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Microbial Indicator Levels in Shellfish, Water,

& Sediments from Upper Narragansett Bay Conditional

Shellfish Growing Area 67 pp

Cabelli (URI)

Narragansett Bay Estuary Program

Microbial Indicator Levels In Shellfish, Water and Sediments from the Upper Narragansett Bay Conditional Shellfish-Growing Area

Final Report of a Study Conducted as Part of the Narragansett Bay Project

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FOREWORD

The United States Congress created the National Estuary Program in 1984, citing its concern for the "health and ecological integrity" of the nation's estuaries and estuarine resources. Narragansett Bay was selected for inclusion in the National Estuary Program in 1984 and designated an "estuary of national significance" in 1988. The Narragansett Bay Project (NBP) was established in 1985. Under the joint sponsorship of the U.S. Environmental Protection Agency and the Rhode Island Department of Environmental Management, the NBP's mandate is to direct a five-year program of research and planning focussed on managing Narragansett Bay and its resources for future generations. The NBP will develop a comprehensive management plan by December, 1990, which will recommend actions to improve and protect the Bay and its natural resources.

The NBP has established the following seven priority issues for Narragansett Bay:

* management of fisheries
* nutrients and potential for eutrophication
* impacts of toxic contaminants
* health and abundance of living resources
* health risk to consumers of contaminated seafood
* land-based impacts on water quality
* recreational uses

The NBP is taking an ecosystem approach to address these problems and has funded research that will help to improve our understanding of various aspects of these priority problems. The Project is also working to expand and coordinate existing programs among state agencies, governmental institutions, and academic researchers in order to apply research findings to the practical needs of managing the Bay and improving the environmental quality of its watershed.

This report represents the technical results of an investigation performed for the Narragansett Bay Project. The information in this document has been funded wholly or in part by the United States Environmental Protection Agency under assistance agreement #CX812768 to the Rhode Island Department of Environmental Management. It has been subject to the Agency's and the Narragansett Bay Project's peer and administrative review and has been accepted for publication by the Management Committee of the Narragansett Bay Project. The results and conclusions contained herein are those of the author(s), and do not necessarily represent the views or recommendations of the NBP. Final recommendations for management actions will be based upon the results of this and other investigations.

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Executive Summary

Surface and bottom water and shellfish (Mercenaria mercenaria) samples were collected from one station just north of and two stations within the conditional shellfish growing area in the upper bay of the Narragansett Bay Estuary. In general, water samples were collected at the time the shellfish were taken and at least once during the previous day. These sampling tours were made three times during the summer, five times during the fall, four times during the winter, and once during the spring. Sediment samples were collected only during the winter and summer and when the shellfish samples were taken. The samples were assayed for fecal coliforms, enterococci, F male-specific bacteriophages (as a viral simulant for the environmental behavior of the Norwalk virus), and Clostridium perfringens (as a conservative tracer). Sediment samples were not assayed for the F phages. In addition, the uptake and retention of the indicators was examined in tank experiments conducted in the laboratory. Sewage was used to contaminate the water in the tanks.

Sampling was conducted when the conditional growing area was open or closed for the harvesting of shellfish; but only the levels of the fecal coliforms in the surface water, the enterococcus levels in the surface and bottom water and C. perfringens levels in the bottom water during the winter were significantly higher when the area was closed than when it was open.

Although the F phage levels in prechlorinated effluents were some 30 times less than those of the fecal coliforms in prechlorinated sewage effluents, they were, on the average, eight times greater in the surface waters and three times greater in the bottom waters. The differences in the levels of the two indicators were greater during the winter and early spring than the summer and fall. These results are consistent with the greater susceptibility of the fecal coliforms than the F phage (viruses) to die-off during chlorination and transport in the receiving waters, especially during the winter. The differences in the levels of the fecal coliforms relative to those of F phages, enterococci and C. perfringens were even more marked in the shellfish, as was the effect of season, suggesting seasonal differences between the fecal coliforms and the other indicators in their uptake by and/or viable retention in the shellfish. The tank experiments confirmed the greater viability of the F phages in the shellfish but were inconclusive with regard to the increased uptake.

The correlation of the indicator levels in the water to the homologous one in the shellfish generaly was best in the fall. The correlation coefficients across stations and seasons were very low, although significance was reached in one case, the F phages in the bottom water and those in the shellfish. Since the F phages were used as a simulant for the environmental behavior of the most frequently identified causative agent of the most prevalent shellfish- and swimming-associated illness, correlation analyses were performed on the levels of all the indicators in the surface and bottom waters against those of the F phages in the shellfish. The correlations were poor in every case. If the initial assumption is correct, the best indicator of the risk of illness from the consumption of raw shellfish propably are is the levels of the F phages in the shellfish themselves.

The most important issues raised, but not resolved, by the findings from the study are the adequacy of the fecal coliform indicator system and the management of the conditional shellfish growing area during the late fall, winter and early spring. Clearly, the results of a prospective epidemiological study are needed, and we cannot overemphasize the importance of findings from the one which is ongoing.

The epidemiological and numerical basis of the existing fecal coliform, shellfish growing are standard is very poor (especially with regard to the risk of viral gastroenteritis). The differences in levels the fecal coliforms and F phages following chlorination, transport in the receiving waters and retention in the shellfish are extremely great. Therefore, a possible limit on the F phages levels in the shellfish which would generally prohibit the harvesting of shellfish from the conditional shellfish harvest area during the late fall, winter and early spring was calculated. It will be of interest to see how realistic this limit in the light of the findings from the on-going epidemiological study.

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FIGURES

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INTRODUCTION

The quality of shellfish relative to the potential for infectious disease from fecalborne pathogens has been assessed historically by the total and/or fecal coliform levels in the growing waters. The assessment is made on the indicator levels in the water rather than in shellfish themselves for several reasons. The most compelling one is that during the winter in temperate zones, when the ambient water temperature falls below 10°C, the coliforms disappear from the shellfish, even in very polluted waters (Cabelli and Heffernan, 1971). This notwithstanding, shellfishborne outbreaks of hepatitis A have occurred during this time (see Cabelli and Heffernan, 1970). The best explanation for these observations is that, during the winter when the animals are minimally active, both the viral pathogens and fecal indicators are physically retained by the animals but that the coliform indicators die-off more rapidly than do the viral pathogens.

The observations noted above have special relevance to "conditional shellfish growing areas", such as the one in upper Narragansett Bay, which are subject to contamination from combined sewer overflows (CSOs). In this specific case, the area is closed for a minimum of seven days following a rainfall in excess of 0.5 inches within a 24 hour period. It is clear from the history of shellfishborne outbreaks of infectious disease (Rippey and Verber, 1986) and by the other waterborne routes as well (Cabelli, 1983a) that the most commonly reported illness is an acute gastroenteritis and that the most frequently associated etiologic agents are viral, the Norwalk-like viruses (Centers for Disease Control, 1987). The possible circumstances which in part prompted this study are the contamination of the shellfish by rainfall induced CSOs in the late fall and the retention and survival of the viral pathogens, but not the fecal coliform indicators, during much of the winter, certainly beyond the seven or so days during which the area is closed to the harvesting of mollucsan shellfish.

A second consideration which led to this study was the incongruity in the results of the bathing beach epidemiological studies (Cabelli, et. al., 1982) and the shellfish growing area standard. In the bathing beach epidemiological studies, the enterococcus levels in the water were much better correlated to the rate of swimming-associated gastroenteritis than either total or fecal coliforms; and, because of this, enterococcus levels in the water are used in the criteria and guideline recently recommended by the USEPA (USEPA 1986). Moreover, there are field data showing that the fecal streptococci, of which the enterococci are a component, are more akin to viruses in their survival in marine waters than are the coliforms (Fattal et al, 1983). Finally, it has been shown that the shellfish/water ratios for fecal streptococci are higher than those for the coliforms (Plusquellec et al, 1986).

The third reason for this study derives from some earlier observations concerning the comparative survival of viruses and coliforms during wastewater chlorination. The viruses are generally more resistant to the cidal effects of chlorine than the coliforms (Scarpino et al, 1972; Lupo, 1979; McBride, 1979). In addition, some recent studies showed that the Norwalk viruses were especially resistant to the cidal effects of combined chlorine, the molecular species found in chlorinated wastewater. Of the viruses examined (including Poliovirus I and the simian rotavirus), only f-2, one of the F male-specific bacteriophages, was comparably resistant to chlorination (Keswick et al, 1985). These results beg

the question of the validity of the coliform and even the enterococcus systems to adequately index the risk of recreational-and shellfishborne illness in situations where there is an acute dependency on wastewater chlorination to meet existing marine water quality standards. In addition, they raise the issue of the adequacy of wastewater chlorination itself with regard to the risk of acute viral gastroenteritis.

OBJECTIVES

The objectives of the present investigation were:

1. To examine the levels of fecal coliforms and enterococci in shellfish, surface and bottom waters, and their underlying sediments relative to those of the F male-specific bacteriophages as a simulant for the Norwalk-like virus and Clostridium perfringens as a conservative tracer.

2. To examine relationships of the levels of the indicators in the shellfish to those in the water as they may be affected by season and other factors.

MATERIALS AND METHODS

FIELD STUDIES:

Study Site and Sample Collection

The general location chosen for the study was the conditional shellfish growing area in upper Narragansett Bay (Figure 1). It was chosen because it is a major harvest area which is subject to fecal contamination from direct or indirect wastewater discharges into the Providence River to its north. Three stations were sited in the general area. One (A) was just north of the conditional area at a location which is closed to the harvesting of shellfish for direct consumption. Stations B and C were within the conditional area. There were two sampling sites for station C as shown. During the fall and winter, a very limited number of samples were collected from two additional sites (D and E) south of the conditional area. Both of these are at sites which, except under extraordinary circumstances, are permanently open to the harvesting of shellfish. Samples were collected from these two sites because of the results obtained from those taken within the conditional area.

Samples were collected at stations A-C on three occasions in summer, five occasions in fall, and five occasions during winter. Surface water samples were taken with a sterilized 1-liter polypropylene sample bottles about six centimeters below the surface of the water. Bottom water samples were obtained with a Kemmerer sampler, the contents of which were transferred to a 1-liter sample bottle which was capped and immediately placed in an ice chest. Sediment samples were taken with a Peterson grab sampler. Upon return of the sampler to the boat, the top 1 cm of sediment was then scraped off with a sterilized tongue depressor into a sterile, capped 8 oz. polypropylene cup which was then placed in an ice chest. A minimum of 12 shellfish were obtained by dredge or rake. They were put in a plastic bag which was then placed in an ice chest. None of the samples was held for more than eight hours prior to assay.

Analysis of samples

Water: One-hundred ml quantities of the samples or appropriate half-log dilutions thereof were assayed for three bacterial indicators. They were fecal

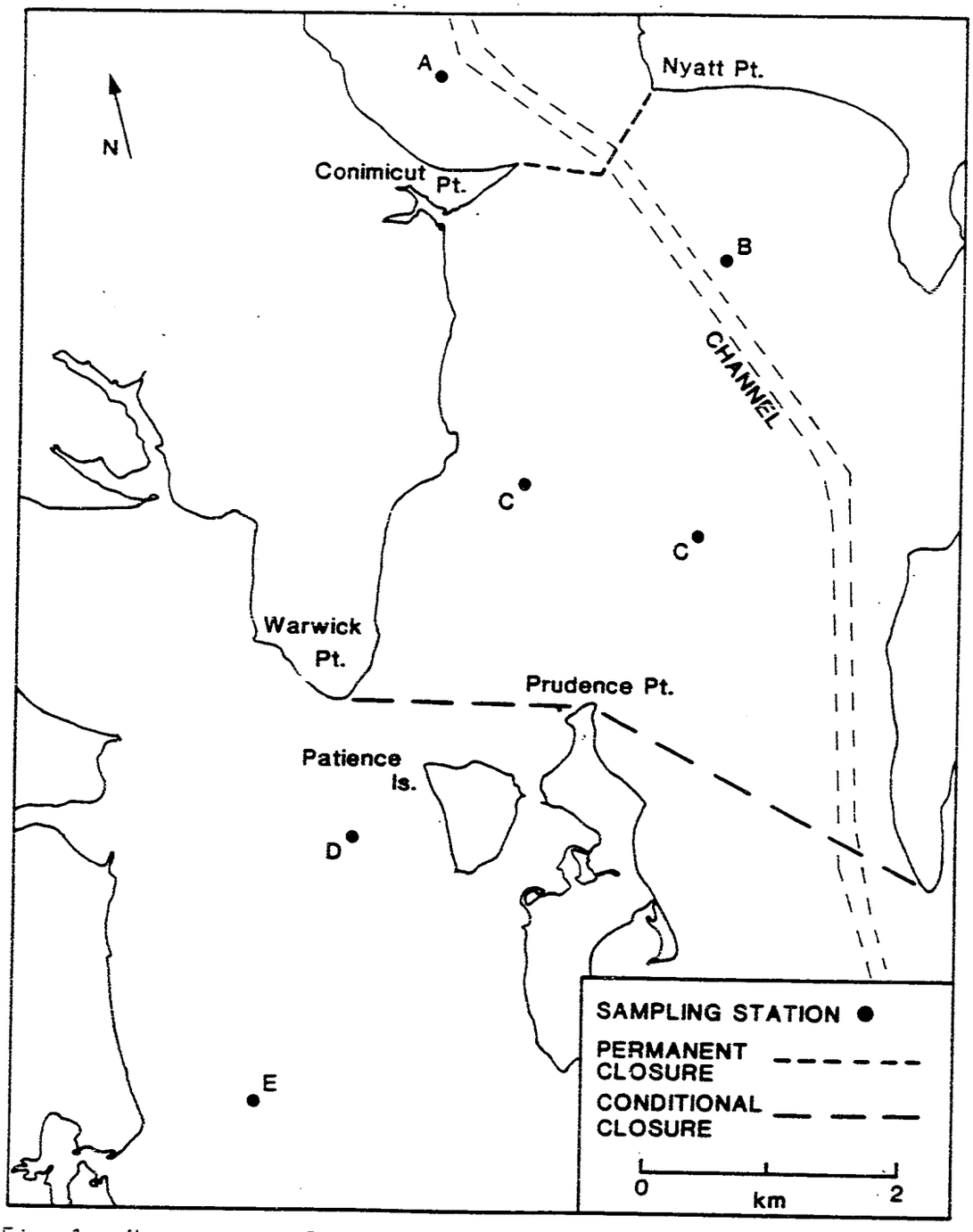

Fig. 1. Narragansett Bay sampling sites above, within and below the closure times for the conditional shellfish growing are in upper Narragansett Bay.

coliforms by the mTEC method (Dufour et al, 1981; Cabelli et al, 1982a), enterococci by the modified mE method (Levin et al, 1975; Dufour, 1980) and C. perfringens by the mCP method (Bisson and Cabelli, 1979). F male-specific bacteriophage levels in the water were determined by a method developed in our laboratory (Debartolomeis, 1988) (see Appendix B). The temperature was measured with a probe which was lowered into the water.

