SURVIVAL OF *VIBRIO VULNIFICUS* AND OTHER VIBRIOS IN RAW OYSTERS (*CRASSOSTREA VIRGINICA*) DURING PROCESSING IN VIRGINIA AND COLD STORAGE

by

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ABSTRACT

The objective of this research was to determine if Vibrio populations, specifically *V. vulnificus* were affected in oysters by the processing methods employed in Virginia. This study was conducted between July and September in 1995 and during the month of August of 1996 when water temperature was expected to be high. Oysters were harvested from Virginia and the Gulf coast and shucked and blown by Virginia processors. They were tested for aerobic plate counts incubated at 35-37^{0}C, salt content, pH, total Vibrios and *V. vulnificus* populations before and after processing. Oysters were stored in crushed ice and maintained an internal temperature of 1^{0}C and tested at 5, 10, and 15 days after processing. Oysters were also stored at -9^{0}C tested every one to two weeks. Procedures described in the Food and Drug Administration's Bacteriological Analytical Manual for identification of *V. vulnificus* were followed. *V. vulnificus* populations were

not significantly affected by blowing. V. vulnificus populations decreased in oysters stored at 1°C and -9°C. V. vulnificus levels decreased faster in blown oysters harvested from the Gulf coast. Vibrio populations were not significantly reduced by blowing in oysters that were 1°C. Oysters stored at -9°C showed decreased Vibrio populations. pH and APC showed an inverse relationship in oysters that were 1°C. In oysters stored at -9°C, pH and APC showed a positive correlation. Significance of these correlations varied.

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INTRODUCTION

In response to concerns over the pathogenic bacterium, Vibrio vulnificus, the United States Food and Drug Administration (FDA) reported that they would prohibit oysters from the Gulf coastal areas to be sold in the half-shell market from April through October. Additionally, shucked oysters sold during that time would have to bear a label stating to cook thoroughly. This regulation would put an economic hardship on Virginia oyster packers since many processors shuck product from other states, principally the Gulf coast (Spalding, 1995).

Almost all cases of V. vulnificus have been associated with shell-stock oysters. A possible reason is that commercially shucked product is usually exposed to fresh water and cold temperature before sale. In Virginia, shucked oysters are exposed to fresh water during blowing. Studies in our laboratory showed that the salt content of the oysters was reduced to approximately 0.10% after blowing (Hackney et al., 1988). This low level of salt in conjunction with cold temperatures may be detrimental to the Vibrios or cause V. vulnificus to enter a viable but nonculturable state.

When Vibrios, including V. vulnificus are exposed to very low salt conditions, they become stressed. Additionally, V. vulnificus and many of the other Vibrios are sensitive to cold. These stresses of low salt and cold

can actually kill many of the Vibrios present. The population is further reduced during cold storage, in part due to competing microflora which lower the pH. Since people do eat raw oysters from commercial containers, this reduction in populations could explain why so few cases of V. vulnificus are associated with shucked product. This work examined the change in V. vulnificus populations in shucked product during refrigerated and frozen storage. In addition, Aerobic Plate Counts, pH, total Vibrios, and oyster salinity were all determined.

SECTION I: REVIEW OF LITERATURE

A. OYSTERS

1. GENERAL

Bivalve mollusks include oysters, clams and mussels (Hackney, 1990). The mollusk industry depends on the natural availability of these animals. These levels may vary from year to year (Hackney, 1990). Oysters and mussels are bottom dwellers, and attach to various structures in the water. Clams live in the mud and sediment (Cook, 1991). Oysters are eco-morphic meaning that they have shells that vary in shape and size and are very irregular (Cook and Ruple, 1994).

Oysters are filter feeders and are naturally contaminated with the natural micro flora occurring in the shallow estuarine waters. The bacteria are present in the shell liquor, intestinal tract, and exterior mucous of the oysters (Cook and Ruple, 1994).

Oysters must be harvested from approved growing waters. The National Shellfish Sanitation Program (NSSP) classifies waters according to their microbial quality. In order to be classified as an approved growing site, fecal coliform levels must have a median most probable number (MPN) value of less than 14/ 100 milliliters (ml) of water (Cook, 1991). Oysters harvested from approved sites must contain a fecal coliform MPN of less

than 230 MPN/gram (g) (Cook, 1991).

Oysters are filter feeders and thus concentrate bacteria in their bodies. Oysters that are to be sold on the wholesale market should have a standard plate count (SPC) of less than 10^5 /g (Cook, 1991). Oyster meats that have SPC's that are greater than 10^6 /g are considered of substandard quality (Cook and Ruple, 1994).

2. PROCESSING PROCEDURES/REQUIREMENTS FOR RAW OYSTERS

In order to maintain safe handling and processing procedures, the NSSP publishes a manual of operations that gives detailed instructions and acceptable practices for operation of a shellfish facility (FDA, 1993). This manual provides practices from harvest and shipping, facility requirements, to distribution.

Once oysters have been harvested, they can undergo wet storage, depuration, or dry storage. Oysters harvested for wet storage must be placed under temperature control within 20 hours of harvest during the months of April to November. Oysters must be under temperature control within 36 hours from December to March. Shell stock must be maintained at 7.2^0C or below until purchased by a customer or consumer (FDA, 1993).

Shell stock that are to be stored under dry storage conditions must

also be maintained at 7.2°C or below and may not be removed from a controlled temperature environment for more than 2 hours (FDA, 1993).

Oysters are shucked or removed from their shells manually because of the irregularity of their shells (Cook and Ruple, 1994). Oysters intended for the raw market are then subject to washing by one of several methods. Further processing of raw oysters is discussed in greater detail in later sections of this review.

3. RISKS ASSOCIATED WITH CONSUMPTION OF RAW OYSTERS

Microbial contaminants, toxins, and decomposition of seafood pose potential serious health problems to humans. Food poisoning associated with consumption of all seafood represents a small percentage of the total reported cases of food poisoning (Benson, 1989). From 1978 to 1987, 10.5% of all food borne outbreaks were caused by fish and shellfish (Liston, 1990). The total number of outbreaks was greater than the total number reported from consumption of meat or poultry (Liston, 1990). However, fewer total people were affected by food poisoning when consuming seafood (Liston, 1990).

Ensuring safety of shellfish is difficult. Harvest waters must be free of fecal contamination or industrial pollution, and also the natural micro flora

and plankton levels must be at levels that are non hazardous to humans (Benson, 1989). Cooked fish is responsible for illness in approximately one out of every million servings of cooked fish. Contrast that with incidence of illness for raw shellfish, one in 25,000 servings of raw shellfish, (Spalding, 1995) and the risks for consuming raw shellfish become apparent.

Less than half of the cases of seafood poisoning are associated with bacterial and viral agents (Liston, 1990). The majority of illness such as paralytic shellfish poisoning (PSP), scombroid fish poisoning, and ciguatera are associated with consumption of various fish and some shellfish such as mussels, clams, and scallops (Liston, 1990).

There are two major causes of food borne illness primarily associated with consumption of raw molluscan shellfish. These causes are Norwalk virus and the pathogenic Vibrios (Liston, 1990). The Vibrios have taken the place of enteric bacteria formerly associated with infection probably because of improved waste treatment and the NSSP (Liston, 1990).

Of the pathogenic Vibrios, several are associated with the consumption of raw shellfish, particularly oysters. Non-O Group 1 Vibrio cholerae is found in shallow estuarine waters and has been associated with the consumption of raw oysters. People becoming ill from Non-O Group 1 V. cholerae suffer from a severe dehydrating diarrhea (Blake, 1980).

V. parahaemolyticus is found naturally in estuarine waters around the world (Blake, 1980). V. parahaemolyticus causes a gastroenteritis that can require hospitalization, but typically is mild (Blake, 1980). V. parahaemolyticus is also associated with the consumption of raw or undercooked seafood, and is naturally found in oysters (Blake, 1980).

Infection caused by V. vulnificus is the most serious (Liston, 1990) of the pathogenic Vibrios. A detailed description of the disease symptoms and disease factors is found later in this review. V. vulnificus is most often associated with the consumption of raw oysters (Hackney et al., 1992; Whitman, 1994; Reyes et al., 1987).

B. INCIDENCE OF MARINE VIBRIOS

1. CHARACTERISTICS

Members of the genus Vibrio are metabolically diverse (Tamplin, 1994). Vibrios are non spore formers, facultatively anaerobic, and have been isolated from marine environments and fresh water environments (Rodrick, 1991; Blake, 1980). Vibrios are Gram negative rods (West, 1989; Rodrick, 1991), and are included in the family Vibrionaceae along with the genera Aeromonas and Plesiomonas (Rodrick, 1991). They can be distinguished from the family Pseudomonadaceae by their ability to ferment

glucose (West, 1989). Vibrios can be distinguished from the family Enterobacteriaceae by their ability to produce cytochrome oxidase (West, 1989; Blake, 1980).

The genus Vibrio contains at least 50 species (Rodrick, 1991), 11 of which are considered pathogenic to humans (Rodrick, 1991; West, 1989). V. cholerae, V. parahaemolyticus, and V. vulnificus are of direct medical concern. V. alginolyticus, V. damsela, V. fluvialis, V. furnissii, V. hollisae, V. mimicus, V. metschnikovii, and V. cincinnatiensis are considered opportunistic pathogens (West, 1989).

These organisms have been associated with various clinical manifestations ranging from mild to severe gastroenteritis, wound infections, ear infections, and primary and secondary septicemia (Rodrick, 1991).

Vibrios occur throughout estuarine and marine environments (Tamplin, 1994; West, 1989) and have been isolated from fresh water, soil, and some warm blooded vertebrates (Rodrick, 1991). Association with shellfish, fish, and plankton in the marine environment allows for prolonged survival of Vibrios (West, 1989). Pathogenic Vibrios produce chitinase (Tamplin, 1994) and the chitin produced by plankton is important to prolonged survival in marine environments (West, 1989).

Pathogenic Vibrios commonly inhabit molluscan shellfish tissues. Since many molluscan shellfish are filter feeders, pathogenic Vibrios can be concentrated in the shellfish tissue (West, 1989). Pathogenic Vibrios have developed many enzymes that allow the organisms to retrieve nutrients from oyster tissues, fish and sediments (Tamplin, 1994). These same enzymes solicit other reactions in humans, resulting in illness and disease (Tamplin, 1994).

Several environmental factors affect the persistence of pathogenic Vibrios. Water temperature is a very important factor affecting the occupance of Vibrios. Vibrios are typically found in waters of temperatures between 10^{0}C to 30^{0}C (West, 1989).

Pathogenic Vibrios are typically halophilic and require salinities between 5 parts per thousand (ppt) and 30 ppt. This range of salinities limits their survival to estuarine waters (West, 1989). Combinations of elevated nutrient and water temperature can provide suitable conditions for survival of pathogenic Vibrios in fresh water areas (West, 1989).

C. VIBRIO VULNIFICUS

1. CHARACTERISTICS

Vibrio vulnificus is a Gram negative non spore forming rod,

facultatively anaerobic (Rodrick, 1991), and halophilic (Rodrick, 1991; Oliver, 1989). It is distinguishable from other members of the family Vibrionaceae by its ability to ferment lactose (Rodrick, 1991; Oliver, 1989). V. vulnificus was first reported in 1970 in a case of wound infection. V. parahaemolyticus was suspect, but later V. vulnificus was confirmed (West, 1989).

The ability of V. vulnificus to ferment lactose has been used to distinguish it from V. parahaemolyticus and V. alginolyticus (Oliver, 1989). This lactose positive Vibrio was originally given the name Beneckea vulnificus (Rodrick, 1991; Oliver, 1989; Bake, 1980). Beneckea vulnificus became Vibrio vulnificus officially in 1980 (Oliver, 1989).

V. vulnificus is ubiquitous in the marine environment having been isolated from seawater, sediment, oysters, crabs (DePaola et al., 1994; Oliver, 1989; Rodrick, 1991), finfish (DePaola et al., 1994), and other marine animals (Oliver, 1989). V. vulnificus exhibits seasonal occurrence and is isolated primarily from waters of warmer temperatures (20 to 30^{0}C) (Oliver, 1989; DePaola et al., 1994; Rodrick, 1991). Water salinity is a factor contributing to the presence of V. vulnificus. Different researchers have concluded that salinities are optimum at 7 to 16 parts per thousand (ppt). Others have concluded that salinities of 16 ppt or higher are optimum

for growth of V. vulnificus (Oliver, 1989).

V. vulnificus levels are higher in oysters compared with surrounding water. Filter feeding by oysters causes a concentration of bacteria found in the surrounding water, explaining higher levels of V. vulnificus in oysters (Oliver, 1989).

Isolation techniques have developed over several years that are specific to isolation of V. vulnificus. Originally, Thiocitrate Bile Salts Sucrose (TCBS) agar was used as the selective media of choice when isolating Vibrio species (Oliver, 1989). TCBS utilizes sucrose as the carbon source in the media. V. vulnificus does not ferment sucrose so produces sucrose negative colonies. V. parahaemolyticus also is sucrose negative on TCBS and can easily be confused with V. vulnificus.

Two other selective media have been developed for isolation of V. vulnificus. Cellobiose Polymyxin B-Colistin (CPC) agar and Sodium Dodecyl Sulfate-Polymyxin-B Sucrose (SPS) agar (Oliver et al., 1992). V. vulnificus hydrolyzes sodium dodecyl sulfate and is resistant to polymyxin B. SPS differentiates V. vulnificus from V. cholerae serogroup non O1. V. vulnificus produces a bluish halo around colonies grown on SPS agar (Oliver, 1989). CPC agar utilizes V. vulnificus' resistance to polymyxin B and colistin and cellobiose fermentation to differentiate from other Vibrio

species. In a study conducted by Sloan et al. (1992), V. vulnificus was isolated 20% more often using CPC agar than when using SPS agar.

2. DISEASE

a. GENERAL

Much of the literature on the illness associated with Vibrio vulnificus describes two distinct clinical manifestations (Sakazaki and Shimada, 1986; Blake et al., 1980; Reed, 1994). In fact, three distinct clinical presentations occur. Infection by V. vulnificus can result in a primary septicemia, wound infection, and occasionally gastroenteritis (West, 1989; Hackney et al., 1992; Whitman, 1994).

b. SEPTICEMIA

Primary septicemia resulting from V. vulnificus infection typically occurs 18 to 48 hours after ingestion (Hackney et al., 1992) of raw oysters or undercooked seafood. In the majority of cases of primary septicemia, most patients had consumed raw oysters (Hackney et al., 1992; Whitman, 1994; Reyes et al., 1987).

