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PUBLIC HEALTH CONSIDERATIONS ASSOCIATED WITH MOLLUSCAN AQUACULTURE SYSTEMS: HUMAN VIRUSES

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ABSTRACT

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The documentation of several recent outbreaks of human virus diseases associated with the consumption of shellfish has reiterated the threat posed by these agents to the shellfish industry. This article reviews pertinent outbreaks, identifies principal viral agents involved, and delineates systems which may be at greatest risk. The results of two recent laboratory studies which sought to define environmental factors that contribute to virus accumulation by shellfish are also discussed. First, the accumulation of environmentally significant levels of feces-associated and monodispersed poliovirus by oysters (Crassostrea virginica) and clams (Mercenaria mercenaria) was investigated. The results of this study suggested that virus accumulation by mollusks may not be significant when water column concentrations are below ≈0.01 plaque-forming units (PFU) per milliliter. The second study focused on the relative contributions of undisturbed sediments versus those in the water column in the accumulation of viruses by epifaunal and infaunal shellfish (C. virginica and M. mercenaria). Viruses were found to be most efficiently accumulated when suspended in the water column.

INTRODUCTION

The continued occurrence of human virus disease outbreaks resulting from the consumption of raw or partially cooked shellfish has served to reemphasize the substantial threat posed by these agents to aquaculture ventures set in coastal or estuarine waters of questionable purity. The occurrence of human viruses in shellfish species residing in light to moderately polluted marine waters is well documented (Morris et al., 1976; Vaughn and Metcalf, 1975; Metcalf and Stiles, 1967; Gerba and Goyal, 1978). Studies have also documented the recovery of viruses from animals residing in waters which had been certified as safe for shellfish harvesting (Fugate et al., 1975; Vaughn et al., 1980; Goyal et al., 1979). Origins of pollution identified during these studies have ranged from continuous point sources such as ocean outfalls and the outlets of sewage-polluted freshwater systems, to sources of a more sporadic
nature [i.e., runoff during periods of heavy rain and flooding, areas under heavy recreational (swimming, boating) use]. Molluscan aquaculture systems situated in waters under the influence of one or more of these sources would appear to be most vulnerable to subsequent contamination by wastewater-associated human viruses.

At present, more than 100 different virus types are known to be excreted by humans in feces or urine. Table I presents the major groups involved, along with some of the diseases with which they are associated. Although there is insufficient epidemiological evidence to support a shellfish-mediated disease potential for all the viral agents listed, two (hepatitis and Norwalk) have been unquestionably linked to disease outbreaks associated with the environmentally contaminated shellfish. The first reported shellfish-related outbreak of hepatitis occurred in Sweden in 1955, resulting in 629 cases (Roos, 1956). Since then, significant outbreaks have occurred in New Jersey, Mississippi, and Alabama in 1961; in Philadelphia and Connecticut in 1963; in North Carolina in 1964; New Jersey in 1966; and in Rhode Island and Massachusetts in 1971 (Portnoy et al., 1975). An outbreak occurred during October and November 1973 affecting 263 individuals from Houston and 15 from Calhoun, Georgia following the consumption of raw oysters from certified waters of Louisiana Bay (Portnoy et al., 1975). Mahoney et al. (1976) detected the presence of Australia antigen (Au), indicative of the presence of type B hepatitis virus, in Maine clams. The clams were taken from waters known to be contaminated with untreated sewage from a local hospital. The antigen could be transmitted to previously uninfected clams, indicating that shellfish could act as both vector and reservoir for Au antigen. Recent studies have identified shellfish carriage of Norwalk or related parvo-like viruses in a series of outbreaks of acute nonbacterial gastroenteritis occurring in Australia (Murphy et al., 1979; Grohmann et al., 1981; Linco and Grohmann, 1980), Great Britain (Appleton et al., 1981) and the United States (Morbidity and Mortality Weekly Report, 1982).

Laboratory and field investigations carried out over the past 20 years have provided a good deal of information concerning basic interactions between human viruses and various molluscan species. The studies have also served to identify several additional areas of pertinent research. Among these are two of considerable ecological import: the role of sediments in the uptake of human viruses by molluscs and the limits of molluscan virus accumulation potential. Few studies to date have focused on the role of virus-contaminated sediments in shellfish accumulation. Metcalf et al. (1974) isolated enteroviruses from estuarine sediments in concentrations ranging from 0.9 to 4.1 PFU per 100 g. Later, Gerba et al. (1977) reported similar virus levels in estuarine sediments impacted by treated domestic wastewater. Sediment-associated viruses were subsequently found to survive for long periods of time under rather hostile environmental conditions (Liew and Gerba, 1980; Smith et al., 1978), leading LaBelle and Gerba (1979) to suggest that sediments acted as a reservoir, or continuous source of viral contamination in polluted marine environments. However, efforts to document the impact of such reservoirs on resident molluscs have not been pursued. The object of one of the studies described herein was to address the relative importance of sediments in the accumulation
TABLE I

Viruses normally excreted by humans

| Group                | Number of types | Disease/symptoms                                      |
|----------------------|-----------------|-------------------------------------------------------|
| Adenovirus           | >30             | Acute respiratory disease                             |
|                      |                 | Pharyngocconjunctival fever                            |
| Astrovirus           | ?               | Infant gastroenteritis                                |
| Calicivirus          | ?               | Winter vomiting disease                               |
| Coronavirus          | ?               | Diarrheal disease                                     |
|                      |                 | Acute gastroenteritis                                 |
| Enterovirus          |                 |                                                       |
| Poliovirus           | 3               | Poliomyelitis, meningitis                             |
| Coxsackievirus (A&B)| 30              | Herpangina, meningitis, respiratory disease           |
| Echovirus            | 34              | Meningitis, acute gastroenteritis, rash               |
| New enteroviruses    | 4               | Meningitis, respiratory disease, encephalitis         |
| Hepatitis A          | 1               | Infectious hepatitis                                  |
| Parvovirus-like      |                 |                                                       |
| Norwalk, Montgomery  |                 |                                                       |
| County, Paramatta,   |                 |                                                       |
| Hawaii, Ditchling    | ?               | Vomiting and diarrhea                                  |
| Agents               |                 |                                                       |
| Rotavirus            | ?               | Vomiting and diarrhea                                  |

of viruses by shellfish using a sediment-associated poliovirus model.

