SURVIVAL OF *VIBRIO VULNIFICUS* AND *ESCHERICHIA COLI* IN ARTIFICIALLY AND NATURALLY INFECTED OYSTER (*CRASSOSTREA VIRGINICA*) TISSUES DURING STORAGE IN SPRAY- AND IMMERSION-TYPE LIVE HOLDING SYSTEMS

by

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APPROVED:

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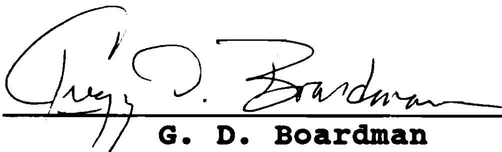

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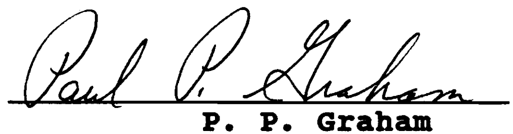

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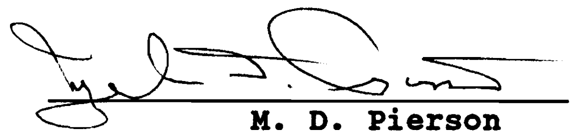

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Survival of *Vibrio vulnificus* and *Escherichia coli* in Artificially and Naturally Infected Oyster (*Crassostrea virginica*) Tissues During Storage in Spray- and Immersion-Type Live Holding Systems

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(ABSTRACT)

Live holding systems are used as temporary storage facilities for shellfish. The potential for mishandling of shellfish stored in these systems is high. The objective of the project was to examine the effects of storing oysters in a spray and an immersion systems on the survival of *Escherichia coli* and *Vibrio vulnificus* within the oysters. The effects of physiological stress imposed on oysters, as a result of interstate shipping, were examined by monitoring the level of *E. coli* in these oysters during storage in a spray tank. The survival rates of naturally-present *E. coli* and *V. vulnificus* in oysters were also observed. The research examined the distribution of artificially- and naturally-present *V. vulnificus* in oyster tissues during storage in an immersion system. There was no significant difference ($p = 0.12$) in the artificially-inoculated bacterial population of oysters after 120 hr of storage in a spray live holding tank. The level of *E. coli* in oysters

which were subject to physiological stress did not change significantly ($p = 0.30$) after 96 hr in the spray tank. Naturally-present E. coli and V. vulnificus in oysters at harvest persisted during the 72 hr storage in the spray tank. V. vulnificus was loosely associated with mucus on the surfaces of the adductor and the mantle tissues in artificially-inoculated oysters. As a result, the bacterial level was reduced on these surfaces during the 72 hr of depuration. V. vulnificus on the gills and the digestive system of artificially-inoculated oysters may become entrapped in cilia and mucus. There was no significant reduction in the bacterial population on the gills ($p = 0.11$) and on the digestive system ($p =0.21$). There was no significant difference in the population of V. vulnificus in the adductor muscle ($p = 0.37$), the mantle ($p = 0.16$), the gills ($p = 0.5$), and the digestive system ($p = 0.5$) of summer oysters naturally-infected with the bacterium. It seems unlikely that depuration of V. vulnificus from oysters naturally harboring the bacterium may be effective.

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TABLE OF CONTENTS

Abstract..ii

Acknowledgements....................................iv

Table of Contents......................................v

List of Figures.......................................viii

Introduction...1

Section I. Literature Review..........................4

A. Oysters

1. General characteristics..................4

2. Microbial flora...............................5

3. Clearance mechanism.......................8

4. Microbiological standards for harvesting sites....................10

5. _Escherichia_ _coli_ as indicator of fecal coliforms....................11

B. _Vibrio_ _vulnificus_

1. General background.........................12

2. Virulence factors.............................13

3. Distribution in the environment........17

4. Incidence of _V_. _vulnificus_ infections.....................................18

5. Clinical manifestations....................19

6. Fecal coliform as an indicator of *V. vulnificus*........................22

C. Depuration

1. Disinfection of depuration water.......23

2. Factors affecting depuration...........26

3. Guidelines on depuration...............27

4. Removal of microorganisms..............28

D. References.................................32

Section II. Survival of *Escherichia coli* and *Vibrio vulnificus* in oyster tissues during storage in a spray live holding system.

Introduction.....................................43

Materials and Methods............................46

Results and Discussion...........................54

References.......................................65

Appendices.......................................69

Section III. Distribution of *Vibrio vulnificus* in tissues of artificially- and naturally-infected oysters (*Crassostrea virginica*) during depuration.

Introduction.....................................74

Materials and Methods............................76

Results and Discussion...........................86

References......................................117

Appendices......................................120

Section IV. Conclusions....................................124

Vitae..126

LIST OF FIGURES

Section II. Survival of *Escherichia coli* and *Vibrio vulnificus* in oyster tissues during storage in a spray live holding system.

Figure 1: Schematic diagram of a live holding tank.......................47

Figure 2: Survival of *E. coli* in oysters during storage in a spray live holding tank................55

Figure 3: Survival of *E. coli* in physiologically-stressed oysters during storage in a spray live holding tank................57

Figure 4: Effect of storing stressed and unstressed oysters with two different initial levels of *E. coli* in the same tank..............................59

Figure 5: Survival of *E. coli* and *V. vulnificus* in unstressed oysters during storage in a spray live holding tank.......................61

Figure 6: Comparison of a spray and an immersion live holding systems on the survival of *V. vulnificus* in oysters and tank water.........................63

Appendix A: Survival of *E. coli* in oysters and water of a spray live holding system...........69

Appendix B: Survival of *E. coli* in physiologically-stressed oysters during storage in spray live holding system..............70

Appendix C: Effect of storing oysters harboring two different levels of *E. coli* in the same tank...........................71

Appendix D: Survival of *E. coli* and *V. vulnificus* in oysters and tank water during storage in a spray live holding system.................................72

Appendix E: Comparison of the survival of *V. vulnificus* in oysters and tank water in spray and immersion systems......................73

Section III. Distribution of *Vibrio vulnificus* in tissues of artificially- and naturally-infected oysters (*Crassostrea virginica*) during depuration.

Figure 1: Schematic diagram of a live holding tank...........................78

Figure 2: Schematic diagram of an oyster...............................82

Figure 3: Efficiency of ultraviolet light disinfection on the reduction of *V. vulnificus* in the immersion live holding tank water.....................87

Figure 4: Uptake of *V. vulnificus* in oyster tissues during artificial inoculation.................88

Figure 5: Distribution of V. vulnificus in artificially-inoculated oyster tissues and tank water..........90

Figure 6: Comparison of the distribution of V. vulnificus naturally present in oyster tissues harvested in fall and summer...........92

Figure 7: Distribution of V. vulnificus in summer oysters during depuration in an immersion live holding system....................94

Figure 8: Distribution of V. vulnificus in fall oysters during depuration in an immersion live holding system.................................95

Figure 9: Presence of V. vulnificus in oyster adductor muscles during depuration in an immersion live holding system.................................96

Figure 10: Presence of V. vulnificus in oyster mantle tissues during depuration in an immersion live holding system....................97

Figure 11: Presence of V. vulnificus in oyster gill tissues during depuration in an immersion live holding system....................98

Figure 12: Presence of V. vulnificus in oyster digestive systems during depuration in an immersion live holding system....................99

Figure 13: Scanning electron micrograph of the adductor tissue....................................101

Figure 14: Scanning electron micrograph of the mantle tissue surface....................................102

Figure 15: Scanning electron micrograph of the epithelial surface of gills....................................103

Figure 16: Scanning electron micrograph of the surface of the oyster stomach....................................104

Figure 17: Transmission electron micrograph of a transverse section of the gill tissue....................................105

Figure 18: Transmission electron micrograph of a transverse section of the gill tissue showing lysosomes......................106

Figure 19: Scanning electron micrograph of a colony of _V. vulnificus_..............................108

Figure 20: Scanning electron micrograph of adductor tissue of oysters artificially-inoculated with _V. vulnificus_.........................109

Figure 21: Another view of a scanning electron micrograph of the adductor muscle of oysters artificially-inoculated with _V. vulnificus_.........................110

Figure 22: Scanning electron micrograph of mantle tissue of oysters inoculated with *V. vulnificus*....111

Figure 23: Scanning electron micrograph of gill surface of oysters inoculated with *V. vulnificus*....112

Figure 24: Scanning electron micrograph of the stomach surface of oysters inoculated with *V. vulnificus*....113

Figure 25: Scanning electron micrograph of the intestine surface of oysters inoculated with *V. vulnificus*....114

Figure 26: Scanning electron micrograph of the rectal surface of oysters inoculated with *V. vulnificus*....115

Appendix A: Uptake of *Vibrio vulnificus* by oysters tissues during artificial inoculation....120

Appendix B: Distribution of *V. vulnificus* in oyster tissues and tank water during depuration in an immersion tank system....121

Appendix C: Effect of depuration of summer oysters in immersion system on the distribution of *V. vulnificus* in oyster tissues and tank water....122

Appendix D: Effect of depuration of fall oysters in immersion system on the distribution of V. vulnificus in oyster tissues and tank water................123

INTRODUCTION

Live holding systems are used as temporary storage facilities for displaying shellfish for retail sales. These systems may hold shellfish either submerged in water or above the water level. Stress imposed on oysters, a result of product handling during transportation, water temperature fluctuations, and/or starvation, may be an important factor in the dynamics of human pathogenic microorganism survival and growth during wet storage (Colburn, 1989; Cook and Ellender, 1986; Eyles and Davey, 1984; Mitchell *et al*., 1966; Power and Collins, 1989).

No reports have been published on the physiological state of oysters prior to their introduction in a spray holding system. Therefore, the first part of this study examined the implications of holding oysters in a spray system. The survival of *Escherichia coli* in the oysters was monitored as an indicator of the effect of stress on the total microbial population in oysters.

One of the dominant genera of the bacterial flora of oysters is *Vibrio* (Colburn *et al*., 1989; Colwell and Liston, 1960). *V*. *vulnificus* is commonly associated with causing primary sepsis through the consumption of oysters by individuals with underlying chronic diseases (Bachman *et al*., 1983; Oliver, 1989). The ability of *V*. *vulnificus* to

survive and multiply in oysters stored in live holding systems for retail sales may be a potential health problem. Oysters naturally harboring the bacterium were placed in the spray live holding tank to examine the population dynamics of _V_. _vulnificus_ during storage.

Differences in the rate of depuration of _V_. _vulnificus_ between artificially-inoculated oysters and oysters naturally harboring the organism have been reported by several investigators (Eyles and Davey, 1984; Kelly and Dinuzzo, 1985; Richards, 1991). The second part of the study concerned the monitoring of _V_. _vulnificus_ in selected oyster tissues during storage in an immersion live holding tank. Oysters were dissected to separate the exterior tissues (adductor, mantle, gills) from the digestive system.

The objectives of this study were:

(1) to examine the level of _E_. _coli_ in oysters stored in a spray live holding system;

(2) to examine the effect of physiological stress on the level of _E_. _coli_ in oysters stored in a spray live holding system;

(3) to examine the effect of storing stressed and unstressed oysters on the level of _E_. _coli_ in oysters during storage in the same spray live holding system;

(4) to observe the levels of _E_. _coli_ and _V_. _vulnificus_

naturally present at harvest in oysters during storage of these oysters in a spray live holding system;

(5) to compare a spray and an immersion spray systems on the level of *V. vulnificus* in oysters during the storage period;

(6) to determine the distribution of *V. vulnificus* in oysters tissues during depuration in an immersion live holding system;

(7) to compare oysters naturally containing *V. vulnificus* and artificially-inoculated oysters to determine whether there is a difference in the distribution of the organism in oyster tissues during depuration in an immersion system;

(8) to observe the relationship between ciliated and non-ciliated surfaces of oyster tissues on the efficiency of depuration of *V. vulnificus* from these surfaces.

SECTION I: LITERATURE REVIEW

A. Oysters

1. General characteristics

Oysters are bivalve mollusks, found in tidal levels or in shallow brackish water along the coasts of temperate and tropical areas. Oysters have dissimilar lower and upper shells or valves, hinged together by an elastic ligament. The upper shell is generally flat, while the lower shell is concave and these shells can close to form a water tight cavity. This is controlled by the adductor muscle, which is attached to both the valves (Wheeler and Hebard, 1981).

Spats or baby oysters swim freely in their first three weeks of life (White and Dewey, 1972). They attach themselves to any support by a slimy secretion near the hinge. A pigment-producing marine bacterium, designated as LST, may also promote the settlement of oyster larvae. LST adheres to surfaces through the synthesis of an acetic polysaccharide exopolymer. This bacterium also produces L-DOPA (Dihydroxyphenylalanine), other melanin precursors, and melanin. Eastern oysters (*Crassostrea virginica*) larvae become attracted to L-DOPA and its polymers. The larvae settle on films of LST formed on surfaces and metamorphose into adults (Weiner *et al*., 1985). Once anchored, oysters never voluntarily move.

Eastern oysters are found in the bays and inlets along the Atlantic and Gulf Coasts. Commercially, this species accounts for the majority of the total oyster production in the U.S. Oyster harvesting is decreasing in Virginia, where over a million bushels annually were harvested 40 years ago. In 1990, only 135,000 bushels, worth 4.7 million dollars, were landed in Virginia (Cohn, 1991).

The decrease in oyster production may be due to over-harvesting, environmental deterioration and diseases, and parasites such as MSX (Haplosporidium nelsoni) and Dermo (Perkinsus marinus). These two parasites can cause high oyster mortalities under high salinity (15 parts per thousand, ppt) and warm water temperatures (Oestrling, 1987). Damage to oyster populations may also come from marine Cytophaga species, which colonize and destroy hinge ligaments during bacterial proliferation (Dungan et al., 1989).

2. Microbial flora

Oysters maintain a steady flow of water through their gills for feeding, respiration, and removal of metabolic products. The volume of water transported depends on shell movements and the width of the opening of the two valves. A similar control of the volume of water can be produced by the edges of the mantle, a soft membrane covering the body

of the oyster. The tentacles on the edge of the opposing mantles interlock while the valves remain open. In this arrangement, no water will pass through the gills. During ideal external conditions, a steady flow of water over the gills can continue for hours without interruption or significant changes in the rate of transport of water. This condition is called steady state and occurs when the temperature, salinity, and food content of the water remain constant.

Oysters obtain their food by pumping large volumes of water (10 L/hr) by ciliary action over the gills, which function as a sieve to remove particulate material including microorganisms (Fleet, 1978). The filtered particles become entrapped in mucus, which is continually secreted by the oyster during the pumping action. The mucus-coated material is directed by the cilia of the gills toward the mouth of the oyster, where it is ingested or directed to the exterior and eliminated as pseudo-feces. Waste material from the alimentary tract is discharged as feces in the form of a fine, mucous thread (Fleet, 1978). Some ingested microorganisms become trapped in the discharged feces. However, many microorganisms may become concentrated in the gut or other tissues as a consequence of the filter-feeding system.

The microflora of an actively-feeding oyster reflects

the flora of the surrounding water. The bacterial population of oysters include the genera Alcaligenes, Bacillus, Cornybacterium, Cytophaga, Flavobacterium, Micrococcus, Pseudomonas, and Vibrio (Vasconcelos and Lee, 1972). The dominant genera are the Pseudomonas and Vibrio spp. (Colburn et al., 1989; Colwell and Liston, 1960; Murchelano and Brown, 1968; Weiner et al., 1985). In another study, Pseudomonas spp. were the dominant organisms, comprising over one third of 321 strains characterized after isolation from the bivalves and seawater (Kueh and Chan, 1985). Gram-positive organisms constitute less than 20% of the isolates.

In addition to these organisms, oysters may be contaminated with microorganisms that transmit human diseases. Infectious bacterial diseases have been transmitted by oysters, such as typhoid from Salmonella typhi, paratyphoid from Salmonella paratyphi, cholera from Vibrio cholerae, and dysentery from Shigella dysenteriae to gastroenteritis caused by Vibrio parahaemolyticus and Salmonella spp. (Fleet, 1978). The number of salmonellae present in sampled west coast oysters was 2.2 organisms/100 g of oyster meats (Fraiser and Koburger, 1984).

Viruses have been isolated from estuary waters, and filter-feeding oysters have been shown to accumulate viruses from these waters. Human pathogenic viruses implicated with

oysters are hepatitis A and Norwalk-type (Speirs et al., 1987; Tierney et al., 1985).

3. Clearance mechanism

The gills play an important role in feeding and filtration processes. Oysters retain suspended particulate material on their gills, which are lamellar and composed of rows of filaments. The cilia on the filaments pump water through the small interfilamentary openings (ostia). The particles are entrapped in mucus and are limited in passage through the gills by the size of the interfilamentary ostia and the action of the large latero-frontal cilia. Oysters filter naturally-occurring particles in the range of 1.0 to 3.0 μm with one-third the efficiency with which large particles are removed (Haven and Morales-Alamo, 1970).

When particles or microorganisms by-pass the filtration process and enter the oyster, cellular responses remove the foreign materials. Particles become clumped in efferent vessels and large number of phagocytes accumulate around them. Most particles are engulfed, removed from circulation, and transported through epithelial layers to the exterior. Once the cells reach the exterior they are carried away in mucus as pseudo-feces by the pumping action of the gills (Hartland and Timoney, 1979).

This process may be supplemented by intracellular

and/or extracellular destruction. Study by Tripp (1960) showed that yeast cells are phagocytized in blood vessels by hemocytes which migrate into the surrounding tissues and later through the epithelial layers to the exterior. A small proportion of the yeast cells that remain in the tissues are digested intracellularly. Bacteria are rapidly destroyed both intra- and extracellularly before the host hemocytes migrate to the exterior. A small number of bacteria may persist in the oyster tissues for several days. Bacterial spores are removed at a slower rate than vegetative cells.

The defense system against foreign particulates is mediated by hemocytes which circulate in the hemolymph. Phagocytosis occurs quickly after contact between the hemocytes and particles (Alvarez et al., 1989). There are two general classes of hemocytes, granular and agranular. Granular cells seem to be more highly phagocytic (Rodrick and Ulrich, 1984). Maximum phagocytic activity by hemocytes is around 10°C to 37°C. There is an inhibition of activity below 8°C (Alvarez et al., 1989). Lysozyme in the hemolymph, as well as in the mantle mucus, is also involved with the mechanisms of internal host defense and digestion (Chu and Peyre, 1989). There is higher lysozyme levels in winter than in summer (Chu and Peyre, 1989).

4. Microbiological standards for harvesting sites

Indicator organisms are used to test the presence of pathogens from possible sewage contamination in water. Coliforms have been most widely used as indicators of sewage. Coliforms are defined as all aerobic or facultative anaerobic, Gram-negative nonsporeforming rods that ferment lactose with the production of gas within 48 hr at 35°C. Organisms in this category include the genera *Escherichia*, *Enterobacter*, *Erwinia*, *Citrobacter*, and *Klebsiella*. The National Shellfish Sanitation Program (NSSP) states that the coliform standard for "approved" shellfish water as the total coliform median or geometric mean Most Probable Number (MPN) count of the water does not exceed 70/100 mL and 10% of the samples does not exceed an MPN of 230/100 mL for a 5-tube decimal dilution test (or 330/100 mL for the 3-tube decimal dilution test) (NSSP, 1989).