The primary septicemia typically begins with symptoms of malaise, fever and chills. Blake et al. (1980) reported that in one study, 21% of the

cases had accompanying nausea and vomiting and 17% had accompanying diarrhea. One third of the cases show signs of hypotension (systolic blood pressure $\leq$ 80 mm Hg) (Blake et al., 1980; Hackney et al., 1992) which can lead to intractable shock and ultimately death (Whitman, 1994). Mortality is extremely high with primary septicemia, often greater than 50% (Hackney et al., 1992; Whitman, 1994).

Secondary skin lesions typically accompany fulminating septicemia (Whitman, 1994; Blake et al., 1980). These lesions typically occur on the lower extremities (Blake et al., 1980). Lesions appear initially as red vesicles and later develop into necrotic ulcers (Blake et al., 1980; Whitman, 1994).

The vast majority of patients developing primary septicemia from *V. vulnificus* have preexisting conditions or underlying liver disease (Whitman, 1994) including such diseases as alcoholism, diabetes, or liver disfunction (Blake et al., 1980). Sixty to 75% of patients developing septicemia have a preexisting liver condition or are heavy alcohol consumers (Whitman, 1994). Fifteen to 20% of patients suffer from a deficiency of the immune system or are taking medications that suppress immune response, or are suffering from serum iron elevation as in the case of hemochromatosis or hemosiderosis (Whitman, 1994; Stelma et al., 1988).

c. WOUND INFECTION

Wound infection can result when pre-existing cuts or wounds are exposed to sea water or wounds that occur while in the presence of seawater (Whitman, 1994; Reed, 1994; West, 1989). Compared to primary septicemia, patients of wound infections frequently have been in contact with crabs or seawater immediately prior to the onset of infection (Sakazaki and Shimada, 1986).

Once infected by V. vulnificus, a rapidly ensuing cellulitis results (Reed, 1994). Typically symptoms begin with redness and intense pain (Whitman, 1994). Vesicles and bullae often are present and progress to tissue necrosis (Whitman, 1994; Blake et al., 1980). V. vulnificus is often isolated from the wound (Blake et al., 1980) though a toxic mediated process has been suggested for those instances when V. vulnificus was not isolated from the wound (Morris, 1994).

Fifty percent of patients developing wound infections require limb amputation (Whitman, 1994). In some instances, a secondary septicemia may result from wound infection and mortality is high in these instances (Whitman, 1994).

Underlying disease is not necessary for development of wound infection. However, mortality rates increase from 25% to 50% in those

cases where patients did have underlying diseases or liver disfunction (Whitman, 1994; Blake et al., 1980).

d. GASTROENTERITIS

The third clinical manifestation of V. vulnificus infection is gastroenteritis. Described as a mild syndrome, typical symptoms such as abdominal cramping, vomiting, and diarrhea are observed (Whitman, 1994). Death is rarely observed in cases of gastroenteritis (Whitman, 1994; West, 1989). Though hospitalization has been required in as many as 50 percent of cases of gastroenteritis associated with V. vulnificus, this may be due to inadequate isolation and identification procedures and a lack of reporting of milder cases (Whitman, 1994). Healthy individuals and individualist with underlying illness are both susceptible to gastroenteritis (Hackney et al., 1992; Whitman, 1994). The majority of patients developing gastroenteritis have consumed raw oysters in the week prior to illness onset (West, 1980; Whitman, 1994; Hackney et al., 1992).

e. INFECTIVE DOSE

The infective dose of V. vulnificus has not been determined, though fatality has been reported with consumption of one raw oyster (Whitman,

1994). Host factors appear to be most important in determining fatality (Morris, 1994).

3. PATHOGENICITY

a. CAPSULE

Two distinct cell morphologies of V. vulnificus have been isolated (Rodrick, 1991; Morris, 1994; Oliver, 1989). One morphology is an encapsulated form. The other form lacks the capsule. The polysaccharide capsule was first described by Kreger et al. (Oliver, 1989). This polysaccharide capsule is antiphagocytic in nature and was discovered to be acidic in nature (Oliver, 1989; West, 1989).

Two distinct colony morphologies have been isolated on Luria agar resulting from an encapsulation factor. The encapsulated V. vulnificus cells appear opaque. Those cells lacking the polysaccharide capsule are translucent. The opaque form of V. vulnificus is the virulent form of this organism (Morris, 1994).

Cells of the same strain of V. vulnificus can transform from opaque to translucent spontaneously. With this transformation from opaque to translucence comes a subsequent loss in virulence. Shifts from translucence to opaque are also possible and show that virulence resumes

(Morris, 1994).

The polysaccharide capsule is antiphagocytic as mentioned before but also provides protection to V. vulnificus from animal serum and from compliment mediated bactericidal activity (Morris, 1994; Rodrick, 1991; West, 1989). Serum resistance was demonstrated when V. vulnificus cells were incubated in human serum for one hour. Strains that were opaque showed little decrease in levels. Those strains that were translucent showed a 4 log decrease in levels and the cells that were recovered from the serum were opaque. This showed a selection for the opaque morphology. V. vulnificus cells that were permanently locked genetically in the translucent form and incubated in the serum were killed and not recovered (Morris, 1994).

Virulence because of the polysaccharide capsule was demonstrated using an iron-loaded mouse model. For opaque strains, the LD_{50} was 3 logs less than the LD_{50} for initially translucent V. vulnificus cells. The cells that were recovered in both instances were opaque. Cells that were permanently genetically locked in the translucent phase had a LD_{50} that was 4 to 5 logs greater than that for the opaque cells (Morris, 1994).

b. EXTRACELLULAR PRODUCTS

V. vulnificus produces several extracellular products or toxins that contribute to the disease symptoms but are not significant factors contributing to virulence (Morris, 1994). Some of these factors that have been described are a cytotoxic hemolysin, protease, elastase, collagenase (Oliver, 1989; Morris, 1994; West, 1989), and siderophore production (Rodrick, 1991; Oliver, 1989). Enterotoxin production has also been reported from some isolated of V. vulnificus (Stelma et al., 1988).

Of these toxins, the cytotoxic hemolysin contributes most significantly to disease symptoms. The hemolysin exhibits cytolytic activity against Red Blood Cells (Erythrocytes) (Oliver, 1989; Morris, 1994) and may provide improved vascular permeability of the organism (Rodrick, 1991). The vascular permeability factor appears to be potent and results in severe vascular fluid loss in laboratory animals (Oliver, 1981). This cytolysin causes skin lesions in animal models and in conjunction with other factors may contribute to skin lesions in humans (Morris, 1994).

Protease(s) active against elastin and albumin have been isolated from V. vulnificus cultures. This protease or elastase is active in destruction of Immunoglobulin G and components of compliment (Oliver, 1989). The elastase causes necrosis, edema, and muscle degradation (Oliver, 1989) and

contributes to disease symptoms. Absence of elastase activity, as with other extracellular products does not affect the virulence of V. vulnificus (Morris, 1994).

Enterotoxin may be the causative agent in diarrhea associated with V. vulnificus disease. Diarrhea is not common with V. vulnificus infections, however gastroenteritis has been associated with V. vulnificus that do not become septic (Stelma et al., 1988).

c. IRON REQUIREMENTS

Elevated serum levels of iron and specifically iron saturated transferrin, an iron binding protein found in serum, appears to be closely linked with occurrence of the disease (Morris, 1994; West, 1989). Alcoholism and other dysfunctional liver diseases can result in hemochromatosis leading to 100% transferrin iron saturation, or thalassemia major, insufficient transferrin production, also resulting in transferrin saturation (Oliver, 1989). These conditions place people at elevated risk for disease.

Under normal serum conditions and transferrin saturation levels below 65%, V. vulnificus is unable to grow (Morris, 1994) and even is killed by the serum defenses (Oliver, 1989). Virulent strains of V. vulnificus are able

to compete with saturated transferrin for iron and thus proliferate (Oliver, 1989).

Virulent strains of V. vulnificus produce two different iron chelating agents known as siderophores. It is the only Vibrio known to produce both chelators. The hydroxamate siderophore is not produced by other Vibrios, and has a greater iron binding ability than the phenolate siderophore seen in other Vibrios (Oliver, 1989). In a study conducted by Stelma et al. (1992), the avirulent strains of V. vulnificus were shown to have decreased phenolate siderophore production and were thus unable to acquire iron from saturated transferrin (Stelma et al., 1988). There was no mention of the hydroxamate siderophore levels produced by these strains in this study.

4. VIABLE BUT NONCULTURABLE STATE

a. GENERAL INFORMATION

Vibrio vulnificus exhibits seasonal variation with most cases of infection occurring during the warm months. Eighty percent of septicemia cases and 94% of wound infections involving V. vulnificus occur between May and October (Whitman, 1994). Isolation of culturable V. vulnificus cells from seawater, sediment, and oysters becomes very difficult during the winter months (Tamplin, 1994; Oliver, 1994; Linder and Oliver, 1989;

Brauns et al., 1991). Often times standard microbiological isolation techniques result in negative detection of the organism (Tamplin, 1994; Linder and Oliver, 1989; Brauns et al., 1991).

V. vulnificus appears to "die off" during the cold winter months and reappears during the warmer months. This phenomenon may be the result of V. vulnificus entering into what is known as a viable but nonculturable (VBNC) state. The VBNC state has been documented for many other organisms such as Aeromonas, Campylobacter, Enterobacter, Escherichia coli, Klebsiella, Legionella, Salmonella, Pseudomonas, and Shigella as well as many other Vibrio species (Oliver, 1994; Oliver et al., 1991).

The induction of the VBNC state is species dependent, but factors such as light, salinity changes, nutrient deprivation, and temperature changes can induce the VBNC state (Oliver, 1994; Linder and Oliver, 1989). V. vulnificus is affected by temperature decreases (Oliver, 1994; Oliver et al., 1991). Entering the VBNC state during the cold winter months appears to be a survival technique employed by V. vulnificus (Linder and Oliver, 1989; Oliver, 1994).

Vibrio vulnificus held at temperatures greater than 10^{0}C appear to remain culturable for long periods of time (Oliver, 1994; Oliver et al., 1991). When the temperature of V. vulnificus cultures is decreased to 5^{0}C,

culturable cells decrease in number. After 40 days, standard microbiological enrichment and isolation techniques produce no culturable results (Oliver, 1994). In a study performed by Oliver et al. (1991), researchers found that when the temperature of V. vulnificus cultures were decreased to 5-7^{0}C, time to reach these temperatures was two hours. Decreases in culturability were noted even after one hour of sampling (Oliver et al., 1991).

V. vulnificus cells begin entering the VBNC state by decreasing in size and changing from rod shaped to cocci (Oliver, 1994). In a study by Linder and Oliver (1989), cells decreased from 1.7um to an average of 0.8um after incubation for 17 days. Studies by Oliver et al. (1991) found that entrance into the VBNC state took longer for stationary phase cells than for logarithmic-phase cells. Stationary phase cells take approximately one month to become nonculturable whereas logarithmic-phase cells become nonculturable in one week (Oliver et al., 1991).

Various macromolecular changes occur within 15 minutes of shifting a culture to 5^{0}C. Decreases in DNA, RNA, and protein synthesis occur (Oliver, 1994). There is a reduction in the total number of ribosomes as V. vulnificus enters the VBNC state (Linder and Oliver, 1989). Specific "cold shock" proteins begin to appear as a result of a temperature decrease to 5^{0}C (Oliver, 1994).

Though many macromolecular changes do occur in transition to the VBNC state, after 15 days, the polysaccharide capsular material of V. vulnificus remains (Linder and Oliver, 1989; Oliver, 1994). V. vulnificus cells in the VBNC state are potentially still virulent.

b. RESUSCITATION

Resuscitation can be defined as "a reversal of those metabolic, physiological, and genetic processes that induced the VBNC state" (Oliver, 1994). Oliver et al. (1991) have reported that resuscitation in the laboratory of nonculturable viable cells of V. vulnificus is possible by raising the temperature of the cultures for two to three days. No additional nutrients are necessary for resuscitation, only an upward temperature shift is required (Oliver, 1994). In other studies, VBNC cells held at 5^{0}C were taken to room temperature, and after one to two days of incubation became culturable (Oliver, 1994).

Studies by McFeters have also been conducted to determine if resuscitation is possible under environmental conditions. A strain of V. vulnificus called a TnphoA insertion mutant contained an inserted gene that provided resistance to kanamycin and allowed production of alkaline phosphatase. This strain was placed in an estuary in a floatable microcosm

chamber. The strain was exposed to the environmental conditions that occurred. Plating on media containing kanamycin and colorometric reagents allowed this strain of V. vulnificus to be differentiated from environmental strains. Results showed that the VBNC state does occur under environmental conditions. Laboratory induced VBNC cells of this same strain were then exposed to spring time conditions in the floating chambers, and results showed that resuscitation also occurred under environmental conditions (Oliver, 1994).

However, other studies question resuscitation and indicate that observed growth is only the growth of a few culturable cells still present (Kaspar and Tamplin, 1993). There is debatable evidence regarding the issue of resuscitation and further research is necessary to determine if this phenomenon is occurring.

c. VIRULENCE

When in the VBNC state, V. vulnificus still processes its polysaccharide capsule and is potentially virulent. Studies have determined that the virulence of VBNC V. vulnificus is greatly reduced compared to culturable cells of V. vulnificus (Linder and Oliver, 1989; Oliver, 1994). LD_{50}s for V. vulnificus when concurrently injected with iron have been

decreased by six logs (Linder and Oliver, 1989). In the virulence studies conducted by Linder and Oliver (1989), subsequent injection of mice with iron and 5 x 10^4 VBNC V. vulnificus cells resulted in no deaths indicating a significant decrease in the virulence of the VBNC cells.

5. FACTORS AFFECTING GROWTH AND SURVIVAL

a. OYSTER PROCESSING

Oysters are usually processed for the live market. These processing techniques are simple and typically involve washing, sorting and packing for shipment (Hackney, 1990).