As previously noted, viruses have been detected in shellfish residing in grossly polluted waters, as well as in those areas certified for the harvesting of shellfish. Vaughn et al. (1979) have reported the sporadic isolation of polio and echoviruses from clams and oysters residing in two approved shellfish harvesting areas located on Long Island. Viral concentrations in these waters averaged 0.001 \( \pm 0.0007 \) plaque-forming units (PFU) per milliliter. In a later report, the authors noted the occasional occurrence of unidentified enteroviruses in clams residing in Raritan Bay, New Jersey (Vaughn et al., 1980). Here, virus levels in the water column were substantially lower than the above concentrations (mean = \( 8.34 \times 10^{-5} \) PFU/ml). In neither case was there evidence to support any correlation between viral occurrence in shellfish and their presence in overlying waters. Similar findings were also reported by Goyal et al. (1979).

A recent study conducted by our laboratory attempted to extend the above findings by investigating the relationship between virus occurrence in the seawater, sediment, and shellfish of a well defined, sewage-impacted marine system (Vaughn et al., 1981). Viruses were frequently isolated from the water column in concentrations averaging 0.001 \( \pm 0.002 \) PFU/ml; however, no isolations were ever made from resident shellfish. These data, together with the sporadic shellfish isolations noted earlier in lightly contaminated waters,
implied that bioaccumulation might not be significant, or detectable when virus concentrations in the water column fall below a certain level. Such shellfish would thus appear to represent a minimal public health hazard.

Experimental evidence to support such contentions has not yet been extensively reported. A survey of previous studies revealed that few have focused on the mode and mechanism of shellfish accumulation at virus concentrations normally found in light to moderately polluted marine systems. Almost invariably, laboratory bioaccumulation studies have employed high virus exposure concentrations (e.g., $10^3$-$10^6$ PFU/ml) in an effort to elucidate the mechanisms of viral uptake. A recent study, which employed more environmentally appropriate virus levels, was conducted by Metcalf et al. (1974). In this study, shellfish were exposed to both free and solid-associated viruses at concentrations ranging from 0.09 to 14.0 PFU/ml. After a brief exposure, viruses were recovered from nearly all shellfish. Bioaccumulation was most evident at the higher viral concentrations, particularly when solid-associated, rather than free viruses were employed. Although these exposure levels represented the lowest reported in laboratory studies, they still exceeded the concentrations of viruses normally encountered in lightly polluted waters. The objective of the second study described here was to analyze the accumulation capabilities of shellfish exposed to "environmentally significant" levels of virus, and determine whether an apparent level existed, below which viral accumulation and carriage was absent, transient, or undetectable. The virus concentrations chosen for these studies ranged from 0.002 to 0.180 PFU/ml, and included both monodispersed and feces-associated types.

**METHODS**

*Viruses, host cells, and assay systems*

Plaque-purified poliovirus type 1 (LSc-2ab) was propagated on Buffalo Green Monkey kidney cells (BGM) (Dahling et al., 1974) and prepared in a monodispersed culture according to the procedure of Jakubowski et al. (1975). Feces-associated viruses were extracted from stool specimens of infants who had recently received trivalent oral polio vaccine. Specimens collected 7-9 days after vaccination were mixed with an equal volume of phosphate-buffered saline (pH 7.2) to dissolve or disperse solids. Specimens were then frozen, thawed, and assayed on monolayers of BGM cells. Periodic assays of frozen stocks indicated that they were extremely stable under -70°C storage.

Low-passage BGM cells obtained from Microbiological Associates (Bethesda, Maryland) were grown in Eagle's minimum essential medium with Earle's balanced salt solution, supplemented with 5% fetal calf serum and antibiotics (penicillin, streptomycin, and gentamycin). Viral enumerations were carried out on monolayers of BGM cells grown in 75-cm$^2$ culture flasks. Four-milliliter sample volumes were placed on replicate cell monolayers and incubated with continuous rocking for 1-2 h. In samples where the virus concentration was expected to be low, at least three-quarters of the entire concentrate was assayed. After decanting excess inoculum, monolayers were overlaid with a plaquing medium containing 0.8% agar and incubated at 37°C.
for 1-3 days. Monolayers were then stained with a second agar layer containing neutral red as previously described (Landry et al., 1978) and incubated at 37°C. Plaque development was observed for a 7-day period. To confirm the viral nature of suspected isolates, plaques were plucked from the agar and passaged on monolayers of BGM cells in 25-cm² flasks.

