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Monitoring Chesapeake Bay Shellfish For Human Enteroviruses

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Sea Grant

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PREFACE

Since the Chesapeake Bay provides a major harvesting ground for edible shellfish, it is important to know how rising threats of pollution and contamination affect the commercial bars that supply us with these shellfish. This study focuses on oysters, the most harvested molluscan shellfish of the Chesapeake, using samples from a number of commercial bars in the Maryland portion of the Bay to look for possible contamination by enteroviruses. Since enteroviruses have been found elsewhere in waters tested to be free of bacteria, checks for bacteria can not be taken as accurate indicators for the presence of enteroviruses; other tests must be used.

To find an accurate test, the researchers infected a control sample of oysters with known enteroviruses, tested several available laboratory techniques, and documented the ability of the Sobsey technique to detect viruses in oyster tissue. They next collected and tested oysters from 11 commercially harvested bars and from one bar closed by the Maryland Health Department because of potential sewage pollution. While the Sobsey technique recovered 56% of the virus planted in the control group, the same test yielded no enteroviruses in Chesapeake Bay oysters, even in those taken from closed bars. It should be noted that none of the shellfish came from waters heavily polluted with raw domestic sewage.

Other studies have found members of the polio, ECHO, and coxsackie virus groups in estuarine and marine waters, and some have isolated enteroviruses in oysters from contaminated waters. Further studies should reveal whether viral contamination relates to bacterial contamination and whether

virological standards should supplement current bacteriological standards to produce more accurate biological indicators of pollution in shellfish growing waters.

On the basis of the current study, however, it seems safe to say that there is no current problem of enterovirus contamination in commercially harvested oysters from the Maryland portion of the Chesapeake Bay.

INTRODUCTION

Shellfish have long been known to take up and accumulate viruses present in polluted waters (5,6, 11), and epidemiological evidence is readily available on the transmission of hepatitis via consumption of contaminated oysters and clams (9,13,14). Enterovirus-contaminated shellfish have also been suspected of posing a public health threat, as many of these viruses are quite stable in the estuarine environment (7). Laboratory experiments have also demonstrated that, in addition to its remaining stable in the environment, poliomyelitis virus can withstand the normal cooking processes used in preparing oysters for human consumption (3).

Documentation has been lacking on the transmission of human disease via enterovirus contaminated shellfish, but this lack of information is probably due to the nature of the enterovirus infection. Many enterovirus infections are subclinical in nature, though infected individuals could transmit the virus to secondary contacts who might develop overt disease. Such cases would rarely, if ever, be traced to a primary infection which may have been shellfish associated. Further, the clinical symptomology of many enteroviral infections is quite nebulous. It may include intestinal disorders, aseptic meningitis, undifferentiated febrile illnesses, respiratory involvement, and rash-like diseases. Individuals developing these syndromes would be unlikely to associate them with eating contaminated food. And since many of these illnesses are of the nonreportable variety, there is no good indicator as to their incidence. For these reasons, retrospective epidemiological studies on this virus group are very difficult to carry out. If one is to examine the potential health hazard of enterovirus infection from shellfish,

one must examine the shellfish directly for enterovirus content. Only recently has the technology and methodology become available to efficiently detect low levels of virus in shellfish.

Although state health officials routinely monitor shellfish growing waters in Maryland for bacteriological and chemical indicators of pollution and restrict commercial harvesting when levels of the indicators pose potential health hazards to the consumer, there is currently no routine monitoring of shellfish for virus contamination. Outbreaks of hepatitis traced to consumption of shellfish harvested from "bacteriologically safe" waters have caused growing concern throughout the country that the virological and bacteriological indicators of human fecal pollution are not always parallel (13).

This project was initiated to evaluate a basic public health question: Do shellfish harvested from the Maryland waters of the Chesapeake Bay contain enterovirus with such frequency as would pose a health threat to the consumer?