The use of the coliform group as a fecal indicator has been subject to criticism because they are ubiquitous in nature and are not limited to fecal origin. As an alternative, fecal coliforms have been used as an indicator under the NSSP. Fecal coliforms are defined as Gram-negative facultative anaerobic nonsporeforming rods that produce gas from lactose within 24±2 hr at 44.5±0.2°C. The fecal coliform standard for shellfish harvesting waters is 14/100 mL of water and not more than 10% of the sample can

exceed an MPN of 43/100 mL for a 5-tube decimal test (NSSP, 1989).

Shellfish growing areas are classified as approved, conditionally approved, restricted, or prohibited (further discussed in Section C.3). This classification is based on sanitary or marine biotoxin survey information, radioactivity, metals, chemical pollutants, and potential environmental pollution. All areas not surveyed are designated as prohibited. In approved sites, fecal material, pathogenic microorganisms, and poisonous and deleterious substances have been shown not to be present in dangerous concentrations. These areas meet either the coliform or fecal coliform standards. Areas subject to intermittent microbiological pollution are classified as conditionally approved. Areas that have a limited degree of pollution are classified as restricted areas. Shellfish harvested from these areas must go through a purification process (Hunt, 1980).

5. *Escherichia coli* as indicator of fecal coliform

Fecal coliform counts do not correlate well with enteric viruses and some bacteria and potentially pathogenic bacteria which are naturally present in the aquatic environment (Paille *et al*., 1987). Due to this lack of relationship, the use of fecal coliform as an indicator of

water quality has been questioned. One organism that is suggested as a better indicator of water quality is Escherichia coli. E. coli generally comprises 95 to 99% of the fecal coliform population (Tardio et al., 1988). Hood et al. (1983a) found E. coli to correlated strongly with fecal coliform levels in both fresh and stored oysters and clams.

There is a lack of correlation of occurrence of Vibrio spp. and sewage contamination and coliform isolation (Colwell et al., 1977; Hood et al., 1983b; Tilton and Ryan, 1987). The classification of seawater for shellfish harvesting is based on bacteriological standards relying primarily on fecal coliform test. Approved sites have a fecal coliform MPN that does not exceed 14/100 mL of seawater in more than 10% of the samples tested. Oyster meat is not to exceed a fecal coliform MPN of 230/100 g of wet weight (NSSP, 1989).

B. Vibrio vulnificus

1. General background

Vibrio vulnificus is a gram-negative curved rod, 0.5 to 0.8 μm in width and 1.4 to 2.6 μm in length (Baumann and Schubert, 1984). V. vulnificus was first reported in 1976 as the "lactose-positive vibrio" by the Centers for Disease Control (Hollis et al., 1976). Biochemically, V. vulnificus

closely resembles *V. parahaemolyticus*, another halophilic marine vibrio. Before the 1970s, the more serious infections attributed to *V. parahaemolyticus* may have actually been due to *V. vulnificus* infections (Morris, Jr., 1988). *V. vulnificus* was identified as a species different from *V. parahaemolyticus*, *V. alginolyticus*, or *V. cholerae* by DNA/DNA hybridization studies (Oliver, 1989).

2. Virulence factors

There is a massive edema (swelling) following subcutaneous injections of *V. vulnificus* in mice. *V. vulnificus* produces hemoconcentration within 3 to 6 hr after subcutaneous or intraperitoneal injection into mice. There is about 1 mL of edema fluid which accumulates at the site of injection. This is equal to two-thirds of the total plasma volume of a mouse. With sublethal injections, the edematous area becomes necrotic. *V. vulnificus* can cross the intestinal mucosa in rabbits and rats, causing bacteremia and death. This suggests that the organism may gain access to the circulatory system from the alimentary tract in humans. Strains that showed virulence when injected in mice intraperitoneally also demonstrated virulence when administered by the oral route (Reyes *et al.*, 1987).

Environmental *V. vulnificus* strains are phenotypically

indistinguishable from clinical isolates and approximately 90% of the environmental strains tested produced in vitro virulence factors and in vivo pathogenicity for mice comparable to those produced by clinical V. vulnificus isolates (Reyes et al., 1987; Tison and Kelly, 1986). Weakly virulent isolates of V. vulnificus do not cause septicemia and death in rabbits. They cause fluid accumulation in the rabbit ileal loops, indicating the existence of an enterotoxin (Stelma et al., 1988).

V. vulnificus produces hemolysin-cytolysin, a heat-labile, antigenic, extracellular toxin (Kreger and Lockwood, 1981). Hemolysin-cytolysin is cytolytic against mammalian erythrocytes, cytotoxic against Chinese hamster ovary cells, possesses vascular permeability factor activity in guinea pig skin, and has lethal activity in mice. The toxin prepared from cultures of a virulent strain of the bacterium was 25 times more toxic than toxin preparations from weakly-virulent strain (Kreger and Lockwood, 1981). The clinical and environmental isolates have identical cytotoxic activity.

Hemolysin-cytolysin is a hydrophobic protein with a molecular weight of about 56 kilodaltons (Gray and Kreger, 1985; Kreger and Lockwood, 1981; Wright et al., 1985). Purified cytolysin preparation was lethal for mice at 3 μg/kg (intravenous 50% lethal dose). The vvhA gene is the

structural gene for the V. vulnificus hemolysin-cytolysin. Regions of the vvhA gene showed homology to the structural gene for the Vibrio cholerae El Torr hemolysin (Yamamoto et al., 1990; Yamanaka et al., 1990).

Other virulence factors of V. vulnificus are protease, elastase, collagenase, DNAse, lipase, phospholipase, mucinase, chondroitin sulfate, hyaluronidase, fibrinolysin, and alkyl sulfatase. Protease exotoxin promotes the production of skin lesions by degrading collagen, elastin and casein and enhances vascular permeability. V. vulnificus adheres to human buccal epithelial cells and possesses hemagglutinating activity (Reyes et al., 1985). The presence of plasmids do not correlate with extracellular hemolysin-cytolysin production, cytotoxicity for Chinese hamster ovary cells, or virulence (Oliver, 1989).

Iron plays an important role in the pathogenesis of V. vulnificus infection. In V. vulnificus infections involving septicemia, most patients have an underlying disease. In cases of patients with septicemia, 80% had liver dysfunction (Oliver, 1989). During liver damage there is a release of stored iron into the circulatory system. Transferrin, the major iron-binding protein in serum, normally binds released iron. The level of free iron in serum is normally 10^{-18} M. Bacterial cell growth requires about 10^{-6} M. During chronic liver damage there may be an overload of the serum iron

binding capacity of transferrin.

There is a strong correlation between serum iron levels and the size of V. vulnificus inoculum required to cause death in mice. Iron is the limiting factor in the survival and growth of V. vulnificus in serum of healthy individuals. Normal human serum is bactericidal for V. vulnificus. The bacterium cannot compete with transferrin or other iron-binding proteins (lactoferrin, ferritin) for iron for cell growth and pathogenesis. In serum of healthy individuals avirulent and virulent strains could not compete with transferrin for iron when transferrin was not saturated. V. vulnificus is unable to obtain iron from transferrin when it is only 30% saturated with iron. Some virulent strains can use transferrin-bound iron when it is 100% saturated because they produce both the hydroxamate and phenolate types of siderophores. The intraperitoneal 50% lethal dose (LD_{50}) in mice is 10^6 to 10^8 organisms but if mice are injected with iron before bacterial challenge, the intraperitoneal LD_{50} is 100 organisms (Bachman et al., 1983; Morris, Jr. et al., 1987; Oliver, 1989; Wright et al., 1981)

Other factors are involved in the virulence of V. vulnificus. This bacterium is resistant to phagocytosis and possesses an antiphagocytic surface component. Virulence of V. vulnificus is attributed to the presence of an acidic polysaccharide. There are some morphological differences

between virulent and avirulent strains. Avirulent strains exhibit only translucent colonies, while virulent strains show opaque colonies. The opacity of a strain is associated with the presence of polysaccharide capsule (Bahrani and Oliver, 1990).

The factors that are linked to virulence are the patients' elevated serum iron levels, production of cytotoxic and cytolytic extracellular factors, siderophores and the resistance of strains to antibacterial activity of human serum (Tamplin et al., 1983).

3. Distribution in the environment

V. vulnificus is associated with estuary and mangrove locations (Eyles, 1986; Rivera et al., 1989). There are a wide range of environmental sources including seawater, sediment and plankton, as well as shellfish from those waters. Shellfish implicated with V. vulnificus are oysters, clams, mussels, and crabs (Kelly and Dinuzzo, 1985; Tilton and Ryan, 1987). There is a direct correlation between water temperature and isolation of V. vulnificus from both seawater and shellfish. V. vulnificus can be found in water when the temperature is greater than 17°C (Tilton and Ryan, 1987). V. vulnificus is sensitive to cold, experiencing metabolic damage at low temperatures (Oliver, 1989). Salinities also affect the prevalence of

the organism. There is no growth at less than 0.1% or greater than 5% NaCl. The organism can be recovered from water when the salinity is 7 to 16 ppt. The optimal temperature is 37°C and the optimal salinity is 10 to 20 ppt (Kelly, 1982; Tamplin et al., 1982).

There are higher numbers and more frequent isolation of V. vulnificus from samples taken during the summer and fall months (Oliver et al., 1983; Tamplin et al., 1982; Tilton and Ryan, 1987) than in the winter months. In the Great Bay Estuary system of New Hampshire and Maine, V. vulnificus was isolated from shellfish in July at levels of $>10^5$ MPN/100 g of meat and remained $>10^3$ MPN/100 g through August. Water samples also had high concentrations of V. vulnificus in August ($>10^4$ MPN/100 mL) but declined through September into fall. The reduction in the cell population in the fall was greater in water samples than in shellfish (O'Neill et al., 1990). In seawater sampled around Galveston Island, Texas, 36% of samples yielded V. vulnificus of up to 500 bacteria/mL of seawater (Kelly 1982). Cook and Ruple (1989) found 10^3 to 10^4 V. vulnificus/g of oysters present at harvest in Louisiana.

4. Incidence of Vibrio vulnificus infections

V. vulnificus infections have been reported in Belgium, Canada, Japan, Senegal (Schandevyl et al., 1984) and the

U.S. (Rodrick et al., 1989). In the U.S., at least 16 states have reported the occurrence of V. vulnificus infections (Rodrick, 1991). From 1975 to 1989, there have been 115 reported cases of shellfish-associated V. vulnificus infections (O'Neill et al., 1990).

Over 80% of these cases either occurred in southern or Gulf coast states or shellfish harvested from these areas (Kaysner et al., 1987). Between 1981 and 1987, 62 cases of V. vulnificus infection were reported to the Florida Department of Health and Rehabilitation Services (Morris, Jr., 1988). Several cases have also been associated with Long Island Sound, in Connecticut, as well as instances in northern New England water and shellfish. There has also been incidences of infection on the Atlantic and Oregon coasts, and in California waters (Oliver, 1982).

5. Clinical manifestations

V. vulnificus can cause primary septicemia or wound infection depending on the means of entry of the bacterium in humans. Primary septicemia may occur in individuals after ingestion of the bacterium. Wound infection occurs when the bacterium enters through a skin lesion that can be as small as an insect bite. V. vulnificus infections result from contact with contaminated seawater or consuming seafood harboring the bacterium (Ratner, 1987). Other clinical

manifestations of atypical _V. vulnificus_ infections are pneumonia and sepsis seen in a drowning victim (Kelly and Avery, 1980), corneal ulcer and endometritis (Tison and Kelly, 1984).

The development of primary septicemia in patients is seen in those individuals with underlying chronic disease, such as liver and blood dysfunctions, diabetes, cancer, AIDS, but also in healthy patients. The diseases are generally liver- or blood-related (Bachman _et al._, 1983). The fatality rate is 40 to 60% (Blake _et al._, 1979; Finegold and Baron, 1986; Tamplin _et al._, 1983). Most cases occur during warm months (May to October) and in men (90%) 40 or more years of age (95%). About 85% of the patients had consumed raw oysters (Oliver, 1989).

The incubation period for the onset of symptoms may be from 7 hr to days with the median incubation time between 16 to 38 hr. The symptoms of the illness are fever (94% of patients), chills (86%), nausea (69%), and hypotension (43%). Intestinal symptoms commonly associated with foodborne infections, such as abdominal pains, vomiting, and diarrhea are less common with _V. vulnificus_ infections. Secondary lesions may occur on the extremities and frequently become necrotic. It may become necessary to amputate the limb. Tetracycline therapy may be an effective treatment (Bachman _et al._, 1983; Blake _et al._, 1980).

In at least two cases, V. vulnificus caused wound infections by entering through a skin lesion (Blake et al., 1979; Rodrick, 1991) due to exposure to seawater and/or shellfish. A wound infection resulted in a male during shrimp peeling (Meadors and Pankey, 1990). In wound infection, there is extensive tissue destruction, usually requiring surgical debridement and skin grafting. Invasion by the bacterium of the skeletal muscles has been documented (Fernandez and Justiniani, 1990).

Symptoms occur more rapidly with wound infection than with primary septicemia. The incubation period may be as short as 4 hr, with an average of 12 hr. The symptoms are intense pain, erythema (redness), and edema at the site of injury. The area rapidly develops lesions, and vesicles or bullae on the lesions. The skin erupts and the adjacent tissues have purple-blue discoloration, which may spread to cover large areas within a few days. Portions of the lesions become necrotic. Severe inflammation of the subcutaneous tissues occurs that can extend into skeletal muscle causing extensive tissue damage. Surgical debridement of the infected area is often necessary and generally the affected limb is amputated. The mortality rate from wound infection is 22%. Twenty-one percent of patients in wound infection cases experienced liver or blood dysfunction and 57% generally had some underlying disease

(Bachman et al., 1983).

6. Fecal coliform as an indicator of V. vulnificus

There is no relationship between the presence of V. vulnificus and the standard fecal coliform MPN value for seawater (14/100 mL) or oyster meats (2.3/g). Both seawater and oysters can serve as a vehicle for transmission of vibrio infections, even when considered safe under the present government guidelines. Thirty-seven percent of seawater samples taken from approved waters (≤14 MPN fecal coliform/100 mL) in Appalachicola Bay, Florida, contained V. vulnificus, whereas 17.6% of seawater samples taken from areas with ≥14 MPN of fecal coliform/100 mL contained V. vulnificus. The percentage of V. vulnificus in oyster meats obtained from the areas were similar with 40.9% for the approved areas, and 42.8% for areas having ≥14 MPN fecal coliform/100 mL seawater (Rodrick et al., 1984). Blake and coworkers (1982) also showed V. vulnificus to be over 33% more common at approved stations than prohibited stations.

Tests used to indicate fecal contamination are not good indicators of Vibrio spp. in foods. Vibrios may be part of the normal flora of the environment from which foods are harvested (Desmarchelier, 1984). There is no correlation of V. vulnificus with fecal coliform levels (Oliver et al., 1983).

C. Depuration

1. Disinfection of depuration water

There are two ways shellfish can be purified to reduce the concentration of contaminants: relaying and depuration. Relaying involves harvesting shellfish from contaminated areas and transferring to clean shellfish growing areas. The rate at which microorganisms are reduced is slower in relayed oysters than in depurated oysters. This is attributed to fluctuations in temperature and salinity, deviation from the ideal temperature and salinities, and mechanical disturbances of animals by currents and other animals (Cook and Ellender, 1986). Shellfish placed in these beds may be subject to recontamination, increased shellfish mortalities from shellfish pathogens, and predation by rays and other animals. Purification under these conditions is subject to other disadvantages. One is a continuing decrease in the availability of clean waters.

Depuration systems may have a continuous flow-through of seawater or recirculation of seawater through the system. In flow-through systems, seawater is continuously pumped from a river or estuarine source through a sterilizing unit, through the depuration tank, and then is discharged. Recirculating systems have water passing through the sterilizing unit, into the depuration tanks, and recirculating through the sterilizing unit. Recirculated

water is disinfected to prevent bacterial accumulation and recontamination of shellfish.

Water can be disinfected using hypochlorite or chlorine, iodophores, ozone, or ultraviolet light (Fleet, 1978; Richards, 1991). Chlorine is effective in microbial reduction but enteric viruses, such as Norwalk, hepatitis A, and polio viruses seem to be less affected. Chlorine also has adverse affects on shellfish feeding and cleansing effectiveness. Even after dechlorination of water using sodium thiosulfate, activated charcoal, or vigorous aeration, water disinfected with chlorine showed a decrease in activity of oysters compared to untreated seawater. The use of chlorine does not seem to be effective, according to Ledo _et_ _al_. (1983). Chlorine was compared as a disinfectant with an untreated seawater system. There was a 62.4% reduction in total viable counts, fecal coliforms, _E_. _coli_, and fecal streptococcus after 48 hr in water disinfected with chlorine compared to a 90.1% reduction in untreated seawater.

Iodine is a powerful antimicrobial agent, and is used in the form of an iodophor as a disinfectant in some European countries. An iodophor is a combination of iodine, hydroiodic acid, and a detergent. Bacterial reduction occurs with 0.1 to 0.4 mg iodophor/L of tank water. There seems to be no effect on the shellfish feeding ability with

the use of iodophores as a disinfectant. Although there is an increase in iodine content in the shellfish flesh by 0.5 to 1.0 mg/kg (Fleet, 1978) the increase may not be objectionable to consumers who are not on low iodine diets.

Ozone is an oxidizing agent that effectively kills bacteria and viruses in the tank water. The use of ozone as a disinfectant is limited, found only in France and Australia, because ozone is toxic to shellfish. Treated water must be aerated before it is added to the tank and any possible residual levels must be closely regulated.

Ultraviolet light irradiation is the most common system in the U.S. and United Kingdom. Microorganisms will only be destroyed if they come in direct contact with the light. Ultraviolet light is only effective to a depth of a few mm and particles can not be greater than 20 μm (Steslow, 1987). Ultraviolet light irradiation is effective in reducing the number of bacteria and viruses in water. The use of this disinfectant does not leave residuals and does not inhibit the feeding activity of the shellfish. High turbidity in water can reduce the effectiveness of the ultraviolet light by impairing light penetration. Sequestered or endogenous microorganisms can not be inactivated with this process. There may also be increases in the number of ultraviolet-resistant bacteria in the treated water.

A new technology of disinfecting depuration tank water

is the use of activated oxygen. Photooxidation of oxygen by ultraviolet light at a wavelength of 180 nm produces activated oxygen (Richards, 1991). Activated oxygen is made up of the hydroxyl radical, atomic oxygen, ozone, hydrogen peroxide and hydrogen dioxide. A number of organic compounds may be formed in water and the effect of these compounds on shellfish has not been investigated.