Recovery of viruses from seawater

To measure the concentration of viruses in flowing seawater systems, 1-liter composite samples were taken at prescribed intervals, diluted with deionized, distilled water (1:4), and supplemented with beef extract (Lab Lemco, K.C. Biological, Lenexa, Kansas) to a final concentration of 3%. Viruses were concentrated by an organic flocculation procedure at pH 3.5 as described by Katzenelson et al. (1976). All concentrates were stored at -70°C until assayed. Efficiency analyses indicated that this technique could recover an average of 67.49% (±5.35) of seeded poliovirus (data not shown).

Recovery of viruses from shellfish

The recovery of viruses from oysters (Crassostrea virginica) was accomplished using a modification of the Cat-Floc-beef extract technique described by Vaughn et al. (1979). An analysis of the efficiency of this procedure has previously been published (Landry et al., 1980; Vaughn et al., 1979).

Hard clams (Mercenaria mercenaria) were also analyzed for their ability to accumulate low concentrations of monodispersed or feces-associated viruses. The virus recovery technique used was a modification of the above oyster procedure (Vaughn et al., 1981).

Seawater systems

Accumulation experiments were conducted in large flowing seawater tables located at Brookhaven National Laboratory (BNL) and the Environmental Systems Laboratory (ESL), Woods Hole Oceanographic Institution (WHOI). These systems have been described in detail (Vaughn et al., 1981).

Sediment experiments

Adsorption/recovery of poliovirus from marine sediments

Fifty- to one hundred-gram portions of clean sediment collected from either Barnstable Harbor, Massachusetts or Wading River, New York were suspended in 200-250 ml of fresh seawater. Suspensions were mixed vigorously on a magnetic stirrer and inoculated with poliovirus stock to a final concentration of 5.0 x 10² - 4 x 10⁴ PFU/ml. Stirring was continued for 30-40 min, at which time the sediments were collected by centrifugation (10,000 g/10 min). The supernatant fluids were decanted and assayed for unadsorbed viruses. The pellets were gently rinsed with seawater, combined, and then blended to uniformity. Sediments were divided into 25- to 50-g aliquots for use in experiments. Viruses were recovered from sediments using a method developed by M.D. Sobsey (personal communication, 1982). Sediments were suspended in a solution of 3% beef extract-0.25 M glycine (pH 9.5) at a ratio of 1:4. The suspension was mixed for 10 min and the pH readjusted, if necessary
to 9.0-9.5. The sample was then centrifuged at 3,000 g for 15 min and the virus-containing supernatant fluid collected and adjusted to pH 3.5. The mixture was then stirred for 15 min, recentrifuged, and the final pellet dissolved in 0.1 M Na<sub>2</sub>HPO<sub>4</sub> (pH 9.0). The extract pH was adjusted to 7.2 and the sample stored (-70°C) until assayed.

**Survival of poliovirus in seawater and marine sediments**

Experiments were conducted to ascertain the relative survival capacities of monodispersed and sediment-associated test viruses in seawater. Inoculated sediments, prepared as previously described (25-g aliquots) were placed in sterile 50 x 9 mm plastic petri dishes and the surface of each carefully smoothed. Dishes were capped, submerged in the BNL flowing seawater chamber and gently uncovered. The characteristics of the flowing seawater were: pH 8.0-8.3; a salinity of 22 ppt; and a temperature of 13.3 ± 1°C. At the desired interval, covers were replaced and the dishes carefully removed. The entire contents of a dish (25 g) was transferred to a beaker containing 100 ml of beef extract-glycine (pH 9.5). A portion of this solution was used to thoroughly rinse the petri dish. Sediment-bound viruses were then extracted as previously described. Virus survival was analyzed by plotting the log of the fraction of survivors (N<sub>T</sub>/N<sub>0</sub>) versus time (where N<sub>T</sub> equals virus titer at time T and N<sub>0</sub> equals virus titer at 0 h). Plots were further defined by an analysis of the variance, followed by an LSD test [Least Significant Difference (Sokal and Rohlö, 1969)] to determine statistically similar groupings.

The survival of sediment-adsorbed viruses was compared to that of free-floating, nonadsorbed viruses exposed to the same conditions. A stock of monodispersed poliovirus type 1 (10<sup>3</sup> PFU/ml) was placed in a membrane diffusion chamber containing 100 ml of seawater. The chamber was bounded by a 10-nm Nucleopore filter with an external 0.45-μm Microweb filter (Millipore) for protection against tearing. The chamber was immersed in the flowing seawater system. At the desired intervals, 4.5 ml of sample was withdrawn, mixed with 0.5 ml of fetal calf serum and frozen until assayed. Survival was plotted by analyzing the log of the fraction of surviving viruses as described earlier.

**Accumulation of sediment-bound viruses by oysters**

Sediments employed in the experiments were collected from shellfish beds located in Greenport, New York. Cores constructed of 5 x 10 cm plastic pipe were filled with a 600-g compact base layer of uninoculated, 2-mm screened sediments held in place with fine mesh screening. Cores were then placed in the 190-liter tank which contained sufficient seawater to saturate each core. Virus-inoculated sediments (100-g aliquots) were then added to the surface of the partially immersed cores. A spatula was used to smooth the surface of the cores, ensuring that the sediments remained even with the top edge of the plastic piping. In experiments using undisturbed sediments, inoculated cores were gently covered with a wetted piece of filter paper (Whatman No. 1) to prevent sediment resuspension during filling of the tank. The water level was gradually raised to a height sufficient to cover both cores and shellfish. During the filling process, the filter paper eventually lifted off the core surface and was carefully removed with forceps. This procedure was effective in controlling the
resuspension of small air-trapped sediment aggregates. Several uninoculated cores were included in the experimental tank. Once the tanks were filled, fresh seawater, collected every 2 days from Long Island Sound, was continually pumped through the system at a flow rate of 14-21 liters/day. In the experiments employing resuspended viruses, inoculated cores were manually agitated during the tank-filling process and the sediments kept in suspension by vigorous aeration via large fish-tank airstones.

