against this disease agent is commonly practiced.

3. Hepatitis antigens can be recovered from oyster tissue. The sensitivity of the test systems must be increased.

4. Bacterial indicators of the quality of shellfish growing waters or shellfish tissues are not capable of specifying the degree of viral contamination of shellfish taken from approved or prohibited oysters.

5. Virus isolations from shellfish are not consistent and are not capable of indicating the presence of bacterial pathogens.

6. Virological studies of the approved areas show the potential for consistent isolations. This should allow a virus classification procedure with a known background count.

7. The fluctuations observed in temperature and salinity measurements did not correlate with any biological parameter.

Recommendations:

1. Shellfish "controls" should be required. Specifically, shellfish from both prohibited and approved areas should be depurated for 14-21 days and sampled in lots consistent with the procedures of contaminated shellfish.

2. Suitable procedures to concentrate hepatitis A virus from shellfish tissues must be developed.

3. More attention should be paid to the extent of viral contamination of "approved" shellfish harvesting sites. These sites are more likely to be implicated in epidemics of shellfish-related disease and seem to have the greatest potential for virological reef classification.

4. Efforts should be made to determine the length of time that viruses survive in both approved and prohibited oysters.

5. Enterovirus extraction procedures should be standardized in a coordinated study with researchers in various locations of the United States.

6. Construct a mathematical model designed to monitor the level of fecal pollution entering an estuarine system and incorporate hydrographic data, especially the effect of dilution, to attempt to correlate physical and biological measurements.

7. More attention should be paid to artifacts in concentrate assays which produce "plaques". The confirmation of "plaques" is very time consuming.

VIRAL EVALUATION OF PROHIBITED OYSTER GROWING WATERS

PART VI. SUPPLEMENTAL INFORMATION

A. Users

1. Researchers whose interest is shellfish virology.

2. State and federal agencies responsible for classification of oyster-growing waters.

3. Local people interested in improving the quality of their product; for example, Mr. Gollott and his new method to relay polluted oysters.

B. Dissemination and Publication of Results

1. Yearly seminar to interested individuals along the Gulf Coast.

2. Paper presented at the annual meeting of the American Society for Microbiology, Los Angeles, 1979.

3. Local presentations (1978 & 1979) at the Mississippi Academy of Sciences.

4. Publication (Journal of Food Protection, 43:105-110, 1980) entitled "Natural Enterovirus and Fecal Coliform Contamination of Gulf Coast Oysters."

5. A separate manuscript which details the results of 1979 and discusses the trends observed during the two-year period is being prepared for publication.

C. Tabulations

1. Number of field exercises: 144

2. Number of person hours expended: 1600

3. Dollars encumbered (1978, 1979) = $57,702

4. Number of publications: 3
   Number of manuscripts being prepared: 1
   Number of local presentations: 2
   Number of national presentations: 1

5. Cooperative efforts with other agencies:
   a) National Marine Fisheries supplied funds for radioimmunological studies.

6. Students receiving degrees sponsored by Sea Grant:
   a) Janet B. Mapp, M.S.; Graduated August, 1979. Thesis Title: A Study to Evaluate Experimental and Natural Enterovirus Levels in Mississippi Oysters.

   b) V. L. Sheladia; Ph.D.; Graduated August, 1980. Dissertation Title: Extraction and Analysis of Enteroviruses from Oxidation Ponds, Shellfish and Sediment Samples

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Natural Enterovirus and Fecal Coliform Contamination of Gulf Coast Oysters

R. D. ELLENDER[1]*, J. B. MAPP[1], B. L. MIDDLEBROOKS[1], D. W. COOK[2] and E. W. CAKE[2]

Department of Microbiology, University of Southern Mississippi, Hattiesburg, Mississippi 39401 and Gulf Coast Research Laboratory, Ocean Springs, Mississippi

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ABSTRACT

The numbers of fecal coliforms and enteroviruses present in oysters and/or their growing waters of two Mississippi reefs were determined over a 12-month period. Bacterial and viral levels reflected the classification of the waters at each location as set by the Mississippi State Board of Health in compliance with the National Shellfish Sanitation Program, but statistically significant correlations between these levels were not observed. Twelve viral isolates were found at an approved oyster harvesting location, eight of which were identified as poliovirus type 1. At the prohibited site, 146 viruses were isolated including poliovirus types 1 and 2, echovirus type 24 and several isolates which remain to be identified. The number of virus isolates from samples from each location represented approximately 35% of the number of plaques observed; however, no consistent ratio of plaque to confirmed virus was demonstrated. The results suggest that the fecal coliform levels in oyster growing waters do not reflect the level of virus contaminaton in either approved or prohibited waters.

