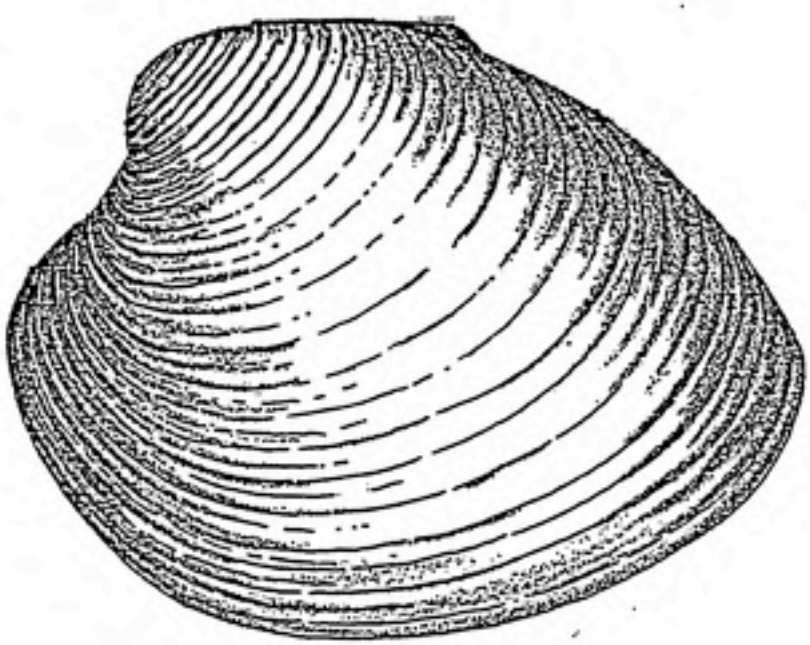
THE EFFECT OF TEMPERATURE AND SALINITY ON THE DEPURATION OF HEPATITIS A VIRUS AND OTHER MICROBIAL INDICATORS FROM CLAMS



JULIE C. MURRAY

ABSTRACT

JULIE C. MURRAY. Effect of Temperature and Salinity on the Depuration of Hepatitis A Virus and Other Microbial Contaminants in Clams.

(Under the direction of Dr. Mark D. Sobsey)

Depuration or the process of self-purification has been used for many years to reduce the enteric microbial contaminants from shellfish harvested from sewage polluted waters. Little is known however about the effectiveness of this process in eliminating enteric viral pathogens. This study compared the reduction of hepatitis A virus (HAV) to that of poliovirus 1, bacteriophage MS2, Escherichia coli, and Streptococcus faecalis, from experimentally contaminated clams (Mercenaria mercenaria). The clams were subjected standard depuration conditions in a model, laboratory-scale system. The effect of temperature (12, 18, 25°C) and salinity (8, 18, 28ppt) on virus and bacteria depuration over period of 4-5 days was studied. The viruses were not depurated as effectively as the bacteria. Elimination rates of test organisms was independent of temperature. Depuration rates were reduced at lower salinity (8 and 18 ppt). The results indicate that the current the practices and conditions of depuration are ineffective in eliminating enteric viruses from clams. These studies further suggest that coliform bacteria is an inadequate indicator of virus elimination from shellfish.

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INTRODUCTION

The pollution of estuaries and other coastal marine waters is threatening an important food source, edible bivalve molluskan shellfish. Shellfish resources are increasingly being closed to harvesting because of the public health risks associated with consumption of contaminated shellfish. Due to expanding populations and development in coastal areas and man's disregard for his environment the economic effects of this loss are great.

Primary sources of sewage pollution include sewage treatment plant effluents and sludges, septic tank seepage, boat waste discharges, and land runoff. Shellfish filter feed in these polluted water and retain suspended particulate matter including pathogenic microorganisms. Thus, shellfish harvested from a fecally contaminated area may contain enteric viruses and bacteria. Shellfish may then transmit disease if eaten raw or partially cooked.

The current standards for shellfish and harvesting waters were developed over 40 years ago and are based on the presence of total or fecal coliform bacteria. Enforcement of this standard has protected consumers from bacterial infection but the ability of coliforms to indicate viral contamination has been questioned. Many outbreaks of

Hepatitis A virus and viral gastroenteritis have resulted from consumption of contaminated shellfish, some harvested from 'approved' beds. Therefore, there is reason for concern that shellfish may accumulate and retain HAV and other enteric viruses more efficiently than they do indicator bacteria.

Depuration is a process in which contaminated shellfish are placed in a clean flowing system and allowed to naturally eliminate or purge thenselves of accumulated contaminants. However, levels of enteric microorganisms may persist in shellfish for a period of time after they have been transferred to clean water; reports of adequate time requirements vary with respect to the environmental conditions of depuration. If the depuration process and the factors which affect it can be understood, commercial scale depuration or relaying could alleviate some of the economic loss of closed marginally polluted harvesting areas and ensure pathogen free shellfish.

Microbial information on depuration is limited, with very little known about the elimination of HAV and most other pathogenic enteric viruses. Hepatitis A virus is probably the most serious viral disease transmitted by contaminated shellfish. Studies are therefore needed to evaluate and characterize the behavior of HAV compared to other enteric viruses, indicator bacteria, and other potential indicators during depuration. Additionally,

studies are needed to determine the effect of environmental factors such as temperature and salinity on depuration to identify the optimum conditions needed for the elimination of microbial contaminants.

OBJECTIVES

To evaluate the reduction of hepatitis A virus compared to poliovirus-1, bacteriophage MS2, Escherichia coli B, and Streptococcus faecalis, from the hardshell clam, Mercenaria mercenaria, at different conditions of temperature and salinity in a lab-scale depuration system.

-To determine the rates of depuration of high levels of HAV, polio, MS2, E. coli, and S. faecalis at temperatures of 12, 18, 25oC.

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

To determine the comparative time period for maximum uptake and concentration from water of bacteria (E. coli B and S. faecalis) and viruses (HAV, poliovirus, and bacteriophage MS2) in clams.

LITERATURE REVIEW

A. Test Organisms

A.1. Viruses

Viruses are the smallest infectious agents, ranging in size from 20-300 nm in diameter. They are obligate intracellular parasites which are capable of replication only in a living host cell.

Viruses contain either single or double stranded nucleic acid, either RNA or DNA and either in one polycistronic molecule or in a different segments. The genome is encased in a protein shell capsid which is specific to the virus. The shell may then be surrounded by a lipid containing membrane.

Viruses have one of three general shapes; spherical (icosahedral), helical (rod-shaped), and complex.

The host range for a specific virus may be broad or extremely limited. Viruses are known to infect unicellular organisms such as mycoplasma, bacteria, and algae, as well as eukaryote cells and multicellular organisms such as plants and animals.

Enteric viruses

Enteric viruses are transient inhabitants of the human alimentary tract. Enteroviruses lack a lipid envelope, and consequently they are stable in the digestive tract environment. The virus remains infectious even after

exposure to acids at pH levels as low as 3.0. Viruses multiply within the gut and are excreted in the feces in large numbers. The titer of some enteric viruses may be as high as $1x10^{12}$ particles per gram/feces.

Disease transmission is by the fecal oral route. This mode of transmission is facilitated through person to person contact and through virus contamination of water and food.

There are more than a hundred types of enteric viruses, these including the enteroviruses, adenoviruses, rotaviruses, reoviruses, Norwalk and Norwalk-like viruses, calciviruses, astroviruses, and hepatitis viruses. The most commonly known enteroviruses are the three types of poliovirus (types 1-3). Other enteroviruses include: 34 types of echoviruses; 30 types of coxackieviruses A + B; and hepatitis A virus, which is now classified as enterovirus 72.

Enteric virus infections are asymptomatic most of the time especially in infants and young children. In some cases, however, they may cause symptoms such as vomiting, diarrhea, gastrointestinal illness, malaise, hepatitis, jaundice, aseptic meningitis, and severe paralysis.

A.1.1. HAV

Based on recent studies HAV, a member of the picornaviridae family, is classified with the enteroviruses as enterovirus 72, although there is growing evidence to

suggest that it may more appropriately belong in a seperate genus of this family (Melnick, 82). Like enteroviruses, HAV remains stable after exposure to ether and acid, but is more resistant to heating at high temperatures (60^{o}c for 1 hour) and to common disinfectants. Therefore, extra protection is needed in dealing with hepatitis A patients and their products. Similar in morphology to other picornaviruses, HAV is a 27 to 32 nm spherical (icosohedral) particle with cubic symmetry. It's physical and chemical characteristics are also like those of enteroviruses, with a linear single stranded RNA genome, having a molecular weight of about $2.25x10^{6}$, positive polarity, a 5'- terminal protein(VPG), and a 3' poly (A) tail. The capsid is composed of four different structural proteins (VP1-VP4; 60 copies each), and 32 capsomeres.

Studies have shown that HAV is persistent in soil, sewage, and the water environment (Sobsey et al, 1987). The most likely mode of transmission of HAV is the fecal-oral route, and both person to person contact and fecally contaminated common-source vehicles, such as water and food are implicated in disease outbreaks. A documented cause of Hepatis A virus disease is the consumption of raw or partially cooked bivalve mollusks (e.g. oysters, clams, mussels, ect.) obtained from fecally contaminated water.

Hepatitis A, or infectious icterus, is a viral infection that begins with symptoms that resemble an

influenza-like illness with an abrupt onset of fever, headache, malaise, fatigue, anorexia, and nausea, followed usually by vomiting, abdominal pain, dark urine, and jaundice. The liver and spleen may be enlarged.

The incubation period of HAV ranges from 15-40 days with a large amount of hepatitis A virus excreted in the stool during the latter part of the incubation before outset of the disease.

The disease is generally more severe and prolonged in adults than in children. Hepatitis A is worldwide in distribution but there is a high endemic prevalence in developing countries.

The wild type hepatitis virus doesn't replicate well in cell culture and this may be due to a defect in its replicative cycle (Locarini et al, 1981). However, adapted strains of HAV have been obtained by multiple passages of virus through cell culture (Provost and Hillman, 1979). One of these serially passaged HAV strains (HM-175), produced a lytic response in two persistently infected cell lines: FRHK-4,and BSC-1 (Cromean et al, 1986). Although such strains don't reflect accurately the responses of wild type HAV, they help simplify the development of methods for the concentration and detection of HAV from environmental samples of water and shellfish as well as for vaccine production and other applied studies on HAV.

A.1.1. Poliovirus-1

Poliovirus is a member of the picornavirus family. The particles are small (28 nm) in diameter and nonenveloped. They contain a single strained RNA genome having a positive polarity and have a molecular weight of 2.5×10^6. The virus is acid stable down to pH 3.0 and has a buoyant density in cesium chloride of about 1.34 g/ml.

Poliovirus has a very restricted host range. Most strains can only be grown on primary or continuous cell line cultures derived from a variety of human or monkey tissues.

There are three antigenic types of poliovirus 1, 2, and 3. They are transmitted via the fecal-oral route through direct or indirect human contact as well as through water and food contamination.

Poliovirus causes poliomyelitis, which occurs worldwide usually in children. Poliomyelitis is caused by anyone of the three serotypes of poliovirus, and the disease occurs generally one of two forms. In the abortive form, poliomyelitis is a minor illness with rapid recovery without paralysis (the nonparalytic form), it is also called aseptic meningitis. The other form causes paralytic meningitis. In this form spinal or meningeal symptoms develop, often accompanied by fever, malaise, nausea or vomiting. Pain in the spine, trunk, or limbs generally occurs.

Due to the success of polio vaccine and improvement in sanitation, there are currently few outbreaks of poliomyelitis in the U.S.A. and other developed countries.

The widespread use of polio vaccine has lead to presence of high concentration of poliovirus in almost all sewage contaminated water (post vaccinal fecal excretion).

Poliovirus is one of the easiest viruses to grow and detect in cell culture. These two characteristics make poliovirus a useful model indicator of virus associated fecal contamination in the environment.

A.2. MS2 Bacteriophage

Bacteriophages are associated with almost all bacterial genera. MS2 phage is host specific to male (F+) or pili producing strains of E. coli.

MS2 is a RNA phage with a particle weight of about 4 x 10^6. It is composed of 180 molecules of a single coat protein forming an icosohedral shell. The shell encloses a single molecule of RNA of 3500 to 4700 nucleotides that are plus sense and fully competent to cause infection and production of virus progeny. The viruses replicate quickly, causing lysis of the host cell in 30 to 40 min and yield a very high level of progeny particles, approximately 10,000 per cell.

A. 3. Bacteria

E. coli and *S. fecaelis* are generally nonpathogenic bacteria that are part of the natural flora of the human intestinal tract. They are excreted in large numbers in human sewage and therefore are adequate indicators of fecal contamination by pathogenic bacteria.

A.3.1. *Escherichia coli*

The concept of using *E. coli* as an indicator was introduced in 1885. The coliform group is defined as bacteria that are aerobic or facultatively anaerobic, Gram-negative, nonspore forming, rod-shaped, and which ferment lactose with acid and gas production within 48 hr. at 35°C.

A.2.2. *Streptococcus fecaelis*

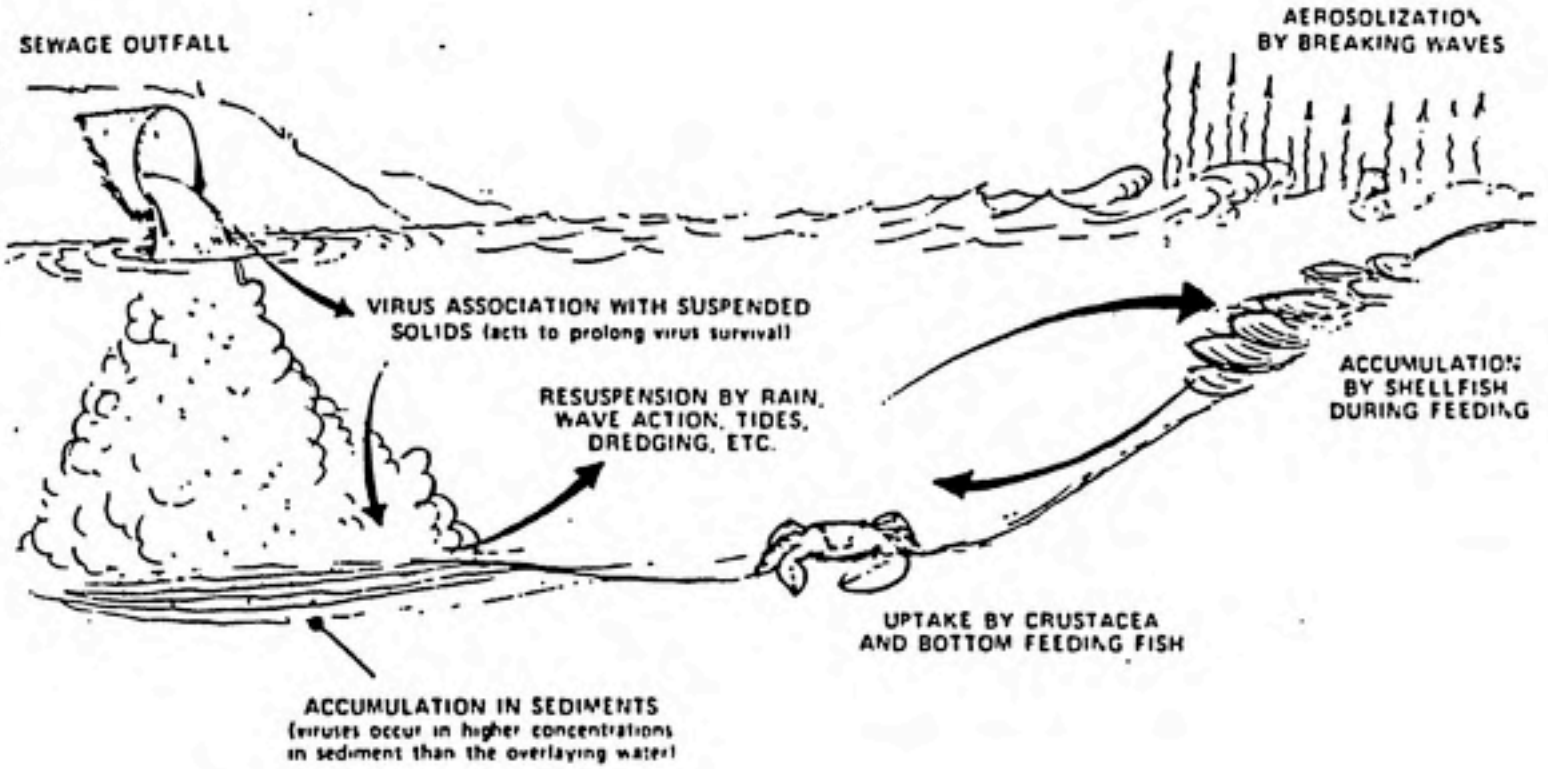
Classification of *S. fecaelis*, on of the fecal streptococci (Lance field group), is dependent on the bacteria's ability to grow in 6.5% NaCl broth in 0.1% methylene blue milk, at pH 9.6, at 10 to 45°C, and to survive at 60°C for 30 min.

B. Enteric Bacteria and Viruses in the Aquatic Environment

B.1. Sources of Contamination

The principle source of enteric viruses in the aquatic environment is the worldwide disposal of sewage and other forms of fecal excreta to surface waters (Figure III.1). Figure III.2. illustrates other modes of viral disease

Figure 1. Model Depicting Fate of Viruses in Coastal Systems



Adopted from Melnick and Gerba (1980)

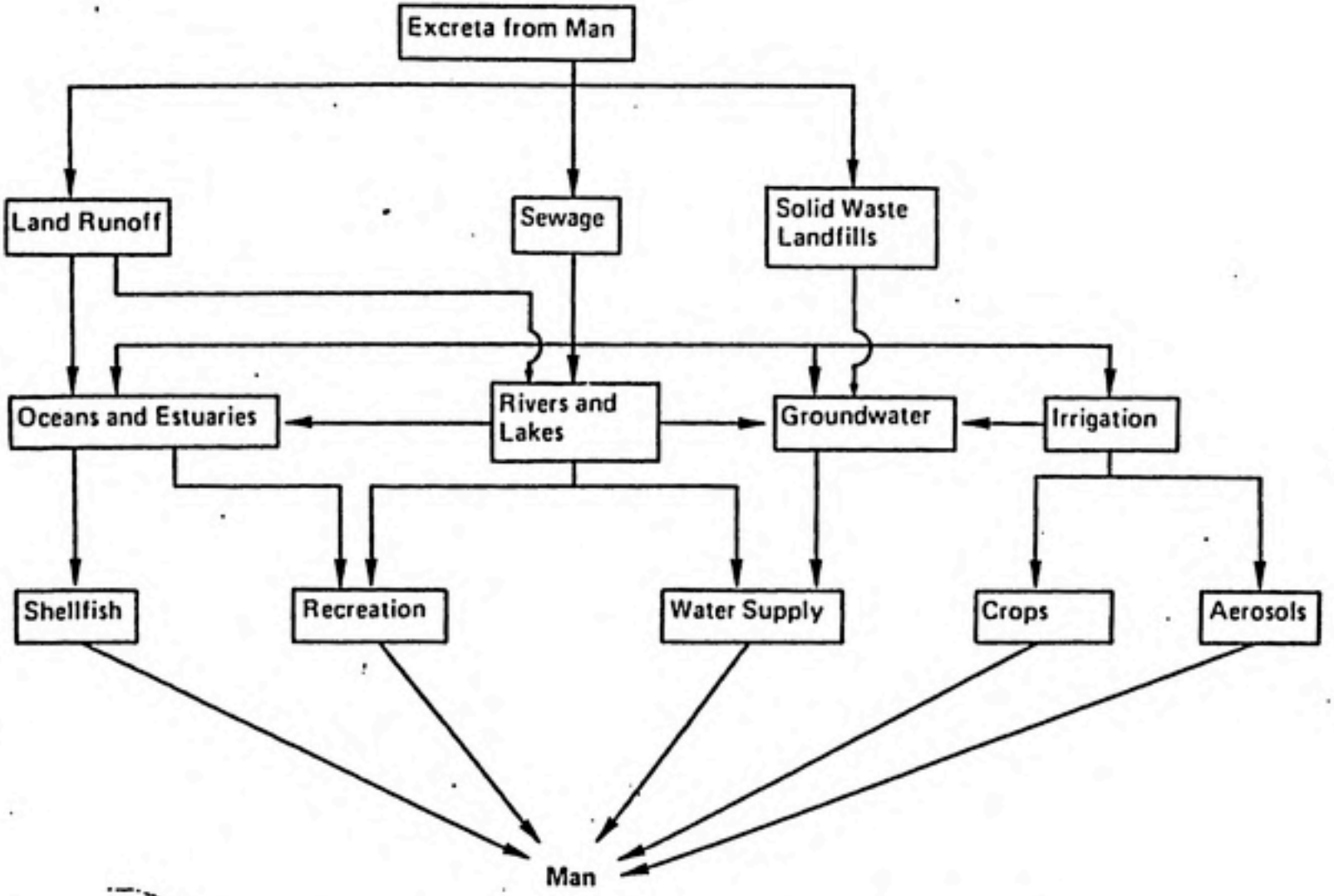


Fig. 2. Routes of enteric virus transmission [*Melnick* et al., 1978].

transmission. Enteric viruses are excreted in the feces of infected individuals at levels as high as 10^{12} particles per gram (Flewett and Woode, 1978). Different wastewater treatment processes remove viruses to varying degrees. The efficiencies of each method are summarized in Table III.1. A combination of these processes can be very effective depending on the extent of treatment used.

The conventional waste water treatment processes comprise primary sedimentation, secondary or biological treatment, and disinfection. Tertiary treatment is also practiced in some cases.

Primary treatment removes the suspended solids from sewage by sedimentation. This step removes up to 50% of bacteria and viruses from sewage; removal is by adsorption of microorganisms to solids with subsequent settling (Gerba and Goyal, 1982).

Secondary or biological sewage treatment utilizes microbial degradation of soluble organics and solids. Trickling filters, activated sludge, and oxidative ponds remove up to 90% of the microbes by inactivation or adsorption and physical removal in sludge (Gerba, 1981).

If treatment is continued through the tertiary stage a high percentage of removal can be expected. This process is facilitated by a coagulant such as aluminum or lime, followed by sedimentation, filtration, activated carbon and disinfection. Disinfection with adequate concentration,

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)

contact time, and quality of influent can succeed in up to 99.99% virus reduction (Gerba, 1981).

Tertiary treatment is rarely used and even conventional primary and secondary treatment followed by disinfection is not always employed. Approximately 5% of the U.S. population still discharge untreated sewage directly via ocean outfalls (Rao & Melnick, 1986). Bitton (1980) estimates that four billion gallons of sewage with only secondary treatment are discharged per day into coastal U.S. waters. These discharges contain an estimated 380 virus PFU/gal in the U.S. (Metcalf, 1987). Although the concentrations of pathogens are supposed to be reduced by dilution and natural degradation, enteric bacteria and viruses have been detected greater than 8 miles from discharge sites (Metcalf, 1974; Dahling and Safferman, 1979).

Sewage sludge disposal also contributes to the presence of enteric pathogens in the marine environment. Bacteria and viruses are concentrated into the sludge during treatment but are not rendered inactive (Goyal, 1984).

Although sewage effluent may meet coliform, suspended solids, BOD, and other quality control standards, treatment processes are ineffective in removing all viral contaminants. It is primarily from these sources, (discharged untreated, 1^{o}, 2^{o} sewage, and sludge); that the

public health risk of shellfish-associated viral disease arises.

B.2. Contamination of Shellfish

Pollution of estuaries and other shellfish habitats by enteric pathogens leads to the contamination of bivalve mollusks. Enteric viruses have been isolated from many edible bivalves including clams, oysters, and mussels (Gerba and Goyal, 1978). Viral contaminants have been found in shellfish harvested from both closed and approved areas (Metcalf and Stiles, 1968b; Goyal et al, 1979; Vaughn et al; 1979b; Ellender et al, 1980; Wait et al, 1983). In many of the above cases no viruses were detected in the overlaying waters.

Shellfish harbor viruses in their tissues and passively transmit them to humans who ingest raw or inadequately cooked shellfish. When survival after typical cooking methods was examined, somewhat better inactivation was observed with steaming and stewing than baking or frying (Di Girolamo, 1970), but overall virus inactivation was not appreciable. After 8 min of stewing 10% of the initial polio remained; 7% remained after 30 min of steaming, while 13% remained after 10 min of frying and 20 min of baking at 121^{o}C. Mazanti (1987) showed that even under pasteurization conditions HAV was not inactivated to an acceptable level. Since it is unlikely that consumers will pasteurize their

shellfish, it is necessary to eliminate the pathogenic microbes before they reach market.

The U.S. has tried to achieve a pathogen free shellfish market by establishing a coliform standard designed to indicate sewage and fecal contamination. The National Shellfish Sanitation Program (NSSP) established these national standards over 40 yr ago. They are based on total and fecal coliform bacteria levels enumerated by the most probable number method. The NSSP standards are summarized in Table III.2. Many shellfish areas have been closed due to failure to meet these standards.

Coliform testing is an inexpensive, relatively easy, and often reliable method of indicating fecal contamination. Enforcement of NSSP standards has successfully limited the number of bacterial disease outbreaks. However, the ability of coliforms to indicate viral contamination of shellfish and harvesting waters has been questioned due to enteric virus isolation from shellfish and overlaying waters in approved areas (Gerba and Goyal, 1978; Morris and Waite, 1981; Larkin and Hunt, 1982; Fugate et al, 1975; Goyal et al, 1979; Vaughn et al, 1980; Portnoy et al, 1975).

The virological quality of shellfish and overlying water is not adequately indicated or controlled by current standards. Other indicators such as poliovirus and other enteroviruses, enterococci (S. faecalis and S. faecium), and

TABLE III.2. NATIONAL SHELLFISH SANITATION PROGRAM STANDARDS

Sample	Indicator (coliform)	Median Conc	Upper 90% Limit
Water (per 100 ml)	Total	< 70	230
	Fecal	< 14	43
Shellfish Meat (per 100 gm)	Fecal	< 230	none

bacteriophages, have been considered but have proven unreliable (Morris and Waite, 1981). It seems that currently, the only valid determination of contamination is direct detection of the specific pathogen of interest. Unfortunately, many of the viral pathogens of interest are difficult or impossible to detect and quantify and for those that can be detected, the methods are unreliable, technically difficult, expensive, and slow to yield results.

B.3. Factors Influencing Survival and Persistence of Enteric Bacteria and Viruses in the Aquatic Environment

The fate of bacteria and viruses in the environment is controlled by physical, chemical, and biological factors. The factors of interest are listed in Table III.3. Rhodes and Kator (1988) showed that enteric microbe survival potential was a function of interacting biological and physical factors.

Temperature, a physical factor, plays the largest role in microbial inactivation. Using membrane dialysis chambers in situ, O'Brien and Newmann (1977) showed the rate of virus inactivation was exponential and affected primarily by water temperature. Additionally, temperature predisposes enteric microorganisms to biological actions such as predation and enzyme production (Bitton, 1980). Rhodes and Kator (1988)

TABLE III.3.

FACTORS AFFECTING ENTERIC BACTERIA AND VIRUS PERSISTENCE IN THE AQUATIC ENVIRONMENT

PHYSICAL

Temperature
Sunlight (ultraviolet light)
Dilution Effects
Adsorption to Particulates and Sediments
Sedimentation
Pollution Presence
Aggregation

CHEMICAL

pH
Salinity
Presence of Cations
Presence of Heavy Metals
Presence of Dissolved Organics

BIOLOGICAL

Virus Type
Predation
Enzymatic Degradation
Shellfish Accumulation

showed that microbial dieoff was inversely related to water temperature.