2. Factors affecting depuration

Factors that affect oyster feeding and microbial removal are water temperature, salinity, turbidity, and dissolved oxygen levels. When the water temperature is lowered there is a reduced feeding activity of oysters. The minimum temperature recommended in the U.S. for depuration is 10°C. Optimal feeding occurs at 20°C. Temperatures above 20°C is not recommended because of the risk of oyster spawning and because of reduced oxygen levels in the water (Perkins et al., 1980). Oysters harvested in warm waters and placed in a depuration tank containing cooler water eliminated coliforms at slower rate than those placed in warmer water.

Salinity can affect the physiological state of shellfish by hindering in the pumping ability. At low salinity, pumping may completely stop. Shellfish seem to depurate well at a salinity of around 30 ppt. The optimal

salinity for oysters is 24 to 25.5 ppt.

Excessive turbidity can reduce the effectiveness of the ultraviolet disinfection unit, as well as reducing the ability for shellfish to pump at an optimal rate. In recirculating systems, water turbidity is less of a problem since oysters remove particulate material through their feeding activities. Moderate to high turbidity levels do not seem to deter the normal functions of the oyster (Perkins et al., 1980). Severe turbidity is a problem when oyster spawning occurs in the tank.

The recommended dissolved oxygen level is above 50 to 100% saturation. Inadequate dissolved oxygen will affect the physiological state of oysters. Haven et al. (1978) recommended a dissolved oxygen level greater than 5.0 mg/L of seawater. Dissolved oxygen levels are generally produced by spraying water over the surface of the tank.

3. Guidelines on depuration

The National Shellfish Sanitation Program is responsible for guidelines on shellfish purification. The level of fecal coliforms in oysters after the conventional 48 hr of depuration are monitored and enforced by state and federal agencies. The fecal coliform MPNs in these oysters may not exceed 100/100 g of sample. Each batch of oysters must comply with this guideline (Richards, 1991).

To increase the frequency of meeting these guidelines, shellfish harvesting waters must meet water quality criteria based on the level of pollution in the water. Shellfish can only be harvested from approved waters (mean MPN of ≤70 total coliforms or ≤14 fecal coliforms/100 mL) or restricted waters (mean MPN of ≤700 total coliforms or ≤88 fecal coliforms/100 mL). Shellfish from approved waters can be sold without depuration. Shellfish from prohibited waters (MPN of >700 total coliforms or >88 fecal coliforms/100 mL) can not be harvested or depurated.

4. Removal of microorganisms

Most of the initial work on elimination of microorganisms by oysters during depuration has been focused on coliforms, since these microorganisms have been used as indicators. Laboratory depuration reduced *E. coli* numbers from 100 cells/g to an undetectable levels within 18 hr (Son and Fleet, 1980). Contaminated bivalve mollusks with high levels of *E. coli*, *Salmonella* (Rowse and Fleet, 1984), and *Clostridium perfringens* were purified to undetectable levels after 48 hr of depuration (Fleet, 1978; Son and Fleet, 1980). Oysters injected with 10^2 to 10^3 CFU of *Campylobacter* spp./g of oyster tissue were effectively cleansed during depuration for 48 hr (Arumugaswamy *et al.*, 1988). However low levels of *E. coli* and *Salmonella* may be

present in oysters after the 48 hr period (Janssen, 1974; Rowse and Fleet, 1982). Fecal coliform elimination may not be a good measure of depuration effectiveness, since it is easily removed from oysters while other microorganisms may not be removed at the same rate (Anonymous, 1988; Richards, 1991).

The commercial depuration process has little effect on the removal of *Vibrio* spp. from oysters. Eyles and Davey (1984) showed that there is no significant difference in the number of *V. parahaemolyticus* between oysters that have been depurated and those that have not been depurated. There is a difference in the efficiency of depuration between oysters naturally infected with *V. vulnificus* and oysters artificially-inoculated with the organism (Kelly and Dinuzzo, 1985).

Rodrick *et al*. (1989) demonstrated 100% reduction of *V. cholerae* and 99.9% reduction of *V. vulnificus* in artificially-inoculated clams placed in depuration tanks for 48 hr. During artificial inoculation, *V. vulnificus* may be so easily removed from the shellfish tissues because they readily migrate through the digestive tract and become entrapped in the feces. Gradual exposure to *V. vulnificus* in natural surroundings may lead to colonization within the cells of the hepatopancreas and digestive diverticulum (Richards, 1991).

Viruses are eliminated at a slower rate than bacteria. Some may not be removed in the standard 48 hr depuration period (Anonymous, 1988; Martinez-Manzanares et al., 1991). Carter and Cantelmo (1989) reported that commercial depuration may not always result in products free from viruses. The viruses of most concern to humans are the enteric virus, such as hepatitis A and Norwalk virus (Cantelmo and Carter, 1989). Most resistant to elimination was bacteriophage f2, a bacterial virus similar in size and composition to the enteric viral pathogens. When oysters accumulated large numbers of virus particles, they failed to eliminate the virus because virus particles readily adsorb to suspended solids in the water. In sewage-polluted estuarine waters, viruses are taken up by oysters by adsorbing to and remaining attached to particulate matter as they pass through the oyster digestive system (Cook and Ellender, 1986). Depuration up to 72 hr may not give a product that satisfactorily reduces bacterial level. Mussels containing initial levels greater than 5 X 10^3 E. coli/100 g may not meet the standard 230 E. coli/100 g after 48 hr of depuration (De Mesquita et al., 1991).

The level of E. coli is a commonly used standard for determining the microbiological quality of shellfish and the efficiency of a depuration system. However, E. coli is not a good indicator of V. vulnificus or viral elimination in

oysters during depuration (Power and Collins, 1989). Group D fecal streptococcus and clostridial spores have been recommended as possible indicators (De Mesquita *et al*., 1991). Fecal streptococci and clostridia spores are more tolerant to the acidic conditions of the digestive tract of bivalve mollusks than *E. coli*. The use of alternate organisms may serve as better indicators of depuration efficiency. Enteric viruses are limited as potential indicators because of the lack of a rapid system for direct detection and identification in shellfish (Richards, 1988).

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SECTION II: Survival of *Escherichia coli* and *Vibrio vulnificus* in Oyster Tissues During Storage in a Spray Live Holding System

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INTRODUCTION

Live holding systems are used as temporary storage facilities for displaying shellfish during retail sales. These systems may hold shellfish submerged in water or above the water level. Oysters commonly are held above the water level in tanks where water is sprayed onto the shells. If oysters have been stressed by temperature fluctuations and mishandling, the survival and growth of pathogenic microorganisms during wet storage may be affected.

A pathogen of increasing concern in oysters is *Vibrio vulnificus*. It is ubiquitous in marine and estuarine environments and virulent to man. Oysters acquire the microorganism as a result of the normal filtration process (Kelly and Dinuzzo, 1985) and these naturally infected oysters are prevalent in harvests from warm waters (Anonymous, 1990; Kelly and Dinuzzo, 1985).

Since *V. vulnificus* is commonly associated with seafood (Kelly, 1982; O'Neill *et al.*, 1990; Tilton and Ryan, 1987),

many individuals have suffered gastroenteritis from consuming contaminated raw or undercooked oysters. From 1975 to 1989, 115 cases of shellfish-associated V. vulnificus infection (O'Neill et al., 1990) were reported. V. vulnificus also has been implicated as a causatory agent of primary sepsis in immunocompromised individuals. Eighty-five percent of the patients suffering from primary septicemia had consumed raw oysters (Oliver, 1989). The mortality rate for infected individuals was between 40 and 60% (Tacket et al., 1984; Finegold and Baron, 1986).

This study examined both stressed and healthy oysters held in the same spray live holding system. The physiological state of the oysters prior to their introduction into a spray live holding tank was emphasized. Survival of E. coli in the system was monitored as an indicator of the effect of stress on the microbial population of oysters.

Oysters used in this experiment may resemble those that are harvested on the coast and sold to inland retail stores. There are limited data on whether interstate shipping increases the microbial population in the oysters. The truck refrigeration units do not always maintain temperatures below 7.5°C and above 4.4°C. As a result, multiplication of the bacterial populations in the oysters during shipping may contribute to an increase in the number

of infections resulting from the consumption of raw oysters (Anonymous, 1989; Cook and Ruple, 1989; U.S. Food and Drug Administration, 1988).

The effect of a spray tank on the survival of _V. vulnificus_ in oysters was also examined. A converted spray holding tank system was used to compare the effect of immersed and conventional depuration tank storage on the survival of _V. vulnificus_ in oysters.

MATERIALS AND METHODS

Preparation of oysters

Oysters, Crassostrea virginica, (30 to 75 mm), were harvested from the Rappahannock River in Virginia (sampled all year long) or harvested on the Gulf Coast of Louisiana and transported to Virginia. Oysters subject to interstate transport and held in dry storage for a minimum of 10 days at ambient temperature were considered physiologically stressed. Oysters harvested in Virginia were considered unstressed. All oysters were scrubbed with a wire brush under running cold tap water to remove surface dirt and foreign material. They were then placed in a spray tank (Marineland, Simi Valley, CA) for live holding studies or in a contamination tank (described below) for inoculation prior to placement in a live holding tank.

The modified spray tank (Fig. 1) was a recirculating system with the following parameters: the filter system was a polyester wool pad placed above activated charcoal (12 cm in depth); the volume capacity was 93.0 L; the flow rate was 160 GPH; the water volume was 32.2 L; and the estimated intensity of illumination of the ultraviolet lamp was 7.1 X 10^2 W/m^2 with a window area of 0.79 cm^2.

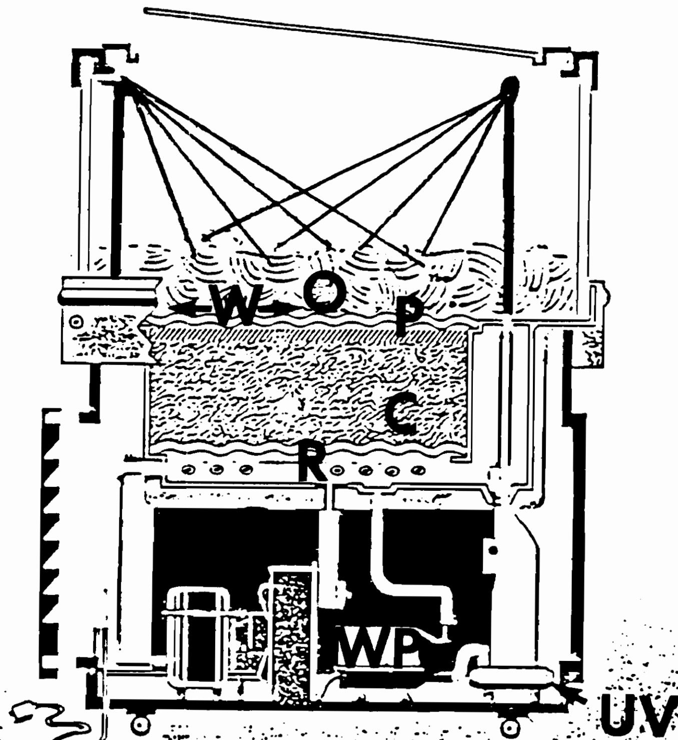

Fig. 1: Schematic diagram of a live holding tank. The tank had the following features: (W) water level; (O) oyster storage area; (P) polyester pad; (C) activated charcoal; (R) refrigeration unit; (WP) water pump; and (UV) ultraviolet disinfection lamp.

The spray nozzles were modified to ensure that the surface of all the live shellfish was sprayed with water.

The system was surface-aerated by spray nozzles. The salinity of the water was 24 ppt and the temperature was maintained at 20°C. The salinity value was determined by measuring the specific gravity of water (read with a hydrometer, Marineland, CA) and converting to salinity in parts per thousand (ppt) using the formula: (specific gravity - 1) x 1323 = salinity (Kelly, 1982).

Laboratory and personal safety measures

The spray tank was placed in a restricted access room under negative air pressure. An ultraviolet light (30 watt GE model # G30T8) was placed above the tank to control any airborne bacteria that may be released as aerosols during spraying of the water. Oyster shucking and bacterial enumeration were restricted to another "authorized personnel only" room. Safety goggles and a gas respirator (Half Mask Silicone Respirator, MA-7523, Laboratory Safety Supply, Janesville, WI) were worn during all contacts with the spray tank, oyster shucking, and *V. vulnificus* enumerations.

Disinfection of spray tank

The spray live holding tank and the inoculation tank were disinfected between experiments with P3-topax 99

(Henkel Corporation Chemical Services Division, Burlington, IA) containing 5.25% sodium hypochlorite. Before each experiment, all the tubing of the tank, the pump motor and the ultraviolet disinfecting lamp were removed from the tank system. The pump and the ultraviolet lamp were disassembled and the parts were scrubbed with a brush and disinfected with sodium hypochlorite solution at 200 ppm. Residual chlorine was removed by rinsing with distilled water.

After each experiment, any remaining oysters were removed and 200 ppm solution of sodium hypochlorite was added to the tank. The spray live holding tank was run continuously for 48 to 72 hr. Then, all parts of the tank including the filter pad, charcoal, and tubes were autoclaved before handling. The system was run for another 48 hr without the above-mentioned parts after which the tank water was discarded and the parts were cleaned and sanitized as described above.

Artificial inoculation of oysters with *Escherichia coli* ATCC 9637

Stock cultures of *E. coli* ATCC 9637 were maintained on Trypticase Soy Agar (TSA) slants at 4°C. The *E. coli* culture used for infection of oysters was prepared by inoculating Trypticase Soy Broth (TSB), and incubating at 37°C for 24 hr. One mL of the culture broth was transferred

to TSB and incubated for 10 hr at 37°C to yield 10^8 to 10^9 CFU/mL. The cell density was determined in the following way: ten mL of broth was spun for 10 min at 10°C and 3,000 x g (Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge), the supernatant was decanted, the pellet was washed with Phosphate Buffered Saline (PBS) (pH 7.6; 0.01 M), and recentrifuged for 10 min at 3,000 x g. The supernatant was decanted and the pellet was resuspended in PBS. The OD values of serial dilutions of cell suspensions in PBS were read on a Bausch and Lomb Spectronic 70 spectrophotometer at 600 nm. The cell density was estimated from plots of optical density vs. log CFU/mL. Cell numbers were estimated from colonies grown on Violet Red Bile Agar (VRBA) incubated for 45°C for 24 hr. During infection, oysters were placed in a single layer in a tank (48 cm x 30 cm x 65 cm) with 10 L of artificial seawater and E. coli at a concentration of 10^4/mL artificial seawater. Oysters were allowed to feed in a medium containing a high concentration of E. coli to simulate an increase in populations occurring from temperature-abuse during handling and storage in live holding tanks. Air stones were placed in the tank to aerate the artificial seawater. Oysters were allowed to feed at 25°C for 12 hr.

Bacterial enumeration

Ten oysters (approximately 100 g of meat) were shucked using aseptic techniques according to the Compendium of Methods for oysters (Hunt et al., 1984). Adductor muscles were separated from the shells using a scalpel sterilized by rinsing with 95% (v/v) ethanol and passing through a flame.

Oyster meat was placed in sterile Stomacher bags (Steward Medical Stomacher 400 Bags, Fisher) and diluted 1:10 with PBS. The mixture was homogenized using a Stomacher Lab-Blender 400 (Steward Medical UAC House, London, England) for 2 min. Samples of 10 mL tank water were also taken and enumerated for E. coli and/or V. vulnificus.

For enumeration of E. coli, homogenized oysters and tank water samples were diluted between $1{:}10^1$ to $1{:}10^6$ with PBS. E. coli was enumerated using the methodology to repair injured cells (Hackney et al., 1979; Ray and Adams, 1984). Samples were poured onto plates of TSA, incubated 1 to 2 hr at 25°C, overlaid with double strength VRBA, and incubated at 45°C for 24 hr (Hackney et al., 1979). Several colonies from the VRBA-overlaid TSA plates were plated on Eosin Methylene Blue Agar (EMB) and incubated for 24 hr at 35°C. Colonies which displayed the following phenotype: dark red colonies with a zone of precipitated bile acids on VRBA-overlaid TSA plates and very dark colonies with metallic

sheen on EMB plates, were confirmed as E. coli using API 20E system biochemical strips (Analytab Products, Plainview, NY).

V. vulnificus was enumerated in oyster samples and tank water using the Most Probable Number (MPN) enumeration technique recommended by the Shellfish Sanitation Branch of the Center for Food Safety and Applied Nutrition of U.S. Food and Drug Administration (U.S. Food and Drug Administration, 1988).

The homogenized oyster samples and water samples were diluted between $1:10^1$ to $1:10^6$ with PBS. The diluted samples were inoculated into Alkaline Peptone Water (pH 8.5) (APW) in a 3-tube MPN series for enrichment and incubated for 12 hr at 37°C. One loopful from APW tubes showing growth in APW were streaked on plates of Thiosulfate Citrate Bile Salt Sucrose Medium (TCBS) (Lotz et al., 1983) and Cellobiose Polymyxin Colistin Medium (CPC) (Massad and Oliver, 1987) and incubated for 24 hr at 37°C to select for V. vulnificus colonies.

Typical V. vulnificus colonies appeared as 2 to 3 mm diameter raised green to blue-green colonies on TCBS and as 2 mm diameter flat, yellow colonies on CPC. Typical V. vulnificus colonies were confirmed biochemically using API Rapid NFT system (Analytab Products, Plainview, NY) with modifications. Colonies were suspended in 2% saline

solution instead of 0.85% as suggested by Analytab Products. The increase in salt concentration allowed for better growth of the organisms in the biochemical tests (MacDonell *et al.*, 1982).

Statistical analysis

The initial and final *E. coli* and *V. vulnificus* populations in oysters stored in a spray live holding system were analyzed using analysis of variance (ANOVA) of the Statistical Analysis System (SAS, 1985). Significances of the differences between the means of initial and final bacterial levels were determined using least significant difference (LSD) (Sokal and Rohlf, 1981). Means with F values ($p < 0.05$) were significantly different from each other.

RESULTS AND DISCUSSION

Survival of *Escherichia coli* in oysters during storage

Artificially-inoculated oysters had an initial *E. coli* concentration of 1.3 X 10^6 CFU/g of oyster tissue (Fig. 2). During the 120 hr of storage in the spray system, there was a gradual 100-fold decrease in *E. coli* populations in the oysters. But the differences in concentrations of *E. coli* in oysters prior to storage and after 120 hr of storage in the spray tank were not significant ($p = 0.12$) (Statistical Analysis System, SAS). Since a spray holding tank had not been previously examined, the results of this study were compared to research data obtained from depuration systems.