The extent to which shellfish growing waters have been polluted by fecal material has been clearly documented in this century by the frequent closure of productive reefs. Since the early 1900's, coliform bacteria have been used to gauge the degree of fecal pollution of water, including marine waters (*25*). Methods for detecting pathogenic bacteria in shellfish or their growing waters are available although the time and expense required may preclude their use in favor of indicator bacteria. In the last 15 years, shellfish viral contaminants have attracted more and more interest as evidenced by the increased number of technical papers dealing with this problem. Compared to bacteria, viruses are not as easily detected nor are the consequences of their presence always understood.

The presence of viruses in shellfish has been documented (*2,8,9,10,17,32,35,36,43*) and usually includes those groups with direct or indirect association with the alimentary tract of man or other homiothermic animals, and whose characteristics permit survival and transmission by feces. The enteroviruses, reoviruses, adenoviruses and hepatitis A virus are considered prime candidates for shellfish contamination. A more complete list and a consideration of the ecological and epidemiological significance of other possible viral contaminants was provided by Carrick and Sobsey (*3*).

[1]*University of Southern Mississippi.*
[2]*Gulf Coast Research Laboratory.*

Viral epidemics attributed to shellfish ingestion most frequently involve a hepatitis virus (*27,31,33,37,38, 40-42*), usually type A. Some evidence for hepatitis B virus transmission by feces or infected shellfish has been reported (*5,15,32*). At present there is no standard technique for isolating and quantifying hepatitis viruses in feces or shellfish, although several proposed methods of fecal detection are under investigation (*14,16,24,32*). The enteric viruses are more easily isolated by routine virological procedures and could perhaps serve as indicators of viral contamination of shellfish. They are important in that they can produce either acute or chronic disease, but most human infections probably remain subclinical. In certain instances, such syndromes as aseptic meningitis, paralysis, herpangina, pleurodynia, myocarditis, skin rash and coryza may occur. In view of the multitude of problems associated with enteric viral infection, it is surprising that so little information exists which supports or negates the importance of polluted shellfish in the transmission of enteric viral diseases.

Methods for detection of viruses in shellfish usually involve the assay of entire shellfish rather than dissection procedures which are designed to isolate infectious particles associated with the feeding, digestive and excretory systems. Viruses that enter oysters from the surrounding water do not reproduce and are often found in the digestive gland (*4,11*). It is possible for viruses to adhere to shellfish due to the charge differences between virus particles and mucous surfaces (*12*). The effect of bioaccumulation by these mechanisms permits viruses to be concentrated from the growing water at least by a factor of 60 (*36*).

Methods for recovery of viruses from shellfish may or may not employ concentration steps. Procedures that do not involve concentration steps are discussed in references 2, 11, 34 and 35, but are not applicable to the analysis of large quantities of shellfish tissue or are unlikely to demonstrate low level contamination. Recent investigations (*17,22,23,28,-30,45-47*) provided data that may reflect the level of contamination in shellfish tissue. Of the methods reported in those investigations, the Sobsey method (*47*) and subsequent revisions (*45,46*) are most often used and have provided the most consistent results.

In Mississippi, coastal estuaries receive the effluents of sewage disposal facilities. Those effluents have been

extremely detrimental to the once viable shellfish industry. This report details the results of a study designed to isolate, enumerate and identify viruses from oysters collected from both approved and prohibited shellfish growing areas. Comparative fecal coliform analyses were also performed.

MATERIALS AND METHODS

Sampling procedures

Water and oyster (*Crassostrea virginica*) samples were collected from approved (Pass Christian reef) and prohibited (Graveline Bayou) shellfish growing areas (Fig. 1) from January through December, 1978. Table 1 outlines the types and numbers of samples taken. Samples were collected monthly, with the exception of oysters from Graveline Bayou for virological analysis, which were collected twice monthly. Surface (upper 0.25 m) water samples were collected in sterile wide-mouth jars while bottom samples were collected in a sterile bottle with the aid of a J-Z sampler. Water samples were collected at each of three locations in Graveline Bayou for 3 days before sampling the oysters and on the actual day of oyster sampling. Water samples were taken at three locations on the Pass Christian reef only on the day of oyster sampling. Oysters (3-5 inches long) were harvested with a hand dredge, culled and placed in an insulated box for shipment. All samples were kept at 4 C until processed.