Oysters (*Crassostrea virginica*), obtained from Dr. R. Mann (WHOI), were transferred to the BNL flowing seawater tank and held at 18°C (±1°C) for a 2- to 7-day acclimation period. The shellfish were observed for pumping activity and only those showing signs of continuous feeding were chosen for bioaccumulation experiments. Following sediment inoculation, selected oysters were carefully positioned on the surface of inoculated or control cores. In each experiment, six oysters were placed on seeded sediments (one per core), while an additional one was added to an uninoculated core. This latter core was located downstream from the inoculated ones and was used to monitor the likelihood of bioaccumulation from the water column. Throughout the experiment, the flowing seawater was periodically monitored for the presence of poliovirus in the water column. After the desired exposure time, which ranged from 48 to 168 h, oysters were carefully collected, and divided into groups, two of which had been exposed to inoculated sediments, and one having been exposed to uninoculated sediments. The surface of each animal was disinfected by dipping in a sodium hypochlorite solution, scrubbed, shucked, and extracted using the previously described method. Concentrates were assayed immediately on BGM cells. At least 65% of each sample concentrate was analyzed. Suspected plaques were picked and passaged on BGM cells for confirmation. Isolates were not considered positive unless they elicited a positive cytopathic effect (CPE) response upon passage in BGM cells.

*Virus accumulation from seawater*

The uptake potential of oysters (*C. virginica*) and hard clams (*Mercenaria mercenaria*) was tested in the BNL seawater system. Experiments involved the use of both monodispersed and feces-associated poliovirus. Initial experiments were carried out at WHOI. Six to eight shellfish were placed in each tank and allowed to equilibrate for periods of 2-7 days prior to the initiation of each experiment. A stock solution of monodispersed or feces-associated viruses was prepared and held at 4°C with constant mixing. The virus solution was continuously metered into a 7-liter chamber at a rate of 1 ml/min, where it was mixed with an appropriate amount of seawater. The inoculated seawater was then allowed to flow into the shellfish tanks for 13- or 28-day periods. All tank effluents were chlorinated by an on-line system before discharge. Following the desired exposure period, several randomly selected shellfish were harvested, decontaminated, and immediately shipped to Brookhaven under ice for processing and assay.

Initial experiments at the BNL facility involved shellfish exposures to purified poliovirus under "static" conditions for 48-h periods. Virus levels used in these experiments were 0.011, 0.0018, and 0.0014 PFU/ml, respectively. At
appropriate times, seawater and oyster samples were collected and processed as previously described. Commencement of a series of flowing seawater experiments using feces-associated virus followed completion of the static experiments. Shellfish were exposed for 48- to 120-h periods, during which time both water (500 ml) and mollusks were periodically collected. Samples were processed and assayed as previously described. Because very low virus numbers were expected, entire shellfish concentrate volumes were assayed.

**Statistical analysis**

Data were statistically analyzed according to the methods described by Sokal and Rohlf (1969) and Steel and Torrie (1960). Most analyses were performed on a Hewlett-Packard HP9845B computer using preprogrammed statistical software.

**RESULTS**

**Survival of poliovirus in marine sediments**

In order to determine appropriate shellfish exposure times in later experiments, the survival of sediment-bound viruses had to be considered. The results of the described survival study, shown in Fig. 1, indicated the extensive survival of sediment-associated viruses. At least 7 days were required to reduce the concentration by 90%. Thereafter, the rate of virus inactivation slowed, with virus survival stabilizing over the last 6 days. Analysis of this latter stage by the LSD test (Sokal and Rohlf, 1969) indicated that no significant difference (p > 0.05) in virus concentration occurred between days 7 and 15. Conversely, the inactivation of monodispersed polioviruses was rapid, with a 90% reduction in virus titer noted after 4 days. No viable viruses could be recovered after day 15. The data suggested that adsorption to particulate matter afforded viruses a degree of protection.

**Accumulation of sediment-associated viruses**

In order to examine the accumulation of sediment-associated viruses by shellfish, two regimes of viral exposure were analyzed. In the first, shellfish were exposed to viruses bound to undisturbed sediments. In the second, shellfish were challenged with a combination of undisturbed sediment-associated viruses, as well as sediment-bound viruses that had been resuspended in the water column.

The results of initial experiments, where viruses occurred solely in undisturbed sediments, are presented in Table II. In the first trial, shellfish failed to accumulate any detectable viruses following prolonged exposure (168 h) to sediment-bound viruses at an initial concentration of 368 PFU per core. Although inactivation reduced this titer by as much as 60% over the experimental period, sufficient viral numbers remained viable to represent a significant source of contamination to the shellfish. In the second trial, when virus concentration was greatly increased (8580 PFU), only one group accumulated any viruses, and this at a relatively low level (2.8 PFU/100 g). Since no viruses were recovered from the water column, the accumulated
viruses could only have come from the undisturbed sediments. However, when this uptake was compared to the amounts of viruses potentially available (8580 PFU), the accumulation appeared to be rather insignificant. No bioaccumulation was observed in the second group of actively pumping shellfish. Apparently, the proximity of the sediment-bound viruses to the shellfish played only a minor role in the bioaccumulation process.