Processing for the raw market can involve several additional steps. After harvesting, oysters are transported to the processing facility, usually by refrigerated truck (Cook, 1991). Oysters are then shucked which is the removal of the oyster from the shell (Hackney, 1990; Cook, 1991). Shucking can be performed by placing a knife between the oyster shell halves and cutting the adductor muscle and removing the oyster body from the open shell (Cook, 1991; Hackney, 1990) or by a method referred to as "popping the hinge." This second method is not usually practiced but involves inserting the knife between the hinge and breaking the oyster open (Hackney, 1990).

Shucking of oysters often results in liquid loss during packing (Cook et al., 1988; Hackney, 1990). One study conducted by Cook et al. (1988) showed that Gulf coast oyster meats lost more liquid after shucking than did East coast oysters. This study also concluded that oysters typically did not have greater than 15% liquid loss when processed according to the Code of Federal Regulations (CFR) (Cook et al., 1988).

Shucked oyster meats are subject to washing by one of several methods. Washing serves to remove any mud or debris from the meats that may have resulted during shucking (Cook, 1994). Oysters can be washed by placing the meats on a perforated tray or skimmer and spraying with water (Cook, 1994). Oysters can also be washed by a method known as blowing. Blowing is the process by which oysters are cleaned in fresh water (water containing less than 0.75% salt) or water containing chlorine. Air is pumped through the bottom of the tanks, agitating the oysters and removing debris and mud (Cook, 1991; 21 CFR 161.130; Hackney, 1990). Oysters may be placed in fresh water for no more than 30 minutes. If blowing is the method of washing employed, the time of contact with fresh water is considered double the actual blowing time (21 CFR 161.130).

Shucking and washing by blowing can reduce the aerobic plate counts by approximately 1 log (Cook, 1991). Changes in V. vulnificus

populations by shucking and blowing has been shown in studies, but a consistent change in population has not been documented (Cook, 1994). A study conducted by Hood et al. (1984) showed that commercial processing by shucking and washing with a skimmer or by blowing produced a cleaner looking product and an overall decrease in microbial load. However, decreases in pathogenic Vibrio species were not significant (Hood et al., 1984). Other studies have also shown that commercial processing by washing on a skimmer do not produce significant reductions in V. vulnificus levels prior to storage (Ruple and Cook, 1992).

Oysters washed by blowing in accordance with the CFR requirements did not loose more liquid compared to unblown oysters within the first 9 to 12 days of storage (Cook et al., 1988).

b. REFRIGERATED STORAGE

Refrigerated temperatures of 7.2°C or less are required by the National Shellfish and Sanitation Program for shucked oyster meats (Kaysner et al., 1989). Several studies indicate that refrigeration of oysters, either shucked or shell stock is an effective means of controlling the growth of Vibrio vulnificus. However, refrigeration is not effective for elimination of the bacteria from oysters.

In a study conducted by Cook (1994), shell stock oysters were held at 10, 13, 18°C, and ambient air temperatures ranging from 23 to 34°C. At 10 and 13°C V. vulnificus did not grow within 30 hours. At temperatures of 18°C and higher V. vulnificus were significantly higher than at the time of harvest.

In a separate study, a sterile oyster broth was produced using oyster homogenate. V. vulnificus cultures were inoculated into the oyster broth and also a sterile buffered salt solution. Oyster broth was incubated at two separate temperatures, 4 and 25°C. The buffered salt solution was incubated at 4°C. Results showed an increase of V. vulnificus in the homogenate incubated at 25°C. V. vulnificus populations decreased rapidly in the oyster homogenate broth incubated at 4°C. The rate of decrease in the buffered salt solution incubated at 4°C was significantly less than the decrease of V. vulnificus levels in the oyster homogenate broth. Oliver concluded that traditional homogenation procedures were releasing a heat stable toxin from the oyster that was lethal to V. vulnificus (Oliver, 1981).

In this same study, Oliver inoculated whole oysters on the surface with V. vulnificus and held them on ice at 0.5°C. A decline was observed in V. vulnificus populations comparable to the decline seen in the buffered salt solution held at 0.5°C. Oliver described this decline as a "gradual die off" of

V. vulnificus (Oliver, 1981).

A study conducted later by Oliver and Wanucha (1989) reevaluated the "die off" observed in other studies. At temperatures below 10°C a rapid decrease in culturability of V. vulnificus was observed. At temperatures of 15°C in the presence of nutrients, V. vulnificus was able to grow. After incubation for 5 days at 10°C when no cells were culturable, a "direct viable count" was conducted using two different metabolic indicator assays, 40 percent of cells remained viable. After incubation for 28 days at 5°C and 10°C, 1 percent of initial inoculum remained viable (Oliver and Wanucha, 1989).

V. vulnificus levels can be decreased by cold storage of shell stock oysters or by keeping shucked packed oysters on crushed ice. Decreases of up to 3 logs in seven days have been reported (Cook, 1994). Another study showed that V. vulnificus levels decreased more rapidly in shucked oyster meats than in shell stock oysters at temperatures of 0 and 4°C. However, the bacteria may be detected for 14 days in the shucked meats and 21 days in shell stock oysters held at 0 and 4°C using the Most Probable Number (MPN) technique (Cook and Ruple, 1992).

c. FROZEN STORAGE

Several studies have been conducted to determine if freezing oysters is an effective means of controlling Vibrio vulnificus. Frozen storage, like refrigerated storage reduces the levels of V. vulnificus but does not completely eliminate the bacteria.

In a study conducted by Cook and Ruple (1992), pure cultures were frozen to -20°C. Numbers of V. vulnificus were reduced more rapidly when stored at -20°C than when cultures were stored at 0°C. In this same study, V. vulnificus could still be isolated from oyster meats frozen to -20°C after 12 weeks of storage at -20°C. Significant reductions of up to three logs were detected in V. vulnificus levels, but Cook and Ruple concluded that though the organism was effectively reduced, freezing could not be relied upon as a valid means of elimination of V. vulnificus (Cook and Ruple, 1992).

Another study looked at the effects of commercial processing and freezing. Shucked meats were blown in fresh water and rapidly cooled to 45°C. Samples were separated and frozen using carbon dioxide (CO_2) cryogenic freezing at -60°F and blast freezing at -20°F. Prior to shucking, oysters were held at 5°C for 12 days. V. vulnificus levels were reduced by commercial processing from 2.4×10^4 MPN/g to levels of 93 MPN/g.

Freezing further reduced V. vulnificus to 100 to 10^{-1} to undetectable levels (Kilgen, 1994).

A second part to this study involved preparing oysters by removing the top shell and placed in groups of six and package quick frozen to a temperature of -60°C. The frozen half shell oysters were then irradiated at gamma radiation levels of 0.5 to 5 Kgy. This study determined that commercial processing techniques of cold water washing, soaking, freezing and irradiation minimally affected the sensory characteristics of the oysters while reducing the levels of V. vulnificus to insignificant and sometimes undetectable levels (Kilgen, 1994). This study made no mention of the Viable But Nonculturable State of V. vulnificus.

Other studies have shown that rapid freezing of pure cultures can be an effective means of culture preservation. The medium used to freeze the cultures significantly affects the longevity of the cultures. Cultures of V. vulnificus stored in shrimp homogenate at -80°C remained relatively stable. Boutin et al. concluded that seafood homogenate could be used to store V. vulnificus cultures as an alternative to traditional agar slants held at room temperature though declines in numbers were observed (Boutin et al., 1985).

d. SALINITY

Vibrio vulnificus is an obligate halophile (Oliver, 1989) requiring low to moderate salinities for growth (Kaspar and Tamplin, 1993). Optimal growth occurs in laboratory media containing 1 to 3 percent sodium chloride (NaCl). Growth has been reported with concentrations of NaCl as low as 0.5 percent. No growth appears at levels of less than 0.1 percent or at levels of 5 percent or higher (Oliver, 1989).

Oysters are considered osmoconformers, which means that the salinity of their environment and surrounding waters is identical to that present in their tissue (Hackney et al., 1988). These salinities are subject to frequent change as rainfall or saltwater intrusion are common in estuaries. Oysters harvested from the same location at different times may have different salinities (Hackney et al., 1988).

In previous studies, an average sodium content of 160mg/100g (wet basis) was measured for the family Osteidae of which Crassostrea virginica is a member (Ward et al., 1983).

Various processing techniques affect the sodium content of the oysters. In a study conducted by Ward et al. (1983), the affect on salinity of three different processing procedures were evaluated. Traditional hand shucking and blowing of oysters showed a significant decrease in total

percent NaCl. An 82-90% decrease in salinity was noted with these processing techniques. Ward et al. postulated that freshly shucked oysters are often still alive when entering the blowing tanks. In an attempt to osmoconform with blowing tank conditions, the oysters discharge sodium and other ions. In this same study, exposure of fresh shell stock oysters to a steam tunnel and retorting of shucked oysters also decreased salinity levels (Ward et al., 1983).

With decreases in salinity of oysters resulting from processing, V. vulnificus levels may also be decreased. However in one study, Ruple and Cook concluded that traditional hand shucking followed by blowing in either tap water, ice water, or water containing chlorine did not appear to significantly reduce initial V. vulnificus levels. They concluded that this could be expected since V. vulnificus are found in the internal tissues of oysters (Ruple and Cook, 1994). However, the decrease in salinity resulting from the blowing processing may contribute to a more rapid decline in culturable V. vulnificus during refrigerated and frozen storage of oysters.

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SECTION II: SURVIVAL OF VIBRIO VULNIFICUS AND OTHER VIBRIOS IN RAW OYSTERS (CRASSOSTREA VIRGINICA) DURING PROCESSING IN VIRGINIA AND COLD STORAGE

ABSTRACT

The objective of this research was to determine if Vibrio populations, specifically Vibrio vulnificus found in oysters were affected by the processing methods employed in Virginia. This study was conducted between July and September in 1995 and during the month of August of 1996 when water temperature was greater than 20°C. Oysters were harvested from Virginia and the Gulf coast and shucked and blown by Virginia processors. They were tested for Aerobic Plate Counts (APC) incubated at 35-37°C, salt content, pH, total Vibrios and V. vulnificus populations before and after processing. Oysters were stored in crushed ice and maintained an internal temperature of 1°C and tested at 5, 10, and 15 days after processing. Oysters were also stored at -9°C tested every one to two weeks. Procedures described in the Food and Drug Administration's Bacteriological Analytical Manual for identification of V. vulnificus were followed. V. vulnificus populations were not significantly affected by blowing. V. vulnificus populations decreased in oysters stored at 1°C and

-9^{0}C. V. vulnificus levels decreased faster in blown oysters harvested from the Gulf coast. Total Vibrio populations remained greater than 2 to 3 logs throughout the study. Total Vibrio populations decreased 1 to 2 logs over storage time at 1^{0}C and -9^{0}C. pH and Aerobic Plate Count showed an inverse relationship in oysters that were stored at 1^{0}C. In oysters stored at -9^{0}C, a positive correlations was observed between pH and APC showed a positive correlation.

INTRODUCTION

Microbial contaminants, toxins, and decomposition of seafood pose potential serious health problems to humans. Oysters are filter feeders and are naturally contaminated with the natural microflora occurring in the shallow estuarine waters. The bacteria are present in the shell liquor, intestinal tract, and exterior mucous of the oysters (Cook and Ruple, 1994). There are two major causes of foodborne illness primarily associated with consumption of raw molluscan shellfish. These causes are Norwalk virus and pathogenic Vibrios (Liston, 1990). The Vibrios have taken the place of enteric bacteria formerly associated with infection probably because of improved waste treatment and the National Shellfish Sanitation Program (NSSP) (Liston, 1990).

Vibrio vulnificus is a Gram negative non spore forming rod, facultatively anaerobic (Rodrick, 1991), and halophilic (Oliver, 1989; Rodrick, 1991). It is distinguishable from other members of the family Vibrionaceae by its ability to ferment lactose (Oliver, 1989; Rodrick, 1991). V. vulnificus was first reported in 1970 in a case of wound infection. V. parahaemolyticus was suspect, but later V. vulnificus was confirmed (West, 1989).

V. vulnificus is ubiquitous in the marine environment having been isolated from seawater, sediment, oysters, crabs (DePaola et al., 1994; Oliver, 1989; West, 1989), finfish (DePaola et al., 1994), and other marine animals (West, 1989). V. vulnificus exhibits seasonal occurrence and is isolated primarily from waters of warmer temperatures (20 to 30°C) (DePaola et al., 1994; Oliver, 1989; West, 1989).

V. vulnificus levels are higher in oysters compared with surrounding water. Filter feeding by oysters causes a concentration of bacteria found in the surrounding water, explaining higher levels of V. vulnificus in oysters (West, 1989). The infective dose of V. vulnificus has not been determined, though fatality has been reported with consumption of one raw oyster (Whitman, 1994). Host factors appear to be most important in determining fatality (Whitman, 1994). Infection by V. vulnificus can result in a primary septicemia, wound infection, and occasionally gastroenteritis (Hackney et al., 1992; West, 1989; Whitman, 1994).

Oysters are usually processed for the live or shucked market. Processing techniques for the live market are simple and typically involve washing, sorting and packing for shipment (Whitman, 1994). Oysters intended for the shucked market can be washed by placing the meats on a perforated tray or skimmer and spraying with water (Cook, 1994). Oysters

can also be washed by a method known as blowing, which is cleaning of the oysters in fresh water (water containing less than 0.75% salt) or water containing chlorine. Air is pumped through the bottom of the tanks, agitating the oysters and removing debris and mud (Cook, 1991; 21 CFR 161.130; Hackney, 1990). Shucking and washing by blowing can reduce the aerobic plate counts by approximately one log (Cook, 1991). Changes in V. vulnificus populations by shucking and blowing has been shown in studies, but a consistent change in population has not been documented (Cook, 1994). A study conducted by Hood et al. (1983) showed that commercial processing by shucking and washing with a skimmer or by blowing produced a cleaner looking product and an overall decrease in microbial load. However, decreases in pathogenic Vibrio species were not significant (Hood et al., 1983). Other studies have also shown that commercial processing by washing on a skimmer do not produce significant reductions in V. vulnificus levels prior to storage (Cook and Ruple, 1994).