Surface water temperatures were measured in situ with a mercury-in-glass thermometer. Salinity measurements were made on a portion of the water samples collected for bacteriological analysis using an AO Goldberg refractometer (No. 10402). Temperature and salinity data are expressed as averages of three replicate measurements.

Samples analysis

Fecal coliform analyses of water and oyster samples were conducted by methods previously described (*39*). Analyses were normally begun within 3 h after collection.

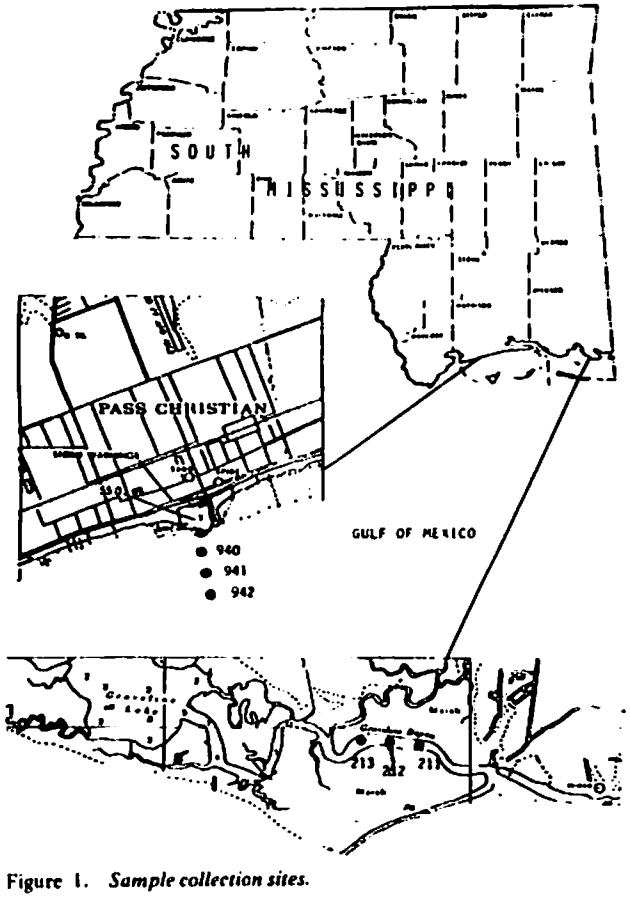

Figure 1. *Sample collection sites.*

TABLE 1. *Nature of samples.*

Location	Type sample	Purpose	Number of samples	Quantity
Pass Christian Reef	Surface water	FC[a]	3	100 ml
	Bottom water	FC	3	100 ml
	Oysters	FC	2	200 ml
	Oysters	V[b]	4	150 ml
Graveline Bayou	Surface water	FC	12	100 ml
	Bottom water	FC	12	100 ml
	Oysters	FC	2	200 g
	Oysters	V	6-7	150 g

[a]Fecal coliform analysis.
[b]Virological analysis.

Oysters (150-g lots) were extracted to determine virus concentration using a modification of the Sobsey procedure (*46*) as shown in Fig. 2.

Tissue culture assay

The Buffalo green monkey kidney cell line (BGM) (*1,6*) was used to analyze all oyster concentrates. Virus samples and/or dilutions (0.2 to 0.5 ml per 25 cm^2 plastic flask) were inoculated onto BGM monolayers (passages 100 to 120) which were incubated for 1 h at 37 C using a rocking apparatus (Bellco) at five rotations per minute. Growth medium for BGM cells consisted of MEM:L15 (1:1), 10% fetal calf serum and 1% L-glutamine (all purchased from Grand Island Biological Company).

Samples were quantitatively assayed by a modification of the plaque method reported by Dahling et al. (*6*). Plaque counts were made on a daily basis for 5 days or until no new plaques appeared for two consecutive days.

Plaque identification

Individual plaques were picked when they were ≥ 1 mm in diameter. A Pasteur pipette, with a bent tip, moistened with 0.05 ml of growth medium was used to transfer an agar plug (area of plaque) to a holding medium (1 ml MEM per tube). Samples were passaged three times in BGM cells with a minimum of one filtration step (0.45 μm). Two blind passages were made of all samples not producing observable cytopathic effect. Plaques identified as viruses were titered and identified serologically (*21*).

