Evaluation of a Flow-Through Depuration System to Eliminate the Human Pathogen *Vibrio vulnificus* from the Eastern Oyster (*Crassostrea virginica*)

by

Matthew Robert Lewis

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
August 9th, 2010

Keywords: *Vibrio vulnificus*, eastern oyster (*Crassostrea virginica*), depuration

Approved by

Cova Arias, Chair, Associate Professor of Fisheries
Yolanda Brady, Associate Professor of Fisheries
Bill Walton, Assistant Professor of Fisheries
Jim Stoeckel, Assistant Professor of Fisheries

Abstract

Evaluation of a flow-through depuration system to eliminate the human pathogen *Vibrio vulnificus* was carried out through altering 3 different parameters: salinity, temperature, and flow rate. Oysters were supplied by Auburn University Shellfish Laboratory (AUSL) and were from hatchery-reared stocks. Determination of *V. vulnificus* numbers in oyster tissues was conducted at 0, 1, 2, 3 and 6 days of depuration.

Results showed that the numbers of *V. vulnificus* were significantly reduced by Day six in seven out of 14 trials. Also, only two out of 14 trials showed a significant difference between the test and control depuration systems. Laboratory-inoculated oysters were depurated from >100,000 CFU/g of *V. vulnificus* in oyster tissue down to 50 CFU/g in six days. Altered temperature and salinity parameters alone and together had very little success with reducing the numbers of *V. vulnificus* in oyster tissues. Flow rate increased to 68 L/m from 11 L/m resulted in a significant reduction of *V. vulnificus* in oysters in six days with an ending concentration of three CFU/g from a starting concentration of 110,000 CFU/g.

Genotyping of the pre- and post-depuration isolates revealed no selection or resistance of by depuration of type A or B *V. vulnificus*. However, Amplified Fragment Length Polymorphism (AFLP®) analysis did show that depuration does select some strains over others, reducing natural heterogeneity.

Acknowledgments

I would like to thank my advisor, Dr. Cova Arias for allowing me the opportunity to join her lab and work on a project that was very enjoyable. I would also like to thank my committee members, Dr. Yolanda Brady, Dr. Jim Stoeckel, and Dr. Bill Walton for their time and expertise. I would like to give a special thanks to Auburn University Shellfish lab, specifically Scott Rikard and Glen Chaplin for their countless hours of help with the depuration system, all of the advice relating to my project, and maintaining the oysters used for my research.

I would also like to thank my lab members, Dr. Oscar Olivares-Fuster, Ryan Wood, Zhen Tao, Andrea Larsen, and Stacy LaFrentz for their technical support and advice. Lastly, I would like to thank the Alabama Agricultural Experiment Station (AAES) for funding.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AFLP	Amplified Fragment-Length Polymorphism
AI-2	Autoinducer-2
APW	Alkaline Peptone Water
BAM	Bacteriological Analytical Manual
BSA	Bovine Serum Albumin
cAMP	cyclic Adenosine Monophosphate
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
CPS	Capsular Polysaccharide
DNA	Deoxyribonucleic Acid
FDA	Food and Drug Administration
HCP	Heat/Cool Pasteurization
HHP	High Hydrostatic Pressure
IQF	Individually Quick Frozen
LD_{50}	Lethal Dose (50%)
LPS	Lipopolysaccharide
MA	Marine Agar
MB	Marine Broth
mCPC	modified Cellobiose-Colistin-Polymyxin B Agar

MPN Most-Probable Number

NBT/BCIP Nitro Blue Tetrazolium Chloride/ 5-Bromo-4-chloro-3-indolyl Phosphate

PCR Polymerase Chain Reaction

RFLP Restriction Fragment-Length Polymorphisms

SDS Sodium Dodecyl-Sulphate

SSC Sodium Isocitrate

TCBS Thiosulfate Bile Salts Sucrose Agar

TNF-α Tissue Necrosis Factor-α

VVA *Vibrio vulnificus* Agar

I. LITERATURE REVIEW

Gulf Coast oyster industry

The eastern oyster (*Crassostrea virginica*) (Gmelin 1791) belongs to the phylum Mollusca, class Bivalvia, order Ostreoida and family Ostreidae. They are filter-feeding bivalves that are found in estuarine environments (55). The eastern oyster is a key organism in coastal marine habitats where it acts as an ecosystem engineer by creating reefs as a habitat for other marine organisms. These reefs sustain specific communities of invertebrates as well as juvenile fish that constitute the food web of a diverse, biologically productive habitat. From a recreational point of view, some fish species that inhabit oyster reef ecosystems are important to sport fishermen (31). The eastern oyster also affects the physical environment by filtering resuspended particles, chemical contaminants, and phytoplankton. This improves water quality for other organisms such as bay grasses. Under ideal conditions, a single oyster can filter up to 18 liters of seawater an hour (81). Historical densities of the eastern oyster in the Chesapeake Bay have been estimated to have had the capacity to filter the entire volume of the Bay in 3.3 days (23); current populations of these oysters are estimated at less than 1% of their historic high levels. This is mainly due to disease and lack of oyster shell for restoration (23).

The eastern oyster is also economically important to consumers and seafood processors and retailers, with an estimated 20 million Americans consuming raw oysters (84). In 2007, the U.S. oyster industry produced 16 million kilograms of oysters valued at over $126 million. The Gulf of Mexico eastern oyster accounts for 63% of the national total, with the non-native Pacific Coast oyster, *Crassostrea gigas,* being the second major oyster species harvested in the U.S.

(31). The state of Washington is the main producer of the Pacific oyster while the states bordering the Atlantic Ocean and the Gulf of Mexico produce mainly the Eastern oyster. However, there has been an overall decline in consumer demand due to safety issues associated with consumption of raw oysters from the Gulf Coast. Eastern oysters harvested from this region are likely to contain the human pathogen *Vibrio vulnificus* (99), especially during the warmer months. Mandatory warning labels and media attention due to the presence of this naturally occurring bacterium have negatively affected consumers demand for Gulf oysters, and therefore the price. These warning labels were put in place to educate the consumer about the potential risks involved when consuming raw oysters. However, it has been found to significantly influence the demand for raw Gulf oysters by reducing the summer price by 50% (52). Since 1991, Atlantic oyster shell prices have exceeded Gulf oyster prices by $4.86 per kilogram (52).

Vibrio vulnificus is an opportunistic bacterium indigenous to estuarine waters that naturally concentrates in the oyster as it filter-feeds (49).Of all the seafood-related deaths in the U.S., *V. vulnificus* is responsible for 95% (68). Consumption of raw oysters containing *V. vulnificus* can result in primary septicemia, with the average mortality rate from these infections exceeding 50% (49). There are several methods currently approved by the FDA for treating raw oysters to eliminate *V. vulnificus*, but all of them have been suggested to affect the organoleptic properties of the oysters (102). In October 2009, the U.S. Food and Drug Administration (FDA) announced that they would ban live oyster sales in the Gulf region during the warmer months to eliminate deaths due to raw oyster consumption containing *V. vulnificus*. This plan, which would take effect in 2011, required all oysters from the Gulf region harvested between May and October to be processed post-harvest (103). In 1994, the FDA had tried a similar approach, but was met with strong opposition from the industry and members of Congress from Gulf States.

Instead, the FDA opted for a “public awareness campaign” to educate those at risk (52). However, this campaign has not yielded the intended results and fatalities linked to the consumption of Gulf oysters have not declined.

The potential economic effects of recent proposed ban alarmed many within the Gulf oyster industry. Avery Bates, vice president of the Organized Seafood Association-Alabama, predicted two-thirds of Alabama's approximately 50 "mom-and-pop” oyster shops would close, due to the costs associated with processing the oysters (17). It is currently unknown by the industry to what extent the consumer will pay higher prices for a taste and texture-altered oyster, or even accept these processed products. Also, it is unknown how many of the small businesses would be able to acquire the capital to invest in the equipment required to process the oysters according to the FDA standards (73). Just several weeks after the FDA announced the proposed ban on oysters for 2011, they withdrew their plan, again under heavy pressure from the oyster industry (32). They announced that they would conduct an independent study to investigate further the post-harvest processing that could be implemented in the fastest and safest way to protect the Gulf Coast economy related to oyster production, while assuring public health. One of their objectives during this period is to validate different post-harvest processing methods that can be implemented to reduce *V. vulnificus* to non-detectable levels while preserving the product’s original taste and texture (32). Therefore, there is an immediate need for a method that would eliminate *V. vulnificus* while keeping the oyster alive, preserving the taste and texture of raw oysters expected by consumers.

Vibrio vulnificus

*Vibrio vulnificus*is an opportunistic human pathogen ubiquitous in estuarine and marine habitats. It is a motile, Gram-negative, curved bacillus with a single polar flagellum. The size

ranges of *V. vulnificus* are 0.5 to 0.8 μm in width and 1.4 to 1.6 μm in length. It was first isolated by the US Centers for Disease Control (CDC) in 1964, but was mistakenly identified as a virulent strain of *V. parahaemolyticus*. In 1976, it had clinical presentations unique from other *Vibrio* species prompting it to be recognized by Hollis and colleagues as a disparate species (44). *Vibrio vulnificus* is facultative anaerobic, and is distinguished from other *Vibrio* spp. by fermentation of lactose and production of β-D-galactosidase (44).

Ecology. *Vibrio vulnificus* is an indigenous member of brackish and marine water microflora. It is free-living and its presence is not associated with pollution or other forms of contamination. In the US it is mainly found along the Gulf Coast where temperatures are subtropical and optimum for its survival. However it has been found as far north as the Great Bay of Maine and Pacific Coast waters where water temperatures are lower year-round relative to the Gulf Coast (51, 76). Its location is not limited to the US, but has also been found in Asia and Europe (43, 64). It has been isolated in waters ranging from 9 to 31°C, but flourishes when water temperature exceeds 18°C (50). This has been corroborated by several depuration trials that showed a temperature of 15ºC greatly reduced the growth of *V. vulnificus* in oysters (21). Lower salinities also encourage the proliferation of *V. vulnificus*. Though known to occur in waters with 1 to 34 parts per thousand (ppt) salt, this species favors waters with 5 to 25 ppt. Salinities greater than 25 ppt have a negative impact on the survival of the bacterium (91).

The optimal conditions for *in vitro* growth of *V. vulnificus* are between 0.5 and 2.0% NaCl, with an optimal temperature of 37ºC. Due to the correlation of these findings with seasonal incidence of *V. vulnificus* in the environment, it has been hypothesized that this bacterium could overwinter in sediments and then re-enter the water column when the water warms again (53). Another *in vitro* study found that survival of the bacterium was optimal in

sterile seawater with a temperature between 13°C and 22°C and a salinity of 10 ppt. Over a 6-day period, the most rapid declines in *V. vulnificus* numbers occurred at 5°C and 35ºC in 10 ppt sterile seawater. When seawater salinity was raised to 30, 35, and 38 ppt, the numbers of *V. vulnificus* decreased by 58, 88, and 83% respectively. Over a broad range of salinities, a group incubated at 14°C had a greater survival rate than a group incubated at 21°C. This finding indicates that temperature may influence the tolerance levels of *V. vulnificus* to salinities that are not optimal for its survival (50).

Temperature and salinity are interrelated and both affect the prevalence of *V. vulnificus* in waters (29). In one study conducted at 5 sites along the Atlantic and Gulf coasts (69), *V. vulnificus* levels at sites along the Gulf coast (where salinities averaged 16 ppt)were positively correlated with water temperature up to 26°C; beyond this temperature, *V. vulnificus* abundance remained constant. The three Gulf sites had median *V. vulnificus* values of 10^3 CFU/g. At the Atlantic coast sites (which had very similar temperatures to the Gulf coast sites but average salinities >26 ppt), *V. vulnificus* were <10 CFU/g in 86% of the samples from these sites. Similarly, at one high salinity Gulf coast site (along the Florida Gulf coast), where salinity was >25 ppt, *V. vulnificus* numbers were low relative to other Gulf sites. Also, in one of the Atlantic sites, in North Carolina, a flooding event temporarily reduced the salinity to levels similar to Gulf sites and correlated to the highest observed levels of *V. vulnificus* in any of the Atlantic sites. Several studies have already shown that salinities higher than 25 ppt do have negative impacts on the levels of *V. vulnificus* in oysters (50, 68, 69). At one of the Atlantic sites, in North Carolina, a flooding event reduced the salinity to levels similar in Gulf sites and correlated to the highest levels of *V. vulnificus* in the Atlantic sites. Therefore, the main difference between *V.*

vulnificus quantities in oysters between the Atlantic Coast and Gulf Coast seems to be more related to salinity rather than temperature (69).

Vibrio vulnificus is not only found in oysters, but has been found in plankton, clams, marine sediment, seawater and marine fish intestines (5, 28, 96, 104). The relationship between zooplankton and *Vibrio* spp. may explain the year-round prevalence of vibrios. The correlation between *Vibrio*s pp. found significantly in the microflora of zooplankton with chitin exoskeletons. The fact that *V. vulnificus* secretes a chitinase may further prove the extent of their association (40, 47, 91).

Pathogenesis. Illness due to *V. vulnificus* caused by consumption of raw oysters can develop rapidly, within as little as 7 h (77). The fact that this infection progresses so rapidly indicates that *V. vulnificus* is able to navigate around host defense mechanisms. Since the most common form of disease originates from the consumption of raw oysters, the very acidic gastric environment is the first host defense that it must evade. *Vibrio vulnificus* does have a *cadBA* operon encoding for a lysine decarboxylase that is up-regulated during acid exposure. This enzyme breaks down lysine to form cadaverine which neutralizes acids (83). This would aid in neutralizing the strongly acidic gastric environment to allow for better survival of *V. vulnificus*.

The first stage of any bacterial infection is usually attachment and colonization. This process typically requires a type of short pilus, or fimbria that are proteins on the cell surface of many bacteria used to initiate attachment (91). *Vibrio vulnificus* does have a type IV pilin that has been shown to contribute to biofilm formation, adherence to epithelial cells, and play a role in virulence. The *pilA* gene is part of an operon that encodes for other pilus biogenesis genes. In a study by Strom and Paranjpye (2005), the authors compared a *pilA* mutant to wild type strains and proved that the mutant cells had reduced adherence to HEp-2 human epithelial cells as well

as reduced biofilm formation on borosilicate glass. They then injected *pilA* mutants into mice and observed a one log higher LD_{50} in the *pilA* mutant compared to the wild type. When they complemented the *pilA* mutant, virulence was completely restored indicating that the protein product of *pilA* is required for virulence and plays a role in attachment to human epithelial cells (80).

Upon attaching to human epithelial cells, *V. vulnificus* invades the bloodstream by secreting a RTX pore-forming toxin that damages the host intestinal epithelial cells. *In vitro* assays of these toxins showed damaged cell membranes resulting in osmotic swelling and eventually cell lysis (59). This RtxA1 toxin has been shown to induce cytoskeletal rearrangement, plasma membrane blebs and hemolytic activity leading to necrosis and cell death. The RtxA1 has also been found to cause mitochondrial membrane depolarization and capsase-3-dependent apoptotic cell death in human intestinal epithelial cells. Therefore, the bacterium can use this virulence factor to cross the gastric mucosal barrier and enter the bloodstream (59).