Shellfish: The shellfish were cleaned, shucked, and homogenized as prescribed (APHA, 1970). Twelve animals were used to prepare the homogenates with the samples collected in 1986. In order to minimize the effect of a high indicator level in a single animal, homogenates were prepared from two pools of six animals each in 1987; and the geometric mean of the levels in the two pools was used. Fecal coliforms were assayed by the five-tube, three-dilution most probable number (MPN) method (APHA, 1985). The enterococcus levels in the shellfish were determined by a five-tube, three-dilution MPN test employing azide-dextrose broth (APHA, 1985) with the exception that the tubes which were turbid after incubation for 48 hours were streaked on a membrane placed on modified mE medium. C. perfringens was assayed by a ten-tube, three-dilution procedure using the iron milk method of Abeyta (1983). The shellfish homogenates were treated and assayed for F bacteriophages by procedures developed in our laboratory (Appendix B).

LABORATORY EXPERIMENTS

Experimental System

The uptake and retention experiments were conducted at the facilities of the Food and Drug Administration laboratory at Davisville, Rhode Island. The experimental animals (quahogs, Mercenaria mercenaria) were collected in the summers of 1986 and 1987 from Narragansett Bay. They were held for at least two months prior to the conduct of the experiments in storage tanks supplied with a flowing source of water at ambient temperatures and a salinity of 30°/000. The water was pumped directly from Allen Harbor. The intake water was sampled at least once a week during the course of the experiments. F phages were never recovered from the intake water. Fecal coliforms and enterococci were detected at levels near the sensitivities of the assays (0.5 CFU/100 ml) on rare occasions or not at all. C. perfringens was consistently recovered from the water, and the levels occasionally were as great at 16 CFU/100 ml; the median level was less than the sensitivity of the assay.

The indicator levels in the animals were examined prior to the uptake experiments or the uptake phase in the retention experiments. These levels were always very low relative to those observed following exposure of the animals to sewage contaminated water (uptake). Those for fecal coliforms and enterococci never exceeded 5 an 7 CFU/100 gm., respectively. F phages were detected in most of the samples, and the levels on occasion were as high as 10-25 PFU/100 gm. C. perfringens was consistently found in the animals prior to uptake, and the levels occasionally reached 100 CFU/100 gm.

The experimental tanks were circular (diameter, 90 cm) and contained standpipes so that the volume of water in the tanks was 127 liters. A circulating pump was used to mix the water in the tanks. The number of animals in a tank never exceeded 300, and the intake water to the tank was regulated to about 4 liters/ min. A thermostatically controlled heating element in the tank

was used to regulate the water temperature as required. Polyvinyl chloride (PVC) piping was used throughout the system.

Raw sewage was obtained from the East Greenwich sewage treatment plant (STP) in 20 liter polypropylene containers on the day contamination was started. Immediately upon their arrival at the laboratory, the containers were placed in a refrigerator to prevent rapid die-off of the indicators. The sewage, which was kept in the refrigerator at about 4-6°C, was fed into the tank at a constant rate by means of a proportioning pump (Technicon). When the contamination period exceeded 72 hours, the sewage was replaced weekly. The sewage was assayed for the four indicators upon receipt at the laboratory by the same methods used for the water samples. The water in the tanks was assayed at the end of the 72 hr. contamination period. In the long term contamination experiment, the water from the tanks was always sampled at the time shellfish were removed for assay and frequently before the sewage was replaced at weekly intervals. The indicator levels in the shellfish were determined by the same methods as used with field samples.

Experimental Design

Cold weather retention: Three experiments were conducted during the following 9/8/86 - 11/1/87, 10/20/86 - 2/10/87 and 10/27/86 - 2/17/87. Little accumulation of the F phages by the quahogs was obtained in some preliminary experiments conducted in June and July of 1986. This, incidentally, was consistent with the data being obtained in the field studies. It was found, however, that placing the animals in the refrigerator for 24 hours prior to their exposure to sewage-contaminated water at the ambient temperature (about 11°C) markedly improved the uptake of the F phages. This practice was followed in these three experiments.

Upon completion of the 72 hr contamination phase, a random sample of 12 animals was assayed to establish a zero time level for the indicators. The water also was assayed at this time; and, because of this, accumulation data also were obtained. The rest of the animals were then taken from the contamination tank, rinsed, and placed in another tank. The water in this tank was maintained at 2-5°C by recirculating it through a chiller unit. The intake water was fed into the tank at approximately 1 liter/min. The densities of the indicators in the shellfish were then determined once a week for the first six weeks and about every other week thereafter.

A fourth experiment was conducted from 1/31/87 - 1/28/88. In this experiment, however, the tank water was allowed to remain at its ambient temperature in order to determine when in the spring the F phages and C. perfringens levels are reduced. Since the animals were inactive at the low ambient water temperature characteristic of this time, they were acclimated for 24 hours at 11°C prior to contamination and contaminated at 11°C. In order to increase the precision of the estimates of the indicator levels, three-pools of 6-8 animals each were assayed; and the geometric mean of the levels from the three pools was calculated. During the uptake phase of the experiment, the levels in the water and the shellfish were determined after 6,12,24 and 48 hours so that accumulation could be examined as well. This experiment could not be completed.

was used to regulate the water temperature as required. Polyvinyl chloride (PVC)

Long Term Contamination 3/26/87 - 1/21/88: Contamination in this experiment was continuous. Sewage was replaced at weekly intervals. The rate of sewage flow was 0.5ml/min into the tank, and that of the water was 2 liters/min. The temperatures of the water in the tank was artificially controlled at 15°C from 3/26/87 to 4/28/87, thereafter the heater was shut off, and the experiment was continued at ambient water temperatures. In general, the indicator levels in the water and in the animals were determined every other week for the duration of the experiment. Three pools of 6-8 animals each were assayed as in the previous experiment. This experiment could not be completed.

Contamination with falling temperatures from 10°-7°C: In this experiment, the animals were acclimated to 10°C for a period of one week. They were then contaminated for a 72 hour period during which the water temperature in the tank was decreased 1°C/day to 7°C. Both the water in the tank and the shellfish were assayed after 72 hours, the latter by three pools of 6-8 animals each.

RESULTS

FIELD STUDIES

Indicator levels in the surface water, bottom water, shellfish and sediments along with the status of the conditional area (open or closed) are given by station and season in Tables A1-A11 of Appendix A. The indicator levels for the surface and bottom water samples generally are the geometric means of the levels in the water collected on the day the shellfish and sediment samples were taken and the day previous to it. During the fall of 1987, sediment samples were not collected; and water samples were taken only at the time the shellfish samples were collected because of logistic problems.

The data were examined statistically by Student's "t" tests to determine whether there were significant differences in the indicator levels when the area was open or when it was closed. This analysis was limited by the number of samples taken, by its dependency on the amount of rainfall which resulted in the closure and by the elapsed time between the rainfall and collection of the samples. This notwithstanding, the data for the closed days were excluded from subsequent analyses only when statistically significant differences were obtained. Such differences were found only during the winter. There were significant differences in the indicator levels in the surface water samples for fecal coliforms and enterococci at all three stations. Significant differences in the bottom water data were obtained only for enterococci at station A and for C. perfringens at all three stations. The indicator levels in the shellfish were not statistically different when the area was closed from when it was open (Tables A1-A10).

The levels of the indicators in the surface water generally were higher than those in the bottom water; but the differences between them decreased as the distance increased from the sources of pollution north of the conditional area (Table 1). In general, the F phages and C. perfringens levels were significantly higher in the winter and early spring than during the summer. This seasonal effect was observed only once with the fecal coliforms or enterococci, possibly because the levels in many of the samples collected during the summer

Table 1 Indicator levels in the surface and bottom water by season

Stn[a] Number	Indicator	Geometric mean indicator levels per 100 ml[b] Surface water				Bottom water			
		Summer (9)[c]	Fall (7)	Winter (13)	Spring (3)	Summer (9)	Fall (7)	Winter (13)	Spring (3)
A	Fecal coliforms	6.8	12.7	5.5	13.7	2.8	1.7	8.0	1.7
	Enterococci	<0.6[d]	1.2	1.1	0.9	<0.9	1.7	1.5	1.4
	F. phage	22.4	61.2	98.5[e]	85.2[e]	7.0	4.8	14.0	5.2
	C. perfringens	37.4	47.7	173[e,f]	271[e,f]	63.8[f]	15.0	88.6[f]	75.6[f]
B	Fecal coliforms	2.2	4.0	2.7	2.9	0.8	2.4	7.7	1.0
	Enterococci	1.0	1.6	<0.8	1.1	0.8	1.3	1.9	1.1
	F. phage	7.4	16.5	28.6[e]	22.0[e]	3.9	4.2	13.3[e]	5.0
	C. perfringens	16.7	31.2[e]	47.3[e]	139[g]	10.4	9.9	47.1[e,f]	36.7[e]
C	Fecal coliforms	1.0	1.0	1.6	1.4	0.8	1.1	10.8[g]	<0.6
	Enterococci	<0.6	0.8	<0.6	<0.5	<0.7	1.0	1.6	1.8
	F. phage	4.8	<3.6	27.7[e,f]	15.8	2.2	1.4	13.1[e,f]	3.8
	C. perfringens	12.8	7.7	32.1[e,f]	110[e,f]	9.5[f]	4.6	48.6[e,f]	41.0[e]

[a] See Figure 1 for location of stations (stns).
[b] Sample collections: Summer, 6/23-24/86, 7/1-2/86, 7/9-10/86; Fall, 10/7-8/87, 10/15/87, 10/29/87, 11/19/87, 11/23/87; Winter, 1/28-29/86, 2/11-12/86, 2/18-19/86, 3/3-4/86; Spring 3/28-29/86
[c] Number of samples
[d] Levels less than sensitivity limits of assay set at the limits; <, more than half the samples less than limits; at least one sample less than limits.
[e] Significantly different from summer at P<0.05.
[f] Significantly different from fall at P<0.05.
[g] Significantly different from other seasons at P<0.05.

frequently were less than the sensitivity of the assay.

The C. perfringens and phage levels were consistently and, at times, markedly higher than those of the fecal coliforms and enterococci. The differences generally were greater in the winter and early spring than in the summer, and greater in the surface than the bottom waters.

The disparity between levels of the F phages and C. perfringens on one hand and those of the coliforms and enterococci on the other is even greater when the relative levels of the indicators in the prechlorinated effluents are considered. This can be seen in Table 2 from the comparisons of the levels of the other indicators to those of the fecal coliforms in the surface and bottom water by season and in the prechlorinated effluent from the Providence and East Providence STPs (data from Cabelli, 1988). The levels of enterococci, F phages and C. perfringens were 0.1 , 0.028 and 0.063 times that of the fecal coliforms in the prechlorinated effluents, respectively. In the surface water, however, they were 0.27, 7.9 and 22.5 times that of the fecal coliforms. The significantly higher F phages/fecal coliforms and C. perfringens/fecal coliform ratios in the surface water samples during the winter and early spring as compared to the summer and fall were probably due to seasonal effects on the die-off of the fecal coliforms relative to those of the F phages and C. perfringens.

Phage assays were not performed on the sediments because of methodological problems. Fecal coliforms could not be detected in any of the sediment samples, and enterococci were recovered from only about half the samples (Table 3). C. perfringens was recovered from all the samples at relatively high levels.