METHODS

Sampling

Since oysters represent the largest molluscan shellfish industry of the Chesapeake Bay, and could be readily obtained from many locations throughout the Bay, they were selected as the shellfish to be monitored for enterovirus content. Routine monthly samplings were taken at twelve oyster bars during the twelve-month period of April, 1977, through

March, 1978 (See Figure 1). Waters throughout the sampling area varied in temperature from 0 to 52 degrees centigrade during the year's monitoring program, and the salinities varied from 6 to 17 parts per thousand (ppt). The sample sites, chosen in consultation with officials of the Maryland State Health Department, represented a variety of water qualities. All oyster bars sampled were open to commercial shellfish harvesting, with the exception of one site, closed because of its proximity to a pollution source (See Table 1). Three of the oyster bars sampled--Bald Eagle, Cook's Point and Norman--were located in some of the cleanest waters of the Chesapeake Bay, as determined by health department monitoring programs. Tolly Point, Herring Bay, and Double Mills, though commercially harvested oyster bars, are located along the edge of pollution lines. Green Marsh, the oyster bar restricted from commercial harvesting, is inside the pollution line, and is approximately 0.25 miles downstream from a municipal waste treatment facility. Though Chinks Point and States Bank--two bars located directly under sewage treatment plant outfalls--were sampled, they could not be tested on a routine monthly basis and are not included among the twelve sampling sites. The other sites were checked regularly, except during January and February of 1978, when ice and severe weather conditions prevented the sampling of some bars.

Oyster samples were collected by dredge on monthly Bay-wide cruises of the University of Maryland research vessel, AQUARIUS, and the Johns Hopkins University research vessel, RIDGELY WARFIELD. A few supplementary samples were collected by hand dredge by the State Health Department and the University of Maryland Horn Point Environmental Lab (HPEL) staff. Once samples were collected, they were kept on ice until transported to the lab. If specimens could not be processed immediately, oysters were stored dry at $4^{o}C$, or were

shucked and stored at -30°C.

Processing oysters for virus assay

Several techniques for enterovirus isolation from oysters were tested in our lab for their efficiency in recovering viruses from artificially contaminated shellfish. Selected as the most suitable was the procedure developed by Sobsey et al. (15). Their method is summarized in Figure 2 and described in the following paragraphs.

Each shellfish sample, which consisted of a pool of three harvestable size oysters, was scrubbed thoroughly in tap water to remove all fouling organisms, then surface-disinfected by dipping in 70% ethanol for one minute. The oysters were then shucked, diluted approximately 1:7 (wt/vol) with distilled water, and homogenized for one minute in a Waring Blender. The salinity of the homogenate was reduced to 1.5 ppt or less, by the addition of distilled water, and the pH of the mixture was adjusted to 5.5 with 1 N HCl. The oyster solids were separated from the mixture by centrifugation at 1,900 x g for 10 minutes. Following centrifugation, the supernatant was discarded and the pellet resuspended in 150 mls of a 0.05M glycine buffer, and the mixture adjusted to a pH of 5.5. The solids were again separated by low speed centrifugation, after which they were washed with 150 mls of 0.05M glycine buffered saline, pH 3.5. The pH of the resulting solids-buffer mixture was approximately 4.5 but was lowered to pH 3.7 by the addition of 1 N HCl. After the mixture was stirred for 15 minutes, the oyster solids were removed by centrifugation at 1,900 x g and discarded. The supernatant was adjusted to pH 7.5 with 1 N NaOH and clarified by filtering through a series of 4 Millipore filters of successively decreasing pore sizes, ending with a 0.22u filter.

Ultrafiltration of the clarified liquid was carried out to reduce the volume of fluid to be assayed for virus content. The liquid, approximately 150 mls, was poured into an Amicon Model 202 Ultrafiltration Cell, fitted with a PM 30 Diaflo membrane. The liquid was concentrated at 30 lbs/in^2 to a final volume of approximately 8 mls. After releasing the pressure from the cell, heat-inactivated fetal bovine serum was added to give a final concentration of 20% serum. The mixture was stirred an additional 15 minutes to elute any virus which may have adsorbed to the membrane. Following this period, the concentrate was aseptically removed from the cell, and the unit was rinsed with a small amount of glycine-buffered saline, which was subsequently added to the concentrate.

Virus assay of processed oyster concentrate

The concentrate resulting from the original pool of oysters processed by the Sobsey technique was assayed for enterovirus directly on cell cultures. A continuous monkey kidney cell line (BGM), known to be sensitive to many enteroviruses, was employed for the virus isolation attempts.

