ABSTRACT

BETH E. BACHUR. Effect of Temperature and Salinity on the Depuration of Hepatitis A Virus and Other Microbial Contaminants in the Eastern Oyster. (Under the Direction of Dr. MARK D. SOBSEY)

Comparative studies were done to evaluate the elimination of hepatitis A virus (HAV), poliovirus 1, MS2 bacteriophage, Escherichia coli, and Streptococcus faecalis in experimentally contaminated Eastern oysters in a laboratory-scale depuration system. The effect of temperature (12, 18, and 25°C) and salinity (8, 18, and 28 ppt) on virus and bacteria depuration was studied. The oysters were contaminated with high levels of organisms in order to follow depuration over several orders of magnitude during several days of depuration. At each day of the depuration period, a sample of contaminated oysters was removed from the tanks and processed for bacteriological and virological analysis. The depuration system was maintained within the range of the environmental conditions required by the U.S. water quality depuration standards. HAV was not effectively depurated and was detected at generally higher levels than the other test organisms at all the conditions tested. Depuration rates were reduced at lower temperature and lower salinity. These results indicate that depuration may not be an effective means of eliminating HAV and other pathogens from contaminated oysters under some depurating conditions.

KEY WORDS: hepatitis A virus (HAV), poliovirus 1, MS2 bacteriophage, Escherichia coli, Streptococcus faecalis, fecal coliform, depuration, bivalves, and oysters.

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ACKNOWLEDGEMENTS

I would like to thank Dr. Mark D. Sobsey for his personal interest and guidance throughout this project. I am also grateful to Dr. Frederic K. Pfaender and Dr. Morris A. Shiffman for their positive criticism and support during the preparation of this report.

Special thanks are due to Greg Lovelace for his valuable assistance with the experiments and to Doug Wait for helpful discussion.

I especially appreciate my husband, Karl Tameler, and my family for their interest, support, and encouragement.

INTRODUCTION

The mouth-watering tradition of eating oysters-on-the-half-shell is threatened by man's disregard of his environment. As the input of pollution to coastal waters continues to increase, more and more shellfish resources are closed to harvesting because of the public health risks associated with consumption of contaminated shellfish.

Sanitation standards for shellfish and shellfish-growing waters based on levels of coliform bacteria were established over 40 years ago. Although enforcement of these sanitation standards has resulted in adequate protection from bacterial disease, the ability of coliform standards to indicate viral contamination of shellfish has been questioned. Many outbreaks of hepatitis A and viral gastroenteritis have resulted from the consumption of contaminated shellfish. In some cases, the viral-contaminated shellfish had been harvested from "approved" waters.

Depuration is a process where contaminated shellfish are placed in clean flowing water and allowed to purge themselves of contaminating microorganisms. So while shellfish connoisseurs continue to devour raw and

inadequately-cooked shellfish, the process of depuration may prove to be a more palatable measure for reducing contaminated shellfish of microbial pollutants. Microbial information on depuration is limited, with very little known about the elimination of HAV. Studies are needed in order to evaluate and characterize the behavior of HAV compared to other enteric viruses, indicator bacteria, and other potential indicators during depuration. In addition, research is needed to determine the effects of environmental factors such as temperature and salinity on the rates of depuration in order to identify the optimum conditions needed to eliminate HAV and other microbial contaminants from shellfish.

OBJECTIVES

To evaluate the elimination of HAV compared to the elimination of poliovirus, MS2 bacteriophage, E. coli, and Streptococcus faecalis at different conditions of temperature and salinity in a laboratory-scale depuration system.

-To determine the rates of depuration of high levels of HAV, poliovirus, MS2 bacteriophage, E. coli, and Streptococcus faecalis at temperatures of 12, 18, and 25°C.

-To determine the rates of depuration of high levels of HAV, poliovirus, MS2 bacteriophage, E. coli, and Streptococcus faecalis at salinities of 8, 18, and 28 ppt.

LITERATURE REVIEW

I. Viruses

Viruses are small, obligate intracellular parasites which have no capacity for independent metabolism or replication outside a host cell. They consist of a nucleic acid genome surrounded by a protein capsid specific to the virus. In addition, some viruses may have an outer lipid envelope derived from the host cell. Viral nucleic acid is unique in that it may be either single- or double-stranded DNA or RNA, but both are not found in the same virus. Viruses range in size from 20nm to 300nm in diameter and have a variety of shapes, including spherical, rod, icosahedral, and complex. Furthermore, viruses vary in host range and can infect and cause disease in the cells of plants and bacteria, as well as in those of animals.

A. Enteric Viruses

Enteric viruses multiply in the alimentary tract and are excreted in the feces in numbers as high as $1x10^{10}$ per gram of feces. Because enteric viruses lack a lipid envelope, they are resistant to the acids and enzymes present in the stomach. Transmission is usually by the fecal-oral route initiated by person-to-person contact or through the ingestion of virus-contaminated food and water.

There are more than 100 types of enteric viruses including the enteroviruses (polioviruses, echoviruses, coxsackieviruses, and hepatitis A virus), adenoviruses, rotaviruses, reoviruses, Norwalk virus, caliciviruses, and astroviruses. Although many enteric virus infections may be asymptomatic, these viruses can cause a variety of illnesses including paralysis, meningitis, hepatitis, diarrhea, and vomiting.

A.1. Hepatitis A Virus

Hepatitis A virus (HAV) is a member of the Picornaviridae family and is classified as Enterovirus 72 (Melnick, 1982). As such, HAV possesses many physical and chemical characteristics of a typical enterovirus. Mature HAV particles are nonenveloped, approximately 27nm in diameter, and have a CsCl buoyant density of 1.33-1.34 g/cc and a sedimentation coefficient of 156-160S. The genome consists of a single strand of RNA having a molecular weight of 2.5×10^6 and positive polarity. The capsid displays icosahedral symmetry and is composed of 32 capsomers made up of four polypeptides (VP1, VP2, VP3, VP4).

Like other enteroviruses, HAV is acid (pH 3) stable and withstands inactivation by ether. But HAV is comparatively more resistant to heating at high temperatures. While other enteroviruses are quickly inactivated at 60°C, HAV is able to survive this temperature for up to 12 hours. Siegl et al. (1984) reported that 50% of HAV particles are inactivated at 61°C in 10 minutes resulting in release of

RNA and loss of infectivity. Information on HAV resistance to chlorination is conflicting. Some studies report that HAV is relatively sensitive to free chlorine (Grabow et al., 1983; Sobsey et al., 1988; Wilson and Sobsey, 1987), while other studies suggest that it is relatively resistant (Peterson et al., 1983; Vaughn et al., 1987). However, all studies to date indicate that HAV is relatively resistant to combined chlorine (Grabow et al., 1984; Sobsey et al., 1988), which is the form of chlorine present in chlorinated sewage effluents. HAV is also persistent in soils, sewage, and the water environment (Sobsey et al., 1987).

The major routes of hepatitis A transmission are contaminated water and food (particulary shellfish), as well as direct and indirect person-to-person contact. For example, Bergeisen et al. (1985) reported a waterborne outbreak of hepatitis A involving 73 cases in Meade County, Kentucky. In addition, O'Mahony et al. (1983), using a case-control study, documented that 25% of the hepatitis A cases in southeast England could be linked to the consumption of shellfish.

Infectious hepatitis or hepatitis A, a viral infection of the liver, varies in severity and duration. The incubation period ranges from 2-8 weeks with large amounts of virus excreted preceding any symptoms. Clinical features may last from 2-5 weeks and include fever, nausea, abdominal pain, vomiting, dark urine, enlargement of the liver and spleen, and jaundice. Infection is often subclinical in

children because the severity of clinical hepatitis A increases with age. In developing countries, hepatitis A is endemic due to overcrowding and poor sanitation and hygiene.

Only recently has HAV been successfully propagated in cell culture. Provost and Hilleman (1979) demonstrated HAV growth in marmoset liver and fetal rhesus monkey kidney (FRhK-6) cells. Today, HAV is cultivated in vitro in a variety of cell lines, including human diploid lung fibroblasts (HDLF) and continuous monkey kidney cell cultures such as BSC-1 and FRhK-4. In vitro infection with HAV does not usually produce cytopathic effects but instead yields a persistent infection of cells.

The development of the radioimmunofocus assay (RIFA) permitted quantification of hepatitis A virus in cell culture (Lemon et al., 1983). This assay utilizes radiolabelled anti-HAV and autoradiography to detect foci of HAV-infected cells on a confluent cell layer. In addition, the number of radioimmunofoci observed are directly related to the amount of virus inoculated. Therefore, the RIFA method provides an accurate and rapid assay for the enumeration of HAV.

A.2. Poliovirus

Poliovirus is a member of the Picornaviridae family and the Enterovirus genus. The virion is a naked icosahedron with a diameter of 27nm and a buoyant density of 1.34 g/cc. In addition, poliovirus is acid-stable like other enteroviruses. The genome is single-stranded RNA of

positive polarity and a molecular weight of approximately 2.5×10^6.

Poliovirus is transmitted via the fecal-oral route, especially through direct and indirect person-to-person contact. The role of fecally contaminated food and water remains uncertain. Poliovirus causes poliomyelitis, usually characterized by symptoms such as fever, sore throat, headache, vomiting, and possibly meningitis. Only about 1% of the cases will develop paralysis which may or may not persist. The incubation period averages two to four days and infection instills lifelong immunity.

Although vaccine campaigns have been successful in the developed nations of the world, polio continues to be endemic throughout many developing countries. Some of the problems encountered by polio vaccination programs include inaccessibility of rural villages and inadequate refrigeration of the vaccine in tropical climates.

II. Enteric Bacteria and Viruses in the Aquatic Environment

A. Sources of Enteric Bacterial and Viral Contamination

Enteric bacteria and viruses enter the aquatic environment from various sources of fecal contamination. Wastewater containing bacterial and viral pathogens can create a significant health problem if released into the environment. The discharge of raw domestic sewage into surface waters has led to polluted drinking water, shellfish, and recreational waters. Approximately 5% of the

U.S. population still discharges untreated sewage directly via ocean outfalls (Rao and Melnick, 1986).

Coliform bacteria make up a relatively high proportion of sewage microbes and are an important indicator of water polluted with fecal material. Untreated domestic sewage may contain more than three million coliforms per 100 ml and from 1×10^3 to 1×10^6 enteric viruses per liter (Bitton, 1980). Although these concentrations are supposed to be reduced by dilution and natural degradation when discharged, enteric bacteria and viruses have been detected as far as 8 miles from the sewage discharge (Metcalf et al., 1974). Persistence at even greater distances has been demonstrated in cold weather (Dahling and Safferman, 1979). Additional sources of enteric bacteria and viruses for coastal waters include septic system leachates, storm water runoff, and boat waste discharges.

Wastewater is treated in order to remove and control the dissemination of biological pathogens, suspended solids, and organic contaminants. The conventional steps in wastewater treatment are primary sedimentation, secondary (biological) treatment, and disinfection. In addition, advanced processes called tertiary treatment may be practiced when water is recycled. Primary treatment removes suspended solids in raw sewage by sedimentation. The primary effluent then undergoes secondary treatment which utilizes microbiological degradation of soluble organics and suspended solids. Tertiary treatment often consists of

coagulation-sedimentation, filtration, activated carbon adsorption, and disinfection.

Although wastewater treatment processes can reduce the concentration of enteric bacteria extensively, they may be inadequate for the removal of enteric viruses. Primary treatment reduces viruses inefficiently with approximately 0-75% removal. Secondary treatment, using trickling filters or oxidation ponds, can achieve from 10 to 95% removal. Coagulation-flocculation seems to be the most efficient treatment method producing a 90-99.99% reduction in viruses. Table 1 summarizes the processes used in sewage treatment and the percent virus removed in each step. Overall, conventional primary and secondary sewage treatment can reduce enteric viruses by 90-99%, but the effluent and solid wastes (sludges) will still contain infective viruses.

Sewage sludge disposal sites also contribute to the input of enteric pathogens into the aquatic environment. During sludge sedimentation, the bacteria and viruses are concentrated in and adsorbed to the sludge solids, yet are not inactivated. Goyal et al. (1984) isolated infective viruses from sediments around a marine sewage disposal site 17 months after all sludge dumping had ended.

Chlorination of primary effluents is an ineffective method of virus inactivation, in addition to forming toxic chlorinated by-products such as trihalomethanes. Sattar et al. (1978) detected 2.7 VIU (viral infective units) per 100 ml in samples of chlorinated primary effluent. This

TABLE 1

VIRUS REMOVAL IN WASTEWATER TREATMENT PROCESSES

TREATMENT	EXPECTED REMOVAL(%)
Primary	
Primary Sedimentation	0-75
Chlorination of Primary Sedimentation effluent	50
Secondary	
Trickling Filter	50
Chlorination of Trickling Filter effluent	50
Activated Sludge	40-90
Chlorination of Activated Sludge effluent	50-90
Tertiary	
Coagulation-Flocculation and Sedimentation	90-99.99
Activated Carbon Adsorption	10-50
Chlorination of Tertiary effluent	99-99.99

Adapted from Mahdy (1979) and Gerba *et al*. (1975)

represented a 10- to 50-fold reduction in VIU compared to the primary treatment. Enteric viruses tend to resist inactivation by disinfection better than coliform bacteria.

Enteric viruses may be inefficiently removed by drinking water processes under typical plant conditions. Enteric viruses, namely poliovirus type 1, were recovered in treated drinking water drawn from a sewage-contaminated river (Payment, 1981). All treated water samples contained viruses and most contained at least 1-10 cytopathic units per 100 liters. The water met coliform, turbidity, and other quality standards. Treatment included prechlorination, flocculation, filtration, ozonation, and postchlorination, and the finished water contained a residual chlorine level and no coliforms.

B. Persistence and Factors Influencing the Survival of Enteric Bacteria and Viruses in the Aquatic Environment

Once bacteria and viruses enter the aquatic environment from various sources, the potential for transmission of disease depends on their survival in the receiving waters. Enteric viruses have been demonstrated to be more persistent in seawater and sediments than coliform bacteria (Bitton, 1980; Kapuscinski and Mitchell, 1980; Sattar, 1981). Moreover, indicator bacteria in surface waters do not reflect the concentration of enteric viruses in the water and sediments (LaBelle et al., 1980). Compared to bacterial pathogens, enteric viruses have a very low infective dose. For example, a dose of 1×10^8 toxigenic E. coli is required to cause disease while a minimum of one infectious unit of

an enterovirus can cause infection (Bitton, 1980). Therefore, even small amounts of enteric viruses should be prevented from entering the aquatic environment.

Physical, chemical, and biological factors can influence the persistence or inactivation of enteric bacteria and viruses in receiving waters. Table 2 shows a summary of these different environmental factors.

The physical factors governing the survival of enteric bacteria and viruses include temperature, sunlight, dilution, and adsorption to solids. Thermal inactivation studies of bacteria and viruses have shown that higher temperatures lead to a rapid loss of infectivity and low temperatures prolong persistence. O'Brien and Newman (1977) demonstrated that a 1-log reduction of poliovirus required 25 hours and coxsackievirus required 7 hours at temperatures ranging from 23-27^{o}C. When tested at 4-8^{o}C, the log reduction times for poliovirus and coxsackievirus increased to 46 and 58 hours, respectively. In addition, temperature affects natural aquatic microbial activities, such as predation and enzyme production, which contribute to enteric bacterial and viral degradation.

Sunlight, specifically ultraviolet radiation, can penetrate the water surface and damage microbial nucleic acid. Ultraviolet light has long been known to inactivate bacteria and viruses, but may be ineffective in waters with a high concentration of suspended and dissolved organic matter. Additionally, concentrations of enteric bacteria

TABLE 2

FACTORS AFFECTING ENTERIC BACTERIA AND VIRUS PERSISTENCE IN WATER

PHYSICAL

1. Temperature
2. Sunlight
3. Dilution Effects
4. Adsorption to Particulates and Sedimentation
5. Aggregation
6. Presence of Pollution
7. Sedimentation

CHEMICAL

1. pH
2. Salinity
3. Presence of Cations
4. Heavy Metals
5. Dissolved Organic Matter

BIOLOGICAL

1. Virus Type
2. Predation
3. Enzymatic Degradation
4. Shellfish Accumulation

Adapted from Sattar (1981)

and viruses are reduced by physical dilution in receiving waters. But the reliance on these "self-purification" processes alone is becoming inadequate to protect the quality of man's drinking water supplies, food-resource waters, and recreational waters.

Because they are charged colloidal particles, viruses are able to adsorb to suspended and particulate solid surfaces. Greater than 99% of enteric viruses rapidly adsorb to estuarine sediments (LaBelle and Gerba, 1980). Moreover, adsorbed viruses retain their infectivity and can accumulate in the sediments.

Numerous studies have demonstrated that adsorption to sediments can protect bacteria and viruses and increase their persistence in the aquatic environment. LaBelle and Gerba (1980) studied the survival of polio- and echoviruses in estuarine sediments near sewage outfalls. Both enteroviruses displayed reduced inactivation rates when adsorbed to sediment. In addition, this study suggested that, in the presence of sewage effluents, viruses persist longer in sediments but not in surface waters. Another study found enteroviruses to survive longer when associated with both suspended and sedimented solids. Poliovirus and rotavirus SA-11 survived for 19 days when attached to solids, but survived only 9 days in seawater alone (Rao et al., 1984). In addition, Gerba and McLeod (1976) showed that E. coli had higher survival rates in sediments than in seawater. Enteroviruses may be protected from thermal

inactivation when adsorbed. Liew and Gerba (1980) demonstrated that poliovirus adsorbed to sediments had longer survival times at 24 and 37°C, but displayed no significant changes in persistence at 4°C.

Chemical factors include pH, salinity, and the presence of cations, heavy metals, and dissolved organics. Most bacteria and viruses are relatively stable at the pH of natural waters (pH 5-9). Siegl et al. (1984) found both HAV and poliovirus to be stable between pH 3-10 at 20°C for 30 minutes. However, alkaline pHs greater than or equal to 11 have been shown to inactivate enteroviruses (Bitton, 1980). Salinity does not seem to play a consistent role in the inactivation of bacteria or viruses, although sudden changes in isotonic conditions, such as in an ocean outfall, may result in greater bacterial and viral instability. The presence of cations, such as Mg^{+2}, can protect enteroviruses against inactivation and enhance adsorption to particulates. Mitchell (1971) found that heavy metals played a role in viral inactivation. The increased survival of the bacteriophage ΘX-174 in both autoclaved and filtered seawater was probably due to the absence of heavy metals. By competing with viruses for adsorption sites on particulates, dissolved organics can limit viral protection by adsorption and can reduce virus survival in the aquatic environment.

Microorganism type, predation, and enzymatic degradation are important biological factors affecting

persistence. Inherent structural and biochemical differences in the many varieties of bacteria and viruses cause them to react to environmental processes in different ways. Predation on enteric bacteria and viruses by indigenous marine bacteria, protozoa, and bacteriophages reduces persistence. These native organisms possess the ability to grow and survive better than enteric organisms at the low nutrient levels found in receiving waters. Shuval et al. (1971) reported a heat-labile, nonfilterable factor in seawater which inactivated viruses and later isolated marine bacteria capable of diminishing poliovirus 1000-fold in 7 days. Furthermore, the enzymatic activity of bacteria contributes to inactivation of enteric viruses in surface waters (Cliver and Herrmann, 1972). In a recent study, proteolytic bacterial enzymes were shown to inactivate echovirus in freshwater by cleaving the protein capsid (Ward et al., 1986). This caused the viral RNA to be susceptible to nuclease digestion.

C. Contamination of Shellfish

Because they live in coastal waters often polluted by fecal wastes, bivalve molluskan shellfish can become contaminated with enteric bacteria and viruses. Shellfish, including clams, oysters, and mussels, feed by filtering suspended particles from the surrounding water, whereby they can concentrate enteric microbes in their bodies. This creates a public health problem since raw or partially

cooked shellfish can act as vehicles of bacterial and viral diseases.

Enteric bacteria and viruses have been isolated from many kinds of edible shellfish (Gerba and Goyal, 1978). Important edible shellfish species are summarized in Table 3. Several studies have isolated enteric bacteria and viruses from both polluted areas closed to shellfishing and approved areas (Goyal et al., 1979; Vaughn et al., 1980; Ellender et al., 1980; Wait et al., 1983). The percentage of virus-positive shellfish ranged from 9-40% for open waters and 13-40% for closed areas. In all these studies, enteroviruses were detected in shellfish where no viruses were isolated from the overlaying waters.

Enteric viruses can survive in shellfish for long periods. Viruses have been reported to survive longer within shellfish than in seawater probably because of protection from virus-inactivating factors (Hedstrom and Lycke, 1964; Metcalf and Stiles, 1965). Lower temperatures seem to prolong virus survival in oysters. Metcalf and Stiles (1968) showed that enteroviruses will persist in oysters for at least four months at temperatures below 7°C. In another study, refrigerated oysters were examined over time for poliovirus survival (DiGirolamo et al., 1970). In chilled Olympia oysters, poliovirus was reduced by only 10% after 5 days of storage at 5°C and by 60% after 15 days. After 30 days of storage at 5°C, 13% of the poliovirus was still infectious.

TABLE 3

IMPORTANT EDIBLE SHELLFISH SPECIES

SCIENTIFIC NAME	COMMOM NAME
Crassostrea virginica	Eastern oyster
Crassostrea gigas	Pacific oyster
Crassostrea commercialis	Sydney Rock Oyster
Clinocardium nuttalli	Cockle
Mya arenaria	Soft Shell Clam
Mercenaria mercenaria	Hard Shell Clam or Northern Quahaug
Mercenaria campechiensis	Southern Quahaug
Mytilus edulis	Blue Mussel
Ostrea lurida	Olympia Oyster
Ostrea edulis	European Oyster
Tapes japonica	Manila clam

Virus survival in typical shellfish cooking methods was also examined (DiGirolamo et al., 1970). Poliovirus survived to a greater degree in fried and baked oysters than in steamed and stewed oysters. After 8 minutes of stewing, 10% of the poliovirus remained infectious, and 13% survived frying for 10 minutes. Baked oysters retained 13% viable poliovirus after 20 minutes in an oven at 121.5°C, while 30 minutes of steaming resulted in a 2-log reduction and approximately 7% of the poliovirus survived. Mazanti (1987) showed that HAV was not inactivated to acceptable levels under pasteurization conditions. At conditions of 62.8°C for 30 minutes and 71.7°C for 15 seconds, HAV was inactivated to 99.8% and 99.95%, respectively. In contrast, poliovirus displayed complete (greater than 99.999%) inactivation at 62.8°C in 1 minute and at 71.1°C in 30 seconds.