Numerous studies have demonstrated that adsorption to sediments can protect bacteria and viruses and increase their persistence in the aquatic environment (Gerba and McLeod, 1976; Kapuscinski and Mitchell, 1980; LaBelle and Gerba, 1980). Adsorption to particulates and sediments shields microorganisms from potentially harmful ultraviolet light and facilitates settling out. Greater than 99% of enteric viruses rapidly adsorb to estuarine sediment (LaBelle and Gerba, 1980; Bitton, 1980), thereby causing pathogens to concentrate in the bottom sediments. The viruses can later be resuspended into the water by turbulence. The concentrated microorganisms then have an enormous infection potential, since viruses in sediment fully retain their ability to initiate infection (Berg, 1983; Bitton, 1980; LaBelle and Gerba, 1980).

An important chemical factor affecting microbial inactivation is heavy metals. The metals compete for adsorption sites on particulates and dissolved organics thereby limiting the protection these agents afford enteric microbes.

Biological factors also contribute to microbial persistence. Bacterial cells, living and dead, act as a haven by providing adsorption sites for viruses (Mitchell, 1971), while predation by marine bacteria, protozoa, and

bacteriophages can reduce the numbers of enteric bacteria and viruses. Shuval et al (1971) isolated a marine bacteria capable of diminishing poliovirus-1 1000-fold in seven days. This antiviral action is closely associated with the marine bacteria's metabolic activity (Bitton, 1980; Shuval, 1971; Mitchell, 1971). Cliver and Hermann (72) found that some enteric viruses are susceptible to proteolytic enzymes. Ward et al (1986) confirmed this; their experiments showed proteolytic bacterial enzymes inactivated echovirus in fresh water by cleaving the protein capsid, exposing the viral RNA to nuclease digestion.

The type of microbe plays a large role in survival. Enteric viruses generally survive longer in seawater than do coliform bacteria (Melnick and Gerba, 1980). Further studies with hepatitis A have shown that it is capable of surviving longer than other enteric viruses (Bosch and Shields, 1987).

C. Epidemiology

Contaminated shellfish pose a public health risk due to the accumulation and persistence of pathogenic microbes in their tissues. Transmission of pathogens by shellfish was demonstrated before the turn of the century (Metcalf, 1987). Large scale shellfish-associated disease outbreaks, such as the New York state outbreak reported by Morse et al (1986), continue to be reported. This outbreak included 1000 cases

of illness and at least 10 cases of hepatitis A or infectious hepatitis.

Between 1900 and 1984, 11600 cases of shellfishborne disease have been documented in the U.S.. Although there are more than 100 known enteric viruses, only a few have been shown to be transimitted by shellfish: hepatitis A, non-A non-B hepatitis, Norwalk, Snow Mountain agent, astrovirus, caliciviruses, and small round viruses (Gerba, 1988).

During the twenty year span from 1961-1982, contaminated shellfish were implicated in over 1000 cases of HAV (Richards, 1985). The number of cases of infectious hepatitis is relatively small compared with the total hepatitis incidence (nearly 30,000/yr); it is none the less one of the most serious viral diseases transmitted by ingestion of contaminated shellfish. Therefore, it is important that public health measures be taken to control this mode of transmission (Mitchell et al, 1966). Mele et al (1989) reports that when comprehensive control measures were introduced in Livorno, Italy, the annual incidence of hepatitis A showed a 10 fold decrease. Two thirds of those cases were directly attributed to raw shellfish consumption.

Table III.4 from the FDA (1990) lists the sources and locations of shellfish-associated HAV epidemics.

In addition to HAV, other enteric viruses have been implicated in nonbacterial gastroenteritis resulting from

Table 4.

III.4. Shellfish-associated HAV Outbreaks

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
1983	01	several species	[illegible]
1983	04	clams	New York
1985	01	clams	New York
1988	51	oysters	Florida

Compiled from FDA Sanitation Program Technical Report. Shellfish Borne Disease Outbreaks. Dr. S. Rippy. February, 1989.

shellfish consumption. Norwalk and rotavirus have been confirmed as the causative agent in many gastroenteritis outbreaks in the U.S. (Richards, 1985). It is also unclear in some cases whether or not viruses were the cause of disease outbreaks, but viruses were the likely etiologic agent in most instances (Richards, 1985).

It is also apparent that the high number of cases in areas such as New York is attributable to increased awareness and better reporting practices concerning shellfishborne disease. It is thus likely that the actual number of shellfish related disease cases nationwide is grossly underestimated, due to lax surveillance and reporting. This is further compounded by the fact that mild cases may go untreated or not recognized as shellfish related.

Many factors have led to current occurrences of enteric disease transmission by shellfish. Current bacteriological standards are inadequate for determining viral contamination in shellfish and overlying waters. Illegal poaching has certainly led to some untraceable cases.
Finally improperly classified growing and harvesting waters has resulted in open but contaminated areas.

Because many outbreaks are due to shellfish harvested from 'approved areas' as well as from shellfish that have purged themselves to levels that met current bacterial standards; it is of the utmost importance to develop

adequate standards and to perfect techniques to ensure pathogen free shellfish. Other approaches to prevent shellfishborne disease outbreaks are listed in Table III.5.

D. Clams

D.1. Introduction

Mercenaria mercenaria or the hard shell clam is a member of the Pelecypoda, a class within the phylum Mollusca. This type of shellfish is set apart from other mollusks by their use of a filter feeding process to obtain food materials. Besides the hard shelled clam, other commercially important bivalves in the U.S. include the Eastern oyster, Crassostrea virginica, the Pacific oyster, Crassostrea gigas, the Olympia oyster, Ostrea lurida, the softshell clam, Mya arenaria, the Manila clam, Tapes japonica, and the blue mussel, Mytilus edulis (Werner, 1983). These clam, oyster, and mussel bivalve forms represent the shellfish most often involved in transmission of virus-caused diseases in the U.S.A. (Metcalf, 1980).

Clams are found in estuaries a few inches below the water-sediment interface. The soft fleshy body is enclosed within two hinged shells called valves. Beneath the calcified valves a mantle structure encloses the body. The clam has fused mantle lobes with siphon structures. The siphons inhale up to 19 l water/hr/oz of tissue (Metcalf, 1980).

Table III.5. APPROACHES TO PREVENT SHELLFISHBORNE DISEASE OUTBREAKS (Guzewich and Morse (1986))

1. Improve shellfish disease surveillance and reporting.
2. Embargo shellfish sold by shippers involved disease outbreaks.
3. Adopt strict state and federal laws to control the sanitary quality of shellfish.
4. Encourage greater participation in the Interstate Shellfish Sanitation Conference.
5. Provide adequate numbers of enforcement officers.
6. Develop microbiological growing water or product standards that assure viral safety.
7. Properly classify shellfish-harvesting areas.
8. Mandate a manifest-type tagging system.
9. Strictly enforce wholesale and retail shellfish-tagging requirements.
10. Require depuration of all shellfish sold.
11. Advise the public against consumption of raw or partially cooked shellfish.
12. Ban the sale of raw shellfish.

Virus pollution of shellfish is often related to association of a virus with a solid. Clams are more likely to take up particles settled onto the uppermost layer of the bottom sediments (Metcalf, 1978; Landry, et al, 1983). Clays, part of the sediment, are among the most important inorganic substances with which viruses associate (Metcalf, 1980).

D.2. Filter feeding

Filter feeding, outlined in figure III.5, is initiated during the pumping of water through the gill slits. Particulate matter is removed by mucus sheets secreted by the gills, with transport to the mouthparts facilitated by ciliary action. The mouth accepts particulates as food based on weight, size, and shape. Clams feed on suspended algae, nanoplankton, and bacteria. Particles rejected as food sources are eliminated from the mantle cavity in the form of pseudofeces (Metcalf, 1980).

Bivalve feeding is influenced by several factors. Water temperature, salinity, pH, turbidity and dissolved oxygen are among the most important factors influencing feeding activity (Metcalf, 1980).

Figure III.5. FILTER FEEDING

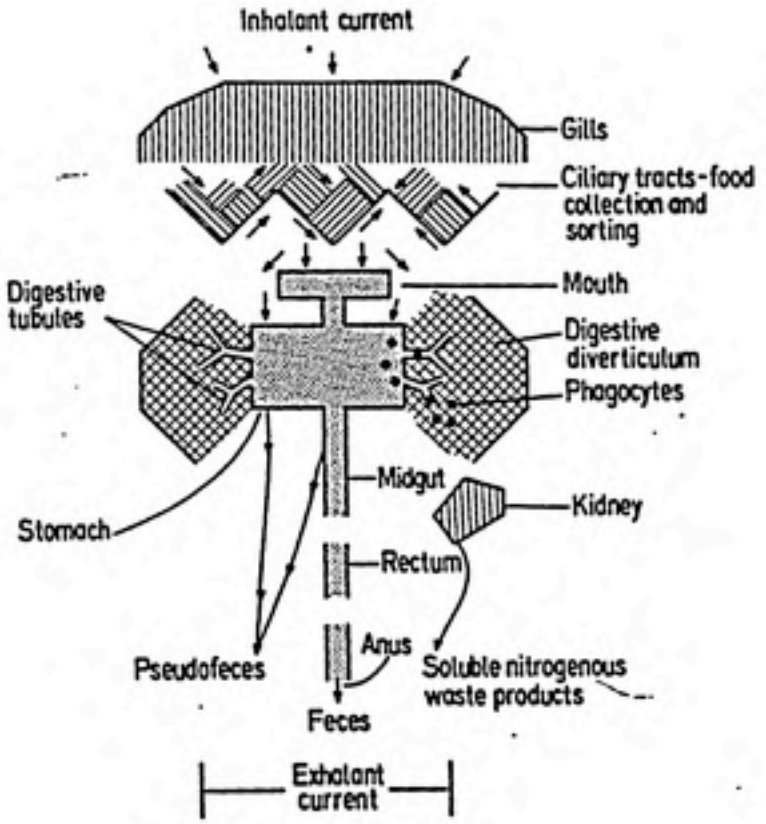


Figure 15.3 A schematic representation of shellfish structures involved in feeding, digestion, and waste elimination processes. Particles swept onto gill structure surfaces are collected, sorted and transported, with the help of mucus and ciliary action, to the mouth region. Rejected material is eliminated in the pseudofeces. Particles accepted as food are passed into the stomach where the digestive processes begin. Digestion may also be carried on, through phagocyte intervention, in digestive diverticula tubules or tissues. Solid wastes are eliminated in the feces, and soluble nitrogenous wastes are excreted through nephridial structures.

Melnick and Gerba (1981)

E. Accumulation of Enteric Bacteria and Viruses by Shellfish

E.1. Mechanism of Uptake

Bacteria and viruses enter the shellfish cavity with the currents of water pumped during feeding. A microbe can either be taken up as a free suspended agent or adsorbed to a suspended solid. Two possible mechanisms exist to explain microbial accumulation in the bivalve's tissue.

Di Girolamo et al (1977) investigated the mechanism of viral uptake by the shellfish mucous. He found that "viruses become attached to secreted mucus and are ingested by the shellfish during feeding." This study indicated that the probable mechanism is one of ionic binding to the mucopolysaccharide fraction of the mucus layer. However, the influence of salinity and pH on uptake indicates that ionic bonding is not the only means of attachment.

A second mechanism of uptake involves the particle to which the microbe is adsorbed. Viruses may be retained on the cilia of the gill surface. Differences in the surface charges of viruses may cause them to accumulate at different rates. According to this theory, viruses with the strongest positive charge should bind most efficiently to the shellfish mucus (Duff, 1967). They will then be transported along the gills to the mouth and enter the digestive system (Metcalf, 1987).

E.2. Concentration of Bacteria and Virus

Although there is no direct correlation of the level of viruses in shellfish to the level in the overlying water (Ellender et al, 1980, Gerba et al, 1979), this comparison is often used to express the level or 'concentration' of viruses in the animals tissues. The extent to which the shellfish is contaminated can be expressed by this terminology (Meinhold, 1982).

Investigators have reported that shellfish can 'concentrate' microbes in their tissues many times above the level in the overlying water (Duff, 1967; Hoff and Becker, 1969; Di Dirolamo et al, 1975). Shellfish can accumulate bacteria 10-30 times higher than the surrounding water (Mitchell, 1966). Virus concentrations up to 60 times greater than overlaying water have been reported (Metcalf, 1987; Mitchell,1966). There appears to be a threshold level of microbial concentration. The most logical explanation is that after a certain microbial titer is reached, elimination balances accumulation. Thus, it appears that this is a dynamic process rather than simple filtration (Mitchell, 1966). In some cases, viral accumulation did not exceeded the exposure level and was several orders of magnitude less (Canzonier, 1971; Hedstrom and Lycke, 1964). It has been suggested that the low level of accumulation observed in these studies was due to the suboptimal conditions for metabolic activity (Hamblet et al, 1969).

The highest concentration of viruses is found in the digestive tract, in the stomach-intestine, and diverticula tissue. This is followed by the mantle fluid, mouth-esophagus, and gills (Metcalf, 1987; Canzonier, 1971). Liu (1966) found that over 90% of poliovirus accumulated by the hard shell clam was concentrated in the gastrointestinal tract. Early reports by Metcalf and Stiles (1965) studying the Eastern oyster, Crassostrea virginica, suggested that the mantle fluid contained the highest concentration of virus with the PFU values equalling that of the seawater. However Meinhold (1982) found that in the Eastern oyster polio 1 was found in the highest concentrations in the digestive tract tissue. In the soft shell clam, Mya arenaria, polio was concentrated in the siphons and digestive diverticula (Metcalf et al, 1979). It is therefore logical to postulate that the area of maximal concentration of viruses in tissues and organs may be specific to the types of shellfish.

There appears to be a threshold level below which viruses are not accumulated. Landry (1982) noted viral accumulation only when water column concentrations exceeded 0.10 PFU/ml. At concentrations below this level, viruses were seldom detected in clams or oysters. Evidence indicated that the lack of accumulation was not due to inefficient extraction or detection methods. This research

presented evidence that an uptake-elimination equilibrium was present at 0.10 PFU/ml.

Enteric viral replication in shellfish has never been demonstrated (Metcalf, 1980; Chang et al, 1985). Metcalf's work showed each virion detected was taken up from the surrounding seawater and did not originate within the shellfish (Metcalf, 1987).

E.3. Factors Affecting Accumulation

The extent of uptake and accumulation of enteric microbes by edible shellfish in their natural habitat and in the laboratory is affected by various factors (see Table III.6). The level of contamination in the water column affects the accumulation of bacteria and viruses; specifically the initial concentration of microbes in the water plays a major role in concentrating these organisms in shellfish tissue (Metcalf and Stiles, 1965). A low concentration of virus will be adsorbed and accepted into the shellfish tissue but will reach equilibrium with the elimination process. Shellfish can harbor a low but consistent concentration of indigenous virus (Landry et al, 1982). However, earlier studies by Canzonier (1971) contradict this observation. Examining the uptake of Coliphage S-13 by Mercenaria mercenaria using low levels of viruses, he found the virus level was 2 to 1000 times the level of virus in the surrounding water after 24 hours of exposure. Landry et al (1982) found, as expected, that

TABLE III.6. FACTORS THAT AFFECT ACCUMULATION AND CONCENTRATION OF ENTERIC BACTERIA AND VIRUSES

POLLUTION CONCENTRATION LEVEL

EXPOSURE PERIOD

VIRUS SURFACE PROPERTIES

ASSOCIATION WITH PARTICULATES AND SUSPENDED SOLIDS

EXCESSIVE TURBIDITY

TEMPERATURE

SHELLFISH INTERSPECIES DIFFERENCES

DISSOLVED OXYGEN

FOOD AVAILABILITY

METABOLIC WASTE DILUTION

pH

SALINITY

uptake increased with increasing concentrations of viruses. Research by Bedford et al (1978) indicated that a maximum concentration level is reached by shellfish. Their work with Rock Oysters indicated that saturation is achieved at 4 X 10^{10} reovirus particles per oyster.

The virus surface properties also play a role in virus concentration. Duff (1967) found that the attachment of viruses to the mucus of the shellfish gills was due to ionic bonding of the virus to the negatively charged sulfate radicals of the mucus. The strongest positively charged particles should bind most efficiently. Investigators have implied that oysters have a large but finite number of adsorption sites (Bedford et al, 1982). Surface characteristics also affect the binding of viruses to particulates and sediments, thereby affecting the likelihood of virus transfer into the shellfish system (Canzonier, 1971).

Virus association with solids present in water has been shown to increase the extent of viral uptake by shellfish (Landry, 1982; Canzonier, 1971; Hamblet, 1969). Hoff and Becker (1969) reported that cell-associated microbes were accumulated 40-60 times greater in Pacific oysters and Manila clams than was free virus. Metcalf et al (1979) similarly showed that feces- and solids associated poliovirus accumulated more efficiently than free virus. Two explanations have been proposed for this increased

accumulation. First, the effect is the direct result of the virus adsorbed particles being accepted as food, and second, that the particulate matter stimulates the pumping and feeding process with the uptake of free or solid-associated viruses as an indirect result (Werner,1983).

The presence of excess turbidity or suspended particulate matter can inhibit microbial uptake. Hamblet (1969) demonstrated that shellfish subjected to low turbidity water (16-24 ppm) for 24 hours accumulated approximately three times as much virus as shellfish subjected to high turbidity (54-77 ppm). The excessive turbidity clogs the gills and palps, thereby interfering with pumping, feeding, and filtration (Lovelace, personal communication; Hamblet, 1969).

Feeding and microbial accumulation normally increase with temperature within the physiological tolerance of shellfish. Meinhold (1982) found that the maximum uptake of poliovirus by the Eastern oyster occurred in 5 hours at 6°C, 2-3 hours at 17°C, and 1-3 hours at 28°C. Shellfish have not been shown to accumulate detectable virus at all in cold water. Metcalf and Stiles (1968) showed oysters do not accumulate enteroviruses below 7°C. These observations are supported by earlier research on the physiological activities of oysters at varying temperatures. The pumping rate of oysters increases steadily up to 30°C and ciliary

action decreases at 4-6°C with no feeding below 4°C (Loosanoff and Nomejko, 1958; Nelson, 1923).

Interspecies differences among shellfish have been associated with varying bioaccumulation rates. Olympia oysters accumulated 86% of the poliovirus in seawater in 12 hours, while Pacific oysters required 48 hours to attain an equal accumulation level (Di Girolamo et al, 1975).

In the laboratory the use of a static system as opposed to a flow through system dramatically affects the level of accumulation. Hamblet et al (1969) notes that differences in experimental observations of accumulation levels (Metcalf and Stiles, 1965; Hedstrom and Lycke, 1964) relate principally to the design of the experimental seawater supply system, i.e., static versus flow-through systems. The use of continuously flowing seawater simulates the natural shellfishes environment and is therefore more conducive to feeding. Static systems do not provide optimal conditions such as adequate dissolved oxygen, food availability, and metabolic waste dilution and therefore inhibit natural feeding and virus accumulation (Hamblet et al, 1969).

Increases in ionic concentrations (salinity) or alteration of pH weakens the virus bond to the mucus of the shellfish gill. Di Girolamo et al observed that decreasing salinity from 28 to 14 ppt caused a 10% increase in viral accumulation. Virus adsorption was therefore affected by

cation concentration. This may be due to competition between cations and viral capsid coats for mucus anions (Di Girolamo et al, 1975).

F. Elimination and Depuration of Enteric Bacteria and Viruses by Shellfish

F.1. Mechanisms of Elimination and Reduction

Shellfish have the ability to get rid of accumulated bacteria and viruses when placed in noncontaminated water. The shellfish replace the microbes with a food source. The bacteria and viruses are then eliminated in feces and pseudofeces in the normal digestive and excretion process. Elimination by this method is closely related to the degree of physiological vigor of the bivalve (Metcalf, 1987). Virus elimination through the intestinal tract, the usual method, is due to the virus being firmly enclosed in the fecal bolus consisting of waste products, undigested materials, and mucus (Perkins, 1980; Metcalf, 1987). A fully infectious virion is eliminated unaffected by the shellfish's digestive processes (Metcalf, 1987).

A second method of microbial elimination is through physical inactivation (Canzonier, 1971; Perkins, 1980). Canzonier (1971) suggests that this reduction is a result of the influence of temperature and other physical factors prevailing during depuration. Canzonier (1971) further noted in cases with low levels of contamination that the

virus persisted for periods commensurate with virus inactivation in seawater at the temperature of his experiments. In this case the stability of the virus appears to be the dominant factor in virus persistence (Canzonier, 1971). "Exceptions to this process occur when viruses are phagocytized and the phagocytes pass through the cell membranes" (Metcalf, 1978). Some removal of the microbes is therefore due to the phagocytic action of the hemolymph. The phagocytes act as a means of intracellular digestion or to protect the cells from foreign substances. Phagocytes eliminate bacteria through enzymatic digestion or exportation to the surrounding water through the epithethial borders. The extruded phagocytes are carried away in the mucus or feces by the water stream set up by the shellfish's natural pumping action (Hartmond and Timoney, 1979).

In contrast to bacteria, phagocytes may transfer viruses to tissue far removed from the normal elimination processes, where they can remain for long periods of time (Canzonier, 1971). Cook and Ellender (1986) offer the explanation that free viruses become entrapped in mucus and are sequestered in the digestive gland and hemolymph. In this tissue, they are refractory to the mechanisms responsible for elimination (Canzonier, 1971). These particles are not dislodged easily and may remain, under ideal conditions for days to weeks (Canzonier, 1971). Fries and Tripp (1970) have demonstrated that shellfish leucocytes

can phagocytize a 60 nm algae DNA virus within two hours of exposure. Others studies have repeatedly confirmed that viruses can be found in the hemolymph (Liu et al, 1966; Di Girolamo, 1975; Metcalf et al, 1979; Metcalf et al, 1980).

F.2. Factors Affecting Elimination Rates

The rate of microbial elimination is dependent on the factors listed in Table III.7. These factors affect the metabolic activity of the shellfish such as the rate of pumping, feeding, and elimination.

A static depuration plant design has resulted in relatively inefficient virus elimination (Hedstrom and Lycke, 1964). A gradual linear decrease of virus was seen but some viruses were detected even after a 100 hours of elimination. Mitchell et al (1966) found that a static system limits the essential factors for shellfish activities such as dissolved oxygen, food, and dilution of metabolic waste. A flow-through system more closely simulates the natural environment. A more suitable environment for natural activities is thus provided and a more rapid and efficient viral elimination can be expected and is observed (Di Girolamo et al, 1975; Hamblet, 1969; Mitchell et al, 1966). Di Girolamo et al, (1975) compared poliovirus Lsc-2ab depuration in Western oysters using both a stationary and a free-flow seawater system. In the static system 15% of the virus was recovered after 120 hours. In contrast,

TABLE III.7. FACTORS AFFECTING ELIMINATION RATES

DEPURATION PLANT DESIGN

SHELLFISH CHARACTERISTICS

LEVEL OF CONTAMINATION

TYPE OF CONTAMINATION

VIRUS SURFACE CHARGE

DISSOLVED OXYGEN CONTENT

pH

TURBIDITY

ASSOCIATION WITH PARTICLES

TEMPERATURE

SALINITY

FOOD AVAILABILITY

the free-flow system had a reduction of 99% after only 72 hours. Other researchers have also observed rapid elimination of virus using flow-through systems. Mitchell et al (1966) demonstrated that poliovirus was reduced greater than 99.9% within 24 hours in Crassostrea virginica. Liu et al (1967b) found polio was not detectable after 3-4 days of elimination by Mercenaria mercenaria. Metcalf et al (1979) reported that feces-associated polio was reduced by 98-100% after 6 days of elimination by M. arenaria using a free flow system.

The biological characteristics of the shellfish are important in the design of the depuration plant and they also affect elimination. Each type of shellfish has its own unique limiting conditions. These parameters define the most suitable conditions of effective purification. They include temperature, salinity, dissolved oxygen, number of organisms per tank, water volume per unit mass of shellfish, and the depth of water over the shellfish (Metcalf, 1987). This can be observed by varying the time necessary for different types of shellfish to depurate to acceptable levels under the same conditions. According to Hoff and Becker (1969), the Eastern oyster can eliminate polio to undetectable levels in 24 hours while 48 hours is necessary for the soft shelled clam.

Additionally, individual activities of shellfish influence the overall elimination rate. Seraichekas et al

(1968) noted "there is no doubt that the majority of shellfish are capable of cleansing themselves when they are subjected to an ideal and clean environment. On the other hand a small number appear not to be functioning well. Thus, a few shellfish still harbor virus after 48 to 72 hr of depuration." Factors such as the age, sex, and size of the shellfish as well as physical injury (e.g. damage to the shell) can influence their activity and thus their elimination rate.

The level and type of contamination appear to play a major role in the efficiency of elimination. Little information is available on comparative viral and bacterial depuration. The relative patterns and rates of elimination of three organisms were studied by Power and Collins (1989). The logarithms of reduction for polio, E. coli, and coliphage were 1.86, 2.9. and 2.16, respectively, within 52 hr of depuration. The differences in the rates of depuration under ideal conditions of poliovirus, E. coli, and a 22-nm icosohedral coliphage suggest that they are eliminated from mussels by different mechanisms. Thus the type of contaminant plays a major role in the efficiency of depuration.

The level of contaminants in the water is generally related to the accumulation level of microorganisms in shellfish. Thus, when the pollutant concentration decreases, so does the accumulated concentration in the

shellfish (Hedstrom and Lycke, 1964; Metcalf and Stiles, 1965). The efficiency of depuration is heavily dependent on the initial contamination level. Less time is required for virus elimination from lightly contaminated shellfish (Liu et al, 1967b; Metcalf and Stiles, 1965; Janssen, 1973; Cook and Ellender, 1986; Metcalf et al, 1979; Perkins et al, 1980; Canzonier, 1971). Liu et al (1967b) found hard shelled clams, contaminated with 10^2 PFU of polio/g of meat, required 24 hr to eliminate to nondetectable levels, while clams contaminated with 10^3 PFU required 72 hr. No apparent difference in the rate of elimination was seen. Many explanations for this occurrence have been postulated. Metcalf et al (1979) stated that depuration effectiveness was dependent upon the number of viruses bioaccumulated and whether these viruses were solid-associated. The associated particles would be quicker to depurate since they were not sequestered in the tissue. Seraichekas et al (1968) proposed that the residual virus in some shellfish are the result of physiological inactivity due to the high contamination level reached. Canzonier (1971) and Mesquite (1988), had slightly contradictory results. They both found that at low initial titers, accumulated over an extended period of time, the retention of virus can be quite prolonged and independent of clam activity as indicated by bacterial elimination. This may mean that the rapid elimination of low titers is due to inactivation by physical

factors such as temperature. Thus the thermal stability of the virus may play a major role in virus persistence. Mesquite (1988) further found that exposure to high titers for a short time resulted in rapid reductions down to a certain level and that some of the bacteia were always retained.

Differences in virus surface charge may cause viruses to eliminate at different rates. Viruses with a strong positive charge may attach more effectively to the negatively charged sulfate radical of the shellfish mucus and therefore depurate more slowly (DiGirolamo et al, 1975; Duff, 1967). Poliovirus depuration occurs quickly with 80-99% removal in 48 hr (Hoff and Becker, 1969; Liu et al, 1967b; Metcalf et al, 1979; Davis, 1986). After the initial drop, the low levels of polio may persist as long as 6 days (Hoff and Becker, 1969). HAV depurates much slower than other viruses studied. Sobsey et al (1987) found Eastern oysters reduced HAV less than 90% under most test conditions even after 5 days.

Elimination of microbes is directly related to the degree of physiologic vigor shown by the bivalve. This vigor is in turn related to environmental factors such as temperature, salinity, pH, dissolved oxygen, turbidity and particulate concentration. Ideal conditions help to optimize normal pumping and feeding activities.