Once there, *V. vulnificus* primarily encounters the complement system, an innate immune response. Research has shown that if the classical complement cascade is blocked, the ability of the serum to kill the bacteria is eliminated. Also, opsonization due to complement activation is crucial to the phagocytosis of *V. vulnificus* by neutrophils. The phagocytosis by neutrophils is an important step in the host defense because it causes a secretion cytokines, which stimulate other leukocytes to the infection site. However, these cytokines are also implemented as a cause of septic shock that occurs in these infections due to an overwhelming response from interleukin 6 (IL-6), IL-8, and tumor necrosis factor alpha (TNF-α) (70). These pro-inflammatory cytokines are greatly expressed during infections and have all been found in high levels in blood of patients infected with *V. vulnificus* (87).

One of the most studied *V. vulnificus* virulence factors has been the extracellular 56-kDa hemolysin encoded by the 3.4 kb fragment designated gene *vvhA* (8). This heat-labile enzyme lyses erythrocytes of mammals and is cytotoxic to certain cell lines. The hemolysin causes pore formation in the cellular membrane of host cells that leads to vascular permeability and hypotension.

Microorganisms need iron to grow, but free iron is rarely found in the human body. To overcome this obstacle, bacteria have evolved siderophores. These iron-chelating compounds take iron from iron transport proteins, such as transferrin and lactoferrin, and deliver them to the bacterium (91). This mode of iron acquisition is extremely important in the pathogenesis of *V. vulnificus* due to a correlation between high iron levels in the serum and risk of infection. *Vibrio vulnificus* produces two siderophores, hydroxymate and phenolate, that when mutated show a reduction in virulence (62). The rapid shift from high to low iron in the environment may also trigger the bacterium to realize it is in a mammalian host. *Vibrio vulnificus* also has a heme receptor, HupA, that functions to uptake iron that is not bound to transferring. This may indicate the importance of HupA in initial growth in a host (49).

Vibrio vulnificus does have a LuxS/LuxR quorum-sensing system that is thought to regulate its virulence. Quorum sensing allows bacteria to communicate with each other and regulate gene expression regarding cell density by secreting autoinducer-2 (AI-2) molecules. This system was first discovered in *V. harveyi* where it plays a role in bioluminescence regulation. When *luxS* and *luxR* in *V. vulnificus* were mutated, there was a decline in cytotoxicity and an increase in the LD_{50}, due to an absence of AI-2 molecules being produced. This indicates that the LuxS quorum sensing system is involved in coordinate virulence expression regulation.

To further illustrate this, *luxS* has also been shown to influence the transcription of the hemolysin, *vvhA*, causing an increase or decrease in the levels of the toxin (49, 58).

Intraspecies Diversity. Currently, *V. vulnificus* is divided into three biotypes classified by different phenotypic and host range characteristics. Biotype 1 strains are normally associated with the colonization of shellfish and therefore consist of most clinical isolates responsible for human disease (29) . They are characterized by indol and ornithine decarboxylase production, and have several serotypes (10, 91). Biotype 2 was described by Biosca *et. al* (1996) to be primarily obligate eel pathogens, but capable of being an opportunistic human pathogen. They can be differentiated from Biotype 1 in that they are negative for indol and ornithine decarboxylase production, and have only one common LPS type (also referred to as serotype E) (10, 91). Biotype 3 was recently discovered in an outbreak in Israel that involved sixty-two individuals with wound infections or septicemia. Further analysis showed that biotype 3 is a hybrid of biotypes 1 and 2. Although biotype 3 has caused human infections, it is currently limited to Israel and individuals who handle tilapia (11, 49).

Since classification at the species level, there have been numerous efforts to subtype *V. vulnificus* strains using genotypic markers, such as cytolysin genes, to suggest which strains may be more likely to cause disease in humans (38, 88). Warner and Oliver (1999) used random amplified polymorphic DNA PCR to identify a 178- to 200 bp segment of DNA that was present primarily in clinical isolates and might be used as a discriminatory method to distinguish human pathogenic strains of *V. vulnificus* from environmental ones (101). However most of these were not adequate methods as there can be over 100 different strains of *V. vulnificus* in individual oysters indicating a very broad genetic diversity among this organism (15). Unfortunately, correlating *V. vulnificus* typed strains with virulence was still very difficult.

All three biotypes can further be divided by a polymorphism present in the16S rRNA gene (7). Nilsson and colleagues (2003) showed that *V. vulnificus* 16S rDNA types (type A or B) could be correlated to virulence. Up to 93% of environmental islolates are type A, while 90% of isolates recovered from clinical cases are type B (74). Ascription to type A or B is carried out by *in vitro* amplification of a 492 bp segment of the 16S rDNA followed by restriction with the endonuclease *HaeIII*. Restricted fragments are resolved by standard gel electrophoresis with results obtained in one day (74).

Epidemiology. The fatality rate for septicemia infections is over 50%, the highest fatality rate of any food-borne pathogen (49, 63, 91). One of the biggest factors in the severity of the disease is host susceptibility. Individuals with a chronic disease affecting the liver or immune function such as cirrhosis, hepatitis, diabetes, cancer and AIDS are the most at risk (91, 92). Susceptibility factors in patients contracting primary septicemia are liver disease and age (over 50 years old) (91). Some evidence suggests that men may be more susceptible to infection than women, with estrogen possibly playing a protective role. Merkel and others (2001) showed that male rats injected with *V. vulnificus* lipopolysaccharide (85) had a fatality rate of 82% compared to only 21% in female rats. When the female rats had their ovaries removed, their fatality rate increased to 75%. When treated with estrogen, the female rats fatality rate decreased down to 38%. Likewise, gonadectomized male rats were treated with estrogen reducing their fatality rate from 82% down to 50%. A further increase in estrogen treatments correlated with lower fatality rates (67).

The CDC has reported an increase of almost 80% in all types of *V. vulnificus* infections from 1996 to 2006 with 121 cases confirmed in 2005 alone (21, 49). Although the mortality rate is high, the rate of infection is low due to healthy individuals having a lower risk of infection and

therefore rarely contracting the disease regardless of its ubiquitous distribution (49). Most cases occur between May and October when waters in the northern hemisphere are warmest. The sources for the majority of the infections due to oyster consumption are Black Bay, LA and Apalachicola Bay, FL. This is thought to be due to the large input of oysters from these areas into the national harvest numbers rather than virulent strains of *V. vulnificus* being concentrated there (69).

There are three major clinical presentations of *V. vulnificus* infections: gastroenteritis, wound infection and primary septicemia. Gastroenteritis is characterized by the isolation of *V. vulnificus* exclusively from stool. This type of infection is usually not life threatening and is therefore thought to be largely underreported. Of all the *Vibrio* spp., *V. parahaemolyticus* is the most common associated with gastroenteritis (29, 91). Symptoms of gastroenteritis are fever, diarrhea, abdominal cramps, nausea, and vomiting (91).

Wound infections are defined by the isolation of *V. vulnificus* from a wound or some other site that is typically sterile. The most common ways of contracting this type of infection are wound exposure to seawater, seafood drippings, or punctures by fins or bones. Naturally the majority of these infections occur in seafood processors and fishermen. In fact, 69% of these infections have shown to be occupational in nature among oyster shuckers and commercial fisherman. Symptoms are inflammation at the wound site that progresses to tissue necrosis. This type of infection often becomes septic resulting in fever, chills and hypotension and can lead to limb amputation and even death in more severe cases (91).

Primary septicemia is most often associated with the consumption of raw shellfish. It is characterized by the isolation of *V. vulnificus* from blood or an otherwise sterile site (29). Patients usually present with symptoms such as fever, chills and pain to the extremities. In as

little as 24 h, secondary lesions such as cellulitis, bullae or ecchymosis are observed on the cutaneous surface of the extremities. These lesions almost always become necrotic and require surgical amputation. After several days, the mental status of patients begins to sharply decline. Tetracycline is the most effective antibiotic used to treat these infections in combination with fluid replacement (91).

There have been reports of atypical infections. In 1990, the first unusual infection was reported as osteomyelitis that resulted from a leg being scraped on a rock in brackish water. It began as a wound infection, but rapidly progressed to a bone infection (97). There has also been a report of an ocular infection caused by *V. vulnificus* when a fisherman was struck in the eye with a fishhook. The infection was a mixed one that included *V. vulnificus*. A fatal case of meningoencephalitis caused by *V. vulnificus* was reported caused by the consumption of raw fish (29).

Isolation and Detection. There are several selective culture media that can be used to isolate *V. vulnificus*. The Cellobiose-Collistin-Polymyxin B (CPC) agar (66) and its modified version known as mCPC (93) were developed to specifically isolate *V. vulnificus* from environmental samples. These media are selective for *V. vulnificus* due to its resistance to polymyxin B and colistin, fermentation of cellobiose as well as the high incubation temperature of 40ºC limiting the growth of other marine bacteria. mCPC has a lower colistin concentration (from 1.4×10^6 U/l to 4×10^5 U/l) than the standard CPC. Colistin and polymyxin B are both peptide antibiotics that differ by a single amino acid, but both affect bacterial membrane permeability leading to cell lysis (42).

Another selective medium frequently used to recover *V. vulnificus* is thiosulphate citrate bile salts sucrose (TCBS) agar. This is a more general medium since it was designed to isolate

pathogenic vibrios. The alkaline pH, bile, and 1% NaCl inhibit background bacteria while encouraging and differentiating between *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae*. However, this medium is not as selective as mCPC for *V. vulnificus* isolation from environmental samples (42).

Direct detection of *V. vulnificus* can be carried out on *V. vulnificus* Agar (VVA) without enrichment. This agar does not contain antibiotics but does have cellobiose. This agar is intended mainly to be followed up with another confirmatory technique such as DNA hybridization (42).

The FDA Bacteriological Analytical Manual (BAM) offers several methods to enumerate *Vibrio vulnificus* from raw shellfish of which the two most commonly used techniques are: the most probable number (MPN) analysis and direct plating on nonselective media followed by colony lift hybridization (33). Following direct plating, typical colonies can be identified as *V. vulnificus* with biochemical tests or alkaline phosphatase-labeled DNA probes (29). These methods are validated and recommended by the FDA, and are intended for *V. vulnificus* quantification in samples. However, they are not necessarily the optimal detection methods for *V. vulnificus*. First of all, they are cumbersome to use. When using direct plating, some of the accompanying microflora present in the samples can grow on the nonselective media. The use of the MPN analysis eliminates this problem, but relies heavily on the ability of the scientist to recognize typical *V. vulnificus* colonies (29). Also, the MPN procedure uses alkaline peptone water, or APW(12), as an enrichment broth, which has been shown to be relatively non-selective. Tamplin and Capers (1992) showed that oysters collected during colder months with no detectable *V. vulnificus* using the MPN method could be incubated at 25ºC for 24 h and yield *V. vulnificus* in large numbers. This indicates that low levels of *V. vulnificus* are present in oyster tissues in the colder months, however the APW enrichment may be relatively unselective (95).

DNA hybridizations and polymerase chain reaction (PCR) are several molecular-based methods that target specific genes by using different techniques that have allowed a more rapid identification of *V. vulnificus* cells. Wright and colleagues (1993) developed a DNA probe for the hemolysin (*vvhA*) gene of *V. vulnificus* that is labeled with alkaline phosphatase. This specific probe allowed for direct discrimination of *V. vulnificus* from other *Vibrio* species (105). The probe was later applied to the DNA colony hybridization to confirm isolates following direct plating on oyster homogenate (29). Another DNA probe was designed by Cerda-Cuellar and others (2000) that is specific to the 16S rDNA gene of *V. vulnificus* that also successfully distinguished it from other *Vibrio* species (20).

PCR, as well as real-time PCR, have been used to detect *V. vulnificus* in oyster and water samples (6, 13, 14, 19, 79, 100). Culturable and nonculturable *V. vulnificus* can be detected by using primers that flank the hemolysin (*vvhA*) gene for a conventional PCR amplification. As little as 31 ng of DNA can be used for detection in unculturable cells and only 72 pg of DNA for culturable cells (14). Several multiplex PCR methods have also been used to simultaneously detect multiple *Vibrio* species based on targeting species-specific genes (13, 29, 100).

Real-time PCR (or quantitative PCR, qPCR) protocols are currently used for detection of *V. vulnificus*. This technique involves simultaneous amplification and detection of target DNA and allows results to be obtained sooner than conventional PCR. Campbell and Wright (2003) developed a real-time PCR assay using TaqMan probes that target the hemolysin (*vvhA*) gene that had a detection limit of 72 fg/μL of genomic DNA. This method has similar sensitivity to the colony lift hybridization (19). Another real-time PCR that utilizes SYBR Green probes targeting the hemolysin had specific detection of *V. vulnificus* among other *Vibrio* species and

had a detection limit of 10^2 CFU/g of oyster homogenate and 10^2 CFU/10 mL of water sample with neither sample requiring enrichment before amplification (79).

Post-harvest processing

Oysters naturally accumulate bacteria and viruses at levels higher than the surrounding water as a result of filtering the seawater. After harvest, these numbers can increase by 2 orders of magnitude if left at temperatures greater than 15ºC for more than 10 hours during transportation and retail storage (21, 60). The FDA requires that all post-harvest processing methods comply with the National Shellfish Sanitation Program's (NSSP) Guide for the Control of Molluscan Shellfish standards. In Section II, Chapter XIV of this guide, under "post-harvest processing," any process must "reduce the level of *Vibrio vulnificus* in the processed product to non-detectable (<30 MPN/gram) and the process achieves a minimum 3.52 log reduction" (34).

Shellfish harvesting waters are currently classified using the fecal coliform index. However, since *Vibrio* spp. are naturally present in the marine environment, the levels of environmental *Vibrios* do not correlate well with the fecal coliform index of control (94). To ensure consumer protection from illnesses associated with consumption of raw oysters, post-harvest treatment can be used to eliminate human pathogens from the oysters. Currently, there are only four methods approved by the FDA in regards to *Vibrio vulnificus*: high hydrostatic pressure processing (HHP), heat/cool pasteurization (HCP), individually quick frozen (IQF), and irradiation. These processes allow the companies that implement them to sell a safety-assured, longer shelf-life product (71).

High hydrostatic pressure processing. This process became commercially available in 1999 in Louisiana. It is a non-thermal process that subjects foods to hydrostatic pressures of up to 1035 MPa (18). Oysters are loaded into a chamber filled with water, then pressurized electronically

(72). High hydrostatic pressure causes changes in essential enzymes of microorganisms and their membrane permeability (39). While some microorganisms can survive at higher pressures (45), *Vibrio* spp. have been shown to be highly susceptible to high pressures. Treatment with hydrostatic pressure of 250 MPa for 10 min at 25ºC have been shown to reduce *V. vulnificus* levels *in vitro* to nondetectable levels. However, if *V. vulnificus* cells are in the viable but nonculturable (VBNC) state, they are more resistant to high hydrostatic pressure (9). In addition, different serotypes of *V. vulnificus* and *V. parahaemolyticus* require different pressure treatments. *Vibrio vulnificus* treated with a pressure of 250 MPa for 120 s reduced the bacterial loads by 5 orders of magnitude colony forming units per gram (CFU/g). However, *V. parahaemolyticus* required a treatment of 300 MPa for 180 s to achieve a similar reduction. This process might also be expected to greatly reduce the risk of infection related to shucking accidents. During the process, a band is wrapped around the shell to contain the oyster once it is subjected to the high heat and pressure. When ready for consumption, the band is cut and the oyster shell is already opened (25).