Results with trends similar to those observed in this study were reported by other researchers. When inoculated with fecal coliforms greater than 10^3 MPN/g, oysters and mussels were not able to reduce the fecal coliform levels in a depuration system (Buisson *et al.*, 1981; Power and Collins, 1989). Even though a high level of *E. coli* was used to inoculate oysters, the level did not increase in oyster tissues during the 120 hr of storage. The increase

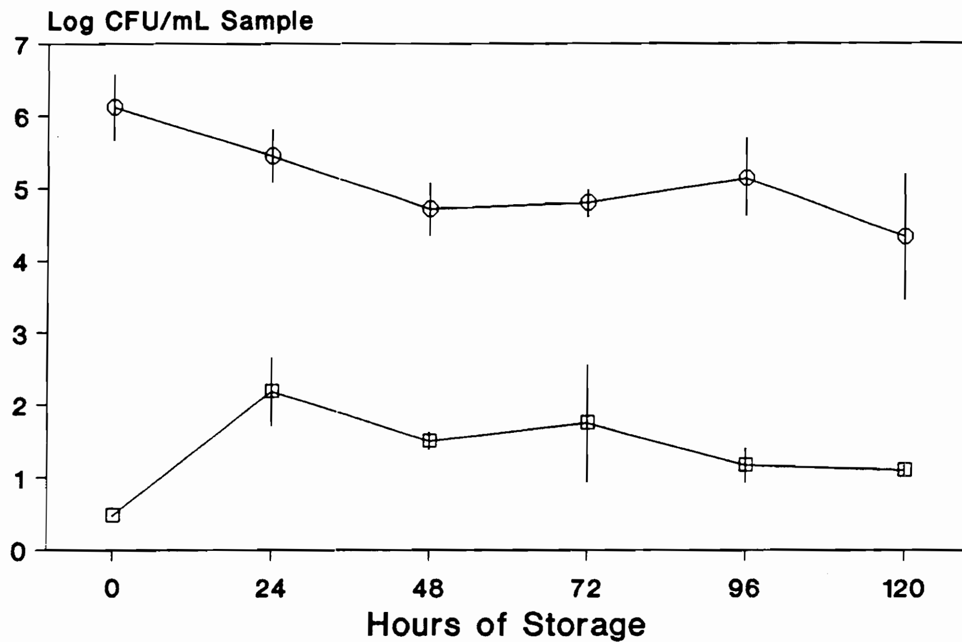

Fig. 2: Survival of E. coli in oysters during storage in a spray live holding tank. Oysters (○) and tank water (□) were enumerated for E. coli. On the Figure, 3 CFU/mL represents any value <3 CFU of E. coli/mL of water.

in the number of E. coli in the tank water may have been the result of feces and pseudo-feces being released into the water or growth of the organism in the water.

Survival of E. coli in stressed oysters

The effect of stress on the survival of E. coli in oysters was examined (Fig. 3). There were no significant increases in the E. coli levels in oyster tissues ($p = 0.30$) during 96 hr of storage in the spray system. E. coli levels in the tank water increased from <3 CFU/mL to 1.7×10^2 CFU/mL. The increase of E. coli in the tank water may indicate that the oysters were physically active. The results of this study were compared to studies of the efficiency of depuration by stressed oysters. Previous studies demonstrated that oysters stressed from temperature fluctuations and mishandling had a decreased efficiency of depuration (Cook and Ellender, 1986; Eyles and Davey, 1984; Mitchell et al., 1966). Power and Collins (1989) also observed that the rate of E. coli elimination by mussels was dramatically affected by stress. In contrast, we found that the physiological stress imposed on the oysters as a result of interstate shipping did not affect the survival of E. coli in oysters during storage in a spray tank. If an

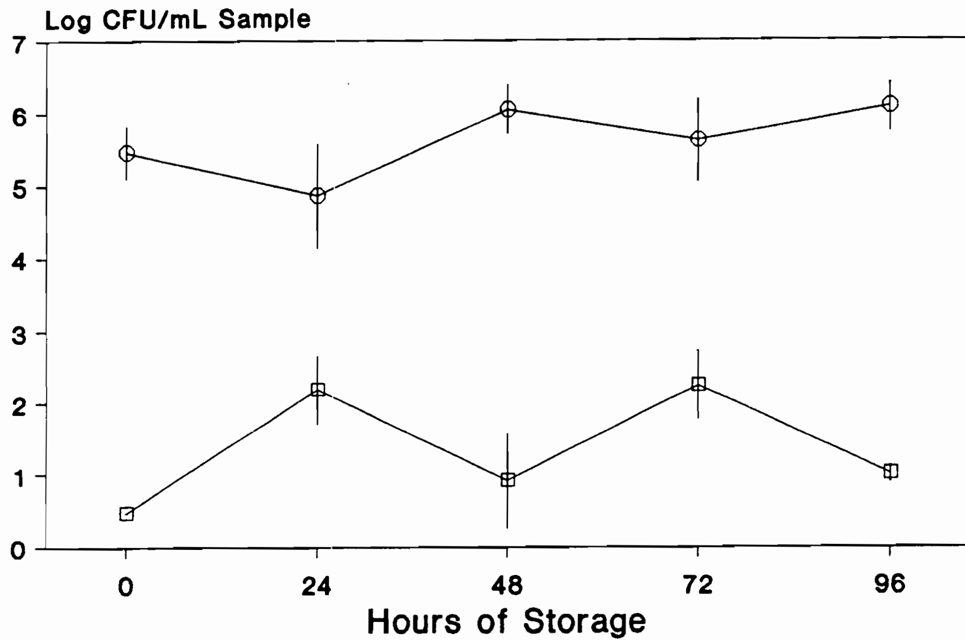

Fig. 3: Survival of E. coli in physiologically-stressed oysters during storage in a spray live holding tank. Oysters (O) and tank water (□) were enumerated for E. coli. On the Figure, 3 CFU/mL represents any value <3 CFU of E. coli/mL of water.

increase did occur, it was offset by a concomitant decrease into the tank water.

Cross-contamination of oysters and water

The effect of storing stressed and unstressed oysters with two different initial levels of E. coli in the same tank was examined (Fig. 4). During 72 hr of storage in the spray tank, E. coli levels in stressed oysters remained relatively constant at 10^5 CFU/mL while the level of E. coli in the unstressed oysters gradually increased from an initial count of 5.8 X 10^2 CFU/mL to 4.5 X 10^4 CFU/mL. The initial level of E. coli in unstressed oysters (5.8 X 10^2 CFU/mL) was significantly different ($p = 0.0001$) from the initial count in the stressed oysters (1.5 X 10^5 MPN/mL). After 72 hr of storage the difference in the level of E. coli in the oysters was not significant ($p = 0.38$). The number of E. coli in the tank water steadily increased from <3 CFU/mL to 2.5 X 10^2 CFU/mL.

Survival of V. vulnificus and E. coli naturally present in oysters

Oysters naturally containing low levels of V. vulnificus and E. coli were placed in the spray live holding tank to duplicate the holding systems used in retail food

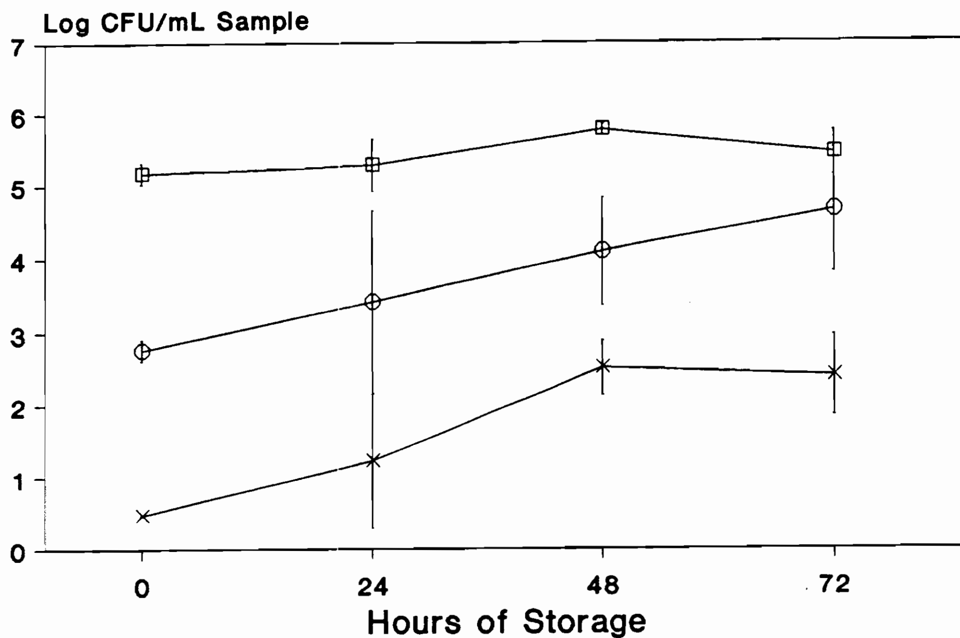

Fig. 4: Effect of storing stressed and unstressed oysters with two different initial levels of E. coli in the same tank. Stressed oysters (□), unstressed oysters (O) and tank water (×) were enumerated for E. coli. On the Figure, 3 CFU/mL represents any value <3 CFU of E. coli/mL of water.

establishments (Fig. 5). V. vulnificus was present in oysters harvested during the summer months. Similar prevalence of V. vulnificus in oysters during warm months has been previously noted (Anonymous, 1990; Kelly and Dinuzzo, 1985). Alvarez (1984) found that oysters purchased in seafood markets in Madison, Wisconsin contained E. coli levels as high as 32 MPN/g.

In this study, there was a significant difference ($p = 0.0001$) between the initial levels of V. vulnificus and E. coli in oysters stored in the spray system. However, after 72 hr of storage there was no difference between the levels of the two organisms. V. vulnificus and E. coli showed similar survival trends in oysters. This stands in contrast to depuration studies where E. coli was easily removed from oyster tissues (Rowse and Fleet, 1984). In laboratory depuration studies, E. coli levels were reduced from 100 cells/g of oyster meat to undetectable levels within 48 hr (Son and Fleet, 1980). The survival of V. vulnificus in oysters stored in the spray system used in this study correlates well with the results of studies of depuration systems (Richards, 1991). V. vulnificus is not easily depurated from oysters because it is a part of the natural flora of oysters, possibly incorporating itself into the tissues of the digestive system (Kueh and Chan, 1985).

V. vulnificus and E. coli naturally present in oysters

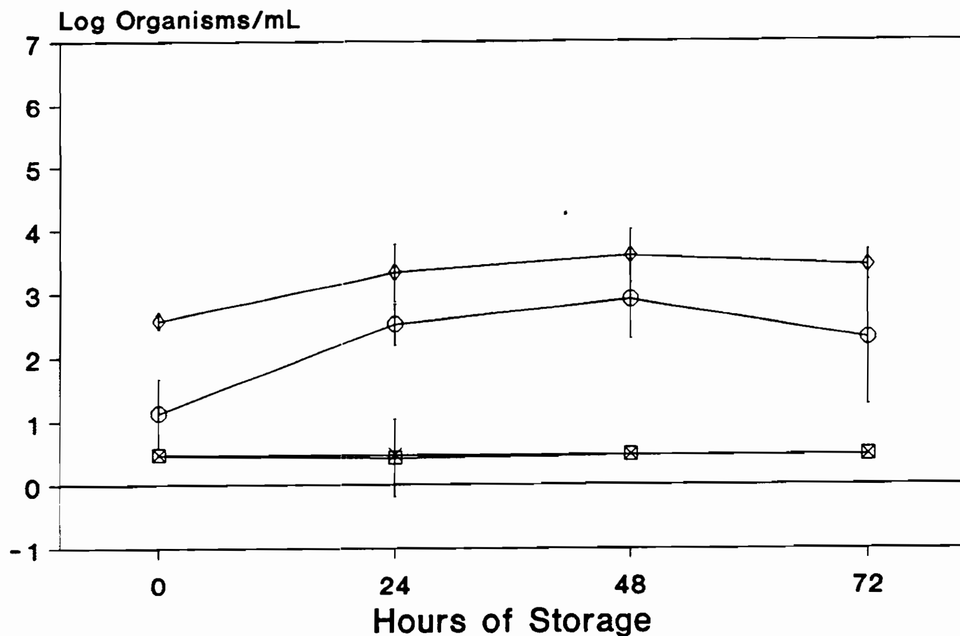

Fig. 5: Survival of E. coli and V. vulnificus in unstressed oysters during storage in a spray live holding tank. E. coli in oysters (○) and tank water (□), and V. vulnificus in oysters (◊) and tank water (×) were enumerated. On the Figure, 3 organisms/mL represents any value <3 organisms/mL of water.

survived during storage in the spray system described in this study. Storage of oysters in a spray live holding tank may not promote the growth of V. vulnificus and E. coli in the oyster tissues. However, oysters held in these systems do not appear to remove either of these two organisms, since they persisted in the tissues after 72 hr of storage.

Comparison of immersion and spray systems

The survival of V. vulnificus in oyster tissues stored in the spray system was compared to the survival of the organism in oysters stored in the immersion system in Figure 6. The initial level of V. vulnificus in oysters placed in the spray tank was not significantly different ($p = 0.34$) from the initial level of the organism in oysters placed in the immersion tank. There was no difference between the two systems on the survival of V. vulnificus in oysters.

The data from this study showed that the level of E. coli remained unchanged in oysters stored in a spray live holding tank. The physiological state of oysters did not appear to affect the survival of E. coli in oysters. But temperature abuse and mishandling prior to storage in spray tanks may still be important factors since they may contribute to increased microbial levels in oysters.

The persistence of E. coli in oysters may indicate the survival of other microbiological populations. E. coli is a

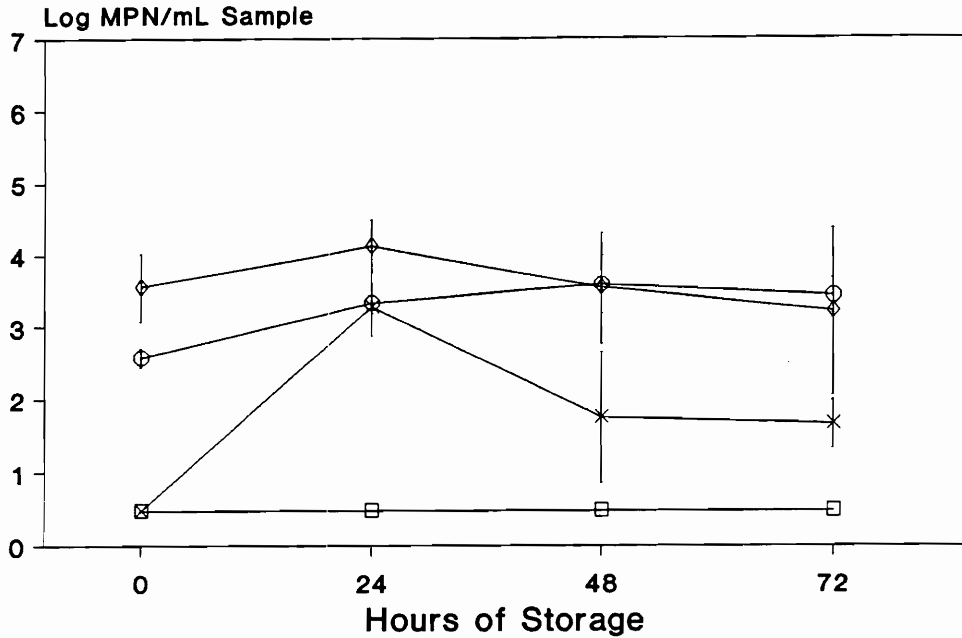

Fig. 6: Comparison of a spray and an immersion live holding systems on the survival of V. vulnificus in oysters and tank water. Oysters (O) and tank water (□) of the spray system and oysters (◊) and tank water (×) of the immersion system are enumerated for V. vulnificus. On the Figure, 3 MPN/mL represents any value <3 V. vulnificus MPN/mL of water.

commonly used standard for determining the microbiological quality of shellfish. *V. vulnificus* also survived in oysters during the storage period. There appeared to be no difference in the survival of *V. vulnificus* in oysters stored in a spray tank and an immersion tank. The level of *E. coli* may indicate *V. vulnificus* levels in oysters stored in a spray live holding tank since neither of these organisms increased or decreased in the oysters.

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APPENDICES

Appendix A: Survival of E. coli in Oysters and Water of a Spray Live Holding System

Time	Oyster		Water	
hr	log CFU/mL	STD+-	log CFU/mL	STD+-
0	6.12	0.45	<.477	0.00
24	5.45	0.36	2.18	0.46
48	4.71	0.36	1.50	0.12
72	4.79	0.18	1.74	0.80
96	5.14	0.54	1.16	0.23
120	4.33	0.86	1.09	0.06

Appendix B: Survival of E. coli in Physiologically-Stressed Oysters During Storage in Spray Live Holding System

Time	Oyster		Water	
hr	log CFU/mL	STD (+-)	log CFU/mL	STD (+-)
0	5.46	0.36	<.477	0.00
24	4.86	0.71	2.18	0.46
48	6.05	0.33	0.91	0.65
72	5.61	0.56	2.24	0.47
96	6.08	0.33	1.01	0.11

Appendix C: Effect of Storing Oysters Harboring Two Different Levels of E. coli in the Same Tank

Time	Stressed Oyster		Non-stressed Oyster		Water	
hr	log CFU/mL	STD (+-)	log CFU/mL	STD (+-)	log CFU/mL	STD(+-)
0	5.17	0.15	2.76	0.15	<.477	0.00
24	5.28	0.36	3.41	1.24	1.22	0.93
48	5.77	0.07	4.09	0.73	2.50	0.37
72	5.44	0.31	4.65	0.85	2.39	0.55

Appendix D: Survival of E. coli and V. vulnificus in Oysters and Tank Water During Storage in Spray Live Holding System

Time	E. coli log CFU/mL				V. vulnificus log MPN/mL			
hr	Oyster	STD+-	Water	STD+-	Oyster	STD+-	Water	STD+-
0	1.13	0.54	<.477	0.00	2.57	0.13	<.477	0.00
24	2.51	0.33	0.43	0.61	3.32	0.45	<.477	0.00
48	2.90	0.62	<.477	0.00	3.58	0.41	<.477	0.00
72	2.29	0.04	<.477	0.00	3.42	0.25	<.477	0.00

Appendix E: Comparison of the Survival of V. vulnificus in Oysters and Tank Water in Spray and Immersion Systems

Time	Spray System				Immersion System			
hr	Oyster	STD+-	Water	STD+-	Oyster	STD+-	Water	STD+-
0	2.57	0.13	<.477	0.00	3.55	0.48	<.477	0.00
24	3.32	0.45	<.477	0.00	4.12	0.37	3.27	0.09
48	3.58	0.41	<.477	0.00	3.53	0.77	1.74	0.89
72	3.42	0.25	<.477	0.00	3.21	1.16	1.65	0.33

All values are in units of log MPN/mL

SECTION III: DISTRIBUTION OF <u>VIBRIO</u> <u>VULNIFICUS</u> IN TISSUES OF ARTIFICIALLY- AND NATURALLY-INFECTED OYSTERS (<u>CRASSOSTREA</u> <u>VIRGINICA</u>) DURING DEPURATION

(formatted for submission to Journal of Food Protection)

INTRODUCTION

Oysters obtain food by pumping water over their gills by ciliary action. The gills function as a sieve to remove particulate material, including microorganisms (Fleet, 1978). The filtered particles become enmeshed in a mucoid material which is continually secreted by the oyster. The mucus-coated material is directed by the cilia of the gills toward the mouth of the oyster, where it can be either ingested or directed to the exterior and eliminated as pseudo-feces. Waste material from the alimentary tract is discharged as feces in a fine, mucous thread (Fleet, 1978; Galtsoff, 1964). Certain species of the ingested microorganisms become concentrated in the gut or other tissues, as a consequence of the filter-feeding system.