The temperature of the water determines the metabolic activity of the mollusk. In general, lower temperatures result in a lower rate of feeding and pumping and therefore lower elimination. The slower cleansing rate is thought to be a result of the stress on the shellfish (Cook and Ellender, 1986). There also appears to be a threshold temperature below which no depuration occurs. Cook and Ellender (1986) found that temperatures above 31°C do not have an adverse effect on the oysters ability to eliminate microorganisms. If the temperature falls below a threshold of 10°C, however, elimination is slowed. Metcalf and Stiles (1968) report the threshold is 7°C. Metcalf (1987) reported that in a North Atlantic estuary during the winter, oysters submerged in 1°C water showed virtually no depuration after 4 months. As long as the temperature stayed below 4.3°C, the shellfish were in a state of 'hibernation'. Within two weeks after the temperature rose above 7°C, there was no virus detectable. Further experiments demonstrate that depuration performance is a function of temperature. Liu *et al* (1967) found that at 18-20°C, enteric viruses were not detected after 48 hr, but at 5-6°C, 96 hr were required to reach a nondetectable level. Rowse and Fleet (1984) showed incomplete depuration of *Samonella charity* and *E. coli* from oysters below 17°C compared to the rapid elimination rate at 18-22 and 24-27°C. Meinhold (1982) showed polio depuration by Eastern oysters was greatest at 28°C. In another study,

Sobsey et al (1987) demonstrated the rate of polio elimination was more efficient at 23°C than at 12 or 17°C in Crassostrea virginica. This report further stated that HAV was not depurated extensively at any of the test temperatures.

Several studies have shown that depuration is inefficient at salinities other than that to which the shellfish are acclimated especially at reduced salinity. Dramatic changes in salinity appear to drastically affect depuration. Liu et al (1967b) showed a decrease in salinity of 50-60% stopped the depuration completely. Rowse and Fleet (1984) reported that low salinity (16-20 ppt) reduced pumping and elimination rates and resulted in some oyster death. Exposure to salinities as high as 32-47 ppt did not affect depuration or cause shellfish mortality. Sobsey et al (1987) reported that oysters depurate HAV more effectively at 28 ppt than 8 or 18 ppt. At 28 ppt less than 5% and 1% of the original HAV was detected after 2-3 days and 5 days, respectively.

Microbial concentrations in depuration water must be low enough to prevent recontamination of shellfish in order for elimination processes to be effective.

Turbidity, particle association, dissolved oxygen, and food availability do not appear appreciably to affect depuration as long as the concentrations do not interfere with normal metabolic functions. High turbidities can

impair or close gills thereby preventing depuration. As long as this is not the case, turbidity plays no role in depuration. Hamblet (1969) found polio depurated equally in low (8-21 ppm) and high (54-80 ppm turbidity waters). Meinhold (1982) reports that although associations with particles affects uptake of viruses it is not a factor in elimination. Hoff and Becker (1968) reported however that crude cell-associated virus persisted longer in Manilla clams and Olympia oysters than free, filtered virus. It should be noted that crude virus are similar to natural environmental forms of viruses. Dissolved oxygen appears to be a passive factor in elimination. As long as sufficient levels are present for shellfish metabolic functions then depuration rates are unaffected. However, Perkins (1980) states that appreciable drops occur in depuration rates when dissolved oxygen is below 8 mg per liter. Powers and Colins (1990) found that food availability affected depuration only when filtered seawater was used and that in that case food addition increased the elimination rate.

In summary, elimination is most effective in those environmental conditions to which the mollusk is already acclimatized, when physical factors are sufficient for normal physiological and metabolic functions, and when contamination levels are lower.

F.3. Commercial Depuration

In the U.S., shellfish intended for depuration or controlled purification can be harvested from waters meeting the requirements for restricted or approved areas. A substantial portion of shellfish are off limits due to the presence of excessive levels of fecal contamination. Moderately contaminated shellfish can be 'reclaimed', however, utilizing a purification method that renders the shellfish free of unacceptable levels of pathogens.

The natural process of self purification can be utilized commercially in two ways: relaying or depuration. Relaying involves transferring the shellfish from polluted (restricted) waters to approved waters (of acceptable microbial quality). The shellfish can then be reharvested from the approved beds after a minimum time of two weeks and sold for public consumption. Depuration plants place shellfish taken from restricted waters in tanks of disinfected, quality controlled, continuous flowing seawater. The seawater is used in a flow-through or recirculating fashion, purified by filtration, UV irradiation, ozonation, or chlorination. Chlorine and ozone are less desirable methods of water disinfection since the residual levels of disinfectant or disinfection by-products act to inhibit the shellfish physiological processes, thereby reducing the elimination rate. Of the two, ozone is

preferable because it is ephemeral in water and rapidly dissipates. Chlorination is now rarely used.

A degree of control can be exercised over depuration which can not be similarly exercised over relaying. Relaying is a slower elimination process, usually requiring 14 days, while depuration in the U.S. is accepted as complete after 2-3 days. In other countries shellfish may be depurated for up to a week.

After a specified time, the depurated shellfish are sampled and depuration effectiveness is tested by bacteriological analysis. Hard shell clams and Eastern oysters must have mean fecal coliform concentrations of less than 20 per 100 grams of meat and no more than 10 % of the samples can exceed 100 fecal coliforms per 100 grams. No single sample is allowed to 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).

Depuration is currently practiced only on a limited scale in the U.S. due to the high cost (Cook and Ellender, 1986). Depuration is widely practiced in Europe and Australia. However, there are only 19 plants in the U.S. depurating clams and oysters; primarily soft shelled clams. The growing importance of shellfish as a food source in the U.S. (Table III.8.) may soon make depuration a more

Table III.8. Important Edible Shellfish Species

Common Name	Scientific Name
Eastern Oyster	Crassostrea virginica
Pacific Oyster	Crassostrea gigas
Olympia Oyster	Osrrea lurida
Hard-shell clam, Northern quahaug	Mercenaria mercenaria
Soft-shell clam	Mya arenaria
Butter clam	Saxidomus giganteus
Blue Mussel	Mytilus edulis

economically viable option for restricted shellfish recovery.

Although depuration may reduce fecal coliforms and other bacteria in shellfish to a safe level and be an adequate indicator of bacterial elimination, pathogenic viruses may not be as easily eliminated. Evidence concerning pathogenic bacteria and viruses is conflicting.

Eyles and Davie (1984) report that coliform and E. coli concentrations were substantially reduced in their commercial depuration system. However they report that on three occasions the E. coli count was not at an acceptable level. They also found that Vibrio parahaemolyticus and V. cholera persisted in oysters after depuration. Janssen (1973) found oysters retained a high level of Samonella typhimurium and Francisella tulatensis for 49 and 11 days, respectively. Son and Fleet's report (1980) was contradictory, stating that effective depuration of Bacillus cereus, Clostridium perfringis, Samonella, and Vibrio parahaemolyitcus was seen within 2-3 days in the Eastern oyster. Thus, it appears from these apparent contradictions that the mode of contaminant uptake and the origin of the contaminant (in vivo or in vitro) can affect the rate of depuration.

The correlation of coliform concentration and viral concentration in shellfish has not been established (Cole et al, 1986). Ellender and Cook (1986) found no proof of a

correlation between poliovirus and fecal coliforms. Virus was present in oysters having less than 50 fecal coliforms per 100 grams. When Grabow (1989) analyzed 610 samples of marine sewage discharge, polluted seawater, and shellfish, the ratio of virus counts to indicator bacteria varied extensively. Further, viruses were detected in a number of samples yielding negative results in conventional bacterial indicator tests.

Fecal coliforms have not been proven to model viral depuration. Canzonier (1971) demonstrated coliphage S-13 persisted for 6 days while E. coli was undetectable after 24-48 hours. Mesquite (1988) found that depuration for 48 hours based on E. coli removal was not sufficient for removing coliphages and therefore was probably not sufficient for removing viruses of public health significance.

Even if coliforms act as an adequate model for some enteric viruses, their importance as a public health indicator must be questioned. Virus types have been reported to depurate at different rates under varying test conditions. Sobsey et al (1987) reports that oysters reduced polio by greater than 98% in 2-3 days, while HAV had depurated only 90% after 72 hours. These reports indicate that reduction of fecal coliforms within 48 hours may not eliminate the health risk of shellfish-associated viral disease.

Depurated and relayed shellfish have been implicated in viral disease outbreaks. Following a Norwalk gastroenteritis outbreak, Grohmann et al (1981) performed a study with 2000 volunteers eating purified oysters. The oysters were either depurated for 48 hours or relayed for 1 week. The oysters met all bacteriological standards. Fifty-two persons became ill with Norwalk gastroenteritis. Another study resulted in 181 volunteers becoming ill after eating raw oysters depurated for 72 hours (Gill et al, 1983). Mele et al (1989) reports that two thirds of the cases reported in Livorno, Italy resulted from consumption of depurated mussels and clams.

Since it has been repeatedly shown that bacteriological quality is an unreliable monitoring criterion for predicting viral contamination and depletion, it is clear that more effective means of monitoring depuration effectiveness and improved controls are needed to ensure 'safe' shellfish.

Depuration is an in vitro phenomemon and therefore it is essential that depuration plants maintain an environment suitable to shellfish biological activity. The important parameters must be studied further to identify the optimal depuration conditions for each species of shellfish.

Further, since shellfish are considered 'safe' based on bacteriological levels, it is important to understand the processes of viral elimination by shellfish. With a better understanding of the processes and factors involved in

depuration we may be better able to ensure that reclaimed purified shellfish are free of unsafe viral as well bacterial pathogens.

Chapter IV. Materials & Methods

A. Media & Components

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

B. Cultivation and Assay Systems

B.1. Cell Culture

Hepatitis A virus and poliovirus type 1 were propagated and assayed in fetal rhesus monkey kidney (FRhK-4) cells and Buffalo Green Monkey (BGMK) cells, respectively.

Cells were grown in Falcon 800 cm^2 roller bottles or 150 cm^2 flasks using 1X MEM containing 10% fetal calf serum. The cell cultures were incubated at 37^oC until confluent (5-7 days). Once the cells formed a confluent layer, the growth medium was aspirated, the cells 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 detached from the surface, the cell suspension was poured into 50 ml centrifuge tubes and centrifuged at 2000 rpm for 10 minutes. The supernatant was poured off and the pellet was resuspended in sufficient growth medium to give a concentration of 5 X 10^4 cells/ml (FRhK-4) or 1.2 X 10^5 cell/ml (BGMK). Five ml of the cells were dispensed per 60 X 15 mm tissue culture dish. The dishes were incubated in

5% CO_2 at 37°C. The cells reached confluency after 5-7 days and were used for viral assay.

B.2. Propagation and Cultivation of Test Organisms

B.2.1 Hepatitis A virus (HAV)

These studies were done using a cytopathic strain of HAV, pHM-175. Confluent monolayers of FRhK-4 cells were rinsed with serum-free maintenance medium and inoculated with stock virus at a multiplicity of infection (MOI) of 0.1 infectious units per cell. The cultures were incubated at 37°C for 30 minutes on roller racks to evenly disperse virus during the adsorption period. Fresh maintenance medium with serum was added and the cells were incubated at 37oc for 5-7 days until the cell layer was destroyed by cytopathogenic effects.

The viruses were harvested from the cells by freezing-thawing the bottles. The cells were scraped from the surface using the partially frozen medium. The thawed cell lysate was vortexed in 250 ml centrifuge tubes with 1/2 volume Freon (trichlorotriflouroethane). The mixture was centrifuged at 5000 G for 20 minutes. Experimental stock HAV was recovered as the resulting supernatant. HAV stocks were stored frozen at -70°C.

B.2.2 Poliovirus- 1

The vaccine-derived LSc strain of poliovirus type was used. Growth medium was drained from roller bottles of

confluent BGMK cells. The cells were inoculated with virus at MOI of 0.01 infectious units per cell. The viruses were allowed to adsorb to the cell cultures at 37oC for 2 hours. The cell layer was rinsed three times with maintenance medium. Fifty milliliters of fresh maintenance medium were added to each roller bottle and the cells incubated for 1-3 days at 37°C. When the cells demonstrated complete cytopathogenic effects, the cultures were frozen and then thawed. The cells were scraped from the bottles surface using the partially frozen medium. The thawed cell lysate was vortexed in 250 ml centrifuge tubes with 1/2 volume Freon. The mixture was then centrifuged at 5000 G for 20 minutes. Experimental polio stock was recovered as supernatant after centrifugation. Polio stocks were stored frozen at -70°C.

B.2.3 Bacteriophage MS2

Bacteriophage MS2, a 25 nm diameter, icosohedral, RNA phage, infectious for male strains of Escherichia coli, was used in these experiments. Stock MS2 was grown on an E. coli C 3000 host by the soft agar overlay method. Three drops of log phase host and 0.2 ml MS2 (approximately 2 x 10^8 total PFU) were added to 3 ml coliphage single strength top agar in tubes, mixed gently, and poured on Coliphage bottom agar plates. The plates were inverted and incubated for 24 hours at 37°C. The plates having confluent lysis were then soaked with 10-15 ml PBS for 15-20 minutes,

which loosened the top agar from the bottom agar. The top agar was then easily scraped from the plate into a sterile beaker. The plate was rinsed once with 5 ml PBS into beaker. The resulting mixture was then vortexed in 50 cc conical bottom centrifuge tubes and centrifuged at 3000 rpm for 20 minutes. Experimental phage stock was recovered as the supernatant and filtered through sterile, 25 mm diameter, 0.45 micron pore size Acrodisk filters (Gelman). Resulting titers of these stocks ranged from 10^9-10^{12} plaque forming units (PFU) per milliliter.

B.2.4. Escherichia coli B., E. coli C 3000, S. faecalis

A stock of Escherichia coli B., E. coli C 3000, and S. faecalis was prepared for each experiment according to the procedure outlined in Fig.1. Generally, one milliliter of the E. coli B and S. fecaelis suspension contained 10^9 colony-forming units (CFU).

B. 3. Assay of Test Organisms

B.3.1 Plaque Assay

B.3.1.1. HAV

HAV was assayed by the plaque technique in FRHK-4 cells (Cromeans et al, 1987). FRHK-4 cells were grown to about 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 several replicates. Virus samples were

Figure 1. Bacterial Stock Preparation Procedures

Stock cultures of E. coli B were maintained on slants of Nutrient Agar #2.

Stock cultures of S. faecalis were maintained on slants of Brain Heart Infusion (BHI).

Stock cultures of E. coli C 3000 were maintained on slants of Nutrient Agar #2.

Inoculate 30 ml sterile Nutrient Broth #2 with a loopful of E. coli B from stock slants.

Inoculate 30 ml sterile BHI Broth with a loopful of S. faecalis from stock slants.

Inoculate 30 ml sterile Nutrient broth #2 with a loopful of E. coli C 3000 from stock slants.

Incubate E. coli and S. faecalis for 5 hours at 37.5°C with agitation.
Centrifuge the cultures at 4500 rpm for 20 minutes.
Discard supernatant.
Resuspend each pellet in 5 ml sterile PBS.
Refrigerate.

Resuspended culture was enumerated by the pour plate method described in figure 3.

Incubate E. coli C overnight at 37.5°C with agitation.
Refrigerate for daily use in coliphage assay.

diluted in serum free MEM diluent for HAV containing anti-poliovirus serum at a concentration of $1x10^{-3}$. The anti-polio serum 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 20 minutes. After incubation, the dishes were overlayed with 5 ml of agarose overlay medium (formulation in Appendix A), the agarose was allowed to solidify, and the dishes were incubated at 37°C in 5% CO_2. After 7 days, the cells received a second agarose overlay with neutral red and were reincubated. After 3-4 days, plaques were counted. HAV concentrations were calculated and expressed as plaque forming units (PFU) per ml.

B.3.1.2. Poliovirus-1

Poliovirus-1 was assayed by the plaque technique in BGMK cells. Confluent layers of BGMK cells in 60 X 15 mm replicate dishes were inoculated 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 sample redistribution every 15-20 minutes. After the adsorption period, an agar overlay medium (see App. A) was added to the dishes and allowed to harden. The dishes were incubated at 37°C for 48 hours. Plaques were countable after 48 hours, and recounted

after 72 hours. Poliovirus concentrations were calculated and expressed as PFU per ml.

B.3.2 MS2 Bacteriophage Assay

The MS2 coliphage plating protocol is outlined in Figure III.2.

B.3.3 Bacteria Plate Counts

E. Coli B and S. faecalis levels in clam homogenate were enumerated by the pour plate technique outlined in figure 3.

C. Clams

Northern quahaugs or hard shell clams (Mercenaria mercenaria) were harvested by manual raking from Calico Creek in Carteret County, NC or bought fresh from local merchants. The clams were washed under cold water to remove mud from the shell and then stored dry at 4°C for a maximum of 2 days after collection. The clams were brought to room temperature before use in experiments.

D. Seawater

Natural seawater pumped from Bogue Sound, NC, was used for the experiments. It was pumped through a series of 3 micron and .45 micron cartridge filters (Filterite) to obtain the desired turbidity of less than 1 NTU. The water was maintained at pH 8.0 by addition of HCl or NaOH. A dissolved oxygen level greater than 5 mg/l was ensured by

Figure 2. Plating procedure for MS2 coliphage.

Pipet either 0.1 or 1.0 ml of appropriate PBS dilution
of 1:2 sample homogenate
and 3 drops of log phase E. coli C 3000 host
into 3 ml of top agar.

Mix gently by rolling tube between palms.

Pour onto bottom agar plate.
Run duplicate plates.

Incubate inverted at 37.5oC for 6-8 hours.

Count plaques and express as PFU/ml.

Figure 3. Plating procedure for *E. coli* and *S. faecalis*

Pipet either 0.2 or 2.0 ml
of appropriate PBS dilution of 1:2 sample homogenate
into 30 ml of agar.

E. coli uses modified MacConkey's agar.
S. faecalis uses M-enterococcus agar.

Pour half the volume (15 ml) into each of two plates
for enumeration.

Allow agar to harden.
Invert plates.

Incubate *E. coli* B at 37.5°C for 24 hours.
Incubate *S. faecalis* at 40°C for 24 hours.

E. coli colonies appear small, disk-shaped,
and pink to brick red in color.

S. faecalis colonies appear round
and purple or maroon in color.

Count colonies and record as Colony forming units (CFU).

continual aeration through an aquarium diffuser stone. The water was brought to the desired salinities of 8, 18, 28 ppt, by diluting Bogue Sound water with distilled deionized water and measuring salinity with a refractometer. The experimental temperatures; 12, 18, 25°C, were controlled by a immersion coil refrigeration unit.

E. Test Organisms Recovery

A sample of five clams was removed daily from the exposure tank for six consecutive days. The first day sample, 'day 0', was to determine the test organism concentration before depuration. The five consecutive days were taken to measure the rate of clam self-purification.

E.1. Bacteria, Bacteriophage MS2, HAV

For the assay of bacteria, MS2 and HAV, a 1:2 homogenate of clam tissue in 0.3M NaCl solution was used. This procedure is described in section A of Figure 4.

E.2. Polio

Following clam homogenation, a concentration procedure was used to separate the virus from the clam tissue and to concentrate it for viral analysis. This procedure is described in section B of figure 4.

F. Overview of Experimental Methods for Depuration Studies

Experiments were conducted using HAV, Polio, MS2, E. coli, and S. faecalis to compare elimination rates at

Figure 4. Clam Processing Preparation for Virus Recovery

Section A

1. Remove 5 clams from depuration tank.

2. Shuck clams, discard liquor.
   Record Weight _____

3. Add equal volume of 0.3M NaCl, blend at low speed for 1 minute, blend at high speed for 2 minutes in semi-micro blender jar.

4. Remove half volume of 1:2 homogenate for bacteriological and HAV analysis.

Section B

5. Measure volume of remaining 1:2 homogenate (_____ml).

6. Pour remaining homogenate into 1 liter blender jar.

7. Measure 6 volumes 7% Beef Extract/0.3M NaCl (_____ml). Rinse semi-micro jar and graduate cylinder with Beef Extract solution, pour into the 1 liter blender jar.

8. Add 0.25 ml Dow Anti-foam and blend at high speed for 30 seconds.

9. Pour into a 2 liter beaker with stir bar.
   Add 0.25 ml Dow Anti-foam. Mix until foam disappears.
   Adjust pH to 7.5.

10. Add correct amount of freon (_____ml). (see equation below)

    step 5 volume X 2 = actual clam weight
    =_____g
    clam weight X 7= Freon volume
    =_____ml Freon.

11. Mix vigorously.
    Centrifuge at 5000 g (5500 rpm) for 20 minutes.

12. Remove eluate supernatant with pipet.
    Adjust pH to 7.2.

13. Measure volume (_____ml).
    Add PEG 8000 to 12% volume weight.

    supernatant volume X 0.12 =_____g PEG 8000

Figure 4. continued

Mix until PEG is completely dissolved.
Incubate overnight in refrigerator without agitation.

14. Centrifuge at 6250 rpm for 20 minutes.

15. Discard supernatant.
Resuspend pellet in small amount of PBS.

16. Add 1 ml of stock GEN-KAN per 100 ml of final sample.
Record final volume(_____ml).

17. Freeze sample at -40°C for viral assay.

different temperatures (12, 18, 25°C) and salinities (8, 18, 28ppt). When temperature was the variable, the salinity was held constant at 28ppt, and when salinity was 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 test water in a 46cm long x 24cm wide x 13cm deep polypropylene tank was seeded with 10^9 organisms each of HAV, Polio, MS2, E. coli, and S. faecalis. This gave an initial level of 10^6 organisms per ml of uptake water. The inoculated water was allowed to mix for one hour. Then 60-70 clams were placed in the tank and allowed to feed for 16-18 hours at room temperature.

After the uptake period, a sample of five clams was processed to determine the initial (day 0) levels of organisms. The remaining clams were divided randomly into two groups and transferred to the two depuration tanks containing filtered depuration water at the designated temperature and salinity.

The depuration system was designed as a once flow-through system with a baffle at the inlet to prevent short circuiting of the feed water. The water flow rate was 12 ml/min/clam. Spent depuration water discharged from the tank was disinfected with chlorine bleach before discarding to the sewer system.

G. Overview of Uptake Experimental Methods

The purpose of this study was to determine the time necessary for maximum uptake of the test organisms, the uptake efficiency of the clams with respect to each organism, and finally to determine if the organisms were concentrated to a level above that in the overlying water. At the beginning of each experiment 5 liters of seawater in a 46 cm long x 24 cm wide x 13 cm deep polypropylene tank were seeded with HAV, Polio, MS2, E. coli, and S. Faecalis, giving an initial concentration of 10^6 organisms per ml of uptake water. The inoculated water at pH 8, dissolved oxygen > 5 ppm, turbidity < 1 NTU, 25°C and 28 ppt salinity was allowed to mix for one hour. Thirty clams were introduced into the water and allowed to naturally feed. Samples were taken at 0, 1, 3, 9, 18, 30 hours. The '0 hour' sample which was not exposed to test organisms was used as a base line indicator of pre-exposure contamination. The samples were removed at set time intervals and processed as described in figure 4. All samples were analyzed for bacteria and virus concentration.

H. Statistical Analysis

The analysis of data was conducted using PC/SAS on a Zenith personal computer. The data for depuration at different temperatures and salinities were analyzed using a combination of simple linear regression and multiple

regression, with temperature and salinity data treated as separate data sets.

The raw data from the depuration experiments consisted of the concentration of each test organism found in the 1:2 clam tissue homogenate on experimental day 0, 1,2,3,4, and 5. These concentrations were converted to the number of organisms per gram of clam meat by multiplying by a factor of 2. These calculated concentrations were normalized to the percent of the initial test organisms per gram of clam meat for each experimental day. This was done by dividing the number of organisms at days 1-5 by the initial number present on day 0 and multiplying the product by 100 to obtain percentage. In an effort to obtain a linear relationship, the percent remaining data was log transformed. The resulting logarithmic data was plotted versus arithmetic time to give an approximation of a straight line.

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

The null hypothesis, that all test organisms are eliminated at the same rate for each experimental condition, was tested by fitting the linear regression of log % remaining over time and using a dummy-dependent-variable

regression to determine whether the slopes of the regression lines were significantly different. This method utilized a pairwise comparison to determine which organisms depurated at the same rate (the lines being coincident or parallel). A second null hypothesis, that the extent of depuration of each organism within the experimental conditions (the three temperatures and salinities) was tested in the same way.

All statistical tests employed a 95% confidence interval level (alpha value = 0.05). This was the basis for acceptance or rejection of the null hypothesis.

IV. RESULTS

IV.A. Depuration Experiments

The results of the depuration experiments, comparing the rates of elimination of HAV, Polio, MS2, E. coli, S. faecalis at different temperatures and salinities, are summarized in Tables 1-3 and 7-9, respectively. Each table shows the percent of the initial organism remaining after 1, 2, 3, 4, and 5 days of depuration for each replicate experiment as well as the mean percent remaining over time for all the experiments. The experimental results of the individual replicate experiments and their mean values in terms of concentrations of viruses and bacteria per unit weight of shellfish meat can be found in appendix B.

IV.A.1. Effect of Temperature

The results of temperature on each test organism can be seen in Figures IV.1-3. The results indicate that temperature had no appreciable effect on the depuration of the individual test organism. As is seen in figure lV.1 the rate of depuration (the decrease in the initial organism concentration over time) is similar for each microbe at the three different temperatures. The graph of the mean percent remaining (fig. IV.2) illustrates the same findings with the initial and final percentage of organisms remaining approximately equal at each temperature. Finally, examining the linear regression graph of the depuration of each organism at the three temperatures (fig.IV.3) we see that

Table 1.

TEMPERATURE: 12 °C

% OF INITIAL LEVELS REMAINING

		DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
E. coli	Trial A	100	38.350	30.009	3.046	0.723	0.430
	Trial B	100	34.995	16.646	8.395	1.365	0.272
	Trial C	100	1.837	0.096	0.032	0.075	0.194
	Trial D	100	2.357	0.404	0.220	0.173	0.094
	MEAN	100	19.385	11.788	2.923	0.584	0.248
S. fecalis	Trial A	100	76.854	24.196	2.153	0.796	0.334
	Trial B	100	56.448	16.461	9.830	2.665	0.357
	Trial C	100	3.380	0.406	0.063	0.184	0.389
	Trial D	100	3.167	0.985	0.220	0.284	0.176
	MEAN	100	34.962	10.512	3.066	0.982	0.314
PHAGE MS2	Trial A	100	121.660	139.568	76.149	77.917	65.393
	Trial B	100	155.511	89.499	126.984	74.971	119.175
	Trial C	100	78.559	96.886	26.432	54.227	40.541
	Trial D	100	60.757	98.400	55.762	35.027	78.378
	MEAN	100	104.122	106.088	71.332	60.536	75.872
POLIO	Trial A	100	52.585	9.917	3.128	1.666	<0.760
	Trial B	100	19.423	8.585	1.557	0.604	0.675
	Trial C	100	80.766	54.774	14.461	4.720	16.114
	Trial D	100	87.617	47.207	57.308	40.489	2.276
	MEAN	100	60.098	30.121	19.113	11.870	<4.956
HAV	Trial A	100	14.865	52.361	16.768	36.865	8.842
	Trial B	100	55.512	82.947	37.977	75.199	30.473
	Trial C	100	14.978	10.489	12.702	20.298	20.652
	Trial D	100	98.894	20.104	7.060	5.245	10.070
	MEAN	100	46.062	41.475	18.627	34.402	17.509

PERCENT OF INITIAL CONCENTRATION OF MICROORGANISMS REMAINING

*Trial A,B,C,D,E,F represent replicate experiments.

Table 2.