Heat/Cool pasteurization. This technique was first implemented commercially in a plant in 1997 in Louisiana. The basic commercial process consists of submerging oysters in a tank of warm water (at least 50°C) for 24 min. The oysters are then submerged into a tank of cold water at 4°C for 15 min (72). Hesselman et al. (1999) conducted a pilot-study on oysters harvested from the Gulf Coast with *V. vulnificus* levels of 10^2 to 10^4 CFU/g that were reduced by up to 4 orders of magnitude in the final product. Oysters were maintained for only 4 min at temperatures above 50°C then shucked and chilled. This process is already used to assist in shucking of oysters, which may also greatly reduce the number of shucking-related *V. vulnificus* cases (41). However, temperatures exceeding 52.5°C affect the oyster meat quality due to protein degradation, which

may cause a decrease in demand from consumers (2). In addition, some *Vibrio* spp. that are pathogenic may be more resistant to the pasteurization process than others (2).

Individually quick frozen (IQF). This technique is the oldest of the available processing methods, beginning in 1988. Oysters are shucked on the half shell and sent through a freezer tunnel that rapidly cools the oysters using liquid carbon dioxide, CO_2 (71, 86). *Vibrio* spp. are known to be extremely susceptible to freezing temperatures. Oysters that have undergone freezing treatments show an up to 5 orders of magnitude reduction in *V. vulnificus* numbers (4). Oysters treated with the IQF process are usually sold on the half shell without requiring shucking from the server, therefore eliminating risk of shucking injury. The shelf life of oysters treated with this method is also extended with treated oysters lasting up to one year (86). One possible disadvantage, however, is that when oysters are subjected to refrigeration temperatures, as they typically are on commercial boats or in warehouses, cold shock proteins are induced in *V. vulnificus*. By producing these proteins, the cells become more resistant to the otherwise lethal effects of freezing (4).

Irradiation. Irradiation has undergone much research before becoming commonly used to eliminate bacterial pathogens in foods. The U. S. has been more cautious about incorporating food irradiation as a food treatment than many other countries. Irradiation is commonly used in Asian and European markets for shrimp (1). *Vibrio* spp. are extremely sensitive to radiation and can be eliminated by doses of less than 0.1 kGy (65). Novak *et al.* (1966) found that a dose of 2 kGy causes no change in organoleptic quality of oyster meat and decreases total bacteria by 99% (75). Whole shell oysters treated with ionizing irradiation doses of 1.0 kGy have reduced *V. vulnificus* levels from 10^7 CFU/g down to nondetectable. Most oysters survive these treatments, and one study found that consumers could not tell a difference (3). One estimate of the added

cost for commercial irradiation is $0.11/kg of oysters (56). However, some other reports show that irradiation produces a yellow pigment on the oyster that may be viewed unfavorablyby most consumers as well as damages vitamins found in oysters. Finally, there is little known about the long-term effects on humans who consume irradiated foods (26).

As mentioned above, all of these methods result in organoleptic changes in the oysters that are processed. There has been interest within the industry to identify and validate alternative methods for eliminating *V. vulnificus*, while maintaining a live, raw oyster. One of the most promising of these methods appears to be depuration.

Depuration. Depuration is defined as the transfer of shellfish containing bacteria and viruses from polluted waters to a controlled, cleaner aquatic environment that permits them to open and function in an optimum physiological mode that favors the elimination of contaminants to non-detectable levels favorable for human consumption without requiring further processing (35, 60, 78). Historically, it is said to have occurred in Mediterranean countries long before the germ concept of disease was established (76). However, the modern concept of depuration is rooted in the late 1800s (76). In 1911, there is record of depuration taking place in Rhode Island with the purpose of eliminating typhoid fever caused by consuming raw shellfish (76). Several years later, the National Shellfish Sanitation Program was established as part of the FDA. To this date, any depuration systems must be approved by the FDA (78). There are currently four states that practice active depuration: Massachusetts, Maine, New Jersey and Florida. Massachusetts has the oldest depuration plant in the U.S. run by the State, and used for depuration of clams. Maine has three plants that depurate softshell clams and oysters. Florida's two plants both process hardshell clams (78). The species of bivalves most commonly subjected to depuration are oysters, mussels and clams. However, of these species, oysters are the most commonly eaten raw. In

consideration of the currently validated post-harvest treatment processes, depuration might be an alternative method that could alleviate the problems of sequestered microorganisms in oysters while keeping the animal alive (60).

The depuration process is very dynamic due to the challenge of maintaining optimum physiological activity of the animals while still achieving parameters that favor removal of microorganisms. Oysters may concentrate *V. vulnificus* from seawater two different ways: ventilation and filtration. Ventilation is defined as the flow rate of water over the gills of the oyster (note: this is not equivalent to the flow rate of the water in the tank). The general process by which oysters feed involves water being ventilated over the cilia-coated gills, which capture some particles from the water column and move them to the labial palps. The filtration, or clearance, rate is defined as the volume of water totally cleared of particles per unit time. The labial palps sort particles as food to be sent to the digestive tract or waste to be expelled as pseudofeces before entering the digestive system (55). It has been shown that as depuration time increases, the majority of *V. vulnificus* organisms are present in the gut, containing 70% of the total *V. vulnificus* found in oyster meats (95). This is probably due to the organisms present in the gut from the filtration process going through the usual digestive processes, unaffected by depuration. However, it might be possible to make the gut environment unsuitable for *V. vulnificus* survival by altering certain water parameters such as salinity and temperature. The organisms from the ventilation process that would reside in the mantle cavity and gills account for less than 10% of endogenous *V. vulnificus* and decrease with depuration time (95). The parameters that encourage removal of microorganisms are not always optimum for ventilation by shellfish (95). At a very basic level, water flow must be high enough to favor and promote the removal of depurated material, feces and pseudofeces from the shellfish (95). However, flow

must also be low enough to prevent any risk of recontamination by feces and pseudofeces being broken up and resuspended in the water. One study showed that a single oyster can expel 10^5 to 10^6 *V. vulnificus* organisms per hour in ventilated water making recontamination easy if the system is not properly designed (95). Ideally, flow must be balanced to allow for settlement of solids below the oysters, but efficient enough to remove purged microorganisms and allow optimum filtration by the bivalves (60).

Basic parameters involved in a depuration system are: water source, flow rate, filtration and disinfecting systems, salinity, and water temperature. However, the main difference is whether a system uses re-circulating or flow-through water. The only depuration methods currently patented within the U.S. are based on closed, re-circulating systems (48, 89). Re-circulating systems require artificial seawater to be constantly cycled through after sterilization. Flow-through systems use natural seawater that passes through once after being sterilized and is discharged. Because of this, flow-through systems are also susceptible to fluctuations in the microbial community composition and abundance and water quality of the source. There are some data that show effluent discharged from pilot-scale, flow-through depuration plants in the US cause only minimal degradation to the estuaries with assumptions that re-circulating plant effluents may be more degradative, although little evidence is given (16, 36, 37, 46). Also, with flow-through systems there would not be costs associated with feeding due to oysters obtaining their nutrition from the natural water being pumped in. Re-circulating systems would require an additional cost to feed the oysters (either cultured live algae or an algae paste); if the oysters are not fed for a prolonged period, the product may undergo changes in quality. The total cost may also be lower to run a flow-through depuration plant as opposed to a re-circulating one, where maintaining water quality could be expensive. The most common method of disinfecting water in

both depuration systems is ultra-violet light (UV). The efficacy of UV-light is dependent upon turbidity, flow rate, and quality of the UV lamps used. Also, in closed systems, where UV is the only disinfection source, bacteria can still become established in the system. UV only kills bacteria at one point within the system; if one bacterium escapes, it can attach to downstream substrate within the system and proliferate, overwhelming the disinfection capacity of the system. In flow-through systems, bacteria may still attach downstream, but with a single-pass application it is irrelevant (30). The temperature determines the quality of bacterial elimination by affecting oyster ventilation rate, which is the volume of water pumped over the gills per unit time, as well as survival of the bacteria within oysters (48).

Salinity, temperature, and dissolved oxygen are the water parameters that must be optimized for maximum shellfish depuration while remaining unfavorable for the propagation of microorganisms (78).This post-harvest process has been well documented in reducing *V. vulnificus* in oysters inoculated with laboratory-grown strains (21, 54, 95). Unfortunately, it has shown little promise for the removal of naturally present *Vibrio* spp (21, 95). This could be due to a possible commensal relationship between *V. vulnificus* and the oyster making depuration more difficult (95). For example, *V. fischeri* is a well-known commensal with another marine animal, the Hawaiian bobtail squid, *Euprymna scolopes*. *Vibrio fischeri* possesses a pilus similar to that found in *V. vulnificus* that competitively allows it to colonize the squid over other marine bacteria (80, 90). The presence of these structures specific for colonization may contribute to their ability to resist depuration. However, if the salinity is high enough to retard *V. vulnificus* (>25 ppt), it could make the internal environment of the oyster inhospitable to *V. vulnificus* proliferation.

Temperature is the most studied parameter in depuration trials, and it has been show that an increase in water temperature correlates with an increase in *V. vulnificus* densities in oysters (21, 69). Temperatures greater than 23°C actually increase *V. vulnificus* populations in oysters (21, 95). Conversely, when the water temperature was decreased to 15°C in a closed, re-circulating system, laboratory-grown *V. vulnificus* numbers were reduced to non-detectable levels in oysters (21). This is probably due to a combination of bacterial growth being halted at a lower temperature as well as a very high rate of oyster ventilation (21). *Vibrio vulnificus* is rarely recovered from oysters in the Gulf of Mexico in the winter months indicating that cooler temperatures are unfavorable for its proliferation (53). It has been suggested that because the bacteria is a transient member of oyster microflora, only present in the warmer months, it should be easily depurated (54).

II. OBJECTIVES

Preliminary data obtained by our group pointed to flow-through depuration as a promising method to eliminate *Vibrio vulnificus* from oysters. Therefore, the objectives of the present study were:

1. Assess flow-through depuration system efficacy in eliminating *V. vulnificus*-artificially inoculated oysters.
2. Asses flow-through depuration system efficacy with oysters containing natural populations of *V. vulnificus*, by manipulating temperature, salinity and flow.
3. Investigate if depuration protocols select for specific *V. vulnificus* strains.

III. MATERIALS AND METHODS

Bacterial Culture

An environmental strain of *V. vulnificus* isolated from oysters from Mobile Bay, Alabama, identified as VV3, was used in depuration experiments requiring inoculation of oysters with bacteria prior to depuration. Cells were cultured in 50 ml conical vials containing Marine Broth (MB) (Difco, Detroit, MI) with shaking at 35°C for 12 hours. Glycerol stocks were made for long-term storage of the strain and maintained at -80ºC.

Oyster Stock

Oysters were obtained from Auburn University Shellfish Lab (AUSL) on Dauphin Island, AL and harvested immediately prior to depuration. These oysters were two years-old and grown in 16 mm mesh bags on an adjustable long line system (BST Oyster Supply, Cowel, South Australia) in an intertidal area below a boat dock about 46 cm off the bottom on the north side of the east end of the island at the following coordinates: 30º 15” 04.68” N, 88º 04” 47.28” W. Oysters were placed into depuration tanks within one hour of harvest.

Artificial Inoculation

For depuration experiments with artificially inoculated oysters, all oysters were placed in a 522 L raceway tank containing 100 ml of seawater. Aeration was provided through air stones. *Vibrio vulnificus* VV3 was cultured in MB for 16 h to achieve exponential growth phase (10^8 CFU/ml); 10 ml of the culture was added to the tank so that the oysters would self-inoculate by filtration overnight to obtain a concentration of approximately 10^5 CFU/g of oyster meat. The

following day, 12 oysters were selected at random for time zero bacterial analysis with the other oysters placed into the depuration system.

Depuration of Oysters

Standard depuration conditions. Table 1 shows all depuration experiments performed during this study. One hundred twelve live eastern oysters, two years old, were used for each depuration experiment. Oysters brought to the lab were immediately scrubbed clean of any fouling organisms and rinsed with freshwater to remove any exogenous material. Oysters were randomly divided between two identical 522 L raceway tanks (Fig. 1), with a water volume of around 300 L on mesh trays suspended 13 cm from the tank bottom(Fig. 2) with fresh seawater piped in from the Gulf of Mexico with a 213m PVC pipeline extending 107m offshore. Oysters were fed once a day with marine microalgae concentrate (Shellfish Diet 1800, Reed Mariculture, Campbell, CA), requiring cessation of water flow for 1 h per feeding. The tanks were drained and cleaned of feces and pseudofeces. Salinity and temperature were measured twice a day with a YSI 85 probe (YSI Inc., Yellow Springs, OH). Mortality was checked every morning before cleaning. One tank was designated as the control and the other as the experimental tank. In the experimental tank, incoming seawater was filtered through a 200 μm bag filter (Aquatic Ecosystems, Inc., Apopka, FL) then treated with a 110 W commercial UV sterilizer (Tropical Marine Centre, Ltd., Chorleywood, UK). Oysters were depurated using a flow-through system with flow rate maintained at 11 L/m for 6 days at the AUSL, unless stated otherwise for a given trial. The approximate water replacement time of the experimental tank under this flow regime was 30 m. Further experimental manipulations are described below.

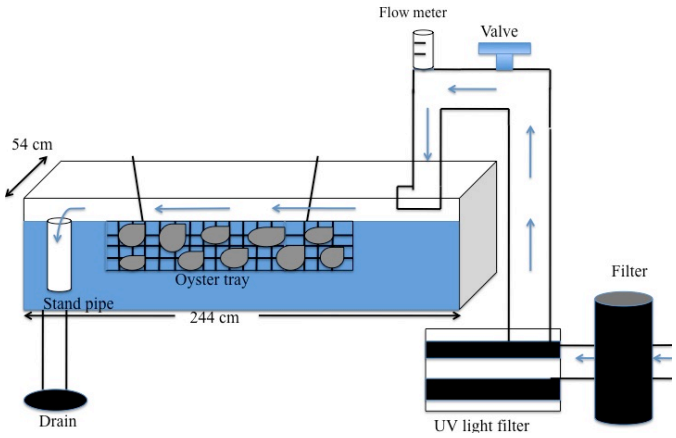


Fig. 1. Schematic diagram of flow-through depuration system illustrating direction of water flow and measurements of tank.

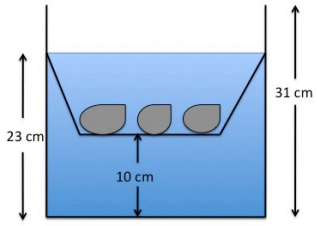


Fig. 2. Suspension of oysters in depuration tank

Test of validity of the control: The first three trials were conducted to evaluate the validity of the control. In these trials, the control tank varied from the experimental tank only with regards to the filtration and UV system. As described above, the water in the experimental tank was filtered and treated with UV sterilization. With each tank cleaning, the filter bag was rinsed with fresh water and any feces/pseudofeces removed from the tank. In these first three trials, oysters were inoculated with laboratory-cultured VV3.