Several experiments designed to assess the role of suspended sediments in bioaccumulation were conducted. Analysis of water samples collected after 24 h (Table III) indicated virus concentrations to be 0.44 and 0.11 PFU/ml in two respective trials. As suggested in a previous report (Vaughn et al., 1981), these concentrations appeared to be sufficient to result in detectable bioaccumulation. The particle-associated nature of the resuspended viruses was confirmed by their removal during a filtration process. It also suggested that significant virus desorption from the sediments did not occur.
TABLE II

Accumulation of sediment-bound poliovirus by oysters (Crassostrea virginica): lack of uptake in the absence of viruses in the water column

| Sample type       | Time (h) | PPFU recovered |          |          |
|-------------------|----------|----------------|----------|----------|
|                   |          |                | Trial 1   | Trial 2   |
| Water (PFU/ml)    |          |                |          |          |
| U\textsuperscript{a} | 0        | --             | 0        |          |
| F\textsuperscript{b} | 0        | --             | 0        |          |
| U                 | 24       | 0              | 0        |          |
| F                 | 24       | 0              | 0        |          |
| U                 | 48       | --             | 0        |          |
| F                 | 48       | --             | 0        |          |
| U                 | 72       | 0              | --       |          |
| F                 | 72       | 0              | --       |          |
| U                 | 144      | 0              | --       |          |
| F                 | 144      | 0              | --       |          |
| Sediment (PFU/100g) |          |                |          |          |
|                   | 0        | 368 (±167)     | 8580 (±806) |          |
|                   | 48       | --             | 2420 (±402) |          |
|                   | 168      | 144 (±35)      | --       |          |
| Shellfish (PFU/100g) |          |                |          |          |
|                   | 48       | --             | 0        |          |
|                   | 48       | --             | 2.8 (±9.7) |          |
|                   | 168      | 0              | --       |          |
|                   | 168      | 0              | --       |          |
|                   | 168      | 0\textsuperscript{c} | -- |          |

\textsuperscript{a}U, unfiltered seawater.
\textsuperscript{b}Assay of filtrate resulting from filtering 250 ml of seawater through a serum-treated 0.22-μm Millipore filter. Only free or non-particle-associated viruses passed through the filter.
\textsuperscript{c}Shellfish residing on an uninoculated core.

Analysis of the undisturbed sediments revealed the initial virus concentrations to be 7124 and 8580 PFU per core, respectively, for two trials. An assay of duplicate cores at 72 h indicated that significant (p < 0.05) viral inactivation had occurred during the exposure period; however, a sufficient number of viruses remained viable to adequately challenge the test animals. Under the described conditions, four of the five oyster groups were found to accumulate viruses at concentrations ranging from 3.7 to 10.2 PFU/100 g of processed homogenate. The remaining oyster group, although receiving an identical exposure, failed to accumulate any viruses. Careful monitoring of the feeding activities of these oysters indicated that the lack of accumulation was not related to cessation of pumping.
TABLE III

Accumulation of sediment-bound poliovirus by oysters (*Crassostrea virginica*): uptake in the presence of high virus concentrations in the water column

| Sample type | Time (h) | PFU recovered |
|-------------|----------|---------------|
|             |          | Trial 1       | Trial 2       |
| **Water column (PFU/ml)** |          |               |
| U<sup>a</sup> | 0        | --            | 0.59(±0.17)   |
| F<sup>b</sup> | 0        | --            | 0.18(±0.30)   |
| U           | 24       | 0.44(±0.09)   | 0.11(±0.09)   |
| F           | 24       | 0.01(±0.02)   | 0.02(±0.03)   |
| U           | 48       | --            | 0             |
| F           | 48       | --            | 0             |
| **Sediments (PFU/100g)** |          |               |
| U           | 0        | 7124(±1461)   | 8580(±806)    |
| F           | 48       | --            | 2420(±402)    |
|            | 72       | 2126(±490)    | --            |
|            | 72       | 4(±7)         | --            |
| *(uninoc)*<sup>c</sup> |          |               |
| **Shellfish (PFU/100g)** |          |               |
| U           | 48       | 0             | 3.70(±13.00)  |
| F†         | 48       | 4.50(±10.08)  | 10.20(±18.50) |
| *(uninoc)*<sup>d</sup> | 48       | 7.60(±25.36)  | --            |

<sup>a</sup>U, unfiltered seawater.

<sup>b</sup>Assay of filtrate resulting from filtering 250 ml of seawater through a serum-treated 0.22-μm Millipore filter. Only free or non-particle-associated viruses passed through the filter.

<sup>c</sup>Assay of 100 g of uninfected sediments collected from a core located downflow of infected cores.

<sup>d</sup>Shellfish harvested from uninoculated cores.

Comparison of this higher incidence (80%) of viral accumulation to the low occurrence (20%) previously noted with the absence of resuspended sediment-associated virus indicated that oysters preferentially accumulated the sediment-associated viruses in the water column. This finding was supported by the recovery of poliovirus from shellfish residing on control cores which had not initially been inoculated with viruses. Later analysis showed that these sediments contained very low concentrations of viruses (4 PFU/100 g), this apparently the result of resettling of viruses from upstream cores. Because the concentration of accumulated viruses was not significantly different (p > .05) in the shellfish placed on lightly contaminated cores (4 PFU/100 g) and those residing on highly contaminated ones (7124 PFU/100 g), it became apparent that uptake from the basal sediments might not be the principal route of contamination. Accumulation from the overlying waters appeared to be the
probable mechanism of contamination. In spite of the higher incidence of viral accumulation noted with resuspended sediments, a statistical comparison, using Poisson means, indicated that the concentration of viruses accumulated under resuspended or undisturbed conditions was not significantly different ($p > .05$). This suggested that when uptake did occur, similar amounts of viruses were accumulated regardless of whether the source was seawater or sediments.