The most striking differences were in the levels of the indicators in the shellfish samples (Table 4). The fecal coliform densities were consistently lower than those of C. perfringens and the F phages; they were also less than those of the enterococci during the summer and fall. During the winter the fecal coliform and enterococcus levels generally were less than the sensitivity of the assay system (an MPN of 2.0/100 g). In general, the levels of the various indicators decreased from stations A through E.

There were some appreciable and statistically significant seasonal differences in the indicator densities in the shellfish. The fecal coliform levels during the fall at stations A and B were significantly greater than those in the summer, winter and spring. This notwithstanding, the mean fecal coliform levels, even that at station A which is in the closed area, were low relative to growing area standard of 14/100 ml of water. Both the mean enterococcus and C. perfringens levels at stations A-C were significantly lower during the winter than during the fall or summer. One explanation for this observation is that the animals are relatively inactive in the winter. During the summer, however, they are actively feeding and may reflect the indicator levels in the bottom sediments. In general, the highest levels of all four indicators were obtained during the fall, and the seasonal differences generally were statistically significant.

The most marked and important seasonal differences were those in the levels of the F phages in the shellfish (Table 4) since the phage were used as simulants for the Norwalk viruses. Their densities were significantly higher during the fall than the winter or summer, and during the winter than the summer. The F

Table 2. Comparison of enterococcus, F phage and C. perfringens levels to those of the fecal coliforms in surface and bottom waters by season.

Sample	Other Indicator	Sta.	Other indicator/fecal coliform ratio Summer	Fall	Winter	Spring	All	Pre-cl_2 sewage[3]
Surface water	Enterococci	X	0.37	0.43	>0.30	0.16	0.27	0.10
		SD	(0.27)	(0.36)	(0.10)	(0.19)		
	F phage	X	3.8	4.2	15.1[c]	8.3[c]		0.028
		SD	(0.8)	(0.6)	(3.9)	(2.6)		
	C. perfringens	X	8.6	9.8	22.9[d]	48.8[d]	22.5	0.063
		SD	(3.8)	(3.3)	(7.4	(29.4)		
Bottom water	Enterococci	X	0.74	0.81	0.19	1.6	0.65	
		SD	(0.37)	(0.24)	(0.05)	(1.2)		
	F phage	X	3.4[e]	2.0	1.6	4.8[e]	3.0	
		SD	(1.3)	(0.8)	(0.3)	(1.6)		
	C. perfringens	X	15.9[f]	5.7	7.2	50[f]	19.7	
		SD	(5.9)	(2.7)	(3.3)	(16)		

[a]Data from Cabelli (1988).

[b]-Approximation because one or more but less than half the levels used in calculating the ratios were less than sensitivity of the assay.

[c]Significantly different from summer and fall ratios (X 4.0) at $p<0.05$.

[d]Same as [c]2 (X 9.2).

[e]Significantly different from fall and winter ratio (X 1.8) at $p<0.05$.

[f]Same as [e] (X 6.5)

Table 3. Indicator levels in bottom sediments.

Stn.	Season	N[a]	Geometric mean indicator level/g (dry wt.)[b] Fecal coliforms	Enterococci	C. perfringens
A	Winter	4	(0)[c]	36.2 (1)	3100 (5)
	Summer	3	(0)	185 (3)	1570 (3)
b	Winter	4	(0)	41.1 (2)	1230 (5)
	Summer	3	(0)	194 (2)	1650 (3)
C	Winter	4	(0)	25.4 (1)	1050 (5)
	Summer	3	(0)	105.1 (1)	580 (3)

[a]Number of samples

[b]Only for measurable levels.

[c]( )-number of samples with measurable levels.

Table 4. Geometric mean indicator levels in the shellfish from the study area by season

Station[a]	Season[b]	N[c]	Indicator level per 100 grams[d] FC	Enterococci	F phage	C. perfringens
A	Summer	3	1.9	17.4	33.8	1280
	Fall	5	22.7[g]	33.8	1440[g]	951
	Winter	4	<2.3	<2.0[f]	146[e]	70.3[f]
	Spring	1	2.0	<2.0	131	270
B	Summer	3	3.1	6.8	<17.4	540
	Fall	4	12.9[g]	41.3[g]	1100[g]	940
	Winter	4	<2.0	<2.0[f]	69.7[e]	50.5[f]
	Spring	1	2.0	<2.0	238	500
C	Summer	3	2.7	11.5	10.1	520
	Fall	5	4.6	19.8	246[g]	883
	Winter	4	<2.3	<2.0[f]	29.5[e]	40.1[f]
	Spring	1	4.0	<2.0	2140	500
D	Fall	3	4.2	7.2	250	321
	Winter	1	<2.0	<2.0	62.5	79.7
E	Winter	1	<2.0	<2.0	12.5	31.6

[a]See Figure 1 for station locations
[b]Sample collection: Summer, 6/24/86, 7/1/86, 7/9/86; Fall 10/7/87, 10/15/87, 10/29/87, 11/19/87, 11/23/87; Winter 1/28/86, 2/11/86, 2/18/86, 3/3/86; Spring 3/28
[c]Number of samples
[d]Levels less than sensitivity limits of assay set at limits; <, more than half of samples less than limit; , at least one sample less than limit
[e]Levels in winter significantly greater than summer and less than fall (P<0.05) for stations A-C.
[f]Level significantly different (P<0.05) than fall or summer
[g]Level significantly different (P<0.05) than winter and summer

phage levels in the shellfish for the single collection during the early spring were intermediate between those in the fall and winter.

Two approaches were taken in the analysis of the data on the indicator levels in the water relative to those in the shellfish. The first was the examination of the association of the levels in the bottom and surface water to those in the shellfish by regression analysis. In the second approach, the ratios of the indicator levels in the bottom water to those in the shellfish were compared in order to examine the accumulation/retention of the indicators to each other and by season.

Linear correlation coefficients were calculated for the indicator levels in the surface and bottom water (Y) against those in the shellfish (*). These analyses were done by season and all seasons combined for the station in the closed area (A), for the two stations in conditional area (B and C), and for all the stations, including the limited data for station D which is the area open for the harvesting of shellfish. The correlation coefficients are given in (Table 5.) Negative correlations were obtained for about one-third of the individual comparisons. One possible explanation for the poor correlations is that, in many instances, the shellfish are reflecting uptake that occurred when water samples were not collected or, conversely, that the shellfish were inactive when the water contained high levels of the indicators. A second one is marked variability in the uptake and retention of the indicators of the shellfish.

The one clear finding was that the best correlations for all the indicators at all the stations occurred during the fall; and, in the fall, better correlations of the indicator levels in the water to those in the shellfish occurred at station A, the station closest to the pollution sources. The best correlation across both stations and seasons was between the F phage levels in the surface water and in the shellfish; and the association was statistically significant. The above not withstanding, less than 50 percent of the data could be explained by the linear regression equations obtained for any of the stations during the fall; that is, in no case did $r^2 = 0.5$.

The most critical relationship with regard to the existing monitoring programs against the risk of shellfishborne disease, aside from that obtained from controlled epidemiological studies, is the relationship of some measure of water quality to the levels of the epidemiologically important pathogens in the shellfish, the viruses that cause acute gastroenteritis and infectious hepatitis. These agents can not be enumerated at present. However, since the F phages were used as simulants for the Norwalk viruses, at least with regard to their survival during treatment, disinfection and transport, the associations of the indicator levels in the water to the phage levels in the shellfish were examined. The correlation coefficients are given in Table 6 by season for all the stations. The correlations were uniformly poor, but somewhat better during the fall. The best one overall was for F phages in the surface water and there was a significant association. If the assumptions concerning the adequacy of the F phages as simulants for the environmental behavior of the Norwalk virus in sewage, marine waters and shellfish are accepted, then the F phage levels in the shellfish is the only good indicator of the risk of illness from their raw consumption. For the present, this speculation can only be and is being examined epidemiologically (Dufour, personal communication).

Table 5. Linear correlation coefficients (r) for the indicator levels in the water (y) against their homologous levels in the shellfish (x) by station and season.

Parameter[a]		Y on X linear correlation coefficients by station and season[b]											
		A				B+C				A-D			
Y	X	Summer (3)	Fall (5)	Winter (5)	All (13)	Summer (6)	Fall (7-9)	Winter (10)	All (25)	Summer (9)	Fall (14-17)	Winter (12-15)	All (35-41)
FC_{sw}	FC_{sh}	0.53	0.80	0.37	0.49	-0.15	0.49	-0.19	0.17	-0.34	0.63*	0.01	0.30
FC_{bw}	FC_{sh}	-0.38[c]	0.93*	0.58	0.23	-0.47	0.42	0.33	-0.08	-0.047	0.68*	-0.16	0.06
Ent_{sw}	Ent_{sh}	0.59	0.49	NC	-0.06	-0.89	-0.09	NC	NC[d]	-0.59	0.17	-0.05	0.06
Ent_{bw}	Ent_{sh}	0.36	0.47	NC	-0.02	-0.43	0.03	NC	NC	0.24	-0.09	-0.02	-0.10
CP_{sw}	CP_{sh}	-0.64	0.59	0.62	-0.41	0.00	0.40	0.60	-0.17	0.52	0.40	0.59*	-0.13
CP_{bw}	CP_{sh}	0.90	0.52	0.37	-0.36	0.51	0.81*	-0.19	-0.56	0.71*	0.47	0.09	-0.37
F ph_{sw}	F ph_{sh}	-0.86	0.51	-0.66	0.48	-0.30	0.39	0.14	0.32	0.16	0.51*	0.16	0.41*
F ph_{bw}	F ph_{sh}	-1.00	0.90*	0.34	0.37	-0.26	0.55	-0.14	0.12	-0.05	0.66*	-0.04	0.21

[a] FC-fecal coliforms; Ent-enterococci; CP-C. perfringens; F ph - F phage; sw-surface water; bw-bottom water; sh-shellfish.
[b] x for $\log_{10}$ levels in the water (Y) against X for $\log_{10}$ levels in the shellfish (X); ( ) number of points.
[c] Negative correlation.
[d] Could not be calculated
[e] Spring data included with winter/
* Association is significant at $p<0.05$.

Table 6 Linear correlation coefficients for indicator levels in the water (Y) against the F phage levels in the shellfish (X) by season for all stations.

Y	X on Y linear correlation coefficient			
	Summer (9)	Fall (17)	Winter (15)	All (41)
FC_{sw}	0.24	0.36	0.11	0.24
FC_{bw}	0.31	0.40	-0.35	0.09
Ent_{sw}	0.22	0.50 *	NC	0.27
Ent_{bw}	-0.11	-0.01	NC	0.18
CP_{sw}	0.49	0.47	0.40	0.21
CP_{bw}	0.32	0.56 *	0.09	0.01
$F\ ph_{sw}$	0.16	0.51 *	0.16	0.41*
$F\ ph_{bw}$	-0.05	0.66 *	-0.04	0.21

See Table 4 for footnotes a-d
*Association is significant at $p<0.05$.

The data on the shellfish/bottom water indicator level ratios by season and station are presented in Table 7. The lowest ratios for the bacterial indicators were obtained during the winter at all the stations. This was not unexpected for the coliforms and enterococci because of the dormancy of the animals and the relatively poor survival of these two indicators in the animals. There are at least two explanations for the low shellfish/bottom water ratios for C. perfringens spores during the winter relative to the fall. The more obvious one is that, although the animals were not actively feeding and hence not accumulating C. perfringens during the winter, they periodically "dribbed-out", as it were, the contents of their digestive tracts. An alternative explanation is that some of the C. perfringens spores started the germination process towards the vegetative state and then died. The significantly lower ratios obtained with the fecal coliforms than C. perfringens probably represent an additional factor, die-off of the coliforms in the animals. The seemingly higher enterococcus than fecal coliform ratios probably is due to better survival of the enterococci in the animals.

The shellfish/water ratios for the F phages were markedly higher during the fall than the winter or summer, and higher during the winter than the summer. High ratios also were obtained for the single sampling tour conducted during early spring.

The data for all stations in Table 7 are repeated in Table 8 along with the statistical comparisons of the shellfish/bottom water ratios for the indicators to each other by season. Accumulation and/or retention, including survival of the indicators in the animals, was generally greatest for the F phages (viruses) and least for the fecal coliforms. The ratios for the enterococci were significantly greater than those for the fecal coliform during the summer and fall. The comparisons of the fecal coliform and enterococcus ratios to those for the C. perfringens spores indicate that die-off of the former in the animals is an important factor. The comparison of the ratios for the phages to those for C. perfringens during the fall, winter and spring suggests that the physical nature of the indicator may also be important in their accumulation/retention by the quahogs. We speculate that some of the virions, possibly those that are not particle associated are accumulated into locations in the quahogs from which their elimination during subsequent feeding activity is more protracted than that of the bacteria.