V. vulnificus levels can be decreased by cold storage of shell stock oysters or by keeping shucked packed oysters on crushed ice. Decreases of up to 3 logs in seven days have been reported (Cook, 1994). In one study V. vulnificus levels decreased more rapidly in shucked oyster meats than in shell stock oysters at temperatures of 0 and 4^{0}C. However, the bacteria

may be detected for 14 days in the shucked meats and 21 days in shell stock oysters held at 0 and 4°C using the Most Probable Number (MPN) technique (Cook, 1994).

In a study conducted by Cook and Ruple (1994), pure cultures of V. vulnificus were frozen to -20°C. Numbers of V. vulnificus were reduced more rapidly when stored at -20°C than when cultures were stored at 0°C. In this same study, V. vulnificus could still be isolated from oyster meats frozen to -20°C after 12 weeks of storage at -20°C. Significant reductions of up to three logs were detected in V. vulnificus levels, but Cook and Ruple concluded that though the organism was effectively reduced, freezing could not be relied upon as a valid means of elimination of V. vulnificus (Cook and Ruple, 1994).

In a different study, oysters were held at 5°C for 12 days prior to shucking (Kilgen, 1994). V. vulnificus levels were reduced by commercial processing from 2.4×10^4 MPN/g to levels of 93 MPN/g. Freezing further reduced V. vulnificus to 100 to 10^{-1} to undetectable levels (Kilgen, 1994).

This study looked at the processing methods employed by Virginia processors to determine if blowing shucked product decreased V. vulnificus levels in oysters harvested from Virginia and the Gulf coast. Also, this study looked at refrigerated and frozen storage of unblown and blown

oysters to determine if processing affected decreases during cold storage. Salinity, pH, microbial quality, and total Vibrio populations were also measured.

MATERIALS & METHODS

Sample collection

Oysters (Crassostrea virginica) were collected from a seafood processing plant in Kinsale, Virginia during the period from July 1995 to October 1995 and during August 1996. Depending on availability, oysters harvested from Virginia and oysters harvested from the Gulf coastal areas were collected. Oysters were collected before blowing and after blowing (Table 1).

Processing Conditions

Processing conditions were the same on all sampling dates. Oysters were shucked into stainless steal containers. After shucking, the oysters were transferred to blowing tanks. Oysters were blown for five to six minutes. Oysters were removed from the blowing tanks but remained in fresh water for 30 minutes before packaging. The blowing tanks contained fresh water without chlorine. The blowing tank temperature ranged from

17.5° C to 18.3° C After processing, the oysters were placed in plastic one gallon containers with plastic lids.

Oysters shucked into stainless steal containers but not receiving any further processing were also collected. These oysters were placed in plastic one gallon containers with plastic lids and labeled unblown.

On Site Testing

Fresh samples were homogenized at the processing plant and a serial Most Probable Number (MPN) using Alkaline Peptone Water (APW) (Elliot et al., 1992) for enumeration of total Vibrios and V. vulnificus populations was inoculated on site. Isolation of Vibrio vulnificus was preformed as described below. The MPN tubes were then transported to Blacksburg, Virginia at approximately 25°C and incubated upon arrival.

Sample Transportation

Samples were packed into one gallon plastic buckets. Three gallons each before and after processing were collected. Gallon containers were packed into ice chests containing ice. Blown and unblown oysters were transported in separate ice chests. Samples were transported to Blacksburg within 6 to 12 hours after processing.

Sample Storage

Samples were separated into 140g to 180g portions and placed in Ziploc^{Tm} brand heavy duty freezer bags. Pint size bags were used for trips 1, 2, and 3. Quart size bags were used for trips 4, 5, 6, and 7. Twenty to thirty bags of blown and unblown oysters were placed in a walk- in refrigerator in chests containing crushed ice. Oysters were maintained at 1^{0}C by replacing ice every five days, or as necessary. Forty to sixty bags of oysters were placed in a walk-in freezer. Freezer temperature was -9^{0}C.

Refrigerated samples were tested at day 5, 10 and 15 after processing date, unless otherwise noted. Frozen samples were tested every one to two weeks as indicated until V. vulnificus was not detected. Frozen samples were thawed by running warm tap water over the bags for 10 to 15 minutes.

Bacterial Analysis

The Most Probable Number technique (MPN) was used in determining total Vibrios and V. vulnificus populations. Isolation procedures outlined in the Food and Drug Administration's Bacteriological Analytical Manual (Elliot et al., 1992) were followed.

Fifty grams of fresh and refrigerated sample was diluted with 450

milliliters (ml) of 1% Bacto peptone (Difco Laboratories, Detroit MI) diluent containing 1% NaCl. Samples were homogenized for two minutes with a stomacher. Serial dilutions were prepared and a three tube MPN series containing Alkaline Peptone Water (APW) (pH 8.5) was inoculated. Serial dilutions were prepared for isolation of V. vulnificus specifically. Total Vibrio populations were calculated from these same dilutions. As a result, total Vibrio populations were not effectively enumerated. As V. vulnificus populations decreased in the frozen samples, 50g of sample was homogenized with 50 ml diluent.

After 12 to 16 hours, all APW tubes showing turbidity were streaked to Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) (Difco Laboratories, Detroit, MI) agar and modified Cellobiose-Polymyxin B-Colistin (mCPC) agar. Total Vibrios was calculated from tubes showing positive grown on TCBS agar. From mCPC agar, two typical V. vulnificus colonies were transferred to sectors of Gelatin Salt (GS) agar, containing six sectors per plate. Gelatinase and oxidase tests were preformed on GS agar. Positive colonies were either tested immediately or inoculated into long term test media and tested at a later date.

All media prepared for later testing contained 2 to 3% NaCl and incubated at 35^{0}C to 37^{0}C unless otherwise indicated. From long term test

media, Tryptic Soy Broth (TSB) (Difco Laboratories, Detroit, MI) containing 2 to 3% NaCl was inoculated and served as the inoculum for the following test broths each containing 2 to 3% NaCl. Bromcresol Purple broths containing lactose, sucrose, trehalose, arabinose, cellobiose, and maltose were inoculated. Salt tolerance was determined by growth in 1% Bacto Tryptone (Difco Laboratories, Detroit, MI) broth containing 0%, 6%, 8% and 10% NaCl. Growth at 42° C was determined by growth in TSB incubated for 24 hours in a 42° C water bath. Voges-Proskauer (VP) test was preformed on cultures grown for 42 hours. Arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase were tested by inoculating decarboxylase basal media supplemented with the appropriate amino acid. MPN tubes giving typical V. vulnificus reactions to the above biochemical tests were considered positive (Elliot et al., 1992).

pH

The pH of each sample was measured by homogenizing 12 to 15g sample with 50 to 55ml of distilled water. A pH meter model 240 (Corning Science Products, Corning, NY) was used. The pH meter was calibrated using buffers (Fisher Chemical, Fisher Scientific, Fair Lawn, NJ) at pH 4, pH 7, and pH 10.

Aerobic Plate Count

Aerobic plate count was determined for each sample. Initial dilutions prepared for MPN tubes were used as the sample. Further dilutions were prepared in peptone containing 2 to 3% NaCl. The spread plate technique was used on plate count agar (PCA) (Difco Laboratories, Detroit MI) containing 2 to 3% NaCl. Plates were incubated at 35°C to 37°C.

Salinity

Salinity of the oysters was determined by direct titration of the chloride ion using 0.171N silver nitrate and dichlorofluorescein as the indicator dye (Hackney et al., 1988).

Statistical Analysis

A randomized complete block design was established, with the block set on harvest location. Statistical analysis was conducted using Statistical Analysis System (SAS) (Statistical Analysis System, version 6.11). Correlation coefficients were calculated using Excel's (Microsoft Excel, version 4.0) Pearson's correlation coefficient.

RESULTS AND DISCUSSION

Refrigerated Storage

Vibrio vulnificus was not significantly affected by the processing methods employed by Virginia processors ($p > 0.05$) (Table 2). Other studies have shown that blowing does not significantly reduce V. vulnificus levels (Cook and Ruple, 1994; Cook, 1994; Hood et al., 1983). Oysters harvested from Virginia showed lower levels of V. vulnificus than in oysters harvested from the Gulf coast ($p < 0.01$). V. vulnificus was detected in unblown oysters harvested from Virginia and the Gulf coast after 15 days of storage at $1^{0}C$. However, in oysters that were washed by blowing, V. vulnificus was not detected at 15 days of storage. In general, V. vulnificus was not detected in blown oysters at 10 to 12 days of storage, except in oysters collected during trip 1 (Table 2). V. vulnificus levels significantly decreased over storage time ($p < 0.01$). V. vulnificus levels were low in all oysters, typically one log or less. Decreases in V. vulnificus levels was typically less than one log. Other studies have documented similar results. V. vulnificus has been cultured using the Most Probable Number (MPN) method from oysters stored at $0^{0}C$ and $4^{0}C$ after 14 days of storage (Cook and Ruple, 1994).

Total Vibrio populations remained high throughout storage, typically

greater than 3 logs (Table 3). Because dilutions were prepared for detection of low levels of V. vulnificus, values for total Vibrio populations were not significantly diluted to obtain exact whole numbers. A study by Hood et al. showed that blowing shucked oyster meats did not significantly reduce pathogenic Vibrio levels (Hood et al., 1983). Vibrio counts were greater than 10^2 to 10^3 at 15 days of storage at $1^{\circ}C$. Total Vibrio populations typically decreased one to two logs over storage time ($p<0.01$).

Log Aerobic Plate Counts (APCs) of oysters showed an increase over storage time in both oysters harvested from Virginia and from the Gulf coast stored at $1^{\circ}C$ ($p<0.01$) (Figure 1). Oysters harvested from the Gulf coast had approximately a 1 log higher APC than oysters harvested from Virginia ($p<0.01$). Washing oysters by blowing caused a 1 to 1.5 log reduction in oysters harvested from the Gulf coast and from Virginia ($p<0.01$), respectively. These results are consistent with other studies (Cook, 1991).

The pH of oysters harvested from the Gulf coast and Virginia stored at $1^{\circ}C$ did not significantly differ ($p>0.05$) (Figure 2). Blown oysters had a higher pH than did oysters that were unwashed ($p<0.01$). Higher pH's in blown oysters have been reported in other studies (Cook et al., 1988). The pH of all oysters stored at $1^{\circ}C$ decreased over storage time ($p<0.01$).

There was a negative correlation between pH and APC for oysters

harvested from Virginia and the Gulf coast stored at 1°C (Table 8). As pH decreased, APC increased. This correlation was only significant for oysters harvested from Virginia and washed by blowing (Table 8).

Frozen Storage

V. vulnificus was not significantly affected by the processing methods employed by Virginia processors in oysters harvested from the Gulf coast and Virginia stored at -9°C ($p > 0.05$) (Table 5). V. vulnificus levels were lower in oysters harvested from Virginia than oysters harvested from the Gulf coast ($p < 0.05$). V. vulnificus was detected in unblown oysters at 28 to 30 days of storage at -9°C for both oysters harvested from the Gulf coast. In blown oysters that were harvested from the Gulf coast, V. vulnificus was detected at 28 to 30 days of storage at -9°C. V. vulnificus was detected in oysters harvested from Virginia and stored at -9°C at 18 to 21 days after processing but not at 28 to 30 days after processing. V. vulnificus levels decreased over storage time ($p < 0.01$) to levels below the limit of detection by methods employed in this research for oysters stored at -9°C except for oysters representing repetition 1 for oysters harvested from the Gulf coast (Table 5). Other studies have reported viability of V. vulnificus after 12 weeks of storage at -9°C (Cook and Ruple, 1994).

Total Vibrio populations remained high in oysters stored -9°C at levels of greater than 2 to 3 logs (Table 6). Total Vibrio populations decreased by 1 to 3 logs over storage time ($p<0.01$).

Log APCs of oysters stored at -9°C decreased 1 to 2 logs over storage time in both oysters harvested from Virginia and the Gulf coast ($p<0.01$) (Figure 3). Oysters harvested from Virginia and from the Gulf coast did not have significantly different APCs ($p>0.05$). Washing by blowing reduced APCs for oysters stored at -9°C and harvested from Virginia and the Gulf coast by 1 to 1.5 logs, respectively ($p<0.05$).

The pH of oysters harvested from the Gulf coast and Virginia stored at -9°C did not significantly differ ($p>0.05$) (Figure 4) . Blown oysters stored at -9°C and harvested from Virginia and the Gulf coast had a higher pH than did unwashed oysters ($p<0.01$). The pH of all oysters stored at -9°C decreased over storage time ($p<0.01$).

Salinity

The salinity of oysters harvested from the Gulf coast and from Virginia was significantly reduced by the blowing methods employed by Virginia processors ($p<0.05$) (Table 4). The reductions in salinity reported in this research are consistent with other studies. Hackney et al. reported

reduction in total percent by blowing to approximately 0.10% (Hackney et al., 1988). The Code of Federal Regulations (CFR) states that oysters are not to be exposed to fresh water for more than 30 minutes. Also, blowing time is considered double the time oysters are agitated in fresh water (21 CFR 161.130). With the processing methods employed during the course of this experiment, oysters were blown for 5 to 6 minutes and then remained in still fresh water for 30 minutes. According to the CFR, total contact with oysters was 40 to 42 minutes. This long exposure of oysters to fresh water may reduce salt levels more than by methods employed in other states.

In oysters harvested from the Gulf coast there was a negative correlation between percent salt of the oysters and the rate of decrease of V. vulnificus ($p<0.05$) (Table 7). The oysters harvested from Virginia had lower salinities than the oysters harvested from the Gulf coast (Table 4). The change in salinity in oysters harvested from Virginia resulting from blowing was not as great as in oysters harvested from the Gulf coast. The greater change in salinity resulted in faster decreases of V. vulnificus levels.

CONCLUSIONS

V. vulnificus levels were not significantly affected by blowing methods employed by Virginia processors in oysters stored at 1°C and

-9^{0}C. Total Vibrio counts remained high in blown and unblown oysters harvested from the Gulf coast and Virginia. Storage of oysters at 1^{0}C and -9^{0}C did significantly reduce total Vibrio levels, specifically V. vulnificus.

This study was unique in that the rate of decline of V. vulnificus was correlated with the change in salinity resulting from blowing. Blowing did not directly reduce V. vulnificus levels, but did have an effect on persistence of V. vulnificus in raw oysters over storage time. Oysters harvested from the Gulf coast showed a greater change in salinity because of blowing compared to oysters harvested from Virginia. This drastic change in salinity showed a positive correlation with the rate of decline of V. vulnificus levels in raw oysters.