Temperature abuse of oysters prior to depuration: One pilot trial (# 4) was conducted to observe whether depuration effectiveness of naturally occurring *V. vulnificus* differed between oysters left at ambient temperatures for 10 h (i.e., temperature abused) and oysters put immediately into the depuration system after harvest. The oysters for immediate depuration were handled as stated above and placed into the control tank, while the oysters left out for 10 h were kept outside in temperatures exceeding 32ºC. After 10 h, oysters were placed into the experimental tank as previously described.

Depuration with cold water: As an additional pilot trial to test the effect of lowered water temperature (# 5) on depuration of naturally occurring *V. vulnificus*, after filtration in the experimental tank, incoming water was chilled to 15º C using a 1¾ hp Delta Star chiller (AquaLogic, Inc., San Diego, CA). The control tank, water remained at the naturally occurring temperature.

Depuration with high salinity: As a pilot trial of the effect of salinity (# 6) on depuration of naturally occurring *V. vulnificus*, salinity was artificially increased in the experimental tank to approximately 35 ppt NaCl. In the experimental tank, water flow was turned off, and the salinity measured using the previously mentioned YSI 85 probe. The measured salinity was subtracted from the target 35 ppt and multiplied by 278 L (water volume for inoculation to give the amount of NaCl to add to the tank in grams). Once the salt was dissolved in the tank, salinity was measured again to confirm the desired salinity. Oysters were left in tank for 12 h at 35 ppt before restoring flow In the control tank, water flow was simply turned off for the duration of the salinity manipulation; water remained at the naturally occurring salinity. This cycle was repeated each day for 6 days.

Depuration at low temperatures and high salinity: These trials (# 7,8,9 and 10) were conducted as described above (with naturally occurring *V. vulnificus*), except when water flow was stopped to bring up salinity in the experimental tank; during this time, the chiller was left running to attempt to maintain a constant temperature of 15ºC. It needs to be noted that in all experiments using the chiller, the unit never functioned at an optimum rate for an extended period of time due to electrical issues. This resulted in periods of temperature warmer than the 15ºC target temperature. The control tank was treated as described above for the prior trials, subjecting the oysters to naturally occurring temperatures and salinities.

Depuration with high flow rate: In four additional trials, flow rate was increased from the standard flow rate of 11 L/m (# 11) to test the effect upon depuration rates. To accommodate the higher flow rates, these trials were conducted in the hatchery to utilize the larger piping of that system. The set-up was exactly the same as the standard depuration with the exception of a clamp-on flow meter (Aquatic Ecosystems, Inc., Apopka, FL) installed in front of the valve controlling water flow. In the first trial (#12), the flow rate was increased to 46 L/m. In additional trials (# 13 and 14), the flow rate was increased further to 68 L/m. The flow was set at the desired rate using the attached flow meter. The variance of flow was 46±4 L/m for trial (#12) and 68±4 L/m for trials (#13 &14). In these experiments, the two tanks were considered replicates. Flow rates and salinity were identical in each tank during a given trial.

Table 1. All depuration trials summarizing the differences in test and control tanks.

Trial	Date	Harvest Temp.	Harvest Salinity	Control	Test	Strain of *V. vulnificus*
A. Test of Validity of Control						
1	02/19/09	16.82ºC	24.17 ppt	No filter/UV	Filter/UV	VV3
2	02/26/09	15.34ºC	26.64 ppt	No filter/UV	Filter/UV	VV3
3	11/11/09	20.08ºC	18.21 ppt	No filter/UV	Filter/UV	VV3
B. Exploratory Trials						
4	05/12/09	26.22ºC	21.04 ppt	No filter/UV; immediate depuration	Filter/UV; 10h at 32ºC before depuration	Natural
5	06/08/09	28.30ºC	15.81 ppt	No filter/UV; no chiller	Filter/UV; chiller	Natural
6	05/25/09	26.34ºC	13.98 ppt	No filter/UV; no added NaCl	Filter/UV; added NaCl	Natural
C. Depuration at low temperatures and high salinity						
7	06/22/09	30.05ºC	24.50 ppt	No filter/UV; no chiller; no NaCl added	Filter/UV; chiller; added NaCl	Natural
8	07/06/09	29.10ºC	25.97 ppt	No filter/UV; no chiller; no NaCl added	Filter/UV; chiller; added NaCl	Natural
9	07/21/09	28.75ºC	27.99 ppt	No filter/UV; no chiller; no NaCl added	Filter/UV; chiller; added NaCl	Natural
10	08/04/09	30.20ºC	26.73 ppt	No filter/UV; no chiller; no NaCl added	Filter/UV; chiller; added NaCl	Natural
D. Depuration with varying salinity and flow						
11	08/19/09	29.44ºC	25.80 ppt	No filter/UV; flow at 11 L/m	Filter/UV; flow at 11 L/m	Natural
12	09/01/09	29.47ºC	30.12 ppt	No filter/UV; flow at 46 L/m	Filter/UV; flow at 46 L/m	Natural
13	09/14/09	26.88ºC	30.49 ppt	No filter/UV; flow at 68 L/m	Filter/UV; flow at 68 L/m	Natural
14	10/12/09	26.51ºC	9.47 ppt	No filter/UV; flow at 68 L/m	Filter/UV; flow at 68 L/m	Natural

Bacteriological Analysis

Vibrio vulnificus numbers in the oysters were enumerated using the FDA MPN procedure (33). Twelve oysters were selected at random before and during depuration at days one, two, three and six. The oysters were scrubbed clean of exogenous material under running tap water before being shucked and weighed in with sterile equipment. Oyster meat and liquid from twelve oysters were pooled together and homogenized using a food-blender for 90s. The homogenate was diluted 1:1 (wt/wt) in phosphate-buffered saline (PBS). Ten-fold dilutions from the homogenate were made with PBS. One ml of each dilution was enriched in triplicate into 10 ml of alkaline peptone water (12) at 35ºC for 18 h. Each tube was then streaked with a 3 mm loop onto modified mCPC agar plates and incubated at 39ºC for 16 h. The following day, *V. vulnificus* typical colonies were selected by picking those that were 1-2 mm in diameter and yellow. The selected colonies were then enriched in 100 μL of APW in a 96-well plate for 3-4 h at 35ºC. Following enrichment, a 48-prong replicator was used to transfer the cultures to a plate containing *V. vulnificus* Agar (VVA) and incubated at 35ºC for 18 h (33).

The MPN procedure was followed by a colony lift and subsequent colony blot hybridization using a *V. vulnificus*-specific oligonucleotide probe (*vvhA*) (DNA Technology, Aarhus, Denmark) that targets the hemolysin gene(33). After 18 h incubation, VVA plate was overlaid with a filter paper (no. 541; Whatman, Inc. Clifton, NJ). Filters with colonies were placed into 1 mL of lysis solution (0.5M NaOH, 1.5M NaCl) to lyse the cells. The filters were then heated in a microwave oven for 1 m to aid in lysis and to fix the DNA to the membrane. Lysis solution was then neutralized with ammonium acetate and washed in standard saline citrate

(27)buffer. To remove any proteins that might be blocking binding sites for the probe, the filter was treated with proteinase K (20 μg/mL) (Sigma Aldrich®, St. Louis, MO), followed by pre-hybridization for 30 m with the hybridization buffer consisting of SSC with 0.5% bovine serum albumin (BSA), 1% sodium dodecyl sulfate (SDS) and 0.5% polyvinylpyrrolidone (PVP-360) at 55ºC to warm to hybridization temperature. The hybridization with the alkaline phosphate-labeled *vvhA* probe was carried out under stringent (55ºC) conditions for 1 h in the hybridization buffer. Excess probe was removed with SSC buffer containing 1% SDS at hybridization temperature. Detection of positive colonies was detected by a colorimetric reaction seen by washing the filters in a NBT/BCIP (nitrobluetetrazolium/5-bromo-4-chloro-3-indolylphosphate toluidinium) substrate (Sigma Aldrich, St. Louis, MO). The BCIP is hydrolyzed by the alkaline phosphatase that produces an indigo precipitate. Positive colonies are those that are dark brown or purple. This resulted in a grid of purple dots on the filter paper. Every three dots represent a tube of the ten-fold serial dilutions. If one of the three bacterial colonies from that tube is positive, the whole tube is considered positive. The number of positive tubes for the three lowest serial dilutions is recorded. The most probable number of *V. vulnificus* CFU/g was then determined by using the chart located in the FDA BAM (33) to interpret the results. This chart has a list of the number of positive tubes correlated to an estimate of CFU/g of oyster meat from the original sample (33).

Comparison of Isolates

During depuration experiments, putative *V. vulnificus* isolates were collected from the mCPC agar before and after depuration and maintained in 0.3% MA tubes for typing using Restriction Fragment-Length Polymorphisms (RFLP) of the 16S rRNA gene. Isolates were streaked as bacterial lawns onto MA plates and incubated at 37°C for 16 h. The bacteria were collected and DNA was extracted using the Pitcher method for rapid extraction of bacterial genomic DNA(82). DNA was resuspended in 200 μL of deionized water (dH_2O) and stored at -20°C. DNA was quantified using the Nanodrop® ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and diluted to 20 ng/μL. Polymerase chain reaction (PCR) using the MJ Research PTC-200 Thermocycler was performed on the DNA samples amplifying a 492-bp segment of the 16S rRNA gene confirmed by a 2% agarose-gel electrophoresis. Remaining DNA was used for a restriction digest with the *HaeIII* restriction endonuclease followed by a 4% agarose gel electrophoresis for confirmation.

AFLP® Analysis

DNA from pre- and post-depuration isolates of *V. vulnificus* were further compared using Amplified Fragment Length Polymorphisms (AFLP) analysis(98). The restriction was carried out using 1 μg of DNA digested with 10 U each of *Hind*III and *Taq*I (Promega, Madison, WI, USA) in a final volume of 30 μL. Following restriction, *Hind*III and *Taq*I adapters were ligated to the restriction fragments using T4 ligase (Promega). AFLP® reactions employed *Hind*III and *Taq*I specific adapters prepared by mixing equimolecular amounts of the partly complementary oligonucleotide sequences 5'-ACGTGGTACGCAGTC-3' and 5'-CTCGTAGACTGCGTACC-3' (for *Hind*III) and 5'-GACGATGAGTCCTGAC-3' and 5'-CGGTCAGGACTCAT-3' (for

*Taq*I). *Hind*III primer H00A was labeled with an IR700 fluorochrome from LI-COR (Lincoln, NE, USA). PCR amplification of specific fragments were performed in a total volume of 10μL containing 6.25 ng of labeled primer, 30 ng of unlabeled primer, 1.5 μL of template DNA, 0.6 U of *Taq* polymerase, 10 mMTris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, and each of the deoxynucleoside triphosphates at 0.2 mM. PCR amplifications were performed using the MJ Research PTC-200 Thermocycler with the following cycle profile: cycle 1, 60 s at 94ºC, 30 s 65ºC, and 60 s at 72ºC; cycles 2 to 12, 30 s at 94ºC, 30 s at annealing temperatures 0.7ºC lower than that used for each previous cycle, starting at 64.3ºC, and 60 s at 72ºC; cycles 13 to 24, 30 s at 94ºC, 30 s at 56ºC, and 60 s at 72ºC. After completion of the cycling program, 5 μL of AFLP® Blue Stop Solution (LI-COR) was added to the reaction mixtures. Prior to gel loading, the samples were heated for 5 m at 94ºC then rapidly cooled on ice to prevent reannealing. The PCR products were electrophoresed on the NEN Global Edition IR2 DNA Analyzer (LI-COR) following manufacturer's instructions. AFLP® images were processed with BioNumerics v 5.0 (Applied Maths Inc., Austin, TX, USA). Following conversion, normalization, and background subtraction with mathematical algorithms, levels of similarity between fingerprints were calculated with the Pearson product-moment correlation coefficient (*r*). Cluster analysis was performed with the unweighted pair-group method using average linkages (UPGMA).

Statistical Analysis

Depuration results from the artificially-inoculated trials (1-3) and the low temperature-high salinity combination trials (7-10) were analyzed using paired t-test in SYSTAT® VERSION 12.0 using the calculated proportion of reduction from the following formula: (1- Day 6/Day 0).

Depuration results from the flow trials (11-14) were analyzed using an ANOVA followed by Tukey (Systat ® ver. 12). Data were square root arcsine transformed for analysis due to data being in the form of proportions.

IV. RESULTS

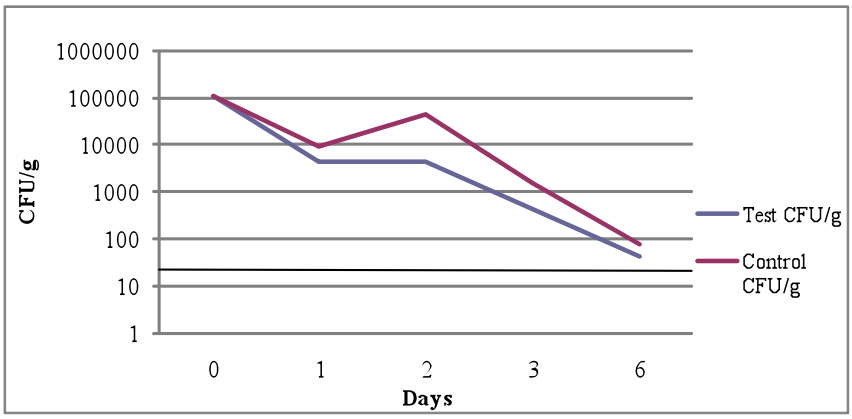
Vibrio vulnificus numbers are low during the colder months of the year, typically below detectable levels. In order to obtain a data set from those months, oysters had to be artificially inoculated with a laboratory-grown culture of *V. vulnificus*. These trials provided a baseline for the depuration system. During the warmer months oysters contained enough *V. vulnificus* cells and were subjected to depuration without artificial inoculation. In total, 14 different depuration trials were carried out testing different variables to observe any impact on the reduction of *V. vulnificus* in oyster tissues (Table 1). These trials tested variables such as salinity, temperature, as well as high flow rates. All trials were performed using the same system as previously described with depuration lasting 6 days. They were conducted at different times of the year with ambient water temperature and salinity varying between trials.

TABLE 2. Summary of depuration trials results using a flow-through system.