If it is assumed that the F phage are a reasonable simulant for the environmental behavior of the Norwalk virus, the worst-case situation with regard to the potential for shellfishborne acute gastroenteritis beyond that indexed by the coliform standard probably occurred during the late fall, winter, and early spring in the conditional area. The sequence of events leading to it was perceived as follows: (i) high indicator levels in the water, especially the bottom water, due to rainfall induced CSOs during the late fall; (ii) greater differential accumulation of the viral pathogens relative to the coliform indicators during this time; (iii) decreasing ambient water temperatures during the late fall and winter; and (iv) greater survival of the viruses than the coliforms in the relatively dormant quahogs. The field data, while it clearly showed appreciably and significantly higher shellfish/bottom water ratios for the viruses than the fecal coliforms, was not conclusive with regard to the factors involved. Three factors were suggested from the data: better uptake of the viruses, at least during the fall; their better retention by the shellfish; and their better survival in the shellfish. These factors were examined in some laboratory tank experiments.

Table 7. Ratios of the indicator levels in the shellfish to those in the bottom water by season.

Station[a]	Season[b]	N[c]	Indicator level per 100 grams[d] FC	Enterococci	F phage	C. perfringens
A	Summer	3	0.70	18.6	4.8	19.8
	Fall	5	8.7[e]	24.8	201[e]	61.9
	Winter	4	<0.22	<1.2[f]	8.8	0.55[f]
	Spring	1	1.2	<1.4	25.5	3.8
B	Summer	3	4.2	8.8[h]	<4.4	52.0[h]
	Fall	4	3.3	58.4	204[e]	125
	Winter	4	<0.27[g]	<1.7[f]	6.7	0.57[f]
	Spring	1	2.0	<1.8	47.0	13.7
C	Summer	3	3.4	13.0	<4.4	54.5
	Fall	5	4.4	26.4	149.[e]	176
	Winter	4	<0.18[f]	<1.9[f]	3.0	0.41[f]
	Spring	1	6.7	<1.1	564.	12.1
All	Summer	9	2.1	12.9[h]	<4.6	38.3[h]
	Fall	12-16	5.0	33.7	182[e]	111
	Winter	9-12	<0.22[f,i]	<1.6[f]	5.7	0.50[f,i]
	Spring	3	2.5	<1.4f	87.3[e]	8.6[f]

[a-d]See Table 1
[e]Significantly different than winter and summer at P<0.05 by Students "t" test.
[f]Significantly different than summer or fall at P<0.05
[g]Significantly different than summer at P<0.05
[h]Significantly different than fall at P<0.05
[i]Significantly different than spring at P0.05

Table 8. Comparison of "accumulation" ratios by indicator

Indicator	GM of shellfish/bottom water ratios			
	Summer	Fall	Winter	Spring
Fecal coliforms	2.1[b]	5.2[d]	<0.22[e]	2.5[f]
Enterococci	12.9[c]	33.7[e]	<1.6[e]	<1.4[e]
F phage	<4.6[b]	182.	5.7	87.3
C. perfringens	38.3	111	0.50[f]	8.6

[a]Geometric means of indicator ratios for shellfish per 100g/bottom water per 100 ml.
[b]Significantly <enterococci and C. perfringens
[c]Significantly < C. perfringens
[d]Significantly < other indicators
[e]Significantly < F phage and C. perfringens
[f]Significantly < F phage
See table 6 for other footnotes

LABORATORY EXPERIMENTS

Three experiments were conducted in the fall and early winter of 1986 in order to examine retention/survival of the indicators. The quahogs, which had been maintained at ambient water temperatures for several weeks, were placed in the refrigerator for 24 hours. Then, they were held in tanks containing sewage contaminated water at 11°C for 72 hours, after which they were transferred to tanks of "clean" water maintained at 2.5-5.0°C for 2-4 months. Assays for the indicator levels in the water and the animals were performed following uptake and periodically while they were held at the low temperature. The results are shown in (Figure 2). Since by definition there was no reduction in the indicator levels at 0 time, the reductions during the first 14-21 days must have been greater than those indicated by the slopes of the regression lines as drawn. That is, single linear regression lines of the log reduction against time do not describe the relationships, at least for the fecal coliforms and enterococci. Because of this, the 7 hour data were not included in the regression analyses; and the estimated reductions at 14 hours ($\hat{Y}_{14}$) as well as the slopes were compared statistically as shown in Figure 2. The fecal coliform levels in the animals were reduced the most rapidly, and the viruses the least rapidly. The enterococcus and C. perrringens reductions were quite variable, and this was due in part to differences between experiments. This notwithstanding, the virus levels clearly decreased more slowly than did those from the C. perfringens spores. These results were consistent with those of the field data and support the notion that at least some of the virions are located at sites within the animals which are less amenable than those of the bacteria to removal during the feeding process.

One of the issues that could become important in the management of the conditional area is, how early in the spring after the animals resume their normal feeding activity is there elimination of the viruses retained during the winter? This was examined in a single experiment in which the quahogs were exposed to sewage contaminated seawater maintained at 11°C and then transferred to "clean" water at about the ambient temperature of the intake water to the tanks. Uptake was performed early in February; and, the 11°C tank temperature notwithstanding, accumulation of the indicators by the quahogs was in the winter mode (the differences between winter and fall accumulation are examined later in this report). The uptake results from this experiment are shown in Figure 3.

The levels of the fecal coliforms, F phages, enterococci and C. perfringens in the shellfish were about 16,13,120 and 250 times those in the water (W/V), respectively, at the end of the 72 hour uptake period. There was a marked decrease in the levels of fecal coliforms, enterococci and even C. perfringens in the animals within ten days of their transfer to the ambient water (2.5°C) (Figure 4); their levels decreased by about 2.9, 2.1 and 1.1 orders of magnitude, respectively, from those following uptake. The F phage levels, however, decreased only slightly (0.36 logs); and this decrease was even less than that obtained with the C. perfringens spores. The F phages concentrations in the animals remained relatively constant until March 20 when there was an appreciable, although not significant, decrease in their levels. At this time, the fecal coliform and enterococcus levels were below the sensitivities of the assays, and that for C. perfringens was at the sensitivity limit. The decrease in the virus levels on March 20 when the ambient water temperature had risen to 4.5°C was probably real since there was a continued decrease on April 28 when the ambient water temperature had risen to 10°C. The intake water to the tanks

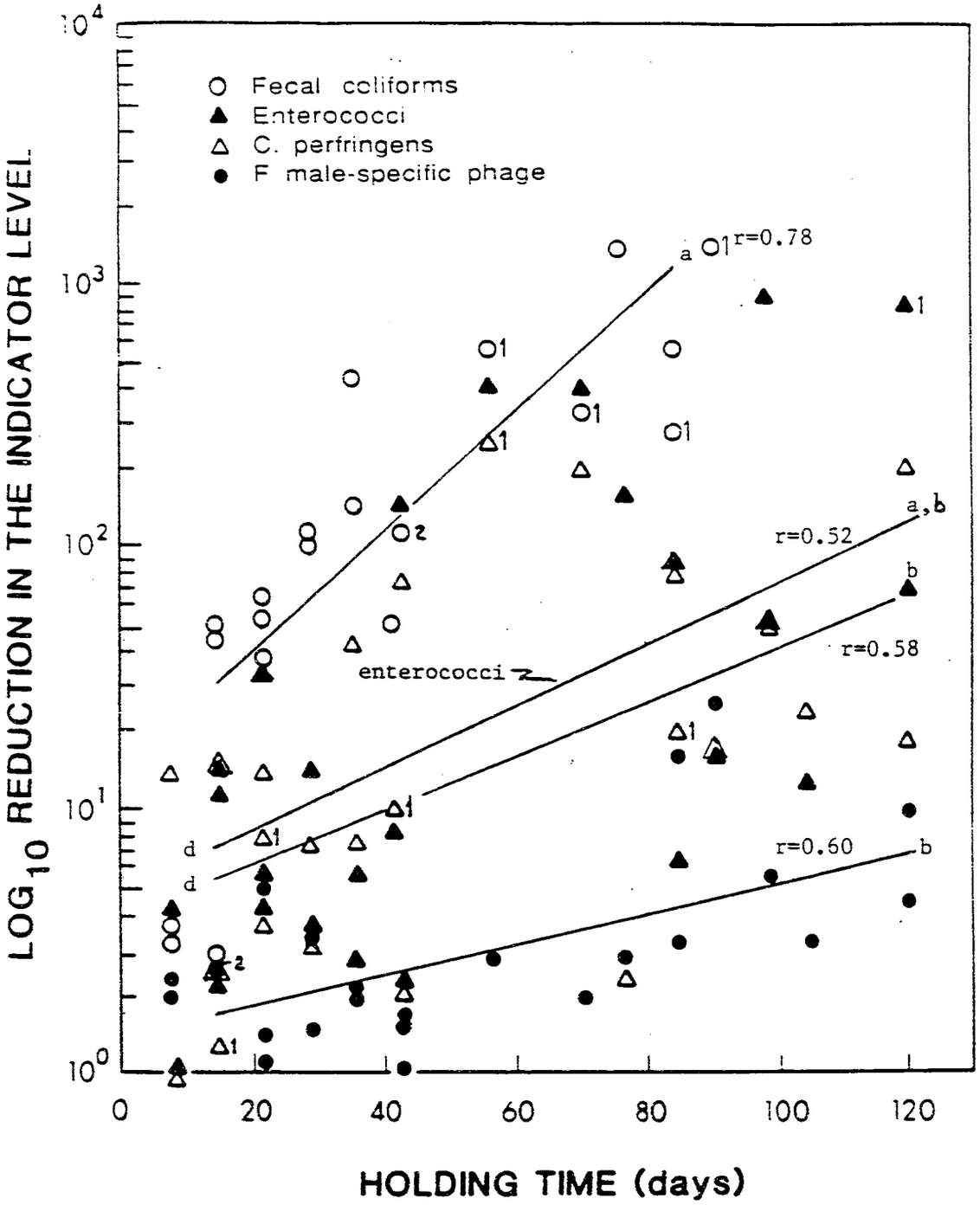

Fig. 2. Log reduction in indicator levels in shellfish maintained in tanks with flowing seawater at 2.5°C. Results from three experiments started 9/8, 10/20 and 10/27/86. | - Reduction greater than value shown, not included in data analysis. Slopes (a-c) and estimated 14 day reductions (d) with same letter not significantly different.

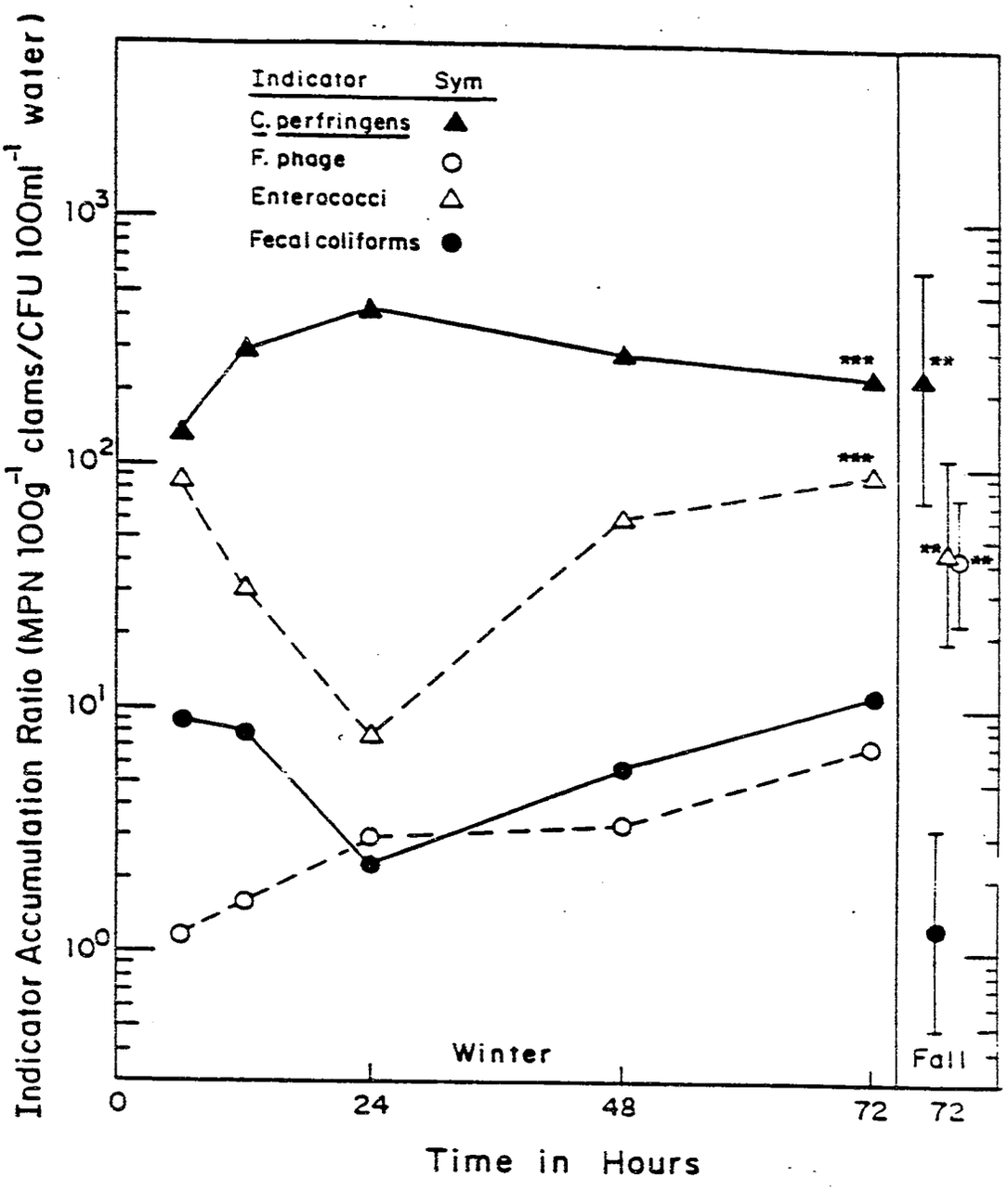

Fig. 3. Comparison of 72 hour accumulation rates for microbial indicators during the winter and fall as obtained in tank experiments. Animals held in sewage contaminated water at 11°C. **, ***, significantly different from accumulation of fecal coliforms at $P < 0.01$ and < 0.001, respectively.