TEMPERATURE: 18 °C

% OF INITIAL LEVELS REMAINING

		DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
E. coli	Trial A	100	10.880	0.461	1.225	7.205	0.021
	Trial B	100	7.913	0.224	0.062	0.047	1.810
	Trial C	100	78.359	84.922	2.188	1.375	0.313
	Trial D	100	47.500	14.271	6.875	0.875	1.437
	MEAN	100	36.163	24.969	2.587	2.376	0.895
S. fecalis	Trial A	100	13.160	0.387	1.186	51.671	0.016
	Trial B	100	7.937	1.020	0.105	0.136	0.658
	Trial C	100	15.759	0.405	0.150	0.081	0.027
	Trial D	100	6.827	1.487	2.052	0.050	0.069
	MEAN	100	10.921	0.825	0.873	12.985	0.192
PHAGE MS2	Trial A	100	66.618	111.471	162.706	132.177	28.059
	Trial B	100	183.824	18.529	42.882	25.059	7.412
	Trial C	100	29.630	165.432	40.000	16.931	12.741
	Trial D	100	240.741	400.000	106.667	7.460	39.519
	MEAN	100	130.203	173.858	88.064	45.407	21.932
POLIO	Trial A	100	2.176	0.398	<0.398	2.148	2.655
	Trial B	100	0.907	<0.502	<0.477	3.466	4.267
	Trial C	100	249.102	9.460	66.230	<4.370	22.157
	Trial D	100	71.153	141.175	17.857	79.880	3.056
	MEAN	100	80.835	<37.884	<21.241	<22.466	8.034
HAV	Trial A	100	93.443	98.216	141.080	125.442	113.227
	Trial B	100	73.473	68.627	88.637	41.626	50.627
	Trial C	100	60.682	58.020	125.299	88.047	41.049
	Trial D	100	68.943	2.774	46.867	58.047	31.566
	MEAN	100	74.135	56.909	100.471	78.291	59.117

PERCENT OF INITIAL CONCENTRATION OF MICROORGANISMS REMAINING

*Trial A,B,C,D,E,F represent replicate experiments.

Table 3.

TEMPERATURE: 25 °C

% OF INITIAL LEVELS REMAINING

		DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
E. coli	Trial A	100	37.847	2.928	0.499	0.077	0.375
	Trial B	100	48.613	3.466	0.179	0.062	0.050
	Trial C	100	395.263	25.685	0.578	-----	1.113
	Trial D	100	40.240	6.892	0.471	-----	0.235
	Trial E	100	1.012	16.170	0.577	0.458	0.488
	Trial F	100	3.865	3.203	1.304	1.088	0.251
	MEAN	100	87.806	9.724	0.601	0.421	0.419
S. fecalis	Trial A	100	41.667	6.443	4.000	2.267	0.267
	Trial B	100	41.333	5.333	2.267	1.467	0.133
	Trial C	100	44.370	1.525	1.878	-----	0.176
	Trial D	100	7.563	0.916	6.422	-----	0.088
	Trial E	100	3.222	80.183	1.635	0.782	1.591
	Trial F	100	2.454	5.777	2.034	1.523	0.568
	MEAN	100	23.435	16.696	3.039	1.510	0.471
PHAGE MS2	Trial A	100	20.128	3.760	20.547	12.123	2.839
	Trial B	100	58.887	5.192	2.946	1.918	0.353
	Trial C	100	254.623	61.021	73.835	-----	6.635
	Trial D	100	111.243	70.118	51.535	-----	16.331
	Trial E	100	25.416	16.989	215.101	93.103	314.625
	Trial F	100	22.027	293.115	204.600	102.735	53.040
	MEAN	100	82.054	75.032	94.761	52.470	65.637
POLIO	Trial A	100	11.130	<0.341	1.023	7.260	<0.418
	Trial B	100	2.955	2.724	0.950	<1.523	<0.459
	Trial C	100	31.208	0.754	17.295	5.205	10.011
	Trial D	100	14.750	0.754	8.539	2.000	18.026
	Trial E	100	8.968	29.697	55.380	<2.640	<2.640
	Trial F	100	16.711	15.338	31.820	10.769	5.262
	MEAN	100	14.287	<8.268	19.168	<4.899	<6.136
HAV	Trial A	100	82.934	28.461	51.842	19.075	------
	Trial B	100	46.721	57.124	18.665	30.287	------
	Trial C	100	65.957	5.319	21.277	68.085	66.489
	Trial D	100	18.191	4.255	21.277	40.766	37.234
	MEAN	100	53.451	23.790	28.265	39.553	51.862

PERCENT OF INITIAL CONCENTRATION OF MICROORGANISMS REMAINING

*Trial A,B,C,D,E,F represent replicate experiments.

Figure 1. DEPURATION OF ORGANISMS AT 3 TEMPERATURES (ORGANISMS / GRAM OF CLAM TISSUE)

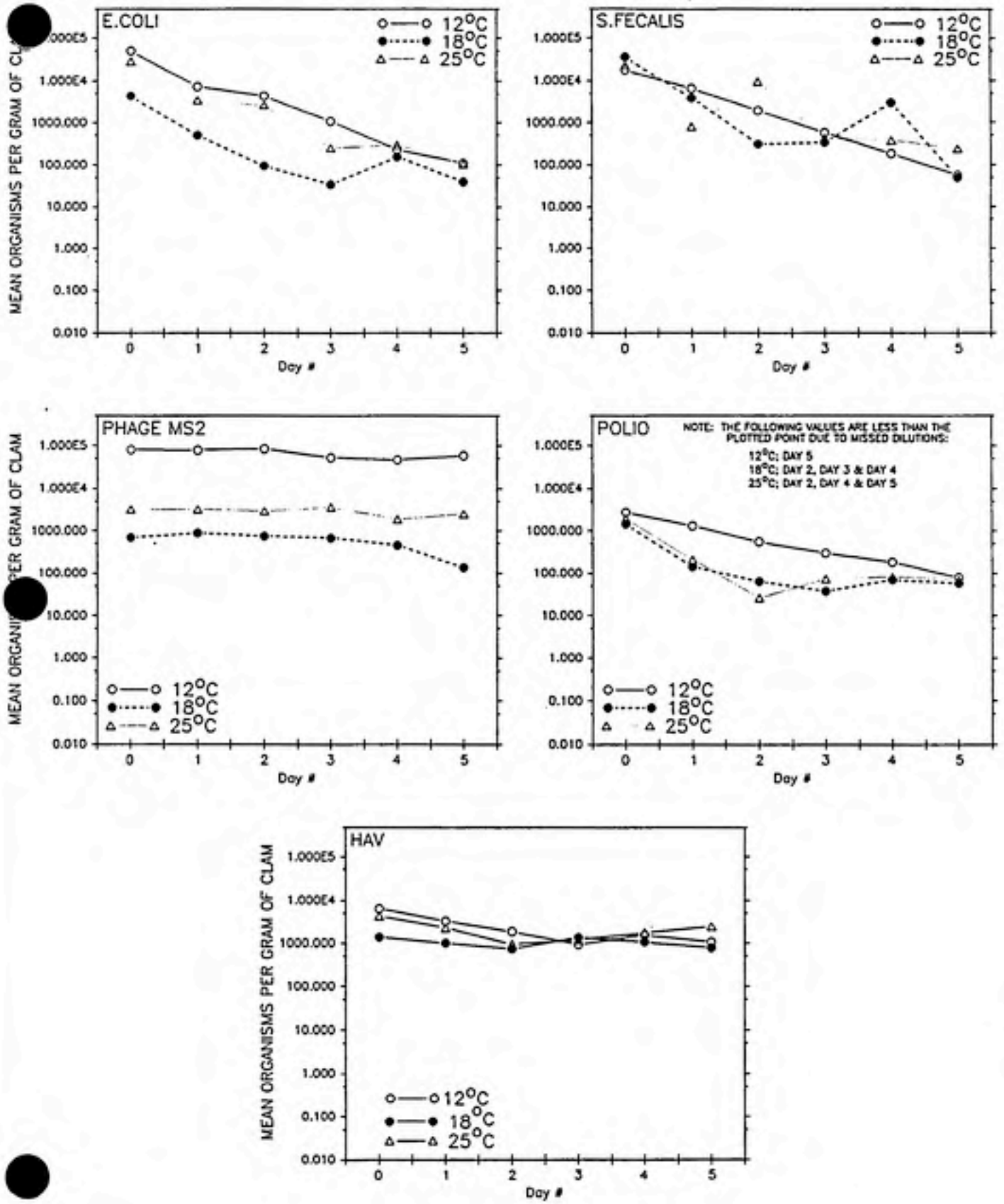


Figure 2. DEPURATION OF ORGANISMS AT 3 TEMPERATURES (MEAN % REMAINING)

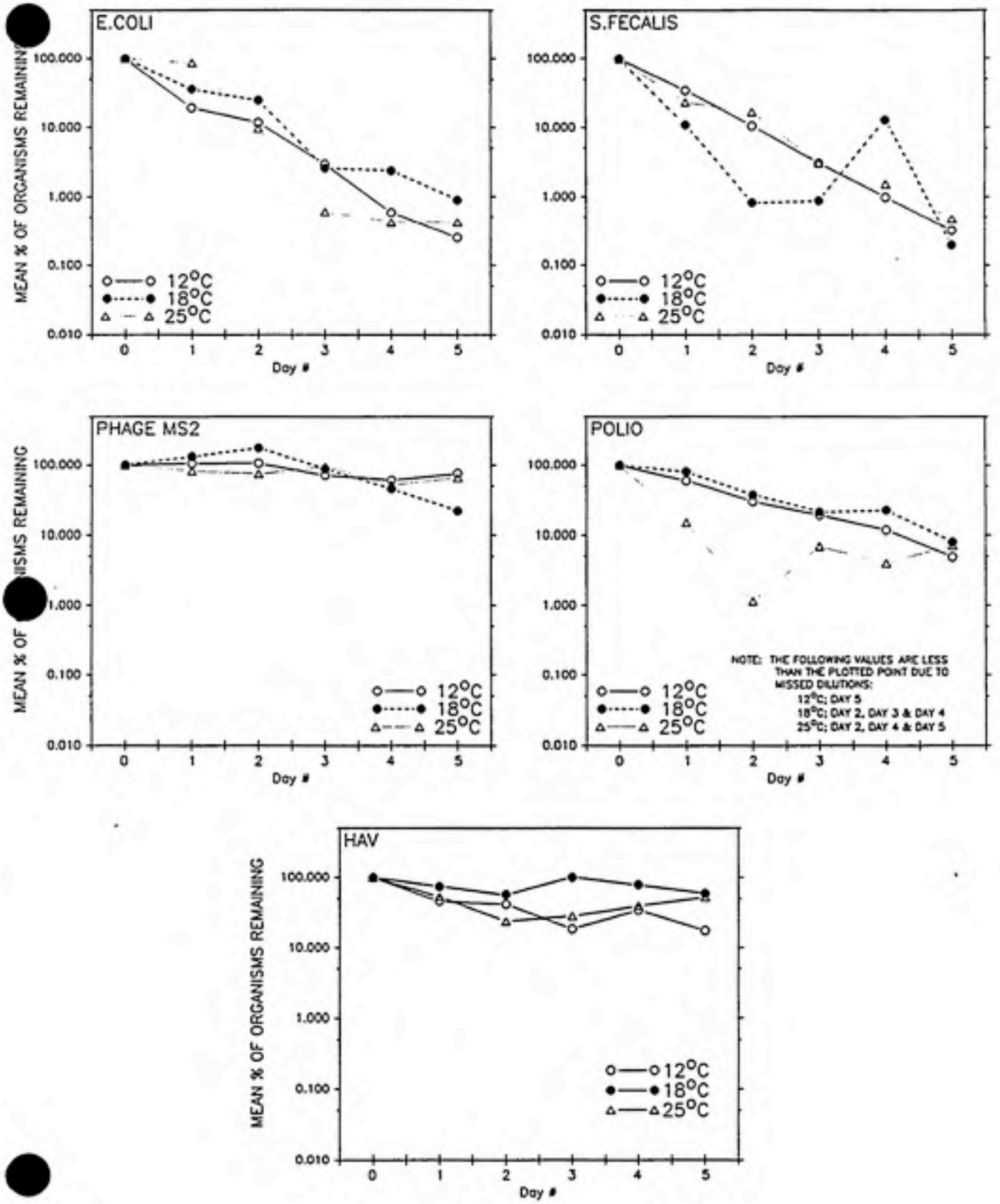
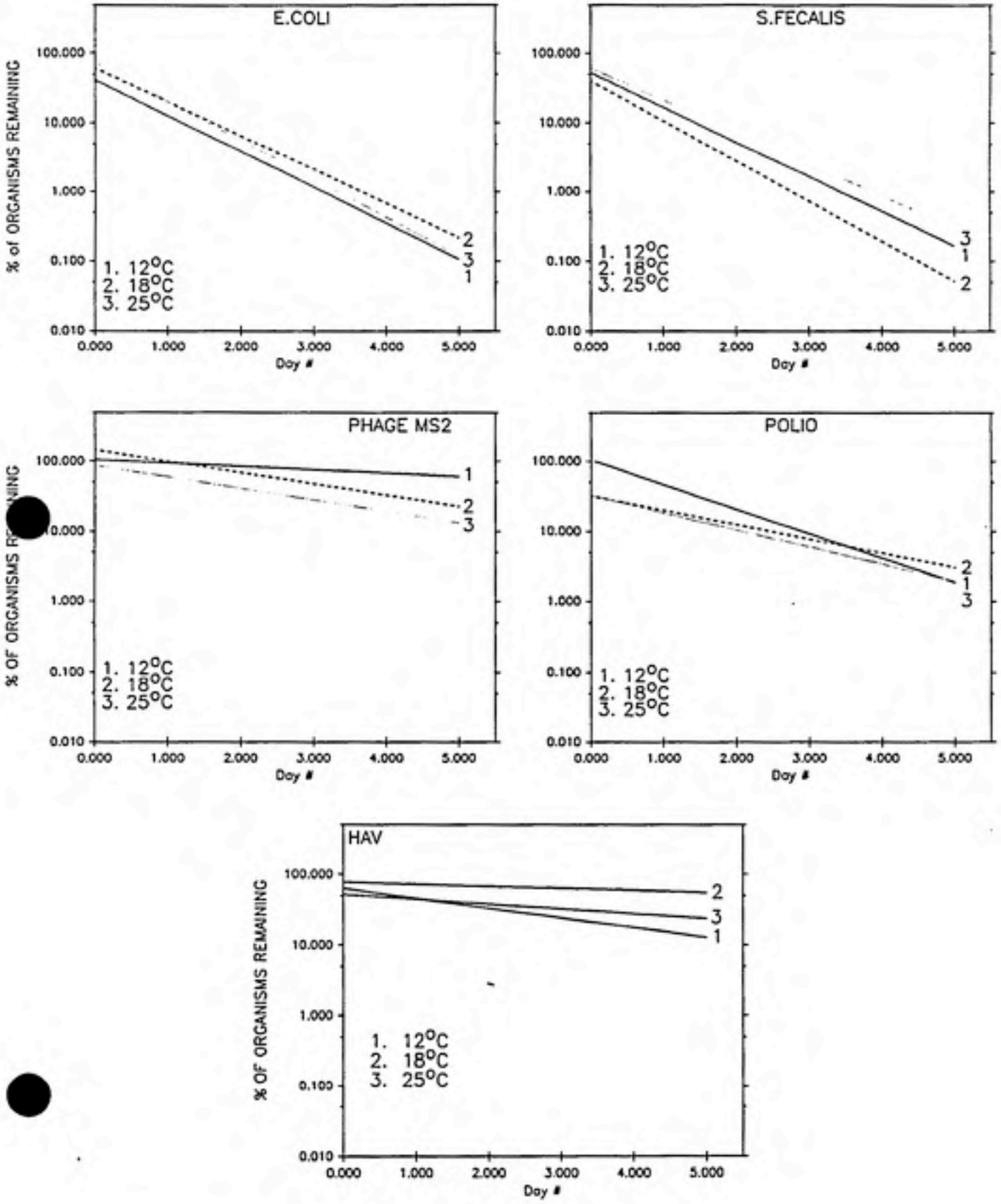


Figure 3. DEPURATION OF ORGANISMS AT 3 TEMPERATURES REGRESSION LINES (MEAN % REMAINING)

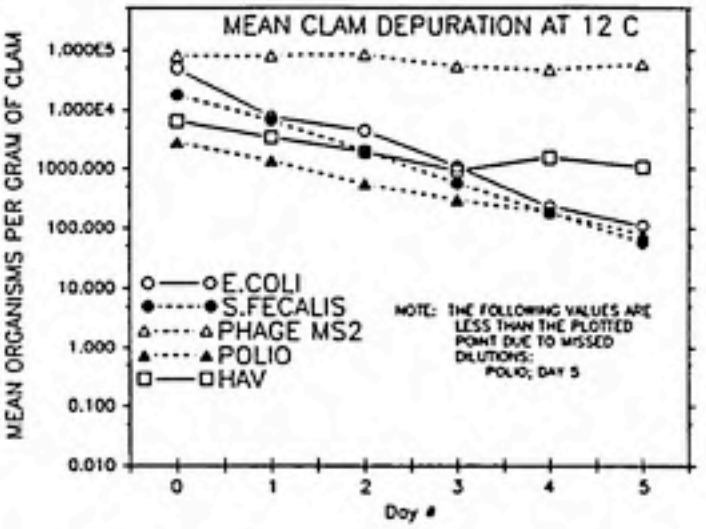


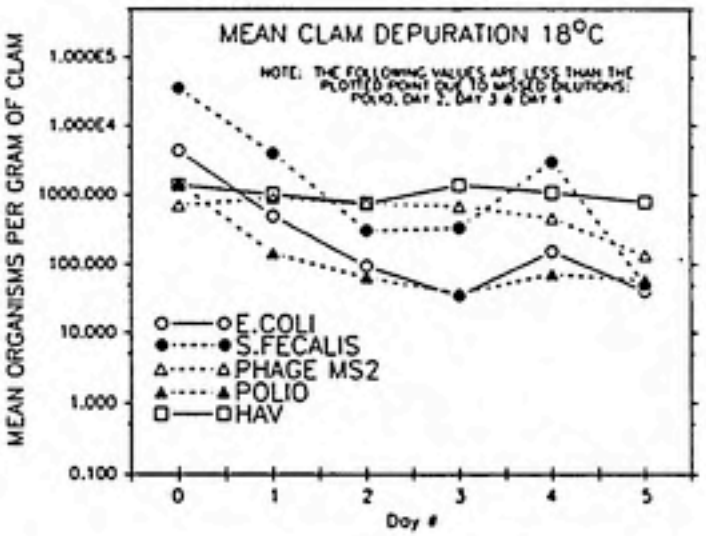
statistically, accounting for standard errors or variations in the data, each organism depurated at the same rate at 12, 18, 25°C.

A comparison of of the depuration of each organism at the different temperatures is depicted in figures IV.4-6. This data indicates that, at each temperature, HAV and MS2 generally depurate less than the other test organisms. At each temperature, the final counts of HAV and MS2 were greater than the other organisms tested and not significantly different from the initial concentration. The percent of organisms remaining (fig. IV.5) further illustrates the difference in the rates of depuration. It is obvious that polio depurates more slowly than the bacteria, and has a depuration rate similar to HAV and MS2. It is clear that once accumulated in the shellfish, the viruses are responding differently than the bacterial indicators. Figure IV.6 shows an absence of depuration of HAV and MS2 by shellfish at all three temperatures. After 5 days of depuration at 12°C, HAV and MS2 have 17.5 and 75.9% of the initial concentration remaining, respectively; at 18°C, 59.1 and 21.9% respectively; and at 25°C, 51.9 and 65.6% respectively. This is in contrast to the bacterial organisms less, where than 0.5% remained after 5 days.

Important information can be obtained from examining only the first 2-3 days of depuration. Since 2-3 days are accepted for U.S. depuration standards, it is necessary to

Figure 4. DEPURATION AT 3 TEMPERATURES OF ORGANISMS (ORGANISMS / GRAM OF CLAM TISSUE)





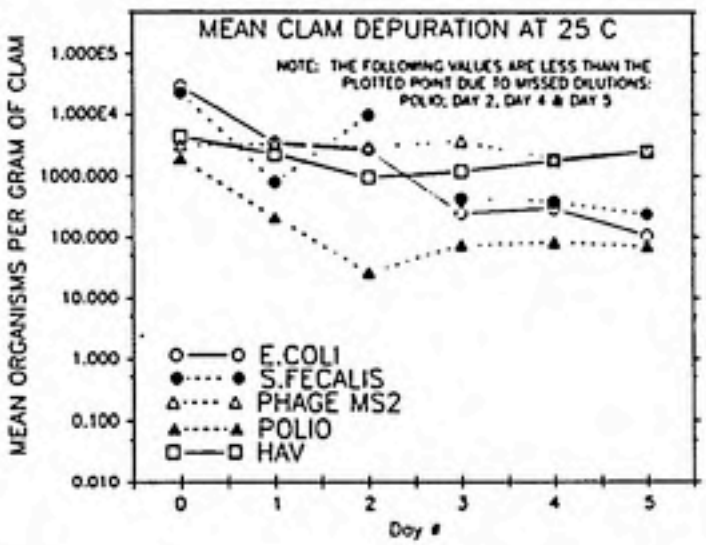


Figure 5. DEPURATION AT 3 TEMPERATURES OF (MEAN % REMAINING)

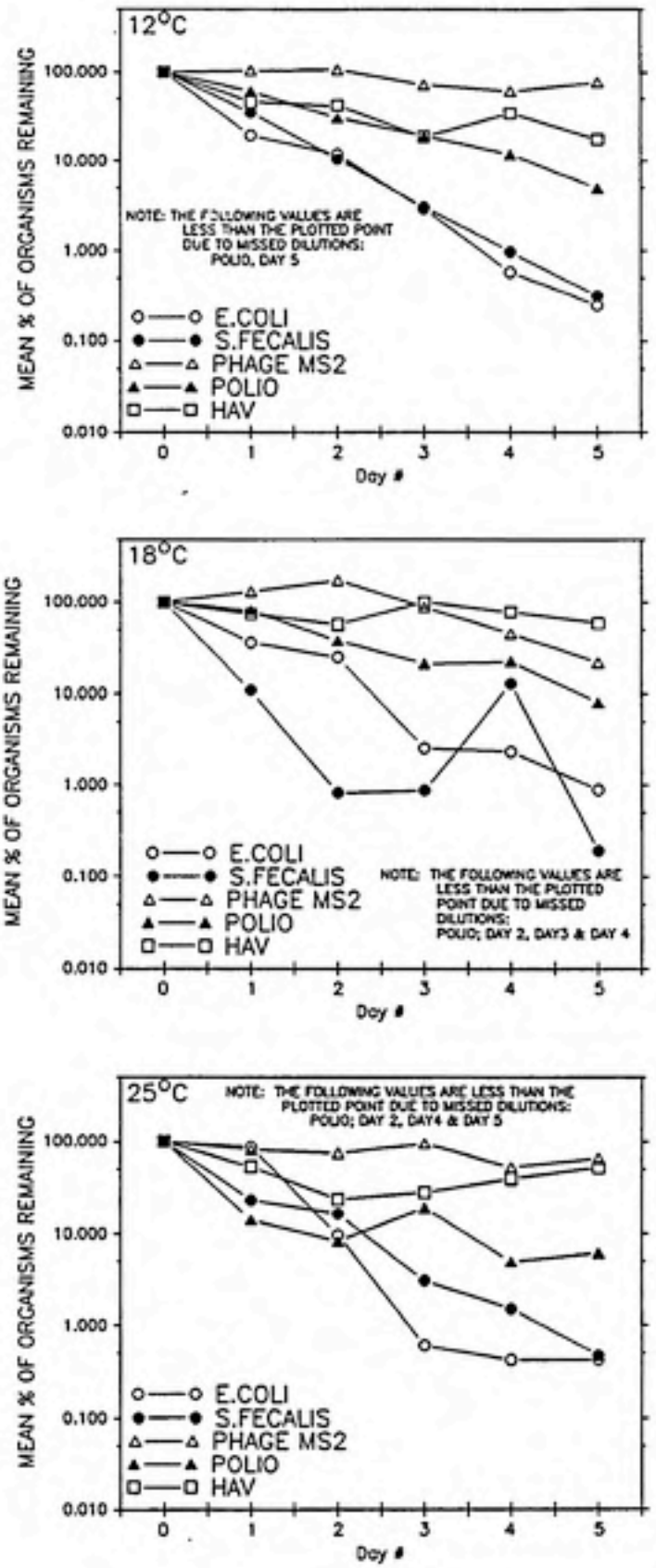
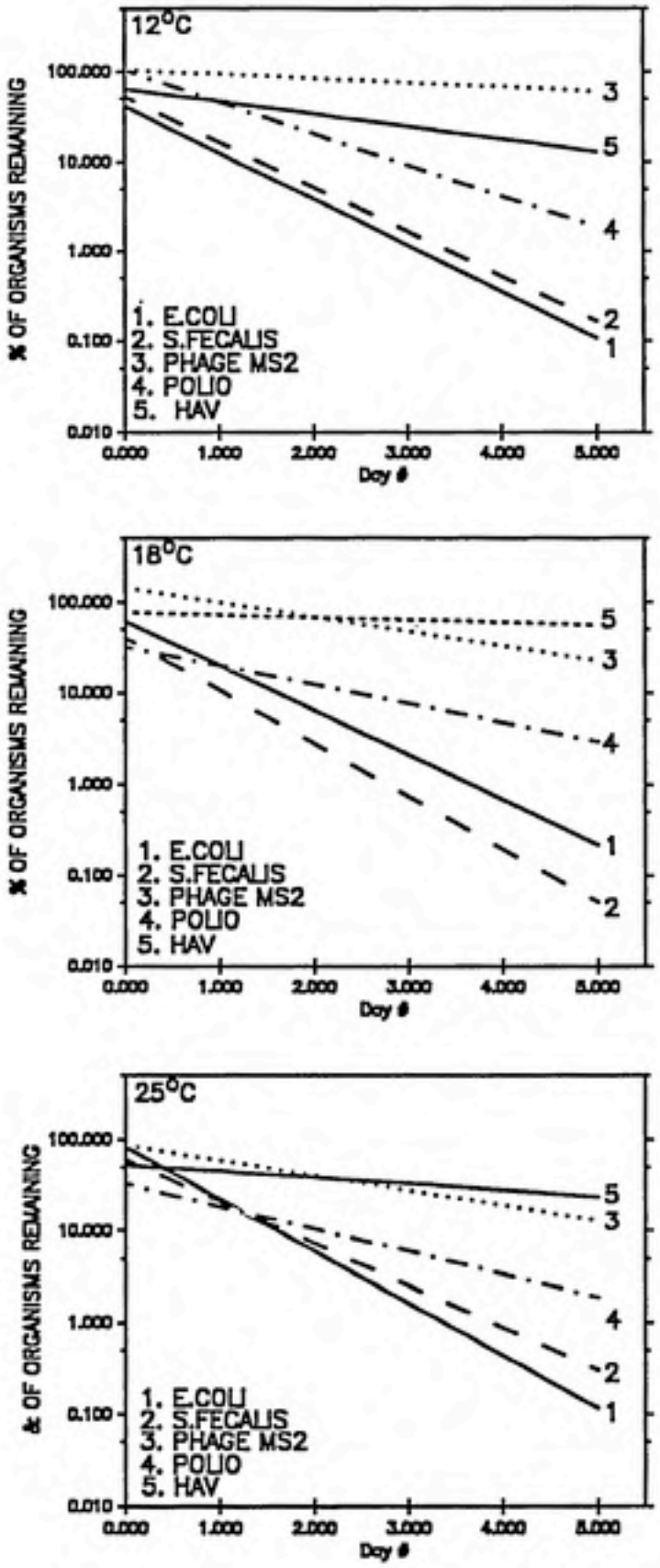


Figure 6. DEPURATION AT 3 TEMPERATURES OF ORGANISMS
REGRESSION LINES
(MEAN % REMAINING)



determine the behavior of the test organisms in this time span. Examining tables 1-3, it is observed that the bacteria have significantly depurated after 3 days. After 3 days at 12°C the E. coli and S. fecaelis have depurated respectively to only 2.92% and 3.06% remaining, at 18°C to 2.59% and 0.87% remaining, and at 25°C to 0.60% and 3.03% remaining. These low bacterial levels are in sharp contrast to the levels of virus present after 3 days of depuration. MS2, polio, and HAV remain in relatively high concentrations, with respective percents remaining of 71.3%, 19.1%, and 18.6% at 12°C, 88.1%, 21.2%, and 100.5% at 18°C, and 94.7%, 19.2%, and 28.3% at 25°C. In general, the concentrations of viruses are a log greater than bacterial concentrations after 3 days of depuration. The differences in depuration rates between the viruses and bacteria after 2-3 days depuration illustrate the same pattern of results as discussed above for 5 days of depuration. Each test organism depurated at similar rates at 12, 18, 25°C, and bacteria depurate significantly greater than viruses.