D. Coliform Standards for Shellfish

There is a need for a reliable indicator of fecal contamination in shellfish and shellfish-harvesting waters because of the potential for transmission of disease-causing pathogens. The National Shellfish Sanitation Program (NSSP) established nationwide sanitation standards for U.S. shellfish and shellfish-growing waters over 40 years ago. The indicator system is based on levels of total and fecal coliform bacteria. The standards for "approved" shellfish waters state that the coliform bacteria Most Probable Number (MPN) should not exceed medians of 70 total coliforms or 14

fecal coliforms per 100 ml, and no more than 10% of the samples can exceed a MPN of 230 and 43 per 100 ml, respectively. In addition, fecal coliform MPN must not exceed 230 per 100 grams of shellfish meat (FDA, 1987). During 1977-1979, 36% of the total shellfish-growing area in 13 U.S. states was closed because of failure to meet these bacterial standards (Rao and Melnick, 1986).

Reasoning behind this system argues that the presence of coliforms is reasonably indicative of fecal contamination and that coliform levels are usually higher than levels of pathogenic organisms. In addition, the methodology involved is fairly easy and inexpensive. Therefore, the use of these standards is an economic means of reducing the risk of disease acquired by the consumption of contaminated shellfish.

Although enforcement of current sanitation standards has resulted in adequate protection from bacterial disease, recent studies have questioned the ability of coliform standards to indicate viral contamination of shellfish and shellfish-harvesting waters (Gerba and Goyal, 1978; Morris and Waite, 1981; Larkin and Hunt, 1982). Enteroviruses have been isolated in water and shellfish from approved harvesting areas (Fugate et al., 1975; Goyal et al., 1979; Vaughn et al., 1980). In addition, no significant relationship has been found between concentrations of enteric viruses or coliform bacteria in shellfish or shellfish-growing waters (Ellender et al., 1980; Gerba et

al., 1980; Wait et al., 1983). Outbreaks of hepatitis A have been associated with the consumption of raw oysters harvested from "approved" waters (Portnoy et al., 1975). Thus, the virological quality of shellfish and their overlying waters and the resulting risk of viral disease is not adequately controlled by current coliform standards.

Because of the unreliability of coliform bacteria to assess the presence of enteric viruses, other indicator organisms have been considered. Fecal streptococci, bacteriophages, and selected enteric viruses (usually poliovirus) have all been evaluated as candidate indicators of viral contamination, but none have proven reliable for the detection of all enteric viruses (Morris and Waite, 1981). Therefore, it seems that the only valid indication of viral contamination at present is the direct demonstration of specific viruses.

III. Epidemiology of Shellfish-Related Disease

Due to the increasing pollution of shellfish-growing waters and the ability of shellfish to concentrate bacteria and viruses in their bodies, shellfish pose a threat to the consumer. The consumption of raw and partially cooked shellfish has been associated with outbreaks of bacterial and viral diseases. Between 1900 and 1983, 198 outbreaks involving 8,659 cases of shellfishborne enteric disease were reported in the U.S..

Several factors contribute to the occurence of enteric disease transmission by shellfish. In the first place, the

current coliform standards are inadequate indicators of viral contamination of shellfish and their overlying waters. In addition, improperly classified growing waters and shellfish poaching can result in the harvesting and distribution of contaminated shellfish. Heavy flooding and rainfall increases storm runoff, overburdens sewage treatment plants, and suspends sediments and can lead to significant quantities of fecal contaminants reaching "approved" areas. Finally, inadequate shellfish-tagging requirements have failed to identify sources of contaminated shellfish.

In the past, outbreaks of typhoid fever have been traced back to the consumption of contaminated shellfish (Richards, 1985). The incidence of bacterial illness caused by ingesting shellfish has been on the decline because of the current shellfish sanitation measures. An indigenous marine bacterium, Vibrio parahaemolyticus, may play a role in recent outbreaks of shellfishborne gastroenteritis. This organism is the most frequent cause of food poisoning in Japan and has been isolated in U.S. coastal waters (Earampamoorthy and Koff, 1975).

Hepatitis A is one of the most important viral diseases transmitted by the consumption of contaminated shellfish. During 1961-1982, the ingestion of contaminated shellfish was responsible for over 1000 cases of hepatitis A in the U.S. (Richards, 1985). Table 4 documents the outbreaks of

shellfish-associated hepatitis A in the U.S.. The transmission of hepatitis A through shellfish has also been investigated in other countries. Between Nov. 1, 1980 and April 30, 1981, 450 cases of infectious hepatitis caused by the consumption of cockles were reported in England (O'Mahony et al., 1983). In addition, O'Hara et al. (1983) retrospectively determined that an outbreak of hepatitis A among the Japanese Overseas Corporation Volunteers stationed in the Phillippines was due to the consumption of contaminated raw oysters.

In addition to hepatitis A virus, other enteric viruses have been implicated in nonbacterial gastroenteritis resulting from shellfish consumption. The first documented outbreak of shellfish-related Norwalk virus gastroenteritis occurred in Australia and involved more than 2000 people (Murphy et al., 1979). In 1982, at least 103 persons were involved in outbreaks of clam- and oyster-associated gastroenteritis in New York state. The causative agent was suggested to be the Norwalk virus (Morse et al., 1986). In London, there were 181 cases of viral gastroenteritis associated with the consumption of depurated oysters contaminated with small round structured viruses (Gill et al., 1983). The work by Grohmann et al. (1981) demonstrated the ineffectiveness of current depuration practices to remove viral contamination. In that study, depurated oysters that met the bacteriological standards caused Norwalk virus gastroenteritis in volunteers.

TABLE 4

OUTBREAKS OF SHELLFISH-ASSOCIATED HEPATITIS A SINCE 1961

YEAR	CASES	SOURCE	LOCATION
1961	84	oysters	Alabama and Mississippi
1961	459	clams	New Jersey, and New York
1961	15	clams	Connecticut
1961	31	oysters	Alabama
1962	3	clams	New York
1963-6	46	oysters/clams	Mass.
1964	123	clams	Conn. and RI
1964	249	clams	Pennsylvania
1964	3	oysters	N.C.
1964	43	clams	New York
1964	3	clams	Wash.,D.C.
1966	4	clams	New Jersey
1966	3	clams	Mass.
1966	4	clams	New Jersey
1967	3	oysters/clams	Texas
1968	3	clams	New York
1969	6	clams	New York
1969	13	oysters	Florida
1971	5	clams	Mass.
1971	3	clams	R.I.
1972	2	clams	Florida,Mass
1973	263	oysters	Texas
1973	15	oysters	Georgia
1973	37	oysters	Louisiana
1973	1	clams	Minnesota
1977	17	shellfish	Washington
1979	10	oysters	Alabama,Fla.
1982	11	clams	New York

Compiled from Gerba and Goyal (1978) and Richards (1985).

IV. The Accumulation of Enteric Bacteria and Viruses in Shellfish

A. The Oyster and Feeding

Oysters are filter-feeding bivalve molluscs of the class Pelecypoda. The eastern oyster, Crassostrea virginica, is found in estuaries attached to hard surfaces and forming beds. The soft, fleshy oyster body is completely enclosed within two hinged shells called valves. Oysters feed on plankton by pumping large volumes of water through the shell cavity. The pumping rate is affected by environmental parameters such as temperature and turbidity. Ciliary action moves the water across the oyster body where small particles (plankton, bacteria, and viruses) are trapped on the mucus sheet secreted by the gills. The mucus is pushed by ciliary action toward the mouth. The chemoreceptive mouth can sort particles on the basis of size and shape. Accepted food particles enter the stomach where they are digested by extracellular enzymes. Any unaccepted particles are expelled in the pseudofeces. From the stomach, dissolved particles are transported to the digestive diverticulum where phagocytosis, intracellular digestion, and storage occurs. Any undigested particles continue through to the intestine and are excreted in the feces.

B. Mechanisms of Uptake

Bacteria and viruses enter the shellfish cavity when currents of water are pumped during feeding. Bacteria and viruses, including those that are adsorbed to particulates, bind to the secreted mucus, are swept by ciliary action to the mouth, and ingested by the oyster. Differences in surface charge among viruses may cause them to accumulate in shellfish at different rates (Duff, 1967). DiGirolamo et al. (1977) investigated the mechanism of viral uptake by the shellfish mucus. They concluded that the ionic bonding of viral particles to sulfate radicals on the mucopolysaccharide fraction of shellfish mucus was the major mechanism of virus entrapment on the mucus layer. In addition, the influence of salinity and pH on the uptake of viruses by shellfish suggested that ionic bonding is unlikely to be the only means of attachment.

C. The Concentration of Enteric Bacteria and Viruses by Shellfish

Filter-feeding shellfish tend to concentrate bacteria and viruses from the water in which they grow. Shellfish can accumulate bacteria to concentrations 10-40 times higher than levels in the surrounding water (Kelly et al., 1960; Mitchell, 1966). Viruses may occur in shellfish at concentrations up to 60 times greater than those found in the overlaying waters (Mitchell et al., 1966). Viruses accumulate mainly in the shellfish hepatopancreas and digestive diverticulum, but phagocytic ingestion can distribute viruses into other tissues (Metcalf and Stiles,

1965; Canzonier, 1971; Richards, 1988). No multiplication of viruses in shellfish is known to occur, yet bacteria may multiply quickly. Within a few hours, bacteria and viruses can accumulate in shellfish to levels equivalent to those in the ambient water, and as long as sufficient numbers of bacteria and viruses occur in the water, these levels will continue to be maintained (Hedstrom and Lycke, 1964; Mitchell et al., 1966; Perkins et al., 1980).

The extent of microbial concentration by shellfish is affected by various factors. The level of contamination in the water column affects the accumulation of bacteria and viruses by shellfish. Studies have shown that the amount of contamination in the water is directly related to the level of accumulation by shellfish (Mitchell et al., 1966; Landry et al., 1982).

Interspecies differences among shellfish have been shown to affect virus bioaccumulation. DiGirolamo et al. (1975) found that at least 86% of poliovirus present in seawater was accumulated within 12 hours by Olympia oysters, while Pacific oysters took 48 hours to attain the same degree of concentration.

The concentration of bacteria and viruses is affected by the temperature of the overlying waters. Metcalf and Stiles (1968) reported that oysters did not accumulate virus at temperatures below 7°C, while temperatures above 7°C initiated pumping and feeding. Therefore, the physiological activities of shellfish can be inhibited by lower

temperatures. A study by Meinhold (1982) found that the maximum uptake of poliovirus by the oyster occurred in 5 hours at 6°C, 2-3 hours at 17°C, and 1-3 hours at 28°C.

The presence of particulate matter seems to affect the uptake rate of shellfish. The effect of turbidity on poliovirus accumulation in the oyster was demonstrated by Hamblet et al. (1969). Highly turbid waters (54-80ppm) were inhibitive to virus accumulation, and oysters concentrated 2-3 times less poliovirus in highly turbid waters than in waters of low turbidity (8-24ppm). Hoff and Becker (1969) reported thet E. coli and cell-associated viruses were accumulated to levels 40-60 times higher in Pacific oysters and Manila clams than were clarified free viruses. In addition, Metcalf et al. (1979) showed that feces- and solid-associated poliovirus was accumulated more efficiently than was free virus. These results may be due to either feeding stimulation by the presence of particulate matter or to more selective acceptance of particles larger than viruses by the shellfish.

V. Elimination and Depuration of Enteric Bacteria and Viruses in Shellfish

A. Mechanisms of Elimination

When placed in clean water, shellfish have the ability to rid themselves of accumulated microorganisms. The mechanisms of elimination include active elimination, physical inactivation, and phagocytic digestion. Enteric bacteria and viruses can be eliminated from shellfish in the feces and pseudofeces as part of the normal digestive and

excretive processes. However, some microorganisms may be ingested by shellfish phagocytes as a means of intracellular digestion or protection from foreign substances. Bacteria are probably more susceptible to elimination by phagocytic action than are viruses. Phagocytes eliminate bacteria from shellfish through either exportation to the surrounding water or digestion by phagocytic enzymes (Hartland and Timoney, 1979; Perkins et al., 1980). In contrast, phagocytes may transport viruses to other tissues where they are removed from normal elimination processes and can remain for long periods of time. Fries and Tripp (1970) demonstrated that oyster leukocytes could phagocytize a 60nm algal DNA virus within 2 hours of exposure. Although a phagocytic mechanism for removal of naturally accumulated viruses has not been demonstrated, viruses have been found in the shellfish hemolymph (Liu et al., 1966; DiGirolamo, 1975; Metcalf et al., 1979; Metcalf et al., 1980). Furthermore, physical inactivation, mainly involving temperature, can also account for the reduction of bacteria and viruses in shellfish (Canzonier, 1971).

B. Depuration

Controlled purification is a process used to eliminate contaminating microorganisms from shellfish. This process relies on the ability of shellfish to "self-purify" when placed in clean flowing water. In the U.S., shellfish intended for purification can only be harvested from waters

meeting the water quality requirements for restricted or approved areas. Thus, these waters must have less than 700 total coliforms per 100 ml or less than 88 fecal coliforms per 100 ml and no more than 10% of the samples can exceed 2,300 per 100 ml (FDA, 1987).

Relaying and depuration are two methods of controlled purification. Relaying involves the transfer of shellfish from polluted to approved waters. Reharvesting of relayed shellfish is allowed after 2 weeks. In depuration processes, shellfish are placed in tanks fed with flowing purified seawater. The seawater is used in a flow-through or recirculating fashion and may be purified by filtration, UV irradiation, ozonation, or chlorination. The use of chlorine or ozone, however, is less desirable because residual levels can inhibit shellfish physiological processes involved in elimination.

In order to optimize shellfish elimination activity, environmental conditions of the depuration system must be controlled. Depuration standards include water quality controls for dissolved oxygen, coliform bacteria, salinity, temperature, pH, turbidity, and flow rate. In addition, shellfish must be depurated for a minimum of 48 hours. Water quality criteria must be developed for each species of shellfish which is commercially depurated. Table 5 summarizes the U.S. water quality depuration standards for oysters.

TABLE 5

DEPURATION WATER QUALITY STANDARDS FOR OYSTERS

	Minimum	Maximum
Temperature	10°C	25°C
Turbidity	----	20 NTU
pH	7.0	8.4
Salinity	within 20% of harvest area	
Dissolved Oxygen	5.0 mg/l	saturate
Coliform bacteria	----	1/100ml
Flow rate	1 gpm/bushel	----

Adopted from FDA (1987)

After the specified time, the depurated shellfish are sampled and the effectiveness of the depuration process is evaluated by bacteriological analysis. Depurated hard shell clams (Mercenaria mercenaria) and eastern oysters (Crassostrea virginica) must not exceed a mean fecal coliform count of 20 per 100 grams of shellfish meat and no more than 10% of the samples may exceed 20 per 100 grams. No single sample may be in excess of 100 fecal coliforms per 100 grams nor may the arithmetic mean of duplicate samples exceed 75 fecal coliforms per 100 grams (FDA, 1987).

Commercial depuration of shellfish is expensive and is practiced only on a limited scale in the U.S. (Cook and Ellender, 1986). Only approximately 19 plants are depurating soft shell clams, hard shell clams, and oysters in the U.S.. However, depuration is widely practiced in Europe and Australia. Depurated shellfish can command a premium price in the marketplace because they are less gritty and may have a more salty taste than nondepurated shellfish.

Although depuration may reduce fecal coliforms in shellfish to acceptable levels, pathogenic bacteria and enteric viruses may not be as readily eliminated. Fecal coliforms have been shown to be eliminated to less than 18 per 100 grams in 48 hours with less than 10% of the samples exceeding 78 fecal coliforms per 100 grams (Perkins et al., 1980). But evidence concerning the effectiveness of

depuration processes on pathogenic bacteria and enteric viruses is conflicting.

Janssen (1973), using a recirculating UV light depuration system, found that the eastern oyster retained high levels of Salmonella typhimurium and Francisella tularensis for 49 and 11 days, respectively. Son and Fleet (1980), however, reported effective depuration of Bacillus cereus, Clostridium perfringens, Salmonella, and Vibrio parahaemolyticus within 2-3 days in the Sydney rock oyster. Concentrations of coliforms and E. coli were substantially reduced in the commercial depuration system used by Eyles and Davey (1984). However, on three occasions, the process failed to reduce E. coli to acceptable levels. They also found that potentially pathogenic bacteria (Vibrio parahaemolyticus and V. cholera) persisted in naturally contaminated depurated oysters. These results disagree with the work by Son and Fleet (1980) who showed rapid depuration of V. parahaemolyticus in laboratory-inoculated oysters. Thus, the mode of contaminant uptake and the origin of the contaminant can affect the rates of depuration in shellfish.

Studies evaluating the elimination of enteric viruses by shellfish depuration have generated different results. Poliovirus has been shown to be rapidly depurated within 24-48 hours in the eastern oyster and hard shell clam (Mitchell et al., 1966; Liu et al., 1967; Metcalf et al., 1979; Sobsey et al., 1987). However, Cook and Ellender (1986) found no correlation between the elimination of poliovirus and fecal

coliforms, with the virus persisting in oysters having less than 50 fecal coliforms per 100 grams. In a study using coliphage S-13, Canzonier (1971) demonstrated persistence of the virus for 6 days, while E. coli was rapidly depurated in 24-48 hours. In addition, hepatitis A virus may persist longer than poliovirus during depuration. While oysters reduced poliovirus by greater than 98% in 2-3 days, HAV generally did not depurate more than 90% after 72 hours (Sobsey et al., 1987). These studies suggest that shellfish depuration based on the reduction of fecal coliforms within 48 hours may not eliminate the risk of shellfish-associated viral disease.

In fact, the consumption of depurated shellfish has been associated with outbreaks of viral disease. Following an oyster-associated outbreak of Norwalk gastroenteritis involving over 2,000 persons in Australia, human volunteers were used to test-consume depurated oysters (Grohmann et al., 1981). The oysters were purified by either relaying into pollution-free waters for 7 days or depuration for 48 hours. Although depurated oysters met the bacteriological standards of less than 230 coliforms for 100 grams of oyster meat, 52 volunteers who test-consumed these oysters became ill with Norwalk gastroenteritis. Another study linked depurated oysters to an outbreak of viral gastroenteritis in England. Viral gastroenteritis occurred in 181 people who had eaten raw oysters that had been depurated for 72 hours (Gill et al., 1983).

C. Factors Affecting Rates of Elimination

The rate of elimination depends on the extent of contamination, the type of shellfish, the specific contaminant, and several environmental factors. The original level of pollution in the water affects the depuration process. When pollutant levels decrease in the shellfish-growing area, so do the acumulated contaminant levels in the shellfish. Thus, virus levels in shellfish will decrease as the virus concentration in the water decreases (Hedstrom and Lycke, 1964; Metcalf and Stiles, 1965). Additionally, the effectiveness of depuration is influenced by the extent of contamination of the shellfish. Heavily contaminated shellfish require longer depuration times than do lightly contaminated ones (Metcalf and Stiles, 1965; Janssen, 1973; Metcalf et al., 1980; Perkins et al., 1980; Son and Fleet, 1980; Cook and Ellender, 1986). Research has shown that shellfish harvested from marginally polluted waters are able to depurate to acceptable market quality after 48 hours, while shellfish contaminated with high levels of bacteria and viruses do not completely depurate during this time (Metcalf et al., 1979).

Differences in virus surface charge may cause viruses to be eliminated at different rates. Because viruses bind to the negatively charged sulfate radicals in the shellfish mucus (DiGirolamo et al., 1977), viruses with a stronger postitive charge may attach more effectively and therefore may not eliminate as readily. In any case, the rates of

elimination for different viruses have been studied and are described here. Poliovirus depuration occurs rather quickly, with approximately 80-99% removed in 48 hours (Hoff and Becker, 1969; Liu et al., 1967; Metcalf et al., 1979; Davis, 1986). After 48 hours, depuration is much slower with low levels of virus persisting even after 6 days. However, other studies have demonstrated more rapid elimination of poliovirus. Mitchell et al. (1966) reported over 95% of poliovirus was removed from Crassostrea virginica within 8 hours and greater than 99.9% was eliminated within 24 hours. Hepatitis A virus can persist longer than poliovirus during depuration. Sobsey et al. (1987) found that Eastern oysters reduced HAV by less than 90% after 5 days, under most conditions tested.

The species of shellfish is another variable determinant of elimination rates. The Eastern oyster can eliminate viruses to undetectable levels in 24 hours, while the soft shell clam requires 48 hours (Hoff and Becker, 1969). In addition, variability in individual shellfish activity during depuration influences the overall elimination rate. Depuration studies using pooled shellfish samples can result in apparent slow elimination rates if a few shellfish are not functioning well and fail to depurate the virus (Seraichekas et al., 1968; Metcalf et al., 1979).

The effectiveness of shellfish depuration depends on several environmental factors. Environmental conditions must be regulated in order to optimize normal pumping and

feeding activities of shellfish. However, each species of shellfish may depurate more effectively in different environmental conditions. Therefore, optimal water quality criteria ranges must be established for each shellfish species.