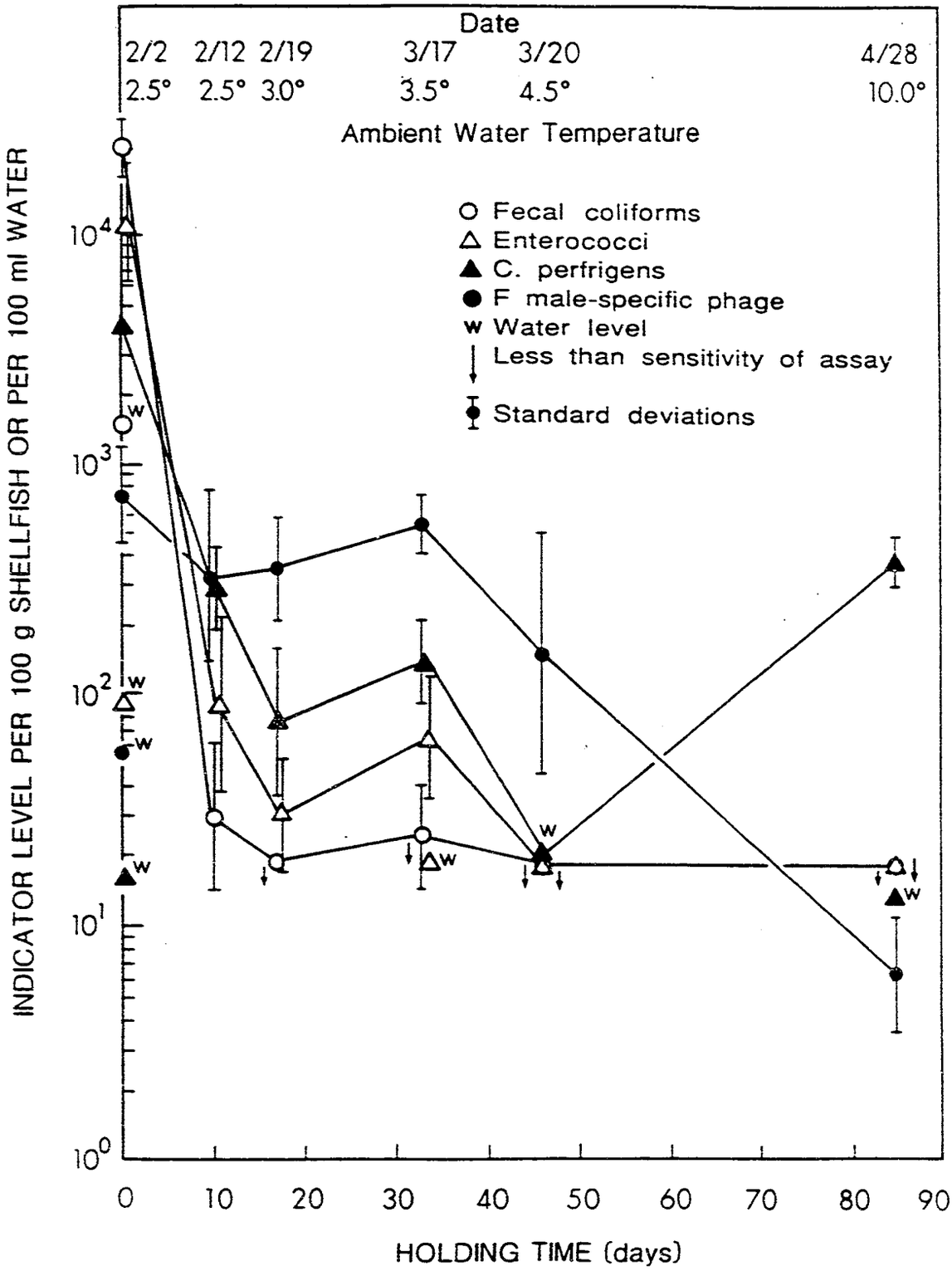

Fig. 4. Indicator levels in sewage-contaminated quahogs held in tanks at the ambient water temperatures during the winter and early spring.

contained low levels of C. perfringens spores during this experiment, but there was no indication of their accumulation by the shellfish until April 28. This too suggests much lower feeding activity prior to April.

The uptake data from the cold weather retention experiments suggested that the greatest accumulation of the F phages occurred during fall. A similar, but not identical, experiment was performed in late January with animals which had been held at ambient temperatures. They were acclimated for 24 hours in water at 11°C after which sewage was fed into the flowing water for an additional 72 hours. Assays for the indicator levels in the water and the animals were performed after 6,12,24,48 and 72 hours of uptake, and the results are shown in Figure 3 along with the uptake data from the cold temperature retention study performed the previous fall. The accumulation ratios for C. perfringens, enterococci and fecal coliforms where comparable to or greater than those obtained during the fall laboratory experiments and higher than those seen from the field data for the spring and summer. This was not so of the viruses, indicating that the requirements for viral accumulation by the shellfish are different from those for the bacteria.

A long term uptake study was conducted with periodic examination of the indicator levels in the water and shellfish. This was done in an attempt to identify seasonal and/or temperature effects on uptake which could explain the field data and to verify the high virus accumulation observed in the laboratory experiments conducted the previous fall. The accumulation factors (indicator level in shellfish $100g^{-1}$/level in water $100m^{-1}$) and the levels in the water are given by date and water temperature in Table 9. Overall, accumulation of the F phages by the animals was comparable to but not greater than that of the fecal coliforms. Both were significantly less than those for the enterococci and C. perfringens which were significantly different from each other. The accumulation ratios resembled those observed during the fall of the previous year (Figure 4) with the notable exception that the high ratios for the viruses were not obtained. The accumulation ratios of the indicators when compared to each other also resembled the field data collected during the summer but not the fall or spring (Table 7).

Inspection of the data in Table 9 suggests that the accumulation of the indicators by shellfish is affected by the indicator levels in the water as well as water temperature. These relationships were examined for all the indicators by linear regression analysis. When the correlation to the tank water temperature was examined, an analysis was also performed in which the data from the experiments in which the water levels were < or > one standard deviation from the mean were excluded. When the relationship of accumulation to the water level of the indicators was examined, only data from the experiments conducted at tank water temperatures between 11 and 20.5°C were included. The results of these analyses also are shown in Table 9.

The correlation coefficients (r values) were somewhat improved by limiting the data used as noted above. Nevertheless, they were quite low, the regression lines generally accounting for less than 30 percent of the data. It can be seen from (Table 10), which summarizes the virus accumulation/retention data from the field monitoring and laboratory experiments, that some factor which was responsible for the high virus accumulations during the fall of 1987 in the bay and in the tanks during the fall of 1986 was not operative in the tanks in 1987.

Table 9. Accumulation ratios from laboratory (tank) experiments. March 1987 - January 1988.

Date 1987	Water[a] Temp, °C	Level in shellfish/level in water[b] (Indicator level in water/100 ml)			
		FC	Enterococci	F phage	C. perfringens
3/26	15.0[oc]	5.5 (76.5)[d]	150 (36.0)	9.9 (95)	210 (4.5)[d]
4/2	15.0[oc]	5.2 (75.0)[d]	150 (73.0)	4.6[d] (35)	390 (3.5)[d]
4/14	15.0[oc]	1.7 (465)	4.7 (192)	0.37 (2000)[e]	28 (16.5)
4/28	15.0[oc]	3.6 (280)	23 (112)	23 (70)	190 (14.5)
5/12	16.0	11.0 (265)	36 (183)	0.60 (225)	240[e] (19)
5/20	15.5	2.1 (285)	6.7 (43.0)	ND[f]	220 (11.0)
5/26	17.0	4.9 (310)	13 (39.5)	2.7 (445)	97 (16.5)
6/2	23.0	3.4 (420)	10 (64.5)	0.46 (90)	140 (7.5)
6/15	20.5	5.5 (140)	130 (5.0)[d]	9.5 (110)	75 (18.0)
6/29	22.0	1.1 (2240)[e]	1.6 (166)	0.91 (1100)[e]	22 (23.5)[e]
7/13	21.5	2.9 (60.0)[d]	6.4 (3.0)[d]	3.1[d] (24)	70 (5.0)
8/3	23.0	0.81 (600)	10 (46.5)	0.68 (117)	140 (3.0)[d]
8/18	27.0	0.80 (825)	0.92 (70)	0.23 (164)	25 (7.0)
8/31	21.0	< 4.9 (1370)[e]	8.0 (90)	1.1 (115)	31 (14)

10/6	14.0	0.95[e] (1040)[e]	15.7[e] (495)[e]	2.5 (110)	> 96[g] (<10)
1/21/88[g]	10.7	3.7 (94.5)[d]	17 (19)	6.0 (644)[e]	110 (5.5)
Accumulation X		3.5[h]	36.4[i]	4.4[h]	130
Water level GM		316	55	165	9.3
SD		105-955	15-205	49-561	4.9-18
r for Acc vs WT[j]					
All data		-0.29(16)	-0.25(16)	-0.39(15)	-0.47(15)
omit d,e,g,		-0.39(9)	-0.55(8)	-0.48(10)	-0.49(9)
r for Acc vs WL[k]					
All data		-0.50(15)	-0.26(16)	-0.30(15)	-0.39(15)
11-20.5°C		-0.46(9)	-0.55(9)	-0.51(8)	-0.72(8)

[a] Temperature in the experimental tanks; except as noted about the same as the ambient water temperature.
[b] Indicator accumulation ratio, level per 100 g in shellfish/level per 100ml in water; water level is that at time shellfish samples taken.
[c] Water maintained at 15°C
[d] Level < one SD from mean for all experiments
[e] Level > one SD from mean for all experiments
[f] No data
[g] Not included in analysis
[h] Significantly less than enterococci and C. perfringens
[i] Significantly less than C. perfringens
[j] Linear correlation coefficients for accumulation ratio against water temperature
[k] Linear correlation coefficients for accumulation ratio against indicator level in water

Table 10. Summary of accumulation/retention data for F phages

Date	Data Source[a]	Stn	Temp °C	Level/100 ml or gm Water	Level/100 ml or gm Shellfish	Shellfish/Water
10/7-11/23, 1987	Field	A	16-6	4.8	1,440	201
		B	16-6	4.2	1,100	204
		C	16-6	1.4	246	182
9/8/86	Tank		11	2440	125,000	51.2
10/20/86	Tank		11	770	17,600	22.9
10/27/86	Tank		11	225	14,300	63.6
10/6/86	Tank		14	110	270	2.5
01/21/88	Tank		10-7	644	3,860	6.0

[a]Field data from Tables 1, 4, and 7. 1986. Tank data summarized in Figure 3. 1987 Tank data from Table 9.

DISCUSSION

It is clear that the levels of F-male specific bacteriophages, which were used as a simulant for the Norwalk viruses as regards their environmental behavior, were greater in both the bottom and surface waters of the conditional shellfish growing area than were those of the coliforms. This is easily explained by the better survival of the F phages during chlorination. The differences were greatest during the winter, and this observation is consistent with the better survival of the viruses during transport in the Providence River and Upper Narragansett Bay during the winter (Cabelli, 1988). The higher F phage levels in the water in the conditional growing area become even more striking when their relative numbers in prechlorinated sewage effluents are considered (Cabelli, 1988).

The greatest differences in the levels of the fecal coliforms and the F phages, however, are in the shellfish themselves, especially during the fall but also during the winter and early spring. The markedly higher shellfish/bottom water ratios for the F phages in the fall than in the other seasons suggests increased accumulation. The laboratory studies did not identify the responsible factor or factors. Changes in the food supply or some subtle effect of temperature are possible explanations. Two additional factors that can be used to explain the higher shellfish/water ratios observed with the F phages than the fecal coliforms are differential die-off of the coliforms and better retention of the F phages in the quahogs. Both of these factors are supported by the data obtained from the laboratory experiments.

The data obtained in the present investigation, using the F male-specific bacteriophages as simulants for the Norwalk viruses, add to an already voluminous literature on the comparative behavior of viruses and coliforms. The former are more resistant to chlorination, at least to chloramines, (Scarpino et. al., 1972); they survive better in marine waters (Fattal et al, 1983); and they are accumulated by shellfish to higher levels and retained by them for longer periods of time (Liu et al, 1966). This leaves the most important question of all. Is there also an appreciable, significant, and unacceptable risk of viral gastroenteritis or even more serious viral diseases from the consumption of clams harvested from approved or conditional areas under the conditions noted above? Because of the much higher F phages than fecal coliform levels in the quahogs when compared to much lower F phages than fecal coliforms in prechlorinated sewage, we speculate that people do become ill with acute gastroenteritis from the consumption of shellfish harvested from some approved areas during the late fall, winter and early spring. Of particular concern are rainfall-managed, CSO-impacted conditional growing areas such as the one in upper Narragansett Bay. A definitive answer to this question can only come from controlled, prospective epidemiological investigations using quahogs harvested under those conditions. The results from such studies is a critical need for the management of shellfish resources.