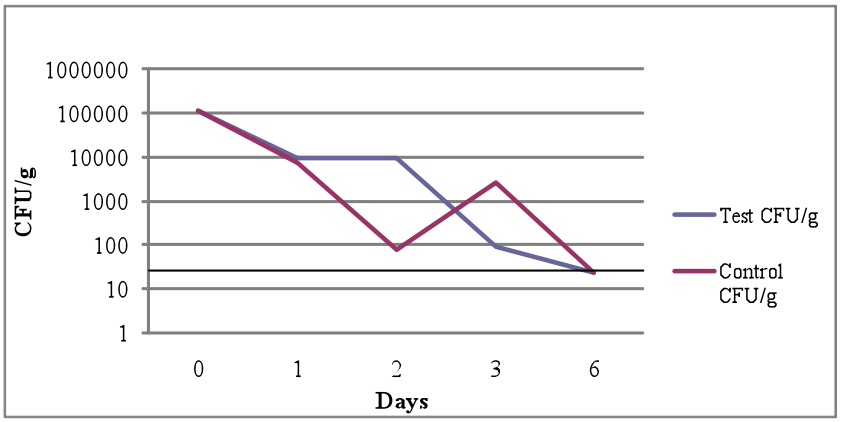
Trial	Date	Parameter	Time 0	Day 6 Control	Day 6 Test	Ambient Average Temp.	Ambient Average Salinity	Trial Average Temp.	Trial Average Salinity
A. Test of Validity of Control									
1	02/19/09	Standard	>1.1X10^5 CFU/g	75 CFU/g	43 CFU/g	22.0 ± 1.7	15.0 ± 2.8	22.0 ± 1.7	15.0 ± 2.8
2	02/26/09	Standard	>1.1X10^5 CFU/g	23 CFU/g	23 CFU/g	15 ± 1.1	27.0 ± 1.7	15 ± 1.1	27.0 ± 1.7
3	11//11/09	Standard	>1.1X10^5 CFU/g	4.3X10^4 CFU/g	2.4X10^4 CFU/g	18.0 ± 0.8	13.5 ± 3.6	18.0 ± 0.8	13.5 ± 3.6
B. Exploratory Trials									
4	05/12/09	Time (10h)	>1.1X10^5 CFU/g	9.3X10^3 CFU/g	7.5X10^3 CFU/g	26.2 ± 2.2	7.7 ± 4.5	26.2 ± 2.2	7.7 ± 4.5
5	06/08/09	Temp. (15ºC)	2.9X10^4 CFU/g	7.5X10^3 CFU/g	9.3X10^3 CFU/g	25.0 ± 2.1	26.0 ± 3.2	18.0 ± 2.4	26.0 ± 3.2
6	05/25/09	Salinity (35ppt)	>1.1X10^5 CFU/g	9.3X10^2 CFU/g	1.1X10^5 CFU/g	25.7 ± 1.0	11.4 ± 4.5	25.7 ± 1.0	35.0 ± 3.3
C. Depuration at low temperatures and high salinity									
7	06/22/09	35 ppt + 15ºC	1.5X10^4 CFU/g	4.6X10^4 CFU/g	4.6X10^4 CFU/g	26.0 ± 1.8	23.0 ± 2.6	17.8 ± 3.9	35.6 ± 2.2
8	07/06/09	35 ppt + 15ºC	1.1X10^5 CFU/g	>1.1X10^5 CFU/g	4.6X10^4 CFU/g	26.0 ± 1.4	20.0 ± 3.3	15.4 ± 1.6	35.2 ± 1.9
9	07/21/09	35 ppt + 15ºC	4.6X10^4 CFU/g	1.5X10^3 CFU/g	1.5X10^3 CFU/g	25.0 ± 1.0	28.9 ± 1.4	15.3 ± 1.3	35.0 ± 1.8
10	08/04/09	35 ppt + 15ºC	1.5X10^4 CFU/g	7.5X10^2 CFU/g	2.7X10^2 CFU/g	26.7 ± 2.0	25.7 ± 3.6	16.4 ± 1.2	31.8 ± 3.2
D. Depuration with varying salinity and flow									
11	08/19/09	Flow rate (11 L/m)	1.4X10^3 CFU/g	9.3X10^2 CFU/g	2.4X10^3 CFU/g	28.3 ± 1.2	24.3 ± 2.3	28.3 ± 1.2	24.3 ± 2.3
12	09/01/09	Flow rate (46 L/m)	>1.1X10^5 CFU/g	2.1X10^4 CFU/g	2.9X10^4 CFU/g	28.5 ± 0.6	27.5 ± 3.4	28.5 ± 0.6	27.5 ± 3.4
13	09/14/09	Flow rate (68 L/m)	1.1X10^5 CFU/g	3 CFU/g	3 CFU/g	27.5 ± 1.7	27.2 ± 1.2	27.5 ± 1.7	27.2 ± 1.2
14	10/12/09	Flow rate (68 L/m)	4.6X10^4 CFU/g	1.1X10^5 CFU/g	>1.1X10^5 CFU/g	25.6 ± 3.1	9.0 ± 2.4	25.6 ± 3.1	9.0 ± 2.4

Depuration of artificially inoculated oysters

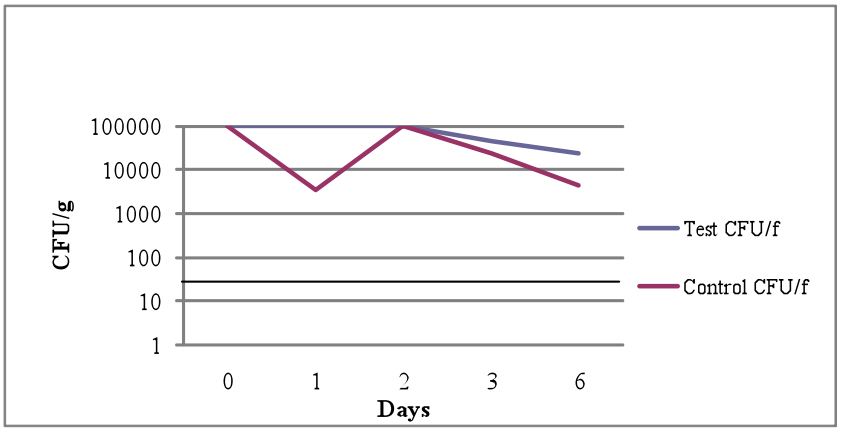
In trials1-3, where artificially inoculated oysters were used, a reduction of 3 to 4 orders-of-magnitude was observed (Fig. 3A & B). Starting concentrations of *V. vulnificus* in oyster tissues were greater than 100,000 CFU/g and over 6 days were reduced to 75 and 43 CFU/g in the control and test tanks respectively. There was a spike in the control tank on day one, while a leveling off was observed in the test tank. Following day 2, there was a decline in *V. vulnificus* numbers in oysters from both tanks (Figure 3A). Figure 3B shows a reduction in *V. vulnificus* numbers in the control tank until a spike was observed on day 2, with reduction continuing on day 3. The test tank never showed a resurgence of *V. vulnificus* in oyster tissues as observed in the control tank, but there was a period of no reduction from day 1 to 2 (Figure 3B). In Figure 3C, *V. vulnificus* concentration in oyster tissues showed a reduction in both test and control tanks after 6 days, but was still well above the target threshold of 30 CFU/g. Statistical analysis (paired t-test, SYSTAT ® ver. 12.0) showed that there was no significant difference in depuration effectiveness of *V. vulnificus* between the experimental and control tanks (t=-0.998, df=2, p=0.424).



A



B



C

Fig. 3. Presence of *Vibrio vulnificus* in Gulf Coast oysters artificially inoculated and subjected to flow-through depuration over a 6-day period as determined by Most-Probable-Number (MPN) analysis. Numbers represent number of colony forming units (CFU) per gram of oyster meat. A line is drawn at the 30 CFU/g threshold of reduction required by the FDA for validation of the system.

Temperature abuse prior to depuration

This was an exploratory trial comparing depuration of oysters left at ambient temperatures (~35ºC) for 10 h and those immediately depurated after harvest. There was little, if any, reduction in the first two days of depuration of both groups. On day 3, *V. vulnificus* numbers in the test tank remained static for the remainder of the experiment while the control tank showed a slight increase over the same period. This single trial provided no qualitative evidence that depuration is less effective against oysters depurated after temperature abuse versus those depurated immediately (Fig. 4A).

Depuration with chilled water

An exploratory flow-through depuration trial with water maintained at 15ºC showed no difference between oysters in the two tanks until day 2. After day 2, a dramatic decrease in *V. vulnificus* numbers in oysters was observed until day 3 in the control tank with respect to the test tank. However, a subsequent spike in *V. vulnificus* numbers followed on day 3 in the control tank to equal the numbers of *V. vulnificus* in oyster tissues from the test tank. The final result was little qualitative evidence that the lower temperature depuration made a difference when compared to the ambient temperature with regards to reduced *V. vulnificus* (Fig. 4B).

Depuration with high salinity

Another exploratory trial of oyster depuration with a 12 h rotation of soaking in 35 ppt seawater and flow-through showed little difference from the beginning of depuration to day 6. After 2 days, the test tank (35 ppt) had slightly lower *V. vulnificus* cells per gram of oyster tissue. After day 3, oysters in the test tank began to show an increase in *V. vulnificus* numbers as the oysters in the control tank showed a decline. There is also very little qualitative evidence to

support that an increase in salinity alone would reduce *V. vulnificus* in oysters from this one trial (Fig. 4C).

The maximum 30 CFU/g concentration of *V. vulnificus* allowed by the FDA was not achieved for any one of the three exploratory trials shown in Figure 4. Therefore, these were not selected as being possible manipulations to the depuration system that would likely promote the rapid removal of *V. vulnificus* from the oyster tissues.

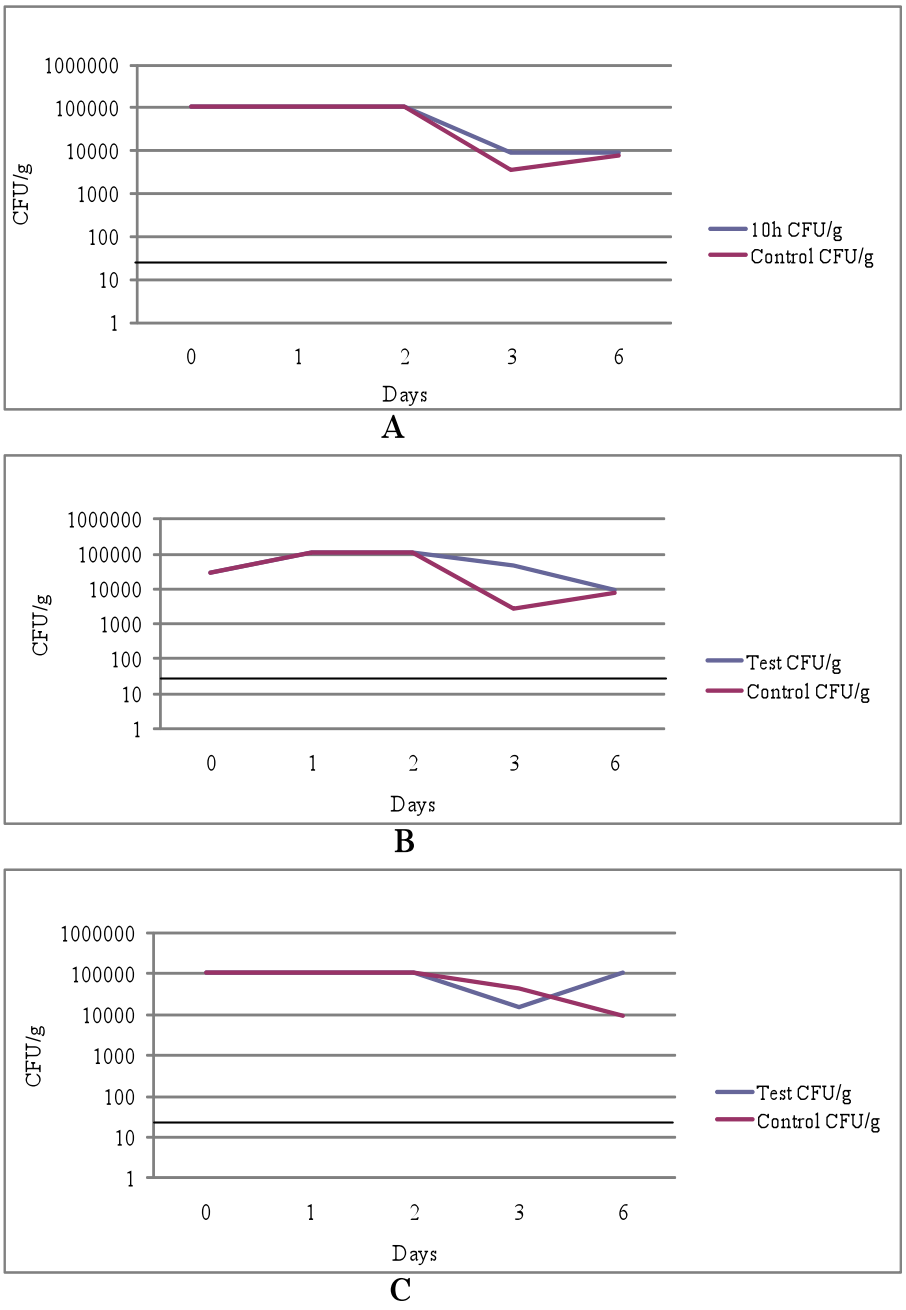


Fig. 4. Single-run exploratory trials. (A) Effects on leaving oysters at ambient temperatures (~35ºC) for 10 h then subjected to depuration for a 6-day period versus oysters immediately placed in depuration after harvest. (B) Persistence of *Vibrio vulnificus* in oyster tissue over a 6-day period in a flow-through depuration system with water maintained at 15ºC. (C) Survival of *Vibrio vulnificus* in oysters with an alternating 35 ppt NaCl brine soak/flow-through depuration every 12 h over a 6-day period.

Depuration with chilled water and high salinity

When flow-through water was maintained at 15ºC for 12 h alternated with a 12 h 35 ppt NaCl brine bath, there was no significant difference (t=1.073, df=3, p=0.362) in reduction of *V. vulnificus* in oysters between test and control tanks (Figure 5, A-D).

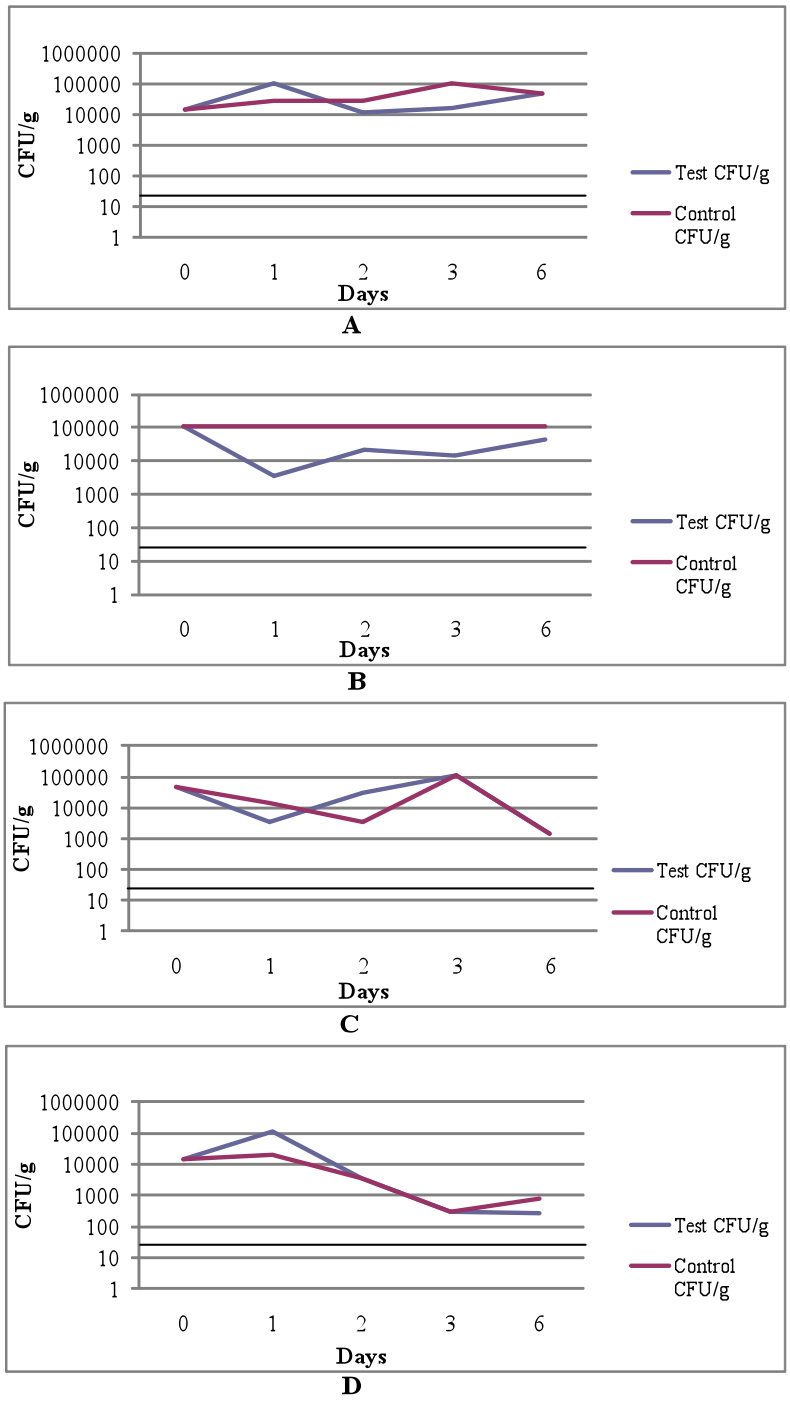


Fig. 5. *Vibrio vulnificus* numbers per gram of oyster tissue over a 6-day period in a flow-through depuration system with water maintained at a constant 15ºC and alternating 12 h brine baths at 35 ppt. Replicate trials indicated as A, B, C and D.