Statistics

Bacterial and viral counts in water and oyster samples were subjected to a square root transformation before calculation of linear correlation coefficients (*48*).

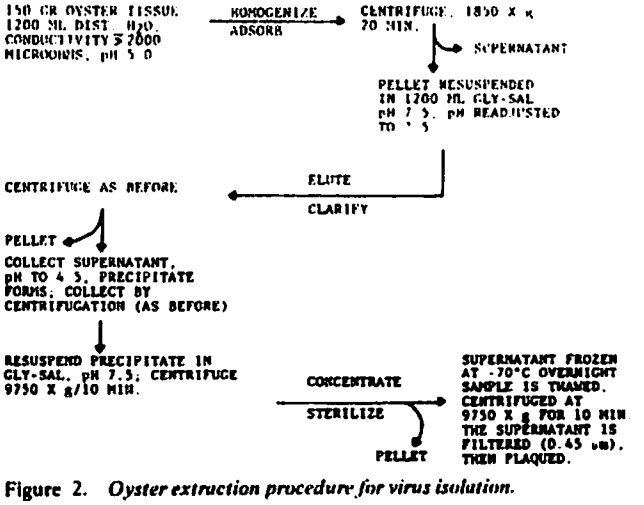

Figure 2. *Oyster extraction procedure for virus isolation.*

RESULTS

This investigation was an extension of a 1976-1977 study designed to compare the viral isolation efficiency of two oyster extraction methods (*13*). The Sobsey

procedure (*47*) was not satisfactory for examination of natural oyster samples and was modified by elimination of the intermediate filtration step and by substitution of the diluent for suspension of the final precipitate. The procedure described in Fig. 2 was tested with seeded oyster samples and yielded recoveries ranging from 61 to 73% (63% average). Oyster concentrates were clear and easy to filter-sterilize before assay. Bacterial and fungal contamination was minimal and individual flasks contained no particulate matter which interfered with visual analysis.

Table 2 presents the results of fecal coliform analyses of water and oyster samples performed during 1978. It is evident that the two areas differ significantly in bacteriological quality. The median fecal coliform MPN for all 71 water samples from the Pass Christian reef was less than 2 per 100 ml with only 8.45% of the samples exceedin an MPN of 43. The Graveline Bayou samples had a median fecal coliform MPN of 23 per 100 ml with 39.9% of the 276 samples exceeding of MPN of 43. These results confirm the approved and prohibited status of Pass Christian reef and Graveline Bayou, respectively.

TABLE 2. *Fecal coliform analysis of water and oyster samples.*

	Pass Christian Reef		Graveline Bayou	
	Water	Oyster	Water	Oyster
Month	Median fecal coliform (MPN/100 ml)	Mean fecal coliform (MPN/100 g)	Median fecal coliform (MPN/100 g)	Mean fecal coliform (MPN/100 g)
January	1400.0	73	1200.0	310.0
February	< 2.0	45	17.0	104.0
March	< 2.0	125	7.8	3,250.0
April	< 2.0	< 20	11.0	765.0
May	< 2.0	< 20	13.0	170.0
June	2.0	61	11.0	44.0
July	3.3	400	130.0	815.0
August	2.0	360	25.0	17,650.0
September	3.3	12,450	28.0	745.0
October	< 2.0	< 20	49.0	230.0
November	17.0	330	170.0	945.0
December	< 2.0	204	22.5	715.0

The fecal coliform counts from oysters (Table 2) also reflected the difference in water quality in those two areas. The median values for all samples taken from Graveline Bayou and Pass Christian were 410 and 78, respectively. There appears to be no apparent relationship between the coliform counts in the water and those in the oysters collected at the same time.

Viral isolates from approved (Table 3) and prohibited (Table 4) oyster samples also reflect the degree of fecal pollution at the two locations examined. Thirty-eight plaque-like isolates from Pass Christian were purified during the 12-month period. Of the 12 plaques confirmed as viruses, eight were identified as poliovirus type 1; four could not be typed. Most of the isolates were picked from April samples but these data could not be correlated with fecal coliform counts of that month.