The poor correlations obtained between the indicator levels in the surface or bottom waters to those of the homologous indicators in the shellfish and especially of the F phages argue against the use of indicator levels in the water as the basis for growing areas classification. The measurement of fecal coliform

or even enterococcus levels in the shellfish is not appropriate for the reasons stated in the Introduction. The measurement of the F phages or possibly C. perfringens densities in the shellfish themselves appears to offer the best hope as a parameter which better reflects the risk of shellfishborne infectious disease.

Since the levels of Norwalk viruses present in the sewage, water or shellfish could not be measured, we attempted to estimate the numbers of these agents using some available information and some not unreasonable assumptions. The calculations suggest that the numbers of Norwalk virions in the prechlorinated sewage were about one-third of those of the F phages and that the number in shellfish harvested from the conditional area during the fall were about 2.7/gm shellfish. The information or assumptions used are:

1. The rate of acute gastroenteritis in the general population is about 14.4 cases/1000 persons/per week or about 2.1 cases/1000 persons/day (Cabelli, 1983); at least 40 percent of the cases are caused by the Norwalk (Centers for Disease Control, 1987); and the duration of the illness is about 2.5 days. Thus, the number of Norwalk virus shedders is estimated at 2.1/1000 persons.

2. An individual ill with viral gastroenteritis sheds about 3.2×10^9 Norwalk virions/gm feces (Blacklow et al, 1972), and an individual passes about 150 g of feces/day.

3. The average water usage (flow/sewered population) for those STPs which discharge directly or indirectly into the Providence River is about 910,000 liters/1000 persons/day.

4. It is assumed that the Norwalk virus levels in the influents to STPs are reduced by secondary treatment similarly to that of the F phages, a mean reduction of about 1.5 orders of magnitude.

5. The mean phage level in the prechlorinated effluents from secondary treatment STPs is about 1.1×10^4 PFU/100 ml, and the mean levels in the shellfish harvested from the conditional area during the fall is 8.4 PFU/gm.

6. The F male-specific bacteriophages simulate the behavior of the Norwalk Viruses in the water and in the shellfish.

The estimated mean Norwalk level in the prechlorinated effluent is obtained as:

$[(2.1 \times 150 \times 3.2 \times 10^9)/9.1 \times 10^5]/32$ or 3.5×10^3 virions/100 ml)

The level in the shellfish harvested from conditional areas (stations B and C) during the fall when the F phage level was 8.4 PFU/gm are estimated as:

$(3.5 \times 10^3/1.1 \times 10^4) \times 8.4$ or 2.7 virions/gm shellfish)

It is assumed that six shellfish of about 30 g each are consumed at a "sitting" to yield an ingested dose of about 480 Norwalk virus virions.

It would seem reasonable from the results of the bathing beach studies that the ingestion of 480 Norwalk virions would produce an appreciable and epidemiologically measurable rate of acute gastroenteritis in a population in which one-third to one-half were children less than 10 years of age since the studies clearly showed children to be the group most susceptible to the illness by far. Children, under ten years of age, however, infrequently consume raw shellfish; and, because of this, we would expect a lower, but nevertheless measurable illness rates from the consumption of such shellfish in an epidemiological study.

The F male-specific bacteriophages would be an effective substitute for the fecal coliform indicator system. Even though they are not found consistently and in high numbers in human feces, these viruses are consistently found in sewage and septic tank contents; and, at least in this country, these are the only sources of human fecal wastes. Thus, a major output of the on-going epidemiological studies could be a criterion based on the levels of the F male-specific bacteriophage levels in the shellfish themselves. There is a need for more data on the levels of the F male-specific phages in shellfish harvested from the conditional and the open areas south of it. At least one station within the conditional area should be selected for intensive monitoring of the bottom water as well.

The abandonment of the fecal coliform standard in the absence of an epidemiologically derived alternative would probably be considered premature by some workers in the field. That it has outlived its usefulness and needs to be replaced can be seen from the history of recent outbreaks of shellfish-associated illness, especially acute gastroenteritis, and data on bacterial-viral relationships such as those produced by this study. These were not unexpected since the development of the standard based upon this bacterial indicator was in response to outbreaks of a bacterial disease, typhoid fever, at a time when wastewater chlorination was not common. The numerical value was both arbitrary and unsupported by epidemiological data. Shellfishborne typhoid fever has disappeared, and the fecal coliform standard may have contributed to its disappearance. We are more inclined to attribute its disappearance to four other factors, the high infectious dose of its etiologic agent, the decrease in the number of shedders (active cases and carriers) in the U.S., the introduction of wastewater chlorination for estuarine and near-coastal discharges and, above all, the prohibition against harvesting shellfish in shadow of an outfall.

The epidemiological data clearly show that, with regard to wastewater derived pathogens, the concern today is with two diseases caused by viruses. Because of the demonstrated differences between viruses and fecal coliforms with regard to die-off during chlorination, transport in marine waters and residence in the shellfish, as well as uptake and retention by the shellfish, a viral indicator is needed and the levels in the shellfish themselves should be measured. If we accept the premise that the fecal coliform standard should be replaced as soon as possible because it inadequately indexes the risk of viral disease and that the levels of F male-specific bacteriophages in the shellfish is the best alternative available, we might speculate from the data obtained as to the numerical limit (standard) that might be used. This would provide the basis for further research as we await the results of the epidemiological studies.

We tentatively conclude, based on the F phage levels in the shellfish relative to those in the prechlorinated effluents, that shellfish should not be harvested from station A, that they could be harvested from the conditional area only during the summer and that station E should be open for the harvesting of shellfish. This would require an F phage standard in excess of 12.5 but less than 30 PFU/100 gm shellfish. Using the foregoing calculations, the 30 PFU/100 gm would extrapolate to an ingested dose of about 17 virions of the Norwalk virus. The USEPA, at their level of concern, was willing to accept 19 cases of swimming-associated gastroenteritis when it published its recommended guideline for marine recreational waters. Thus, an unacceptable illness rate from an epidemiological study might be a differential rate of 19 cases of acute gastroenteritis/1000 persons, when quahogs containing about 30 PFU/100 g and control shellfish from a pristine area are fed to a population of first time shellfish consumers at an inland location. This expectation could be examined in the course of some further epidemiological studies conducted with quahogs taken from the conditional area when it is open to harvesting according to the existing management practices. In view of the importance of the findings to Rhode Island and the USEPA, this study should be a high priority item for the Narragansett Bay Project.

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APPENDIX A

Table A-1. Indicator levels in surface water, bottom water, sediment, and shellfish, samples collected from station A during the summer.

Date[a]	Status[b]	Sample[c] Type	GM Indicator level/100ml or 100g[d] FC	Entero-cocci	C. perfringens	F phage	Temp C°
6/23-24/86	open	SW(3)	4 (1.2-12.8)	< 0.9 (0.3-2.2)	36.6 (14.4-93)	18 (8.8-37)	18.0
		BW(3)	1.3 (0.4-4.4)	1.7 (0.7-4.3)	71.4 (59-86)	6.9 (2.8-17)	
		Sediment	<60	120	359	ND	
		Shellfish	2	23	3500	40	
6/30-7/1/86	Open	SW(3)	21.6 (15-31.5)	<0.5 -	34.4 (25-48)	32.7 (9.2-116)	18.0
		BW(3)	4.4 (1.5-13)	0.8 (0.5-1.2)	68.3 (50-93)	10.4 (7.0-15.6)	
		Sediment	<76.5	<22.8	3300		
		Shellfish	2.0	11	1200	6.2	
7/8-9/86	Closed						
		SW(3)	3.7 (1.5-9.6)	<0.5 -	41.7 (18.5-94.0)	19.3 (9.0-41.4)	19.6
		BW(3)	3.7 (1.7-8.6)	0.6 (0.4-0.9)	53.3 (30-95)	4.8 (2.2-10.4)	
		Sediment	<77.6	233	3260		
		Shellfish	1.8	21	500	156	
GM	Open	SW	9.2	<0.7	35.5	24.3	
		BW	2.5	1.2	69.8	8.5	
		Sediments	<67.7	165.4	1088.4	ND	
		Shellfish	2.0	16.0	2049	16	
GM	Closed	SW	3.7	<0.5	41.7	19.3	
		BW	3.7	0.6	53.3	4.8	

Sediments	<77.6	233	3260	
Shellfish	1.8	21	300	156

[a] Shellfish and sediment samples collected on second day
[b] Whether conditional area was open or closed to the harvesting of shellfish
[c] SW-surface water; BW-bottom water; ( ) number of samples
[d] <, at least half of samples less than sensitivity limits of assay; some samples less than sensitivity limits of assay; ( ) upper and lower 1 SD
[e] Indicator levels of samples when conditional area closed to the harvesting of shellfish significantly different at ($P<0.05$).

Table A-2. Indicator levels in surface water, bottom water, sediment, and shellfish, samples collected from station B during the summer.

Date[a]	Status[b]	Sample[c] Type	GM Indicator level/100ml or 100g[d] FC	Entero-cocci	C. perfringens	F phage	Temp C°
6/23-24/86	Open	SW(3)	1.4 (0.8-2.6)	1.7 (1.0-2.8)	16.5 (13-21)	7.0 (4.0-12)	18.0
		BW(3)	0.7 (0.4-1.4)	0.9 (0.5-1.6)	15.1 (8.6-27)	2.5 (1.7-3.8	
		Sediment	<68.1	<68.1	1430	ND	
		Shellfish	8	5	790	<20	
6/30-7/1/86	Open	SW(3)	2.4 (1.0-5.7)	<0.6 (0.4-0.9)	14.1 (7.6-26)	10.0 (6.4-16)	18.0
		BW(3)	0.63 (0.4-0.9)	1 (0.5-1.2)	9.3 (6.4-14)	5.7 (3.2-10)	
		Sediment	<56.5	480	2050	ND	
		Shellfish	2	7.8	600	< 6.25	
7/8-9/86	Closed	SW(3)	3 (2.0-4.5)	1.0 (0.7-1.3)	20 (19.5-20.5)	5.8 (4.5-7.7)	19.6
		BW(3)	1 (0.5-2.0)	0.5 -	8.0 (4.5-14)	4.2 (2.4-7.3)	
		Sediment	<39.3	78.6	1530	ND	
		Shellfish	< 2.0	8	330	42	
GM	Open	SW	1.9	1.0	15.3	8.3	
		BW	0.7	1.0	11.9	3.8	
		Sediments	<62	<181	1712		
		Shellfish	4.0	6.2	688.5	<11.2	
GM	Closed	SW	3.0	1.0	20	5.8	
		BW	1.0	0.5	8.0	4.2	

Sediments	<39.3	78.6	1530	
Shellfish	< 2.0	8	330	42

[a]Shellfish and sediment samples collected on second day
[b]Whether conditional area was open or closed to the harvesting of shellfish
[c]SW-surface water; BW-bottom water; ( ) number of samples
[d]<, at least half of samples less than sensitivity limits of assay; some samples less than sensitivity limits of assay; ( ) upper and lower 1 SD
[e]Indicator levels of samples when conditional area closed to the harvesting of shellfish significantly different at ($P<0.05$).

Table A-3. Indicator levels in surface water, bottom water, sediment, and shellfish, samples collected from station C during the summer.

Date[a]	Status[b]	Sample[c] Type	GM Indicator level/100ml or 100g[d] FC	Entero-cocci	C. perfringens	F phage	Temp C°
6/23-24/86	Open	SW(3)	1.1 (0.9-1.4)	1.0 (0.5-2.0)	8.9 (5.6-14)	3.9 (1.1-14)	18.0
		BW(3)	0.6 (0.4-0.9)	0.72 (0.4-1.4)	7.6 (5.5-10.5)	1.3 (0.8-1.9)	
		Sediment	<37	<37	306	ND	
		Shellfish	5	6.2	460	<20	
6/30-7/1/86	Open	SW(3)	0.5 -	<0.5 -	12.8 (7.1-23)	8 (5-13)	18.0
		BW(3)	0.72 (0.4-1.4)	< 0.6 (0.4-0.9)	14.9 (9.2-24)	7.1 (3.2-16)	
		Sediment	<34.8	105	900	ND	
		Shellfish	<2.0	13	500	<6.25	
7/8-9/86	Closed	SW(3)	1.6 (1.0-2.4)	< 0.5 -	18.4 (14.4-24)	3.4 (2.1-5.5)	19.6
		BW(3)	1.2 (0.5-2.8)	<0.8 (0.4-1.8)	7.6 (6.6-8.8)	1.3 (0.8-1.9)	
		Sediment	<70.6	<70.6	635	ND	
		Shellfish	2	9.3	600	<8.3	
GM	Open	SW	0.8	0.7	10.7	5.6	
		BW	0.7	0.7	10.6	3.0	
		Sediments	<35.9	<62.3	547.6		
		Shellfish	3.2	9.0	480	<11.2	
GM	Closed	SW	1.6	<0.5	18.4	3.4	
		BW	1.2	0.8	7.6	1.3	

Sediments	<70.6	<70.6	635	
Shellfish	2.0	9.3	600	< 8.3

[a]Shellfish and sediment samples collected on second day
[b]Whether conditional area was open or closed to the harvesting of shellfish
[c]SW-surface water; BW-bottom water; ( ) number of samples
[d]<, at least half of samples less than sensitivity limits of assay; some samples less than sensitivity limits of assay; ( ) upper and lower 1 SD
[e]Indicator levels of samples when conditional area closed to the harvesting of shellfish significantly different at ($P<0.05$).