The water temperature can determine the physiological activity of shellfish with generally lower temperatures resulting in lower activity or inactivity. Liu et al. (1967) studied the effects of water temperature on viral depuration in Mercenaria mercenaria, and demonstrated depuration performance to be a function of temperature. At 18-20°C, enteric viruses were not detectable within 48 hours, but at 5-6°C, approximately 96 hours were required for depuration. The elimination of Salmonella charity and Escherichia coli from Crassostrea commercialis was shown to be more rapid at the water temperature range of 18-22°C than at 24-27°C, and incomplete depuration resulted at temperatures below 17°C (Rowse and Fleet, 1984). At higher temperatures, fecal coliforms and poliovirus are eliminated at a faster rate in relaid Eastern oysters (Crassostrea virginica) (Cook and Ellender, 1986). At temperatures less than 20°C, oysters failed to eliminate poliovirus within 2 weeks, whereas complete depuration occurred in less than one week at temperatures greater than 25°C. In another study, Sobsey et al. (1987) demonstrated that the rate of poliovirus elimination was more efficient at 23°C than at 17

and 12°C in Crassostrea virginica, while HAV was not extensively depurated at any temperature.

Several studies have demonstrated that purification is ineffective at salinities below those to which the shellfish are acclimated. Liu et al. (1967) showed that the reduction of salinity to 75% of the original level did not affect depuration, while a further reduction to 50-60% of the original salinity stopped the depuration process. Rowse and Fleet (1984) demonstrated reduced pumping rates, ineffective purification, and death in oysters exposed to lower salinities (16-20 ppt). Exposure to higher salinities (32-47 ppt) did not result in weakened pumping activity or mortality and produced effective depuration. Sobsey et al. (1987) found that oysters depurated HAV more effectively at 28 ppt than at lower salinities (8 and 18 ppt). At 28 ppt, less than or equal to 5% and 1% of the original HAV remained in oysters depurated after 2-3 days and 5 days respectively, whereas 10-41% remained after 5 days at 8 and 18 ppt. In addition, poliovirus was eliminated more slowly at 8 ppt than at the higher salinities. These studies provide evidence for a guideline in which the depuration water should maintain salinities above a minimum level to assure maximum shellfish depuration. Differences in depuration rates at high and low salinities may be a result of increased shellfish activity at higher salinities and greater ionic retention of viruses at lower salinities.

Turbidity does not affect the ability of shellfish to eliminate contaminants, provided the turbidity is not so excessive that water disinfection processes are impaired or gill closing of shellfish occurs. Hamblet (1969) found that Crassostrea virginica depurated poliovirus to nondetectable levels within 48 hours at both low (8-21 ppm) and high (54-80) turbidities in a flow-through system. Hoff and Becker studied the differences in the depuration of cell-associated and filtered poliovirus in Manila clams and Olympia oysters. They demonstrated that the crude cell-associated virus persisted longer than did the free filtered virus in both species of shellfish. Crude virus preparations are probably more similar to viruses found in the natural environment.

Depuration occurs more rapidly in a flow-through system than in a static system. Comparing the two systems, DiGirolamo et al. (1975) found that Olympia and Pacific oysters eliminated 99% of poliovirus after 3 days in a flow-through system, whereas only 74-84% was eliminated in a static system after 5 days. Mitchell et al. (1966) demonstrated poliovirus to be reduced to greater than 99.9% within 24 hours in Crassostrea virginica using a flow-through system. Limited oxygen and food and the concentration of metabolic wastes in a static system may impair the feeding activities of shellfish. A flow-through system is more likely to simulate the natural environmental conditions; therefore leading to maximum feeding and elimination activities in shellfish.

D. Summary

Current literature contains much information on the depuration of E. coli and poliovirus from contaminated shellfish. However, relatively little is known about the elimination characteristics of HAV by shellfish under depurating conditions. Previous depuration studies compared HAV elimination to that of poliovirus in a laboratory-scale depuration system using synthetic sea water (Sobsey et al., 1987). In order to more effectively evaluate the depuration of HAV, the use of internal bacterial and viral controls in depuration experiments would help to compare the results with previous studies. In addition, the use of natural sea water may simulate natural conditions better than synthetic sea water, and may improve the pumping efficiency of the shellfish.

MATERIALS AND METHODS

I. Media and Components

All media and their formulations are described in the Appendix A.

II. Cultivation and Assay of Test Organisms

A. Cell Culture

Hepatitis A virus was propagated and assayed in fetal rhesus monkey kidney (FRhK-4) cells. Poliovirus type 1 was grown and assayed in Buffalo Green Monkey Kidney (BGMK) cells. Described below is the procedure used for the production and maintenance of these cell lines.

Cells were grown in Falcon 800 cm^2 roller bottles or 150 cm^2 flasks using Eagle's Modified Minimum Essential Medium (1x MEM) containing 10% fetal calf serum. The cell cultures were incubated at 37°C for 5-7 days and allowed to grow to confluency. Once the cells formed a confluent layer, the old growth medium was decanted, the cell were rinsed with warm PBS, and a 0.05% Trypsin-0.02% EDTA solution was added to the flask or roller bottle. After the cell layer was removed from the surface, the cell suspension was poured into 50 ml centrifuge tubes and centrifuged at 2000 rpm for 10 minutes. The cell pellet was resuspended in sufficient growth medium to give a concentration of 1.2 x

10^5 cell/ml (BGMK) or 5 x 10^4 cells/ml (FRhK). Five milliliters of the cells were dispensed into 60 x 15 mm tissue culture dishes and incubated in 5% CO_2 at 37°C. After 5-7 days, the cells reached confluency and were used for virus assay.

B. Propagation of Test Organisms

B.1. Hepatitis A Virus

A cytopathic strain of HAV, known as pHM-175, was used in these experiments. For HAV propagation, confluent monolayers of FRhK-4 cells were rinsed with serum-free maintenance media and inoculated with the virus at a multiplicity of infection (MOI) of 0.1-0.01 infectious units per cell. The cultures were incubated at 37°C for 1 hour and then fresh maintenance media (with serum) was added. The cells were incubated at 37°C for 5-7 days until they showed at least 50% cytopathology.

The virus was harvested from the cells by freeze-thawing and scraping the cells from the flask surface with the frozen medium. The thawed cell lysates were pooled into 250 ml centrifuge tubes and sonicated for 3 minutes total with 15 second bursts not above the 70-75 Watts setting. The HAV preparation was then extracted with 1/2 volume of Freon and centrifuged at 4000 rpm for 20 minutes. Experimental stock HAV was recovered in the supernatant. The cell debris and Freon material was extracted with 0.1% SDS in PBS at room temperature to obtain additional seed stock HAV. SDS was then removed by centrifugation at 10,000

x g for 15-20 minutes at 4°C. HAV stocks were stored frozen at -70°C until use.

B.2. Poliovirus-1

The vaccine-derived LSc strain of poliovirus was used in all the experiments. Roller bottles of confluent BGMK cells were drained of the growth medium and inoculated with the virus at a MOI of 0.1-0.01 infectious units per cell. The virus was allowed to adsorb to the cell cultures at 37°C for 2 hours and then cell layers were rinsed three times with maintenance media. Fresh maintenance media was added to the roller bottles and the cells incubated at 37°C for 1-3 days. When the cells reached nearly complete cytopathology, the cultures were frozen and thawed three times and clarified by centrifugation at 5000 rpm for 20 minutes. The virus-containing supernatant was stored at -70°C for assay and experimental use.

B.3. Bacteriophage MS2

The bacteriophage MS2, which infects male-specific strains of Escherichia coli, was used in these experiments. Stock bacteriophage MS2 was grown on an E. coli C3000 host by the soft agar overlay plate lysis method and partially purified from infected cell debris and soft agar by fluorocarbon extraction and filtration through a 0.2 um porosity filter (Rovozzo and Burke, 1973). The phage stock was stored at -70°C until use. The stock used contained 5.9 x 10^{10} plaque forming units (PFU) per ml.

B.4. E. coli B

Stock cultures of E. coli B were maintained on slants of Nutrient Agar #2. A loopful of E. coli B was inolculated into 30 ml of sterile Nutrient Broth #2 in a shaker flask. The flask was incubated at 37°C for 5 hours with rapid agitation. The log phase cultures were then centrifuged at 4500 rpm for 20 minutes, and the cell pellet was resuspended in 5 ml of sterile PBS. The resuspended culture was enumerated by the pour plate method on Modified MacConkey's Agar at 37°C for 24 hours and stored at 4°C until experimental use. Generally, one ml of the suspension contained 10^9 colony-forming units (CFU).

B.5. Streptococcus faecalis

Stock cultures of Strep. faecalis were maintained on slants of Brain Heart Infusion (BHI) agar. Strep. faecalis was inoculated into 30 ml of sterile BHI broth in a shaker flask and incubated at 37°C for 5 hours with rapid agitation. The log phase cultures were then centrifuged at 4500 rpm for 20 minutes and the cell pellet resuspended in 5 ml of sterile PBS. The resuspended culture was enumerated by the pour plate method on m-Enterococcus agar at 40°C for 24 hours and stored at 4°C until experimental use. One milliliter of the suspension usually contained 10^9 CFU/ml.

C. Assay of Test Organisms

C.1. Radioimmunofocus Assay

Hepatitis A virus was assayed by the radioimmunofocus assay (RIFA) (Lemon et al., 1983). FRhK-4 cells were grown

to 85% confluency in 60 x 15 mm dishes at 37°C and 5% CO_2. Growth medium was aspirated from individual dishes and 0.2 ml of a sample dilution was inoculated onto the cells. Samples were inoculated in duplicate. Virus samples were diluted in serum-free virus diluent (HAV) containing sheep anti-poliovirus serum at a concentration of 1×10^{-3}. The anti-polio was added to the diluent to inhibit poliovirus infectivity during HAV assay. Inoculated dishes were incubated for 1 hour at 37°C with the sample redistributed over the cell layer every 15 minutes. After incubation, the dishes were overlaid with 5 ml of agarose overlay medium (formulation in Appendix), the agarose was allowed to solidify, and the dishes were incubated at 37°C and 5% CO_2. After 4 days, the cells received a second agarose overlay medium and reincubated.

Seven to ten days after initial inoculation, the agarose overlay medium was gently removed from the dish, and the cell layer was washed with 2 ml of warm (37°C) PBS. After being completely air dried, the cells were fixed with 2 ml of cold acetone for 2 minutes. The fixative was removed and the cells were allowed to dry. To each dish 1.5 ml of I^{125}-labelled anti-HAV serum (500,000 cpm/ml) was added and the dishes incubated at 37°C for a minimum of 4 hours. The labelled antibody was then aspirated from the dishes and the cells rinsed 2 times with PBS to remove residual label. The dishes were air dried overnight. The bottom of the dishes were cut out and placed in contact with

x-ray film (Kodak X-AR5) in film cassettes. The film was exposed for 4 days at -70°C and then developed. The HAV foci, appearing as black, circular spots on the exposed film, are then counted and the HAV concentrations were calculated and expressed as radioimmunofocus units (RFU) per ml.

C.2. Plaque Assay

Poliovirus-1 was assayed by the plaque technique in BGMK cells. Confluent layers of BGMK cells in 60 x 15 mm dishes were inoculated in duplicate with 0.2 ml of each sample dilution. Virus samples were diluted in PBS containing 2% heat-inactivated fetal calf serum. The dishes were incubated at 37°C for one hour with the dishes being tilted every 15 minutes to redistribute the sample. After the adsorption period, an agar overlay medium (formulation in Appendix A) was added to the dishes and then allowed to harden. The dishes were incubated at 37°C for 2-3 days. Plaques were counted on day 2 and 3 of the incubation. Poliovirus concentrations were calculated and expressed as plaque-forming units (PFU) per ml.

C.3. MS2 Bacteriophage Assay

E. coli C3000 host stock was maintained on slants of Nutrient Agar #2. A loopful of host cells was inoculated into 30 ml of Nutrient Broth #2 in a shaker flask. The culture was incubated at 37°C overnight with agitation and then stored at 4°C until dispensed. Phage samples were diluted in sterile PBS. An appropriate amount of sample

dilution was added to 3 ml of coliphage single strength top agar (nutrient broth #2 with 0.7% agar) in a 16 x 125 screw cap test tube. Then 0.1 ml of host E. coli was added to the sample mixture and mixed gently by rolling between the palms. The sample-agar-host mixture was poured onto plates containing a base layer of coliphage bottom agar (nutrient agar #2). Plates were allowed to harden and then incubated at 37°C inverted. Plaques were counted after 4-6 hours of incubation. Phage concentrations were expressed as PFU/ml.

C.4. Bacterial Plate Counts

E. coli B levels in oyster homogenate were enumerated by the pour plate technique. An appropriate amount of sample was inoculated into 30 ml of molten Modified MacConkey's Agar, mixed, and then half the volume (15 ml) poured into each of two plates. The agar was allowed to harden and the plates were inverted and incubated at 37°C for 24 hours. Small, disk-shaped, brick-red colored colonies were identified as E. coli B. Colonies were counted and expressed as colony-forming units (CFU) per ml.

Strep. faecalis was enumerated by the pour plate technique using m-Enterococcus (ME) agar. An appropriate amount of sample was inoculated into 30 ml of molten m-Enterococcus agar, mixed, and then half the volume was poured into each of two plates. The agar was allowed to harden, and the plates were inverted and incubated at 40°C for 24 hours. Strep. faecalis colonies appeared round and

dark maroon-colored. Colonies were counted and expressed as CFU/ml.

III. Oysters

Eastern oysters (Crassostrea virginica) were harvested from Calico Creek in the lower Newport River estuary near Morehead City, NC. The oysters were scrubbed under cold running water to remove mud and barnacles from the shell and then stored dry at 4°C for a maximum of 3 days after collection. The oysters were taken out of the refrigerator and brought to room temperature before use in the uptake tank.

IV. Seawater

Natural seawater used for the experiments was pumped from Bogue Sound, NC. Salinities in Bogue Sound were 28 parts per thousand (ppt) or greater during all experiments. Salinity was controlled by diluting the incoming seawater with distilled deionized water and measured with a refractometer. The pH was maintained at 8.0 by addition of HCl or NaOH. Seawater was filtered sequentially through 5 um and 0.45 um pore size pleated cartridge filters. After filtering, turbidity of the seawater was less than 0.1 NTU.

V. Test Organism Recovery From Oysters

For the assay of bacteria and bacteriophage MS2, a sample of oysters was removed from the depuration tank, aseptically shucked with the liquor discarded, and the oyster meats weighed in a sterile tared beaker. A sample of 5 pooled oysters was used for each analysis. An equal

volume:oyster weight of distilled water was added and the mixture blended at low speed for 1 minute, followed by high speed for 2 minutes in a semi-micro blender jar. An appropriate amount of the 1:2 oyster homogenate was removed for bacteriological analysis, and the remaining homogenate was processed by the UNC Oyster Concentration Procedure (Sobsey et al., 1978; Wait et al., 1981). This method is a modified adsorption-elution-precipitation method used to concentrate enteroviruses from oysters.

In this virus concentration procedure, 3.5 volumes of cold distilled water was added to the remaining homogenate to dilute the initial oysters 1:7 overall. The mixture was blended at high speed for 30 seconds. The homogenate was then transferred to a sterile beaker with a magnetic stir bar. While magnetically mixing the homogenate, the pH was adjusted to 5.0 and the conductivity corrected to less than or equal to 2000 mg/l as NaCl by adding distilled water. In this step, viruses are initially adsorbed to homogenized oyster tissue at a low salt concentration and pH. The homogenate was then centrifuged at 2000 x g and 5°C for 15 minutes and the supernatant discarded.

Viruses were eluted from the sediment by resuspending it in 7 volumes of 0.05M glycine-0.15M saline and adjusting the pH to 7.5. The samples were then centrifuged at 2000 x g at 5°C for 15 minutes and the pellet discarded. Next, viruses in the supernatant were acid precipitated. The supernatant was transferred to a beaker and the pH adjusted

to 4.5. The sample was mixed very slowly for 10 minutes and then centrifuged at 2000 x g for 15 minutes. The resulting pellet was resuspended in a minimum amount of 0.1M Na_2HPO_4, and the pH was adjusted to 7.3. The sample was further treated with filter sterilized Cat-Floc at a final concentration of 0.1% and slowly mixed on a rotary platform shaker for 15 minutes. Cat-floc is a cationic, polyelectrolyte used as a clarifying agent by precipitating shellfish-associated components that are toxic to cell cultures used for virus assay. After mixing, the sample is then centrifuged at 10000 x g for 20 minutes and the pellet discarded. The resulting supernatant concentrate is treated with antibiotics and frozen at -70°C until virus assay.

VI. Experimental Methods

Experiments were conducted with hepatitis A virus, poliovirus-1, MS2 bacteriophage, E. coli B, and Strep faecalis to compare elimination rates at different temperatures (12, 18, 25°C) and different salinities (8, 18, 28 ppt). When temperature was the variable, the salinity was maintained at 28 ppt, and when salinity was the variable, the temperature was held at 25°C. All experiments were conducted under a laminar flow biohazard hood.

At the beginning of each experiment, 10 liters of natural seawater were added to a 23 x 44 x 13 polypropylene tank and allowed to sit, covered and aerated, under the hood for 24 hours to dissipate any residual chlorine that may be present. Then the uptake water was seeded with HAV,

poliovirus, MS2 bacteriophage, E. coli B, and Strep faecalis to give initial levels of 10^4-10^6 organisms of each per ml in the uptake water. The inoculated water was allowed to mix for about 1 hour. Then 65 oysters were placed in the uptake water and allowed to naturally accumulate the test organisms for 14-16 hours at room temperature. The initial number of oysters included 10 "extras" to replace any that died during an experiment.

After the uptake period, the contaminated oysters were removed from the uptake tank and a sample of 5 oysters was processed to determine initial (Day 0) organism levels. The remaining oysters were divided equally and transferred to two tanks (23 x 44 x 13) containing filtered depuration water at the designated temperature and salinity. The depuration tanks were designed as a flow-through system with a baffle at the the inlet end to prevent short cicuiting of the feed seawater. An aquarium stone aerated the depuration tank water to keep the dissolved oxygen level greater than 5 mg/l. The water flow rate in the depuration tanks was maintained at 12 ml/minute/oyster. In order to control temperature, depuration tanks were immersed in larger tanks fed with tempered water from a recirculating cooling bath. Used depuration water was disinfected with chlorine before discarding.

A sample of 5 oysters was removed from each depuration tank at 1, 2, 3, 4, and 5 days of depuration and processed for bacteriological and virological analysis. The flow rate

of the depuration feed water was reduced proportionally at each sampling time in order to maintain the flow rate at 12 ml/minute/oyster.

VII. Statistical Analysis

The raw data from the depuration experiments consisted of the concentration of each organism found in the oyster homogenate for each day of the experiment. These concentrations were converted to the number of organisms per gram of oyster meat for the different organisms for each day of the experiment. These calculated concentrations were normalized to percent of initial organism remaining on each day by dividing the number of organisms per gram of oyster meat at each day of the experiment by the initial number of organisms per gram of oyster meat (Day 0) and multiplying by 100. The percent remaining data were then log transformed and plotted versus arithmetic time to give an approximation of a straight line.

Linear regression analysis of the depuration data from each temperature and each salinity experiment was performed. A correlation coefficient (r) was calculated to determine the linear relationship between log % remaining and time (in days) for the different organisms at each experimental condition.

The null hypothesis that HAV is eliminated at the same rate as the other test organisms for each experimental condition was tested by fitting linear regressions of log % remaining over time and using a pairwise, two-sided t-

statistic to test if the slope of these regression lines significantly differ from each other. Also, the null hypothesis that there was no difference in the extent of elimination of each organism at the three different temperatures and the three salinities was tested in the same way. All the statistical tests employed a 95% confidence level and was the basis for acceptance or rejection of the null hypothesis.

RESULTS

The results of the depuration experiments, comparing the rates of elimination of HAV, poliovirus 1, MS2 bacteriophage, E. coli, and S. faecalis at different temperatures and salinities, are summarized in Tables 6 and 7. Since each experiment was performed in duplicate, the results were averaged and are presented as percent of initial organism remaining after 1, 2, 3, 4, and 5 days of depuration.