Future research is needed regarding longer blowing times and greater decreases in salinities, and the affects that these methods have on V. vulnificus levels.

Table 1. Harvest and processing data for oysters.

Oysters (Repetition)[a]	Harvest Date	Processing Date	Processor Location	Harvest Location
Gulf (1)	7/6/95 and 7/7/95	7/12/95	Kinsale	Port Sulfur, Louisiana
Gulf (2)	9/14/95 (blown only) 9/16/95 and 9/17/95	9/19/95	Kinsale	American Bay, Louisiana
Gulf (3)	7/31/96	8/6/96	Kinsale	Louisiana
Gulf (4)	8/19/96 and 8/20/96	8/21/96	Kinsale	Louisiana
Virginia (1)	8/1/95	8/3/95	Kinsale	Yoekamico River, Virginia
Virginia (2)	9/18/95	9/19/95	Kinsale	Yoekamico River, Virginia
Virginia (3)	Unavailable	8/30/96	Kinsale	Yoekamico River, Virginia

[a] Refers to statistical analysis.

Table 2. Log MPN/g values for total Vibrio vulnificus counts in oysters harvested from the Gulf coast and from Virginia in oysters stored at 1°C.

Harvest Location	Rep.	Unblown Day[a] 0	5[b]	10[c]	15	Blown Day[a] 0	5[b]	10[c]	15
Gulf	1	0.71	1.32	1.25	0.48	0.71	1.01	1.01	<0.48[d]
	2	1.92	1.06	<0.48[d]	<0.48[d]	1.30	1.39	<0.48[d]	<0.48[d]
	3	<0.48[d]	0.47	<0.48[d]	<0.48[d]	0.47	<0.51	<0.48[d]	<0.48[d]
	4	1.02	<-0.52	---[e]	<-0.52[d]	2.20	<-0.52[d]	---	<-0.52[d]
Virginia	1	0.66	1.68	<-0.52[d]	<-0.52[d]	1.12	1.25	<-0.52[d]	<-0.52[d]
	2	1.41	1.44	0.55	-0.45	0.55	<0.51	< 0.47[d]	<-0.52[d]
	3	0.66	0.02	<-0.52[d]	<-0.52[d]	<0.66	-0.21	<-0.52[d]	<-0.52[d]

[a] Day indicated testing on that number of days after processing. Day 0 represents testing on the day of processing.
[b] Day 5 includes data from days 5 to 7 after processing.
[c] Day 10 includes data from days 10 to 12 after processing.
[d] Below the limit of detection for this assay.
[e] Data not collected.

Table 3. Log MPN/g values for total Vibrio counts in oysters harvested from the Gulf coast and from Virginia in oysters stored at 1°C.

Harvest Location	Rep.	Unblown Day[a] 0	5[b]	10[c]	15	Blown Day[a] 0	5[b]	10[c]	15
Gulf	1	>3.04	>3.04	>3.04	>3.04	>3.04	>3.04	>3.04	>3.04
	2	>3.04	>3.04	>3.04	>3.04	>3.04	>3.04	>3.04	>3.04
	3	>6.04	>5.85	5.13	5.17	5.66	5.28	4.27	3.30
	4	5.66	5.38	---[d]	3.90	4.62	4.09	---	3.02
Virginia	1	>3.04	>2.71	>2.04	>2.04	>3.04	>3.04	>2.04	>2.04
	2	>3.04	>3.04	>3.04	>2.04	>3.04	>3.04	<1.05	>2.04
	3	5.34	3.88	3.71	>4.04	4.30	2.96	2.27	3.04

[a] Day indicated testing on that number of days post processing. Day 0 represents testing on the day of processing.

[b] Day 5 includes data from days 5 to 7 after processing.

[c] Day 10 includes data from days 10 to 12 after processing.

[d] Data not collected.

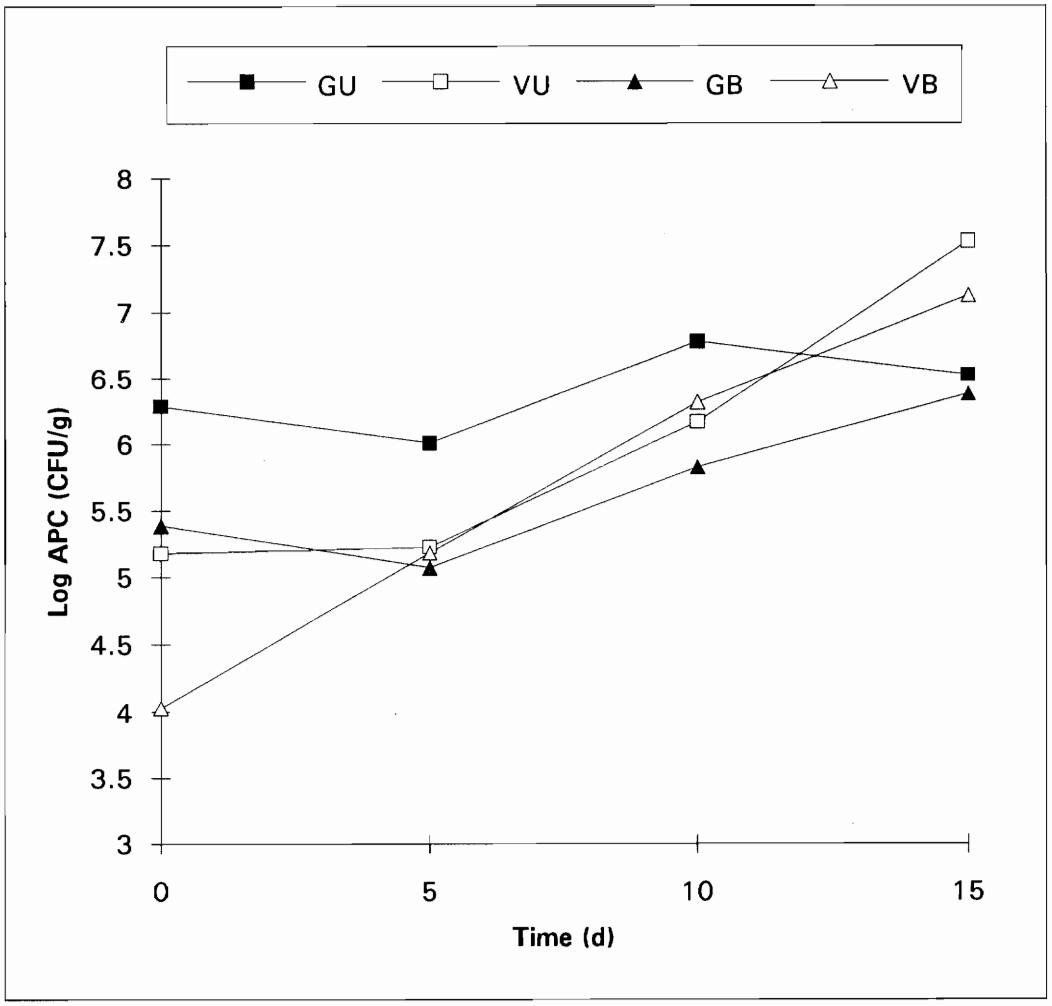

Figure 1. Log Aerobic Plate Counts (APCs) of unblown (U) and blown (B) oysters harvested from the Gulf coast (G) and Virginia (V) stored at 1 C.

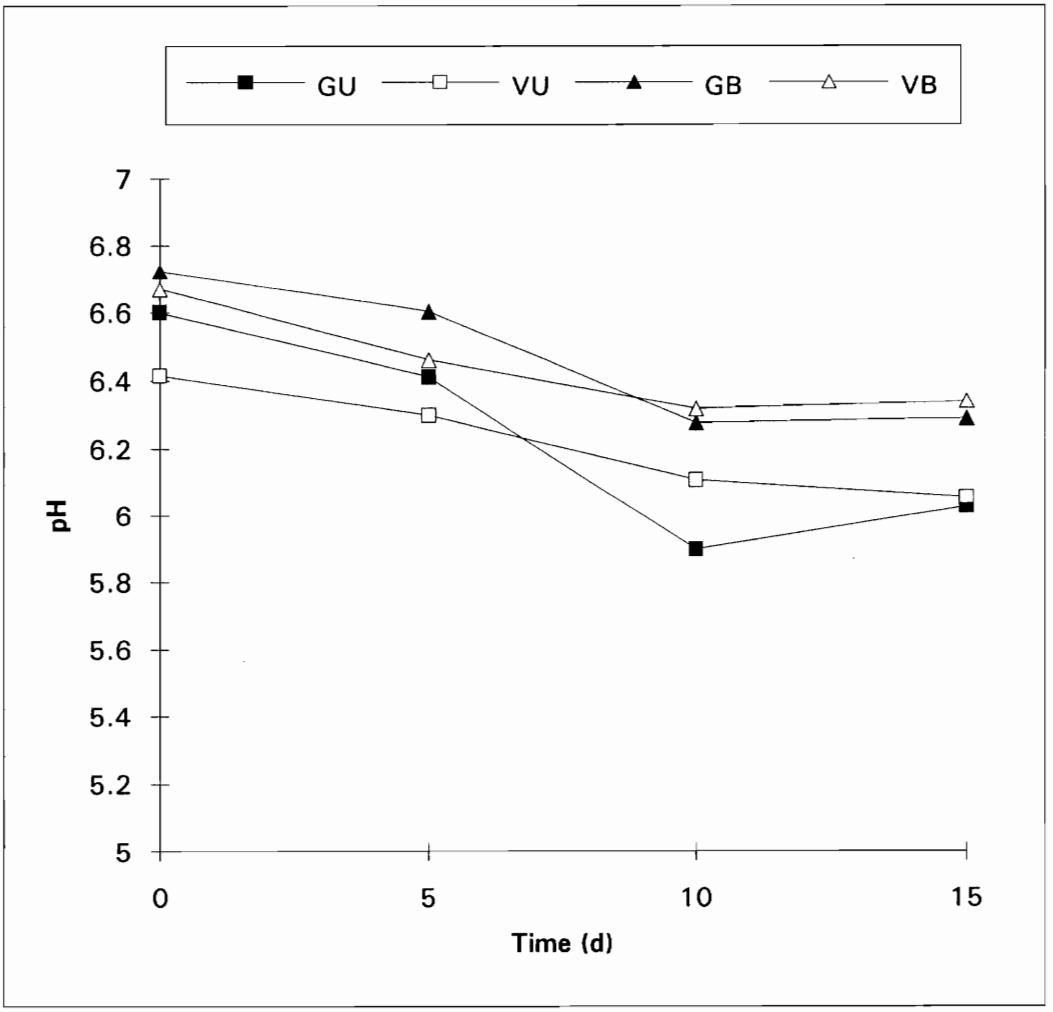

Figure 2. pH of unblown (U) and blown (B) oysters harvested from the Gulf coast (G) and Virginia (V) stored at 1 C.

Table 4. Percent salt of unblown and blown oysters harvested from Virginia and the Gulf coast.

		% Salt					
		Unblown			Blown		
Harvest Location	Rep.	Trial 1	Trial 2	Avg.[a]	Trial 1	Trial 2	Avg.
Gulf	1	0.28	0.23	0.26	0.11	0.10	0.11
	2	0.62	0.59	0.61	0.15	0.17	0.16
	3	0.41	0.39	0.40	0.12	0.14	0.13
	4	0.40	0.36	0.38	0.24	0.22	0.23
Virginia	1	0.20	0.18	0.19	0.18	0.15	0.17
	2	0.54	0.53	0.54	0.19	0.17	0.18
	3	0.24	0.16	0.20	0.11	0.12	0.12

[a] Average calculated from two repetitions.

Table 5. Log MPN/g values for total Vibrio vulnificus counts in oysters harvested from the Gulf coast and from Virginia in oysters stored at -9°C.

Harvest Location	Rep.	Week[a]	Unblown 0	1	2	3	4[d]	Blown 0	1	2	3	4
Gulf	1		0.71	0.78	0.66	0.88	-0.29	0.58	<0.48[b]	<0.48[b]	0.48	0.01
	2		1.94	<-0.52[b]	---[c]	0.34	<-0.52[b]	1.30	<-0.52[b]	---	<-0.52[b]	<-0.52[b]
	3		0.48	<-0.52[b]	0.40	---	<-0.52[b]	0.48	<-0.52[b]	0.07	---	<-0.52[b]
	4		1.02	0.04	0.32	- 0.11	---	2.20	0.63	0.26	0.29	---
Virginia	1		0.66	<-0.52[b]	<-0.52[b]	- 0.21	<-0.52[b]	1.12	<-0.52[b]	<-0.52[b]	-0.21	<-0.52[b]
	2		1.41	<-0.52[b]	<-0.52[b]	<-0.52[b]	<-0.52[b]	0.55	<-0.52[b]	<-0.52[b]	<-0.52[b]	<-0.52[b]
	3		0.66	- 0.07	0.41	---	---	0.66	0.16	0.11	---	---

[a] Week indicated testing on that number of weeks after processing. Week 0 represents testing on the day of processing.

[b] Below the limit of detection for this assay.

[c] No data collected.

[d] V. vulnificus was not detected after 11 weeks of storage for Gulf Rep. 1.

Table 6. Log MPN/g values for total Vibrio counts in oysters harvested from the Gulf coast and from Virginia in oysters stored at - 9°C.

Harvest Location	Rep.	Week[a]	Unblown 0	1	2	3	4	Blown 0	1	2	3	4
Gulf	1		>3.04	>3.04	>3.04	>3.04	>2.04	>3.04	>3.04	>3.04	>2.29	>2.04
	2		>3.04	2.35	---[b]	>2.04	>2.04	>3.04	1.48	---	>2.04	2.04
	3		>6.04	4.42	4.07	>4.04	>4.04	5.66	<3.48	3.50	3.85	>4.04
	4		5.66	2.52	>3.85	3.85	---	4.62	<2.48	2.11	2.80	---
Virginia	1		>3.04	2.04	2.04	1.17	0.52	>3.04	1.61	1.39	1.85	0.36
	2		>3.04	1.36	---	1.67	0.18	>3.04	<0.48[c]	---	>1.85	0.37
	3		5.34	3.52	3.28	---	---	4.30	2.31	2.71	---	---

[a] Day indicated testing on that number of weeks after processing. Week 0 represents testing on the day of processing.