Confluent monolayers of BGM cells, grown in 25 cm2 plastic tissue culture flasks, were washed once with Hanks' Balanced Salt Solution (HBSS) and then inoculated with 0.5 ml concentrate per flask. Following a one-hour adsorption period at 37°C, 4.5 ml of Eagles Minimum Essential Medium with Earle's Salts (EMEM) were added to each flask. Uninoculated control flasks were included with each sample. In most cases, 90% of the total concentrate obtained from an oyster sample was used for virus assay; the remaining 10% was stored at -30°C for possible future reference.

Flasks were incubated at 37°C for seven days,

during which time the cells were examined microscopically for evidence of cytopathic effects (CPE). If no CPE was observed after one week, the cultures were blind-passaged one time to fresh BGM cells, which were incubated for another seven-day period. If no viral CPE was evident after that period, the sample was considered negative for enterovirus. Throughout the year's monitoring program, routine procedural checks of the Sobsey method were conducted by determining the percent of virus recovery from artificially contaminated oysters.

The Sobsey technique, then, uses buffers of different salinity and pH to effect alterations in the adsorption properties of the virus. The virus is adsorbed to the oyster solids throughout the initial wash steps, and then eluted from the oyster solids into the supernatant fluid by increasing the salinity of the buffer to 8.5 ppt and decreasing the pH to 3.7. Following the removal of oyster solids and microbial contaminants by centrifugation and filtration, virus in the fluid is then concentrated by ultrafiltration. Efficiency of virus recovery was evaluated from artificially contaminated oysters. Control experiments were performed using a homogenate of three oysters inoculated with poliovirus type I to yield an initial titer of 10^4 PFU/ml. The fractions obtained at various steps during the procedure were sampled and assayed for virus content.

RESULTS

Although the test is effective, there was some loss of virus, especially during the discarding of the pellet (see Table 2). During the initial wash steps the low salt and acid conditions permit

adsorption of the virus particles to the oyster solids, and the supernatant wash fluid can be discarded without significant loss of virus; but loss of virus occurred during the elimination of the pellet, despite alterations of time or temperature. Also, the virus-infected supernatant resulting from the elution step was sometimes milky in appearance, and a precipitate frequently formed when the pH of the fluid was increased to 7.5, making the liquid difficult to filter. It became necessary to filter the fluid through several Millipore filters, which resulted in a loss of about 5% of the input virus.

The final ultrafiltration step proved to be very efficient. Initial tests of the unit alone, using clarified filtrate inoculated with poliovirus, yielded a final concentrate containing 96-100% of the input virus. As indicated in Table 2, the procedure used was found to have an overall recovery of 65% input virus.

Similar control experiments, using artificially contaminated oysters, were performed routinely throughout the year's monitoring program. The average recovery of virus from all such experiments was found to be 56%.

Control experiments to test the sensitivity of the methods used were also run throughout the year. Three shucked oysters were inoculated directly with a low level of virus, and then processed according to Sobsey's technique. The final concentrate obtained was assayed for virus on BGM cells in a manner identical to that used on actual oyster samples. Such experiments indicated that the methods used were sensitive enough to detect infectious virus from three oysters artificially contaminated with a total of three PFU polio viruses.

From April, 1977, through March, 1978, a total

of 143 oyster samples were collected and processed for enterovirus examination, as indicated in Table 3. The result: No virus was detected in any of the oyster samples examined for enterovirus. Of these samples, 120 came from oyster bars which are harvested commercially, while the remaining 23 were obtained from oyster bars closed to harvesting due to nearby sewage treatment outfalls. Some of the sample concentrates, most frequently those obtained from polluted oysters, were cytotoxic to the BGM cells used for virus assay, which may have been due to accumulation of chemical agents in the oyster tissue. This problem, when suspected, could be diminished by reducing the concentrate inoculum from 0.5 ml per BGM culture, to 0.25 or 0.1 ml per culture.

DISCUSSION

No virus isolates were made from any of the 143 oyster samples collected from the Bay and examined for enterovirus content during the period of April, 1977, through March, 1978. The apparent lack of enterovirus in the shellfish samples examined should have direct application to the shellfish industry in Maryland, since many published studies have reported pollution in other states, especially in the southern U.S. The presence of enterovirus in estuarine waters receiving human fecal pollution has been well documented, and isolation of polio, ECHO, coxsackie, and reoviruses from estuarine and marine waters (2,4,12), as well as from marine sediments (2), has been reported. Both field and laboratory studies have demonstrated that oysters growing in waters which contain enteroviruses will also exhibit enterovirus contamination.