I. Effect of Temperature on Organism Depuration

The results of the temperature experiments are showm in Table 6 and Figures 1-3. In addition, the regression results are shown in Table 8. At 12°C, oyster depuration was slow and ineffective with high numbers persisting even after 5 days. Depuration of HAV at 12°C was incomplete and much slower than for the depuration of the other test organisms at the same temperature. After 2 days of depuration, 80.6% of the initial HAV remained, while 29.0% of poliovirus, 47.9% of MS2, 35.5% of E. coli, and 46% of S. faecalis remained. The depuration rate decreased after day 2, and by day 5, 3.2% of poliovirus, 58.4% of MS2, 13.0% of E. coli, and 8.7% of S. faecalis remained. In contrast, HAV

TABLE 6

MEAN RESULTS OF TEMPERATURE EXPERIMENTS

ORG	DAY	MEAN PERCENT INITIAL ORGANISM REMAINING TEMPERATURE (°C)		
		12	18	25
HAV	0	100.00	100.00	100.00
	1	222.94	14.36	12.66
	2	80.56	<3.48	14.53
	3	61.91	24.98	10.13
	4	309.30	5.14	4.54
	5	248.58	44.56	2.72
POLIO	0	100.00	100.00	100.00
	1	35.61	2.19	2.73
	2	29.00	0.70	0.32
	3	5.79	1.53	0.14
	4	10.47	0.80	<0.09
	5	3.22	<0.03	<0.02
MS2	0	100.00	100.00	100.00
	1	120.42	33.92	30.52
	2	47.91	11.10	9.22
	3	64.63	41.17	3.32
	4	39.25	24.58	0.30
	5	58.42	16.08	0.65
EC	0	100.00	100.00	100.00
	1	60.29	11.82	0.89
	2	35.52	0.77	0.36
	3	5.42	6.72	0.32
	4	7.86	13.81	0.22
	5	13.01	1.83	0.03
SF	0	100.00	100.00	100.00
	1	95.45	25.70	1.88
	2	46.26	1.24	20.78
	3	14.21	6.09	2.69
	4	11.00	<5.51	1.36
	5	8.67	6.21	0.24

TABLE 7

MEAN RESULTS OF SALINITY EXPERIMENTS

ORG	DAY	MEAN PERCENT INITIAL ORGANISM REMAINING SALINITY (PPT) 8	18	28
HAV	0	100.00	100.00	100.00
	1	61.98	150.15	33.61
	2	80.97	41.35	53.48
	3	110.03	63.78	24.35
	4	19.81	72.03	20.39
	5	9.09	12.44	27.24
POLIO	0	100.00	100.00	100.00
	1	69.71	8.55	<1.41
	2	16.15	0.67	0.06
	3	10.16	2.63	<0.04
	4	5.21	0.50	4.38
	5	1.97	0.59	0.37
MS2	0	100.00	100.00	100.00
	1	86.42	54.53	30.72
	2	44.72	13.26	16.65
	3	31.22	5.48	6.28
	4	6.92	0.35	2.04
	5	8.66	0.35	0.18
EC	0	100.00	100.00	100.00
	1	100.83	2.90	0.07
	2	187.16	1.18	0.11
	3	132.97	3.75	9×10^{-5}
	4	464.01	0.25	7×10^{-5}
	5	250.53	0.35	6×10^{-5}
SF	0	100.00	100.00	100.00
	1	94.22	7.69	17.40
	2	50.85	8.29	8.32
	3	95.64	21.57	0.80
	4	18.47	2.18	0.62
	5	7.43	12.93	0.61

FIGURE 1

MEAN OYSTER DEPURATION AT TEMPERATURE 12 C

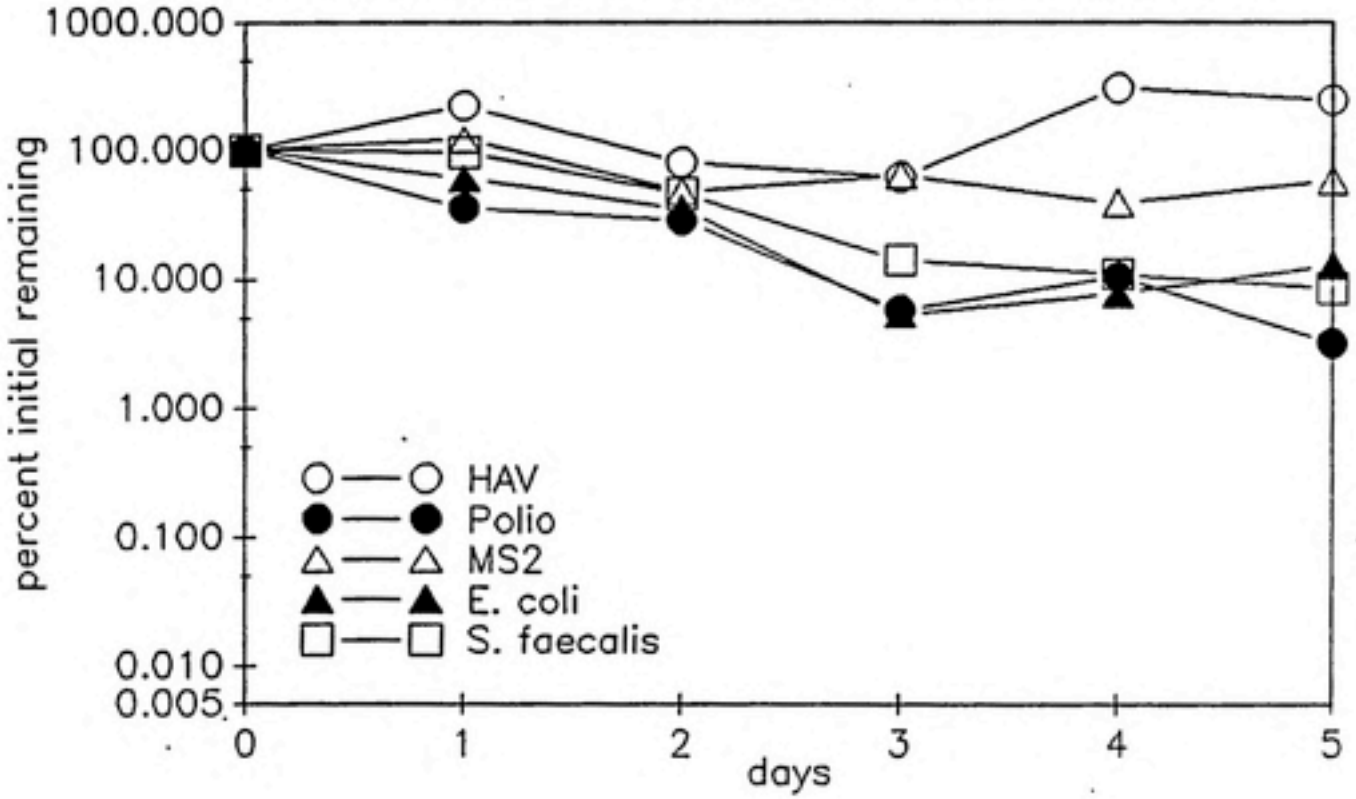


FIGURE 2

MEAN OYSTER DEPURATION AT TEMPERATURE 18 C

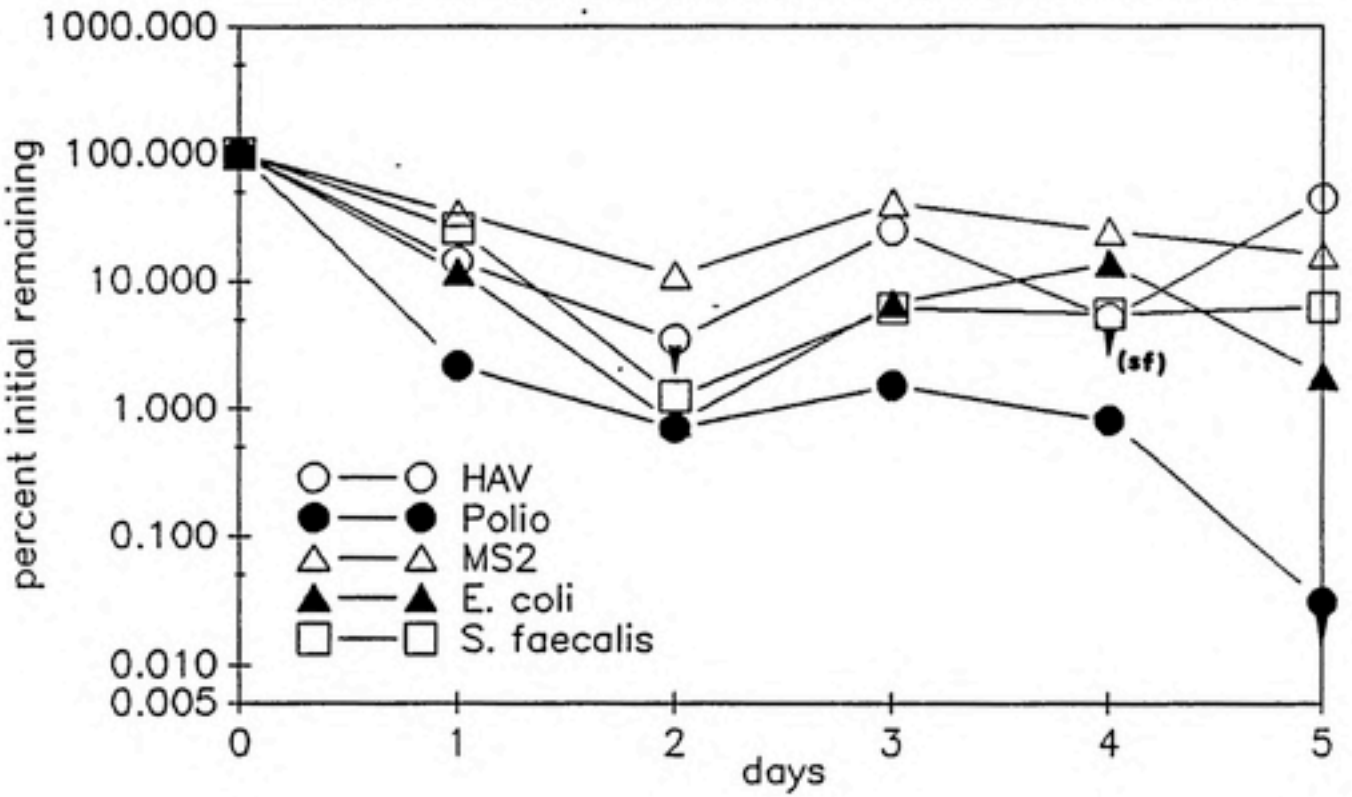


FIGURE 3

MEAN OYSTER DEPURATION AT TEMPERATURE 25 C

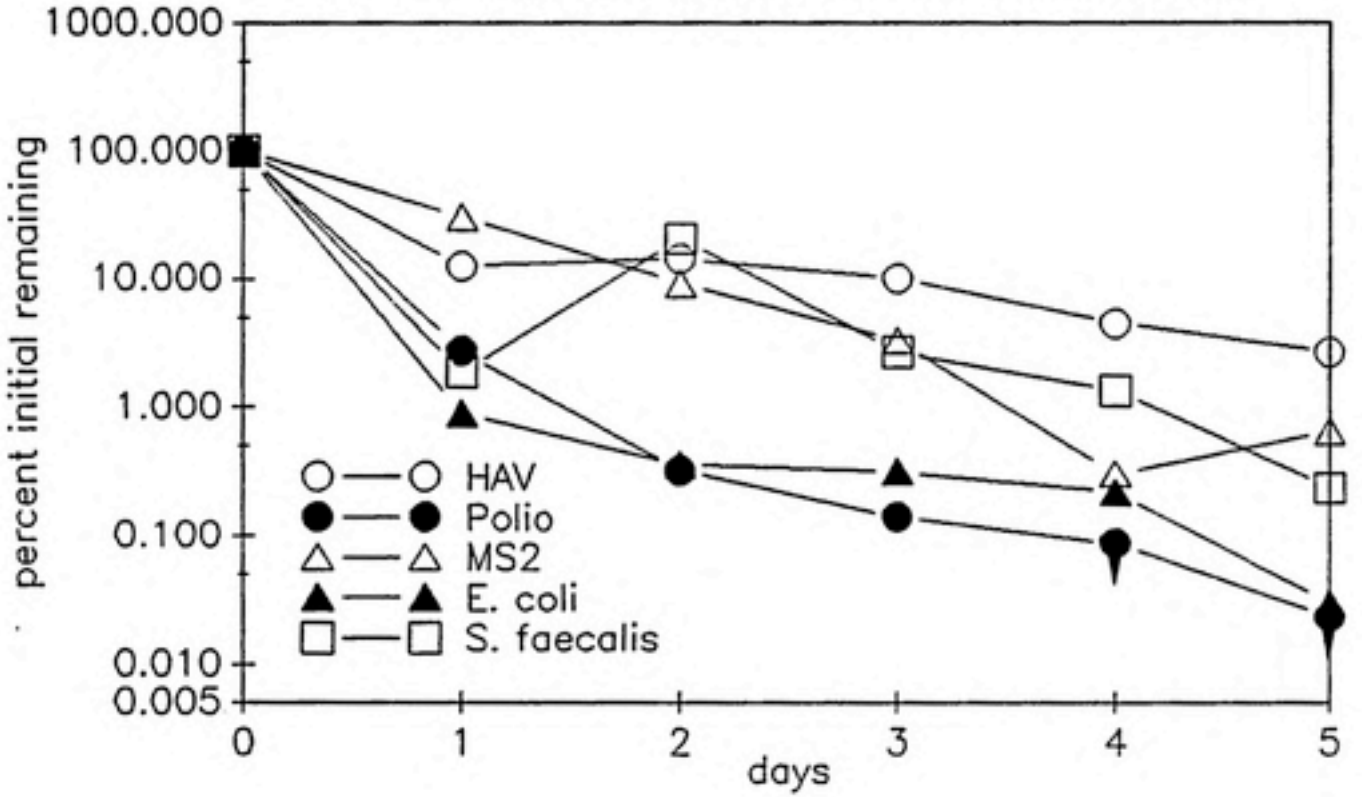


TABLE 8

SUMMARY OF LINEAR REGRESSION FOR TEMPERATURE EXPERIMENTS

TEMP	ORGANISM	SLOPE	STND ERROR	R
12	HAV	0.0861	0.0671	0.3760
12	POLIO	-0.2858	0.0396	0.9159
12	MS2	-0.0756	0.0334	0.5823
12	EC	-0.2802	0.0673	0.7965
12	SF	-0.2564	0.0407	0.8939
18	HAV	-0.0623	0.1243	0.1565
18	POLIO	-0.5440	0.0985	0.8679
18	MS2	-0.1088	0.0467	0.5935
18	EC	-0.2199	0.1079	0.5418
18	SF	-0.2051	0.0976	0.5534
25	HAV	-0.2858	0.0484	0.8814
25	POLIO	-0.6769	0.0907	0.9208
25	MS2	-0.5036	0.0545	0.9462
25	EC	-0.5709	0.0961	0.8828
25	SF	-0.4261	0.0964	0.8134

"increased" to 248.6% of initial virus remaining at 12°C after 5 days. This apparent increase in the % initial HAV remaining value is probably a result of the variability in the concentration of organisms from pooled samples of oysters and in the efficiency of virus recovery. Unfortunately, these fluctuations are seen throughout the temperature and salinity experiments. This virus concentration method has been developed and evaluated as a reliable method for detecting and enumerating HAV and poliovirus in oysters (Sobsey et al., 1978; Keating, 1985). However, the variabilities in the recovery efficiencies may have reduced the accuracy with which depuration of HAV in oysters was followed.

Depuration at 18°C provided similar patterns of depuration, however, the reductions were more rapid and extensive than at 12°C. Poliovirus was rapidly reduced with 0.7% and <0.03% remaining after 2 and 5 days, respectively. The bacteria, E. coli and S. faecalis, were both eliminated to about 1% remaining after 2 days. But after 5 days of depuration at 18°C, the percentages increased to 1.8 and 6.2% remaining, respectively. The MS2 bacteriophage was eliminated more slowly than poliovirus and the bacteria, with 11.0% remaining at day 2 and 16.1% remaining at day 5. HAV persisted to a greater extent than other test organisms in oysters depurated at 18°C, with 44.6% of the initial HAV remaining after 5 days of depuration.

The experiments performed at the highest temperature of 25°C gave the most effective depuration rates occurring for all test organisms at the three temperatures. Poliovirus and E. coli were eliminated most rapidly at 25°C, with about 0.3% initial organisms remaining after 2 days. Elimination proceeded more slowly after 2 days, with <0.02% of poliovirus and 0.03% of E. coli remaining after 5 days. The elimination of MS2 and S. faecalis proceeded more slowly than for polio and E. coli. After 2 days, 9.2% of initial MS2 remained and 20.8% of initial S. faecalis remained. By day 5, both organisms were reduced extensively to <1% of initial levels. Depuration of HAV at 25°C was relatively slow, with 14.5% initial virus remaining after 2 days of depuration and 2.7% remaining after day 5.

For the temperature experiments, Figures 4-8 show the effects of temperature on the depuration of each organism. All the organisms depurated more efficiently at higher temperatures. Hav was depurated more slowly and less extensively at 12°C than at 25°C. At day 2, HAV reductions to 80.6%, <3.5%, and 14.5% of initial virus levels occurred for temperature 12, 18, and 25°C, respectively. By day 5, HAV was eliminated to 248.6%, 44.5% and 2.7% of initial virus levels at 12, 18, and 25°C, respectively. Poliovirus elimination proceeded faster at both 18 and 25°C than at 12°C, with 29.0% remaining at 12°C, 0.7% remaining at 18°C, and 0.32% remaining at 25°C after 2 days of depuration. MS2 bacteriophage and E. coli depuration occurred at a faster