**Bioaccumulation in shellfish exposed to low virus levels in the water column**

Initial uptake experiments involved the extended exposure of oysters to low concentrations of monodispersed poliovirus. The results of two such experiments, which proceeded for 13 and 28 days, respectively, are presented in Table IV. The mean virus concentration for the entire experiment was 0.104 PFU/ml ($\pm 0.55$). Viruses were recovered from shellfish on days 6, 10, and 13, in concentrations ranging from 2.1 to 29.5 PFU/100 g. Statistical analysis of the data indicated that no significant difference existed in the levels of viral accumulation (mean $= 18.96 \pm 12.05$), indicating that an equilibrium had been reached between accumulation and depuration. In the second experiment, virus input levels were reduced, with concentrations averaging 0.011 ($\pm 0.008$) PFU/ml. On two occasions, viruses were isolated from shellfish (days 4 and 14) in rather low concentrations (2.1 and 5.7 PFU/100 g, respectively). The lack of viral isolations during the latter stages of the study suggested that the prolonged exposure did not appreciably enhance the likelihood of efficient viral carriage. When compared to the overall accumulations noted in the 13-day experiment, the decreased frequencies of viral occurrence and low concentrations of shellfish-bound viruses noted in the 28-day experiment implied that the viral exposure levels were approaching a lower limit of an efficient bioaccumulation capability.

Data resulting from the above experiments indicated the need to further reduce the virus exposure levels in order to delineate the range over which inefficient accumulation occurred. Several experiments were first undertaken in a small (20-liter), static system, in which oysters were exposed to monodispersed poliovirus at concentrations of 0.0110, 0.0018, and 0.0014 PFU/ml, respectively. Following a 48-h uptake period, shellfish extractions yielded no virus isolates. This inability to detect viruses was not judged to be associated with any obvious deficiencies in the extraction method, as previous experiments had indicated its ability to detect as few as 1-2 PFU per animal (data not shown).

The final series of low-level uptake experiments involved the exposure of shellfish to feces-associated polioviruses in a 190-liter flowing seawater system. In the initial experiment, shellfish were exposed to viruses, ranging in concentrations from 0.007 to 0.038 (mean $= 0.022$), for a 48-h period (Table V). When the oysters were extracted and extensively assayed, only a single viral particle was isolated. Testing of all other pools failed to recover any virus particles, suggesting that the input levels used were at or below the proposed critical level of efficient accumulation.

In a subsequent experiment, viral levels were decreased to a range of 0.005-0.007 PFU/ml in an attempt to completely eliminate accumulation. Following a
TABLE IV

Accumulation of low-level monodispersed poliovirus by oysters (Crassostrea virginica) in a large-scale flowing seawater system

| Experiment no. | Exposure period (days) | Virus recovery |  |
|---------------|------------------------|----------------|---|
|               |                        | Seawater (PFU/ml) | Oysters (PFU/100 g) |
| 1             | 0                      | 0.032 (±0.059)   | --  |
|               | 3                      | 0.180 (±0.221)   | 0   |
|               | 6                      | 0.154 (±0.154)   | 25.3 (±58.2) |
|               | 10                     | 0.082 (±0.095)   | 29.5 (±88.2) |
|               | 13                     | 0.071 (±0.095)   | 2.1 (±24.5)  |
| 2             | 4                      | 0.002 (±0.006)   | 2.1 (± 2.0)  |
|               | 7                      | 0.004 (±0.013)   | 0   |
|               | 14                     | 0.014 (±0.024)   | 5.7 (±12.8)  |
|               | 21                     | 0.026 (±0.030)   | 0   |
|               | 25                     | 0.012 (±0.025)   | 0   |
|               | 28                     | 0.009 (±0.026)   | 0   |

TABLE V

Inability of physiologically active shellfish to accumulate low concentrations of feces-associated poliovirus

| Experiment no. | Exposure time (h) | Total PFU recovered |
|---------------|------------------|---------------------|
|               | Water Column (PFU/ml) | Shellfish pools (PFU/50 g) |
|               | 24 h | 48 h | 24 h | 48 h | Oysters | Clams |
| 1             | 48   | 0.038 (±0.050) | 0.007 (±0.022) | 0 | 0 | 0 | 1 |
| 2             | 48   | 0.007 (±0.016) | 0.005 (±0.012) | 0 | 0 | 0 | -- |
| 3             | 120  | 0.0116 (±0.016) | 0.020 (±0.031) | 0<sup>a</sup> | 0<sup>b</sup> | -- | 0<sup>c</sup> |

<sup>a</sup>Shellfish harvested at 48 h.
<sup>b</sup>Shellfish harvested at 120 h.

48-h exposure period, three oyster groups (5-6 animals/group) were processed and assayed. Complete assay of each resulting concentrate yielded no virus isolates. As all experimental animals were known to be active during the exposure period, it was concluded that the virus challenge level (mean = 0.006 PFU/ml) was below that required for any measurable uptake by oysters.

To further define these limits and analyze the effect of a longer (120-h)
exposure period, a third experiment was conducted using an exposure range of 0.0116-0.020 PFU/ml. Both oysters and hard clams (*M. mercenaria*) were included in the experiment to determine whether critical accumulation levels were peculiar to a single group of bivalves. During the course of the study, animals exposed to a mean virus concentration of 0.016 PFU/ml were harvested and processed at 48 and 120 h. Assay of shellfish concentrates once again yielded no virus isolates (Table V). These data suggested that a critical level might indeed exist, below which viruses could not be effectively accumulated by shellfish. Moreover, on the basis of the studies presented here, this level appeared to occur at a virus concentration of approximately 0.01 PFU/ml.