Results of the oyster monitoring study indicate that there currently does not seem to be a problem of enterovirus contamination in commercially harvested oysters from the Maryland portion of the Chesapeake Bay. (Shellfish from the Virginia waters of the Bay were not evaluated in this project.) Oyster samples taken from waters closed to shellfish harvesting due to nearby sewage treatment plant outfalls were also negative for enterovirus contamination, although no shellfish samples were obtained from waters which are heavily polluted with raw domestic sewage.

The Sobsey technique used for examining the oysters for enterovirus had a minimum detection limit of three PFU polio viruses per batch of three oysters and yielded an average recovery of 56% of input virus in control experiments using oysters contaminated with 10^4 PFU poliovirus/ml. Reports of viral contamination in shellfish harvested from "bacteriologically safe" waters indicate a possible poor or inconsistent correlation between the bacterial and viral indicators of human fecal pollution (13).

Since enterovirus contamination of shellfish can present a potential health hazard to the shellfish consumer, it is important to ascertain the relationship between these two biological indicators of pollution and to determine the virological quality of commercially available shellfish. Preliminary experiments done in collaboration with Colwell and Austin (1), who examined oyster samples for both coliform and enterovirus content, indicated relatively low coliform counts, even in the oyster samples collected from oyster bars located under sewage treatment plant outfalls. Similar studies on other estuarine systems are currently underway, in which experiments have been designed

to evaluate the relationship between the coliform index and the enterovirus isolation as measures of fecal pollution (16). Once studies of this sort have been completed, intelligent decisions can be made as to whether bacteriological standards can remain as the sole biological indicator of pollution in shellfish growing waters, or whether additional virological standards should also be applied.