FIGURE 4

MEAN OYSTER DEPURATION OF HAV AT 3 TEMPERATURES

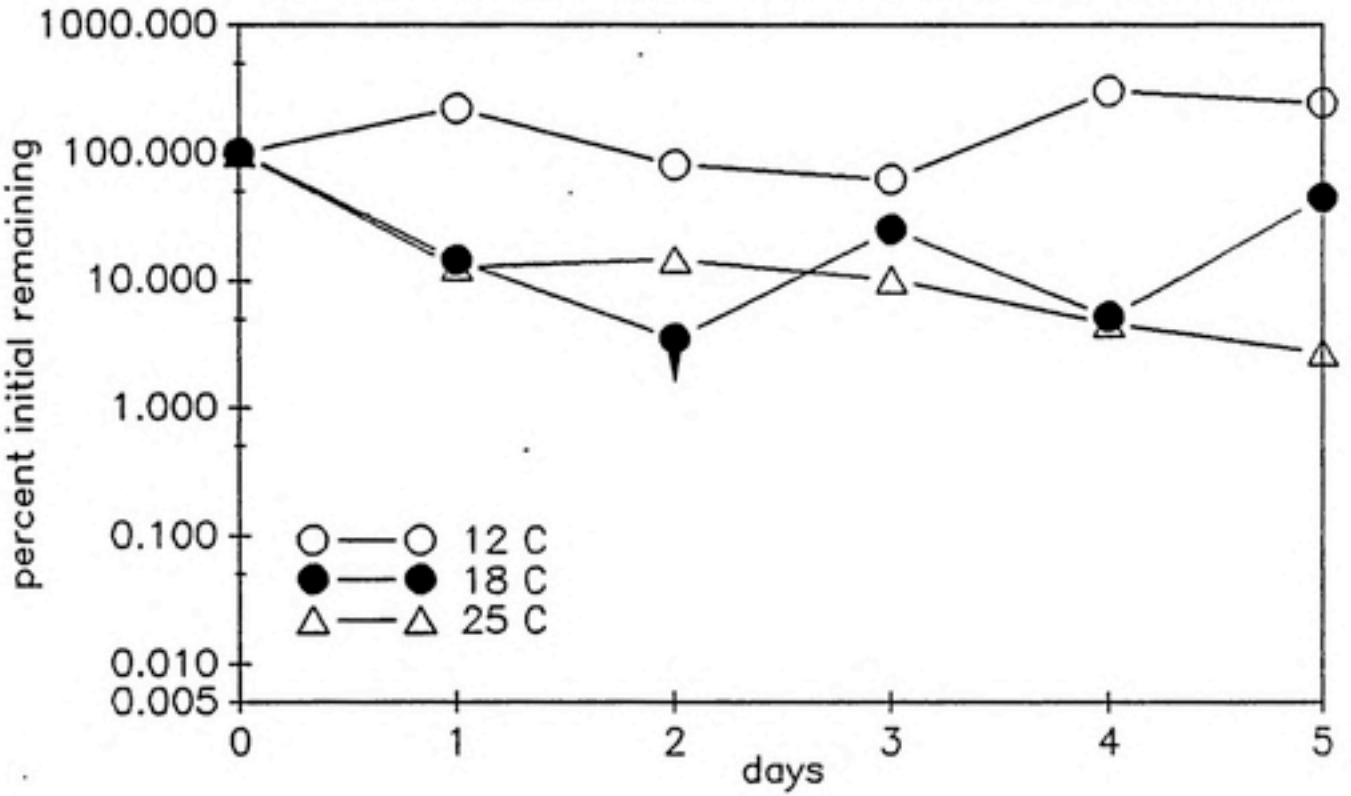


FIGURE 5

MEAN OYSTER DEPURATION OF POLIO AT 3 TEMPERATURES

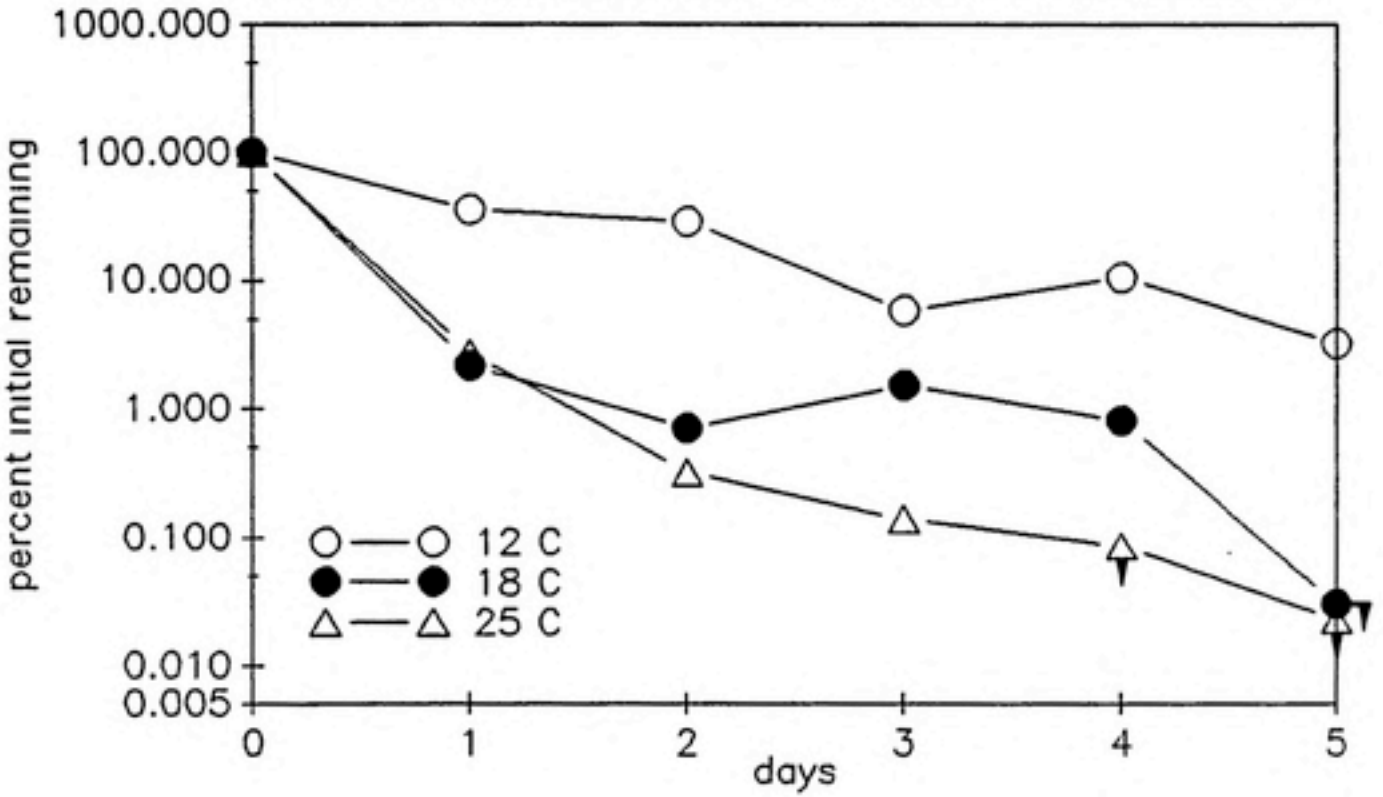


FIGURE 6

MEAN OYSTER DEPURATION OF MS2 AT 3 TEMPERATURES

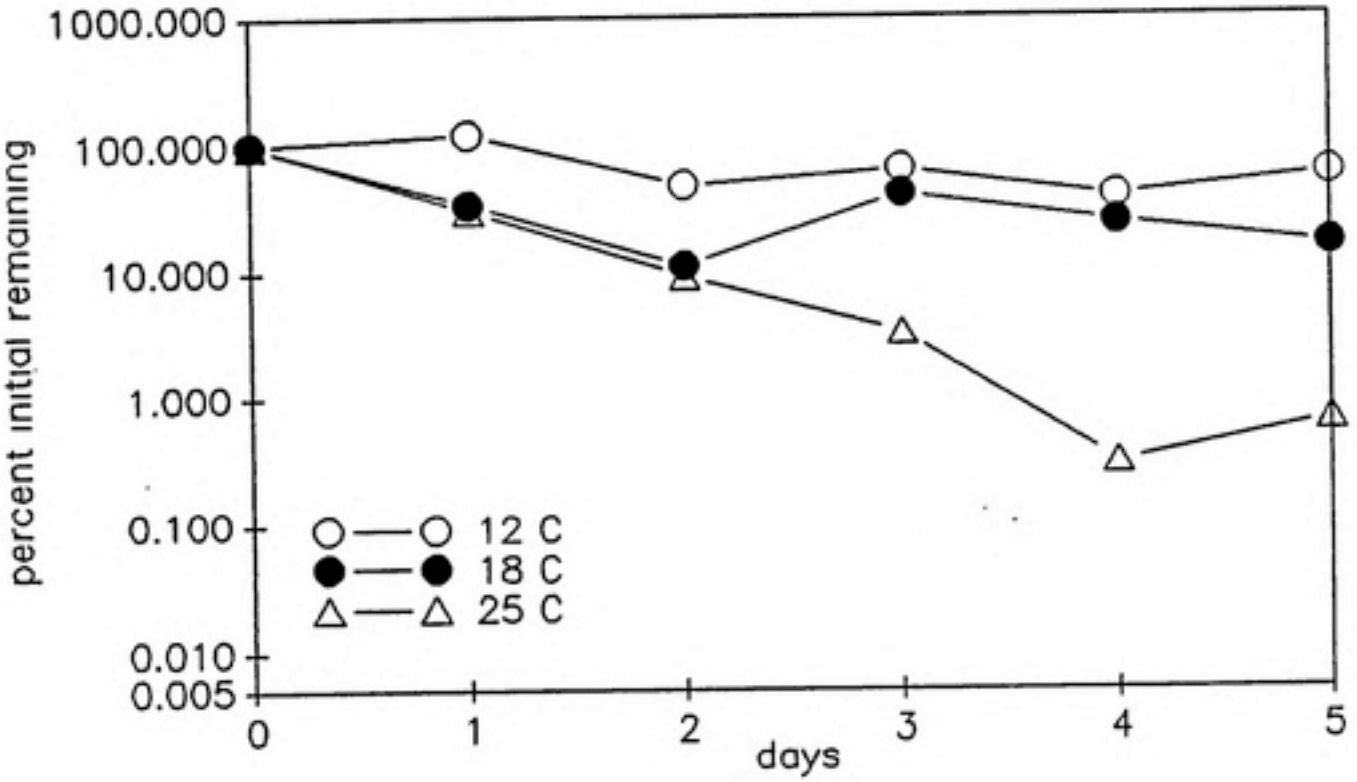


FIGURE 7

MEAN OYSTER DEPURATION OF E. COLI AT 3 TEMPERATURES

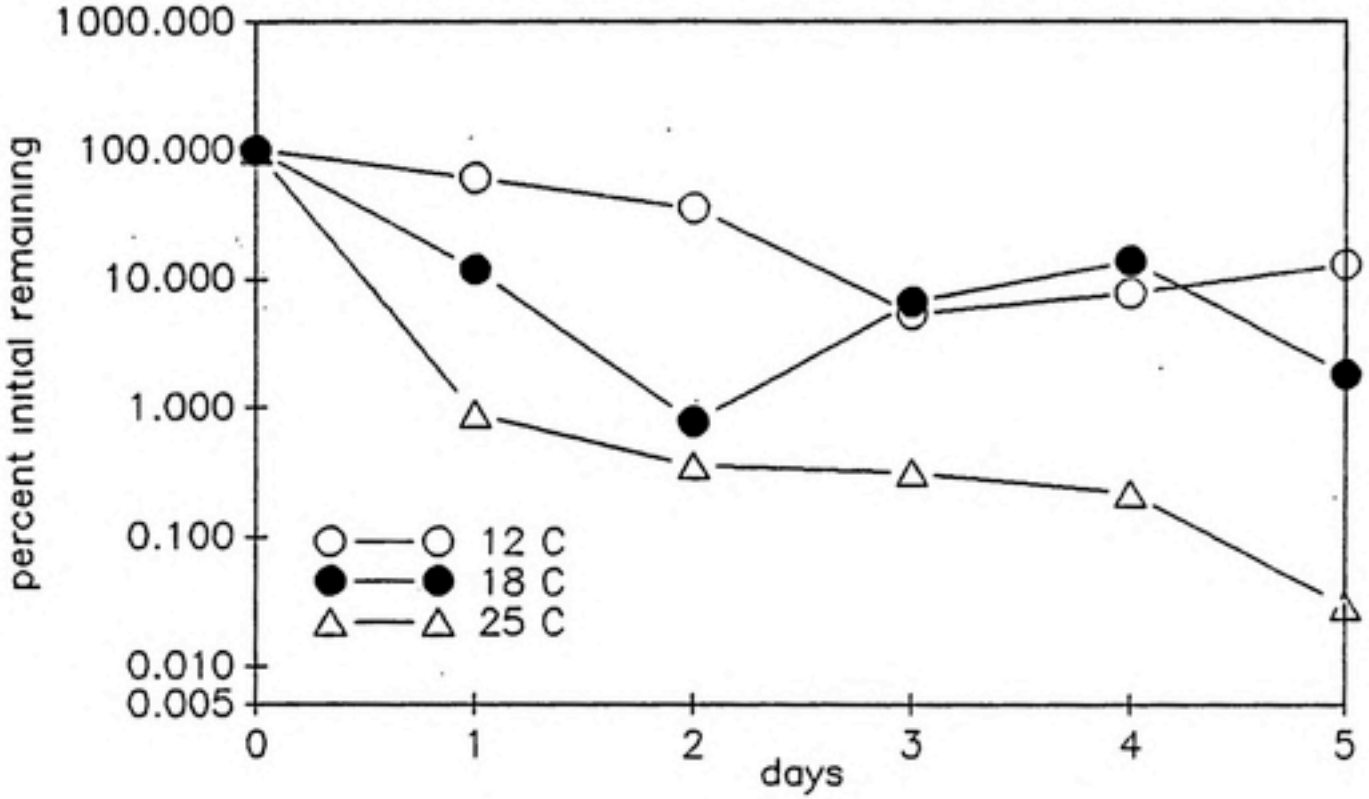
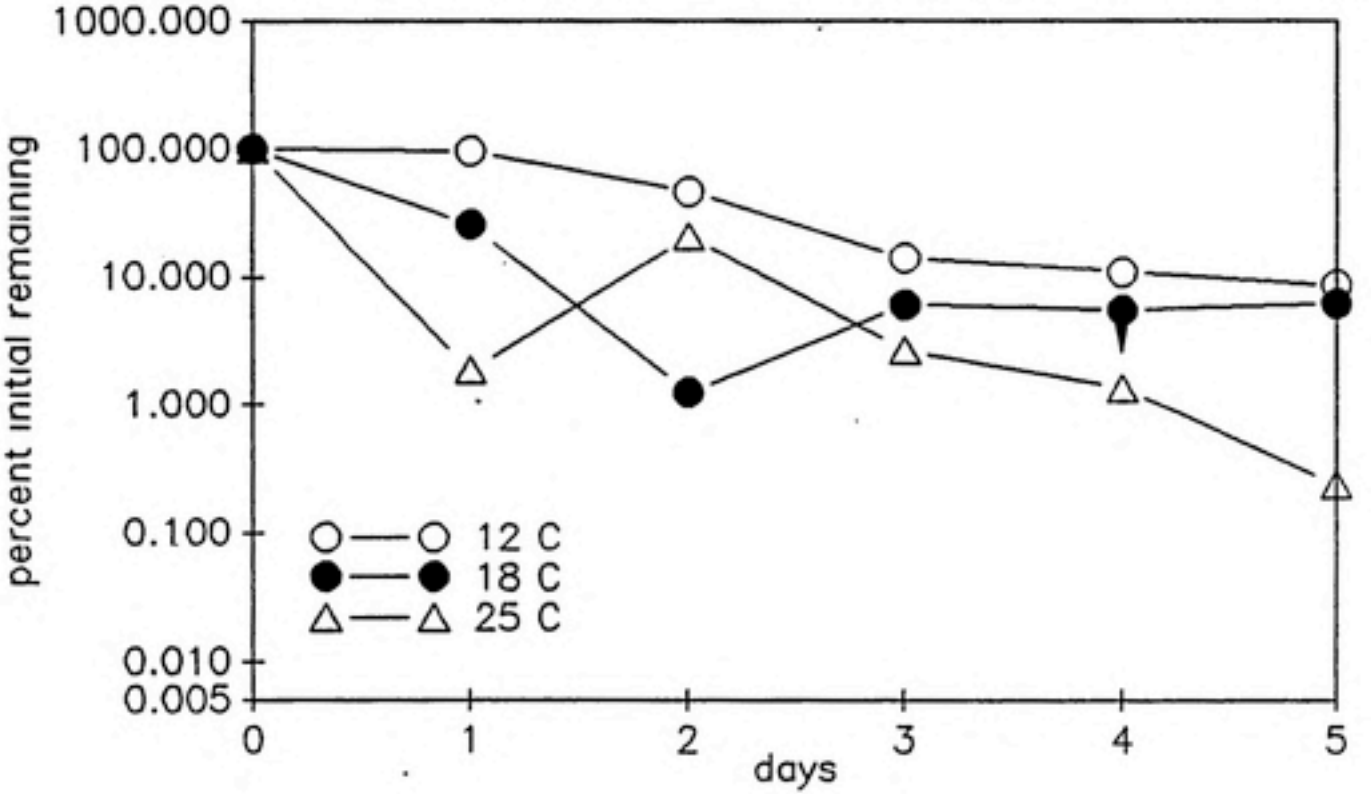


FIGURE 8

MEAN OYSTER DEPURATION OF S. FAECALIS AT 3 TEMPERATURES



rate at 25°C than at either 18 or 12°C. After 5 days of depuration, MS2 was eliminated to 58.4%, 16.0% and 0.65% of initial levels and E. coli was reduced to 13.0%, 1.8%, and 0.03% of initial levels at temperatures 12, 18, and 25°C, respectively. S. faecalis reductions were more extensive at 25°C than at either 12 or 18°C. After 5 days, S. faecalis was eliminated to 8.7%, 6.2%, and 0.24% of initial levels at 12, 18, and 25°C, respectively.

Statistical analyses of the temperature experiments are shown in Table 9. A Student's T test statistic was used to test the null hypothesis that there was no difference in the rates of depuration (slope) between HAV and the other test organisms at each temperature. This test utilized a 95% confidence level as the criterion for significance. At the temperature 12°C, analyses of the data showed a significant difference between the rate of elimination of HAV and the rates of elimination of poliovirus, MS2, E. coli, and S. faecalis. In each case, HAV was eliminated more slowly than the other organism. At 18°C, HAV was eliminated at a significantly slower rate than was poliovirus ($p=0.006$). However, there were no significant differences in the rates of elimination between HAV and MS2, E. coli, and S. faecalis at 18°C. Finally, HAV had a significantly slower rate of elimination at 25°C than poliovirus ($p=0.001$), E. coli ($p=0.01$), and MS2 bacteriophage ($p=0.007$). No significant differences were shown for HAV and S. faecalis at 25°C.

TABLE 9

STATISTICAL COMPARISON OF THE EFFECT OF TEMPERATURE ON THE DEPURATION OF HAV AND OTHER TEST ORGANISMS

TEMP	TEST	P VALUE
12	HAV VS POLIO	0.00009
12	HAV VS MS2	0.04211
12	HAV VS EC	0.00086
12	HAV VS SF	0.00025
18	HAV VS POLIO	0.00603
18	HAV VS MS2	0.72941
18	HAV VS EC	0.34857
18	HAV VS SF	0.37599
25	HAV VS POLIO	0.00097
25	HAV VS MS2	0.00678
25	HAV VS EC	0.01463
25	HAV VS SF	0.20693
12 & 18	HAV VS HAV	0.30465
18 & 25	HAV VS HAV	0.10790
12 & 25	HAV VS HAV	0.00018
12 & 18	POLIO VS POLIO	0.02353
18 & 25	POLIO VS POLIO	0.33159
12 & 25	POLIO VS POLIO	0.00068
12 & 18	MS2 VS MS2	0.56840
18 & 25	MS2 VS MS2	0.00002
12 & 25	MS2 VS MS2	0.000001
12 & 18	EC VS EC	0.64039
18 & 25	EC VS EC	0.02373
12 & 25	EC VS EC	0.02130
12 & 18	SF VS SF	0.63220
18 & 25	SF VS SF	0.12140
12 & 25	SF VS SF	0.11900

Statistical analyses were also performed to determine the effect of depuration water temperature on the elimination of each test organism. The Student's T test was used to test the null hypothesis that there was no difference in the rates of depuration for each test organism at the three temperatures. A 95% confidence level was the basis for acceptance or rejection of the null hypothesis. For HAV, there was a significant difference in the depuration rates between 12 and 25°C ($p=0.0002$), with HAV depuration occurring more rapidly at 25°C than at 12°C. Poliovirus was depurated significantly more quickly at both 18 and 25°C than at 12°C, with p values 0.02 and 0.0007, respectively. In addition, the analyses showed that MS2 and E. coli were eliminated at a significantly higher rate at 25°C than at 18 and 12°C. For S. faecalis, there was no significant difference between the slopes of the three temperatures.

II. Effect of Salinity on Organism Depuration

The results of the salinity experiments are presented in Table 7 and Figures 9-11. The regression results from the salinity experiments are shown in Table 10. Overall, depuration at 8 parts per thousand (ppt) resulted in gradual reductions, although elimination of E. coli was inconsistent showing increasing numbers over time. HAV was not rapidly or extensively reduced in oysters depurated at salinity 8 ppt, with about 81% of the initial viruses remaining after 2