Table A4. Indicator levels surface water, bottom water, sediment, and shellfish, samples collected from station A during the fall.

Date[a]	Status[b]	Sample[c] Type	GM Indicator level/100ml or 100g[d] FC	Entero-cocci	C. perfringens	F phage	Temp C°
10/6-7/87	Closed	SW(3)	9.1 (1.8-46)	2.2 (0.9-5.2)	37.1 (20-70)	14.4 (3.7-56)	15.8 ± 0.6
		BW(3)	<0.63 (0.4-0.9)	3.0 (1.1-7.9)	14.2 (9.9-20)	1.8 (1.0-3.2)	16.1 ± 0.5
		Shellfish	4.5	49.0	190	866	
10/15/87	Open	SW(1)	4.0	<0.5	22.0	350	14.0
		BW(1)	1.0	<0.5	5.5	13	14.2
		Shellfish	1.9	11.0	830	857	
10/29/87	Closed	SW(1)	110	0.5	79.5	21.0	13.1
		BW(1)	10.5	2.5	9.5	1.0	13.0
		Shellfish	310	58.0	855	81.0	
11/19/87	Closed	SW(1)	29.0	<0.5	152	1410	10.0
		BW(1)	6.0	2.5	34.0	18.0	9.2
		Shellfish	43.0	27.5	3500	4850	
11/23/87	Open	SW(1)	5.5	9.5	41.5	106	6.2
		BW(1)	3.0	0.5	34.0	44	6.2
		Shellfish	53.0	51.5	1650	21400	
GM	Open	SW	4.7	2.2	30.2	193	
		BW	1.7	0.5	13.7	24	
		Shellfish	10.0	23.8	1170	4282	

GM	Closed	SW	19.0	1.2	57.2	39.0
		BW	1.7	4.7	15.6	2.5
		Shellfish	39.1	42.8	826	698

[a]Shellfish and sediment samples collected on second day
[b]Whether conditional area was open or closed to the harvesting of shellfish
[c]SW-surface water; BW-bottom water; ( ) number of samples
[d]<, at least half of samples less than sensitivity limits of assay; , some samples less than sensitivity limits of assay; ( ) upper and lower 1 SD
[e]Indicator levels of samples when conditional area closed to the harvesting of shellfish significantly different at ($P<0.05$).

Table A-5. Indicator levels in surface water, bottom water, sediment, and shellfish, samples collected from Station B during the fall.

Date[a]	Status[b]	Sample[c] Type	GM Indicator level/100ml or 100g[d] FC	Entero-cocci	C. perfringens	F phage	Temp C°
10/6-7/87	Closed	SW(2)	<1.3	1.3	28	4	15.6 ± 0.3
		BW(2)	1.0	5.0	19	3.5	
		Shellfish	ND	ND	ND	ND	
10/15/87	Open	SW(1)	1.0	<0.5	20	73	13.7
		BW(1)	4.0	<0.5	1	3.0	14.3
		Shellfish	<1.8	<18.0	130	25.5	
10/29/87	Closed	SW(1)	26.5	2.5	29.5	2.0	12.7
		BW(1)	3.5	2.0	7.0	1.0	12.7
		Shellfish	110	170	1000	2122	
11/19/87	Closed	SW(1)	38.0	0.5	113.5	722	9.5
		BS(1)	5.0	0.5	15.5	10.0	9.0
		Shellfish	2.9	38	2400	4383	
11/23/87	Open	SW(1)	4	15	25	16	5.9
		BW(1)	3.5	<0.5	25	29	6.3
		Shellfish	47.5	25.0	2145	6320	
GM	Open	SW(1)	2.0	2.7	22.4	34.2	
		BW(1)	3.7	<0.5	5.0	9.3	
		Shellfish	9.2	21.2	528	401	
GM	Closed	SW(1)	5.6	1.2	37.9	11.5	
		BW(1)	1.9	2.2	14.0	2.8	
		Shellfish	17.9	80.4	1549	3050	

[a] Shellfish and sediment samples collected on second day
[b] Whether conditional area was open or closed to the harvesting of shellfish
[c] SW-surface water; BW-bottom water; ( ) number of samples
[d] <, at least half of samples less than sensitivity limits of assay; some samples less than sensitivity limits of assay; ( ) upper and lower 1 SD
[e] Indicator levels of samples when conditional area closed to the harvesting of shellfish significantly different at ($P<0.05$).

Table A-6. Indicator levels in surface water, bottom water, sediment, and shellfish, samples collected from station C during the fall.

Date[a]	Status[b]	Sample[c] Type	GM Indicator level/100ml or 100g[d] FC	Entero-cocci	C. perfringens	F phage	Temp C°
10/6-7/87	Closed	SW(3)	0.63 (0.42-0.94)	<0.63	9.1 (5.7-14.4)	<1 -	15.6 ± 0.4
		BW(3)	<0.72 (0.38-1.4)	0.63 (0.42-0.94)	3.8 (1.5-9.3)	1 -	15.5 ± 0.1
		Shellfish	2.0	49.0	330	119	
10/15/87	Open	SW	<0.5	<0.5	1	3	14.2
		BW	<0.5	<0.5	6	1	13.8
		Shellfish	2.0	<18	600	208	
10/29/87	Closed	SW	3.5	1.0	3.0	<1	12.8
		BW	1.0	<0.5	1.5	<1	12.6
		Shellfish	3.25	52.5	945	46.5	
11/19/87	Closed	SW	1.0	0.5	50.5	128	9.7
		BS	2.5	1.5	8.5	1	9.0
		Shellfish	9.4	9.4	2050	1053	
11/23/87	Open	SW(1)	2.0	4.5	14	21	6.0
		BW(1)	1.5	1	11	12	6.4
		Shellfish	17.5	6.95	1400	738	
GM	Open	SW	1.0	1.5	3.7	7.9	
		BW	0.9	0.7	8.1	3.5	
		Shellfish	5.9	11.2	917	392	
GM	Closed	SW	1.0	0.7	10.3	2.6	
		BW	1.0	0.7	3.7	1.0	
		Shellfish	3.9	29.0	861	180	

[a] Shellfish and sediment samples collected on second day

[b] Whether conditional area was open or closed to the harvesting of shellfish

[c] SW-surface water; BW-bottom water; ( ) number of samples

[d] <, at least half of samples less than sensitivity limits of assay; some samples less than sensitivity limits of assay; ( ) upper and lower 1 SD.

[e] Indicator levels of samples when conditional area closed to the harvesting of shellfish significantly different at ($P<0.05$).

Table A-7. Indicator levels in surface water, bottom water, sediment, and shellfish, samples collected from station D during the fall.

Date[a]	Status[b]	Sample[c] Type	GM Indicator level/100ml or 100g[d] FC	Entero-cocci	C. perfringens	F phage	Temp C°
10/6-7/87	Closed	SW(2)	0.5	0.5	2.25	<1	15.5
		BW(2)	<0.5	<0.5	4.25	<1	15.7 ± 0.5
		Shellfish	ND	ND	ND	ND	
10/15/87	Open	SW	<0.5	<0.5	6.5	3	13.4
		BW	<0.5	<0.5	4.5	<1	13.8
		Shellfish	<1.8	<1.8	40	9	
11/19/87	Closed	SW	0.5	<0.5	5.5	6.0	9.3
		BS(1)	1.5	5.0	5.0	1	9.0
		Shellfish	2.0	15	1045	659	
11/23/87	Open	SW	1.0	<0.5	6.5	20	5.8
		BW	2.5	7.5	0.5	2.0	5.3
		Shellfish	20	14	790	2,625	
GM	Open	SW	0.7	<0.5	6.5	7.7	
		BW	1.1	1.9	1.5	1.4	
		Shellfish	6.0	5.0	178	154	
GM	Closed	SW	0.5	0.5	3.0	1.8	
		BW	0.7	1.1	4.2	1	
		Shellfish	2.0	15	1045	659	

[a] Shellfish and sediment samples collected on second day
[b] Whether conditional area was open or closed to the harvesting of shellfish
[c] SW-surface water; BW-bottom water; ( ) number of samples
[d] <, at least half of samples less than sensitivity limits of assay; some samples less than sensitivity limits of assay; ( ) upper and lower 1 SD

[e]Indicator levels of samples when conditional area closed to the harvesting of shellfish significantly different at ($P<0.05$).

Table A8 . Indicator levels in surface water, bottom water, sediment, and shellfish, samples collected from station A during the winter.

Date[a]	Status[b]	Sample[c] Type	GM Indicator level/100ml or 100g[d] FC	Entero-cocci	C. perfringens	F phage	Temp C°
1/27-28/86	Closed	SW(4)	32.4 (4.1-255)	25.7 (60-109)	221 (101-486)	47.7 (12.1-186)	3.0
		BW(4)	20.1 (2.2-181)	11.9 (3.8-37.4)	336 (112-1004)	25.3 (15-43)	
		Sediment	<46	<46	5670	ND	
		Shellfish	4.0	ND	170	300	
2/10-11/86	Open	SW(3)	6.6 (1.4-30.2)	1.35 (0.4-4.4)	49.2 (20.4-118.5)	228 (120-434)	2.0
		BW(3)	6.6 3.1-14)	1.0 0.5-2.0)	85 (77-93)	25 (9.4-66)	
		Sediment	<44.5	<44.5	3100	ND	
		Shellfish	< 2.0	< 2.0	45	125	
2/17-18/86	*Day 1 open Day 2 closed	SW(3)	63.3 (30.8-130)	8.45 (5.4-13.3)	294 (179-482)	99.6 (15-663)	1.8
		BW(3)	14.6 (9.7-22)	3.2 (1.5-6.8)	165 (59.3-457)	10.6 (3.4-35)	
		Sediment	<16.7	<16.7	741	ND	
		Shellfish	< 2.0	< 2.0	79.7	175	
3/2-3/86	Open	SW(3)	1.08 (0.5-2.4)	0.63 (0.4-0.9)	165.1 (108-254)	117.3 (40-342)	2.1
		BW(3)	7.3 (5.4-9.8)	1.44 (1.0-2.0)	58.5 (48-71.3)	11.3 (6.9-18.5)	
		Sediment	<14.2	<14.2	3073	ND	
		Shellfish	< 2.0	<2.0	40	68.8	

GM	Open	SW	3.7	1.2	98.4	112
		BW	7.7	1.5	88.6	15.8
		Shellfish	2.0	<2.0	42.4	92.7
GM	Closed	SW	46.2[e]	19.4[e]	268	86.7
		BW	18.2	6.9[e]	233[e]	20
		Shellfish	2.8	<2.0	116	229

[a] Shellfish and sediment samples collected on second day
[b] Whether conditional area was open or closed to the harvesting of shellfish
[c] SW-surface water; BW-bottom water; ( ) number of samples
[d] <, at least half of samples less than sensitivity limits of assay; some samples less than sensitivity limits of assay; ( ) upper and lower 1 SD
[e] Indicator levels of samples when conditional area closed to the harvesting of shellfish significantly different at ($P<0.05$).

Table A9 . Indicator levels in surface water, bottom water, sediment, and shellfish, samples collected from station B during the winter.

Date[a]	Status[b]	Sample[c] Type	GM Indicator level/100ml or 100g[d] FC	Entero-cocci	C. perfringens	F phage	Temp C°
1/27-28/86	Closed	SW(4)	31.5 (3.1-321)	19.5 (6.2-61)	38.1 (20.5-71)	27.0 (9.5-76.5)	3.0
		BW(4)	10.2 (1.0-102)	5.4 (1.1-27.3)	96.2 (43-215)	19.9 (7.0-57)	
		Sediment	<74	<74	548	ND	
		Shellfish	<2.0	ND	80	225	
2/10-11/86	Open	SW(3)	6.1 (4.5-8.4)	<0.5 (0 -0.5)	20.1 (8.1-50)	69.0 (50-94.4)	2.0
		BW(3)	38.5 (10.9-135)	3.5 (1.6-9.0)	37.5 (19.2-73)	14.0 (4.7-42)	
		Sediment	<24.1	<24.1	1100	ND	
		Shellfish	<2.0	<2.0	9.4	37.5	
2/17-18/86	*Day 1 open Day 2 closed	SW(3)	22 (13.4-36)	4.3 (0.97-19.4)	55.4 (45-68.5)	18.3 (12.3-52)	1.8
		BW(3)	7.3 (2.9-18.7)	1.0 (0.3-3.3)	85.7 (32.4-227)	6.9 (3.4-21.3)	
		Sediment	<16.4	<16.4	1000	ND	
		Shellfish	< 2.0	< 2.0	31.6	150	
3/2-3/86	Open	SW(3)	0.5 -	0.5 -	43 (21.2-87.5)	10.5 (1.8-62)	2.1
		BW(3)	1.1 (0.9-1.4)	0.5 -	52.7 (45-62)	6.0 (2.2-16)	
		Sediment	<18.1	<36.2	2500	ND	
		Shellfish	< 2.0	<2.0	40	18.8	

GM	Open	SW	2.7	0.8	31.3	27.1
		BW	7.2	1.2	47.1	10.8
		Shellfish	<2.0	<2.0	56.6	26.6
GM	Closed	SW	25.6[e]	6.9[e]	45	30.3
		BW	8.4	3.4	96.5[e]	13.5
		Shellfish	<2.0	<2.0	50.3	183.7

[a]Shellfish and sediment samples collected on second day
[b]Whether conditional area was open or closed to the harvesting of shellfish
[c]SW-surface water; BW-bottom water; ( ) number of samples
[d]<, at least half of samples less than sensitivity limits of assay; some samples less than sensitivity limits of assay; ( ) upper and lower 1 SD
[e]Indicator levels of samples when conditional area closed to the harvesting of shellfish significantly different at ($P<0.05$).