[b] Data not collected.

[c] Below the limit of detection for this assay.

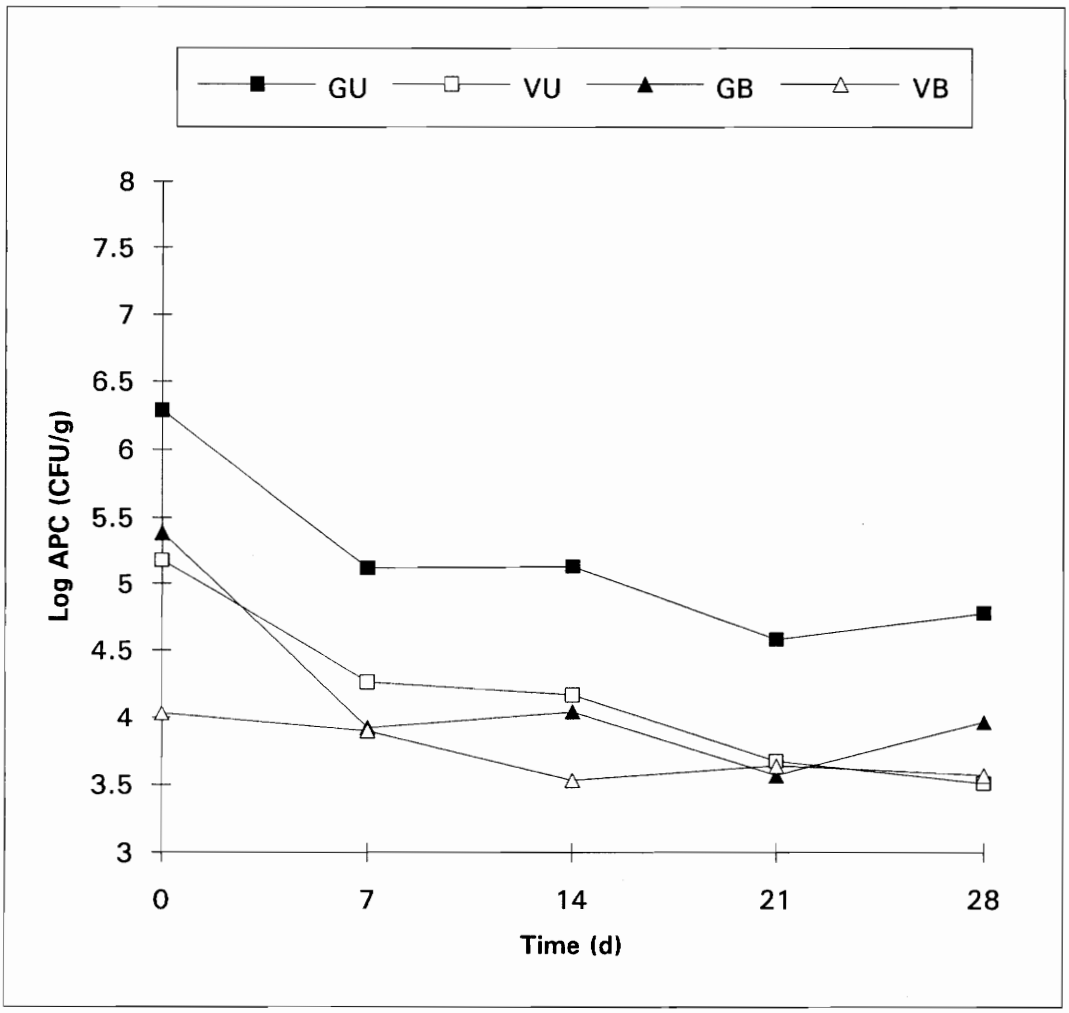

Figure 3. Log Aerobic Plate Counts (APCs) of unblown (U) and blown (B) oysters harvested from the Gulf coast (G) and Virginia (V) stored at -9 C.

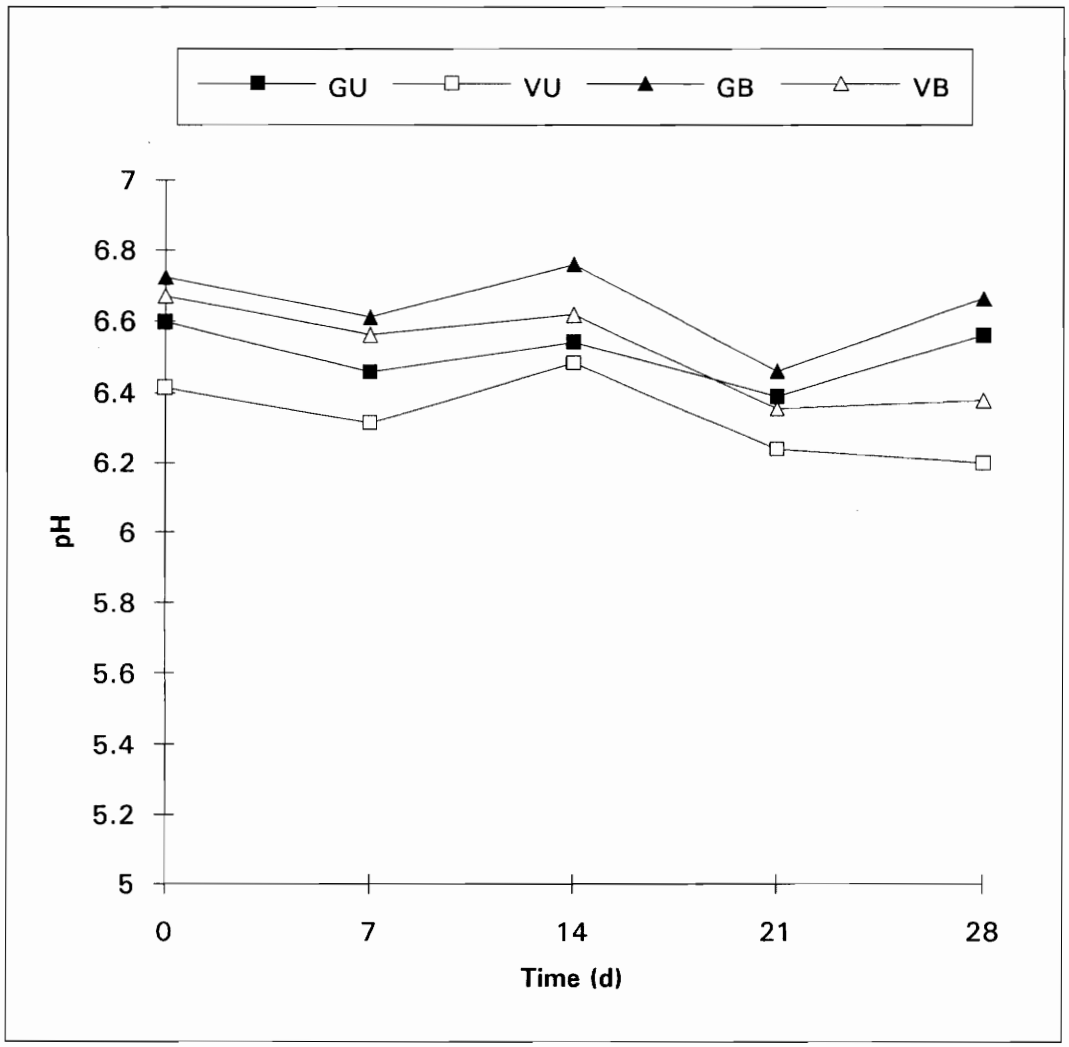

Figure 4. pH of unblown (U) and blown (B) oysters harvested from the Gulf coast (G) and Virginia (V) stored at -9 C.

Table 7. Correlation values for the rate of decrease of V. vulnificus with percent salt of blown and unblown oysters harvested from the Gulf coast and Virginia stored at 1°C and - 9°F.

		Unblown	p-value	Blown	p-value
1°C					
	Gulf	-0.63	>0.05	-0.97	<0.05
	VA	-0.70	>0.05	-0.20	>0.05
-9°C					
	Gulf	-0.92	>0.05	-0.97	<0.02
	VA	-0.99	<0.05	-0.51	>0.05

Table 8. Correlations of Aerobic Plate Counts and pH of blown and unblown oysters harvested from the Gulf coast and Virginia stored at 1°C and -9°C.

		Unblown	p-value	Blown	p-value
1°C					
	Gulf	-0.82	>0.05	-0.81	>0.05
	VA	-0.89	>0.05	-0.96	<0.05
-9°C					
	Gulf	0.67	>0.05	0.60	>0.05
	VA	0.71	>0.05	0.56	>0.05

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APPENDICES

A. Vibrio vulnificus data. All statistical analysis was conducted using the Most Probable Number values (MPN) obtained from the Food and Drug Administration's Bacteriological Analytical Manual table of MPN values. The log was taken of the MPN values. For MPN numbers of 0, 0, 0, a value of less than 3 was assigned. During calculations, all less than signs were dropped, and the numbers were calculated as actual values, ie, less than 3 was analyzed as 3.

Table 9. Slopes calculated from decreasing Vibrio vulnificus levels for unblown oysters stored at 1°C. These slope values were used in the correlation between salt levels and decreasing V. vulnificus levels.

Harvest Location	Rep.	Slope	R square value
Gulf	1	-0.015	0.06
	2	-0.098	0.86
	3	....[a]	.....
	4	-0.092	0.60
VA	1	-0.115	0.88
	2	-0.129	0.88
	3	-0.081	0.89

[a] No V. vulnificus was detected.

Table 10. Slopes calculated from decreasing Vibrio vulnificus levels for blown oysters stored at 1°C. These slope values were used in the correlation between salt levels and decreasing V. vulnificus levels.

Harvest Location	Rep.	Slope	R square value
Gulf	1	-0.014	0.13
	2	-0.068	0.75
	3	-0.008	0.07
	4	-0.163	0.60
VA	1	-0.134	0.77
	2	-0.065	0.66
	3	-0.077	0.79

Table 11. Slopes calculated from decreasing Vibrio vulnificus levels for unblown oysters stored at -9°C. These slope values were used in the correlation between salt levels and decreasing V. vulnificus levels.

Harvest Location	Rep.	Slope	R square value
Gulf	1	-0.020	0.32
	2	-0.057	0.36
	3	-0.043	0.79
	4	-0.092	0.65
VA	1	-0.018	0.40
	2	-0.055	0.50
	3	-0.018	0.12

Table 12. Slopes calculated from decreasing Vibrio vulnificus levels for blown oysters stored at -9°C. These slope values were used in the correlation between salt levels and decreasing V. vulnificus levels.

Harvest Location	Rep.	Slope	R square value
Gulf	1	-0.043	0.42
	2	-0.051	0.50
	3	-0.042	0.89
	4	-0.087	0.74
VA	1	-0.032	0.70
	2	-0.077	0.60
	3	-0.039	0.81

B. Total Vibrios data. All data was analyzed by dropping the greater than or less than signs. (Example: values of >110 were analyzed as 110).

C. Aerobic Plate Count Data.

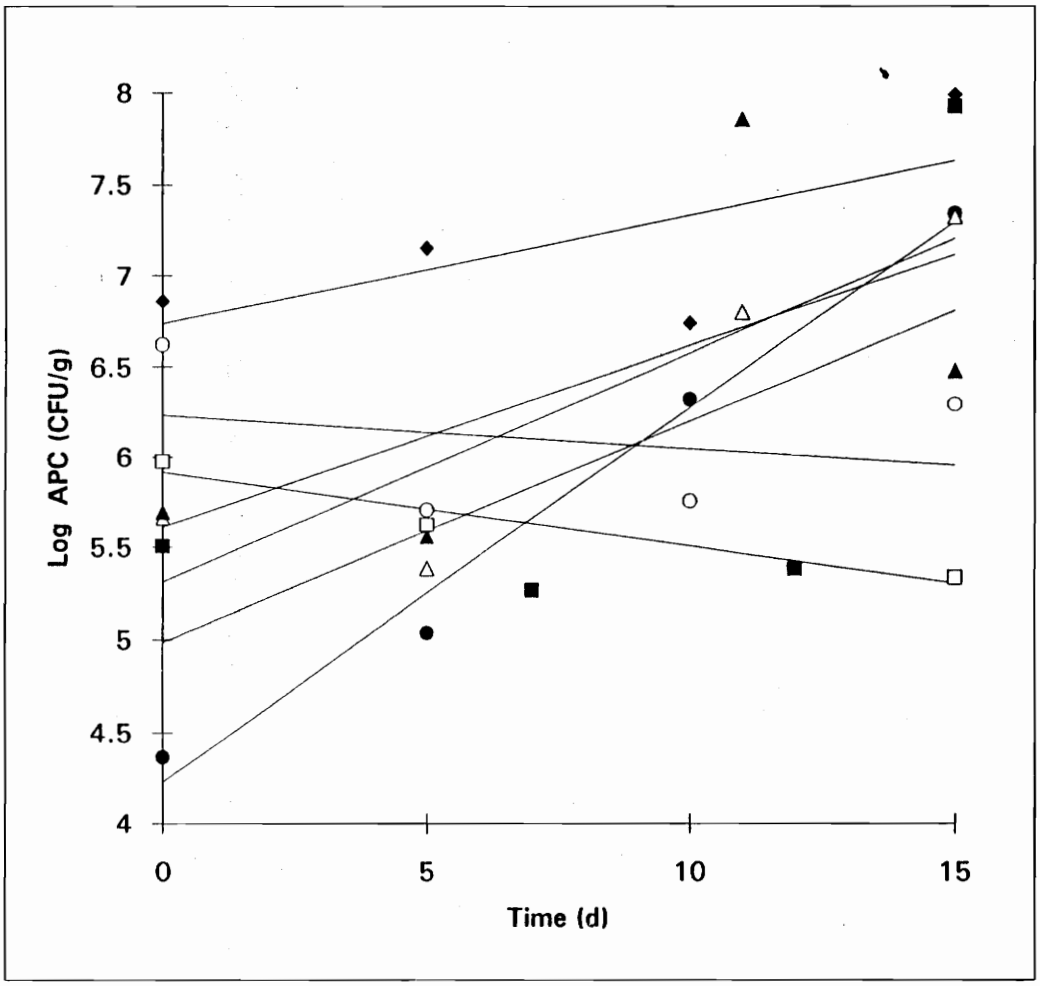

Figure 5. Log Aerobic Plate Counts for all repititions, before taking averages for unblown oysters harvested from Virginia and the Gulf coast and stored at 1 C.