The statistical analysis of the data for the temperature experiments are shown in Table 4-6. The data were grouped by temperature and subgrouped by organism yielding a corresponding data set. Correlations were performed between log % remaining and day for each of these data sets, and simple linear regressions were run using the

model log % remaining=B_o+B_1[day]. The results of those tests are summarized in Table 4.

The next step in the analysis involved the use of Backward Selection Multiple Regression to find the least squares solution for a model containing log % remaining, day, time/temperature, and/or [day X time/temperature]. The resulting model was tested for coincidence and then parallelism of the test organisms according to the methods of Kleinbaum and Kupper (1988). If any two lines were coincident, the analysis was complete. If the lines were not coincident, they were tested for parallelism. The values of the temperatures (or salinities in later analysis) were entered in the equation and solved, yielding the model log % remaining=B_o+B_1[day]. This allowed for the direct comparison of the models. This test utilizes a 95% confidence interval as the criterion for acceptance.

The depuration data were further tested using the above model and pairwise comparisons among the temperatures. In this manner all parallels in depuration rates at the test temperatures could be detected. The conclusions of these tests are summarized in Table 5. Each organism showed the same rate of depuration at all temperatures ($p > 0.05$) with the exception of MS2 which showed a discrepancy in the multiple and linear regression results. In this case, the multiple regression model showed MS2 depurated faster at 25^{o}C ($p = .0001$). This contradiction is not a problem,

TABLE 4

SUMMARY OF LINEAR REGRESSION FOR TEMPERATURE EXPERIMENTS

Temp.	org.	slope	Intercept	R
12	E. coli	-0.5168	1.6114	-0.7749
	S. faecalis	-0.5003	1.7211	-0.8122
	MS2	-0.0477	2.0203	-0.0440
	Polio	-0.3506	2.0168	-0.7840
	HAV	-0.1380	1.8042	-0.5762
18	E. coli	-0.4894	1.7866	-0.7451
	S. faecalis	-0.5797	1.6055	-0.8155
	MS2	-0.1608	2.1608	-0.5905
	Polio	-0.2074	1.5028	-0.3874
	HAV	-0.0279	1.8888	-0.1433
25	E. coli	-0.5576	1.8578	-0.8676
	S. faecalis	-0.4611	1.7846	-0.8786
	MS2	-0.1638	1.9393	-0.4020
	Polio	-0.2480	1.5184	-0.5664
	HAV	-0.0681	1.7217	-0.2889

TABLE 5

EFFECT OF DIFFERENT TEMPERATURES ON ORGANISMS

ORGANISM	P VALUE	RESULT	
E. coli	>.25	ALL COINCIDENT	*
S. faecalis	<.05	NOT COINCIDENT	
	>.25	ALL PARALLEL	*
MS2	>.05	ALL COINCIDENT	*
Polio	>.25	ALL COINCIDENT	*
HAV	<.01	NOT COINCIDENT	
	>.10	ALL PARALLEL	*

*CONCLUSION=TEMPERATURE HAS NO EFFECT ON DEPURATION RATES

however, since 25°C was chosen as the model temperature in all studies and analyses where temperature was not the variable under investigation. This model was then used to determine if there was a significant difference in the rate of depuration among test organisms at any given temperature.

The difference in the behaviors of microbes at 25°C was determined using the above model and pairwise comparisons among the organisms. Similarities in depuration rates between organisms were detected. E. coli and S. faecalis depurated at the same rate ($p > .25$), while MS2, Polio, and HAV depurated at parallel rates ($p > .10$). The results of these tests are summarized in Table 6.

IV.A.2. Effect of Salinity

The results of salinity on each organism are summarized in Tables 7-9 and can be inspected visually in figures 7-9. The results indicate that salinity does play a role in the rate of depuration of some test organisms. For the bacterial indicators tested, the rates of depuration at 8 and 18 ppt were equal while the rate at 28 ppt was greater. This, however, is not the case with the viruses, which showed no significant difference in the depuration rates at the different salinities ($p > .05$). Figure 8 illustrates this decrease in concentration at 28 ppt in E. coli and S. faecalis, while no such decrease is seen in the depuration rates of the viruses. Figure 9, the linear regression graph, clearly illustrates this decrease. By 5 days, the

TABLE 6

EFFECT OF TEMPERATURE ON EACH ORGANISM

MODEL TEMPERATURE = 25°C

ORGANISM	P VALUE	RESULT	
E. coli vs *S. faecalis*	>.25	COINCIDENT	*
vs MS2	<.001	NOT COINCIDENT	
	<.001	NOT PARALLEL	
vs Polio	<.001	NOT COINCIDENT	
	<.001	NOT PARALLEL	
vs HAV	<.001	NOT COINCIDENT	
	<.001	NOT PARALLEL	
S. faecalis vs MS2	<.001	NOT COINCIDENT	
	<.001	NOT PARALLEL	
vs Polio	<.01	NOT COINCIDENT	
	<.01	NOT PARALLEL	
vs HAV	<.001	NOT COINCIDENT	
	<.001	NOT PARALLEL	
MS2 vs Polio	<.001	NOT COINCIDENT	
	>.25	PARALLEL	+
vs HAV	>.25	COINCIDENT	+
Polio vs HAV	<.001	NOT COINCIDENT	
	>.10	PARALLEL	+

CONCLUSION = VIRUSES BEHAVE DIFFERENTLY THAN BACTERIA
* E. coli and S. Feacalis are coincident.
+ MS2, Polio, and HAV are parallel.

Table 7.

SALINITY: 28ppt

% OF INITIAL LEVELS REMAINING

		DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
E. coli	Trial A	100	37.847	2.928	0.499	0.077	0.375
	Trial B	100	48.613	3.466	0.179	0.062	0.050
	Trial C	100	395.263	25.685	0.578	-----	1.113
	Trial D	100	40.240	6.892	0.471	-----	0.235
	Trial E	100	1.012	16.170	0.577	0.458	0.488
	Trial F	100	3.865	3.203	1.304	1.088	0.251
	MEAN	100	87.806	9.724	0.601	0.421	0.419
S. fecalis	Trial A	100	41.667	6.443	4.000	2.267	0.267
	Trial B	100	41.333	5.333	2.267	1.467	0.133
	Trial C	100	44.370	1.525	1.878	-----	0.176
	Trial D	100	7.563	0.916	6.422	-----	0.088
	Trial E	100	3.222	80.183	1.635	0.782	1.591
	Trial F	100	2.454	5.777	2.034	1.523	0.568
	MEAN	100	23.435	16.696	3.039	1.510	0.471
PHAGE MS2	Trial A	100	20.128	3.760	20.547	12.123	2.839
	Trial B	100	58.887	5.192	2.946	1.918	0.353
	Trial C	100	254.623	61.021	73.835	-----	6.635
	Trial D	100	111.243	70.118	51.535	-----	16.331
	Trial E	100	25.416	16.989	215.101	93.103	314.625
	Trial F	100	22.027	293.115	204.600	102.735	53.040
	MEAN	100	82.054	75.032	94.761	52.470	65.637
POLIO	Trial A	100	11.130	<0.341	1.023	7.260	<0.418
	Trial B	100	2.955	2.724	0.950	<1.523	<0.459
	Trial C	100	31.208	0.754	17.295	5.205	10.011
	Trial D	100	14.750	0.754	8.539	2.000	18.026
	Trial E	100	8.968	29.697	55.380	<2.640	<2.640
	Trial F	100	16.711	15.338	31.820	10.769	5.262
	MEAN	100	14.287	<8.268	19.168	<4.899	<6.136
HAV	Trial A	100	82.934	28.461	51.842	19.075	-----
	Trial B	100	46.721	57.124	18.655	30.287	-----
	Trial C	100	65.957	5.319	21.277	68.085	66.489
	Trial D	100	18.191	4.255	21.277	40.766	37.234
	MEAN	100	53.451	23.790	28.265	39.553	51.862

PERCENT OF INITIAL CONCENTRATION OF MICROORGANISMS REMAINING

*Trial A,B,C,D,E,F represent replicate experiments.

Table 8.

SALINITY: 18ppt

% OF INITIAL LEVELS REMAINING

		DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
E. coli	Trial A	100	46.016	40.743	[-EQUIPMENT FAILURE-]		
	Trial B	100	97.963	36.549	[-EQUIPMENT FAILURE-]		
	Trial C	100	60.860	113.200	17.116	46.915	5.026
	Trial D	100	114.919	28.271	67.622	54.556	36.944
	Trial E	100	2.202	2.045	0.879	0.404	1.271
	Trial F	100	2.220	4.376	0.542	1.189	0.589
	MEAN	100	54.030	37.531	21.540	25.766	10.957
S. fecalis	Trial A	100	18.345	19.482	[-EQUIPMENT FAILURE-]		
	Trial B	100	32.310	19.578	[-EQUIPMENT FAILURE-]		
	Trial C	100	16.596	26.277	5.059	10.254	4.279
	Trial D	100	36.669	7.311	30.520	41.765	12.937
	Trial E	100	10.743	10.820	4.911	1.507	1.086
	Trial F	100	7.118	9.978	0.381	3.168	0.644
	MEAN	100	20.297	15.574	10.217	14.174	4.736
PHAGE MS2	Trial A	100	94.380	21.344	[-EQUIPMENT FAILURE-]		
	Trial B	100	75.872	38.275	[-EQUIPMENT FAILURE-]		
	Trial C	100	98.429	48.334	17.745	44.239	39.187
	Trial D	100	51.460	38.299	28.343	26.433	72.705
	Trial E	100	55.254	49.153	20.881	13.966	30.508
	Trial F	100	28.588	21.921	4.339	11.601	18.983
	MEAN	100	67.330	36.221	17.827	24.060	40.346
POLIO	Trial A	100	91.897	361.173	[-EQUIPMENT FAILURE--]		
	Trial B	100	113.649	175.829	[-EQUIPMENT FAILURE--]		
	Trial C	100	45.545	24.087	52.587	22.003	26.527
	Trial D	100	42.425	206.176	42.387	35.653	94.775
	Trial E	100	13.963	5.992	30.218	6.753	6.708
	Trial F	100	62.046	10.321	1.016	13.506	5.470
	MEAN	100	61.588	130.596	31.552	19.479	33.370
HAV	Trial A	100	11.765	-------	[-EQUIPMENT FAILURE-]		
	Trial B	100	64.706	45.099	[-EQUIPMENT FAILURE-]		
	Trial C	100	10.526	31.579	19.297	78.947	28.072
	Trial D	100	33.335	13.158	8.421	21.053	2.105
	Trial E	100	50.000	21.213	15.153	3.333	20.513
	Trial F	100	40.600	20.833	7.140	20.800	16.667
	MEAN	100	35.155	26.376	12.503	31.033	16.839

PERCENT OF INITIAL CONCENTRATION OF MICROORGANISMS REMAINING

*Trial A,B,C,D,E,F represent replicate experiments.

Table 9.

SALINITY: 8ppt
% OF INITIAL LEVELS REMAINING

		DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
E. coli	Trial A	100	694.737	229.323	168.233	300.188	87.914
	Trial B	100	252.632	589.774	294.286	180.451	35.470
	Trial C	100	19.565	2.725	0.483	0.326	------
	Trial D	100	75.903	6.541	1.192	1.217	------
	Trial E	100	68.915	48.305	11.576	13.390	13.051
	Trial F	100	152.825	38.017	5.085	23.588	4.201
	MEAN	100	210.763	152.448	80.142	86.527	35.159
S. fecalis	Trial A	100	320.325	30.000	22.161	31.936	14.032
	Trial B	100	87.339	20.807	299.906	13.227	7.742
	Trial C	100	152.830	3.962	3.396	21.934	------
	Trial D	100	336.085	16.509	4.151	6.297	------
	Trial E	100	95.144	299.040	50.288	23.374	430.398
	Trial F	100	173.937	75.819	14.815	29.136	12.420
	MEAN	100	194.277	74.356	65.786	20.984	116.148
PHAGE MS2	Trial A	100	100.035	31.249	46.087	38.435	84.870
	Trial B	100	79.217	78.059	56.348	42.522	75.043
	Trial C	---	-------	------	------	------	------
	Trial D	---	-------	------	------	------	------
	Trial E	100	24.245	175.824	19.780	176.264	76.868
	Trial F	100	118.590	80.789	16.248	108.022	54.441
	MEAN	100	80.522	91.503	34.616	91.311	72.806
POLIO	Trial A	100	68.605	68.831	119.433	129.697	163.710
	Trial B	100	150.511	156.021	90.649	73.881	66.133
	Trial C	100	25.568	8.596	21.633	3.554	-------
	Trial D	100	45.148	9.406	4.663	6.920	-------
	Trial E	100	40.471	23.027	21.955	10.259	5.611
	Trial F	100	35.803	12.516	11.044	6.840	11.099
	MEAN	100	61.018	46.399	44.896	38.525	61.638
HAV	Trial A	100	9.996	27.001	12.000	16.873	6.432
	Trial B	100	257.158	54.374	15.001	5.628	<3.000
	Trial C	100	<16.640	<12.480	16.640	33.360	------
	Trial D	100	233.360	142.880	33.360	300.000	------
	Trial E	100	155.573	22.187	28.587	171.413	55.573
	Trial F	---	-------	-------	------	-------	------
	MEAN	100	<134.55	<51.784	21.118	105.455	<21.669

PERCENT OF INITIAL CONCENTRATION OF MICROORGANISMS REMAINING

*Trial A,B,C,D,E,F represent replicate experiments.

Figure 7. DEPURATION OF ORGANISMS AT 3 SALINITIES (ORGANISMS / GRAM OF CLAM TISSUE)

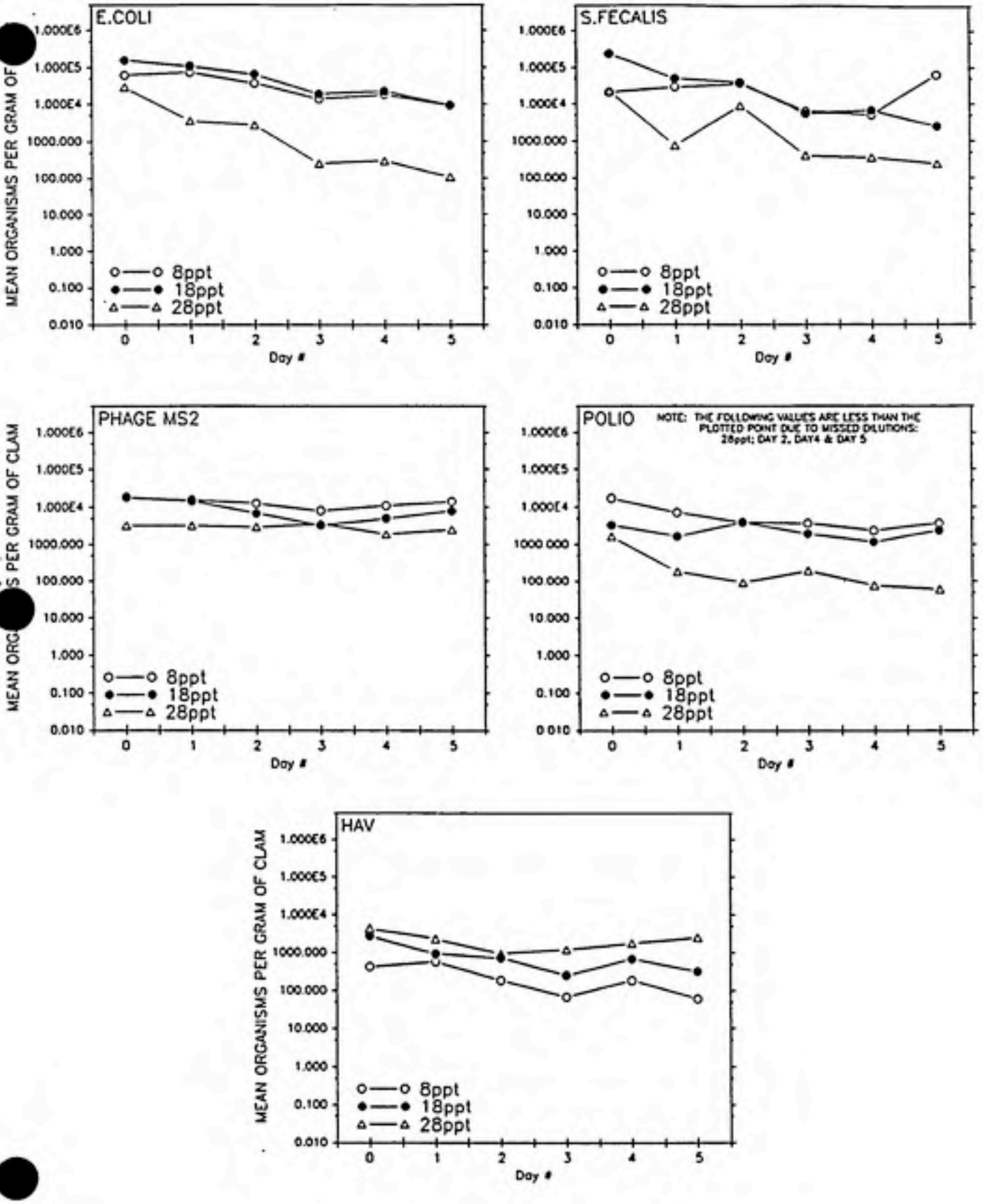


Figure 8. DEPURATION OF ORGANISMS AT 3 SALINITIES (MEAN % REMAINING)

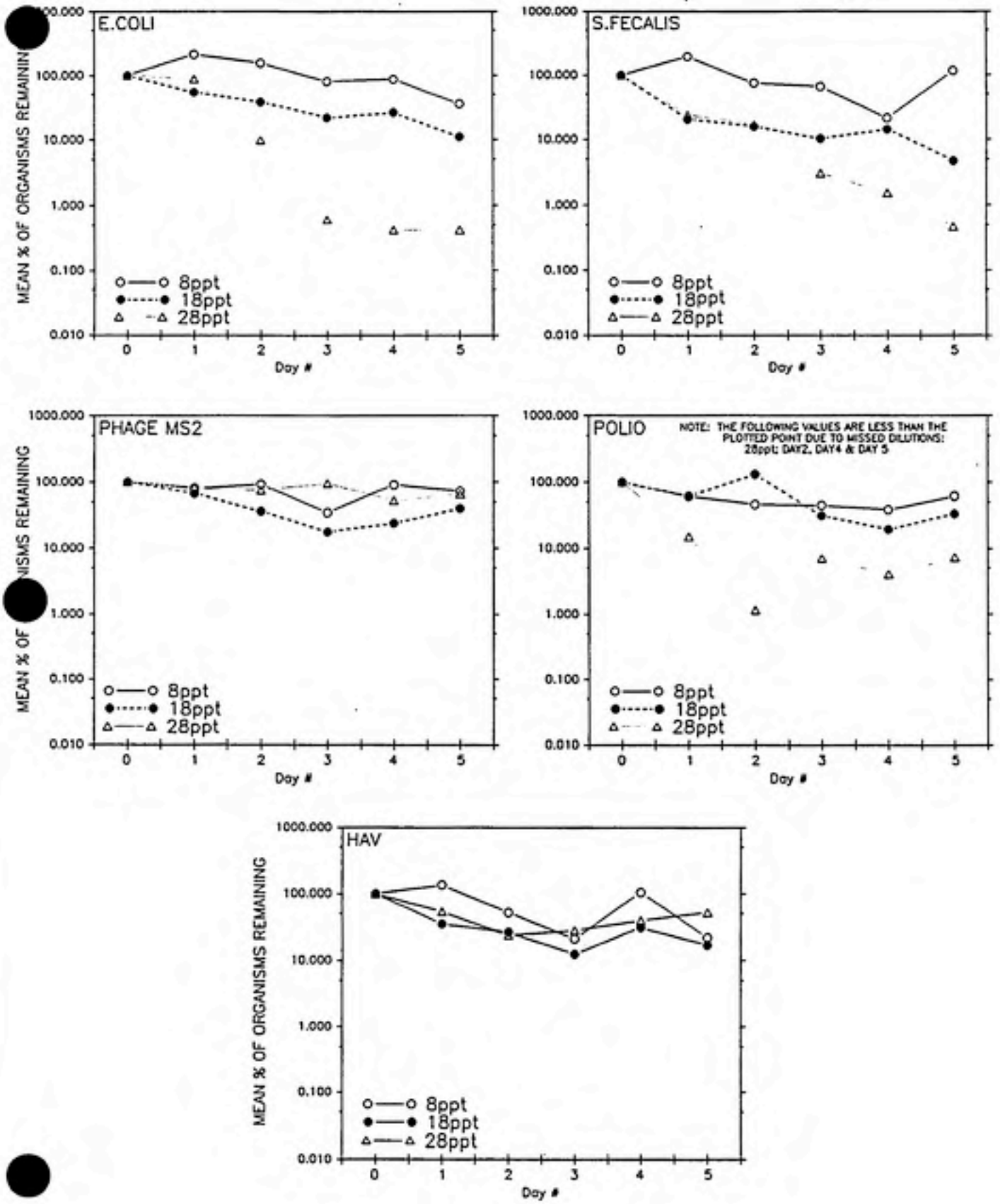
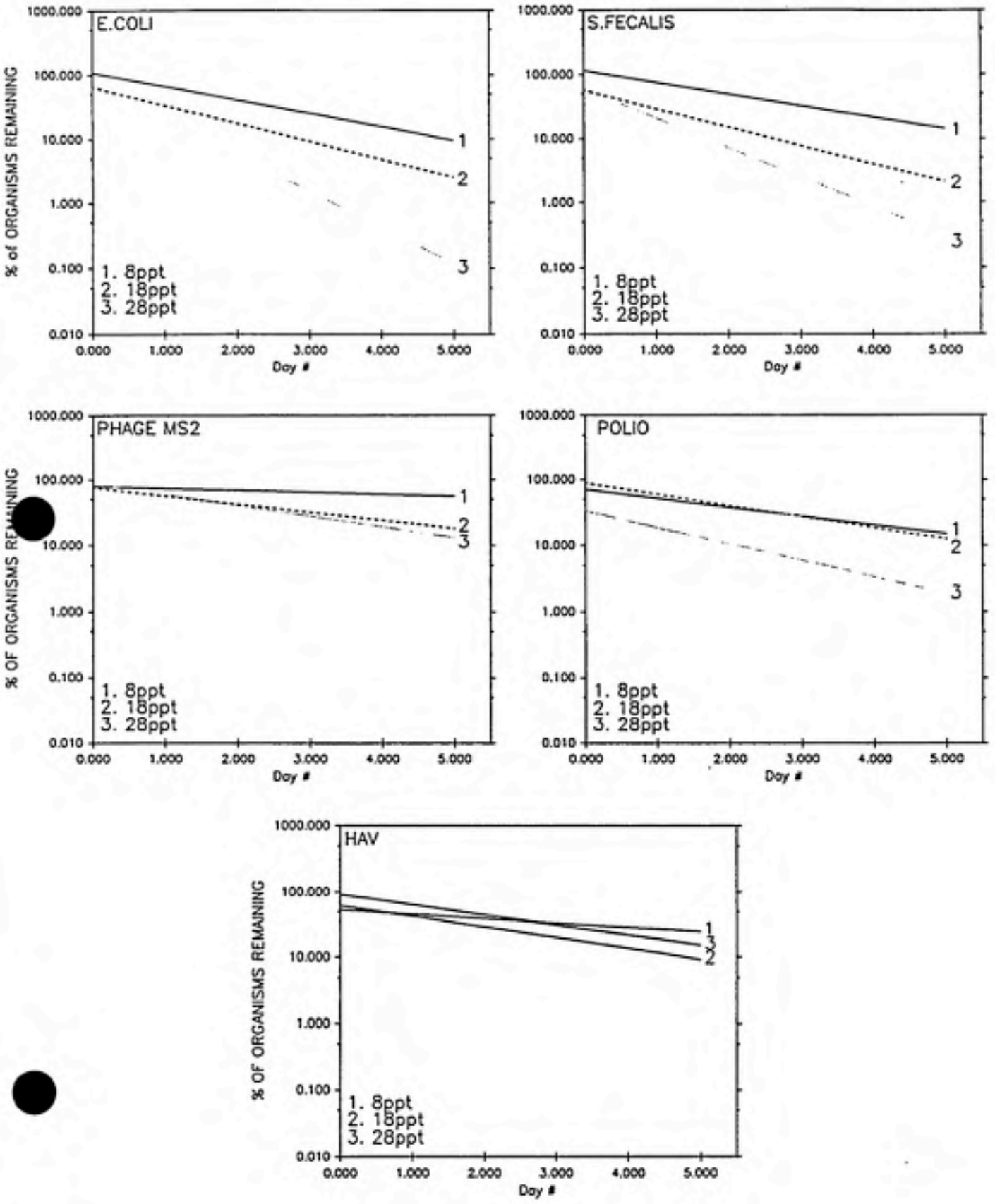


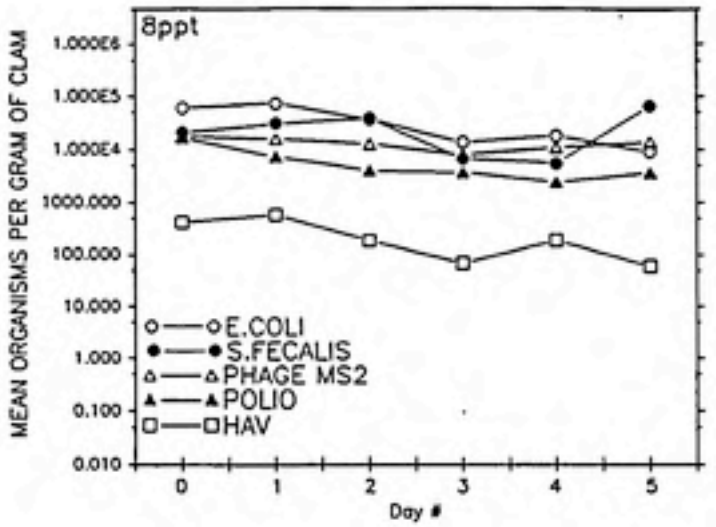
Figure 9. DEPURATION OF ORGANISMS AT 3 SALINITIES
REGRESSION LINES
(MEAN % REMAINING)

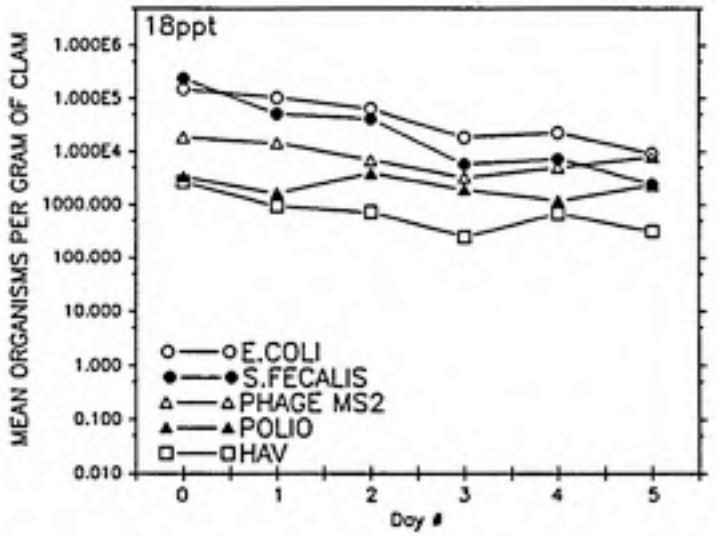


concentrations of both bacteria have decreased to less than 0.5% of the initial concentration while HAV has decreased to 51.8%, Polio to 6.1%, and MS2 only to 65.6% of the initial quantity (100%).