Table A10. Indicator levels in surface water, bottom water, sediment, and shellfish, samples collected from station C during the winter.

Date[a]	Status[b]	Sample[c] Type	GM Indicator level/100ml or 100g[d] FC	Entero-cocci	C. perfringens	F phage	Temp C°
1/27-28/86	Closed	SW(4)	26.8 (3.2-226)	12.6 (3.6-43.6)	10.9 (3.7-32)	19.7 (6.4-60.5)	3.0
		BW(4)	13 (1.4-115)	5.5 (1.7-18)	174 (91-330)	10.6 (4.8-23.5)	
		Sediment	<40	<40	417	ND	
		Shellfish	<2.0	ND	40	38	
2/10-11/86	Open	SW(3)	2 (0.6-6.6)	<0.5 -	21.5 (15-30.5)	78 (61-99)	2.0
		BW(3)	6.5 (2.8-15)	0.72 (0.4-1.4)	44.8 (19-104)	44.8 (19-104)	
		Sediment	<25.4	<25.4	681	ND	
		Shellfish	< 2.0	< 2.0	30	87.5	
2/17-18-86	*Day 1 open Day 2 closed	SW(3)	6.9 (1.8-26)	2.1 (1.3-3.5)	80.5 (61-107)	31.0 (12.6-75)	1.8
		BW(3)	5.5 (4.2-7.2)	0.9 -	65 (27-155)	5.1 (2.1-12)	
		Sediment	<13.9	<13.9	1100	ND	
		Shellfish	< 2.0	2	31.6	12.5	
3/2-3/86	Open	SW(3)	1.25 (0.25-6.2)	0.63 (0.4-0.9)	81.5 (56-119)	10 -	2.1
		BW(3)	27.1 (15-49)	1.74 (0.9-3.3)	54.1 (41-71)	13 (11.6-14)	
		Sediment	< 7.1	< 7.1	1700	ND	
		Shellfish	< 2.0	<2.0	20	62.5	

GM	Open	SW	1.6	<0.6	44.0	28.3
		BW	12.2	1.0	48.6	21.2
		Shellfish	<2.0	<2.0	24.5	74.0
GM	Closed	SW	22.0[e]	7.0[e]	22.2	27.2
		BW	9.3	3.0	133.0[e]	8.1
		Shellfish	<2.0	2.0	35.6	22

[a]Shellfish and sediment samples collected on second day
[b]Whether conditional area was open or closed to the harvesting of shellfish
[c]SW-surface water; BW-bottom water; ( ) number of samples
[d]<, at least half of samples less than sensitivity limits of assay; some samples less than sensitivity limits of assay; ( ) upper and lower 1 SD
[e]Indicator levels of samples when conditional area closed to the harvesting of shellfish significantly different at ($P<0.05$).

Table A11. Indicator levels in surface water, bottom water, sediment, and shellfish, sample collected from stations A-C during the spring.

Date[a]	Station[b]	Sample[c] Type	Geometric Mean Indicator level/100 ml or 100g[a] FC	Entero-cocci	C. perfringens	F phage	Temp C°
3/27-28/86	A	SW(3)	13.7 (4.5-41.4)	0.9 (0.3-2.6)	271 (244-301)	85.2 (40-182)	11.6
		BS(3)	1.7 (1.1-2.70)	1.4 (0.5-3.7)	71.6 (57-90)	5.2 (4.1-6.6)	
		Sediment	<18.1	36.1	7442	ND	
		Shellfish	<2.0	<2.0	270	131	
3/27-28/86	B	SW(3)	1.4 (0.5-3.7)	<0.5 _	110 (53-227)	13.8 (4.9-39)	
		BW(3)	0.6 (0.4-0.9)	1.8 (0.9-3.7)	41.1 (34-50)	3.8 (2.5-5.6)	
		Sediment	<12.9	<12.9	2470	ND	
		Shellfish	4	<2	500	2144	
3/27-28/86	C	SW(3)	2.9 (1.0-8.6)	1.1 (0.3-4.4)	139 (117-165)	22 (6.0-80)	
		BW(3)	1.0 (0.3-3.3)	1.1 (0.3-4.8)	36.7 (23-58)	5.0 (3.4-7.5)	
		Sediment	<15.6	46.7	1680	ND	
		Shellfish	2.0	<2	500	238	

[a] Conditional area open.

[b] Whether conditional area was open or closed to the harvesting of shellfish

[c] SW-surface water; BW-bottom water; ( ) number of samples

[d] <, at least half of samples less than sensitivity limits of assay; some samples less than sensitivity limits of assay; ( ) upper and lower 1 SD

*

APPENDIX B

ASSAY METHODS FOR F MALE-SPECIFIC BACTERIOPHAGES

Media

1. Tryptone Broth (per liter)

Bacto Tryptone	10.0 g
Dextrose	1.0 g
NaCl	5.0 g
Distilled water	1000 ml

Dissolve the ingredients and dispense in 100 ml quantities to 250 ml Erlenmeyer flasks and in 10 ml quantities to tubes as required. Autoclave at 121°C for 15 min and store in the refrigerator.

2. Antibiotic solution

Ampicillin	150 mg
Streptomycin sulfate	150 mg
Distilled water	100 ml

Filter sterilize the solution and store in the refrigerator. Prepare fresh every week.

3. Bottom Agar (Tryptone Agar)

Add 15.0 gm of agar to the ingredients in Tryptone broth (for 1 liter of medium). Autoclave at 121°C for 15 min. Cool to 50°C; add 20 ml of the antibiotic solution, mix, and dispense in about 38 ml quantities to sterile 150 x 15 mm petri plates.

4. Double strength (2X) Tryptone Soft Agar

Tryptone	20.0 g
Dextrose	2.0 g
NaCl	1.0 g
Agar	14.0 g
Distilled water	1000 ml

Heat to dissolve the ingredients. Add 1.0 of a 1 M $CaCl_2$ solution and dispense in 4 ml quantities to 16 x 150 mm tubes. Cap the tubes and store them in the freezer.

5. Single strength (1X) Tryptone Soft Agar

Reduce all the ingredients in the 2X Tryptone Agar by a factor of two.

Stock Cultures of Host Strain - Maintain the strain on slants of the bottom agar held in the refrigerator.

Concentration of Water Samples

C-1. During the late afternoon of the day before the assays are to be performed, aseptically add the antibiotic solution in 0.3 ml quantities to the number of tubes (10 ml) and in 3.0 ml quantities to the number of flasks (100 ml) of Tryptone broth needed. Inoculate them from the stock of the host strain, and incubate the cultures overnight at 37°C.

C-2. The following morning, inoculate flasks containing 100 ml amounts of Tryptone Broth (no antibiotics) with 10 ml quantities of the overnight culture of the host strain. Incubate the flasks for 3.5 ± 0.5 hours at 37°C to obtain log phase cells.

C-3. For each sample to be concentrated, add 1 g of Tryptone and 1 g of powdered beef extract to a sterile, capped, 250 ml centrifuge bottle.

C-4. For each sample, add 100-200 ml of the water and 10 ml of log phase culture to one of the centrifuge bottles. Incubate the mixture in a water bath for 30 min at 30.5 ± 0.5 C with very gentle shaking.

C-5. Centrifuge the bottles at 9000 x g for 10 min in a refrigerated centrifuge at 4°C.

C-6. Decant the supernatant, leaving 7-8 ml behind in which to resuspend the pellet. Place bottle in the 50°C water bath for about one min and proceed to assay.

Assay of Concentrate

A-1. Remove the needed number of plates containing the bottom agar from the refrigerator the day before they are to be used. Remove an equal number of tubes of 2X soft agar from the freezer the morning of the day they are to be used.

A-2. Autoclave the tubes of soft agar at 121°C for 15 min. Place them in a water bath at 50°C.

A-3. Remove 4 ml of the resuspended pellet (step C-6) to a tube of 2X soft Tryptone Agar. Gently mix the contents of the tube by rolling it in the palms of your hands. Do this rapidly so that the agar will not harden, and avoid bubbles. Immediately pour the contents over a hard agar plate. Gently swirl the plate to distribute the soft agar over the surface of the hard agar. Similarly prepare a second plate from the remainder of the resuspended pellet. Allow the agar to harden; invert and incubate the plates at 37°C for at least 9 hours and no more than 18 hours.

A-4. Count the plaques and record the data.

Direct Assay of Water Samples or Sewage Effluents (no concentration)

a) Direct assay of duplicate 4 ml samples can be done with water samples collected near the sewage outfalls.

DA-1. Same as A-1

DA-2. Same as A-2

DA-3. Add 4 ml of the warmed (equilibrated to room temperature or 1 min at 50°C) water sample and 1 ml of the 3 hour culture of the host strain to a tube of 2X soft agar. Mix and pour the contents of the tube over a hard agar plate. Assay a second 4 ml portion of the sample in the same manner. Incubate as in A-3.

DA-4. Same as A-4

b) Direct assay of 1 ml (duplicate 0.5 ml quantities) or decimal dilutions thereof usually is required only with sewage effluents since samples with phage levels up to 1.0×10^4/100 ml can be assayed by direct plating of duplicate 4 ml portions.

DDA-1. 100 x 15mm plates containing about 17 ml of bottom agar are prepared as described in the Media Section.

DDA-2. 13 x 100 mm tubes containing 4.0 ml of single strength soft agar are prepared as described in the Media Section.

DDA-3. Same as A-2

DDA-4. Add 0.5 ml of the sample and 0.02 ml of the 3 hour culture of the host strain to a tube of single strength soft agar. Mix and pour the contents of the tube over a 100 x 15 mm bottom agar plate. Assay a second 0.5 ml portion. Incubate as in A-3.

DDA-5. Same as A-4

sampled during any given tour, and 5-7 such tours should be conducted during the six month period. The sampling frequencies are given in Table A-1.

C. Microbial Assays

1. Water and effluents: C. perfringens (mCP); enterococci (mE as modified by use of indoxyl β D glucoside); E. coli and fecal coliforms (mTEC); F male-specific phages (Cabelli method).
2. Sediments: Assays for C. perfringens by the "sonicate and settle" extraction method followed by membrane filtration by mCP. If the metaphosphate extraction method is used, MF assays can be done for enterococci (mE) and E. coli/fecal coliforms (mTEC) as well.
3. Shellfish: Clean, shuck and homogenize 9-12 animals as described in "Standard Methods for the Examination of Shellfish and Shellfish-Growing Waters." Then perform 5 tube, 3 dilution (1.0, 0.1 and 0.01 ml) MPN assays by the appropriate methods for C. perfringens, enterococci and fecal coliforms/E. coli. The MPNs/100 ml can be converted to MPN/100 g by weighing a given volume of the homogenate and using the values obtained for the conversion.
   a. E. coli/fecal coliforms: Inoculate tubes of LST broth as described in "Standard Methods." Then transfer to EC as they specify only use EC MUG (Difco) instead of EC. Read the EC tubes for fecal coliforms as they specify. Then place the tubes under "black light", long wave UV, and record fluorescence. Those that fluoresce contain E. coli. MUG is methyl umbellifervl glucuronide.
   b. Enterococci: Inoculate tubes of Azide Dextrose Broth as given in "Standard Methods for the Examination of Water and Wastewater." After incubation, streak from the turbid tubes on the surface of a membrane filter placed on a MF plate of modified mE medium. Use half or a third of a plate per tube. Record blue growth on the membranes after incubation at 41°C for 24-36 hours as positive.
   c. C. perfringens: Inoculate tubes of Iron-Milk medium, incubate and read as described by Matches et al.
   d. F male-specific bacteriophages: Weigh 100 g of the homogenate into a sterile, screw-cap centrifuge tube, and centrifuge at 13000 x g for 15 min. Decant the supernatant to a sterile tube and record the volume. Let the fluid warm to room temperature. Add 5 ml

to 5 ml of 2 x soft agar; add 1 ml of the host cells and pour over the large plates. Incubate and count as given in the appended method for the phage assay.