**DISCUSSION**

Evidence for shellfish-mediated transmission of human virus diseases such as hepatitis (Portnoy *et al.*, 1975) and Norwalk-induced gastroenteritis (Murphy *et al.*, 1979) has emphasized the importance of developing a rudimentary understanding of factors that influence the accumulation of viruses by shellfish. Two little-studied areas, the role of virus-contaminated marine sediments, and the limits of virus accumulation in shellfish exposed to environmentally significant virus levels, were the subject of the present investigation.

Data presented here and by others (Landry *et al.*, 1980; Liew and Gerba, 1980) have suggested that particle association significantly extends the survival capacity of viruses and enhances their potential for interaction with local marine organisms. The ramifications of the existence of a sediment-based viral reservoir for neighboring shellfish beds appears to depend on the state of the sediments. Under conditions where sediments remained undisturbed, and no viruses occurred in overlying waters, the incidence of accumulation was quite low. Accumulation was noted in three of nine trials (1.60-20.3 PFU/100 g), but when the concentration of accumulated viruses was compared to the total number available (0.03-5.88 x 10^4 PFU), the process appeared to be relatively inefficient. The failure of oysters to routinely accumulate viruses at concentrations 36-2000 times reported ambient levels (Gerba *et al.*, 1977; Metcalf *et al.*, 1974) indicated that undisturbed sediments may be a minor source of viral contamination to shellfish.

A possible explanation for the few instances of accumulation noted under these conditions in the present study might be related to the pumping and feeding activities of the molluscs (e.g., opening and closing), which could result in localized sediment resuspension and subsequent viral accumulation from the water column. This type of accumulation was clearly evident in those experiments where virus-contaminated sediments were intentionally resuspended, allowing the viruses to permeate overlying waters. Under these conditions the occurrence of viral accumulation was substantially increased. A statistical analysis of all accumulation data indicated that no significant differences existed in the concentrations of viruses accumulated by shellfish under disturbed or undisturbed conditions. However, because the incidence of uptake was clearly higher with resuspended sediments, the latter appeared to represent a more important source of viral contamination.
The second study described here was one of the first to define the nature of low-level virus carriage by shellfish. Our inability to routinely recover both monodispersed (Table IV) and feces-associated viruses (Table V) from actively pumping shellfish exposed to approximately 0.01 PFU/ml led to the speculation that such levels might be beyond the limits of efficient accumulation by the animals. Although the experimental results support the concept of inefficient virus accumulation by shellfish residing in waters containing less than 0.01 virus particles per milliliter, several field studies have documented instances of virus-contaminated shellfish in marine waters seemingly devoid of viruses (Gerba and Goyal, 1978; Vaughn et al., 1979; Vaughn et al., 1980).

Although these reports appear to contradict the proposed limit, several arguments can be advanced to explain both occurrences. First, under natural field conditions, rates of viral accumulation and depuration proceed at a slower rate than those observed in an experimental system. It is therefore possible for shellfish to accumulate viruses during periods when viruses are present in overlying water, and maintain these levels long after the waters have been cleared of viruses. Second, the seawater virus concentrations reported from field studies may have been overly conservative due to inefficient concentration techniques or samplings conducted at inappropriate depths. In the latter case, water samples collected at surface or middepths may not have accurately reflected the concentrations that shellfish were exposed to at the watersediment interface.

Although innumerable studies have addressed the accumulation of viruses by shellfish (Gerba and Goyal, 1978), several aspects of the present study have distinguished its uniqueness.

1. In general, most of the previous studies were carried out in small-scale, static, seawater systems which were seeded with monodispersed laboratory strains of virus. The present study made use of large-scale, flowing systems which were available at both Woods Hole and Brookhaven Laboratory. The use of these systems provided an environment which was more consistent with the normal habitat of the shellfish. Furthermore, it allowed the segregation of active and inactive animals.

2. With the exception of the work of Metcalf et al., 1980, which dealt solely with accumulation in soft-shell clams, no previous study had considered the accumulation of feces-associated viruses.

3. In addition to being unique in its use of oysters and hard clams, the present study assessed accumulation potentials at human virus exposure levels which, although far lower than any previously used, were consistent with levels typically recovered in light to moderately polluted marine waters.

Overall, the study presents reasonable evidence to indicate that at concentrations normally encountered in lightly polluted seawater, both monodispersed and feces-associated viruses were not efficiently accumulated by resident shellfish. Additional studies should be designed to further pursue this concept focusing on extended uptake periods, followed by appropriate depuration studies. Such investigations may have important implications in the certification and regulation of shellfish and shellfish-raising waters.
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REFERENCES

Appleton, H., Palmer, S.R., and Gilbert, R.J., 1981. Food-borne gastroenteritis of unknown aetiology: a virus infection? British Med. Jour., 282:1801-1802.

Dahling, D.R., Berg, G. and Berman, D., 1974. BGM, a continuous cell line more sensitive than Primary Rhesus and African Green kidney cells for the recovery of viruses from water. Health Lab. Sci., 4:275-282.

Fugate, K.J., Cliver, D.O. and Hatch, M.T., 1975. Enteroviruses and potential bacterial indicators in Gulf Coast oysters. Jour. Milk Food Technol., 38:100-104.