In the same manner as for the temperature experiments, the salinity data were then analyzed to determine if the organisms depurated at different rates at the three salinities. Figures 10-12 illustrate the effect of salinity on each test organism. At each salinity the same conclusion is reached as with temperature; the bacteria depurate at similar rates that differ from those of the viruses, which depurate at slower rates. The viruses also depurate at parallel rates. The distinction among the depuration rates can be most easily seen in figure 12 where the increased depuration of the organisms at 28 ppt results in a separation of the lines for percent initial organisms remaining rather than the cluster of depuration lines as seen in figures 10 and 11. This separation allows the parallelism of lines 3, 4, and 5, (the viruses) to be distinguished from lines 1 and 2 (the bacteria). The different behavior between viruses and bacterial after 5 days of clam depuration is clear: the test viruses are retained relative to the initial virus concentration with equal efficiency and are retained to a relatively greater extent than bacteria.

Figure 10. DEPURATION AT 3 SALINITIES OF ORGANISMS (ORGANISMS / GRAM CLAM TISSUE)





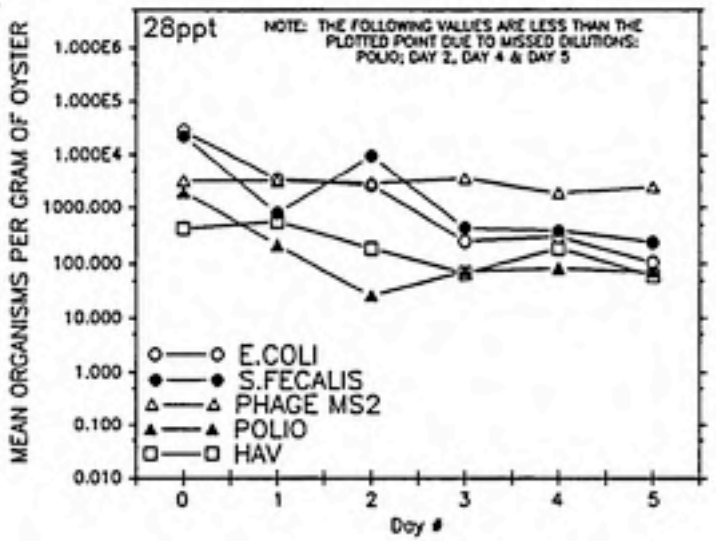
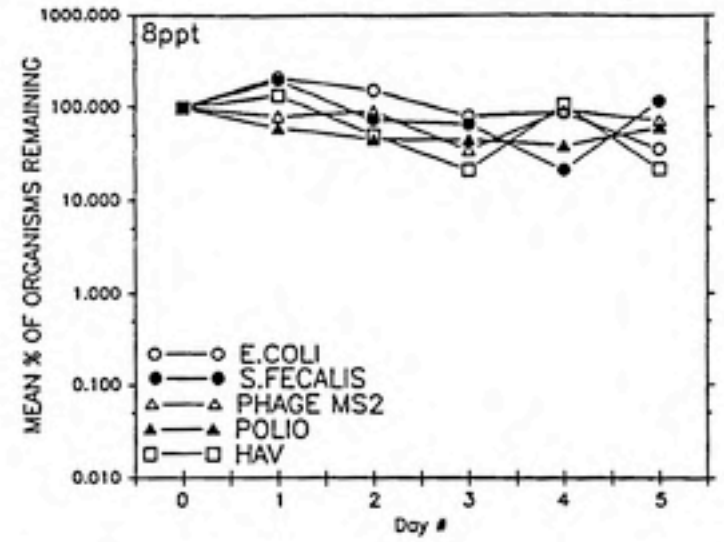
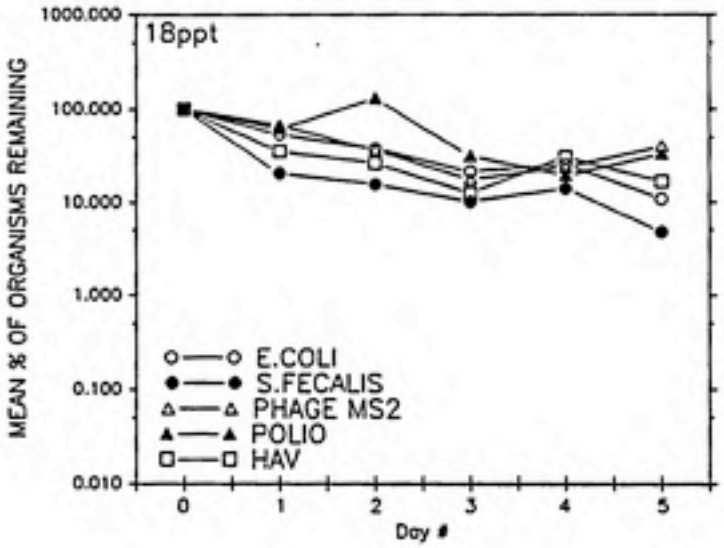


Figure 11. DEPURATION AT 3 SALINITIES OF ORGANISMS (MEAN % REMAINING)





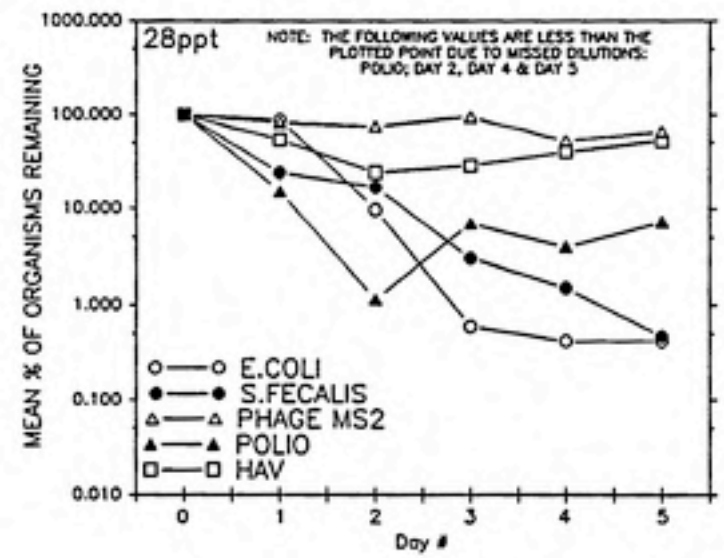
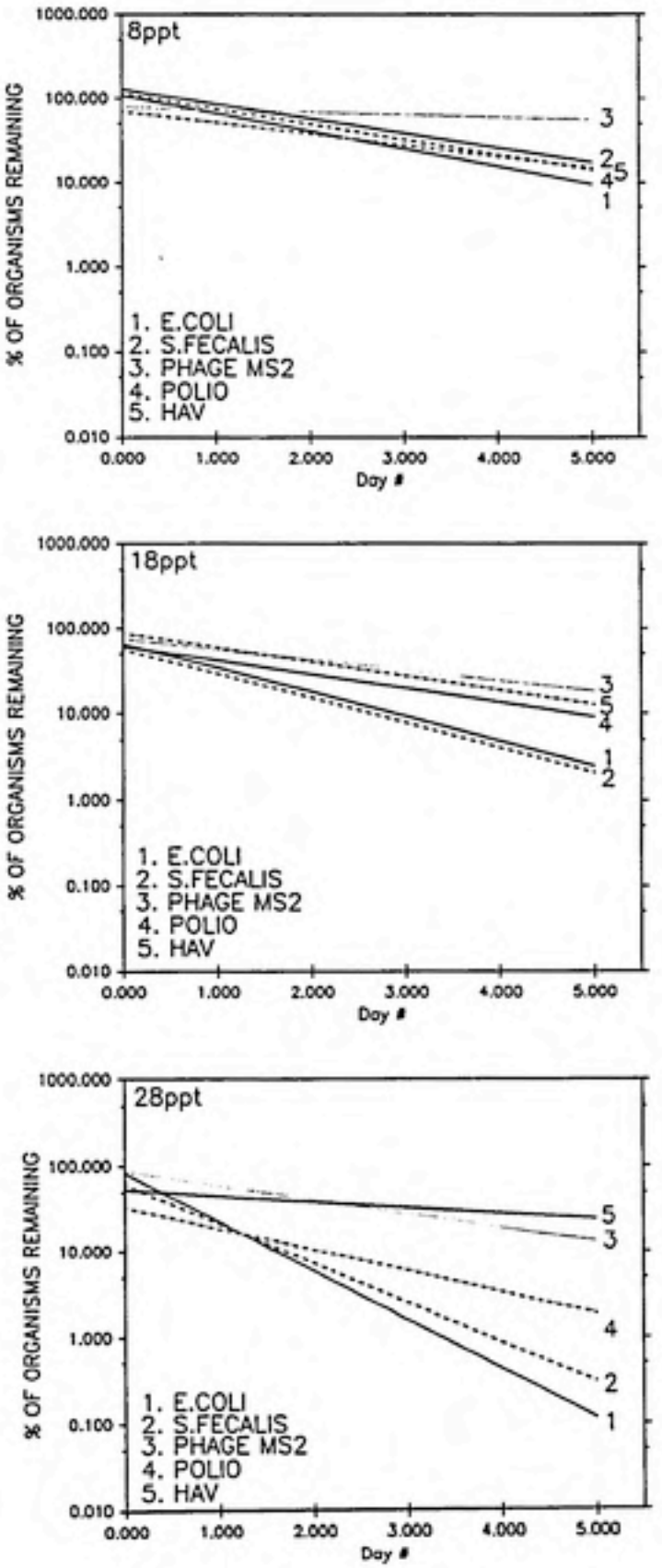


Figure 12. DEPURATION AT 3 SALINITIES OF ORGANISMS
REGRESSION LINES
(MEAN % REMAINING)



Examining the data for 2-3 days of depuration, it is noteworthy that only at salinity 28 ppt were the test organisms depurated to a significant degree. At salinities 8 and 18 ppt, less than a log reduction was observed in any of the organisms. After 3 days of depuration at 28 ppt, the same conclusions are observed as in the temperature experiments: that the bacteria have significantly depurated after 3 days while the viruses have not. After 3 days at 28ppt, E. coli and S. fecaelis have depurated to 0.60% and 3.03% of the initial levels, while MS2, polio, and HAV remain at relatively high levels of 94.7%, 19.2%, and 28.3% remaining, respectively. A 10-fold greater quantity of viruses than bacteria is detected after 3 days of depuration. After only 2 days of depuration, bacterial concentrations are not as greatly reduced, so the differences in the depuration rates between the bacteria and viruses is not as obvious. The bacteria are not depurated to an acceptably low level and therefore a third day of depuration would be mandated. In general, however the depuration rate differences among the organisms after 2-3 days of depuration illustrate the same results as discussed above for 5 days of depuration; bacteria depurate appreciably more than viruses.

The statistical analysis of the salinity data are shown in Tables 10 - 12. E. coli and S. fecaelis depurate at equal rates (coincident lines, $p > 0.25$). The bacteria have

Table 10

SUMMARY OF LINEAR REGRESSION FOR SALINITY EXPERIMENTS

Sal.	org.	slope	Intercept	R
8ppt	E. coli	-0.2107	2.0366	-0.4022
	S. faecalis	-0.1814	2.0606	-0.5040
	MS2	-0.0292	1.8953	-0.1849
	Polio	-0.1338	1.8474	-0.4307
	HAV	-0.1562	1.9573	-0.4712
18ppt	E. coli	-0.2830	1.8156	-0.5621
	S. faecalis	-0.2870	1.7546	-0.7368
	MS2	-0.1240	1.8770	-0.6188
	Polio	-0.1679	1.9423	-0.5043
	HAV	-0.1650	1.7871	-0.6367
28ppt	E. coli	-0.5576	1.8578	-0.8676
	S. faecalis	-0.4611	1.7846	-0.8786
	MS2	-0.1638	1.9393	-0.4020
	Polio	-0.2480	1.5184	-0.5664
	HAV	-0.0681	1.7217	-0.2889

TABLE 11

EFFECT OF DIFFERENT SALINITY ON ORGANISMS

ORGANISM	SAL	P VALUE	RESULT
E. coli	8 vs 18	>.05	PARALLEL
S. faecalis	8 vs 18	>.05	PARALLEL
MS2	ALL	>.05	ALL COINCIDENT
Polio	ALL	>.05	ALL PARALLEL
HAV	ALL	>.05	ALL PARALLEL

*CONCLUSION=HIGHER SALINITY EFFECTS DEPURATION RATES OF BACTERIA
=8 AND 18 ppt SALINITY BEHAVE THE SAME FOR BACTERIAL DEPURATION
=SALINITY HAS NO EFFECT ON VIRAL DEPURATION

TABLE 12

EFFECT OF SALINITY ON EACH ORGANISM

MODEL SALINITY = 28ppt

ORGANISM	P VALUE	RESULT	
E. coli vs S. faecalis	>.25	COINCIDENT	+
vs MS2	<.001	NOT COINCIDENT	
	<.001	NOT PARALLEL	
vs Polio	<.001	NOT COINCIDENT	
	<.001	NOT PARALLEL	
vs HAV	<.001	NOT COINCIDENT	
	<.001	NOT PARALLEL	
S. faecalis vs MS2	<.001	NOT COINCIDENT	
	<.001	NOT PARALLEL	
vs Polio	<.01	NOT COINCIDENT	
	<.01	NOT PARALLEL	
vs HAV	<.001	NOT COINCIDENT	
	<.001	NOT PARALLEL	
MS2 vs Polio	<.001	NOT COINCIDENT	
	>.25	PARALLEL	*
vs HAV	>.25	COINCIDENT	*
Polio vs HAV	<.001	NOT COINCIDENT	
	>.10	PARALLEL	*

CONCLUSION = + E. coli and S. Feacalis are coincident.
* MS2, Polio, and HAV are parallel.

parallel depuration rates at salinities 8 and 18 ppt ($p > 0.05$). At salinities 8 and 18 ppt, bacterial depuration rates were similar (slope = -0.18 - -0.29) however at 28 ppt there was significantly greater depuration of test organisms (slope = -0.46 - -0.56). The virus depuration rates were not effected by salinity: depuration rates of each test organism were similar at all salinities (slope = -0.06 - -0.24). All the viruses also depurated at rates parallel or coincident to each other (all lines coincident or parallel, $p > .05$). (fig. 12).

IV. B. Uptake Experiments

The results from the three replicate uptake experiments are shown in Tables IV.13 and 14. The results are also depicted graphically in figures IV.13 and 14. The three uptake replicates were conducted under the same conditions: salinity = 28 ppt, temperature = 25°C, turbidity = < 0.1 NTU, pH 8, and a dissolved oxygen content = > 5 mg/l. The quantity of test organisms inoculated into the water were targeted to achieve concentrations of approximately 10^{12} infectious units per milliliter (I.U./ml) for MS2 and 10^{9} I.U./ml for polio and HAV.

The concentration of MS2, polio and HAV found in clams at each sampling time is expressed as PFU/gr and the initial concentration in the water is reported as PFU/ml.

Table 13.

UPTAKE OF MS2, POLIOVIRUS, AND HEPATITIS A VIRUS BY CLAMS
(plaque forming units/ gram clam tissue)

	MS2	POLIOVIRUS	HAV
exp. 1			
0 hr	175.	0.0	0.0
1 hr	393000	150	150
3 hr	816000	939.4	0.0
9 hr	781000	782.6	680
18 hr	2673000	303	818
30 hr	999000	212	100
water (counts/ml)	226000000	2333000	5416.7
exp. 2			
0 hr	5090.1	0.0	0.0
1 hr	11525	250	50
3 hr	21085.6	750	250
9 hr	345455	50	318.2
18 hr	100500	1250	909
30 hr	136818	50	545
water (count/ml)	1770000	90000	10909
exp. 3			
0 hr	50	0.0	0.0
1 hr	310000	250	0.0
3 hr	370000	730	625
9 hr	28000	50	187.5
18 hr	29000	1650	687.5
30 hr	18000	450	250
water (counts/ml)	2570000	7000	10900

Table 14.

UPTAKE OF MS2, POLIOVIRUS, AND HEPATITIS A VIRUS BY CLAMS
(percent remaining relative
to initial organism concentration in water)

	MS2	POLIOVIRUS	HAV
exp. 1			
0 hr	0.0000774	0.0	0.0
1 hr	0.174	0.0064	0.92
3 hr	0.36	0.0403	0.46
9 hr	0.345	0.0335	12.2
18 hr	1.18	0.13	15.1
30 hr	0.44	0.0091	1.85
exp. 2			
0 hr	0.29	0.0	0.0
1 hr	0.65	0.28	0.46
3 hr	1.19	0.83	2.3
9 hr	19.5	0.06	2.9
18 hr	5.68	1.4	8.3
30 hr	15.6	0.06	5.0
exp. 3			
0 hr	0.002	0.0	0.0
1 hr	12.1	3.6	0.0
3 hr	14.4	10.4	5.7
9 hr	1.1	0.71	1.7
18 hr	1.13	23.6	6.3
30 hr	0.70	6.4	2.3
Mean % remaining of replicate experiments			
0 hr	0.10	0.0	0.0
1 hr	4.3	1.3	0.46
3 hr	5.3	3.8	2.82
9 hr	6.99	0.80	5.6
18 hr	2.66	9.3	9.9
30 hr	5.58	2.2	3.1

Figure 13.

MS2, POLIO, and HAV UPTAKE BY CLAMS

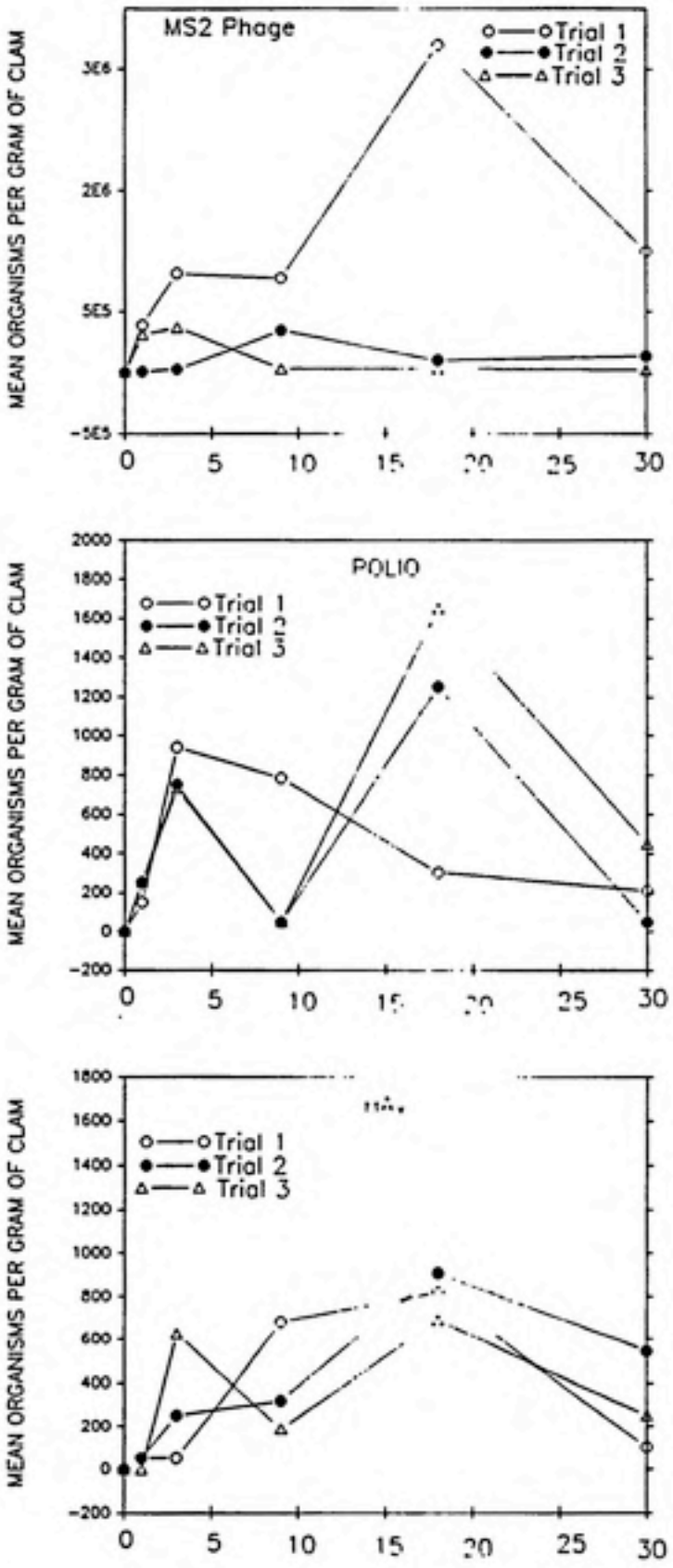
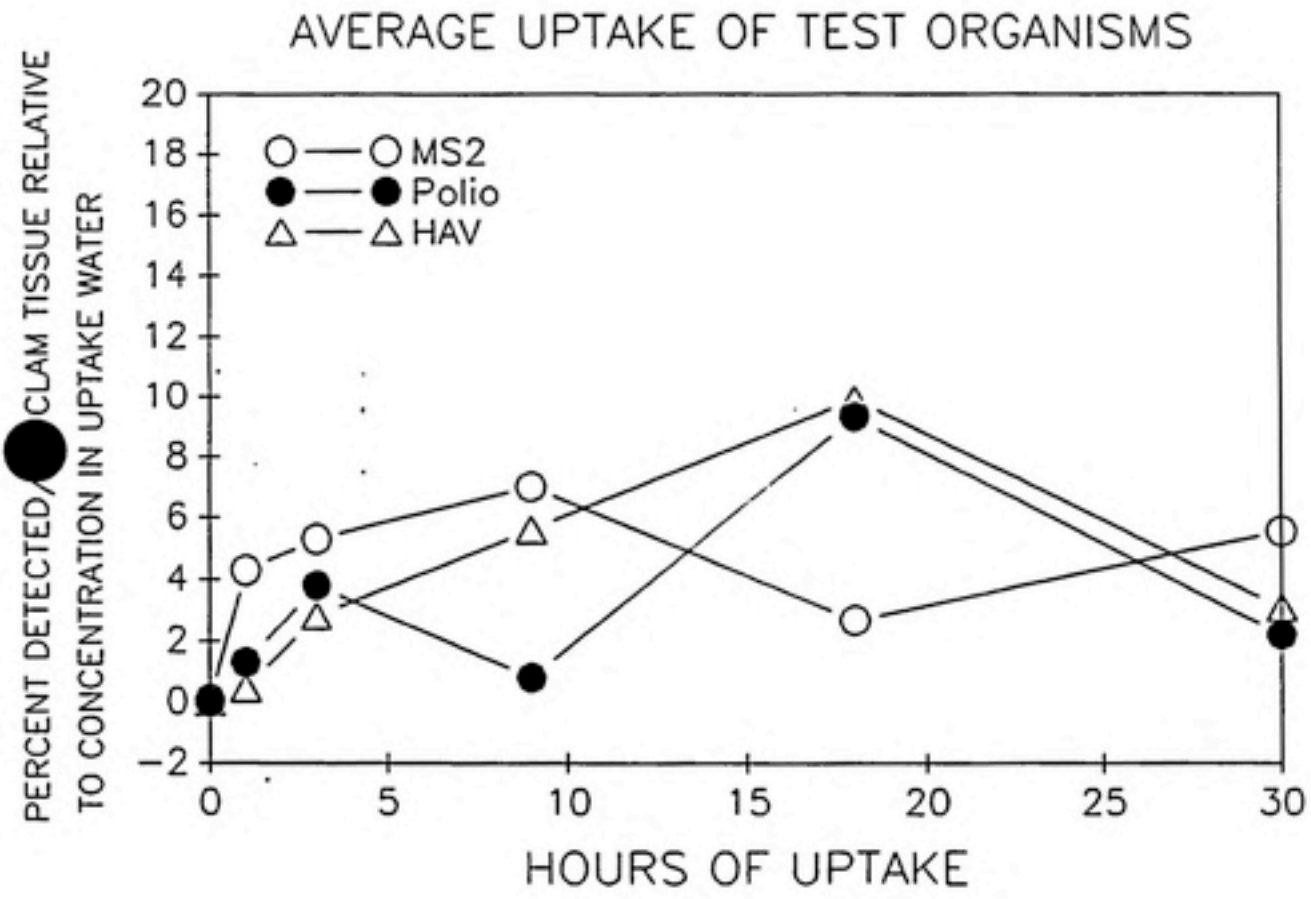


Figure 14.


AVERAGE UPTAKE OF TEST ORGANISMS
PERCENT DETECTED/CLAM TISSUE RELATIVE TO CONCENTRATION IN UPTAKE WATER
MS2
Polio
HAV
20
18
16
14
12
10
8
6
4
2
0
-2
0
5
10
15
20
25
30
HOURS OF UPTAKE

As shown by the results in table 14, the concentration of each organism in clams increases as a function of time in each replicate experiment reaching a maximum ranging from 3-18 hr and then declining thereafter.

For experiment 1 the maximum uptake was at 18 hours for MS2 and HAV with concentrations of 2.7 X 10^6 PFU/ml and 818.2 PFU/ml respectively. Polio was recovered at a maximum concentration at 3 hr, with a concentration of 939.4 PFU/ml. In experiment 2, MS2 has maximum uptake at 9 hr (345455 PFU/ml), polio at 18 hr (1250 PFU/ml), and HAV at 18 hr (785.7 PFU/ml). Experiment three has maximal uptakes at 3 hr for MS2 (3.7 X 10^5PFU/ml), at 18 hr for polio (1650 PFU/ml), and at 18 hr for HAV (687.5 PFU/ml). In summary, MS2 was maximally recovered at 3, 9, or 18 hr, polio at 3 or 18 hr, and HAV at 18 hr in the replicate experiments.

In order to compare the uptake of the organisms by the clams, the data for virus concentrations at each exposure time were normalized by dividing these numbers by the initial water concentration of the respective virus and multiplying by 100. This calculation gives the percentage of the virus in the clams relative to the initial concentration in the water (Table IV.14).

The mean relative maximum uptake of MS2 is at 9 hr (6.99%), polio at 18 hr (9.3%), and HAV at 18 hr (9.9%). Polio and HAV are taken up to maximal levels in the same time frame but MS2 had the greatest level of uptake relative

to the concentration in water at 9 hr. This may be due to the greater initial MS2 concentration in the uptake water than polio or HAV. If however there are significant differences in the uptake and retention of MS2 and the human enteric viruses then MS2 would be an unacceptable indicators of viral contamination.

Public health safety can not be assured without using an indicator that behaves kinetically similar to human viral pathogens in both uptake, persistence, and depuration in shellfish.

V. DISCUSSION

V.A. DEPURATION

V.A.1. Overview of Findings

Contaminated shellfish continue to cause infectious hepatitis A and viral gastroenteritis outbreaks in the U.S. and around the world, despite the existence of bacteriological criteria and standards for them and their harvest waters. Therefore, the current operational and bacteriological standards for commercial depuration are being questioned and reevaluated. Studies such as the present one are designed to determine what factors are important to consider in establishing new or improved depuration criteria, setting new standards and determining if the current criteria for standards are acceptable. Only with such studies will it be possible to fully understand the public health aspects of shellfish depuration. With improved understanding, it should be possible to reduce shellfishborne viral disease and further ensure public safety for this food commodity.