In comparison, plaque-like isolates from oysters collected from Graveline Bayou totalled 416. Of this number, 146 or 35%, were identified as viruses. Of 55 random isolates identified, 50 were polio type 1, one was polio type 2, one was echovirus type 24 and three were unidentifiable by the procedures employed. Plaque-like isolates were not evenly distributed among the samples for a given month. The percent of positive 150-g samples (Table 4) ranged from 10 to 55%. In July, one of 10 samples contained virus as compared to five of nine samples in October.

TABLE 3. *Viral analysis of approved oysters. Pass Christian Reef.*

Month	# Oysters/ # samples	*	# Plaque-like isolates Total/100 g	# Plaques identified as viruses Total/100 g
Jan.	24/2	0	2/0.6	0/0
Feb.	32/2	0	4/1.3	0/0
Mar.	24/2	0	6/2.0	0/0
Apr.	30/2	1	19/6.3	11/3.6
May	21/1	0	0/0	0/0
June	38/2	0	0/0	0/0
July	32/2	0	0/0	0/0
Aug.	36/2	0	2/0.6	0/0
Sept.	33/2	0	0/0	0/0
Oct.	26/2	1	2/0.6	1 0.3
Nov.	21/1	0	0/0	0/0
Dec.	33/2	0	3/1.0	0/0
TOTAL	350/22		38	12

*Number of samples containing confirmed virus isolates.

TABLE 4. *Viral analysis of prohibited oysters. Graveline Bayou.*

Month	# Oysters/ # Samples	*	# Plaque-like isolates Total/100 g	# Plaques identified as viruses Total/100 g
Jan.	139/10	5	88/5.8	31/2.2
Feb.	65/5	1	38/5.0	5/0.7
Mar.	53/3	1	18/3.0	3/0.5
Apr.	56/3	1	5/1.1	3/0.7
May	102/6	3	51/5.6	18/2.0
June	200/13	3	38/1.9	12/0.6
July	180/10	1	44/2.9	23/1.5
Aug.	89/7	3	39/5.0	11/1.4
Sept.	191/10	5	46/2.7	26/1.5
Oct.	129/9	5	15/1.1	10/0.7
Nov.	88/8	1	9/0.8	3/0.3
Dec.	35/3	1	25/5.5	1/0.2
TOTAL	1327/87		416	146

*Number of samples containing confirmed virus isolates.

Generally, in both areas bottom water salinities were higher than surface salinities (Tables 5 and 6). The salinities of the waters over the Pass Christian reef remained fairly constant during the year except for two periods following heavy rainfall when the salinities were significantly reduced. The water salinities in Graveline Bayou ranged from a low of 2.0 to a high of 28 ppt. On one occasion, the salinity fluctuated as much as 15 ppt over the 4-day sampling period.

Temperatures of surface waters at the approved reef ranged from 8 C in January to 31 C in June. The same general trend in temperature fluctuation was observed at Graveline Bayou.

Correlation coefficients which compared fecal coliforms in surface and bottom waters, fecal coliforms in oyster tissue and plaque-like and actual virus isolates from oyster samples are presented in Table 7. A significant ($P \leq 0.1$) positive correlation was found between fecal coliforms in bottom and surface waters but not between those and fecal coliforms in oyster tissue.

TABLE 5. *Physical data on water samples from Pass Christian reef.*

Date	Surface Temperature[a] (C)	Surface Salinity[a] (ppt)	Bottom Salinity[a] (ppt)[b]
1/27/78	8.0	2.0	5.5
2/28/78	14.0	16.0	19.6
3/30/78	19.0	14.6	15.6
4/27/78	21.0	14.6	15.0
5/31/78	30.0	7.0	8.3
6/28/78	31.0	13.8	14.6
7/3/78	28.0	18.0	18.0
8/30/78	29.0	14.6	18.6
9/28/78	27.0	17.3	17.3
11/2/78	23.0	18.0	18.3
11/30/78	17.0	17.6	18.3
12/14/78	11.0	15.3	20.0

[a]An average of three measurements.
[b]Parts per thousand.

Significant correlations between fecal coliforms in waters or oysters and plaque-like or virus isolates in oysters were not observed. Although the number of confirmed viral isolates began as plaque-like isolates, no correlation could be found to indicate a relationship on a month-to-month basis.