Oysters acquire <u>Vibrio</u> <u>vulnificus</u> as a result of normal filtration processes (Kelly and Dinuzzo, 1985). From 1975 to 1989, there have been 115 reported cases of shellfish-associated <u>V</u>. <u>vulnificus</u> infections (O'Neill <u>et</u> <u>al</u>., 1990).

Commercial depuration has been shown to be ineffective in removing this pathogen from oysters. Studies have demonstrated that Vibrio spp. can even multiply in shellfish depuration tank water and pumping systems (Barrow and Miller, 1969; Eyles and Davey, 1984; Greenberg et al., 1982). Depuration may not remove V. vulnificus from oyster tissues because it may be sequestered in the organs of the oyster associated with the digestive system, predominately in the digestive diverticulum and the lower intestine (Kueh and Chan, 1985).

The objective of this study was to examine the distribution of V. vulnificus in oyster tissues during the depuration process. Oysters naturally harboring V. vulnificus at harvest were compared to artificially-inoculated oysters to observe the distribution of the organism in oyster tissues during depuration.

Oysters were dissected into four tissue sections (adductor, mantle, gills, and digestive system). The exterior tissues, adductor, mantle and gills, were separated but the digestive system was kept intact. This technique allowed for surface contamination and internal infection to be differentiated. Scanning electron microscopy was used to examine the ultrastructure of the oyster surfaces. Micrographs were compared to the depuration study data to attempt to determine the nature of the association of V.

vulnificus with these surfaces.

MATERIALS/METHODS

Preparation of oysters

Oysters (30 to 75 mm), from the Rappahannock River in Virginia (sampled all year long) were scrubbed with a wire brush under running cold tap water to remove surface dirt and foreign material. They were then placed in a modified spray tank (Marineland, Simi Valley, CA) for live holding studies or in a contamination tank (described below) for inoculation prior to placement in a live holding tank. The spray tank was modified to an immersion system by submerging oysters below the water level. The recirculating tank had the following parameters: the filter system was a polyester wool pad placed above activated charcoal (12 cm in depth); the volume capacity was 93.0 L; the flow rate was 160 GPH; and the water volume was 53.7 L (Fig. 1). The estimated intensity of illumination of the ultraviolet lamp was 7.1 X 10^2 W/m^2 with a window area of 0.79 cm^2. The spray nozzles were modified to ensure that the entire surface of the tank was sprayed with water.

The system was surface-aerated by spray nozzles. Sea salt mix (Marineland, CA) was added (2,148 g in 53.7 L of

distilled water) to achieve a salinity of 24 parts per thousand (ppt). Salinity was determined from the specific gravity reading of tank water using a hydrometer (Marineland, CA) and the formula (specific gravity - 1) x 1323 = salinity (Kelly, 1982). The temperature was maintained at 20°C and 10 oysters and 10 mL of tank water were removed as samples every 24 hr for 72 hr during depuration. The contamination tank was a tub with the following dimensions: 65 cm x 48 cm x 30 cm. Four hundred grams of sea salt mix was added to 10 L of distilled water, yielding a salinity of 24 ppt. The temperature of tank water was 25°C and the water was aerated with air stones.

Laboratory and personal safety measures

The immersion tank and the inoculation tank were placed in a restricted access room. An ultraviolet light (30 watt, GE model # G30T8) was placed above the tank to control any airborne bacteria that may be released as aerosols during spraying of water. Oyster shucking and bacterial enumeration were restricted to another "authorized personnel only" room. Safety goggles and gas respirator (Half Mask Silicone Respirator, MA-7523, Laboratory Safety Supply, Janesville, WI) were worn during all contacts with oysters and *V. vulnificus*.

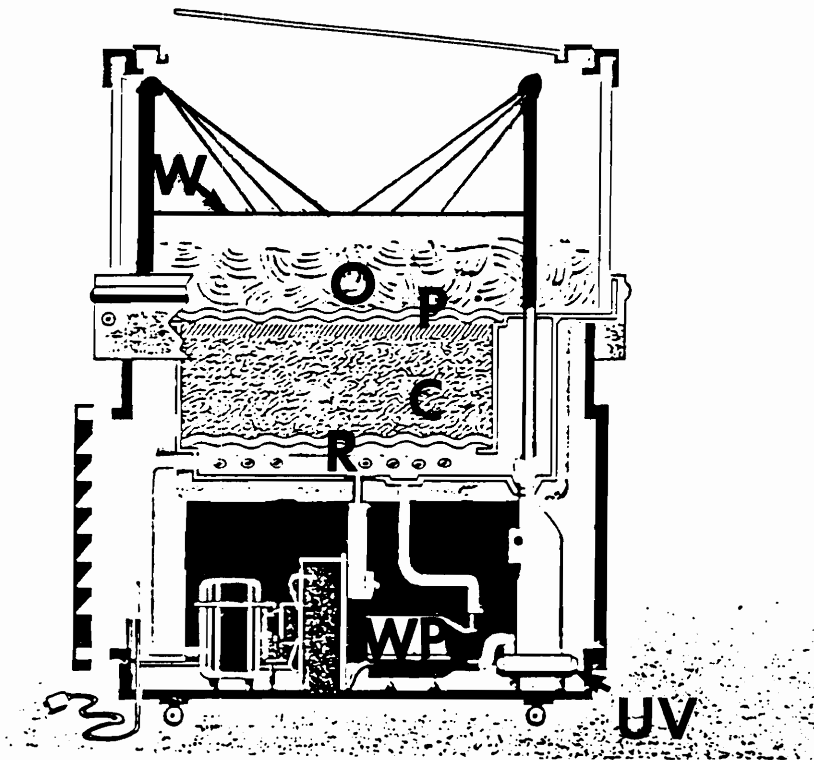


Fig. 1: Schematic diagram of a live holding tank. The tank had the following features: (W) water level; (O) oyster storage area; (P) polyester pad; (C) activated charcoal; (R) refrigeration unit; (WP) water pump; and (UV) ultraviolet disinfection lamp.

Disinfection of immersion tank

The immersion tank and the inoculation tank were disinfected between experiments with P3-topax 99 (Henkel Corporation Chemical Services Division, P.O. Box 927, Burlington, IA) containing 5.25% sodium hypochlorite. Before each experiment, all of the tubing of the immersion tank, the pump, and the ultraviolet disinfecting system were removed from the tank system. The pump and the ultraviolet lamp were disassembled and the parts scrubbed with a brush and disinfected with sodium hypochlorite solution at 200 ppm. Residual chlorine was removed by rinsing with distilled water.

After each experiment, any remaining oysters were removed and a 200 ppm solution of sodium hypochlorite was added to the tank. The tank was run continuously for 48 to 72 hr. Next all removable parts including the filter pad, charcoal and tubes were autoclaved before handling. The tank system was run for another 48 hr without the above components before water was discarded and the parts were cleaned as described above.

Artificial inoculation

A *V. vulnificus* strain, CPC 71, isolated from oysters and shown to be virulent in mice was donated by J. D. Oliver (University of North Carolina at Charlotte). Stock cultures

of V. vulnificus were maintained on Long Term Preservation Medium (LTPM) stabs at room temperature. The V. vulnificus culture used for infection of oysters was prepared by inoculating into 200 mL Brain Heart Infusion Broth supplemented with 2% NaCl (BHI+2% NaCl), and incubating for 24 hr at 37°C. One mL of the culture broth was transferred to 200 mL BHI+2% NaCl and incubated for 18 hr to yield a V. vulnificus cell density of 10^8 to 10^9 organisms/mL (Oliver, 1989). The cell density was determined in the following way: ten mL of broth was spun for 10 min at 10°C and 3,000 x g (Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge), the supernatant was decanted, the pellet was washed with Phosphate Buffered Saline (PBS) (pH 7.6; 0.01 M), and recentrifuged for 10 min at 3,000 x g. The supernatant was decanted and the pellet was resuspended in PBS. The OD values of serial dilutions of cell suspensions in PBS were read on a Bausch and Lomb Spectronic 70 spectrophotometer at 600 nm. The cell density was estimated from plots of optical density vs. log CFU/mL. Cell numbers were estimated from colonies grown on Thiosulfate Citrate Bile salt Sucrose (TCBS) incubated at 37°C for 24 hr. During infection, oysters were placed in a tank filled with artificial seawater (24 ppt, 25°C), with 10^4 CFU V. vulnificus/mL. Oysters were allowed to feed in this tank for 12 hr after which they were placed in the depuration

tank.

Bacterial enumeration

Ten oysters were shucked aseptically at each sampling point (Hunt et al., 1984) and dissected with surgical scissors and scalpels to separate the adductor, the mantle, the gills and the digestive system of each oyster (Fig. 2). After every incision and cut, 100 mL of PBS was gently poured on the tissues to reduce cross-contamination. The surgical instruments were rinsed with 95% ethanol (v/v) and passed through a flame after each handling of the oyster tissues. The term "digestive system" is used here to describe all tissues other than the adductor, the mantle and the gills. The mantle fluid was not used.

V. vulnificus was enumerated in oyster samples and tank water using the Most Probable Number (MPN) enumeration technique recommended by Shellfish Sanitation Branch of the Center for Food Safety and Applied Nutrition for Food and Drug Administration (U.S. Food and Drug Administration, 1988). Oyster tissues were weighed and appropriate volumes of PBS were added to achieve 1:10 dilution of the samples. Ten mL of the tank water samples were diluted with 90 mL of PBS for 1:10 dilution.

Oyster tissue parts were homogenized using a Stomacher Lab-Blender 400 (Steward Medical UAC House, London, England)

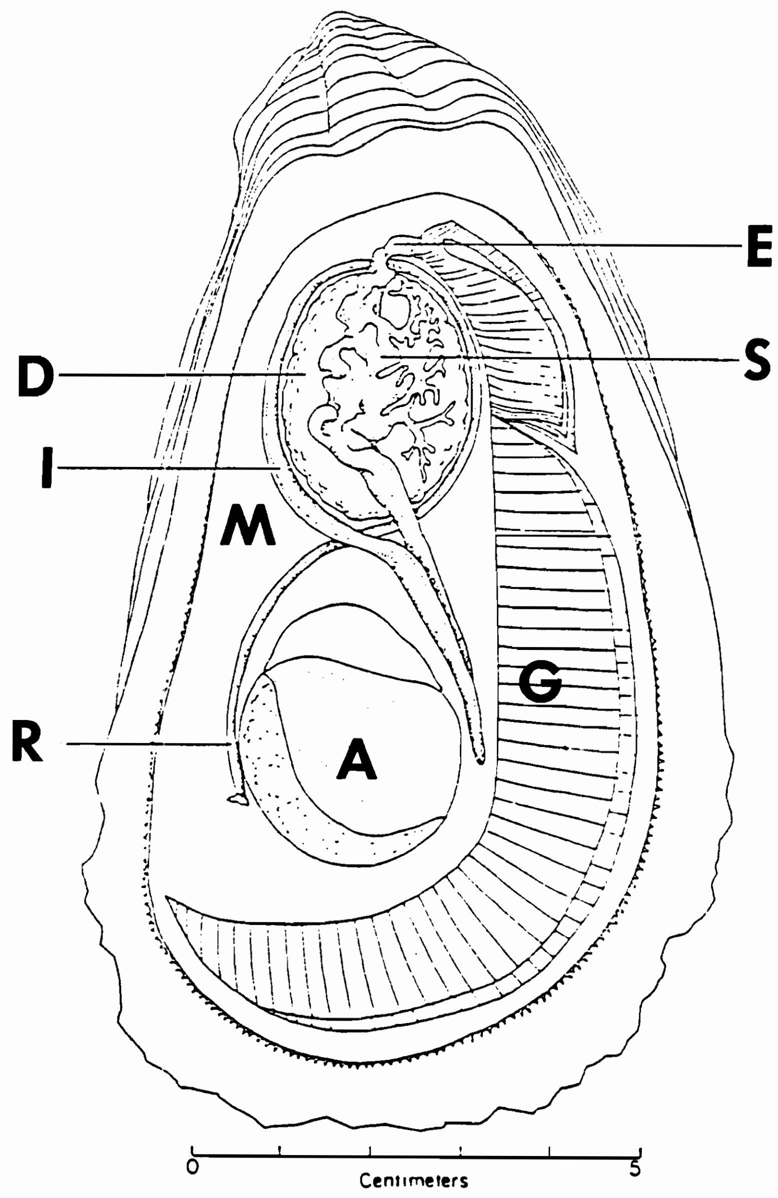


Fig. 2: Schematic diagram of an oyster. (E) esophagus; (S) stomach; (D) digestive diverticula; (I) intestine; (M) mantle; (A) adductor; (R) rectum; (G) gills.

for 2 min. The homogenates and water sample was diluted 10^1- to 10^6-fold with PBS. One mL of each dilution was inoculated into Alkaline Peptone Water (pH 8.5) (APW) in a 3-tube MPN series for enrichment and incubated for 12 hr at 37°C. One loopful from each APW tube showing growth was streaked on plates of TCBS (Lotz et al., 1983) and Cellobiose Polymyxin Colistin Medium (CPC) (Massad and Oliver, 1987) and incubated for 24 hr at 37°C. Typical V. vulnificus colonies appeared as 2 to 3 mm diameter raised green to blue-green colonies on TCBS and as 2 mm diameter flat, yellow colonies on CPC. Typical V. vulnificus colonies were confirmed biochemically using API Rapid NFT system (Analytab Products, Plainview, NY) with modifications. The colonies were suspended in a 2% saline solution instead of the 0.85% solution suggested by Analytab Products. The increased salt concentration allowed for better growth of the organism in the biochemical tests (MacDonell et al., 1982).

Statistical analysis

The initial and final V. vulnificus populations in oysters stored in an immersion live holding system were analyzed using analysis of variance (ANOVA) of the Statistical Analysis System (SAS, 1984). Significances of the differences between the means of initial and final

bacterial levels were determined using least significant difference (LSD) (Sokal and Rohlf, 1981). Means with F values ($p \leq 0.06$) were significantly different from each other.

Preparation of samples for electron microscopy

The methodology for electron microscopy was modified from procedures of Tall and Nauman (1981). Artificially-inoculated and uninoculated oysters were aseptically shucked. Whole oysters were placed in 1% glutaraldehyde/filtered artificial seawater (pH 6.0, salinity of 14 ppt) and tissue parts (gills, mantle, adductor, and digestive system) were excised. Artificial seawater was obtained by adding sea salts (Marineland, CA) to distilled water to a salinity of 14 ppt. Artificial seawater was filtered through a 0.2 µm membrane filter (Millipore Corp., Bedford, MA), adjusted to pH 6.0, and autoclaved.

Oyster tissues were prefixed in 1% glutaraldehyde/filtered seawater for 24 hr at 4°C. The specimens were washed three times in filtered artificial seawater and postfixed in 1% osmium tetroxide/filtered artificial seawater for 24 hr at 25°C. After fixation, the tissues were washed three times in artificial seawater and dehydrated with a graded series of ethanol in distilled

water (10%, 50%, 70%, 80%, 100% ethanol).

Scanning electron microscopy

After the dehydration of the samples, they were critical point dried with CO_2 (Ladd Critical Point Dryer, Cat. No. 28000, Ladd Research Industries, Burlington, VT), and sputter-coated with gold palladium (Hummer X, Anatech LTD, Alexandria, VA). The specimens were viewed in a Philips 505 scanning electron microscope (Holland). Micrographs were recorded on Polaroid film (type 55 P/N). Photographs were processed on Kodak Ektamatic processor and fixed permanently on Kodak Ektamatic processing paper.

Transmission electron microscopy

After the specimens were dehydrated, they were infiltrated with graded series (10%, 30%, 50%, 80%, 100% resin) of Spurr low-viscosity embedding media epoxy resin in 100% ethanol (Polysciences Inc., Paul Valley Industrial Park, PA) for 30 min at 25°C for each series, and embedded at 100% resin for 24 hr at 71°C. They were then thin sectioned with a glass knife on an MT-6000 microtome (Research and Manufacturing Company, Inc., Tucson, AZ). Thin sections were mounted on copper grids, stained with lead citrate and uranyl acetate, and examined in a Zeiss High-Resolution transmission electron microscope (EM 10CR,

Germany). Micrographs were recorded with Kodak electron microscope film 4489. (Estar Thick Base).

RESULTS/DISCUSSION

Efficiency of ultraviolet light during depuration

The ultraviolet light in the depuration tank was effective in causing a 2.5 X 10^3 MPN reduction of V. vulnificus/mL of tank water (Fig. 3). Within one hour of the inoculation of the tank, the bacterial population was reduced to undetectable levels (<3 V. vulnificus MPN/mL of tank water). The design of the holding system appeared effective in either destroying or inactivating V. vulnificus in the tank water.

Artificial inoculation

After 6 hr of uptake of V. vulnificus, there was a 100-fold increase in the numbers of the organism in each of the tissue sections: adductor, mantle, gills and digestive system (Fig. 4). Levels did not increase upon longer exposure. Similar results were shown by Perkins et al. (1980), who also noted a steady state of uptake of V. vulnificus by oysters after 6 hr. Son and Fleet (1980) also performed artificial inoculation by feeding oysters for up

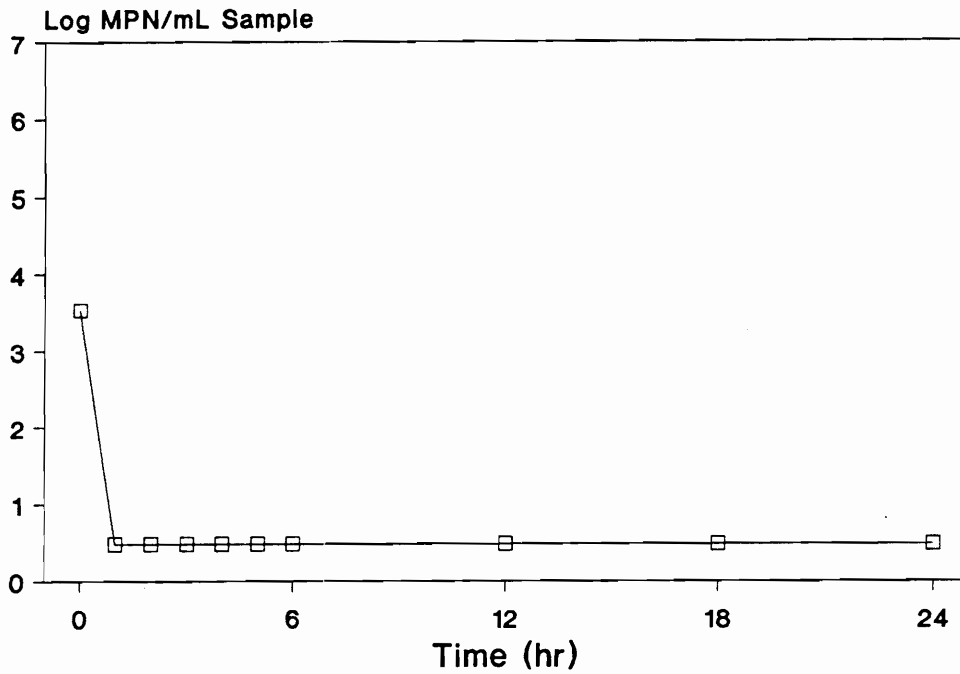


Fig. 3: Efficiency of ultraviolet light disinfection on the reduction of V. vulnificus in the immersion live holding tank water. On the Figure, 3 MPN/mL represents any value <3 V. vulnificus MPN/mL of tank water.