Gerba, C.P. and Goyal, S.M., 1978. Detection and occurrence of enteric viruses in shellfish: a review. J. Food Protect., 41:743-754.

Gerba, C.P., Smith, E.M. and Melnick, J.L., 1977. Development of a quantitative method for detecting enteroviruses in estuarine sediment. Appl. Environ. Microbiol., 34:158-163.

Goyal, S.M., Gerba, C.P. and Melnick, J.L., 1979. Human enteroviruses in oysters and their overlying waters. Appl. Environ. Microbiol., 37:572-581.

Grohmann, G.S., Greenberg, H.B., Welch, B.M. and Murphy, A.M., 1981. Oyster-associated gastroenteritis in Australia. The detection of Norwalk virus and its antibody by immune electron microscopy and radioimmunoassay. J. Med. Virol., 6:11.

Jakubowski, W., Hill, W.F., Jr. and Clarke, N.A., 1975. Comparative study of four microporous filters for concentrating viruses from drinking water. Appl. Microbiol., 30:58-65.

Katzenelson, E., Fattal, B. and Kostovsky, T., 1976. Organic flocculation: an efficient second-step concentration method for the detection of viruses in tap water. Appl. Environ. Microbiol., 32:638-639.

LaBelle, R.L. and Gerba, C.P., 1979. Influence of pH, salinity, and organic matter on the adsorption of enteric viruses to estuarine sediment. Appl. Environ. Microbiol., 38:93-101.

Landry, E.F., Vaughn, J.M., Thomas, M.Z. and Vicale, T.J., 1978. Efficiency of beef extract for the recovery of poliovirus from wastewater effluents. Appl. Environ. Microbiol., 36:544-548.

Landry, E.F., Vaughn, J.M. and Vicale, T.J., 1980. Modified procedure for extraction of poliovirus from naturally-infected oysters using Cat-Fluc and beef extract. Jour. Food Protect., 43:91-94.

Liew, P.F. and Gerba, C.P., 1980. Thermostabilization of enteroviruses by estuarine sediment. Appl. Environ. Microbiol., 40:305-308.

Linco, S.J. and Grohmann, G.S., 1980. The Darwin outbreak of oyster-associated viral gastroenteritis. Med. J. Aust., 1211.

Mahoney, P., Fleischner, G., Millman, L., London, W.T., Blumberg, B.S. and Arias, I.M., 1976. Australia antigen: detection and transmission in shellfish. Science, 183:80-81.

Metcalfe, T.G., Moulton, E. and Eckerson, D., 1980. Improved method and test strategy for recovery of enteric viruses from shellfish. Appl. Environ. Microbiol., 39:141-152.

Metcalfe, I.G. and Stiles, W.C., 1967. Survival of enteric viruses in estuary waters and shellfish. In: G. Berg (Editor), Transmission of viruses by the water route. Intersciences, New York.

Metcalfe, T.G., Walls, C. and Melnick, J.L., 1974. Virus enumeration and public health assessments in polluted surface water contributing to transmission of virus in nature. In: J.F. Malina and B.P. Sagik (Editors), Virus Survival in water and Wastewater Systems. Center for Research in Water Resources. Austin, Texas.
Morbidity and Mortality Weekly Report, 1982. Center for Disease Control, 31:449-451.
Morris, R.L., Mears, A.J., and Kim, J., 1976. Viruses-bacteria in coastal waters and shellfish. Ann. Rept. of the Southern Calif. Coastal Waters Res. Proj.
Murphy, A.M., Grohmann, G.S., Christopher, P.J., Lopez, W.A., Davey, G.R. and Millsom, R.H., 1979. An Australia-wide outbreak of gastroenteritis from oysters caused by Norwalk virus. Med. J. Aust., 2:329-333.
Portnoy, B.L., Mackowiak, P.A., Caraway, C.T., Walker, J.A., McKinley, T.W. and Klein, C.A., 1975. Oyster-associated hepatitis: failure of shellfish certification programs to prevent outbreaks. JAM, 233:1065-1068.
Roos, B., 1956. Hepatitis epidemic conveyed by oysters. Sven Lakartidningen, 53:989-1003.
Smith, E.R., Gerba, C.P. and Melnick, J.L., 1978. Role of sediment in the persistence of enteroviruses in the estuarine environment. Appl. Environ. Microbiol., 35:685-689.
Sokal, R.R. and Rohlf, F.J., 1969. Biometry. W.H. Freeman and Co., San Francisco.
Steele, R.G.D. and Torrie, J.H., 1960. Principles and Procedures of Statistics. McGraw-Hill, New York.
Vaughn, J.M., Landry, E.F., Thomas, M.Z., Vicale, T.J. and Penello, W.F., 1979. Survey of human enteroviruses occurrence in fresh and marine surface waters on Long Island. Appl. Environ. Microbiol., 38:290-296.
Vaughn, J.M., Landry, E.F., Vicale, T.J. and Dahl, M.C., 1980. Isolation of naturally occurring enteroviruses from a variety of shellfish species residing in Long Island and New Jersey marine embayments. J. Food Protect., 43:95-98.
Vaughn, J.M., Landry, E.F., Vicale, T.J. and Mann, R., 1981. Factors affecting the accumulation of human viruses by filter-feeding bivalve molluscs. Brookhaven National Laboratory Report No. 30767. Upton, New York.
Vaughn, J.M. and Metcalf, T.G., 1975. Coliphages as indicators of enteric viruses in shellfish and shellfish raising estuarine waters. Water Res., 9:613-616.