When the results of fecal coliform and virus analyses of Graveline Bayou oysters are graphically compared (Fig. 3), they reflect the lack of statistical correlation. The variations observed in three particular months are of interest. In March, the number of fecal coliforms rose while the number of plaque-like and confirmed virus isolates decreased or remained constant. In May, the number of plaque-like and confirmed virus isolates increased, but did not correlate with the decreasing number of fecal coliforms. The numbers of both fecal coliforms and plaque-like isolates increased in August, but the number of confirmed virus isolates remained at approximately the level of the previous month.

DISCUSSION

The two locations chosen for this study were selected because previous observations had shown that each was ecologically, topographically and bacteriologically distinct. The Pass Christian reef lies in open waters of the Mississippi Sound and is not readily influenced by rapid environmental changes. Conversely, Graveline Bayou is greatly influenced by local rainfall and tidal flushing and may change rapidly within the short time period. The maximum sampling effort was expended at this location primarily to increase the probability of virus recoveries. Oyster harvesting has not been permitted in Graveline Bayou since 1975. The bayou begins at the Mississippi Sound and runs 4389 m to Lake Graveline (95 hectares). Bayou depth varies from 0.6-3.7 m. The average sill depth at the bayou's mouth is 15 cm at mean low tide. Several sources of sewage pollution contribute to the closure of Graveline Bayou to shellfishing harvesting: Del Flore treatment plant, 1097 m east of the bayou's mouth and the Gautier Point treatment facility, 3474 m east of Graveline. Septic tanks near the lake and new housing development near the bayou's mouth also

TABLE 7. *Correlation coefficients. Graveline Bayou isolates.*

	FCWS	FCWB	FCO	PLI	VI
FCWS	1.000				
FCWB	0.748**	1.000			
FCO	0.052	0.414	1.000		
PLI	0.079	0.069	0.191	1.000	
VI	0.405	0.446	0.290	0.455	1.000

**$P < 0.1$ level of significance
FCWS, FCWB, FCO, PLI, VI represent fecal coliform water surface, MPN 100 ml; fecal coliform water bottom, MPN 100 ml; fecal coliform oyster, MPN 100 gr^{-1}; plaque-like isolates, 100 gr^{-1}; viruses identified 100 gr^{-1}.

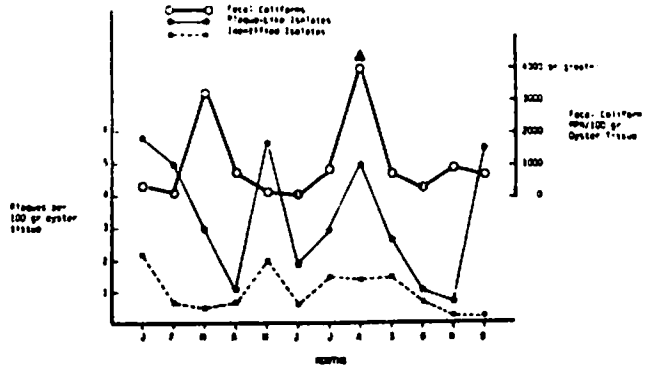

Figure 3. *Fecal coliform, plaque-like isolates and confirmed virus levels per 100 g of oyster tissue. Samples collected in Graveline Bayou, 1978.*

TABLE 6. *Physical data on Graveline Bayou water samples.*

Dates[a]	Surface Temperature[b] (C)	Surface Salinity[b] (ppt)[c]	Bottom Salinity[b] (ppt)[c]
1/24/78 to 1/27/78	9.0 to 12.5	2.0 to 7.0	2.0 to 10.3
2/25/78 to 2/28/78	11.0 to 14.5	6.0 to 12.0	10.3 to 17.6
3/28/78 to 3/31/78	17.5 to 19.5	11.6 to 21.3	17.0 to 22.0
4/25/78 to 4/28/78	19.0 to 23.5	14.6 to 19.6	15.0 to 19.6
5/28/78 to 5/31/78	27.0 to 30.0	4.0 to 5.8	4.8 to 6.0
6/25/78 to 6/28/78	29.0 to 32.0	8.5 to 17.3	10.0 to 17.3
7/31/78 to 8/3/78	28.0 to 32.0	6.2 to 18.6	7.5 to 22.0
8/27/78 to 8/30/78	27.0 to 30.0	14.0 to 16.0	14.0 to 16.0
9/25/78 to 9/28/78	27.0 to 29.0	16.0 to 16.6	16.0 to 16.6
10/30/78 to 11/2/78	21.0 to 23.0	24.0 to 25.6	24.0 to 25.6
11/27/78 to 11/30/78	17.0 to 20.0	21.0 to 22.0	21.0 to 22.0
12/11/78 to 12/14/78	8.0 to 12.0	20.0 to 28.0	22.0 to 28.0

[a]Represents the last 4 days of each month.
[b]An average of three measurements.
[c]Parts per thousand.

contribute sewage. From the sound, sewage enters Graveline by the westerly current drift in conjunction with tidal currents and prevailing southeast winds.