Gulf Rep. 1 — VA Rep. 1 — VA Rep. 2 — Gulf Rep. 2

Gulf Rep. 3 — Gulf Rep. 4 — VA Rep. 3

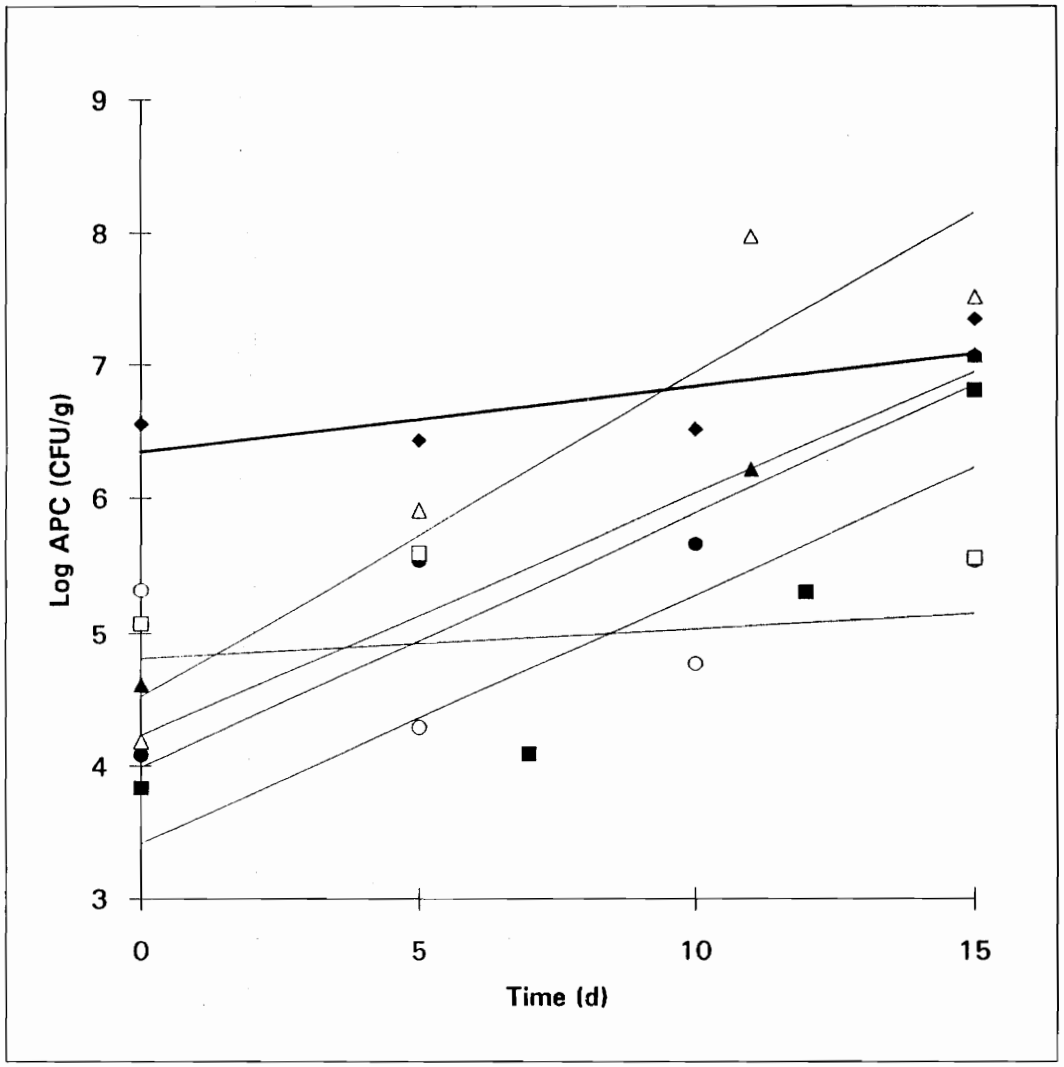

Figure 6. Log Aerobic Plate Counts for all repititions, before taking averages for blown oysters harvested from Virginia and the Gulf coast and stored at 1 C.

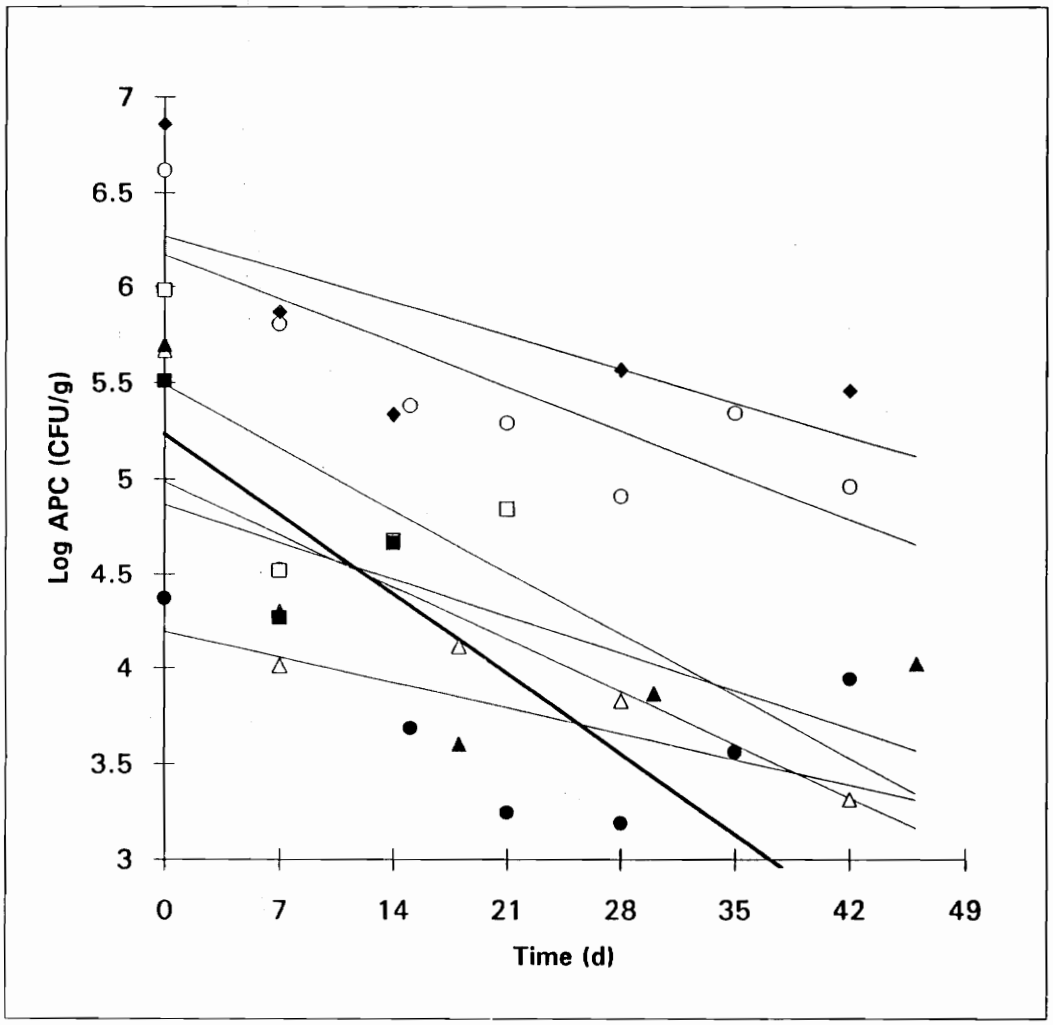

Figure 7. Log Aerobic Plate Counts of all repititions before all averages of unblown oysters harvested from Virginia and the Gulf coast and stored at -9 C.

Gulf Rep. 1 — VA Rep. 1 — VA Rep. 2 — Gulf Rep. 2

Gulf Rep. 3 — Gulf Rep. 4 — VA Rep. 3

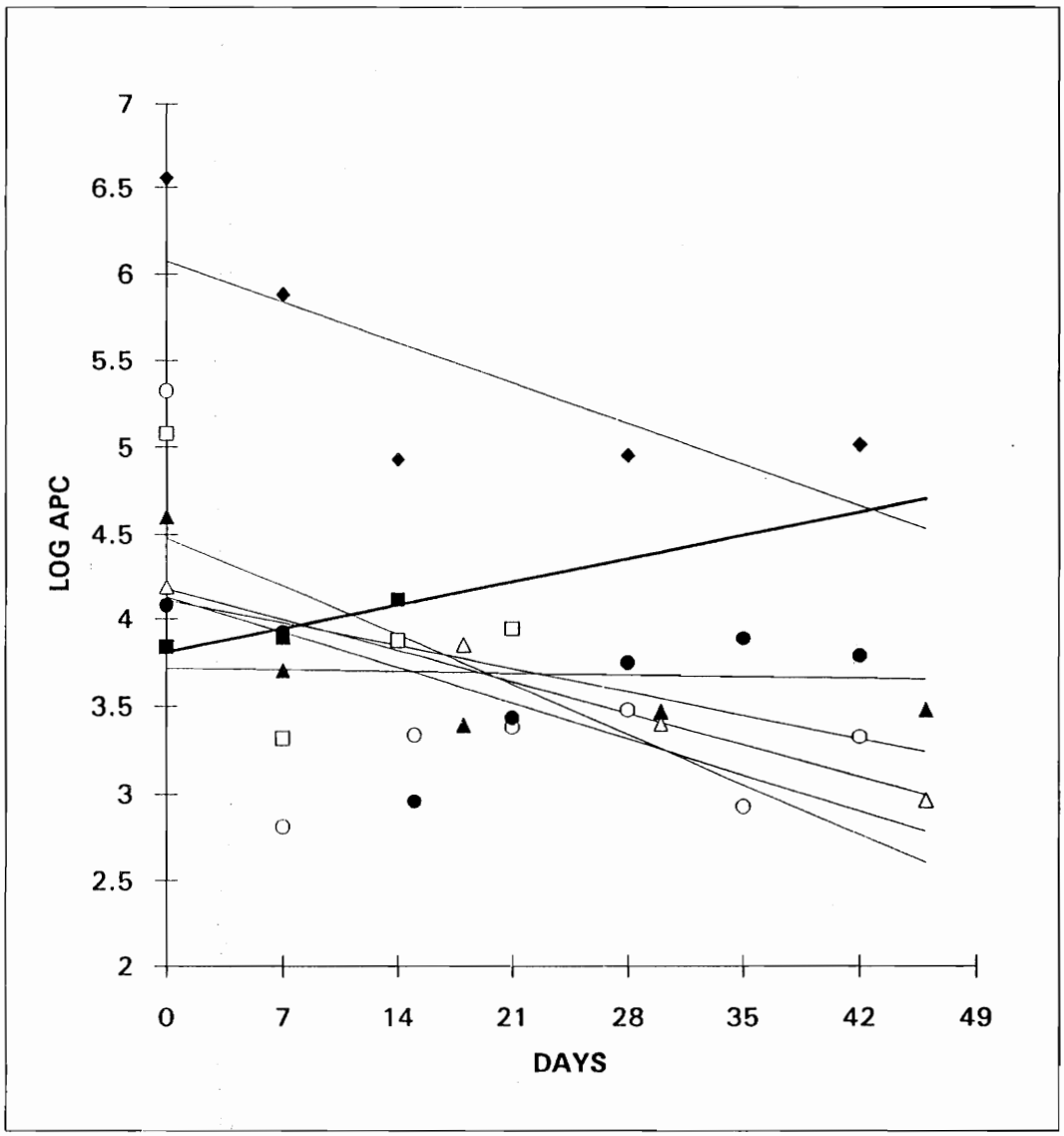

Figure 8. Log Aerobic Plate Counts of all repititions before all averages of blown oysters harvested from Virginia and the Gulf coast and stored at -9 C.

Gulf Rep. 1 — VA Rep. 1 — VA Rep. 2 — Gulf Rep. 2

Gulf Rep. 3 — Gulf Rep. 4 — VA Rep. 3

D. pH Data.

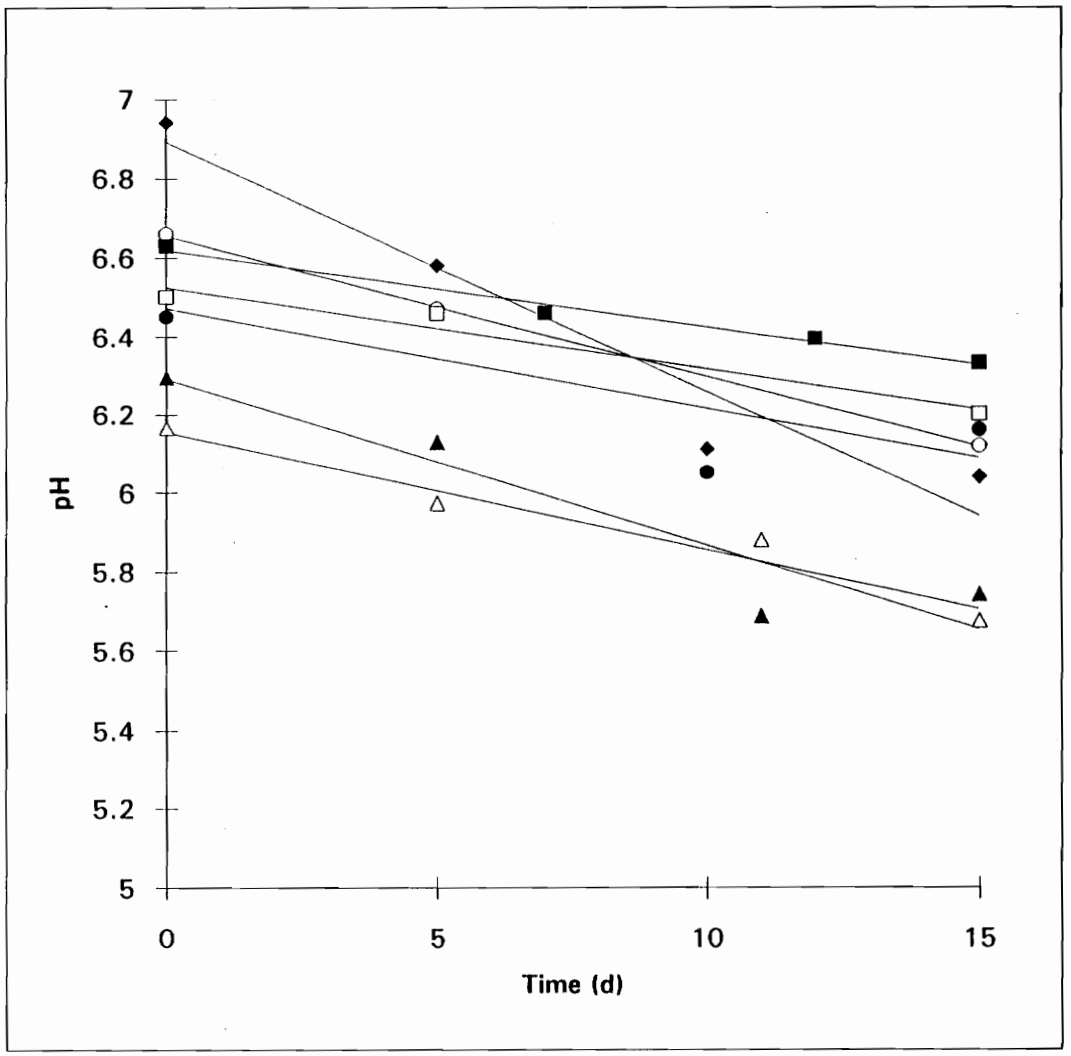

Figure 9. pH values for all repititions before taking averages for unblown oysters harvested from Virginia and the Gulf coast and stored at 1 C.