The results of this study indicate that viruses persist longer in depurated clams than the test indicator bacteria. Interestingly, temperature had no effect on the rates or efficiency of viral and bacterial depuration, but for bacteria, depuration was a function of salinity. High salinities caused bacterial depuration in the hard shell

clam to proceed more rapidly. The depuration of viruses was not affected by salinity.

The viruses showed a consistent, statistical difference in depuration from E. coli and S. fecaelis, with the rate of viral depuration slower than bacteria at all temperatures and salinities. E. coli and S. fecaelis were not useful as indicators of viral reduction under the depuration conditions studied. These results are consistent with previous studies which found no relationships between the elimination of fecal coliforms and viruses either in a depuration system or in relaying (Power et al, 1989; Canzonier, 1971; Cook and Ellender, 1986). In the present study it was found that under the study conditions, HAV, polio, and MS2 were depurated at statistically equivalent rates. The findings are different from those of earlier studies comparing the reduction of HAV and poliovirus by Eastern oysters (Sobsey et al, 1987). In that study, poliovirus was rapidly reduced in 2-3 days and HAV persisted in oysters for up to 5 days of depuration. This may be simply explained by differences in shellfish species.

The present study indicated that HAV and the other test viruses persisted extensively even after 5 days depuration, while E. coli and S. fecaelis were reduced at more rapid rates. The bacteria were reduced to levels of <0.5% of their initial concentration after 5 days of depuration under all test conditions. These results have important

implications for current and proposed depuration practices. Operators and regulators of commercial depuration facilities need to be aware of the findings that even after the indicator organisms (E. coli and other fecal coliforms) are depurated to acceptably low levels, there may still be a potential public health risk due to the considerable persistence of HAV and other enteric viruses.

The results of this study indicated that the rate of depuration for enteric bacteria and viruses was not dependent on temperature. The lack of a temperature effect may be because the range of study temperatures were well within the tolerance limits of the clams. The study temperatures were above 7°C, the temperature reported to cause 'no shellfish metabolic activity' (Metcalf, 1987; Metcalf and Stiles, 1968), and high temperatures (above 31°C) have not been reported as having any adverse effect on viral and bacterial depuration (Cook and Ellender, 1986). The low temperatures may not have adversely affected microbial reduction because the clams were acclimatized to cooler water temperatures due to the season of the year. The lower temperature experiments were conducted in early summer,and therefore, the clams may have been acclimatized to water temperatures similar to that of the study conditions. If this was the case, then no temperature stress was induced and the clams could depurate efficiently.

Depuration of enteric bacteria was affected by salinity, with greater depuration at the high salinity (28ppt) than at lower salinities (8 and 18 ppt). However, this was not the case with the viruses, which showed no appreciable effect on depuration rates due to salinity. Reduced shellfish activity or inactivity at lower salinities may at least partially explain the differences in the depuration behaviors of bacteria and viruses. Bacterial depuration did decrease at the lower salinities compared to higher salinity, thus suggesting a salinity effect. This may be due to poor survival of bacteria at higher salinities. Overall however clams depurate bacteria more efficiently than viruses.

U.S. commercial depuration standards allow for a range of conditions with respect to temperatures (10-25^{o}C) and salinities (within 20% of the harvest site). These ranges of conditions need to be tested for viral and bacterial depuration by each species of shellfish since the findings of different studies vary with respect to the effects of lower temperatures and salinities (Bachur, 1988; Cook and Ellender, 1986; Metcalf, 1987; Liu et al, 1967; Rowse and Fleet, 1984). Previous studies found that stresses at unfavorable temperature and salinity conditions caused ineffective viral and bacterial reductions. Thus, commercial depuration under some operating conditions may lead to inefficient shellfish cleansing.

In the present study, reductions of low initial levels of enteric bacteria and viruses were not examined. Thus, it is possible that at more realistic, naturally occurring levels of contamination, clams may require shorter depuration periods. However, the results indicate that at some conditions the time for effective viral reduction may be very long, even at low initial virus levels. This may be a result of viruses becoming sequestered in tissues away from the elimination mechanisms (Cook and Ellender, 1986). These viruses are then protected and may persist even after long depuration periods. This study also indicates that high initial levels of contamination are clearly undesirable because of inefficient viral depuration. Thus, shellfish destined for depuration should be harvested only from lightly contaminated (ie restricted) waters.

V.A.1. Effect of Temperature on Organism Depuration

This study evaluated the reduction of HAV, polio, MS2, E. coli, and S. faecalis at three temperatures (12, 18, 25^{o}C). These temperatures are within the U.S. water quality standards for clams, which require commercial depuration water to be in the range of 10-25^{o}C (FDA, 1989).

Overall, these studies have shown that temperature does not appreciably effect the rate or efficiency of bacterial or viral depuration by clams. Each organism studied depurated at statistically equivalent rates at all

temperatures. However, bacteria depurated at a significantly faster rate than viruses. After 5 days of depuration, the bacteria were reduced to less than 1% of their initial concentration under each test condition but the percentage of initial virus remaining ranged from 4-75%. HAV persisted at levels of 17-59% of the initial concentration. This high level of virus persistence poses a potential health risk that would not be detected using current bacterial indicators of depuration effectiveness.

The results of this study indicate that the current range of water temperatures is adequate for bacterial depuration by hardshell clams, although effective virus depuration can not be assured at any temperature.

V.A.2. Effect of Salinity on Organism Depuration

This study also evaluated the elimination of HAV, polio, MS2, E. coli, and S. fecaelis at three salinities: 8, 18, and 28 ppt. Although, the salinities are within the range experienced in the environment and perhaps used commercially, no effort was made to restrict test salinities to +/- 20% of the salinities of the waters from which the clams were harvested. The water quality standards of U.S. depuration regulations require that the salinity be within 20% of the harvest area. The salinities encountered by the study clams in the waters from which they were harvested were 28 - 36ppt.

The results of the salinity experiments generally indicated that the salinity of the depuration water had an effect on the ability of clams to reduce bacteria; however, virus elimination was not affected by salinity.

In this study, the bacteria depurated at a statistically greater rate than the viruses at all test salinities. While bacteria depurated to <12% of the initial concentration, the virus concentration remained at 6 - 73% of the initial concentration. These results are supported by those of Power and Collins (1990) who found that *E. coli* was reduced by 99.99% while coliphage reduction was relatively inefficient under the same conditions.

In this study bacteria elimination was less effective at lower salinities (8 and 18 ppt) than at 28 ppt. This decrease in elimination may be due to the shellfish not being acclimatized to the salinity of the depuration water. During the present study the ambient harvest area salinity was between 30 - 35 ppt. The depuration at 28 ppt more closely simulated the clams natural environment, and therefore metabolic functions may have not been stressed. These results are supported by findings in the previously published literature. Liu *et al* (1967b) showed that a salinity decrease of 50 - 60% stopped depuration completely in the Northern quahaug. In addition, Galtsoff (1964) found that oyster activity was reduced when salinities were different by 10% from the ambient harvest area salinity.

Rowse and Fleet (1984) reported reduced pumping and elimination from the Sydney Rock oysters at lower salinities (16-20 ppt).

Viruses showed no decrease in depuration rate with decreased salinity but they were depurated poorly at all salinities tested. These findings are in contrast to those of previous studies, which report HAV to be eliminated by oysters more effectively at a salinity 28 ppt than at 8 or 18 ppt (Sobsey et al, 1987). Furthermore, the previous study was conducted 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 in actual depuration plants.

The reductions in bacteria that occured at low salinities may possibly be related to the dilution of natural seawater to lower salinities, which could have diluted natural antagonists present in the seawater. Poor viral elimination and reduced bacterial elimination at low salinities may have been the result of increased attachment of enteric microbes to the shellfish mucus. Di Girolamo et al (1977) showed that ionic bonding to shellfish mucus by poliovirus was increased at low salinities. This bonding provided both protection from inactivation and resistance to elimination. Therefore, decreased depuration at lower salinities may have been a consequence of increased survival.

From the results presented, it appears that the use of waters having lower salinities in a depuration plant may lead to ineffective depuration of indicator bacteria. Despite this effect, however, these bacteria do not act as an adequate indicator for viral reduction, in any case. In order to assure maximum bacterial depuration, salinities should be maintained above a minimum level, specific to the shellfish type and the harvest environment at the time of harvest. Thus, it is also necessary to determine an indicator that accurately models viruses under all depuration conditions and to identify conditions under which viruses are depurated efficiently by clams.

V.B. UPTAKE

In order to determine whether HAV, polio, and MS2 accumulate at the same rate with the same efficiency in the normal feeding process of the clams, three replicate uptake experiments were performed as described in Chapter III.

In interpreting the data for these experiments, it is assumed that each clam is feeding and that they are feeding at the same rate. It is further assumed that the viruses are randomly distributed with equal exposure to all clams. Finally it is assumed that no virus is lost due to adsorption to the tank or clam shells.

Over the 30 hour experimental uptake period, all viruses reached a maximum concentration and then began to

decline. The maximum time for HAV and polio was 18 hours while MS2 reached a maximum at 9 hr. These results suggest that HAV and polio and possibly MS2 may uptake by clams kinetically similar and therefore polio or MS2 may serve as an adequate model for HAV uptake.

Finally no concentration was seen in the shellfish tissue above the the concentration in the water. This may be due to the short uptake period relative to that of natural feeding in slightly contaminated waters. Also the literature reports that optimal conditions are necessary for viruses to concentrate in shellfish tissues above the concentrations in the water (Duff, 1967; Hoff and Becker, 1969; Di Girolamo et al, 1975). In particular, bioconcentration of viruses and other microbes occur when shellfish are in a dynamic or flow through hydraulic condition. There also appears to be a threshold above which no uptake occurs; at this point elimination balances uptake. Thus, the lack of concentration may be due to the clams excreting the viruses back into the water at the same time they were feeding, especially in a static uptake system. A longer uptake period with lower initial titers and a flow through system may have resulted in the previously reported virus concentration. Thus, this difference in uptake efficiency may be due to differences in experimental design. Where a flow through system was used, concentration effect was observed (Hamblet, 1969; Mitchell et al, 1966;

Canzonier, 1971). This study, which used a static uptake system, and other studies utilizing a static system have also not observed virus concentration above ambient levels in the water (Keating, 1985; Liu et al, 1966; Meinhold, 1982; Werner, 1983).

V.C. Factors Contributing to Experimental Variation

Several sources of error may have contributed to the experimental variation seen in this study. Although study conditions were held constant in regard to factors such as pH, turbidity, dissolved oxygen, other biological variations such as clam size, age, sex, and reproductive activity were not controlled for in this study. These factors may have affected the ability of the clams to depurate. The individual variation could then result in large differences in the averaged concentrations of organisms when comparing among shellfish samples (Seraichekas et al, 1968). In the data of this present study, there is occasionally an apparent increase in the percent of the initial microbes remaining or an increase above the initial levels (taken as 100%). The variability in the concentrations of organisms observed in the individual shellfish samples is probably the result of pooling small numbers of shellfish in these samples and/or of variable bacteria recovery and generally

low and variable efficiency in viral recovery and detection.

Different methods of virus recovery were used in this study. In the depuration experiments, the UNC method of virus recovery from clam tissue was used for polio detection, while for MS2, HAV, and the indicator bacteria detection utilized direct plating of a 1:2 mixture of homogenized tissue with 0.3M NaCl was used. In the uptake experiments both HAV and polio were detected using the UNC method. The differences in the recovery and detection methods may obscure the true virus concentrations in the tissue and make accurate comparisons difficult. It is clear, however, that a more consistent and efficient recovery and detection method would strengthen the conclusion that bacterial indicators do not model virus reductions.

Another factor that may have resulted in uptake experimental variations is that MS2 was inoculated with a higher initial titer than the enteric viruses. This difference may have caused differential clam uptake and subsequent depuration behavior for the viruses.

Additionally, results of the individual replicate experiments varied considerably with the maximum uptake times varied drastically among the replicate experiments. In order to more reliably determine the factors affecting uptake and uptake kinetics more replicates need to be conducted.

The extent of the initial contamination (uptake of contaminants) at 'day 0' may also have influenced depuration. Although the clams were allowed to uptake bacteria and viruses for similar times, the extent of contamination was drastically different between some replicate experiments. Previous studies have shown the effectiveness of depuration is influenced by the extent of contamination (Cook and Ellender, 1986; Metcalf et al, 1980; Son and Fleet, 1980). Longer depuration is required for heavily contaminated shellfish. However this was not consistently the case in this study.

Fluctuation or variability is seen throughout the temperature and salinity experiments as well as the uptake experiments. Such variability may prove important in improving depuration criteria and standards. If it is clear that not all shellfish uptake and depurate viruses and other pathogens identically, then it will be necessary to incorporate safety factors that will ensure adequate time and conditions for depuration, despite such variability. Appropriate research on candidate indicators will also be necessary to continue the search for adequate viral indicators.

V.D. Implications for Commercial Depuration

The results of these experiments indicate that bacteria do not act the same as viruses in clams. The virological quality of the depurated clams and resulting risk of disease was not adequately reflected by the behavior of indicator bacteria. The results of this study have demonstrated that depuration based on the reduction of fecal coliforms may not render the shellfish acceptable with respect to enteric viruses. This finding is supported by the reports of hepatitis A and gastroenteritis resulting from consumption of depurated shellfish (Grohmann et al, 1981,; Gill, 1983). While it is clear that E. coli is a good indicator of bacterial behavior in shellfish, it is also apparent that it is an inadequate indicator for HAV and other enteric viruses. Organisms such as MS2 or polio may prove to be a more reliable alternative for indicating the behavior of viruses in clams and other shellfish.

This study showed that viruses were not as readily depurated as bacteria. In all experiments the test bacteria were reduced at a faster rate and more extensively than were the viruses. Further research is needed to determine an indicator that models enteric viruses in shellfish under a variety of conditions. Only with an adequate indicator can depuration provide shellfish products of acceptably low risk of viral contamination.

Whether or not laboratory scale depuration systems can adequately portray viral depuration behavior in a commercial

setting is uncertain. In this study shellfish were contaminated with high levels of virus, while naturally contaminated shellfish, such as those commercially depurated, typically contain low levels of viruses and other pathogens. Also, only a single layer of shellfish were depurated in this study, while commercial plants depurate large, densely packed tanks or baskets containing multiple layers of shellfish. Thus, there is thus a need for pilot scale depuration studies.

Until a reliable indicator of viral contamination of shellfish is identified, depuration may not be an effective means of eliminating the public health risk of shellfish-associated viral disease. Preventing fecal pollution from entering coastal waters and shellfish resources is a more direct and fool-proof solution. However, given the unlikelihood of a total cessation of sewage discharge into estuarine waters, perhaps shellfish farming or agriculture under controlled conditions is the appropriate direction for future commercial shellfishing.

CONCLUSIONS

* HEPATITIS A VIRUS, POLIOVIRUS, and MS2 BACTERIOPHAGE ARE NOT REDUCED AS READILY AS BACTERIA FROM EXPERIMENTALLY CONTAMINATED CLAMS

* FECAL COLIFORMS SUCH AS E. COLI AND FECAL STREPTOCOCCI (ENTEROCOCCI) SUCH AS S. FAECALIS DO NOT ADEQUATELY INDICATE THE DEPURATION BEHAVIOR OF VIRUSES SUCH AS HAV

* WATER TEMPERATURE IN THE RANGE OF 10 TO 25°C DOES NOT APPRECIABLY EFFECT VIRAL OR BACTERIAL DEPURATION IN CLAMS

* LOW WATER SALINITY (8 and 18 ppt) LOWERS THE RATE OF BACTERIAL ELIMINATION FROM CLAMS BUT HAS NO EFFECT ON VIRAL REDUCTIONS WHICH ARE POOR AT LOW, MEDIUM, AND HIGH SALINITIES

* DEPURATION MAY NOT CURRENTLY BE AN EFFECTIVE MEANS OF ELIMINATING VIRAL PATHOGENS FROM CONTAMINATED SHELLFISH

* PUBLIC HEALTH RISKS OF SHELLFISH-ASSOCIATED VIRAL DISEASE ARE NOT ADEQUATELY INDICATED BY FECAL COLIFORMS

* PUBLIC HEALTH RISKS OF SHELLFISH-ASSOCIATED VIRAL DISEASE ARE NOT ADEQUATELY CONTROLLED BY DEPURATION

* BETTER INDICATORS OF VIRUSES NEED TO BE IDENTIFIED AND IMPLEMENTED INTO SHELLFISH REGULATORY PROGRAMS

ADDITIONAL RESEARCH

DEPURATION

* TO BETTER DEFINE THE EFFECTS OF TEMPERATURE AND SALINITY ON DEPURATION OF CLAMS BY PERFORMING MORE REPLICATE EXPERIMENTS WITH MORE RELIABLE VIRUS DETECTION METHODS
* TO STUDY THE EFFECTS OF OTHER ENVIRONMENTAL AND BIOLOGICAL FACTORS ON DEPURATION RATES
* TO COMPARE RATE OF HAV DEPURATION TO OTHER ENTERIC VIRUSES, FECAL COLIFORMS, AND OTHER POTENTIAL INDICATORS
* TO DETERMINE THE PERSISTENCE AND BEHAVIOR OF LOW LEVELS OF VIRAL CONTAMINANTS IN DEPURATION OF SHELLFISH
* TO DETERMINE THE OPTIMUM DEPURATION TIME REQUIRED TO ENSURE PATHOGEN-FREE SHELLFISH
* TO DETERMINE IF POLIOVIRUS AND MALE-SPECIFIC COLIPHAGES SUCH AS MS2 ARE AN ADEQUATE INDICATOR OF HAV DEPURATION

UPTAKE

* TO DETERMINE THE TIMES FOR MAXIMUM UPTAKE OF HAV AND OTHER ENTERIC VIRUSES AND INDICATOR BACTERIA
* TO DETERMINE IF VIRAL CONCENTRATION ABOVE THE LEVEL OF OVERLYING WATERS OCCURS IN A FLOW-THROUGH OR DYNAMIC EXPOSURE SYSTEM.
* TO DETERMINE IF CLAMS UPTAKE DIFFERENT MICROBES DIFFERENTLY WITH RESPECT TO TIME, MAXIMUM CONCENTRATIONS IN SHELLFISH AND HYDRAULIC CONDITIONS

IN GENERAL

* TO DEVELOP MORE EFFICIENT METHODS FOR HAV RECOVERY AND DETECTION IN CLAMS

REFERENCES CITED

REFERENCES CITED

Bachur, Beth E. 1988. The Effect of Temperature and Salinity on the Depuration of Hepatitis A Virus and other Microbibal Contaminants From the Eastern Oyster. Masters Technical Report. University of NOrth Carolina at Chapel Hill.

Bedford, Anne J., Gillian Williams, and A.R. Bellamy. 1978. Virus Accumulation by the Rock Oyster Crassostrea glomerata. Applied and Environmental Microbiology 35:1012-1018.

Bitton, G. 1980. Adsorption of Viruses to Surfaces : Technological and Ecological Implications. In: Bitton Madshall (eds.) Adsorption of Microorganisms to Surfaces. John Wiley and Sons, Inc. New York.

Bosch, A. and P.A. Shields. 1987. Survival of hepatitis A virus and poliovirus in seawater and marine sediments. Abstracts Annual Meeting for American Microbiologists. p.295.

Canzonier, W.J. 1971. Accumulation and elimination of Coliphage S-13 by the hard shell clam, Mercenaria mercenaria. Applied Microbiology 21:1024-1031.

Chang, P., O. Liu, L. Miller, and S. M. Li. 1971. Multiplication of human enteric viruses in Northern quahaugs. Proc. Soc. Exp. Bio. Med. 136:1380-1384.

Cliver, D.O. and J.E. Herrman. 1972. Proteolytic and microbial inactivation of enteroviruses. Water Research 6:797-805.

Cole, M.T., M. Kilgen, L.A. Reily, C.R. Hackney. 1986. Detection of Enteroviruses and Bacterial Indicators and Pathogens in Lousiana Oysters and their overlying waters. Journal of Food Protection 49(8):596-601.

Cromeans, T., M.D. Sobsey, H.A. Fields. 1986. Studies of Rapidly Replicating Hepatitis A Virus from a cell culture. In: "Abstracts of the Annual Meeting of American Society for Microbiologists." p.322.

Cromeans, T., M.D. Sobsey, H.A. Fields. 1987. Development of a plaque assay for a cytopatic, rapidly replicating isolate of hepatitis a virus. Journal of Medical Virology 22:45-56.

Dahling, D.R. and R.S. Safferman. 1979. Survial of enteric viruses under natural conditions in a subartic river. Applied and Environmental MIcrobiology 38:1103-1110.

Davis, A.L. 1986. Elimination of hepatis A virus and poliovirus from the Eastern Oyster, Crassostrea virginica. Technical report. University of North Carolina, Chapel Hill.

Dienstag, J.L., I.D. Gust, C.R. Lucas, D.C. Wong, and R.H. Purcell. 1976. Mussel- associated viral hepatitis, type a: serological confirmation. The Lancet i:561-564.

DiGirolamo, R., J. Liston, and J. Matches. 1975. Uptake and elimination of poliovirus by the West Coast oyster. Applied Microbiology 24:260-264.

DiGirolamo, R., J. Liston, and J. Matches. 1977. Ionic bonding, the mechanism of viral uptake by shellfish mucus. Applied and environmental Microbiology 33:19-25.

Dismukes, W.E., A.L. Bisno, S. Katz, and R.F. Johnson. 1969. American Journal of Epidemiology 89(5):555-561.

Duff, M.F. 1967. The uptake of enteroviruses by the New Zealand marine blue mussel Mytilus edulis aoteanus. American Journal of Epidemiology 85(3):486-493.

Dulbecco, Renalto and H. S. Ginsberg. Virology. 2nd ed. 1988. J. B. Lippincott Co., Philadelphia.

Ellender, R.D., J.B. Mapp, B.L. Middelbrooks, D.W. Cook, and E.W. Cake. 1980. Natural enterovirus and fecal coliform contamination of gulf coast oysters. Jounal of Food Protection 43(2):105-110.

Eyles, M.J. and G.R. Davey. 1984. Microbiology of Commercial Depuration of the Sydney Rock Oyster, Crassostrea commercialis. Journal of Food Protection 47:703-706.

Flehming, B. 1980. hepatitis a-virus in cell culture: I. propagation of different hepatitis a-virus isolates in a fetal Rhesus monkey kidney cell line (Frhk-4). Medical Microbiology Immunology 168:239-248.

Flewett, T.H. and G.N. Woode. 1978. The Rotaviruses. Archives of Virology 57:1-23.

Food and Drug Administration. 1990. National Shellfish Santiation Program Manual of Operations, Part I and II, Public Health Service, Washington, D.C..

Fries, C.R. and M.R. Tripp. 1970. Uptake of viral particulates by oyster leucocytes in vitro. Journal of Invertabrate Pathology 15:136-137.

Fugate, K.J., D.O. cliver, and M.T. Hatch. 1975. Enteroviruses and potential bacteriel indicators in Gulf Coast oysters. Journal of milk and food Technology 38(2) :100-104

Galstoff, P.S., 1964. The American oyster Crassostrea virginica. U.S. Fish Wildlife Serv. Fish Bulletin 64:1-480 Yonge, C.M., 1960. Oysters. Collins Clear-Type Press, London.

Gerba, C.P., 1981. Virus survival in wastewater treatment, pp. 39-48. In: Viruses and Wastewater Treatment, M. Goddard and M. Butler, eds. Pergamon Press, New York.

Gerba, C.P. and S.M. Goyal, 1982. Methods of Environmental Virology, Marcel Dekker, New York.

Gerba, C.P., and J.S. McLeod. 1976. Effect of sediments on the survival of Escherichia coli in marine waters. Applied and Environmental Microbiology 32(2):114-120.

Gerba,P.G., and S.M. Goyal. 1978. Detection and occurence of enteric viruses in shellfish : a review. Journal of Food protection 41(9):743-745.

Gerety, R.J. 1984. Hepatitis a. Academic press, Inc., Orlando, Florida.

Gill, O.N., W.D. Cubitt, D.A. McSwiggen, B.M. Watney and C.L.R. Bartlett. 1983. Epidemic of gastroenteritis caused by oysters contaminated with small round structured viruses. Brittish Medical Journal 287:1532-1534.

Goyal, S.M., C.P. Gerba, and J.L. Melnick. 1979. Human enteroviruses in oysters and their overlying waters. Applied and Environmental Microbiology 37(3):572-581.

Grabow, W.O.K., G.K. Idema, P. Coubough, B.W. Bateman. Selection of Indicator Systems for Human Viruses in Pollution in Seawater and Shellfish. 1989. Water Science Technology 21(3):111-117.

Grohmann, G.S., A.M. Murphy, P.J. Christopher, E. Auty, and H.B. Greenburg. 1981. Norwalk virus gastroenteritis in volunteers consuming depurated pysters. Australian Journal of Experimental Biology and Medical Science 59:219-228.

Gust, I.D., N.I. Lehmann, S. Growe, M.Mc. Crorie,S.A. Locarini, and C.R. Lucas. 1985. The origin of the HM175 strain of hepatitis a virus. The Journal of Infectious Diseases 151(2):365-367.

Guzewich, J.J. and D.L. Morse. 1986. Sources of Shellfish Outbreaks of Probable Viral Gastroenteritis: Implications for control. Journal of Food Protection 49(5):389-394.

Hamblet, F.E., W.F. Hill, Jr., E.W. Akin, and W.H. Benton. 1969. Oysters and human viruses: effects of seawater turbidity on poliovirus uptake and elimination. American Journal of Epidemiology 89:562-571.

Hartland, B.J. and J.F. Timoney. 1979. In vivo clearence of enteric bacteria from the hemolymph of the hard shell clam and the American oyster. Applied Environmental Microbiology 37:517-520.

Hedstrom, C.E., and E. Lycke. 1964. An experimental study on oysters as virus carriers. American Journal of Hygiene 79:134-142.

Herrmann, J.E., and D.O. Cliver. 1968. Methods for detecting food-borne enteroviruses. Applied and Environmental Microbiology 16(10):1564-1569.

Hoff, J.C. and R.C. Becker. 1969. The accumulation and elimination of crude and clarified poliovirus suspensions by shellfish. American Journal of Epidemiology 90(1):53-61.

Hunt, D.A. 1980. Biological standards for shellfish growing areas- What do they mean ? Journal of Food Protection 43(2):127-128.

Janssen, W.A. 1973. Oysters: retention and excretion of three types of human waterborne disease bacteria. Health Laboratory Sciences 11:20-24.

Jaweta, E., J.L. Melnick, E.A. Adelberg. REview of Medical Microbiology (17th ed) Appleton and Lange, Norwalk, Conn.. p.442-456.

Kapuscinski, R.B. and R. Mitchell. 1980. Processes controlling virus inactivation in coastal waters. Water Research 14:363-371.

Keating, M.H. 1985. Evaluation of methods for recovering HAV from the eastern oyster Crassostrea virginica. Master's Thesis, University of North Carolina, at Chapel Hill.

Kleinbaum, D.G., L.L. Kupper, K.E. Muller. Applied Regression Analysis and Other Multivariate Methods. PWS-Kent Pub. Co. Coston. p.250, 324-325, 599.

LaBelle, R.L., and C.P. Gerba. 1979. Influence of pH, salinity, and organic matter on the adsorption of enteric viruses to estuarine sediment. Applied and Environmental Microbiology 38(1):93-101.

LaBelle, R.L. ans C.P. Gerba. 1980. Influence of pH, salinity, and organic matter on the adsorption of enteric viruses to estuarine sediment. Applied and Environmental Microbiology 38:93-101.

Landry, E.F., J.M. Vaughn, T.J. Vicale and R. Mann. 1983. Accumulation of sediment-associated viruses in shellfish. Applied and Environmental Microbiology 45(1):238-247.

Landry, E.F., J.M. Vaughn, T.J. Vicale and R. Mann. 1982. Inefficient accumulation of low levels of monodispersed and feces-associated poliovirus in oysters. Applied and Environmental Microbiology 44:1362-1369.