Depuration with high flow rate

As a starting point, a low flow (11 L/m) and high salinity (>25 ppt) depuration trial was conducted (Fig. 6A). This trial demonstrated very little reduction of *V. vulnificus* in oyster tissues from beginning of depuration to day 6 in both replicate tanks. As the concentrations of *V. vulnificus* in oysters from replicate tank two began to decline, the opposite was observed in replicate tank one. This same trend was observed throughout the experiment (Fig. 6A). The maximum final concentration (<30 CFU/g) of *V. vulnificus* required by the FDA to validate the system was not reached in either tank.

When a medium flow (46 L/m) and high salinity (>25 ppt) were combined for a depuration trial, a time zero concentration of *V. vulnificus* was 110,000 CFU/g of oyster meat and was depurated to 29,000 CFU/g and 21,000 CFU/g in replicate tanks one and two respectively by day six (Fig. 6B). A decline of *V. vulnificus* occurred in both tanks until day two and then they slowly began to increase again by day three. The goal threshold of <30 CFU/g was not achieved in either replicate tank.

As Figure 6C shows, when flow rate was increased to 68 L/m from 11 L/m, with ambient salinity being high (>25 ppt) there is a large reduction of *V. vulnificus* cells in oysters from both tanks. On day 1, oysters in both tanks began to show depuration until on day 2, the oysters in replicate tank one began to increase in their concentration of *V. vulnificus* while the oysters in replicate tank two maintained a steady reduction. After day 3, replicate tank one oysters began to reduce their numbers of *V. vulnificus* steadily for the remainder of the trial. When this trial was repeated with high flow (68 L/m) and a low ambient salinity (<10 ppt) (Figure 6D), the results were not similar to those shown in Figure 6C. *Vibrio vulnificus* numbers started at ~10,000 CFU/g in oysters but actually by day 6 had increased in both tanks. There was a temporary

decline in *V. vulnificus* numbers in oysters from both tanks on day 2, but on day 3 quickly returned to concentrations greater than the time 0 samples.

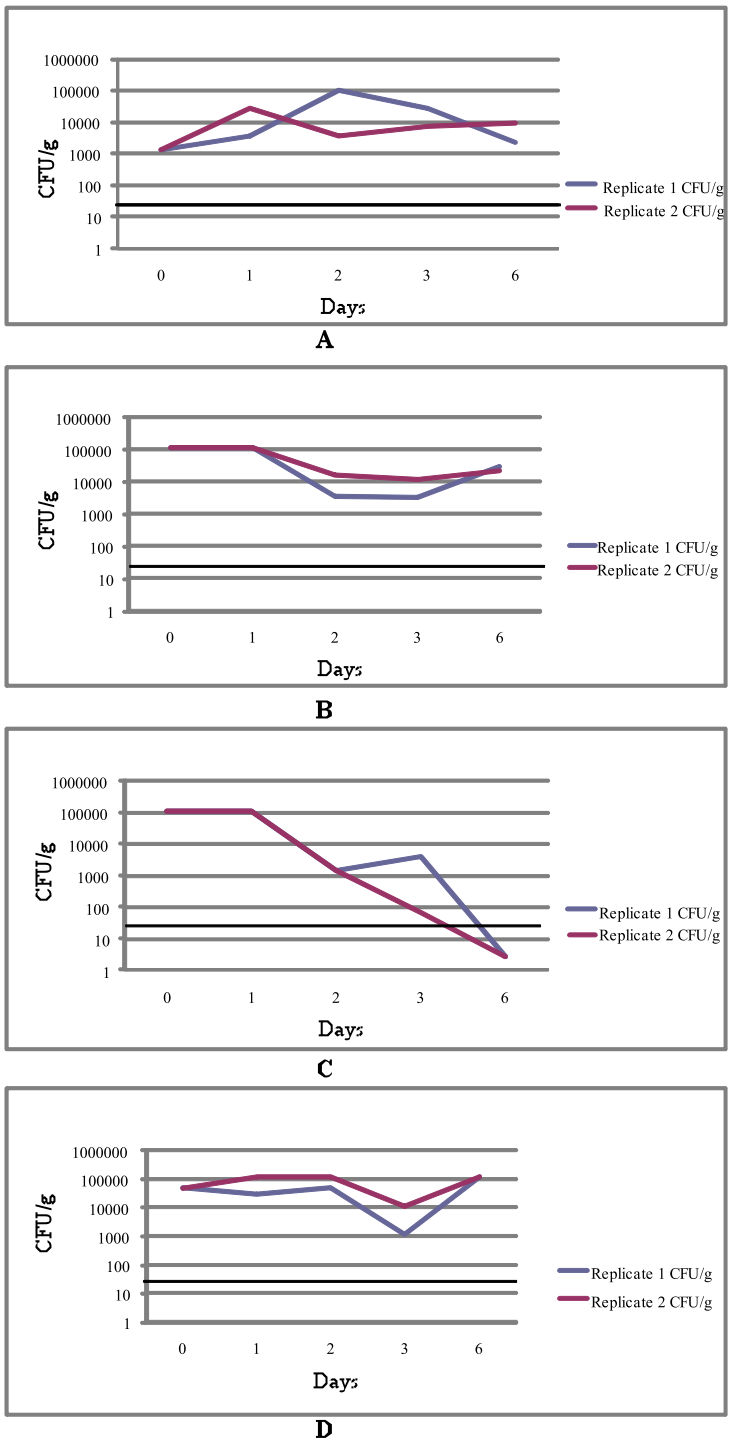


Fig. 6. Reduction in CFU/g of *V. vulnificus* in four different flow and salinity treatments: (A) low flow (11 L/m) and high salinity (>25 ppt); (B) medium flow (46 L/m) and high salinity (>25 ppt); (C) high flow (68 L/m) and high salinity (>25 ppt); (D) high flow (68 L/m) and low salinity (<10 ppt) over a 6-day period in a flow-through depuration system.

Analysis of Salinity and Flow Rate Relationship

When the four depuration trials shown in Figure 6 were analyzed further with an ANOVA followed by a post-hoc pairwise Tukey test, there was a significant effect of treatment. There was no significant difference ($P > 0.05$) between the low flow:high salinity and high flow:low salinity treatments, which showed no effective reduction. However, the medium flow:high salinity treatment had significantly higher depuration effectiveness than either of these two treatments ($P < 0.05$), and the high flow:high salinity treatment was significantly more effective than any of the treatments ($P < 0.05$).

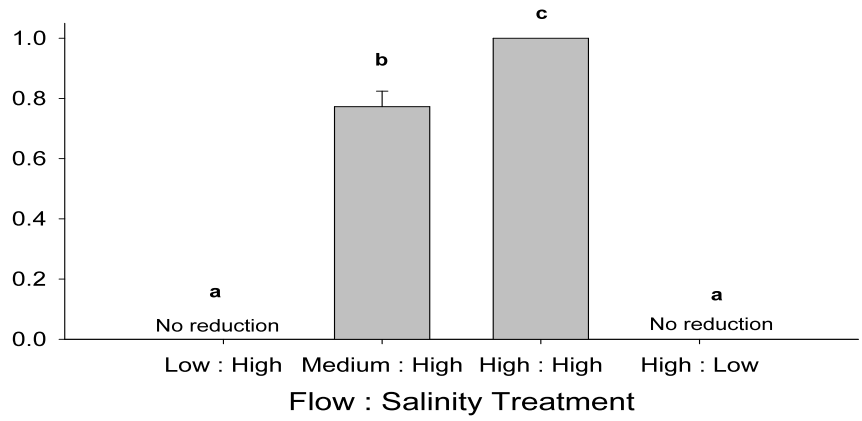


Fig.7. Proportional reduction in CFU/g of *V. vulnificus* in four different flow and salinity treatments: flow rates were 11, 46 and 68 L / min (low, medium, high). Salinity levels were ≥ 25 ppt and < 10 ppt (low, high). Error bars represent ± 1 standard deviation. Letters indicate significant differences between treatments.

Ribotyping of depuration isolates

Figure 8 shows a typical example of both 16S-RFLP types (A and B) commonly obtained for *V. vulnificus* following a digestion with *HaeIII* ran on a polyacrylamide gel. They both share bands at 204 bp and 120 bp but differ in that type B has two other bands at 147 bp and 21 bp, while type A has just one other band at 168 bp.

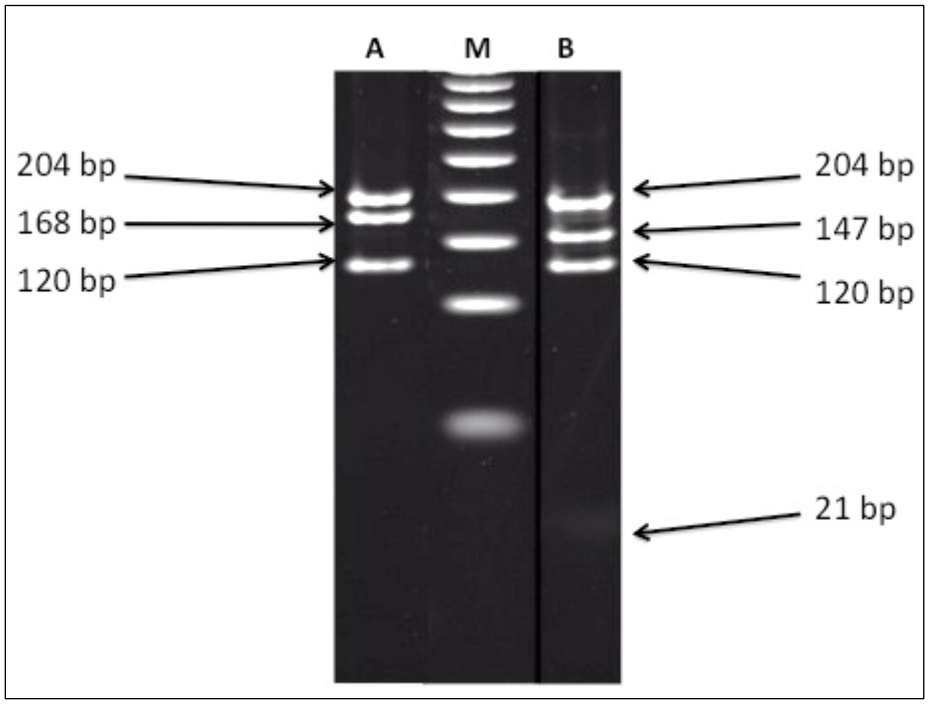


Fig. 8. Polyacrylamide RFLP gel electrophoresis (50 bp ladder) showing digestion of *Vibrio vulnificus* 16S rDNA with *HaeIII*.

Figure 9 shows an example of a typical RFLP pattern when a 492 bp segment of *V. vulnificus* 16S rDNA is amplified and digested with *HaeIII* and ran on a 3% agarose gel. There are 3 different banding patterns observed. Lanes 1, 2,10, 12 and 13 are *V. vulnificus*16S rDNA A, while lanes 5 and 6 are typical type B patterns. Lanes 3,4, 7, 8, 9 and 11 are suspected *Vibrio parahaemolyticus* strains.

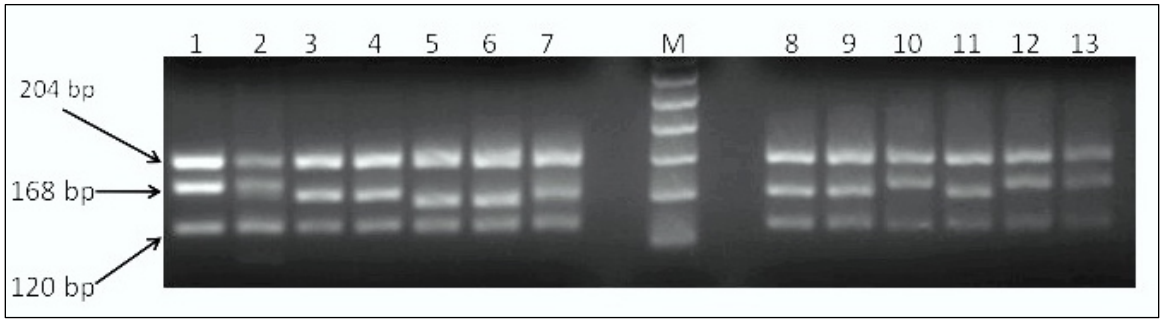


Fig. 9. RFLP digest of *Vibrio vulnificus* 16S rDNA with *HaeIII* ran on 3% agarose gel (50 bp ladder).

A total of 41 pre-depuration and 56-depuration strains were typed using the 16S-RFLP methodology. In total, 51 strains were 16S type A, while 46 were 16S type B. Type A strains were predominant before depuration (26 out of 41) and type B strains were more abundant after depuration (31 out of 56). However, this was not statistically significant and the conclusion is that both types A and B were equally distributed before and after depuration.

TABLE 3. Amounts of *Vibrio vulnificus* ribotypes A and B before and after depuration as determined by restriction fragment length polymorphisms (RFLP) analysis of the 16S rDNA sequences.

Ribotype:	**Pre-depuration**	**Post-depuration**
Type A	26	25
Type B	15	31

Figure 10 shows an AFLP® gel image with a corresponding dendogram generated from a cluster analysis using BioNumerics software. Two main clusters defined at 55% similarity were defined as are indicated as I and II. It is noteworthy that this technique yielded isolate-specific patterns of 65-80 distinct bands ranging from 100 bp to 900 bp (because of the molecular marker used for normalization only bands below 700 bp were included in the analysis).