FIGURE 9

MEAN OYSTER DEPURATION AT SALINITY 8 PPT

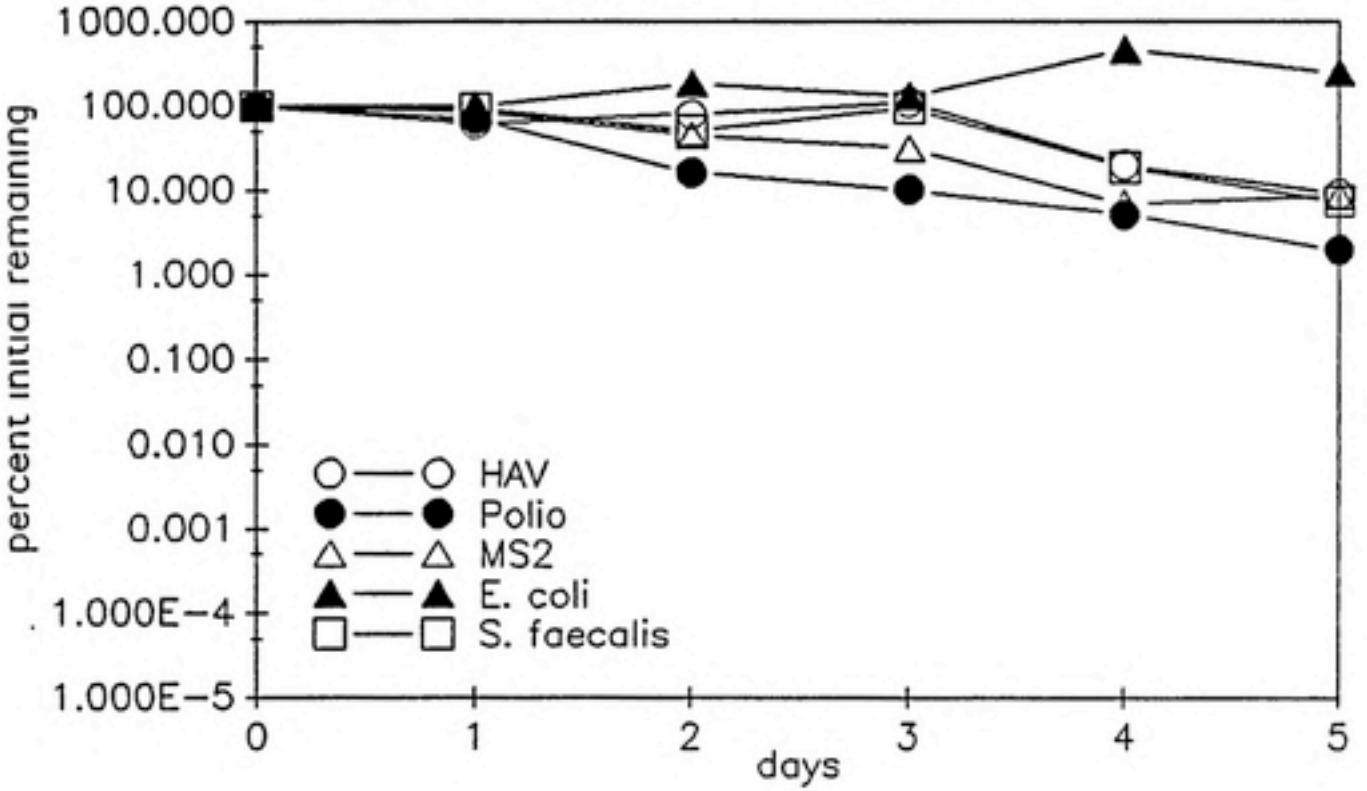


FIGURE 10

MEAN OYSTER DEPURATION AT SALINITY 18 PPT

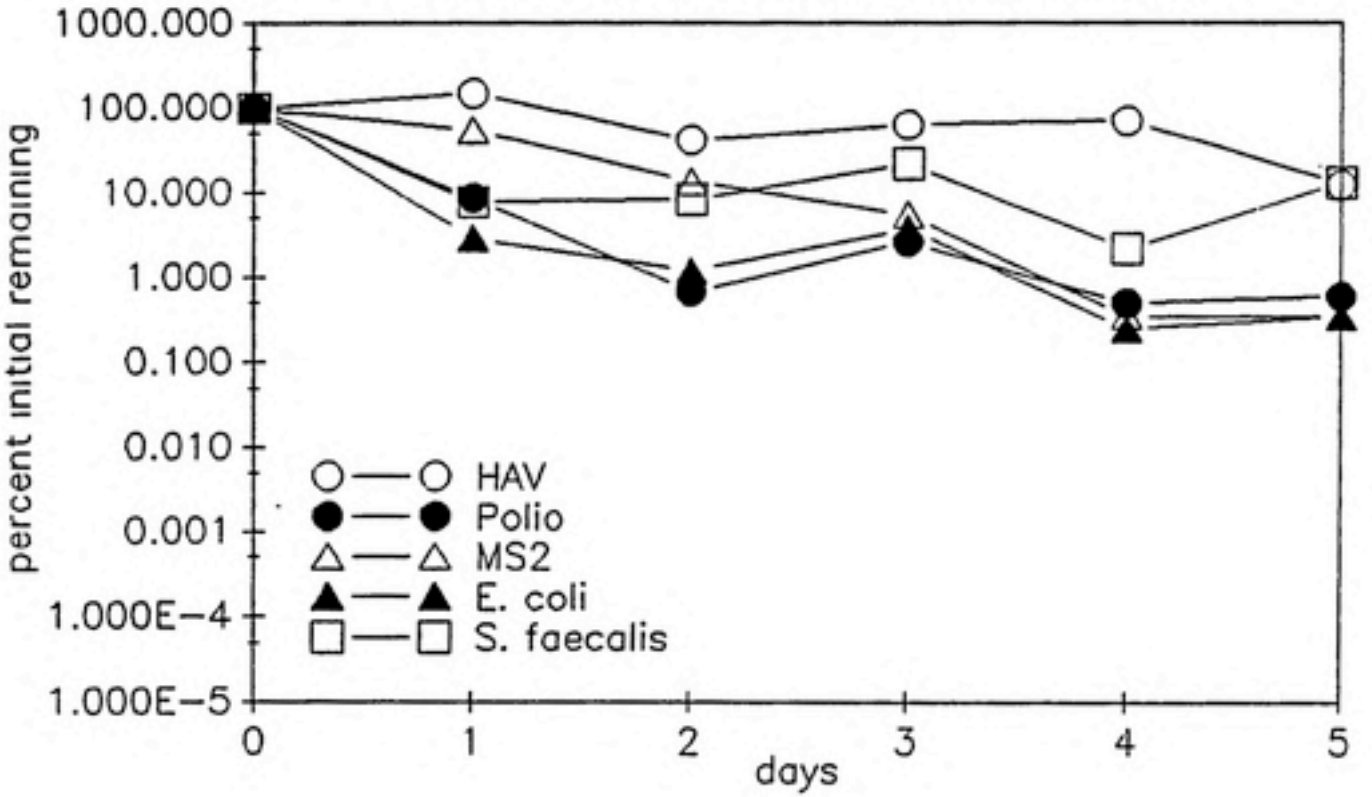


FIGURE 11

MEAN OYSTER DEPURATION AT SALINITY 28 PPT

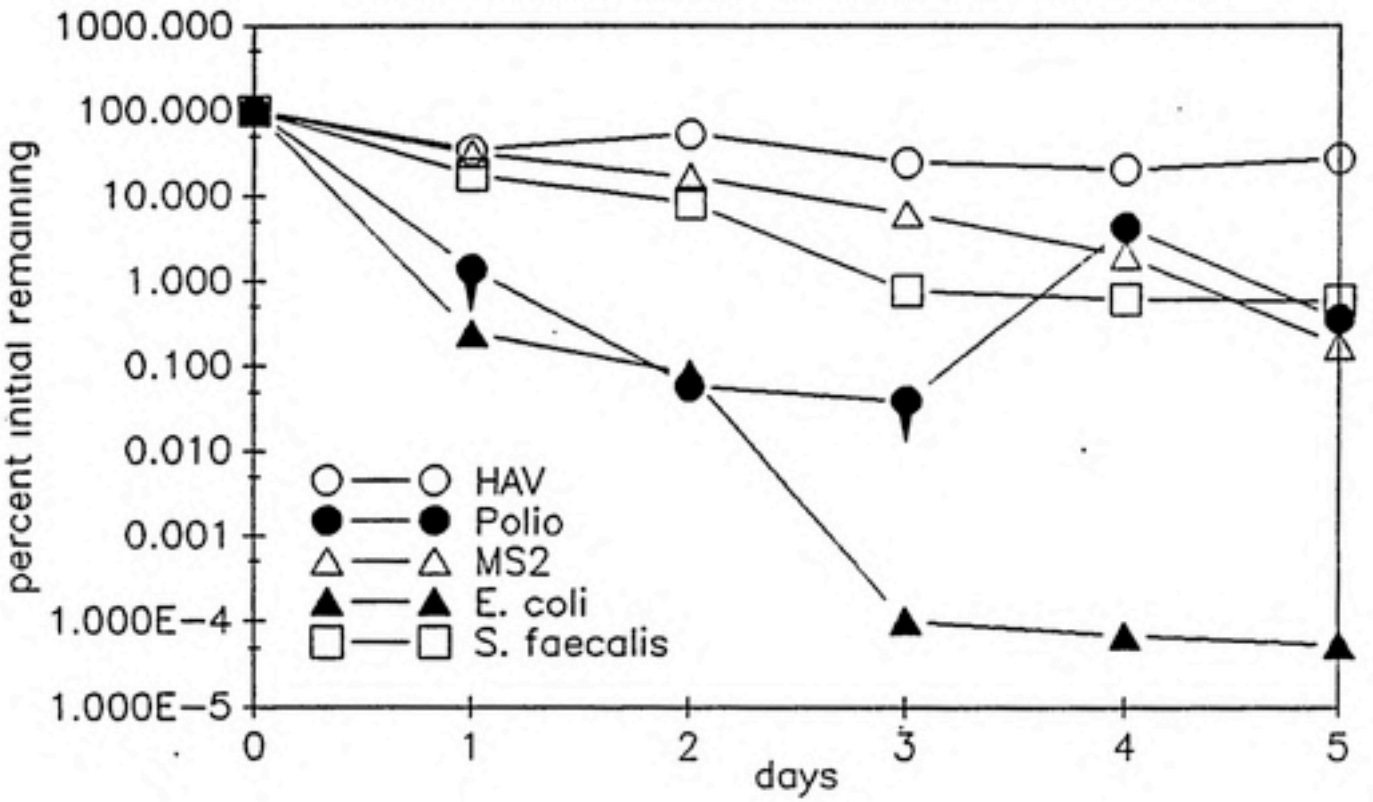


TABLE 10

SUMMARY OF LINEAR REGRESSION FOR SALINITY EXPERIMENTS

SAL	ORGANISM	SLOPE	STND ERROR	R
8	HAV	-0.1665	0.0870	0.5181
8	POLIO	-0.4006	0.0572	0.9114
8	MS2	-0.3066	0.0686	0.8161
8	EC	-0.0915	0.1433	0.1980
8	SF	-0.2599	0.0782	0.7244
18	HAV	-0.1667	0.0635	0.6388
18	POLIO	-0.4320	0.0943	0.8230
18	MS2	-0.5593	0.0438	0.9707
18	EC	-0.4290	0.0863	0.8437
18	SF	-0.1697	0.0835	0.5405
28	HAV	-0.1033	0.0399	0.6528
28	POLIO	-0.2836	0.2091	0.4121
28	MS2	-0.5038	0.0387	0.9745
28	EC	-1.2451	0.1908	0.8999
28	SF	-0.4719	0.0697	0.9143

days and 9.1% remaining after 5 days of depuration. In contrast, poliovirus was depurated more efficiently than HAV at 8 ppt, with 16.2% and 2.0% remaining after 2 and 5 days, respectively. MS2 bacteriophage and S. faecalis were more slowly eliminated having 44.7% and 50.9% of initial organisms remaining at day 2 and 8.7% and 7.4% remaining at day 5, respectively. Initial E. coli increased to 187.2% after 2 days and to 250.5% after 5 days. This increase in organism concentration may be due to differences in the pumping activity of individual oysters in the pooled samples or to stress on the oysters induced by the low salinity.

Depuration at 18 ppt resulted in somewhat faster and more extensive reductions than at 8 ppt, although high numbers persisted after 2 days for some organisms. HAV persisted in oysters depurated at 18 ppt, with 41.4% of initial viruses remaining after 2 days and 12.4% remaining after 5 days of depuration. Moreover, MS2 bacteriophage and S. faecalis were reduced relatively slowly at 18 ppt, with 13.3% and 8.3% of initial organisms remaining after 2 days and 0.35% and 12.9% remaining after 5 days, respectively. Poliovirus was rapidly and extensively eliminated during the first two days, with 0.67% remaining after 2 days of depuration. After this initial reduction, poliovirus depurated very little, with 0.59% remaining after 5 days. Reduction of E. coli at 18 ppt was rapid and consistent, with 1.2% and 0.35% of initial bacteria remaining after 2 and 5 days, respectively.

Depuration at salinity 28 ppt was generally more efficient than at either 8 or 18 ppt. However, oyster depuration of HAV was not effective. After 2 days of depuration, 53.5% of the initial HAV remained and 27.2% remained after 5 days. Similarly, MS2 bacteriophage and S. faecalis were eliminated somewhat slowly, although not as slow as HAV. After 2 days of depuration, 16.7% and 8.3% initial MS2 and S. faecalis remained, respectively. In contrast, poliovirus and E. coli were rapidly reduced in oysters depurated at 28 ppt, with about 1% remaining after 1 day and <0.4% remaining after 5 days of depuration.

Figures 12-16 show the effects of salinity on the depuration of each organism. Salinity did not influence HAV elimination, although more extensive reductions of poliovirus, MS2, E. coli, and S. faecalis were seen at the highest salinities of 28 and 18 ppt. Gradual viral reductions occurred for HAV at all three salinities. Even after 5 days of depuration, 9.1%, 12.4%, and 27.2% initial HAV remained at 8,18, and 28 ppt, respectively. In contrast, oyster purification of the other test organisms was more effective at higher salinities. Poliovirus depuration was more extensive with increasing salinity, with 16.2% remaining at 8 ppt, 0.67% remaining at 18 ppt, and 0.06% remaining at 28 ppt after 2 days of depuration. MS2 bacteriophage was depurated more extensively at higher salinities, with 44.7%, 13.3%, and 16.7% remaining at 8, 18, and 28 ppt, respectively, after 2 days. By day 5, extensive