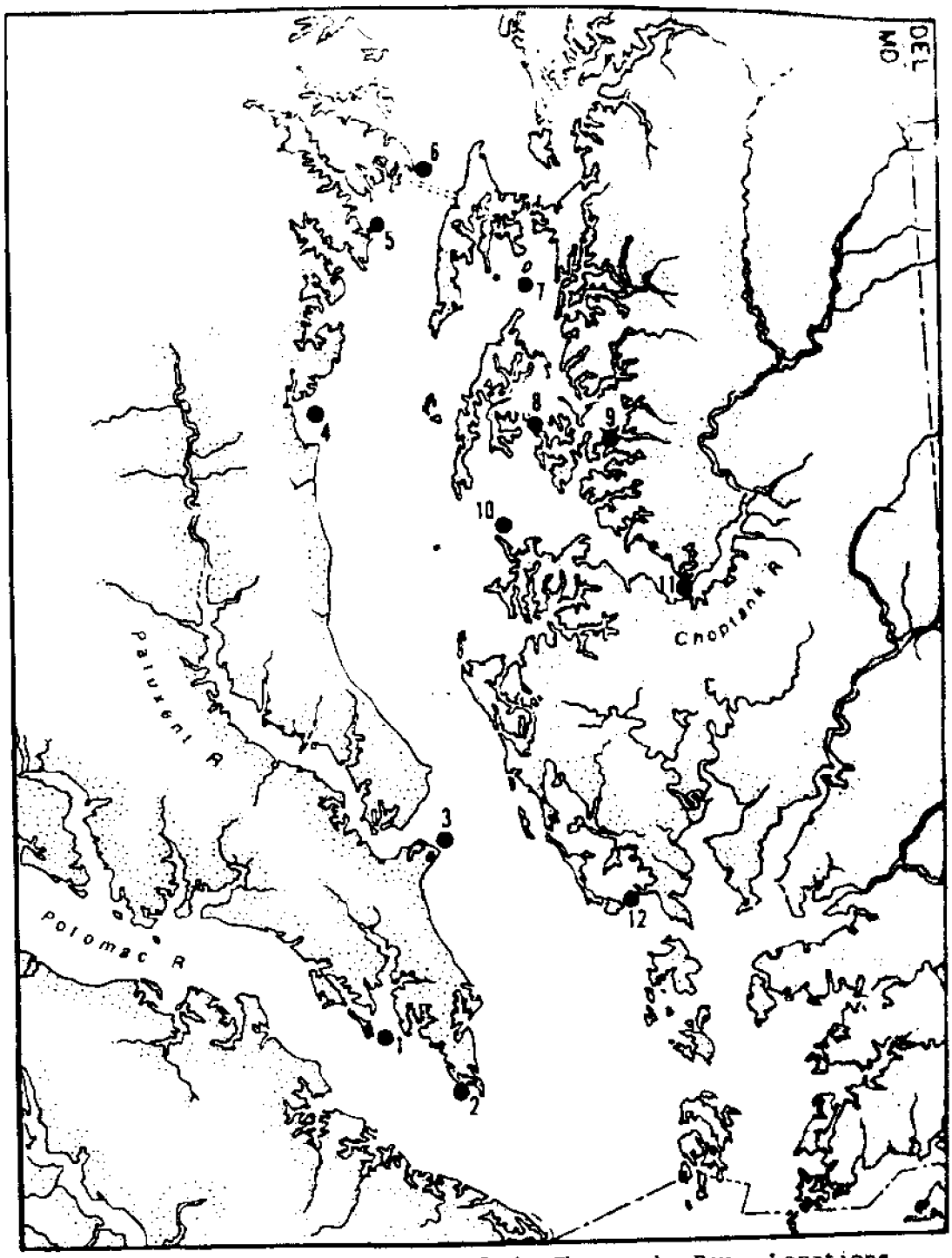

Figure 1. Shellfish sampling area of the Chesapeake Bay. Locations of routinely sampled oyster bars indicated by closed circles (●).
1) Chickencock; 2) Cornfield Harbor; 3) Hog Island; 4) Herring Bay; 5) Tolly Point; 6) Sandy Point, N.; 7) Bald Eagle; 8) Deep Neck; 9) Double Mills; 10) Cook's Point; 11) Green Marsh; 12) Norman.

Oyster bar names according to Merritt (10).