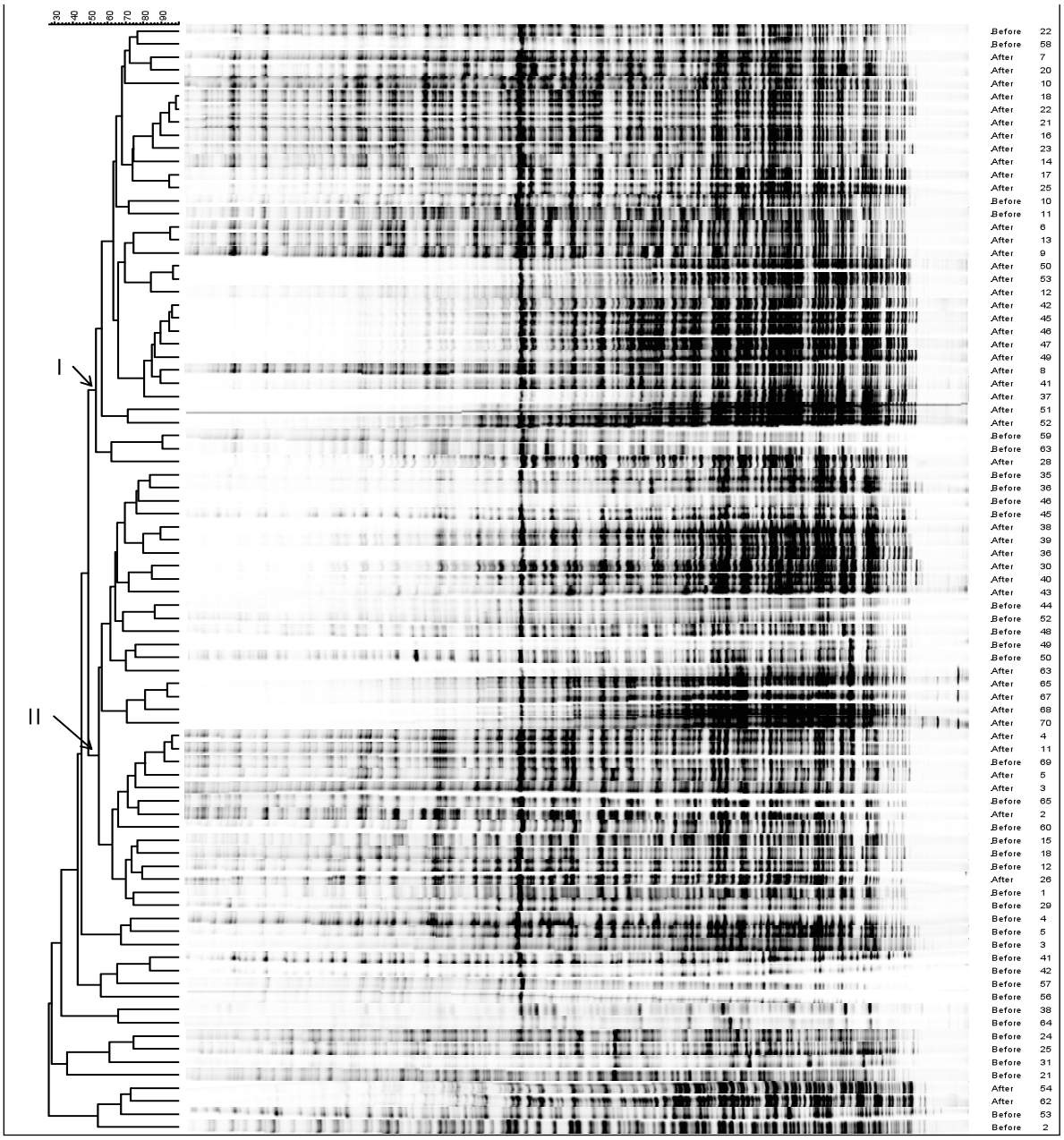


Fig. 10. AFLP® patterns of *V. vulnificus*. The dendogram was derived by UPGMA cluster analysis of the AFLP profiles of 46 *S. agalactiae* strains and reference strains. The tracks show the processed band patterns after conversion, normalization, and background subtraction. Levels of linkage are expressed as the Pearson product-moment similarity coefficient.

Figure 11 shows a multidimensional scaling (MDS) 3-D plot of isolates from the AFLP® gel analysis of pre- and post-depuration isolates generated from the BioNumerics software program. The two clusters that are circled represent the two clusters generated in Figure 10. This graph shows that the pre-depuration isolates are more evenly scattered among the plot. However, the after depuration isolates are mainly grouped in the two circled groups indicating they are more genetically homogenous.

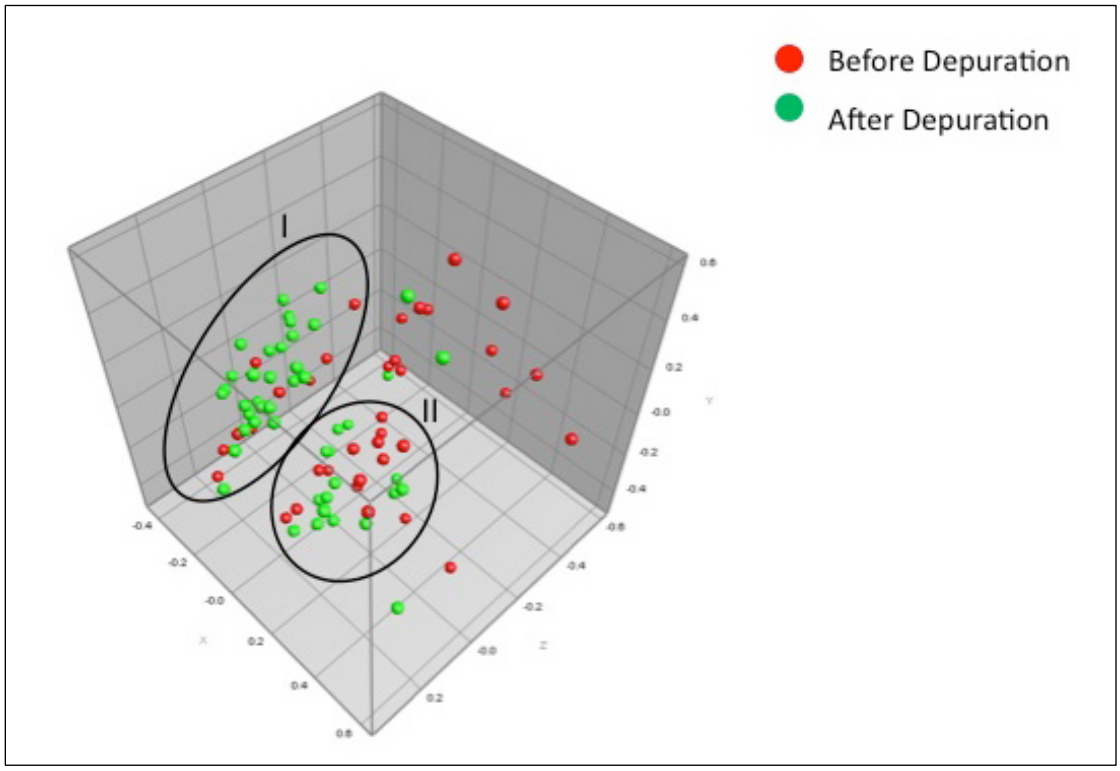


Fig. 11. Multidimensional scaling (MDS) plot showing the relatedness of isolates taken before and after depuration trials using AFLP data. Distance between entries represents graphic dissimilarities obtained from the similarity matrix. Before depuration and after depuration isolates are indicated by red and green dots respectively.

V. DISCUSSION

These findings demonstrate that it is possible to depurate *V. vulnificus* from oyster tissues using a flow-through system in accordance with FDA guidelines for the system. It is well known that depuration is highly dynamic and involves a delicate interplay between the physiology of oysters and bacteria (95). Previous studies primarily utilize recirculating depuration systems for elimination of *V. vulnificus* from oyster tissues (21, 48, 50, 95). There has been little investigation into the possibilities of elimination of *V. vulnificus* using flow-through depuration systems. Flow-through depuration systems allow for constant water removal to prevent any recontamination issues. It also constantly brings in food to encourage oysters to remain open and ventilation rate is constant, thereby allowing for constant removal of *V. vulnificus* cells. The presented study is the first pilot study evaluating the efficacy of a flow-through system with different variables for the elimination of *V. vulnificus* from oysters.

Reduction in artificially inoculated oysters (Figures 3A and B) is typical in oysters inoculated with laboratory-incubated strains of *V. vulnificus*. The oysters quickly take up the *V. vulnificus* cells from artificially inoculated water, but never becomes established in the oyster and are readily cleared (21, 54, 95). This is probably due to the loss of pili or other structures needed for attachment in the oyster by culturing in a laboratory. The spike in *V. vulnificus* cells seen on day 2 may be due to temporary shutdown of the flow to clean tanks of feces and pseudofeces. In contrast, Figure 3C shows no little reduction in *V. vulnificus* numbers over the 6-day trial period. This could be due to the lower average salinity (~14 ppt) during this trial

compared with the average salinity (~25 ppt) during the previous two experiments (Figures 3A and B)

As oysters are exposed to elevated temperatures during transportation to processing plants and the lag in arrival between processing for cold storage, *V. vulnificus* numbers could increase in oyster tissues from 10- to 100-fold in 10 h in warmer months (24, 106). When oysters were left at ambient temperatures (~35ºC) for 10 h after harvest followed by depuration for 6 days, there was no difference in *V. vulnificus* counts in those oysters versus those that were immediately depurated upon harvest (Figure 4A), meaning that depuration was equally effective in reducing *V. vulnificus* numbers in temperature-abused oysters as it was in freshly harvested stocks, although the reduction was not at the 30 CFU/g minimum required by the FDA.

Chae and colleagues (2009) reported that oysters greatly reduced their pumping rate when water was 3ºC, but pumping rate increased rapidly from 8 to 16ºC with no further increase after 16ºC. This indicated that oysters pumped water at its highest rate at 15ºC and when combined with the fact that *V. vulnificus* growth was halted at the same temperature, significant reductions should be observed (21). Tamplin and Capers (1992) also showed depuration seawater maintained at 15ºC resulted in no growth of *V. vulnificus* within oyster tissues (95). However, this was not the outcome of the present study. The oysters were taken from water averaging 30ºC and plunged into 15ºC water, so the lack of reduction seen in Figure 4B may be due to the oysters in the test tank (15ºC) having their pumping rate negatively affected by the rapid change in low water temperature which could result in a decrease of expelling *V. vulnificus* cells (21). It is worth mention that Chae and colleagues (2009) used oysters that were artificially inoculated, while the present study used oysters that were naturally colonized by *V. vulnificus* in the environment, which may also account for the lack of reduction.

It is well documented that salinities greater than 25 ppt negatively impacts the growth of *V. vulnificus* (50, 68, 69). This bacterium is rarely recovered from high salinity sites, while both *in vitro* and *in situ* experiments have shown that *V. vulnificus* growth is favored by relatively lower salinities (<16 ppt) (53). The salinity was raised to 35 ppt in the test tank with flow turned off for 12 h to allow oysters to soak in the 35 ppt water before restoring water flow. However, this resulted in very little reduction in *V. vulnificus* numbers in oyster tissues over a 6-day period (Figure 4C). The unusually high salinity could have shocked the oysters physiologically to where they needed the first 2 days to acclimate before resumption of normal pumping rate due to the optimum salinity of oysters being 15-18 ppt (99). The subsequent spike in *V. vulnificus* numbers seen in the test tank on day 6 could have been due to the 12 h periods of flow-through which could have allowed their numbers to increase without the selective pressure of high salinity.

To further investigate the relationship between salinity and temperature and the effects they have on reduction of *V. vulnificus* in oyster tissues, oysters with naturally occurring concentrations of *V. vulnificus* were depurated with a combination of lowered temperature (15ºC) and high salinity (35 ppt). This was achieved by using a chiller to lower water temperature while maintaining flow, then every 12 h flow was ceased and tank water was brought up to 35 ppt salinity for a brine soak. The high salinity should discourage the multiplication of *V. vulnificus* within the oysters and combined with the 15ºC water, significant reductions should be observed. However, none of the experiments testing this theory resulted at or below the target 30 CFU/g maximum final concentration (Figure 5). This could be due to the physiological stress of salinity that was rapidly increased along with the chiller not functioning properly causing drastic fluctuations in water temperature with the chiller maintaining at 15ºC and then breaking allowing water to rapidly climb back up to summer temperatures. This could cause the oysters to close and

experience a cessation in pumping activity, thereby preserving the *V. vulnificus* cells in their tissues, allowing for an increase in their numbers. The control tank average salinities were between 20 and 29 ppt in all four test experiments combining low temperature and high salinity. There is data that show that lower temperatures may have an effect on the resistance of *V. vulnificus* to salinities up to 25 ppt. This lower temperature (15ºC) may have increased the tolerance of *V. vulnificus* to the higher salinity, explaining why there was little reduction overall in all 4 experiments (Figure 5), even though average salinity was high (53).

The last variable to investigate was flow rate. It was hypothesized that an increased flow rate could increase the rate of bacteria carried away that is expelled by the oyster, and therefore result in higher reductions of *V. vulnificus* in oysters. The first increase was to 45 L/m from the previous 11 L/m (Figure 6B). This showed a significant ($P<0.05$) difference in *V. vulnificus* from time zero to the final day of the trial. However, the final concentration of *V. vulnificus* cells in oysters tissues were still at 29,000 CFU/g. It was then hypothesized that a further increase in flow rate might have even better reduction over the 6-day test period. This was demonstrated in Figure 6C where naturally occurring *V. vulnificus* cells in oyster tissues were reduced from an initial 10^5 CFU/g to 3 CFU/g in 6 days. The flow rate was 68 L/m, an increase from the original 11 L/m, and the previous 45 L/m. Interestingly, when this experiment was repeated one month later (Figure 6D), *V. vulnificus* concentrations actually increased by day 6 from their initial numbers in oyster tissues. The water temperature was the same for both experiments, but the second experiment (Figure 6D) was conducted during a time when the seawater had a consistent lower salinity (~6 ppt) for the entire trial period than the first (~28 ppt) (Figure 6C). This could allow for the proliferation of *V. vulnificus* as opposed to the higher salinity water that discourages the growth of the microorganism.

The depuration trial data show that only one trial showed promise as a viable means of *V. vulnificus* depuration from oysters utilizing a high flow and high salinity combination (Figure 6C). The UV/filter system was used in only one tank for all experiments. Experiments 1-3 showed no effect of the filter and UV-disinfection system, and may not be playing a large role in the removal of *V. vulnificus* from oyster tissues in the rest of the experiments. This was also observed by Kelly and colleagues (1985) when they observed marked reductions in *V. vulnificus* in depuration using seawater without bactericidal treatment (54). Figure 7 shows data that supports the idea that high salinity and high flow rate are both required for successful depuration to <30 CFU/g. When salinity was kept at high values, there were significant increases in depuration success with increasing flow indicating flow alone might have been the major factor. However, when flow was maintained at a high rate, but salinity was low, depuration was not successful. Thus, high flow alone does not guarantee depuration success.