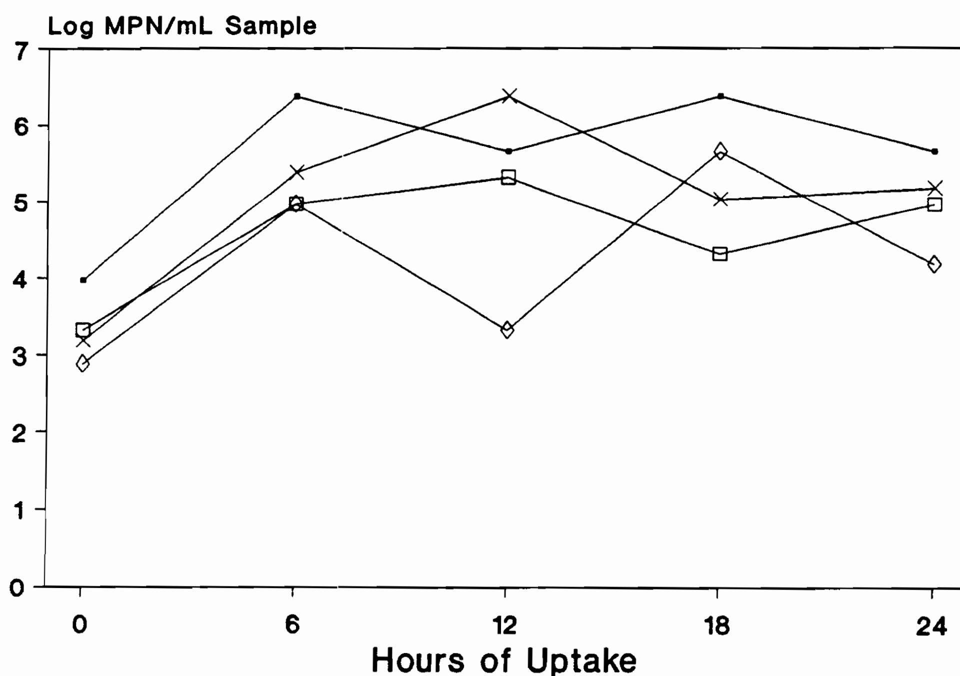


Fig. 4: Uptake of V. vulnificus in oyster tissues during artificial inoculation. Adductor muscle (◊), mantle tissue (×), gill tissue (•), and digestive system (□) were dissected from ten oysters and enumerated for the presence of V. vulnificus.

to 6 hr. Rodrick *et al*. (1989) reported clams accumulated 10^5 units of *Vibrio* spp. during the first 6 hr of feeding. Martinez-Manzanares *et al*. (1991) reported a steady state of microbial accumulation was reached between 6 to 12 hr of exposure to microorganisms.

Depuration of artificially-inoculated oysters

After 72 hr of depuration there was a significant reduction in the level of *V. vulnificus* in the adductor ($p = 0.004$) and the mantle tissues ($p = 0.06$) (Fig. 5). This decrease may have been due to the release of organisms from the adductor and the mantle tissues and may imply that the gills were actively pumping water over these muscles. There was no significant reduction in the bacterial population of the gills ($p = 0.11$) and the digestive systems ($p = 0.21$).

The release of *V. vulnificus* from the adductor and the mantle tissues may have increased the number of organisms in the tank water. There was an increase from <3 MPN/mL to 9 MPN/mL after 24 hr of depuration and then a decrease to 2 *V. vulnificus* MPN/mL in the tank water. A possible reason for the low number of organism in water may be the effectiveness of the ultraviolet disinfection unit. Similar trends of *V. vulnificus* reduction in artificially-inoculated oysters were shown by other investigators (Kelly and Dinuzzo, 1985; Steslow *et al*., 1987). These researchers, however

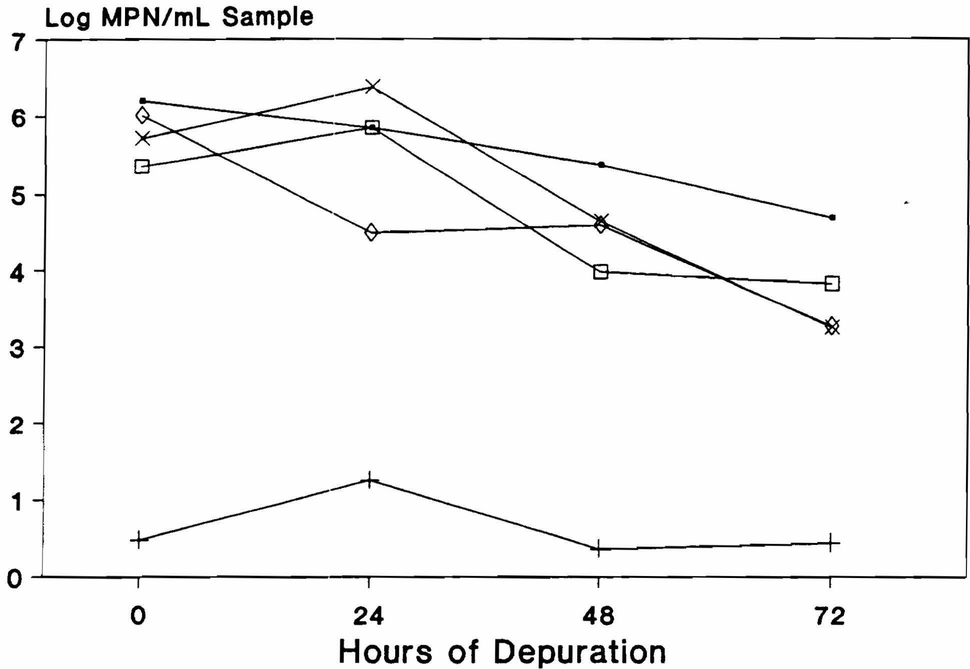


Fig. 5: Distribution of V. vulnificus in artificially-inoculated oyster tissues and tank water. Adductor muscle (◊), mantle tissue (×), gill tissue (•), and digestive system (□) of oysters and the tank water (+) were enumerated for the presence of V. vulnificus. On the Figure, 3 MPN/mL represents any value <3 V. vulnificus MPN/mL of tank water.

enumerated the presence of V. vulnificus from whole oysters, and not individual tissue parts. Kelly and Dinuzzo (1985) reported the reduction of V. vulnificus from oysters from 10^6 cells/g of oyster to 25 cells/g after 24 hr. Rodrick et al. (1989) showed a 99% reduction of V. vulnificus in artificially-inoculated clams placed in depuration tanks for 48 hr.

Depuration of naturally-infected oysters

There is a great frequency of V. vulnificus in oyster tissues during the summer months. During May, some stations in Florida reported 4.6 X 10^4 MPN/100 mL seawater. In July, the number of V. vulnificus was 2.3 X 10^3 MPN/mL of oyster. In August, there was 3 X 10^3 MPN/mL of V. vulnificus in oyster tissues (Tamplin et al., 1982). In this study, high numbers of V. vulnificus were also naturally present in oysters harvested during the summer months. The concentrations of V. vulnificus found in the oyster tissues harvested in during the fall months were 100 times lower than in summer months (Fig. 6). A bar graph illustrates the distribution of V. vulnificus in the adductor muscle (9.5 X 10^2 MPN/mL), the mantle (6.2 X 10^3 MPN/mL), the gills (2.0 X 10^3 MPN/mL), and the digestive system (1.8 X 10^4 MPN/mL) of naturally-infected summer oysters. During summer months, the digestive system had the highest concentration of V.

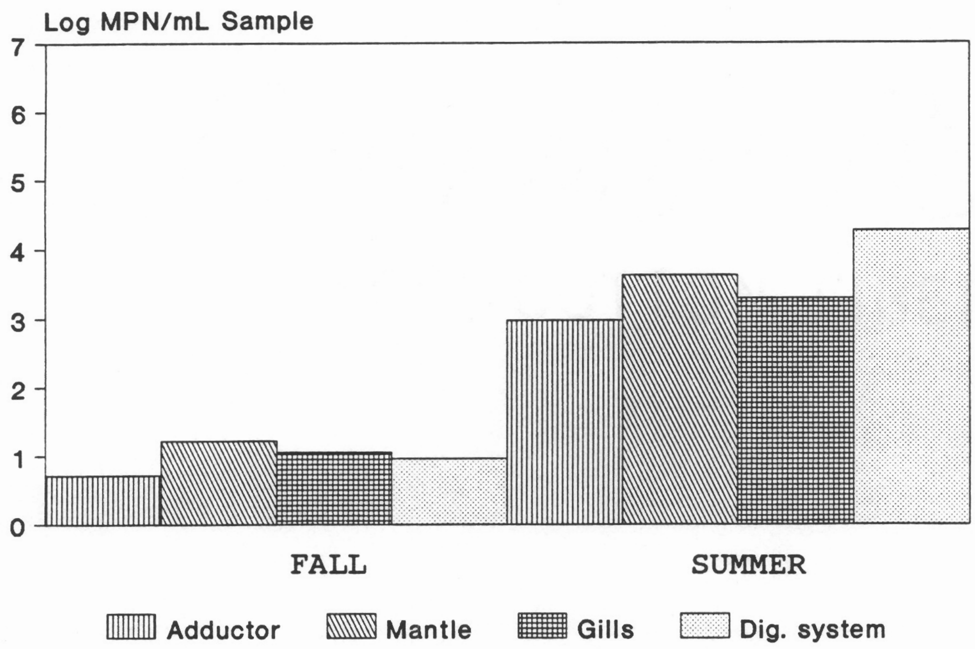


Fig. 6: Comparison of the distribution of V. vulnificus naturally present in oyster tissues harvested in fall and summer.

vulnificus. This observation was similar to those made by Rodrick et al. (1989) who stated that V. vulnificus was found to have the highest concentration in the digestive glands.

Summer oysters with high levels of V. vulnificus were placed in the immersion tank (Fig. 7). There were no significant reduction between the initial V. vulnificus levels and those present after 72 hr of depuration on the adductor muscle ($p = 0.37$), the mantle ($p = 0.16$), the gills ($p = 0.5$), and the digestive system ($p = 0.5$). There was an increase in cell number from <3 V. vulnificus MPN/mL to 1.9×10^3 V. vulnificus MPN/mL in the tank water after 24 hr of depuration. This increase may be due to the release of V. vulnificus by oysters during filter-pumping.

Fall oysters harboring naturally present V. vulnificus in their tissues were placed in the immersion tank for depuration studies (Fig. 8). There were no significant changes in the numbers of the organism in the adductor muscle ($p = 0.5$), the mantle ($p = 0.84$), the gills ($p = 0.75$), and the digestive system ($p = 0.75$) after the depuration period of 72 hr.

Figures 9, 10, 11, and 12 represent the levels of V. vulnificus found in adductor, mantle, gill, and digestive system tissues during 72-hr depuration studies. On each graph, three sets of data are presented: results obtained

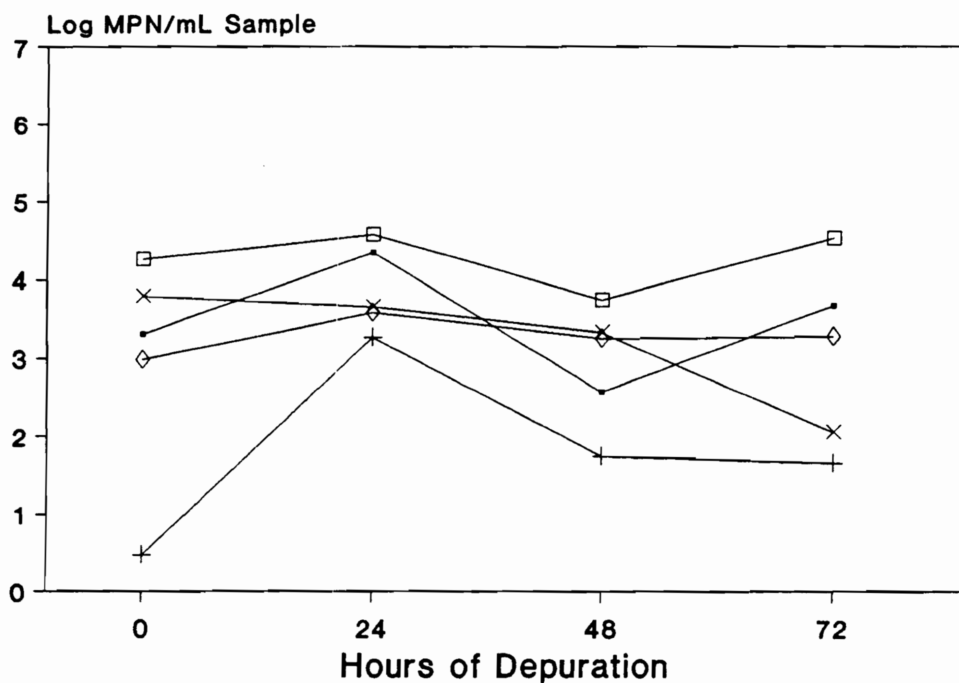


Fig. 7: Distribution of V. vulnificus in summer oysters during depuration in an immersion live holding system. Adductor muscle (◊), mantle tissue (×), gill tissue (▪), and digestive system (□) of oysters and the tank water (+) were enumerated for the presence of V. vulnificus. On the Figure, 3 MPN/mL represents any value <3 V. vulnificus MPN/mL of tank water.

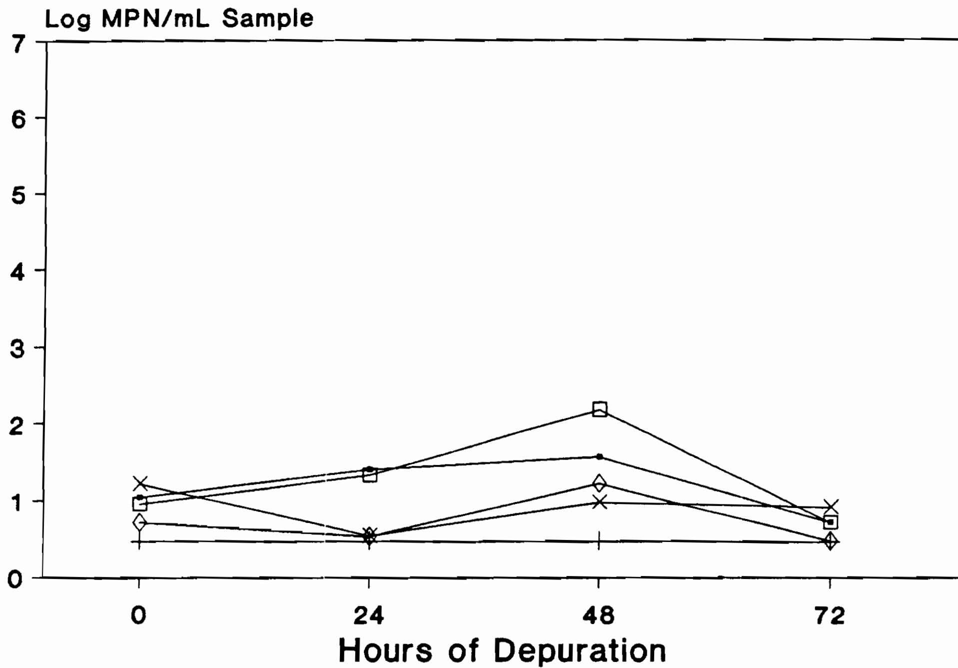


Fig. 8: Distribution of V. vulnificus in fall oysters during depuration in an immersion live holding system. Adductor muscle (◊), mantle tissue (×), gill tissue (•), and digestive system (□) of oysters and the tank water (+) were enumerated for the presence of V. vulnificus. On the Figure, 3 MPN/mL represents any value <3 V. vulnificus MPN/mL of tank water.

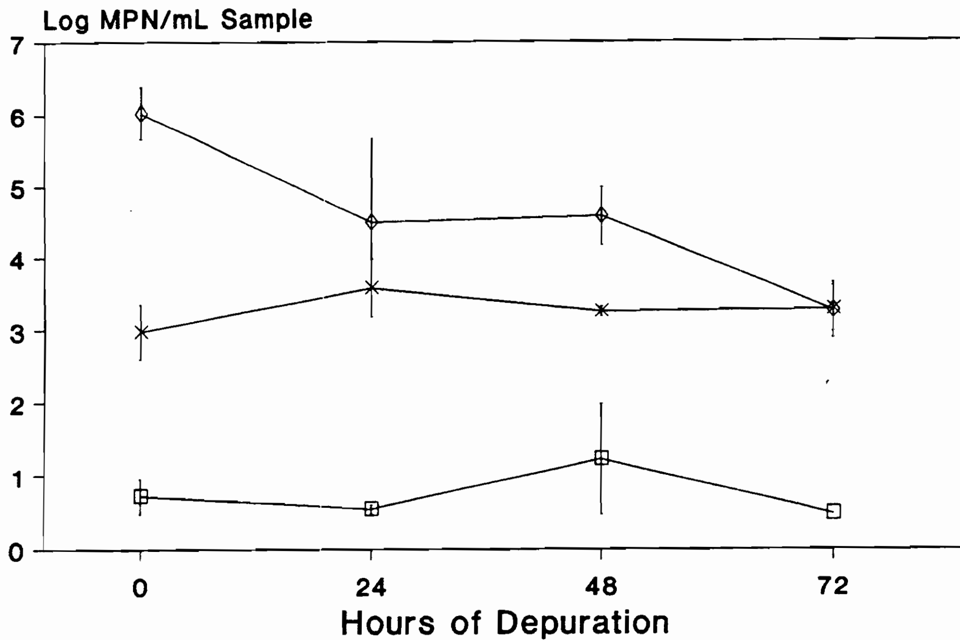


Fig. 9: Presence of V. vulnificus in oyster adductor muscles during depuration in an immersion live holding system. Adductor muscles from artificially-inoculated oysters (◊), naturally-infected summer oysters (×), and naturally-infected fall oysters (□) were compared for the presence of V. vulnificus during 72 hr of depuration.