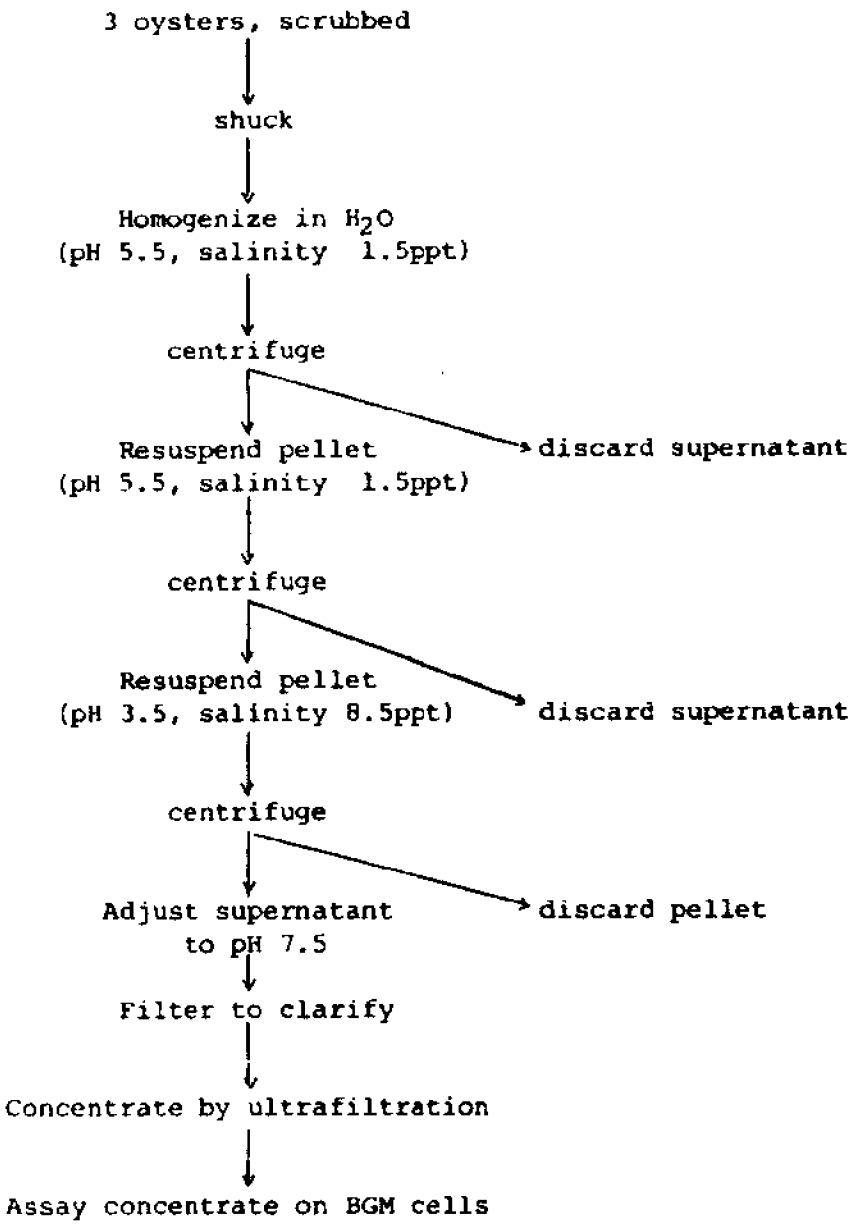

Figure 2. Outline of the Sobsey procedure used for processing oysters for enterovirus content.

Table 1. OYSTER BARS MONITORED FOR ENTEROVIRUS, 1977-1978

Oyster Bar	River System	Approved for Commercial Harvesting[a]
Chickencock	St. Mary's R.	yes
Cornfield Harbor	Potomac R.	yes
Hog Island	Patuxent R.	yes
Herring Bay	Herring Bay	yes
Tolly Point	Severn R.	yes
Sandy Point, N.	Chesapeake Bay, W.	yes
Bald Eagle	Eastern Bay	yes
Deep Neck	Broad Creek	yes
Double Mills	Tred Avon R.	yes
Cook's Point	Choptank R.	yes
Green Marsh	Choptank R.	no
Norman	Honga R.	yes
Supplementary Sampling Sites (not routinely monitored):		
Piney Point Hollow	Potomac R.	yes
Farmers	Patuxent R.	no
Chinks Point	Severn R.	no
Hollicutts Noose	Eastern Bay	yes
Ash Craft	Miles R.	no
States Bank	Choptank R.	no
Todds Point	Choptank R.	yes
Marumsco	Pocomoke Sound	yes

(a) Maryland State Department of Health and Mental Hygiene (8).

Table 2. Recovery of Poliovirus from oyster homogenates seeded with 10^4 PFU Poliovirus/ML

Sample	% of Original[a]
Seeded Oyster Homogenate	100%
Supernatant #1 (discard)	<1%
Supernatant #2 (discard)	<1%
Final Pellet (discard)	12%
Supernatant #3 - Prefiltrate	73%
Filtrate	68%
Concentrate	65%

(a) Values listed represent a composite of two experiments.

Table 3. Oyster Sampling Schedule--April, 1977 through March, 1978

Oyster Bar	Sampling Dates											
	1977									1978		
	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar
Chickencock	-[a]	S	S	S	S	S	S	S	S	S	S	-
Cornfield Harbor	-	S	S	S	S	S	S	S	S	S	S	S
Hog Island	-	S	S	S	S	S	S	S	S	S	S	S
Herring Bay	S	S	S	S	S	S	S	S	S	S	S	S
Tolly Point	-	S	S	S	-	S	S	S	S	S	S	S
Sandy Point, N.	-	S	S	-	S	-	S	-	S	-	S	-
Bald Eagle	-	S	S	S	-	S	S	S	S	S	-	S
Deep Neck	S	S	S	S	S	S	S	S	S	S	S	S
Double Mills	S	S	S	S	S	S	S	S	S	S	S	S
Cook's Point	S	S	S	S	S	S	S	S	S	S	S	S
Green Marsh	S	S	S	S	S	S	S	S	S	S	-	S
Norman	S	S	S	S	S	S	S	S	-	S	S	S

[a] indicates not sampled.

Additional samples examined (by month):

Chinks Point 6/77; 10/77; 12/77 (2 samples); 2/78.

States Bank 5/77; 6/77; 8/77; 1/78; 3/78.

Piney Point Hollow 8/77.

Farmers 2/77.

Ash Craft 9/77.

Todds Point 2/77.

Hollicutts Noose 2/77.

Marumsco 2/77.

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