FIGURE 12

MEAN OYSTER DEPURATION OF HAV AT 3 SALINITIES

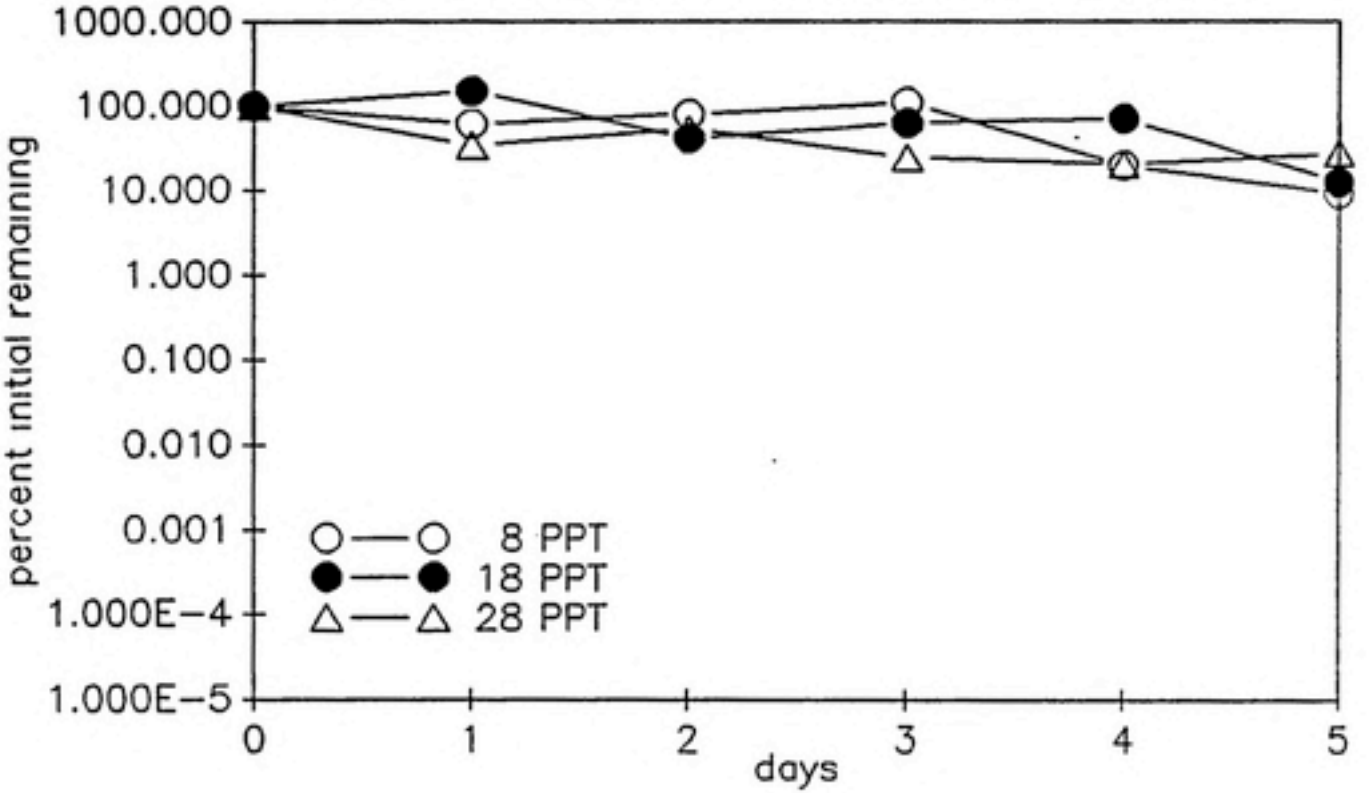


FIGURE 13

MEAN OYSTER DEPURATION OF POLIO AT 3 SALINITIES

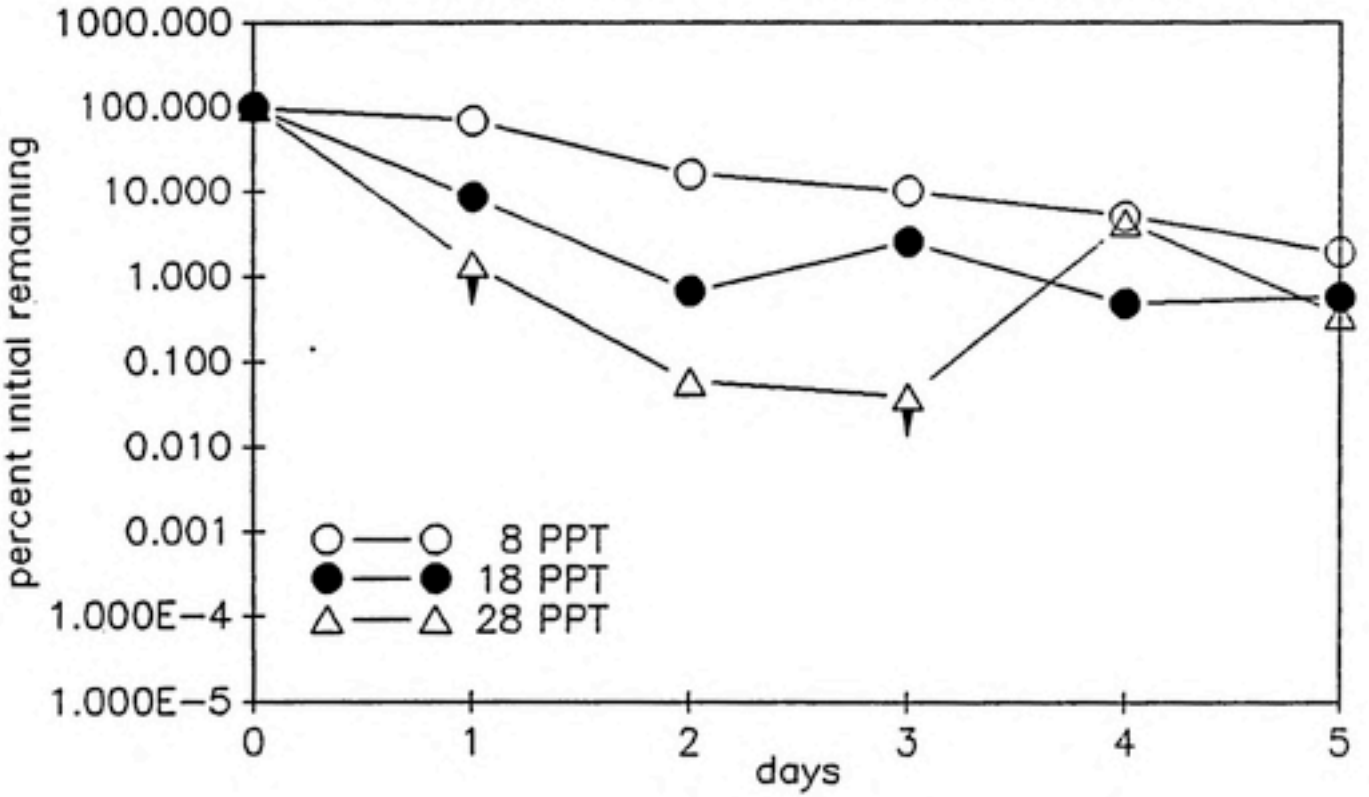


FIGURE 14

MEAN OYSTER DEPURATION OF MS2 AT 3 SALINITIES

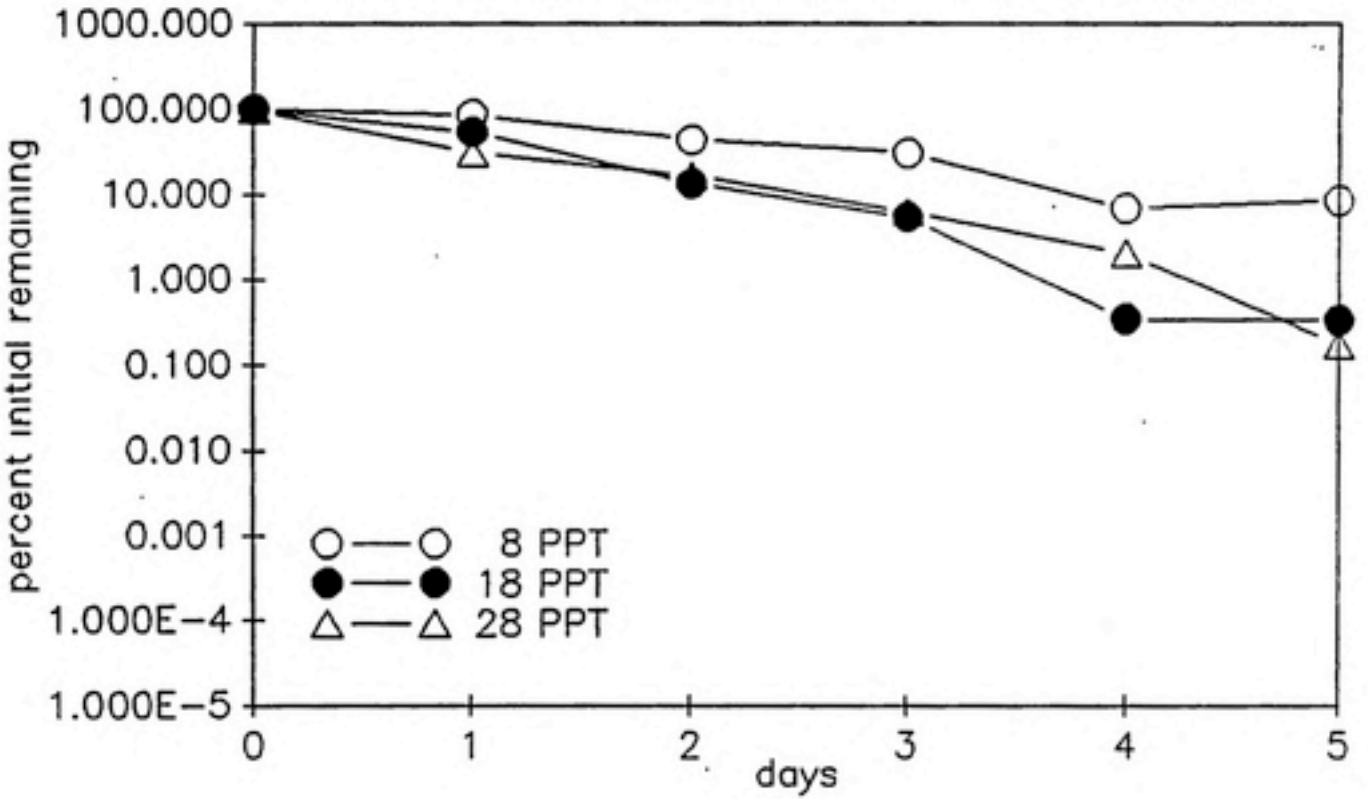


FIGURE 15

MEAN OYSTER DEPURATION OF E. COLI AT 3 SALINITIES

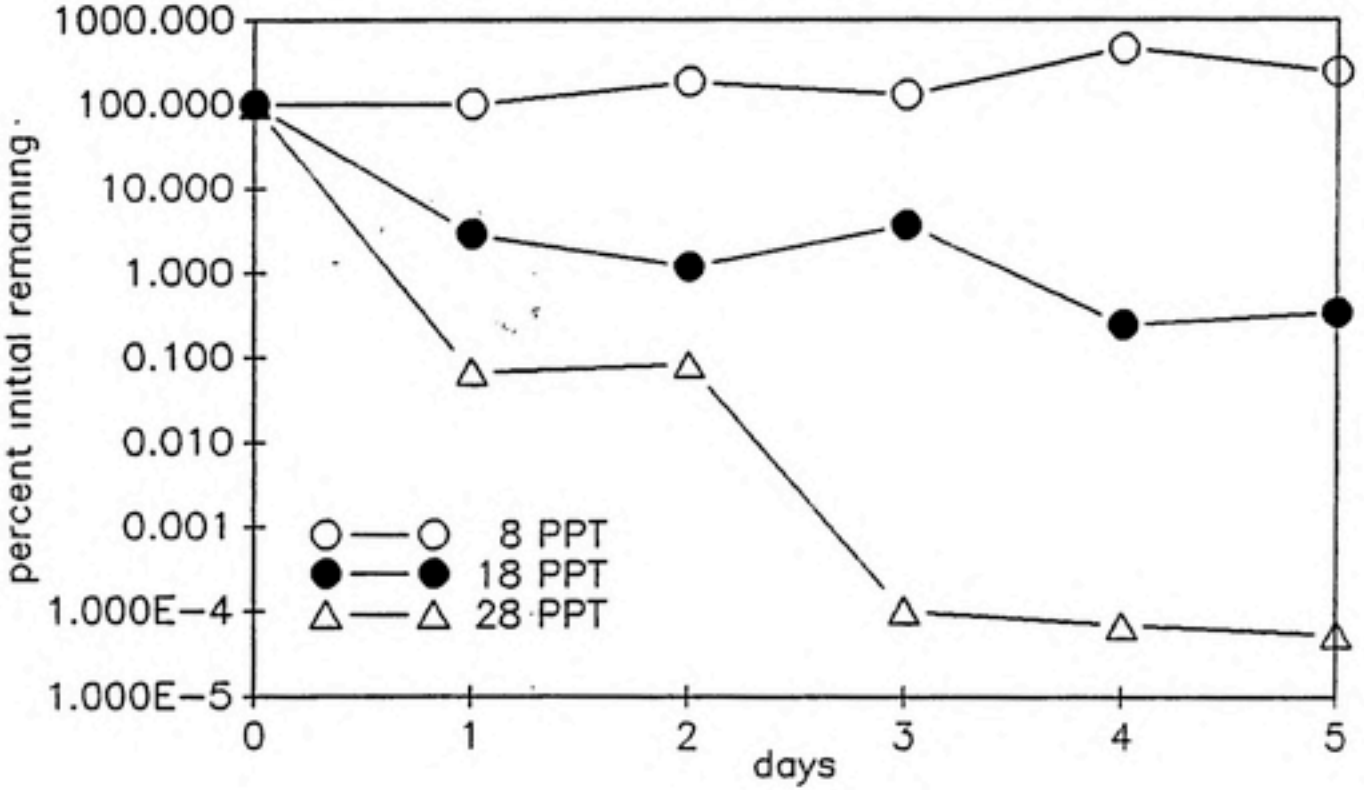
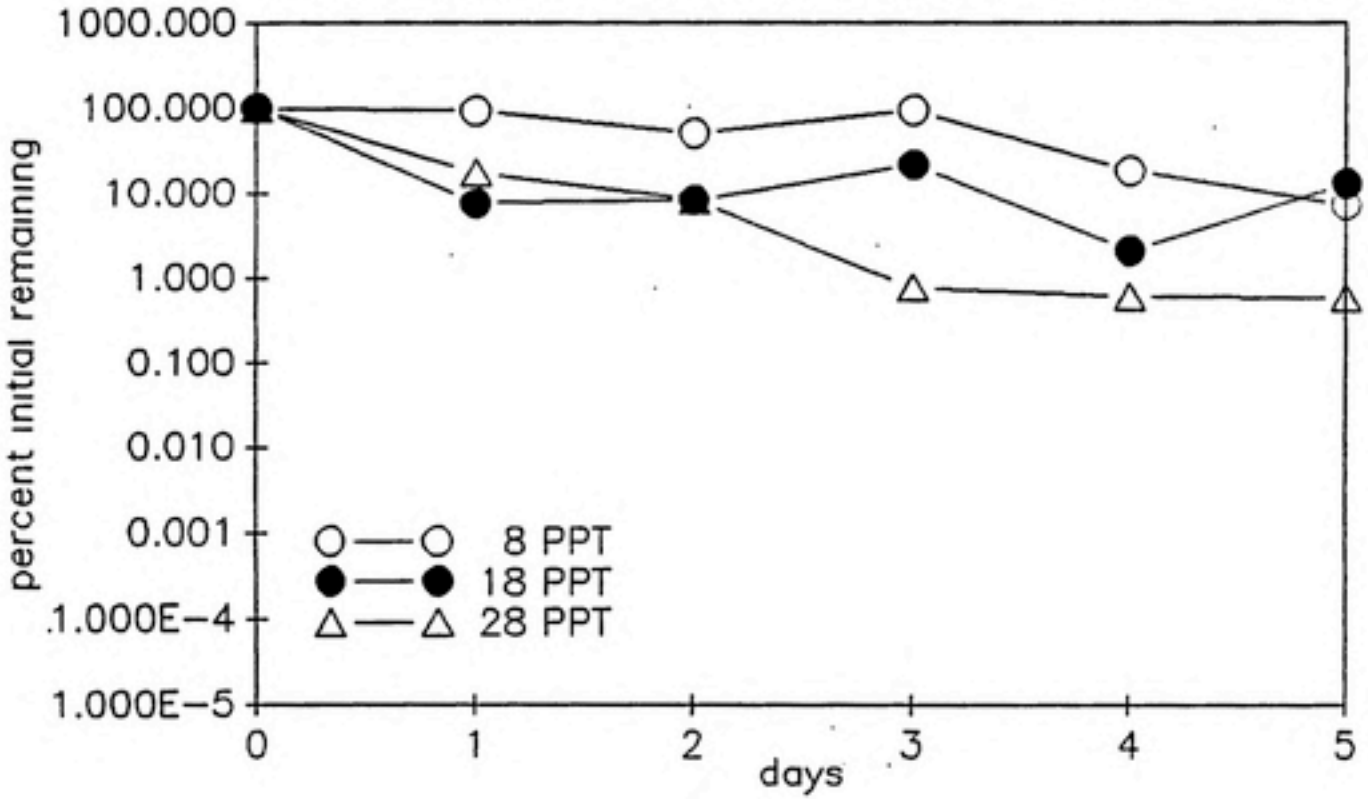


FIGURE 16

MEAN OYSTER DEPURATION OF S. FAECALIS AT 3 SALINITIES



MS2 reductions were observed at 18 and 28 ppt, with <0.4% remaining, while oysters depurated at 8 ppt contained 8.7% of initial MS2 remaining. E. coli elimination proceeded more rapidly and more extensively as the salinity was increased, with 250.5% remaining at 8 ppt, 0.35% remaining at 18 ppt, and 0.00006% remaining at 28 ppt after 5 days of depuration. S. faecalis reductions were more efficient at 28 ppt than at either 8 or 18 ppt. After 5 days, oysters depurated at 8, 18, and 28 ppt contained 7.4%, 12.9%, and 0.61% of initial organisms, respectively.

Statistical analysis of the salinity experiments are shown in Table 11. A Student's T test statistic was used to test the null hypothesis that there was no difference in the rates of depuration (slope) between HAV and the other test organisms at each salinity. A 95% confidence level was used for the basis of acceptance or rejection of the hypothesis. At a salinity of 8 ppt, analyses of the data showed a significant difference in the rate of depuration of HAV and poliovirus ($p=0.03$). Thus, poliovirus was eliminated at a significantly more rapid rate than was HAV at 8 ppt. There were no other significant differences between HAV and the other test organisms at 8 ppt. At 18 ppt, HAV was eliminated at a significantly slower rate than poliovirus ($p=0.03$), MS2 ($p=0.00004$), and E. coli ($p=0.02$). HAV and S. faecalis had similar slopes at 18 ppt. Finally, HAV had a significantly slower rate of depuration at 28 ppt than MS2,

TABLE 11

STATISTICAL COMPARISON OF THE EFFECT OF SALINITY ON THE DEPURATION OF HAV AND OTHER TEST ORGANISMS

SAL	TEST	P VALUE
8	HAV VS POLIO	0.03487
8	HAV VS MS2	0.21946
8	HAV VS EC	0.65895
8	HAV VS SF	0.43324
18	HAV VS POLIO	0.02908
18	HAV VS MS2	0.00004
18	HAV VS EC	0.02278
18	HAV VS SF	0.97720
28	HAV VS POLIO	0.40603
28	HAV VS MS2	0.00000
28	HAV VS EC	0.000007
28	HAV VS SF	0.00014
8 & 18	HAV VS HAV	0.99908
18 & 28	HAV VS HAV	0.40723
8 & 28	HAV VS HAV	0.51554
8 & 18	POLIO VS POLIO	0.77852
18 & 28	POLIO VS POLIO	0.52434
8 & 28	POLIO VS POLIO	0.59483
8 & 18	MS2 VS MS2	0.00518
18 & 28	MS2 VS MS2	0.35298
8 & 28	MS2 VS MS2	0.02021
8 & 18	EC VS EC	0.05605
18 & 28	EC VS EC	0.00078
8 & 28	EC VS EC	0.00008
8 & 18	SF VS SF	0.43898
18 & 28	SF VS SF	0.01098
8 & 28	SF VS SF	0.05537

E. coli, and S. faecalis. At 28 ppt, there were no significant differences between poliovirus and HAV.

Statistical analyses were also performed to determine the effect of salinity on the elimination of each test organism. The Student's T test was used again to test the null hypothesis that there was no difference in the rates of elimination for each test organism at the three salinities, and a 95% confidence level was used as the criterion for significance. There were no significant differences in the depuration rates in pairwise comparisons among the three salinities for either HAV or poliovirus. MS2 bacteriophage was depurated significantly more rapidly at both 18 and 28 ppt than at 8 ppt, with p values 0.005 and 0.02, respectively. E. coli depurated more efficiently at 28 ppt than at either 8 or 18 ppt. In addition, S. faecalis was depurated significantly more rapidly at 28 ppt than at 18 ppt, but there was no difference between the depuration rates at 8 and 18 ppt or 8 and 28 ppt.

DISCUSSION

The results of this study indicate that HAV persisted longer in depurated oysters than the other test organisms at all the conditions tested. In addition, lower temperatures and lower salinities generally caused depuration of the Eastern oyster to proceed more slowly.

HAV was not as readily depurated as the other organisms at any of the temperatures or salinities studied. Although HAV consistently had slower rates of depuration than the other test organisms, significant differences between the rates were not always shown. This lack of statistical significance was probably due to the variability in the efficiency of virus recovery, in the pumping activity of individual oysters from pooled samples, and because of the low numbers of replicate experiments for each variable.

Nevertheless, these results agree with a previous study comparing the elimination of HAV and poliovirus (Sobsey et al., 1987). In that study, poliovirus was rapidly reduced in 2-3 days, but HAV persisted in oysters for up to 5 days of depuration. In addition, other investigators have found no correlation between the elimination of fecal coliforms and viruses in a depuration system (Canzonier, 1971) and in relaying (Cook and Ellender, 1986).

The present study has indicated that HAV persisted even after 5 days of depuration, while poliovirus, coliphage MS2, E. coli, and Streptococcus faecalis are generally eliminated from oysters at more rapid rates. Commercial depuration plants need to be aware that even after the indicator organism (E. coli and other fecal coliforms) is removed by depuration, HAV may not have been effectively eliminated. In addition, poliovirus, MS2 bacteriophage, E. coli, and S. faecalis were not useful as indicators of HAV elimination under depurating conditions.

The results of this study indicate that the rate at which enteric bacteria and viruses was slower in depurating oysters at low temperature and salinity. Reduced shellfish activity or inactivity may be induced by stresses experienced by the oysters when exposed to lower temperature and salinity. Although this was not specifically investigated in this study, previous studies have shown that the exposure of shellfish to lower temperature and salinity can inhibit the bivalve's ability to depurate (Liu et al., 1967; Rowse and Fleet, 1984; Cook and Ellender, 1986). In addition, Cook and Ellender (1986) demonstrated that higher temperature and salinity than normal did not result in physiological stress to the oysters. U.S. depuration standards allow for a wide range of temperature (10-25^{o}C) and salinity (within 20% of harvest site) in the depuration water. However, this study implies that oysters experiencing stresses at low temperature and salinity may

depurate ineffectively. Thus, depuration under some operating conditions may lead to inefficient elimination of enteric microorganisms.

Furthermore, the elimination of viruses was probably due to physical inactivation as well as active elimination. Meinhold (1982) showed that the reduction of poliovirus in oysters at 28°C was due to physical inactivation, probably due to thermal stress, while active elimination of the virus was responsible for most of the reduction at 6 and 17°C. Comparing the depuration of HAV and poliovirus, Davis (1986) found that the elimination of poliovirus at 18 ppt. was due to both active elimination and pysical inactivation, while the elimination of HAV was due to physical inactivation alone.

In the present study, shellfish were contaminated with high levels of organisms to follow elimination over several orders of magnitude for several days of depuration. Naturally-contaminated shellfish would usually contain much lower virus levels and would require shorter depuration times. However, as indicated in this study, high levels of contamination in shellfish are undesireable because of inadequate depuration. Thus, shellfish to be used for depuration should only be harvested from lightly contaminated (restricted) sites. However, at some temperatures and salinities the time for effective removal may be very long, even at low initial HAV levels.

I. Effect of Temperature on Organism Depuration

This study evaluated the elimination of HAV, poliovirus, MS2 bacteriophage, E. coli, and S. faecalis at three temperatures (12, 18, and 25°C) commonly found in commercial depuration systems. These temperatures are within the U.S. depuration water quality standards for oysters which require the water temperature to be in the range of 10-25°C (FDA, 1987).

Overall, these experiments demonstrated that depuration water temperature affects the rate at which enteric bacteria and viruses were eliminated from the Eastern oyster. All the organisms were eliminated at a faster rate at higher water temperatures. In addition, depuration of all the organisms except S. faecalis showed a significantly more rapid rate of elimination at 25°C than at 12°C. Despite the lack of a statistical difference, examination of the data showed that S. faecalis elimination proceeded more rapidly and extensively at 25°C compared to the lower temperatures (12 and 18°C). Furthermore, depuration of all the organisms at the lower temperatures was generally incomplete, with high numbers persisting even after 5 days. These results demonstrating that temperature directly influences the efficiency of depuration are supported by the existing literature (Liu et al., 1967; Canzonier, 1971; Perkins et al., 1980; Rowse and Fleet, 1984; Cook and Ellender, 1986; Sobsey et al., 1987).

Although HAV was not rapidly or extensively reduced at lower temperatures (12 and 18°C), depuration of HAV at 25°C was marginally effective, with no more than 3% of initial viruses remaining after 5 days of depuration. In contrast to these results, Sobsey et al. (1987) reported inefficient depuration of HAV at 12, 18, and 23°C and salinity 18 ppt. However, rapid and extensive reductions of HAV at high temperatures (23°) and a higher salinity of 28 ppt were observed in their study. The present study followed depuration at different temperatures at a high salinity of 28 ppt. Consideration of this information leads to the assumption that HAV may depurate more efficiently at higher temperatures when combined with higher salinities.

In any case, oyster depuration activity was dependent on water temperature in this study. An important interpretation from these findings is the implication that ineffective depuration and a definite risk to public health can result from depuration of the Eastern oyster at lower temperatures. Therefore, maintenance of a minimum water temperature is crucial for adequate depuration. This may require many depuration facilities to install water-heating equipment during the winter months. In addition, longer depuration times at lower temperatures may not effectively reduce the risk of shellfishborne bacterial or viral diseases.

II. Effect of Salinity on Organism Depuration

The depuration of HAV, poliovirus, MS2 bacteriophage, E. coli, and S. faecalis was also evaluated at salinities 8, 18, and 28 ppt. These salinities are encountered by the oysters in the waters from which they were harvested (5-32 ppt). Depuration water quality standards require that the salinity be within 20% of the harvest area.

The results of the salinity experiments generally indicated that salinity of the depuration water had an effect on the ability of the oysters to eliminate microbes. Depuration was shown to be ineffective at the lowest salinity (8 ppt) studied. This finding is supported by the work of Sobsey et al. (1987) which found that poliovirus was eliminated more slowly at 8 ppt. than at higher salinities (18 and 28 ppt.). However, HAV was not extensively reduced at any salinity in the present study. The slow rate which occurred at salinity 28 ppt. contradicts the findings of Sobsey et al. (1987). They found that HAV was more efficiently depurated at 28 ppt. than at lower salinities (8 and 18 ppt.). However, the previous study was done in artificial seawater, while the present study was done in natural seawater. The results in natural seawater are probably a better model for microbial reductions by oysters in actual depuration plants. However, the dilution of the natural seawater to get the lower salinities may have also diluted any natural inactivating factors present in the seawater.

Previous studies have shown that depuration is ineffective at salinities below the levels at which the shellfish are acclimated (Liu et al., 1967; Rowse and Fleet, 1984). In addition, Galtsoff (1964) found that oyster activity was reduced when salinities were different by 10% from the harvest area. During the present study, the ambient harvest area salinity was 18 ppt. Thus, depuration at 18 and 28 ppt may have been more effective because these salinities were most similar to the natural environment of these oysters. Reduced elimination at low salinities may have been the result of increased attachment of enteric microbes to shellfish mucus. DiGirolamo et al. (1977) showed that the mechanism of poliovirus attachment to shellfish mucus was ionic bonding which increased at lower salinities.

From the results presented, it appears that the use of waters having lower salinities in a depuration plant may lead to ineffective depuration and an increased risk of shellfishborne enteric disease. In order to assure maximum depuration, salinities should be maintained above a minimum level and be near the salinity at which the shellfish were acclimated. This would mean that many depuration plants may require salinity adjustments during periods of high rainfall.

III. Factors Contributing to Experimental Variation

Although important observations were made on the effects of temperature and salinity on depuration of health-

related microbial contaminants by oysters in these experiments, several sources of error may have contributed to experimental variation. These laboratory studies were conducted in a flow-through system designed according to specifications for commercial depuration plants. In this depuration system, certain water quality factors such as flow rate, pH, turbidity, dissolved oxygen, temperature, and salinity were controlled. However, the size, age, and reproductive activity of the oysters may have affected the ability of the oysters to depurate. Any variability in shellfish activity during depuration may result in large differences in the rate of depuration by individual shellfish. Therefore, depuration studies using pooled shellfish samples may result in large differences in the concentration of organisms when comparing shellfish samples (Seraichekas et al., 1968; Metcalf et al., 1979).

Variations in the virus recovery method is another source of error when comparing different shellfish samples. The UNC method of virus recovery was used to recover HAV and poliovirus from oyster meats. Evaluation of the UNC method found that average HAV and poliovirus recovery efficiencies ranged from 20-78% and 35-65%, respectively (Keating, 1985; Sobsey et al., 1978). These kinds of variations in virus recovery may contribute to concentration differences when comparing shellfish samples and may cloud the true effects of factors such as temperature and salinity. In addition, the results of the duplicate depuration experiments

sometimes varied considerably. In order to more reliably evaluate the effect of temperature and salinity on depuration, more replicate experiments need to be conducted.

Variations in the extent of contamination of oysters at day 0 between experiments may have influenced the effectiveness of depuration. Although the oysters were allowed to uptake for similar lengths of time, the viral concentrations of day 0 samples were sometimes very different between experiments. Previous studies have shown that the effectiveness of depuration is influenced by the extent of contamination (Metcalf et al., 1980; Son and Fleet, 1980; Cook and Ellender, 1986). This relationship has not yet been demonstrated for the depuration of HAV. Nevertheless, longer depuration times may have been required for heavily contaminated oysters than for lightly contaminated ones. However, this was not usually observed in these experiments.

IV. Implications for Commercial Depuration

This study demonstrated that HAV was not readily depurated by shellfish and that lower water temperatures and salinities caused reduced depuration rates in the Eastern oyster. In addition, the virological quality of the depurated oysters and the resulting risk of disease was not sufficiently reflected by the levels of indicator bacteria. The results of this study have demonstrated that shellfish depuration based on the reduction of fecal coliforms may not eliminate the risk of viral disease. This finding is

supported by other investigations (Canzonier, 1971; Sobsey et al., 1987). In fact, the consumption of depurated shellfish has been associated with outbreaks of hepatitis A (Grohmann et al., 1981; Gill et al., 1983). Careful consideration of this information indicates that depuration may not be an effective means of eliminating the public health problem caused by HAV in oysters. Perhaps a better method of dealing with this public health problem is to prevent pollution from entering our coastal waters and shellfish resources.

Additional research is needed to better define the effects of temperature and salinity on the depuration of the Eastern oyster. Furthermore, the effects of other environmental factors on the rates of depuration need to be determined in order to optimize the conditions for viral depuration. Studies are needed to determine and compare the rate and extent of elimination of HAV, other enteric viruses, fecal indicator bacteria, and other potential indicators by other shellfish under depurating conditions. Finally, studies are needed to determine the persistence of HAV and other viruses at low initial levels of contamination in shellfish under depurating conditions.

CONCLUSIONS

1. Hepatitis A virus was not readily eliminated from the Eastern oyster in a flow-through depuration system.

2. Poliovirus, MS2 bacteriophage, E. coli, and Streptococcus faecalis were more rapidly depurated than HAV.

3. Poliovirus, MS2 bacteriophage, E. coli, and S. faecalis are not adequate indicators of the depuration behavior of HAV.

4. Lower water temperature (12°C) caused depuration to proceed more slowly than at higher temperatures (18 and 25°C).

5. Lower salinity (8 ppt.) caused reduced depuration rates compared to higher salinities (18 and 28 ppt.).

6. Depuration may not be an effective means of eliminating HAV or other pathogenic microbes from contaminated shellfish.

APPENDIX A

FORMULATIONS FOR MEDIA AND COMPONENTS

COMPONENTS AND MEDIA FORMULATIONS

Agar Overlay Medium (HAV-RIFA):

1. 1/2 total required volume of 1% agarose in distilled-deionized water (autoclave to melt agarose).

2. Add 1/2 total required volume of prewarmed (37°C) Eagle's 2X MEM to molten agarose.

Per 100 ml of resulting 0.5% agarose medium:

Fetal calf serum (heat inactivated)	2.0 ml
L+ Glutamine (200 mM)	1.0 ml
Sodium Bicarbonate (7.5%)	1.5 ml
Hepes (1.5M)	1.0 ml
NEAA (100X)	1.0 ml
Gen/Kan (100X)	1.0 ml
Nystatin (as needed)	1.0 ml
$MgCl_2$ (4M)	1.0 ml

Agar Overlay Medium (Poliovirus):

1. 1/2 total required volume of 1.5% Bacto-agar in distilled-deionized water (autoclave to melt agar).

2. Add 1/2 total required volume of prewarmed (37°C) Eagle's 2X MEM to molten agar.

Per 100 ml of resulting 0.75% agar medium:

Fetal calf serum (heat-inactivated)	2.0 ml
L+ Glutamine (200 mM)	1.0 ml
Sodium Bicarbonate (7.5%)	1.5 ml
Hepes (1.5M)	1.0 ml
NEAA (100 X)	1.0 ml
Gen/Kan (100 X)	1.0 ml
Pen/Strep (1000 X)	0.1 ml
Nystatin (as needed)	1.0 ml
$MgCl_2$ (4M)	0.75 ml
Neutral Red (100 X filtered)	1.0 ml

Brain Heart Infusion Broth

Per liter of distilled-deionized water:

Brain Heart Infusion Broth	37.0 gm

Sterilize by autoclaving.

Brain Heart Infusion Agar

Per liter of distilled-deionized water:

Brain Heart Infusion Agar	52.0 gm

Sterilize by autoclaving.

Cat-Floc

Per 10 ml distilled-deionized water:

Concentrated Cat-Floc	0.1 ml

Filter sterilize.
Use 1.0 ml for every 10 ml of sample.
Do not store. Make up day of use.

Coliphage Bottom Agar

Per liter of distilled-deionized water:

Nutrient Broth	8.0 gm
NaCl	8.0 gm
Bacto-agar	15.0 gm
$CaCl_2$	0.29 gm

Sterilize by autoclaving.
Pour plates with 15 ml in each plate.

Coliphage Single Strength Top Agar

Per 500 ml of distilled-deionized water:

Tryptone	5.0 gm
NaCl	4.0 gm
Yeast Extract	0.5 gm
Glucose	0.5 gm
$CaCl_2$	0.14 gm
Bacto-agar	3.75 gm

Boil to dissolve and dispense 3 ml into 16 X 125 screw top test tubes.
Autoclave to sterilize.

Disodium Phosphate (0.1M)

Per 90 ml distilled-deionized water:

$Na_2HPO_4 \cdot 7H_2O$	2.68 gm

Adjust to pH 9.0-9.5.
Bring to 100 ml.
Sterilize by autoclaving.
Store at 4°C.