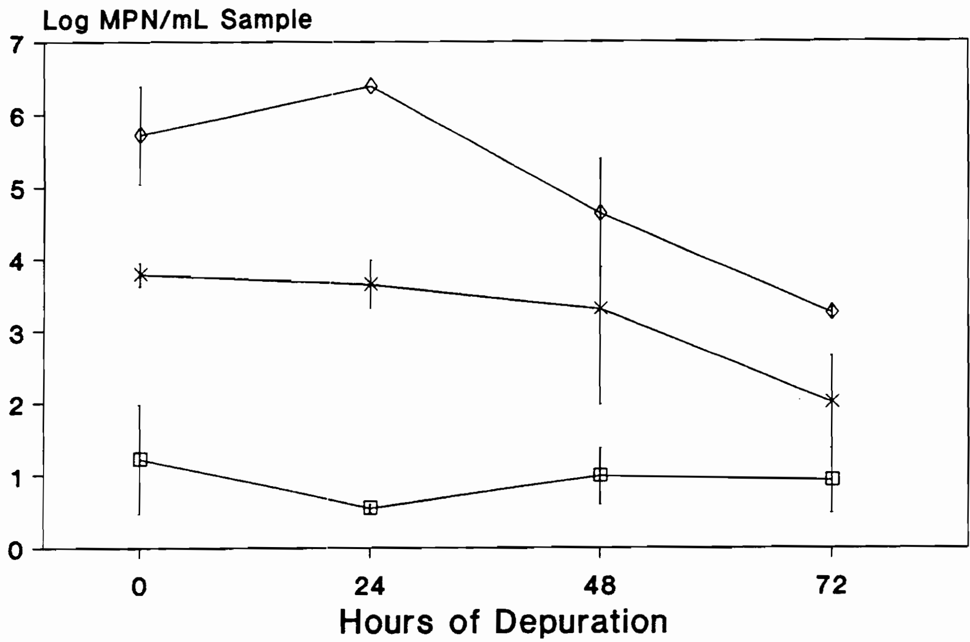


Fig. 10: Presence of V. vulnificus in oyster mantle tissues during depuration in an immersion live holding system. Mantle tissues from artificially-inoculated oysters (◊), naturally-infected summer oysters (×), and naturally-infected fall oysters (□) were compared for the presence of V. vulnificus during 72 hr of depuration.

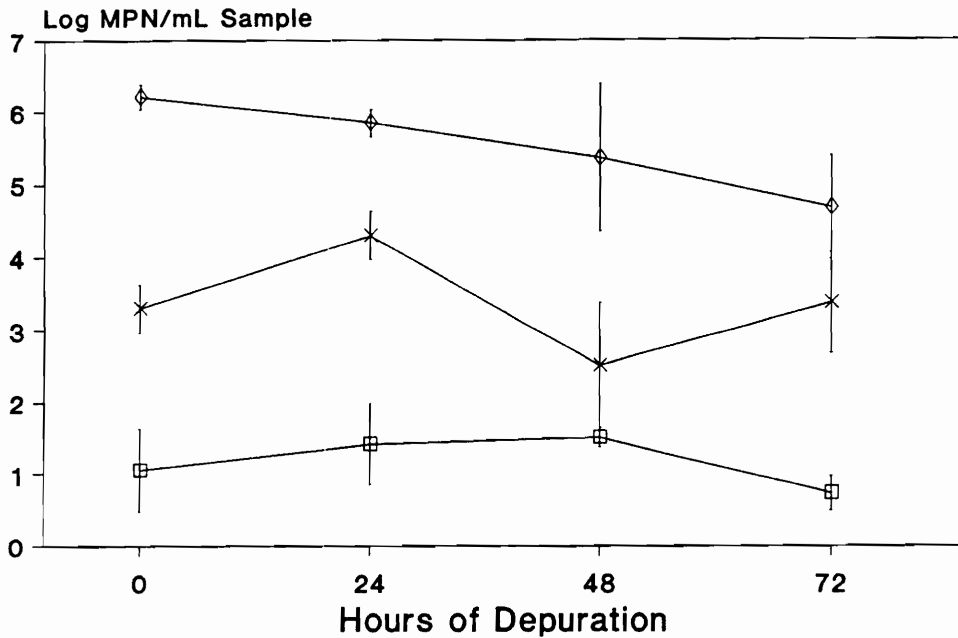


Fig. 11: Presence of V. vulnificus in oyster gill tissues during depuration in an immersion live holding system. Gill tissues from artificially-inoculated oysters (◊), naturally-infected summer oysters (×), and naturally-infected fall oysters (□) were compared for the presence of V. vulnificus during 72 hr of depuration.

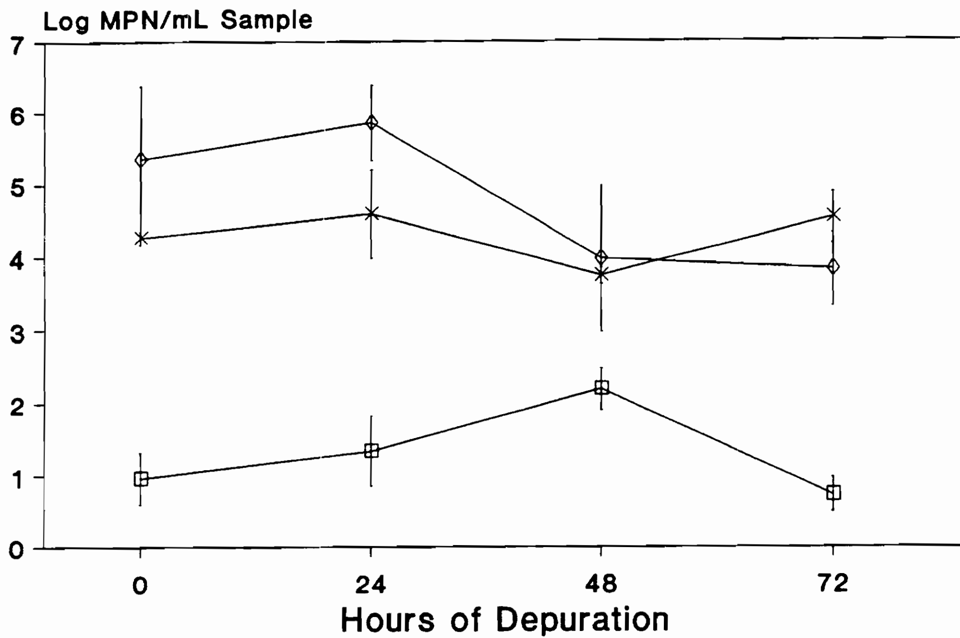


Fig. 12: Presence of V. vulnificus in oyster digestive systems during depuration in an immersion live holding system. Digestive systems from artificially-inoculated oysters (◊), naturally-infected summer oysters (×), and naturally-infected fall oysters (□) were compared for the presence of V. vulnificus during 72 hr of depuration.

from summer oysters, from fall oysters, and from artificially-inoculated oysters. These Figures further illustrated that V. vulnificus counts decreased only in the mantle and the adductor tissues of the inoculated oysters. There was no change in bacterial population in the fall and summer oyster tissues.

Scanning and transmission electron microscopy

The adductor, the mantle, the gills, and the stomach were each examined by scanning electron microscopy for surface-associated material (Figs. 13, 14, 15, 16, respectively). The adductor and the mantle were nonciliated surfaces, while the gill and the intestine surfaces were ciliated. The structural unit of the gill is a cylindrical section of ciliated epithelium. All of the surfaces were covered predominately with mucus and mucocytes. Similar observations were seen by Garland et al. (1982).

A transmission electron micrograph of the gill tissues showed the secretion of mucocytes through the ciliated surface (Figs. 17A, 17B). Lysosomes (Rhodin, 1963) were seen in a micrograph of the gill tissues. Lysosomes play an important role in the internal inactivation of microorganisms in oysters.

Oyster tissues were artificially inoculated with V vulnificus. A micrograph depicting the vibrioid shape of

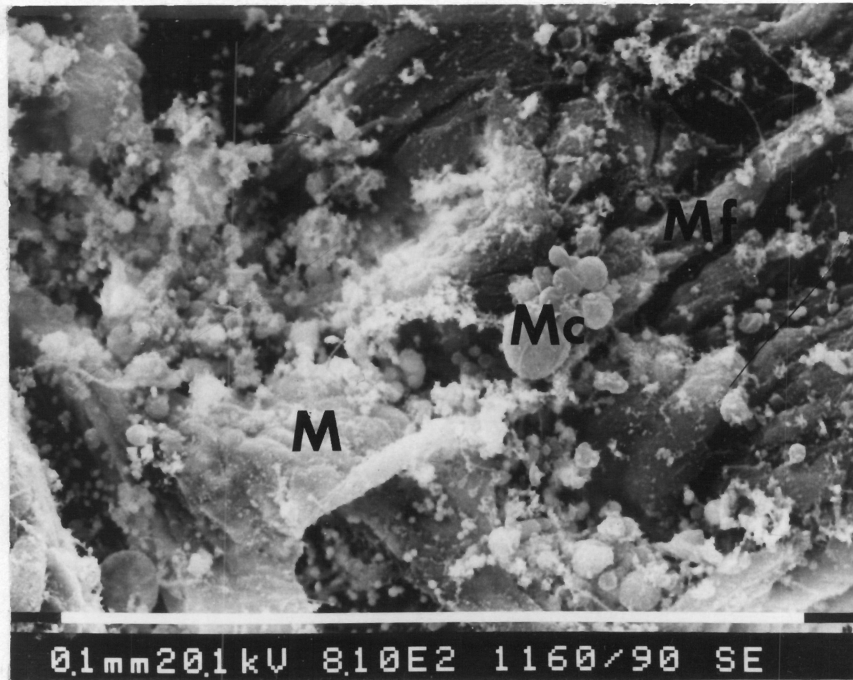


Fig. 13. Scanning electron micrograph of adductor tissue. Portions of the muscle fibers (Mf) are covered with a sheet of mucus (M) and mucocytes (Mc) on the surface. Bar represents 0.1 mm. Magnification is 810 X.

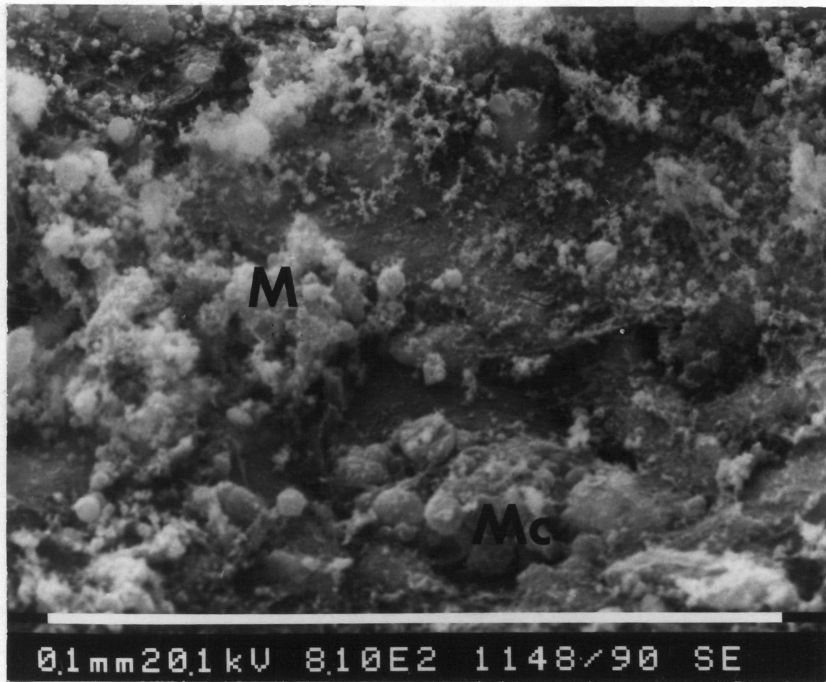


Fig. 14. Scanning electron micrograph of mantle tissue surface. This nonciliated surface is covered predominately with mucus (M) and mucocytes (Mc). Bar represents 0.1 mm. Magnification is 810 X.

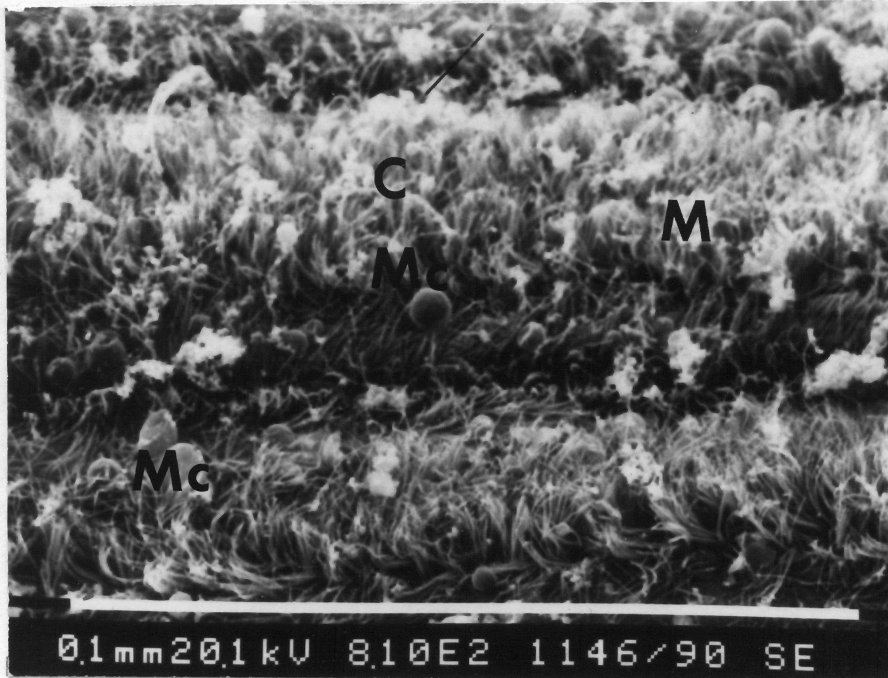


Fig. 15. Scanning electron micrograph of epithelial surface of gills. The surface is covered with cilia (C), mucus (M) and mucocytes (Mc). Bar represents 0.1 mm. Magnification is 810 X.

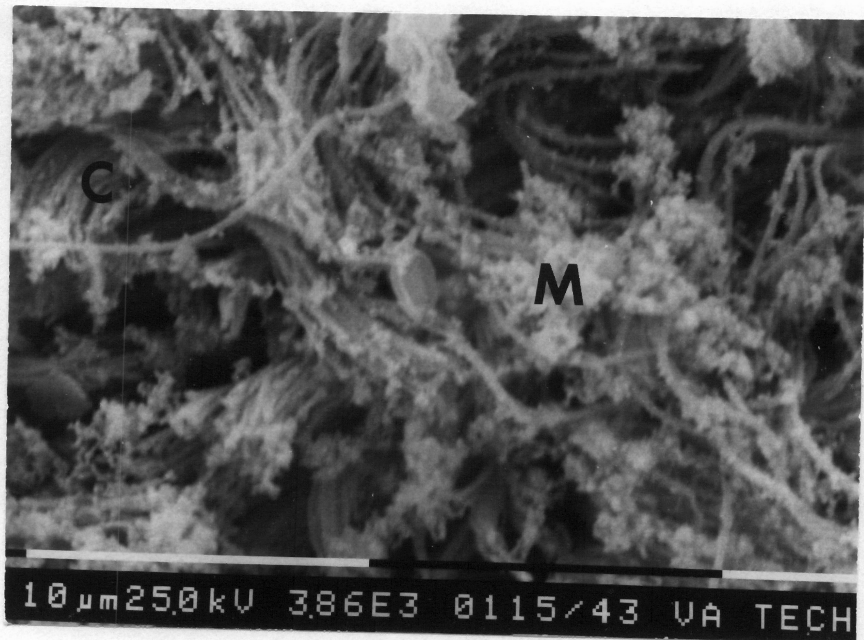
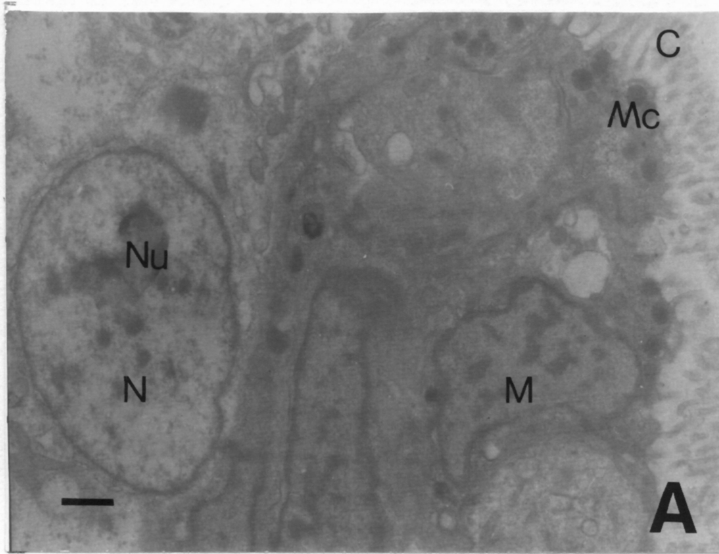


Fig. 16: Scanning electron micrograph of the surface of the oyster stomach. The ciliated surface (C) is covered with mucus (M). Bar represents 10 μm. Magnification is 3,860 X.



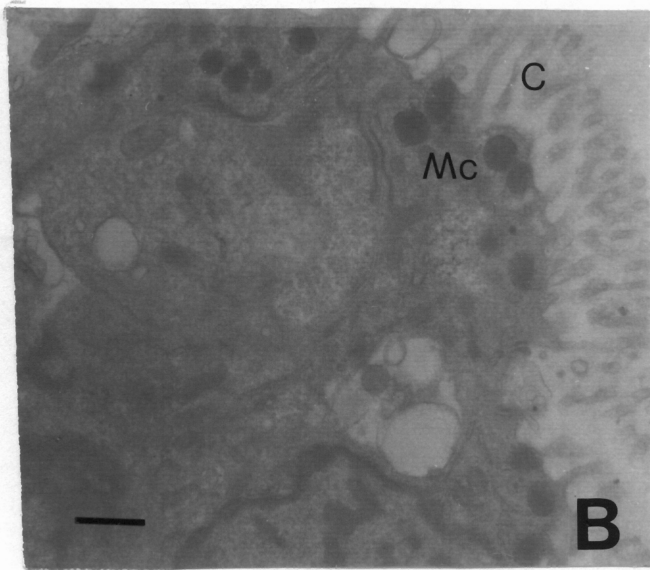


Fig. 17: Transmission electron micrograph of a transverse section of gill tissue. (A): Cellular components such as nucleus (N), nucleolus (Nu), mitochondria (M), cilia (C), and mucocytes (Mc) are evident. Magnification is 6,000 X. (B): Cilia (C) and mucocytes (Mc) at higher magnification of 9,000 X. Mucocytes are being secreted through the ciliated surface. Bars represent 1 μm.