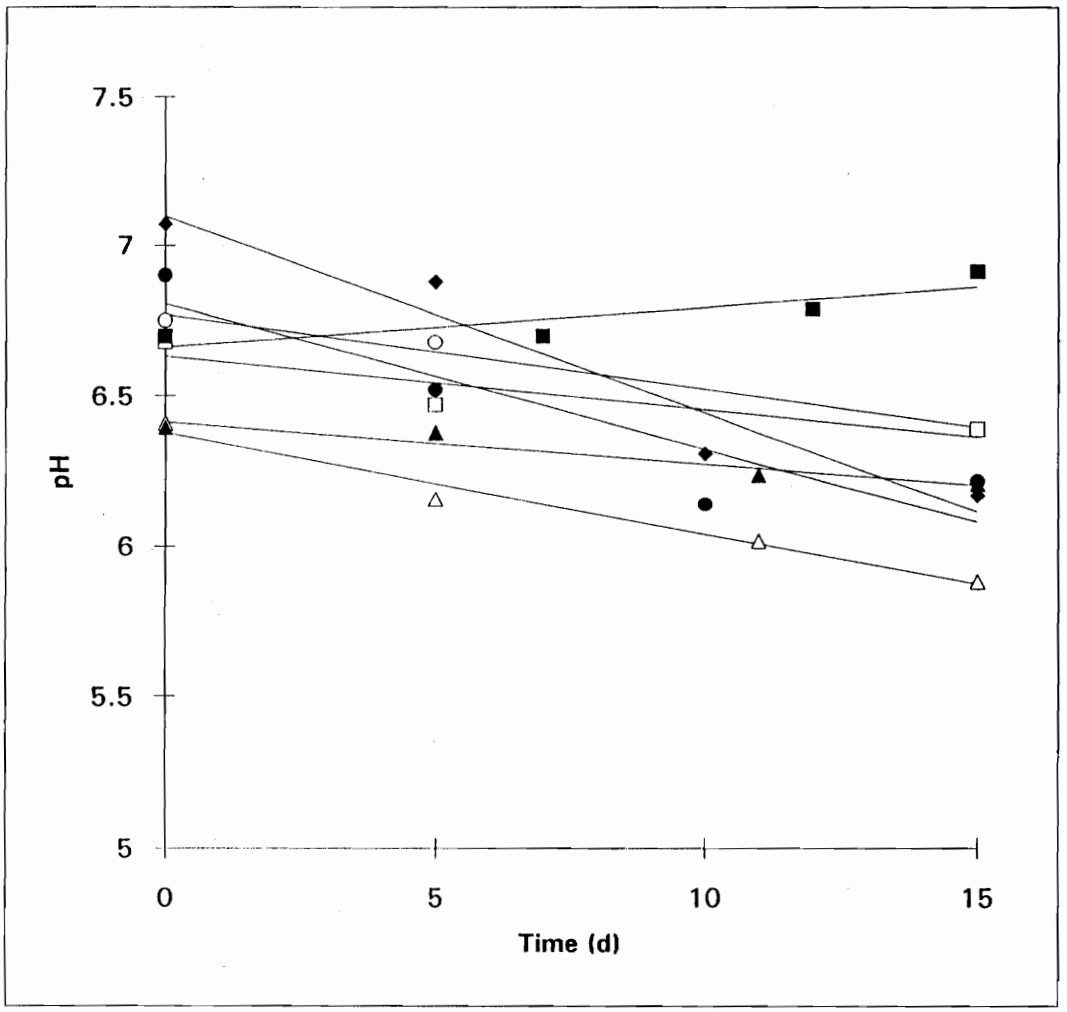

Figure 10. pH values for all repititions before taking averages for blown oysters harvested from Virginia and the Gulf coast and stored at 1 C.

Gulf Rep. 1
VA Rep. 1
VA Rep. 2
Gulf Rep. 2
Gulf Rep. 3
Gulf Rep. 4
VA Rep. 3

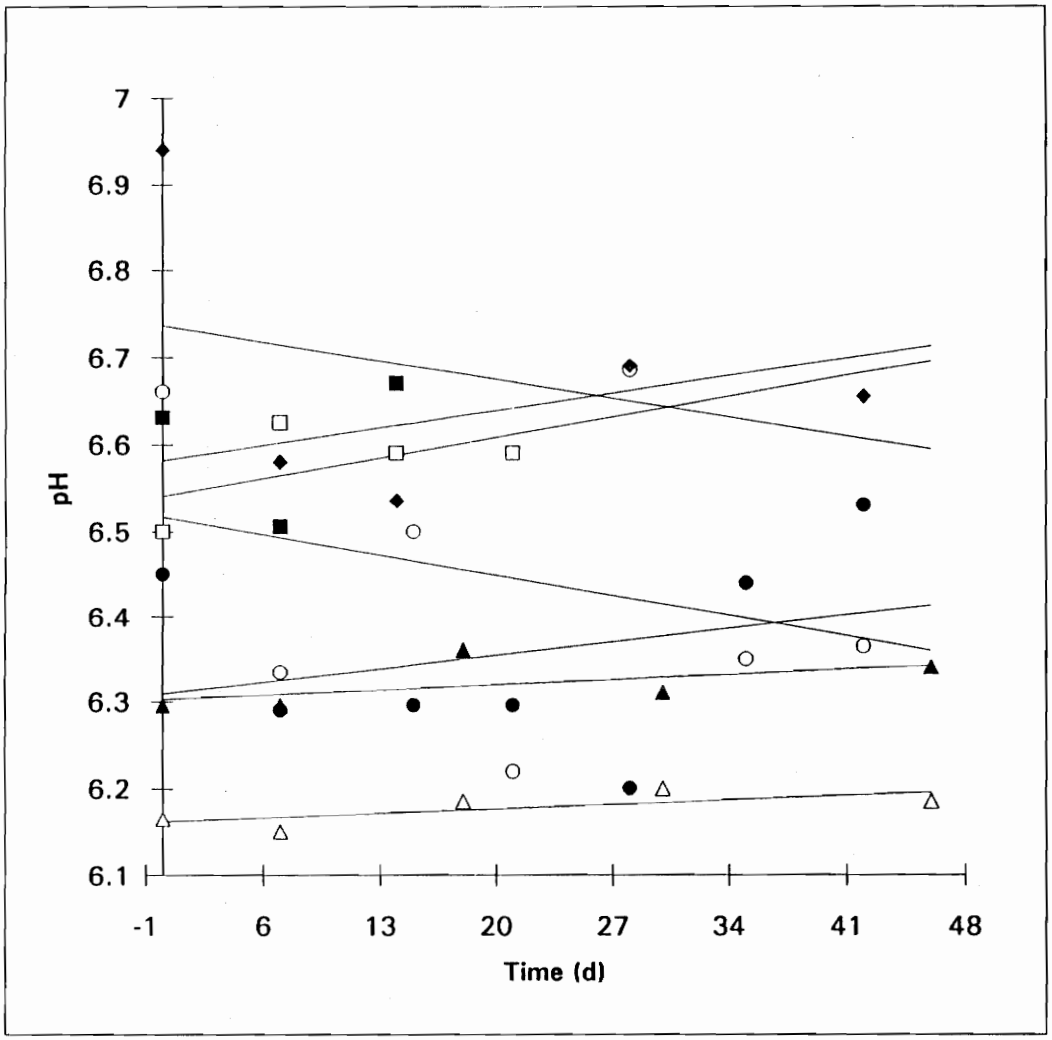

Figure 11. pH for all repititions before taking averages for unblown oysters harvested from Virginia and the Gulf coast and stored at - 9 C.

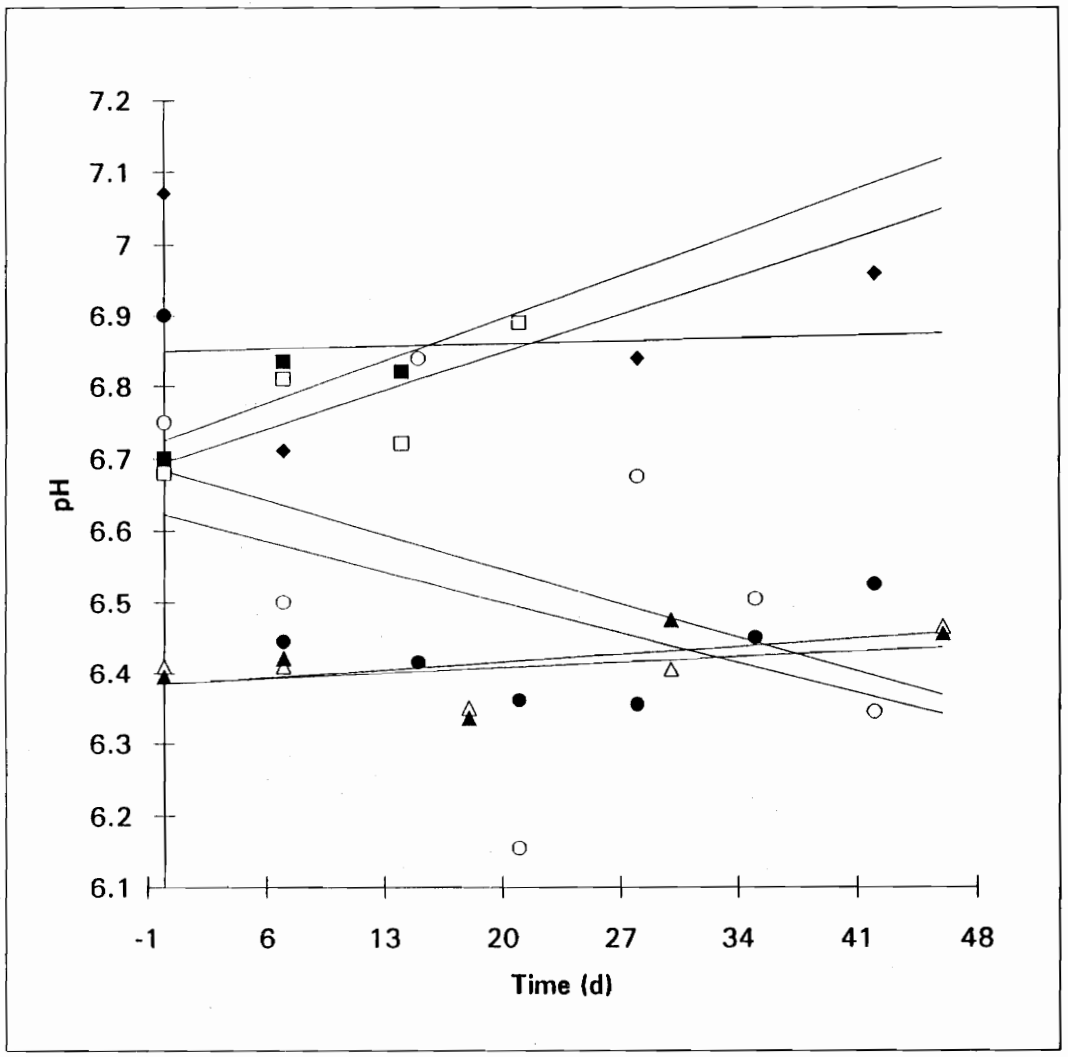

Figure 12. pH for all repititions before taking averages for blown oysters harvested from Virginia and the Gulf coast and stored at - 9 C.

Gulf Rep. 1 — VA Rep. 1 — VA Rep. 2 — Gulf Rep. 2

Gulf Rep. 3 — Gulf Rep. 4 — VA Rep. 3

E. Future Research

Some areas of possible interest in future research would be to look at the affect of change in salinity of raw oysters with the affect on Vibrios and Vibrio vulnificus populations. It would be of possible interest to identify the maximum or most effective change in salinity that produced the most notable rate of decline of V. vulnificus populations.

Also, it may be of interest to determine if raising the salinities of oysters before shucking and then subjecting them to longer blowing and washing times was effective in reduction of V. vulnificus populations.

VITAE

Vicki Ostrander was born November 2, 1972 in Montgomery County, Virginia. She grew up in Narrows, Virginia and graduated from Narrows High School as valedictorian in 1990. Vicki received her Bachelor of Science in Biology from Virginia Polytechnic Institute and State University in May of 1994.

Vicki decided to further her education, focusing her studies in Food Science and Technology after taking a Food Microbiology class offered at Virginia Polytechnic Institute and State University. She began her Masters program in January 1995.

Vicki was a member of the Institute of Food Technologists while attending Virginia Polytechnic Institute and State University. She presented her research at the 1996 Annual Institute of Food Technologists meeting and Food Expo in New Orleans, Louisiana.