Eagle's Modified Minimum Essential Medium (1X MEM)

Per liter of distilled-deionized water:

Autoclavable Eagle's MEM with Earle's balanced salts and phenol red.	9.4 gm

Prepare as directed.
Store at 4°C.

Eagle's Modified Minimum Essential Medium (2X MEM)

Per liter of distilled-deionized water:

Autoclavable Eagle's MEM with Earle's balanced salts; without phenol red.	9.4 gm

Prepare as directed.
Store at 4°C.

Gentamycin/Kanamycin (100 X)

Per liter of distilled-deionized water:

Gentamycin (powder)	5.0 gm
Kanamycin (powder)	25.0 gm

Sterilize by autoclaving.
Store at -20°C.

Glutamine (200 mM, 100 X)

Per liter of distilled-deionized water:

L+ Glutamine	29.5 gm

Filter sterilize.
Store at -20°C.

Glycine (50 mM)-Saline (0.14M)

Per 900 ml distilled-deionized water:

Glycine	3.75 gm
NaCl	8.18 gm

Adjust to pH 7.5 with 1N NaOH.
Bring to 1000 ml.
Sterilize by autoclaving.
Store at 4°C.

Growth Medium

Per 100 of 1X MEM:

Fetal Calf Serum (heat inactivated)	10.0 ml
L+ Glutamine (200 mM)	1.0 ml
Sodium Bicarbonate (7.5%)	1.5 ml
Hepes (1.5 M)	1.0 ml
Non-Essential Amino Acid (NEAA)	1.0 ml
Gen/Kan (100 X)	1.0 ml

Hepes Buffer (1.5M)

Per liter of distilled-deionized water:

Hepes	356 gm

Sterilize by autoclaving.
Store at 4°C.

Magnesium Chloride (4M)

Per liter of distilled-deionized water:

$MgCl_2 \cdot 6H_2O$	813 gm

Sterilize by autoclaving.
Store at 4°C.

Maintenance Medium

Per 100 ml of 1X MEM:

Fetal Calf Serum (heat inactivated)	2.0 ml
L+ Glutamine (200 mM)	1.0 ml
Sodium Bicarbonate (7.5%)	1.5 ml
Hepes (1.5 M)	1.0 ml
NEAA (100 X)	1.0 ml
Gen/Kan (100 X)	1.0 ml
Nystatin (as needed)	1.0 ml

m-Enterococcus Agar

Per liter of distilled-deionized water:

Bacto Tryptose	20.0 gm
Yeast Extract	5.0 gm
Dextrose	2.0 gm
K_2HPO_4	4.0 gm
Sodium Azide	0.4 gm
Bacto-Agar	10.0 gm
Triphenyltetrazolium Chloride	0.1 gm

Boil solution briefly.
Store molten at 45°C.

Modified MacConkey Agar (Single-Strength)

Per liter of distilled-deionized water:

Peptone	17.0 gm
Proteose Peptone	3.0 gm
Lactose	10.0 gm
Bile Salts No. 3	0.75 gm
Bacto-Agar	10.0 gm
Neutral Red[a]	0.03 gm
Crystal Violet[b]	0.001 gm

[a]Prepare a 0.3% (0.3g/100ml) solution of neutral red in distilled water and use 10 ml per liter of single-strength medium.
[b]Prepare a 0.01% (0.01g/100ml) solution of crystal violet in distilled water and use 10 ml per liter of single-strength medium.
Boil medium briefly and store molten at 45°C.

Neutral Red (1:300)

Per liter of distilled-deionized water:

Neutral Red (powder)	3.33 gm

Filter sterilize.
Store at 4°C, wrapped in foil.

Nutrient Broth No. 2

Per liter of distilled-deionized water:

Nutrient broth	8.0 gm
NaCl	5.0 gm

Sterilize by autoclaving.

Nutrient Agar No. 2

Per liter of distilled-deionized water:

Nutrient broth	8.0 gm
NaCl	5.0 gm
Bacto-agar	15.0 gm

Sterilize by autoclaving.

Nystatin (100 X)

Per liter of distilled-deionized water:

Nystatin (powder, 5420 USP Units/mg)	0.55 gm

Filter sterilize.
Store at -20°C.

Penicillin/Streptomycin (1000 X)

Per 100 ml of distilled-deionized water:

Penicillin G	1×10^7 IU
Streptomycin	1×10^7 ug

Filter sterilize.
Store at -20°C.

Phosphate Buffered Saline, pH 7.5

Per liter of distilled-deionized water:

NaCl	8.0 gm
KCl	0.2 gm
KH_2PO_4	0.12 gm
Na_2HPO_4 (anhydrous)	0.91 gm

Sterilize by autoclaving.
Store at 4°C.

Sodium Bicarbonate (7.5%)

Per liter of distilled-deionized water:

Sodium Bicarbonate (Arm and Hammer)	75.0 gm

Sterilize by autoclaving.
Store at 4°C.

Trypsin-EDTA (10 X)

Per liter of distilled-deionized water:

Trypsin 1:250 (Difco)	5.0 gm
EDTA (di-sodium salt)	2.0 gm

Filter sterilize.
Store at -20°C.

Virus Diluent (HAV)

Per 100 ml of 1X MEM:

Sodium Bicarbonate (7.5%)	1.5 ml
Hepes (1.5 M)	1.0 ml
Gen/Kan	1.0 ml
Nystatin (as needed)	1.0 ml
Antisera (as needed)	0.1 ml

Virus Diluent (Poliovirus)

Per 100 ml of PBS:

Fetal calf serum (heat inactivated)	2.0 ml
Gen/Kan (100 X)	1.0 ml
Nystatin (as needed)	1.0 ml

APPENDIX B

RAW DATA FOR TEMPERATURE AND SALINITY EXPERIMENTS

TABLE 12

ELIMINATION OF TEST ORGANISMS AT TEMPERATURE 12°C

ORG	DAY	ORGANISMS PER GRAM			% INITIAL ORGANISM REMAINING		
		TRIAL			TRIAL		
		A	B	MEAN	A	B	MEAN
HAV	0	190.37	190.37	190.37	100.00	100.00	100.00
	1	54.75	794.07	424.41	28.76	417.13	222.94
	2	237.84	68.87	153.36	124.94	36.18	80.56
	3	64.95	170.75	117.85	34.12	89.70	61.91
	4	828.49	349.11	588.80	435.21	183.39	309.30
	5	324.23	622.19	473.21	170.32	326.84	248.58
POLIO	0	976.58	976.58	976.58	100.00	100.00	100.00
	1	273.77	421.85	347.81	28.03	43.20	35.61
	2	185.51	380.82	283.17	19.00	38.99	29.00
	3	68.77	44.40	56.58	7.04	4.55	5.79
	4	94.69	109.72	102.20	9.70	11.24	10.47
	5	16.21	46.66	31.44	1.66	4.78	3.22
MS2	0	3.4×10^4	3.4×10^4	3.4×10^4	100.00	100.00	100.00
	1	2.2×10^4	5.9×10^4	4.0×10^4	64.78	176.06	120.42
	2	1.1×10^4	2.1×10^4	1.6×10^4	33.73	62.09	47.91
	3	1.8×10^4	2.5×10^4	2.2×10^4	54.33	74.93	64.63
	4	1.1×10^4	1.5×10^4	1.3×10^4	32.84	45.67	39.25
	5	9.9×10^3	2.9×10^4	2.0×10^4	29.67	87.16	58.42
EC	0	2.8×10^4	2.8×10^4	2.8×10^4	100.00	100.00	100.00
	1	1.1×10^4	2.3×10^4	1.7×10^4	38.63	81.95	60.29
	2	4.7×10^3	1.5×10^4	9.8×10^3	17.00	54.04	35.52
	3	1.7×10^3	1.3×10^3	1.5×10^3	6.12	4.73	5.42
	4	848.00	3.5×10^3	2.2×10^3	3.06	12.66	7.86
	5	376.00	6.8×10^3	3.6×10^3	1.36	24.66	13.01
SF	0	4.9×10^4	4.9×10^4	4.9×10^4	100.00	100.00	100.00
	1	2.0×10^4	7.5×10^4	4.7×10^4	40.08	150.81	95.45
	2	1.3×10^4	3.3×10^4	2.3×10^4	26.52	65.99	46.26
	3	7.4×10^3	6.6×10^3	7.0×10^3	14.98	13.44	14.21
	4	3.9×10^3	7.0×10^3	5.4×10^3	7.91	14.09	11.00
	5	1.5×10^3	7.1×10^3	4.3×10^3	3.04	14.31	8.67

TABLE 13

ELIMINATION OF TEST ORGANISMS AT TEMPERATURE 18°C

ORG	DAY	ORGANISMS PER GRAM			% INITIAL ORGANISM REMAINING		
		TRIAL			TRIAL		
		A	B	MEAN	A	B	MEAN
HAV	0	901.64	901.64	901.64	100.00	100.00	100.00
	1	61.16	197.85	129.51	6.78	21.94	14.36
	2	59.94	<2.84	<31.39	6.65	<0.31	<3.48
	3	305.13	145.28	225.20	33.84	16.11	24.98
	4	61.04	31.60	46.32	6.77	3.50	5.14
	5	633.20	170.42	401.81	70.23	18.90	44.56
POLIO	0	2.7×10^3	2.7×10^3	2.7×10^3	100.00	100.00	100.00
	1	76.45	42.04	59.25	2.83	1.55	2.19
	2	20.98	17.01	19.00	0.78	0.63	0.70
	3	70.94	11.62	41.28	2.62	0.43	1.53
	4	25.64	17.86	21.74	0.95	0.66	0.80
	5	<0.37	1.26	<0.82	<0.01	0.05	<0.03
MS2	0	7.6×10^3	7.6×10^3	7.6×10^3	100.00	100.00	100.00
	1	1.9×10^3	3.2×10^3	2.6×10^3	25.46	42.39	33.92
	2	784.00	908.00	846.00	10.29	11.92	11.10
	3	3.9×10^3	2.4×10^3	3.2×10^3	51.44	30.91	41.17
	4	2.2×10^3	1.6×10^3	1.9×10^3	28.66	20.50	24.58
	5	1.2×10^3	1.2×10^3	1.2×10^3	16.19	15.97	16.08
EC	0	1.8×10^4	1.8×10^4	1.8×10^4	100.00	100.00	100.00
	1	1.2×10^3	2.9×10^3	2.1×10^3	6.86	16.78	11.82
	2	106.00	166.00	136.00	0.60	0.94	0.77
	3	808.00	1.6×10^3	1.2×10^3	4.59	8.85	6.72
	4	3.3×10^3	1.5×10^3	2.4×10^3	18.94	8.69	13.81
	5	200.67	442.00	321.34	1.14	2.51	1.83
SF	0	2.5×10^4	2.5×10^4	2.5×10^4	100.00	100.00	100.00
	1	1.1×10^3	1.2×10^4	6.5×10^3	4.41	46.98	25.70
	2	265.50	365.33	315.42	1.05	1.44	1.24
	3	1.4×10^3	1.7×10^3	1.5×10^3	5.49	6.70	6.09
	4	$<1.4\times10^3$	1.4×10^3	$<1.4\times10^3$	<5.51	5.51	<5.51
	5	558.00	2.6×10^3	1.6×10^3	2.20	10.22	6.21

TABLE 14

ELIMINATION OF TEST ORGANISMS AT TEMPERATURE 25°C

ORG	DAY	ORGANISMS PER GRAM			% INITIAL ORGANISM REMAINING		
		TRIAL			TRIAL		
		A	B	MEAN	A	B	MEAN
HAV	0	1.5×10^3	1.5×10^3	1.5×10^3	100.00	100.00	100.00
	1	201.20	167.43	184.31	13.83	11.50	12.66
	2	297.85	125.18	211.51	20.47	8.60	14.53
	3	117.96	176.78	147.37	8.11	12.15	10.13
	4	99.52	32.76	66.14	6.84	2.25	4.54
	5	15.01	64.22	39.61	1.03	4.41	2.72
POLIO	0	2.8×10^4	2.8×10^4	2.8×10^4	100.00	100.00	100.00
	1	1.3×10^3	226.72	754.45	4.64	0.82	2.73
	2	67.69	109.53	88.61	0.25	0.40	0.32
	3	36.30	41.16	38.73	0.13	0.15	0.14
	4	43.79	<4.10	<23.95	0.16	<0.02	<0.09
	5	<3.00	9.63	<6.32	<0.01	0.04	<0.02
MS2	0	3.0×10^3	3.0×10^3	3.0×10^3	100.00	100.00	100.00
	1	967.00	840.00	903.50	32.67	28.38	30.52
	2	318.00	228.00	273.00	10.74	7.70	9.22
	3	103.00	93.33	98.17	3.48	3.15	3.32
	4	14.00	4.00	9.00	0.47	0.14	0.30
	5	20.00	18.33	19.17	0.68	0.62	0.65
EC	0	9.5×10^3	9.5×10^3	9.5×10^3	100.00	100.00	100.00
	1	100.00	70.00	85.00	1.05	0.74	0.89
	2	34.00	34.00	34.00	0.36	0.36	0.36
	3	36.67	23.33	30.00	0.39	0.25	0.32
	4	35.20	6.40	20.80	0.37	0.07	0.22
	5	3.20	2.40	2.80	0.03	0.03	0.03
SF	0	6.4×10^3	6.4×10^3	6.4×10^3	100.00	100.00	100.00
	1	156.00	84.00	120.00	2.45	1.32	1.88
	2	452.60	2.2×10^3	1.3×10^3	7.11	34.45	20.78
	3	276.80	65.40	171.10	4.35	1.03	2.69
	4	120.29	52.44	86.37	1.89	0.82	1.36
	5	6.40	23.80	15.10	0.10	0.37	0.24

TABLE 15

ELIMINATION OF TEST ORGANISMS AT SALINITY 8 PPT

ORG	DAY	ORGANISMS PER GRAM TRIAL A	ORGANISMS PER GRAM TRIAL B	ORGANISMS PER GRAM MEAN	% INITIAL ORGANISM REMAINING TRIAL A	% INITIAL ORGANISM REMAINING TRIAL B	% INITIAL ORGANISM REMAINING MEAN
HAV	0	1.4×10^4	170.38	7.3×10^3	100.00	100.00	100.00
	1	1.4×10^3	195.01	778.72	9.51	114.45	61.98
	2	907.77	265.12	586.44	6.34	155.60	80.97
	3	3.2×10^3	337.08	1.8×10^3	22.22	197.84	110.03
	4	1.6×10^3	48.93	805.84	10.91	28.72	19.81
	5	857.14	20.79	438.97	5.98	12.20	9.09
POLIO	0	2.2×10^4	1.7×10^4	1.9×10^4	100.00	100.00	100.00
	1	1.3×10^4	1.4×10^4	1.3×10^4	57.67	81.75	69.71
	2	3.8×10^3	2.5×10^3	3.2×10^3	17.52	14.78	16.15
	3	1.0×10^3	2.7×10^3	1.8×10^3	4.73	15.58	10.16
	4	357.20	1.5×10^3	926.99	1.63	8.79	5.21
	5	61.91	623.78	342.85	0.28	3.66	1.97
MS2	0	2.5×10^3	1.2×10^4	7.1×10^3	100.00	100.00	100.00
	1	1.1×10^3	1.5×10^4	8.1×10^3	44.90	127.93	86.42
	2	528.00	8.0×10^3	4.2×10^3	21.55	67.89	44.72
	3	173.33	6.5×10^3	3.3×10^3	7.07	55.37	31.22
	4	114.00	1.1×10^3	595.34	4.65	9.18	6.92
	5	18.70	1.9×10^3	980.35	0.76	16.56	8.66
EC	0	1.8×10^4	1.6×10^3	9.6×10^3	100.00	100.00	100.00
	1	2.3×10^4	1.1×10^3	1.2×10^4	129.92	71.74	100.83
	2	8.8×10^3	5.1×10^3	6.9×10^3	50.06	324.26	187.16
	3	1.2×10^3	4.1×10^3	2.6×10^3	6.65	259.28	132.97
	4	588.00	1.5×10^4	7.5×10^3	3.34	924.68	464.01
	5	561.00	7.8×10^3	4.2×10^3	3.19	497.87	250.53
SF	0	2.9×10^4	1.0×10^4	2.0×10^4	100.00	100.00	100.00
	1	2.0×10^4	1.2×10^4	1.6×10^4	68.60	119.84	94.22
	2	7.4×10^3	7.7×10^3	7.6×10^3	25.32	76.38	50.85
	3	1.6×10^3	1.9×10^4	1.0×10^4	5.60	185.68	95.64
	4	895.00	3.4×10^3	2.2×10^3	3.05	33.88	18.47
	5	892.00	1.2×10^3	1.0×10^3	3.04	11.82	7.43

TABLE 16

ELIMINATION OF TEST ORGANISMS AT SALINITY 18 PPT

ORG	DAY	ORGANISMS PER GRAM			% INITIAL ORGANISM REMAINING		
		TRIAL			TRIAL		
		A	B	MEAN	A	B	MEAN
HAV	0	396.94	396.94	396.94	100.00	100.00	100.00
	1	197.73	994.30	596.02	49.81	250.49	150.15
	2	74.00	254.29	164.15	18.64	64.06	41.35
	3	346.74	159.58	253.16	87.35	40.20	63.78
	4	54.45	517.34	285.90	13.72	130.33	72.03
	5	26.00	72.73	49.36	6.55	18.32	12.44
POLIO	0	1.1×10^4	1.1×10^4	1.1×10^4	100.00	100.00	100.00
	1	1.4×10^3	477.74	929.35	12.70	4.39	8.55
	2	74.00	72.66	73.33	0.68	0.67	0.67
	3	179.35	391.69	285.52	1.65	3.60	2.63
	4	10.89	97.61	54.25	0.10	0.90	0.50
	5	98.81	30.30	64.56	0.91	0.28	0.59
MS2	0	286.67	286.67	286.67	100.00	100.00	100.00
	1	133.30	179.33	156.32	46.50	62.56	54.53
	2	34.60	41.44	38.02	12.07	14.46	13.26
	3	15.80	15.60	15.70	5.51	5.44	5.48
	4	1.00	1.00	1.00	0.35	0.35	0.35
	5	1.50	0.50	1.00	0.52	0.17	0.35
EC	0	2.0×10^4	2.0×10^4	2.0×10^4	100.00	100.00	100.00
	1	456.00	726.00	591.00	2.24	3.56	2.90
	2	317.59	163.93	240.76	1.56	0.80	1.18
	3	725.83	803.32	764.53	3.58	3.94	3.75
	4	62.00	38.00	50.00	0.30	0.19	0.25
	5	73.33	68.00	70.67	0.36	0.33	0.35
SF	0	5.6×10^3	5.6×10^3	5.6×10^3	100.00	100.00	100.00
	1	421.00	448.00	434.50	7.46	7.93	7.69
	2	188.60	747.40	468.00	3.34	13.24	8.29
	3	1.4×10^3	1.0×10^3	1.2×10^3	24.90	18.24	21.57
	4	115.20	130.60	122.90	2.04	2.31	2.18
	5	344.40	1.1×10^3	730.00	6.10	19.76	12.93

TABLE 17

ELIMINATION OF TEST ORGANISMS AT SALINITY 28 PPT

		ORGANISMS PER GRAM			% INITIAL ORGANISM REMAINING		
		TRIAL			TRIAL		
ORG	DAY	A	B	MEAN	A	B	MEAN
HAV	0	8.0×10^3	8.0×10^3	8.0×10^3	100.00	100.00	100.00
	1	4.2×10^3	1.2×10^3	2.7×10^3	51.85	15.37	33.61
	2	4.3×10^3	ND*	4.3×10^3	53.48	ND	53.48
	3	2.0×10^3	1.9×10^3	2.0×10^3	25.48	23.22	24.35
	4	1.1×10^3	2.2×10^3	1.6×10^3	13.49	27.29	20.39
	5	1.9×10^3	2.5×10^3	2.2×10^3	23.84	30.64	27.24
POLIO	0	1.5×10^3	1.5×10^3	1.5×10^3	100.00	100.00	100.00
	1	41.91	<0.72	<21.32	2.77	<0.05	<1.41
	2	0.88	ND	0.88	0.06	ND	0.06
	3	0.65	<0.62	<0.63	0.04	<0.04	<0.04
	4	90.61	41.87	66.24	5.99	2.77	4.38
	5	6.28	4.79	5.54	0.41	0.32	0.37
MS2	0	1.0×10^4	1.0×10^4	1.0×10^4	100.00	100.00	100.00
	1	3.3×10^3	2.8×10^3	3.1×10^3	32.97	28.47	30.72
	2	1.7×10^3	ND	1.7×10^3	16.65	ND	16.65
	3	605.00	650.00	627.50	6.05	6.50	6.28
	4	178.67	228.33	203.50	1.79	2.28	2.04
	5	17.80	18.00	17.90	0.18	0.18	0.18
EC	0	1.7×10^7	1.7×10^7	1.7×10^7	100.00	100.00	100.00
	1	1.6×10^4	6.5×10^3	1.1×10^4	0.10	0.04	0.07
	2	1.8×10^4	ND	1.8×10^4	0.11	ND	0.11
	3	30.00	1.33	15.67	2×10^{-4}	8×10^{-6}	9×10^{-5}
	4	7.00	18.00	12.50	4×10^{-5}	1×10^{-4}	7×10^{-5}
	5	8.67	10.00	9.34	5×10^{-5}	6×10^{-5}	6×10^{-5}
SF	0	4.1×10^3	4.1×10^3	4.1×10^3	100.00	100.00	100.00
	1	1.1×10^3	281.50	713.25	27.93	6.87	17.40
	2	341.00	ND	341.00	8.32	ND	8.32
	3	49.00	16.40	32.70	1.20	0.40	0.80
	4	9.80	41.20	25.50	0.24	1.01	0.62
	5	30.40	19.20	24.80	0.74	0.47	0.61

*No Data (ND) available because temperature rose in homogenate during blending and cooked the organisms

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