Larkin, E.P. and C.P. Gerga. 1980. Thermostabilization of enteroviruses by estuarine sediment. Applied and Environmental Microbiology 40:305-308.

Liu, O.C., H.R. Seraichekas, and B.L. Murphy. 1966a. Fate of poliovirus in Northern Quahaug. Proc. Soc. Exp. Biol. Med. 121:601-607.

Liu, O.C., H.R. Seraichekas, and B.L. Murphy. 1967. Viral depuration of the northern quahaug. Applied Microbiology 15(2):307-315.

Liu, O.C., H.R. Seraichekas, and B.L. Murphy. 1966b. Viral pollution of shellfish. 1. Some basic facts of uptake. Proceeding of Society of Experimental Biology and Medecin 123:481-486.

Locarini, S.A., A.G. Coulepis, E.G. Westaway, and I.D. Gust. 1981. Restricted replication of human hepatitis a virus in cell culture : Intracellular biochemical studies. Journal of Virology 37(1):216-225.

Loosenoff, V.L. and C.A. Nomejko, 1951. Existence of physiologically different races of oysters, Crassostrea virginica. Biol. Bull. 101: 151-156.

Manzanti, L.E. 1987. Thermal Inactivation of Hepatitis A virus and poliovirus in the Eastern Oyster, Crassostrea virginica. Masters Thesis. University of North Carolia at Chapel Hill.

Meinhold, A.F. 1982. The uptake, elimination, and tissue distribution of poliovirus in the Eastern oyster, Crassostrea virginica. Master's Thesis. University of North Carolina, Chapel Hill.

Mele, A., M.G. Rastelli, O.N. Gill, D. di Biscegelie, F. Romini, G. Pardelli, C. Valtriani, Patriarchi. Recurrent endenic hepatitis A association with consumption of raw shellfish, probably controoled through public health measures. 1989. Am J of Epid 130(3):540-546.

Melnick, J.L. and C.P. Gerba. 1980. The Ecology of Enteroviruses in Natural Waters. CRC. Crit. Rev. Envron. Control 10:65.

Melnick, J.L. 1984. Etiologic agents and their potentiel for causing waterborne virus disease. In: Viruses in the water environment a revue. Monogr. Virol.(Karger, Basel) (15):1-16.

Melnick, J.L. 1982. Classification of hepatitis a virus as enterovirus type 72 and of hepatitis b as hepadnavirus type 1. Intervirology 18:105-106.

Mesquita, M.M.F. 1988. Effects of Seawater contamination level and Exposure Period on Bacterial and Viral Accumulation and Elimination Processes by Mytilus Edulis. Water Science Technology 20(11/12):265-270.

Metcalf, T.G., E. Moulton, and D. Eckerson. 1980. Improved method and test strategy for recovery of enteric viruses from shellfish. 39(1):141-152.

Metcalf, T.G. 1987. Bioaccumulation and disposition of solids-associated virus by shellfish. pp 77-89. In: Human viruses in sediments, sludges, and soils. by: Rao, V.C., and J.L. Melnick. CRC Press, Inc. Boca Raton, Florida.

Metcalf, T.G., and W.C. Stiles. 1965. The accumulation of enteric viruses by the oyster, Crassostrea virginica. Journal of Infectious Disease 115:68-76.

Metcalf, T.G. and W.C. Stiles. 1968b. Enteroviruses within an estuarine environment. American Journal of Epidemiology 88:379-391.

Metcalf, T.G., B. Mullin, D. Eckerson, E. Moulton, and E.P. Larkin. 1979. Bioaccumulation and depuration of enteroviruses by the soft-shelled clam, Mya arenaria. Applied and environmental Microbiology 38(2):275-282.

Metcalf, T.G., 1978. Indicators of viruses in shellfish, pp. 383-415. In: Indicators of viruses in water and food, G. Berg, ed. Ann Arbor Science, Ann Arbor.

Mitchell, J.R., M.W. Presnell, E.W. Akin, J.M.Cummins and O.C. Liu. 1966. Accumulation and elimination of poliovirus by the Eastern oyster. American Journal of Epidemiology 84:40-50.

Mitchell, R. 1971. Destruction of bacteria and viruses in seawater. Journal of the Sanitary Engineering Division, Proceedings of the American Society of Civil Engineers pp. 425-432.

Morris, R. and W. M. Waite. 1981. Indicators, viruses, and water quality. In: M. Goddard and M. Butler (eds.) Viruses and Wastewater Treatment. Pergamon Press, New York.

Nelson, T.C. 1923. On the feeding habits of the oyster. Proc. Soc. Exp. Biol. Med. 21:90-91.

O'Brien, R.T., and J.S. Newman. 1977. Inactivation of poliovirus and coxsackieviruses in surface water. Applied and Environmental Microbiology 33(2):334-340.

Perkins, F.O., D.S. Haven, R.M. Alamo, and M.W. Rhodes. 1980. Uptake and elimination of bacteria in shellfish. Journal of Food Protection 43(2):124-126.

Portnoy, B.L., P.A. Mackowiak, C.T. Caraway, J.A. Walker, T.W. McKinley, C.A. Klein,jr. 1975. Oyster- associated hepatitis .Failure of shellfish certification programs to prevent outbreaks. Journal of American Medical Association 233(10):1065-1068.

Power, U.F. and J.K. Collins. 1989. Differential depuration of poliovirus, Escherichia coli, and a coliphage by the common mussel, Mytilus edulis. Applied and Environmental Microbiology 55:1386-1390.

Power, U.F. and J.K. Collins. 1990. Elimination of Coliphage and E. coli from Mussels during Depuration under Varying Conditions of Temperature and Salinity and Food Availability. Journal of Food Protection 53(3):208-212.

Power, U.F. and J.K. Collins. 1989. Differential depuration of poliovirus, Escherichia coli, and a coliphage by the common mussel, Mytilus edulis. Applied and Environmental Microbiology 55:1386-1390.

Provost, P.J., and M.R. Hilleman. 1979. Propagation of human hepatitis a virus in cell culture in vitro (40422). Proceedings of The Society for Experimental Biology and Medicine 160:213-221.

Rao, V.C. and J.L. Melnick. 1986. Human Viruses in Sediments, Sludge, and Soils. CRC. Press, Inc. Boca Raton, Fl.

Rao,V.C., K.M. Seidel, S.M. Goyal, T.G. Metcalf, and J.L. Melnick. 1984. Isolation of enteroviruses from water, suspended solids, and sediments from Galveston Bay: Survival of poliovirus and rotavirus adsorbed to sediments. Applied and Environmental Microbiology 48(2):404-409.

Rhodes, M.W. and H. Kator. 1988. Survival of Escherichia coli and Samonella spp. in Estuarine Environments. Applied and Environmental Microbiology 54:2902-2907.

Richards, Gary. 1988. Microbial Purification of Shellfish: A Review of Depuration and Relaying. Journal of Food Protection 51(3):218-250.

Rhodes, M.W. and H. Kator. 1988. Survival of Escherichia coli and Samonella spp. in Estuarine Environments. Applied and Environmental Microbiology 54:2902-2907.

Richards, G.P. 1985. Outbreaks of shellfish-associated enteric virus illness in the united states: requisite for development of viral guidelines. Journal of Food Protection 48:815-823.

Richards, G.P. 1987. A review of Methods for the Analysis of Selected Microorganisms from Marine Sediments, Shellfish, and Seawater. unpublished.

Rowse, A.J. and G.H. Fleet. 1984. Effects of temperature and salinity on the elimination of Samonella charity and Escherichia coli from Sydney Rock oysters (Crassostrea commercialis). Applied and Environmental Microbiology 48:1061-1063.

Sattar, S.A. and J.C.N. Westwood. 1981. Viral pollution of surface waters due to chlorinated primary effluents. Applied and Environmental Microbiology 36:427-431.

Seraichekas, H.R., D.A. Brasear, J.A. Barrick, P.F. Carey, and O.C. Liu. 1968. Viral depuration by assaying individual shellfish. Applied Microbiology 16:1865-1871.

Shuval, H.I., M. Asce, A. Thompson, B. Fattal, S. Cymbalista and Y. Wiener. 1971. Natural virus inactivation processes in seawater. Journal of Sanitary Engineering Division, Proceedings of the American Society of Civil Engineers pp.587-601.

Sobsey, M.D., A.L. Davis, and V.A. Rullman. 1987. Persistance of hepatitis A and other viruses in depurated Eastern Oysters. Proceedings Oceans '87, The Marine Technology Society, Washington, D.C., 5:1740-1745.

Sobsey, M.D., C.R. Hackney, R.J. Carrick, B. Ray, and M.L. Speck. 1980. Occurence of enteric bacteria and viruses in oysters. Journal of Food Protection 43(2):111-113.

Son, N.T. and G.H. Fleet. 1980. Behavior of pathogenic bacteria in the oyster, Crassostrea commercialis, during depuration, relaying, and storage. Applied and Environmental Microbiology 40: 994-1002.

Vaughn, J.M., E.F. Landry, M.Z. Thomas, T.J. Vicale, and W.F. Penello. 1979. Survey of human enterovirus occurence in fresh and marine surface waters on Long Island. Applied and Environmental Microbiology 38(1):290-296.

Wait, D.A., C.R. Hackney, R. J. Garrick, G. Lovelace, M.D.Sobsey. 1983. Enteric Bacterial and viral pathogens and enteric bacteria in hard shell clams. Journal of Food Protection 46:493-496.

Ward, R.L., D.R. Knowlton, and P.E. Winston. 1986. Mechanisims of inactivation of enteric viruses in fresh water. Applied and Environmental Microbiology 52:450-459.

Werner, K. 1983. Rotavirus uptake and elimination by oysters. Master's Thesis. University of North Carolina at Chapel Hill.

APPENDIX A

FORMULATIONS FOR MEDIA AND COMPONENTS

Components and Media Formulations

Agar Overlay Medium (HAV):

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
Nonessential Amino Acids (100X)	1.0 ml
Gen/Kan (100X)	1.0 ml
Nystatin (as needed)	1.0 ml
$MgCl_2$ (4M)	0.75 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
Nonessential Amino Acids (100X)	1.0 ml
Gen/Kan (100X)	1.0 ml
Pen/Strep (1000X)	0.1 ml
Nystatin (as needed)	1.0 ml
$MgCl_2$ (4M)	0.75 ml
Neutral Red (100X filtered)	1.5 ml

Beef Extract:

Per liter of distilled-deionized water:

BBL Beef Extract V	70.0 gm
NaCl	17.53 gm

Beef Heart Brain Infusion:

Chloroform:

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 per plate.

Coliphage 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 16X 125 screw top test tubes.
Autoclave to sterilize.

Dow Antifoam:

Eagle's Modified MEM 1X:

Per liter of distilled-deionized water:

Autoclave Eagle's MEM with Earle's balanced salts and phenol red. 9.4 gm
Prepare as directed.
Store at 4°C.

Eagle's Modified MEM 2X:

Per liter of distilled-deionized water:

Autoclave Eagle's MEM with Earle's balanced salts: without phenol red. 9.4 gm
Prepare as directed.
Store at 4°C.

Freon: trichloro triflouro methane

Gentamycin/Kanamycin (100X):

Per liter of distilled-deionized water:

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

Sterilize by autoclaving. Store at -20°C.

Growth Medium:

Per 100 ml 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 (1.5M)	1.0 ml
Nonessential Amino Acids (100X)	1.0 ml
Gen/Kan (100X)	1.0 ml

Hepes Buffer:

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.

Maintainance 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 (1.5M)	1.0 ml
Nonessential Amino Acids (100X)	1.0 ml
Gen/Kan (100X)	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 -deionised water:

Neutral Red (powder)	8.0 gm
NaCl	5.0 gm

Sterilize by autoclaving

Nutrient Agar No.2

Per liter of distilled -deionised 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:

Nyastatin (powder, 5430 USP Units/mg)	0.55 gm

Filter sterilize.
Store at -20°C.

Penicillin /Streptomycin (1000 X)

Per liter of distilled-deionised water :

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

Filter sterilize.
Store at - 20°C.

Polyethyleneglycol:

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	1.0 ml
Antisera	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	1.0 ml

APPENDIX B

EXPERIMENTAL RESULTS

TEMPERATURE: 12 °C

ORGANISMS PER GRAM OF CLAM MEAT

		DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
E. coli	Trial A	36766.67	14100.00	11033.33	1120.00	266.00	158.00
	Trial B	36766.67	12866.67	6120.00	3086.67	502.00	100.00
	Trial C	62783.33	1153.33	60.00	20.00	46.80	122.00
	Trial D	62783.33	1480.00	253.33	138.00	108.40	59.20
	MEAN	49775.00	7400.00	4366.67	1091.17	230.80	109.80
S. fecalis	Trial A	18650.00	14333.33	4512.50	401.50	148.50	62.33
	Trial B	18650.00	10527.50	3070.00	1833.33	497.00	66.67
	Trial C	17260.00	583.33	70.00	10.80	31.73	67.07
	Trial D	17260.00	546.67	170.00	38.00	48.93	30.40
	MEAN	17955.00	6497.71	1955.63	570.91	181.54	56.62
PHAGE MS2	Trial A	67866.67	82566.67	94720	51680	52880	44380
	Trial B	67866.67	105540	60740	86180	50880	80880
	Trial C	92500.00	72666.67	89620	24450	50160	37500
	Trial D	92500.00	56200.00	91020	51580	32400	72500
	MEAN	80183.33	79243.33	84025	53472.5	46580	58815
POLIO	Trial A	4050.95	2130.18	401.75	126.70	67.48	<30.79
	Trial B	4050.95	786.83	347.76	63.07	24.47	27.35
	Trial C	1420.18	1147.02	777.89	205.36	67.03	228.85
	Trial D	1420.18	1244.32	670.42	813.87	575.02	32.32
	MEAN	2735.56	1327.09	549.46	302.25	183.50	<79.83
HAV	Trial A	3363.60	500.00	1761.20	564.00	1240.0	297.40
	Trial B	3363.60	1867.20	2790.00	1277.40	2529:4	1025.0
	Trial C	9533.40	1427.90	1000.00	1210.90	1935.1	1968.8
	Trial D	9533.40	9428.00	1916.60	673.10	500.00	960.00
	MEAN	6448.50	3305.78	1866.95	931.35	1551.1	1062.80

*Trial A,B,C,D,E,F represent replicate experiments.

TEMPERATURE: 18 °C

ORGANISMS PER GRAM OF CLAM MEAT

		DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
E. coli	Trial A	8425.00	916.67	38.86	103.20	607.00	1.80
	Trial B	8425.00	666.67	18.86	5.20	4.00	152.50
	Trial C	320.00	250.75	271.75	7.00	4.40	1.00
	Trial D	320.00	152.00	45.67	22.00	2.80	4.60
	MEAN	4372.50	496.52	93.78	34.35	154.55	39.98
S. fecalis	Trial A	23100.00	3040.00	89.33	274.00	11936	3.60
	Trial B	23100.00	1833.33	235.67	24.20	31.40	152.00
	Trial C	47750.00	7525.00	193.33	71.67	38.60	13.00
	Trial D	47750.00	3260.00	710.00	980.00	24.00	32.80
	MEAN	35425.00	3914.58	307.08	337.47	3007.50	50.35
PHAGE MS2	Trial A	1133.33	755.00	1263.33	1844.00	1498.00	318.00
	Trial B	1133.33	2083.33	210.00	486.00	284.00	84.00
	Trial C	270.00	80.00	446.67	108.00	45.71	34.40
	Trial D	270.00	650.00	1080.00	288.00	20.14	106.70
	MEAN	701.67	892.08	750.00	681.50	461.96	135.78
POLIO	Trial A	2768.27	60.24	11.03	<11.03	59.47	73.48
	Trial B	2768.27	25.11	<13.89	<13.22	95.94	118.13
	Trial C	151.83	378.22	14.36	100.56	<6.64	33.64
	Trial D	151.83	108.03	214.35	27.11	121.28	4.64
	MEAN	1460.05	142.90	<63.41	<37.98	<70.83	57.48
HAV	Trial A	1244.40	1162.80	1222.20	1755.60	1561.00	1409.00
	Trial B	1244.40	914.30	854.00	1103.00	518.00	630.00
	Trial C	1514.30	918.90	878.60	1897.40	1333.30	621.60
	Trial D	1514.30	1044.00	42.00	709.70	879.00	478.00
	MEAN	1379.35	1010.00	749.20	1366.43	1072.83	784.65

*Trial A,B,C,D,E,F represent replicate experiments.

TEMPERATURE: 25 °C

ORGANISMS PER GRAM OF CLAM MEAT

		DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
E. coli	Trial A	8050.00	3046.67	235.67	40.20	6.20	30.20
	Trial B	8050.00	3913.33	279.00	14.40	5.00	4.00
	Trial C	2336.00	9233.33	600.00	13.50	----	26.00
	Trial D	2336.00	940.00	161.00	11.00	----	5.50
	Trial E	75450.00	763.33	12200.00	435.00	345.67	368.50
	Trial F	75450.00	2916.00	2416.67	984.00	821.00	189.50
	MEAN	28612.00	3468.78	2648.72	249.68	294.47	103.95
S. fecalis	Trial A	30.00	12.50	1.93	1.20	0.68	0.08
	Trial B	30.00	12.40	1.60	0.68	0.44	0.04
	Trial C	1983.33	880.00	30.25	37.25	----	3.50
	Trial D	1983.33	150.00	18.17	127.37	----	1.75
	Trial E	65600.00	2113.33	52600.00	1072.80	512.75	1043.50
	Trial F	65600.00	1610.00	3790.00	1334.20	999.25	372.75
	MEAN	22537.78	796.37	9406.99	428.92	378.28	236.94
PHAGE MS2	Trial A	1303.33	262.33	49.00	267.80	158.00	37.00
	Trial B	1303.33	767.50	67.67	38.40	25.00	4.60
	Trial C	4506.67	11475	2750.00	3327.50	-----	299.00
	Trial D	4506.67	5013.33	3160.00	2322.50	-----	736.00
	Trial E	3737.78	950.00	635.00	8040.00	3480.00	11760.0
	Trial F	3737.78	823.33	10956.00	7647.50	3840.00	1982.50
	MEAN	3182.59	3215.25	2936.28	3607.28	1875.75	2469.85
POLIO	Trial A	2992.14	333.03	<10.20	30.62	217.23	<12.52
	Trial B	2992.14	88.42	81.51	28.41	<45.56	<13.74
	Trial C	916.90	286.15	6.92	158.58	47.72	91.79
	Trial D	916.90	135.24	6.92	78.30	18.34	165.28
	Trial E	989.30	88.72	293.79	547.87	<26.11	<26.11
	Trial F	989.30	165.32	151.74	314.79	106.53	52.06
	MEAN	1632.78	182.81	<91.85	193.09	<76.92	<60.25
HAV	Trial A	3952.80	3278.20	1125.00	2049.20	754.00	------
	Trial B	3952.80	1846.80	2258.00	737.80	1197.20	------
	Trial C	4700.00	3100.00	250.00	1000.00	3200.00	3125.00
	Trial D	4700.00	855.00	200.00	1000.00	1916.00	1750.00
	MEAN	4326.40	2270.00	958.25	1196.75	1766.80	2437.50

*Trial A,B,C,D,E,F represent replicate experiments.

SALINITY: 8ppt

ORGANISMS PER GRAM OF CLAM MEAT

		DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
E. coli	Trial A	13300.00	92400.0	30500.00	22375.0	39925.0	11692.5
	Trial B	13300.00	33600.0	78440.00	39140.0	24000.0	4717.5
	Trial C	51750.00	10125.0	1410.00	250.0	168.57	-------
	Trial D	51750.00	39280.0	3385.00	616.67	630.00	-------
	Trial E	118000.0	81320.0	57000.00	13660.0	15800.0	15400.0
	Trial F	118000.0	180333	44860.00	6000.0	27833.3	4957.5
	MEAN	61016.67	72843.1	35932.50	13673.6	18059.5	9191.88
S. fecalis	Trial A	41.33	132.40	12.40	9.16	13.20	5.80
	Trial B	41.33	36.10	8.60	123.96	5.47	3.20
	Trial C	3533.33	5400.00	140.00	120.00	775.00	------
	Trial D	3533.33	11875.0	583.33	146.67	222.50	------
	Trial E	60750.00	57800.0	181666.7	30550.0	14200.0	261467
	Trial F	60750.00	105667	46060.00	9000.00	17700.0	7545.00
	MEAN	21441.56	30151.7	38078.50	6658.30	5486.03	67255.2
PHAGE MS2	Trial A	28750	28760	8984	13250	11050	24400
	Trial B	28750	22775	22442	16200	12225	21575
	Trial C	-----	-----	-----	-----	-----	-----
	Trial D	-----	-----	-----	-----	-----	-----
	Trial E	7280.00	1765.00	12800.00	1440.00	12832.0	5596.00
	Trial F	7280.00	8633.33	5888.00	1182.86	7864.00	3963.33
	MEAN	18015.0	15483.3	12528.50	8018.22	10992.8	13883.6
POLIO	Trial A	3069.54	2105.85	2112.80	3666.05	3981.10	5025.14
	Trial B	3069.54	4620.00	4789.12	2782.50	2267.82	2029.97
	Trial C	1789.67	457.58	153.83	387.17	63.60	-------
	Trial D	1789.67	808.00	168.33	83.45	123.84	-------
	Trial E	46318.50	18745.6	10665.60	10169.3	4752.00	2598.75
	Trial F	46318.50	16583.4	5797.40	5115.20	3168.00	5141.00
	MEAN	17059.23	7220.07	3947.85	3700.60	2392.73	3698.71
HAV	Trial A	833.30	83.30	225.00	100.00	140.60	53.60
	Trial B	833.30	2142.90	453.10	125.00	46.90	<25.00
	Trial C	125.00	<20.80	<15.60	20.80	41.70	------
	Trial D	125.00	291.70	178.60	41.70	375.00	------
	Trial E	187.50	291.70	41.60	53.60	321.40	104.20
	Trial F	------	------	------	------	------	------
	MEAN	420.82	<566.08	<182.78	68.22	185.12	<60.93

*Trial A,B,C,D,E,F represent replicate experiments.

SALINITY: 18ppt

ORGANISMS PER GRAM OF CLAM MEAT

		DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
E. coli	Trial A	333800	153600	136000	[-EQUIPMENT FAILURE--]		
	Trial B	333800	327000	122000	[-EQUIPMENT FAILURE--]		
	Trial C	87250	53100	98766.67	14933.3	40933.3	4385
	Trial D	87250	100267	24666.67	59000.0	47600.0	32336.3
	Trial E	36180	796.667	740.00	318.0	146.0	460.0
	Trial F	36180	803.333	1583.33	196.0	430.0	213.0
	MEAN	152410	105298	63959.44	18611.8	22277.3	9322.8
S. fecalis	Trial A	521000	95575	101500	[-EQUIPMENT FAILURE--]		
	Trial B	521000	168333	102000	[-EQUIPMENT FAILURE--]		
	Trial C	42300	7020	11115	2140	4337.5	1810.0
	Trial D	42300	15511.1	3092.5	12910	17666.7	5472.5
	Trial E	150333	16150.0	16266.7	7382.5	2266.0	1632.0
	Trial F	150333	10700.0	15000.0	573.3	4762.0	968.0
	MEAN	237877	52214.9	41495.69	5751.46	7258.0	2470.63
PHAGE MS2	Trial A	25800	24350	5506.667	[-EQUIPMENT FAILURE--]		
	Trial B	25800	19575	9785.000	[-EQUIPMENT FAILURE--]		
	Trial C	27050	26625	13074.33	4800	11966.7	10600
	Trial D	27050	13920	10360.00	7666.67	7150.0	19666.7
	Trial E	2950	1630	1450.00	616.00	412.0	900.0
	Trial F	2950	843.33	646.67	128.00	342.22	560.0
	MEAN	18600	14490.6	6818.78	3302.67	4967.72	7931.67
POLIO	Trial A	1034.12	950.33	3734.97	[-EQUIPMENT FAILURE--]		
	Trial B	1034.12	1175.28	1818.29	[-EQUIPMENT FAILURE--]		
	Trial C	7715.51	3514.00	1858.40	4057.34	1697.68	2046.72
	Trial D	7715.51	3273.31	15907.50	3270.34	2750.80	7312.40
	Trial E	1292.02	180.40	77.42	390.42	87.25	86.67
	Trial F	1292.02	801.65	133.35	13.13	174.50	70.67
	MEAN	3347.22	1649.16	3921.66	1932.81	1177.55	2379.12
HAV	Trial A	4250.00	500.00	-------	[-EQUIPMENT FAILURE--]		
	Trial B	4250.00	2750.00	1916.70	[-EQUIPMENT FAILURE--]		
	Trial C	2375.00	250.00	750.00	458.30	1875.00	666.70
	Trial D	2375.00	791.70	312.50	200.00	500.00	50.00
	Trial E	1500.00	750.00	318.20	227.30	50.00	307.70
	Trial F	1500.00	609.00	312.50	107.10	312.00	250.00
	MEAN	2708.33	941.78	721.98	248.18	684.25	318.60

*Trial A,B,C,D,E,F represent replicate experiments.

SALINITY: 28ppt

ORGANISMS PER GRAM OF CLAM MEAT

		DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
E. coli	Trial A	8050.00	3046.67	235.67	40.20	6.20	30.20
	Trial B	8050.00	3913.33	279.00	14.40	5.00	4.00
	Trial C	2336.00	9233.33	600.00	13.50	----	26.00
	Trial D	2336.00	940.00	161.00	11.00	----	5.50
	Trial E	75450.00	763.33	12200.00	435.00	345.67	368.50
	Trial F	75450.00	2916.00	2416.67	984.00	821.00	189.50
	MEAN	28612.00	3468.78	2648.72	249.68	294.47	103.95
S. fecalis	Trial A	30.00	12.50	1.93	1.20	0.68	0.08
	Trial B	30.00	12.40	1.60	0.68	0.44	0.04
	Trial C	1983.33	880.00	30.25	37.25	----	3.50
	Trial D	1983.33	150.00	18.17	127.37	----	1.75
	Trial E	65600.00	2113.33	52600.00	1072.80	512.75	1043.50
	Trial F	65600.00	1610.00	3790.00	1334.20	999.25	372.75
	MEAN	22537.78	796.37	9406.99	428.92	378.28	236.94
PHAGE MS2	Trial A	1303.33	262.33	49.00	267.80	158.00	37.00
	Trial B	1303.33	767.50	67.67	38.40	25.00	4.60
	Trial C	4506.67	11475	2750.00	3327.50	-----	299.00
	Trial D	4506.67	5013.33	3160.00	2322.50	-----	736.00
	Trial E	3737.78	950.00	635.00	8040.00	3480.00	11760.0
	Trial F	3737.78	823.33	10956.00	7647.50	3840.00	1982.50
	MEAN	3182.59	3215.25	2936.28	3607.28	1875.75	2469.85
POLIO	Trial A	2992.14	333.03	<10.20	30.62	217.23	<12.52
	Trial B	2992.14	88.42	81.51	28.41	<45.56	<13.74
	Trial C	916.90	286.15	6.92	158.58	47.72	91.79
	Trial D	916.90	135.24	6.92	78.30	18.34	165.28
	Trial E	989.30	88.72	293.79	547.87	<26.11	<26.11
	Trial F	989.30	165.32	151.74	314.79	106.53	52.06
	MEAN	1632.78	182.81	<91.85	193.09	<76.92	<60.25
HAV	Trial A	3592.8	3278.2	1125.00	2049.2	754.00	------
	Trial B	3952.8	1846.8	2258.00	737.8	1197.2	------
	Trial C	4700.0	3100.0	250.0	1000.0	3200.0	3125.00
	Trial D	4700.0	855.0	200.0	1000.0	1916.0	1750.00
	MEAN	4326.4	2270.0	958.25	1196.75	1766.8	2437.5

*Trial A,B,C,D,E,F represent replicate experiments.