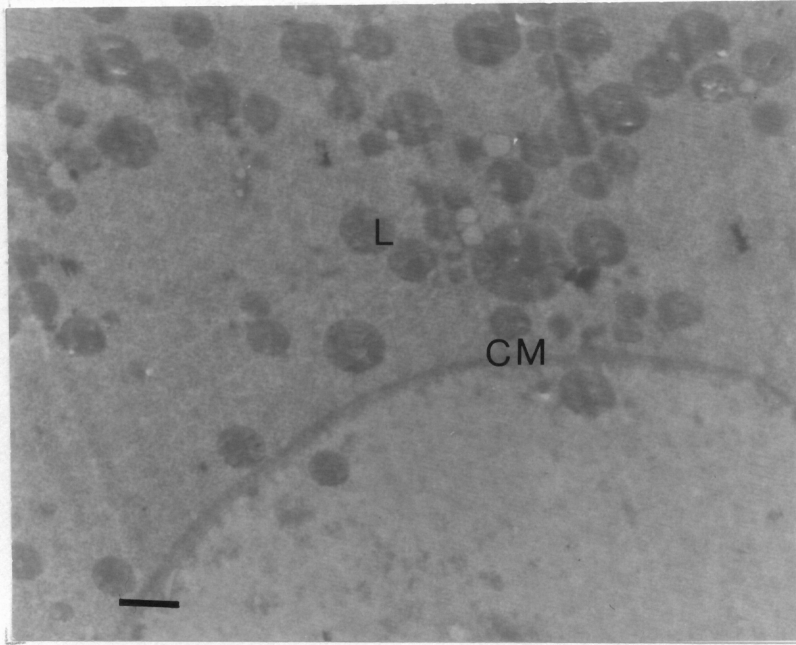


Fig. 18: Transmission electron micrograph of a transverse section of gill tissue showing lysosomes. (A): Shows the cellular components such as lysosomes (L). CM represents cell membrane. Bar represents 1 μm. Magnification is 7,875 X.

this organism can be seen in Figure 19. Vibrioid organisms were associated with the mucus on the epithelial surfaces of the adductor (Figs. 20, 21), the mantle (Fig. 22) and the gills (Fig. 23). An abundance of mucus was observed covering a large part of the surfaces. On the gill tissues, clusters of vibrioid organisms were observed to the right of the cilia in Figure 23. The stomach of an oyster is a ciliated surface but no vibrioid organisms were found (Fig. 24). The intestine and the rectum were also ciliated surfaces mostly covered with mucus with vibrioid organisms entrapped in the mucus cluster (Figs. 25 and 26).

The results of this research showed that the reductions in _V. vulnificus_ counts occurred only during depuration of artificially-inoculated oysters. The decrease in the bacterial population was due to the release of the organism from the adductor and the mantle tissues. The adductor and the mantle tissues are nonciliated surfaces, as confirmed by scanning electron micrographs. _V. vulnificus_ may have been loosely entrapped by mucus on these surfaces during inoculation. This could account for the ease with which _V. vulnificus_ levels in these tissues could be released.

There was no change in the bacterial population in the tissues of the fall and the summer oysters during the 72-hr depuration period. Naturally present _V. vulnificus_ in oysters may be sequestered within the tissues. Even on the

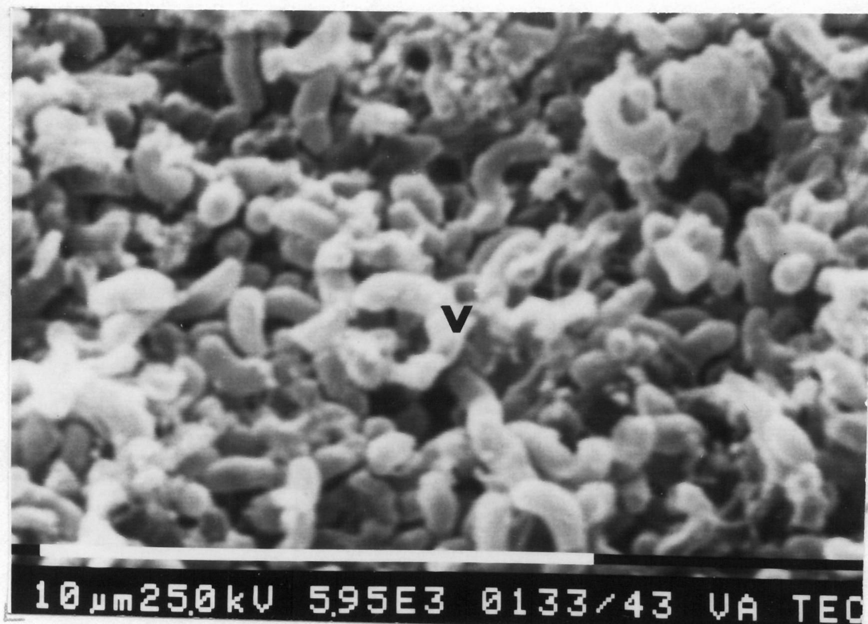


Fig. 19: Scanning electron micrograph of a colony of V. vulnificus. The micrograph shows the vibrioid shape (v) of this organism. Bar represents 10 µm. Magnification is 5,950 X.

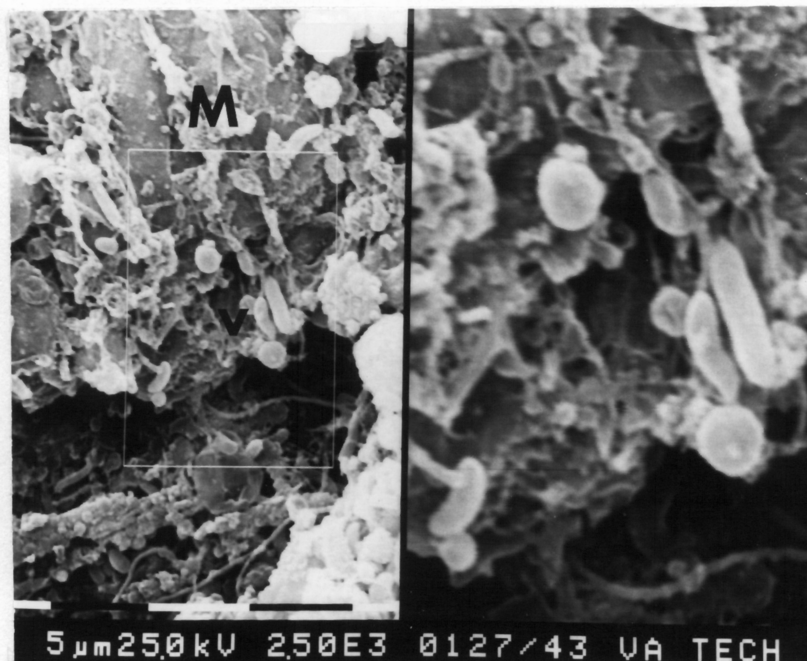


Fig. 20: Scanning electron micrograph of adductor tissue of oysters artificially-inoculated with *V. vulnificus*. Vibrioid organisms (v) are associated with the mucus (M) on the surface. The right micrograph is a further magnification of the vibrioid organisms. Bar represents 5 μm. The left micrograph is 2,500 magnification and the right micrograph is 6,000 magnification.

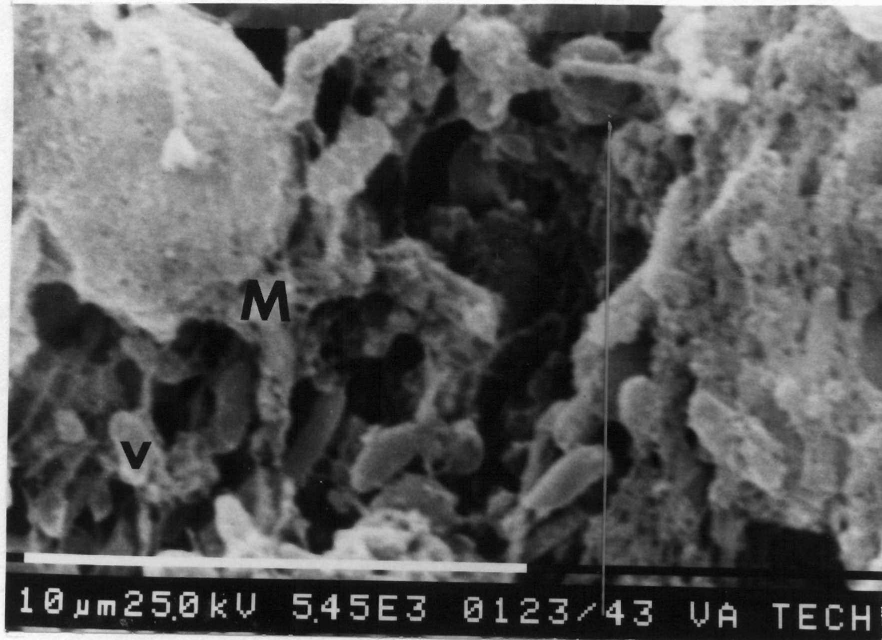


Fig. 21: Another view of a scanning electron micrograph of adductor muscle of oysters artificially-inoculated with V. vulnificus. Vibrioid organisms (v) can be seen entrapped in mucus (M) on the surface of the adductor muscle. Bar represents 10 μm. Magnification is 5,450 X.

Fig. 22: Scanning electron micrograph of mantle tissue of oysters inoculated with V. vulnificus. Clusters of vibrioid organisms (v) and mucus (M) are seen on the surface. Bar represents 10 μm. Magnification is 4,400 X.

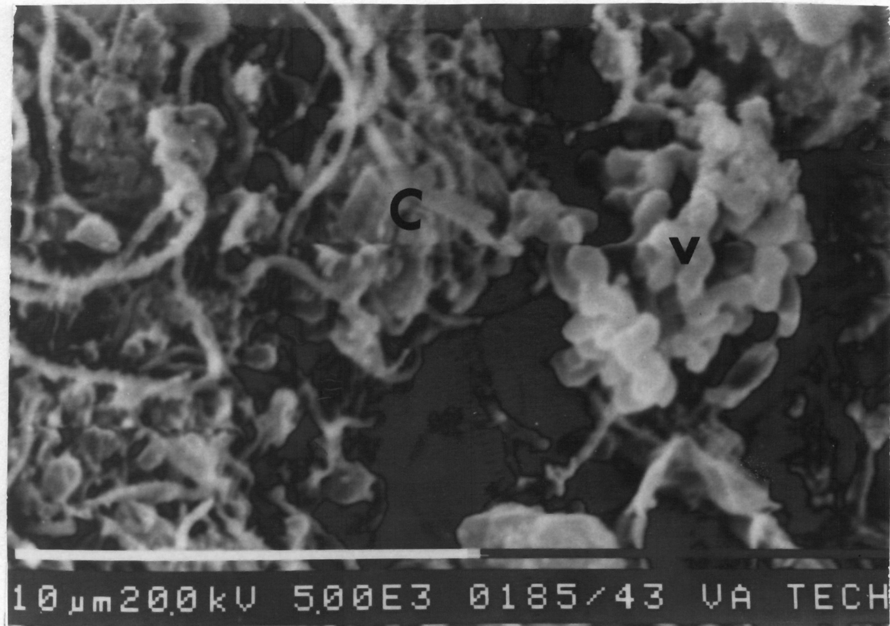


Fig. 23: Scanning electron micrograph of gill surface of oysters inoculated with V. vulnificus. Vibrioid organisms (v) are present to the right of the cilia (C). Bar represents 10 μm. Magnification is 5,000 X.

Fig. 24: Scanning electron micrograph of the stomach surface of oysters inoculated with V. vulnificus. No vibrioid organisms were seen on the ciliated surface (C). Bar represents 10 μm. Magnification is 3,860 X.

Fig. 25: Scanning electron micrograph of the intestinal surface of oysters inoculated with V. vulnificus. There appear to be vibrioid organisms (v) entrapped in mucus (M) and cilia (C) cluster. Bar represents 10 μm. Magnification is 2,500 X.

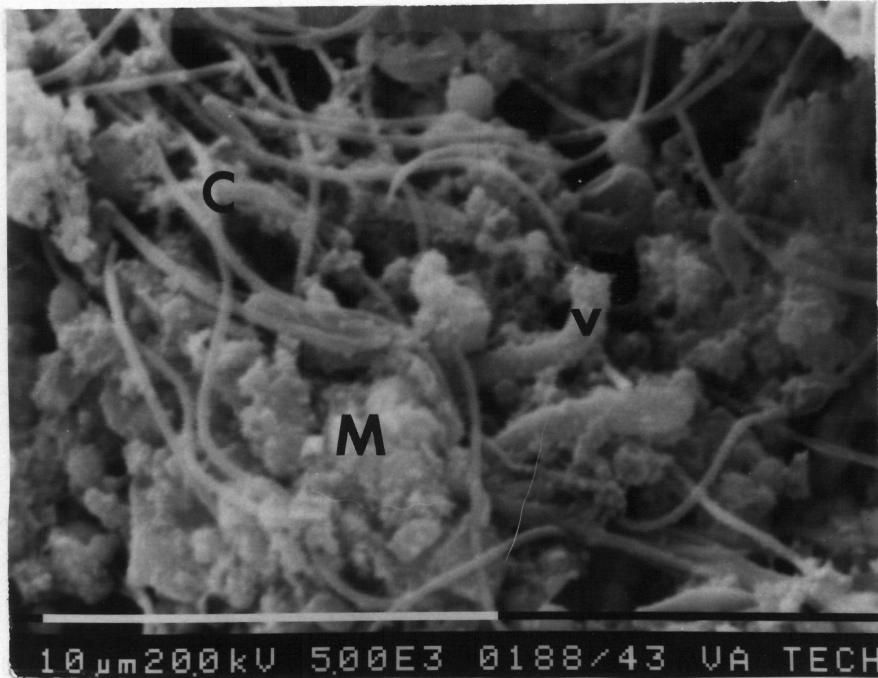


Fig. 26: Scanning electron micrograph of rectal surface of oysters inoculated with V. vulnificus. There appear to be vibrioid organisms (v) entrapped in cilia (C) and mucus (M). Bar represents 10 μm. Magnification is 5,000 X.

surfaces of the adductor, the gill, and the mantle tissues, *V. vulnificus* may be more closely associated with the cilia and the mucus than with the tissues that were exposed to the bacterium during artificial inoculation. It seems unlikely that depuration of *V. vulnificus* from oysters naturally harboring the bacterium may be effective.

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APPENDICES

Appendix A: Uptake of Vibrio vulnificus by Oyster Tissues During Artificial Inoculation

Time	Adductor	Mantle	Gills	Dig. System
hours		log MPN/mL tissue		
0	2.88	3.18	3.97	3.32
6	4.97	5.38	6.38	4.97
12	3.32	6.38	5.66	5.32
18	5.66	5.04	6.38	4.32
24	4.18	5.18	5.66	4.97

Appendix B: Distribution of V. vulnificus in Oyster Tissues and Tank Water During Depuration in an Immersion Tank System

(Oysters were Inoculated with Vibrio vulnificus prior to Depuration)

Time	A	STD+-	M	STD+-	G	STD+-	D	STD+-	Water	STD+-
hr					log MPN/mL					
0	6.02	0.36	5.71	0.67	6.21	0.17	5.35	1.03	<.477	0.00
24	4.49	1.17	6.38	0.00	5.85	0.19	5.85	0.53	1.25	0.89
48	4.58	0.40	4.63	0.75	5.37	1.01	3.97	1.00	0.36	0.26
72	3.26	0.38	3.25	0.07	4.68	0.71	3.82	0.50	0.44	0.35

A (adductor): M (mantle); G (gills); D (digestive system)

Appendix C: Effect of Depuration of Summer Oysters in Immersion System on the Distribution of V. vulnificus in Oyster Tissues and Tank Water

Time	A	STD+-	M	STD+-	G	STD+-	D	STD+-	Water	STD+-
hr					log MPN/mL					
0	2.98	0.38	3.79	0.16	3.30	0.33	4.27	0.09	<.477	0.00
24	3.58	0.40	3.65	0.33	4.30	0.33	4.58	0.61	3.27	0.09
48	3.25	0.07	3.30	1.33	2.50	0.87	3.74	0.11	1.74	0.89
72	3.28	0.31	2.00	0.64	3.37	0.70	4.53	0.35	1.65	0.33

A (adductor); M (mantle); G (gills); D (digestive system)

Appendix D: Effect of Depuration of Fall Oysters in Immersion System on the Distribution of V. vulnificus in Oyster Tissues and Tank Water

Time	A	STD+-	M	STD+-	G	STD+-	D	STD+-	Water	STD+-
hr					log MPN/mL					
0	0.72	0.24	1.22	0.75	1.05	0.58	0.96	0.36	<.477	0.00
24	0.54	0.06	0.54	0.06	1.41	0.56	1.33	0.48	<.477	0.00
48	1.22	0.75	0.98	0.38	1.50	0.14	2.18	0.29	<.477	0.00
72	0.48	0.00	0.92	0.44	0.72	0.24	0.72	0.24	<.477	0.00

A (adductor); M (mantle); G (gills); D (digestive system)

SECTION IV: CONCLUSIONS

Oysters stored in live holding systems may harbor pathogens. If the bacterial population of oysters prior to storage in these tanks are high, this level may persist throughout the storage period. The level of E. coli did not change in oysters held in a spray live holding system. There was no significant difference ($p = 0.12$) in the bacterial population of oysters with 10^6 CFU E. coli/mL after 120 hr of storage.

The level of E. coli in oysters subject to physiological stress did not significantly change ($p = 0.30$) after 96 hr in the spray tank. But temperature abuse and mishandling of oysters prior to storage may be important factors since they may promote increased microbial levels in oysters.

The survival of E. coli in oysters may suggest the survival of other microbiological populations. E. coli is a commonly used standard for determining the microbiological quality of shellfish. The level of E. coli and V. vulnificus naturally found in oysters at harvest persisted during storage in the spray system.

Oysters held in spray tanks for retail sales may not eliminate V. vulnificus. There is a prevalence of V. vulnificus in oysters during the summer months. V.

vulnificus was found at concentrations of 10^4 MPN/mL in oysters harvested in summer. The health implications of this microbial retention are presently unknown.

Oysters may be depurated to reduce the level of microorganisms before they are sold in retail markets. There was a difference in the efficiency of depuration of V. vulnificus in oysters naturally- and artificially-inoculated with the organism. V. vulnificus artificially introduced into oysters may have become loosely associated with mucus on the surfaces of the adductor muscles and the mantle tissue. As a result, the bacterium was easily removed from these surfaces during depuration. V. vulnificus on the gills and the digestive system became entrapped with cilia and mucus, therefore was more difficult to remove. It seems unlikely that depuration of oysters naturally harboring the bacterium may be effective.

A possible subject of future study involves heating oysters before placing them in the depuration tank and seeing how this affects the levels of V. vulnificus in oyster tissues. Keeping in mind that oysters must not be killed by any proposed treatment, the effect of temperature and time factors on the destruction of V. vulnificus could be evaluated.

VITAE

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PUBLICATIONS:

Colby, J.W. and G.J. Flick, Jr., 1991. Distribution of *Vibrio vulnificus* in oyster (*Crassostrea virginica*) tissues during artificial inoculation and depuration, Proceedings from the 16th Annual Tropical and Subtropical Fisheries Technology Conference, Raleigh, North Carolina (in press).

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PRESENTATIONS:

Presentation at Shellfish Institute of North America (SINA) and National Blue Crab Industry Association (NBCIA) Convention. Survival of _Vibrio vulnificus_ in oyster tissues during storage in spray and immersion live holding tanks. February 24, 1992. Baltimore, Maryland.

Presentation at the 16th Annual Tropical and Subtropical Fisheries Technology Conference. Distribution of _Vibrio vulnificus_ in oyster (_Crassostrea virginica_) tissues during artificial inoculation and depuration. September 30, 1991. Raleigh, North Carolina.

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