In our literature survey, we could find few previous studies which analyzed the virological content of oysters over an extended period. The lack of such studies is surprising when one considers the inability of the coliform standard to adequately predict increased viral contamination and the possible epidemiological consequences. Undoubtedly hepatitis outbreaks transmitted by shellfish are well documented (*18*), but similar occurrences of enterovirus disease transmission are by their very nature difficult to identify. The ingestion of raw or incompletely cooked oysters presents the potential for enterovirus transmission and the isolation of these viruses from shellfish has been observed on more than one occasion (*17,21,34,35*).

The modifications of the Sobsey extraction procedure, which have been made in our laboratory over the last 3 years, resulted from a desire to process naturally polluted oyster samples. During 1978, a wide seasonal variation of environmental parameters occurred, but no significant changes in the procedure were required. Most problems usually occurred when the final precipitate was suspended. Heavy contamination and/or inability to filter the concentrate before assay demanded the most attention. The more turbid the final concentrate, the more likely that the plaque assay would be adversely affected. When the concentrate was frozen, then thawed, centrifuged and filtered, less than 0.1% of the virus was lost to the precipitate and the filter.

Fecal coliform levels are used to verify the classification of shellfish-growing waters. The present classification system does, in general, protect the public from diseases transmitted by shellfish, but it is by no means considered infallible, especially in regard to the level of viral contamination (*17,20,21*). The problem is compounded by the lack of valid correlations between fecal coliform levels in waters and oyster samples taken simultaneously at the same site (Table 2). One factor which could account for that lack of correlation is salinity which on occasion varied as much as 15 ppt during the 4-day sampling period. Similar fluctuations were noted in the fecal coliform counts in water which in one instance changed by 2 logs in one 3-day period.

The expected rise and fall of surface water temperatures during the year did not correspond to the fluctuation of either fecal coliform or virus levels. At the Pass Christian reef, the highest recorded values for temperature, fecal coliform and virus counts occurred in the months of June, January and April, respectively. The same parameters recorded at Graveline Bayou corresponded to the months of August and January. Although our studies and those of other investigators (*21*) do not indicate correlations between salinity and temperature versus fecal coliforms in water and oysters and virus in oyster, fluctuation of those parameters would affect indicator ratios and could produce significant variation in the data used for sanitary surveys.

The plaque procedure used contributed to the problems of sample assay. For all samples examined, over 60% of all plaques were not of viral origin. This discrepancy could be due to artifacts in the flasks, limited chemical or biological contamination or failure of the isolate to replicate in the BGM cell line. This complication can be avoided using an all-or-none quantal assay in addition to the plaque assay. Studies of minimal viral contamination of oysters that compare all or none versus plaque assay methods would more clearly define the most appropriate method.

Most of the purified viral isolates (85%) was identified as poliovirus type 1. This observation is not unusual (*21*) and probably reflects the wide-spread distribution of oral polio vaccine. What is surprising is the very low numbers of other polio types observed. Perhaps environmental factors or certain aspects of the oyster extraction procedure contributed to the failure to detect viruses that are shed by the fecal route. Although Katzenelson and Kedmi (*26*) did not express this particular concern, they did suggest that additional research be done to develop a cell system with greater potential for multiple-virus assay.

These data again emphasize the need to re-evaluate the use of the coliform standard for verification of shellfish growing waters. As the fecal coliform standard is routinely used, consideration for keeping the total coliform standard should be given since Goyal et al. (*21*) have demonstrated a relationship between viruses in estuarine water and the total coliform counts in water and oysters. The relationships of viruses in shellfish to viruses in estuarine sediments should be defined since recent studies (*7,19,44,49*) confirmed that sediments can contribute large numbers of viruses to the water column and possibly to feeding shellfish.

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