Table 3 shows the results obtained from 16S rDNA-RFLP typing with the numbers of 16S-A *V. vulnificus* remaining constant throughout depuration. Studies looking at *V. vulnificus* typing have shown that the majority of environmental isolates are type A while most clinical isolates are type B (74). Approximately, 30% of isolates recovered pre-depuration were type B, which does not correlate with previous studies from the same geographic region. Nilsson *et al.* (2003) found that only 6% of isolates from oysters in the Gulf of Mexico and Atlantic Coasts were type B (74). Another group, (Kim and Jeong 2001) found that 65% of their environmental isolates from oysters, sediment, and seawater off the southern coast of Korea were type B. These findings coupled with the present study indicate geographical location is not the sole determinant of type A/B ratios in oysters (57). It is more likely seasonal, which is indicated by a study by Lin *et al.* (2003) that showed the prevalence of 16S type B was more common in the summer months

which also correlates with the higher incidence of disease (61). Interestingly, type B isolates doubled from 15 to 31 by the end of the 6-day period (Table 3), although the increase was not significant ($P>0.05$). Finally, one study suggested that Florida isolates do not follow the rule A=environmental and B=clinical as they had some clinical strains result in environmental (type A) profiles using rep-PCR testing (22).

The results of the AFLP cluster analysis (Figure 12) shows 2 main clusters that when compared to the multidimensional scaling (MDS) plot, indicate that the isolates obtained post-depuration are more genetically homogenous than those obtained prior to depuration. Logically one strain would be more likely to have specific adaptations to colonize the oysters making that strain more difficult to depurate.

VI. CONCLUSIONS

The depuration treatment that seemed to show the most promise is the high flow rate (68 L/m) in conjunction with a high salinity (28-32 ppt). When this trial was performed, a starting concentration of 10^5 CFU/g *V. vulnificus* in oyster tissues was reduced to 3 CFU/g in 6 days. When this was repeated with lower salinity, the numbers of *V. vulnificus* actually increased over the 6-day period. With these results, a new flow-through depuration system needs to be designed to accommodate a high flow rate while incorporating and maintaining a high salinity. This would require a multi-disciplinary approach by incorporating microbiology, engineering, and oyster physiology. This would allow for an efficiently designed system with regard to the dynamics between bacterial and oyster physiology. Oysters may be acclimated before being depurated in the high salinity and low temperature trials to prevent any physiological shock that would prompt them to close, ceasing pumping activity. This preliminary data shows promise that a system with these attributes could revolutionize the oyster industry by providing a safe, live eastern oyster that is safe for anyone to eat.

REFERENCES

1. **Andrews, L. S., M. Ahmedna, R. M. Grodner, J. A. Liuzzo, E. A. Murano, R. M. Rao, S. Shane, and P. W. Wilson.** 1998. Food Preservation Using Ionizing Radiation. Reviews in Environmental Contamination Toxicology **154:**1-53.
2. **Andrews, L. S., S. DeBlanc, C. D. Veal, and D. L. Park.** 2003. Response of *Vibrio parahaemolyticus* 03:K6 to a Hot Water/Cold Shock Pasteurization Process. Food Additives and Contaminants **20:**331-334.
3. **Andrews, L. S., M. Jahncke, and K. Millikarjunan.** 2003. Low-Dose Gamma Irradiation to Reduce Pathogenic *Vibrios* in Live Oysters (*Crassostrea virginica*). Aquatic Food ProtectionTechnology **12:**71-82.
4. **Archer, D. L.** 2004. Freezing: An Underutilized Food Safety Technology? International Journal of Food Microbiology **90:**127-138.
5. **Arias, C., M. Majcian, R. Aznar, E. Garay, and M. Pujalte.** 2001. Low Incidence of *Vibrio vulnificus* Among *Vibrio* isolates from Seawater and Shellfish of the Western Mediterranean. Applied Microbiology **86:**125-134.
6. **Arias, C. R., E. Garay, and R. Aznar.** 1995. Nested PCR method for rapid and sensitive detection of *Vibrio vulnificus* in fish, sediments, and water. Applied and Environmental Microbiology **61:**3476-3478.
7. **Aznar, R., W. Ludwig, and K. H. Schleifer.** 1993. Sequence determination of rRNA genes of pathogenic Vibrio species and whole-cell identification of *Vibrio vulnificus* with rRNA-targeted oligonucleotide probes. International Journal of Systematic Bacteriology **44:**330-337.
8. **Bang, Y. B., S. E. Lee, J. H. Rhee, and S. H. Choi.** 1999. Evidence that Expression of the *Vibrio vulnificus* Hemolysin Gene Is Dependent on Cyclic AMP and Cyclic AMP Receptor Protein. Bacteriology **181:**7639-7642.
9. **Berlin, D. L., D. S. Herson, D. T. Hicks, and D. G. Hoover.** 1999. Response of pathogenic Vibrio species to high hydrostatic pressure. Applied and Environmental Microbiology **65:**2776-80.
10. **Biosca, E., J. Oliver, and C. Amaro.** 1996. Phenotypic Characterization of *Vibrio vulnificus* Biotype 2, a Lipopolysaccharide-Based Homogenous O Serogroup within *Vibrio vulnificus*. Applied and Environmental Microbiology **65:**918-927.
11. **Bisharat, N., V. Agmon, R. Finkelstein, R. Raz, G. Ben-Dror, L. Lerner, S. Soboh, R. Colodner, D. N. Cameron, D. L. Wykstra, D. L. Swerdlow, and J. J. Farmer.** 1999. Clinical, epidemiological, and microbiological features of *Vibrio vulnificus* biotype 3 causing outbreaks of wound infection and bacteraemia in Israel. The Lancet **354:**1421-1424.
12. **Boeckmann, B., A. Bairoch, R. Apweiler, M.-C. Blatter, A. Estreicher, E. Gasteiger, M. J. Martin, K. Michoud, C. O'Donovan, I. Phan, S. Pilbout, and M. Schneider.** 2003. The SWISS-PROT protein knowledgebase and its supplement TrEMBL in 2003. Nucleic Acids Res. **31:**365-370.

13. **Brasher, C. W., A. DePaola, D. D. Jones, and A. K. Bej.** 1998. Detection of microbial pathogens in shellfish with multiplex PCR. Current Microbiology **37:**101-7.
14. **Brauns, L., M. Hudson, and J. Oliver.** 1991. Use of the Polymerase Chain Reaction in Detection of Culturable and Nonculturable *Vibrio vulnificus* Cells. Applied and Environmental Microbiology **57:**2651-2655.
15. **Buchrieser, C., V. V. Gangar, R. L. Murphree, M. L. Tamplin, and C. W. Kaspar.** 1995. Multiple *Vibrio vulnificus* Strains in Oysters as Demonstrated by Clamped Homogenous Electric Field Electrophoresis. Applied and Environmental Microbiology **61:**1163-1168.
16. **Bugg, J. C., Jr.** 1969. Presented at the Gulf and South Atlantic States Shellfish Sanitation Research Conference.
17. **Burdeau, C.** 2009. FDA to Ban Sale of Raw Oysters from Gulf of Mexico. Associated Press.
18. **Calik, H., M. T. Morrissey, and H. An.** 2002. Effect of high-pressure processing on *Vibrio parahaemolyticus* strains in pure culture and pacific oysters. Journal of food Science **67:**1506-1510.
19. **Campbell, M. S., and A. C. Wright.** 2003. Real-time PCR analysis of *Vibrio vulnificus* from oysters. Applied and Environmental Microbiology **69:**7137-7144.
20. **Cerda-Cuellar, M., J. Jofre, and A. R. Blanch.** 2000. A selective medium and specific probe for detection of *Vibrio vulnificus*. Applied and Environmental Microbiology **66:**855-859.
21. **Chae, M. J., D. Cheney, and Y.-C. Su.** 2009. Temperature Effects on the Depuration of *Vibrio parahaemolyticus* and *Vibrio vulnificus* from the American Oyster (*Crassostrea virginica*). Journal of Food Science **74:**62-66.
22. **Chatzidaki-Livanis, M., M. A. Hubbard, K. Gordon, V. J. Harwood, and A. C. Wright.** 2006. Genetic distinctions among clinical and environmental strains of *Vibrio vulnificus*. Applied and Environmental Microbiology **72:**6136-6141.
23. **Coen, L. D., and M. W. Luckenbach.** 2000. Developing Success Criteria and Goals for Evaluating Oyster Reef Restoration: Ecological Function or Resource Exploitation? Ecological Engineering **15:**323-343.
24. **Cook, D.** 1997. Refrigeration of Oyster Shellstock: Conditions Which Minimize the Outgrowth of *Vibrio vulnificus* Journal of Food Protection **60:**349-52.
25. **Cook, D.** 2003. Sensitivity of *Vibrio* Species in Phosphate-Buffered Saline and in Oysters to High-Pressure Processing. Food Protection **66:**2276-82.
26. **Danila.** 2002. Food Irradiation FAQ. Organic Consumers Association.
27. **Decostere, A., F. Haesebrouck, E. Van Driessche, G. Charlier, and R. Ducatelle.** 1999. Characterization of the adhesion of *Flavobacterium columnare* (*Flexibacter columnaris*) to gill tissue. Journal of Fish Diseases **22:**465-474.
28. **DePaola, A., G. M. Capers, and D. Alexander.** 1994. Densities of *Vibrio vulnificus* in the intestines of fish from the U.S. Gulf Coast. Applied and Environmental Microbiology **60:**984-8.
29. **Drake, S. L., A. DePaola, and L.-A. Jaykus.** 2007. An Overview of *Vibrio vulnificus* and *Vibrio parahaemolyticus*. Comprehensive Reviews in Food Science and Food Safety **6:**120-144.
30. **Eccleston, B.** 1998. UV Intensity Levels Affected by Water Quality. Water Technology **21:**61-68.

31. **EOBRT.** 2007. Status Review of the Eastern Oyster (*Crassostrea viriginica*). Report. National Marine Fisheries Service.
32. **FDA.** 2009. FDA Statement on *Vibrio vulnificus* in Raw Oysters.
33. **FDA.** 2002. MPN procedure for the enumeration of *Vibrio vulnificus* using gene probe for identification. FDA/Office of Seafood.
34. **FDA.** 2007. The Guide for the Control of Molluscan Shellfish.
35. **Fox, J. M., and A. L. Chauvin.** 1988. Presented at the OCEANS '88: A Partnership of Marine Interests, Baltimore, MD.
36. **Furfari, S. A.** 1966. Depuration Plant Design. **999-FP-7**.
37. **Furfari, S. A.** 1976. Shellfish Purification: A Review of Current Technology FAO Conference on Aquaculture.
38. **Gray, L. D., and A. S. Kreger.** 1989. Detection of *Vibrio vulnificus* Cytolysin in *Vibrio vulnificus* infected Mice. Toxicon **27:**459-464.
39. **He, H., R. M. Adams, D. E. Farkas, and M. T. Morrissey.** 2002. Use of high-pressure processing for oyster shucking and shelf-life extension. Journal of Food Science **67:**640-645.
40. **Heidelberg, J. F., K. B. Heidelberg, and R. R. Colwell.** 2002. Bacteria of the ϒ-Subclass *Proteobacteria* Associated with Zooplankton in Chesapeake Bay. Applied and Environmental Microbiology **68:**5498-507.
41. **Hesselman, D. M., M. L. Motes, and J. P. Lewis.** 1999. Effects of a Commercial Heat-Shock Process on *Vibrio vulnificus* in the American Oyster, *Crassostrea virginica*, Hravested from the Gulf Coast. Journal of Food Protection **11:**1266-69.
42. **Hoi, L., I. Dalsgaard, and A. Dalsgaard.** 1998. Improved Isolation of *Vibrio vulnificus* from Seawater and Sediment with Cellobiose-Colistin Agar. Applied and Environmental Microbiology **64:**1721-1724.
43. **Hoi, L., J. L. Larsen, I. Dalsgaard, and A. Dalsgaard.** 1998. Occurrence of *Vibrio vulnificus* Biotypes in Danish Marine Environments. Applied and Environmental Microbiology **64:**7-13.
44. **Hollis, D. G., R. E. Weaver, C. N. Baker, and C. Thornsberry.** 1976. Halophilic *Vibrio* Species Isolated from Blood Cultures. Clinical Microbiology **3:**425-31.
45. **Hoover, D. G., C. Metrick, A. M. Papineau, D. F. Farkas, and D. Knorr.** 1989. Biological effects of high hydrostatic pressure on food microorganisms. Food Technology **43:**99-107.
46. **Huntley, B. E., and R. J. Hammerstrom.** 1971. An Experimental Depuration Plant: operation and Evaluation. Chesapeake Science **12:**4.
47. **Huq, A., A. B. Small, P. A. West, M. I. Huq, R. Rahman, and R. R. Colwell.** 1983. Ecological Relationship between *Vibrio cholerae* and Planktonic Crustacean Copepods. Applied and Environmental Microbiology **45:**275-283.
48. **Jamison, J. L.** 1992. Laboratory and Field Evaluations of Commercial Oyster Depuration in the Gulf of Mexico. NOAA.
49. **Jones, M. K., and J. D. Oliver.** 2009. *Vibrio vulnificus*: Disease and Pathogenesis. Infection and Immunity **77:**1723-1733.
50. **Kaspar, C. W., and M. L. Tamplin.** 1993. Effects of Temperature and Salinity on the Survival of *Vibrio vulnificus* in Seawater and Shellfish. Applied and Environmental Microbiology **59:**2425.

51. **Kaysner, C. A., C. Abeyta, Jr., M. M. Wekell, A. DePaola, Jr., R. F. Stott, and J. M. Leitch.** 1987. Virulent strains of Vibrio vulnificus isolated from estuaries of the United States West Coast. Applied and Environmental Microbiology **53:**1349-51.
52. **Keithly, W. R., and H. Diop.** 2001. The Demand for Eastern Oysters, *Crassostrea virginica*, from the Gulf of Mexico in the Presence of *Vibrio vulnificus*. Marine Fisheries Review **63:**47-53.
53. **Kelly, M. T.** 1982. Effect of temperature and salinity on *Vibrio (Beneckea) vulnificus* occurence in a Gulf Coast environment. Applied and Environmental Microbiology **44:**820-824.
54. **Kelly, M. T., and A. Dinuzzo.** 1985. Uptake and Clearance of *Vibrio vulnificus* from Gulf Coast Oysters (*Crassostrea virginica*). Applied and Environmental Microbiology **50:**1548-1549.
55. **Kennedy, V. S., R. I. E. Newell, and A. F. Eble.** 1996. The Eastern Oyster: *Crassostrea virginica*. Maryland Sea Grant College, University of Maryland System, College Park.
56. **Kilgen, M. B., M. T. Hemard, D. Duet, and S. Rabalais.** 2001. Collaborative Evaulation of Commercial Irradiation for *Vibrio vulnificus* Control in Louisiana Oysters. International Atomic Energy Agency Technical Document.
57. **Kim, M. S., and H. D. Jeong.** 2001. Development of 16S rRNA targeted PCR methods for the detection and differentiation of *Vibrio vulnificus* in marine environments. Aquaculture **193:**199-211.
58. **Kim, S. Y., S. E. Lee, Y. R. Kim, C. M. Kim, P. Y. Ryu, H. E. Choy, S. S. Chung, and J. H. Rhee.** 2003. Regulation of *Vibrio vulnificus* Virulence by the LuxS Quorum-Sensing System. Molecular Microbiology **48:**1647-1664.
59. **Lee, B. C., S. H. Choi, and T. S. Kim.** 2008. *Vibrio vulnificus* RTX Toxin Plays an Important Role in the Apoptotic Death of Human Intestinal Epithelial Cells Exposed to *Vibrio vulnificus*. Microbes and Infection **10:**1504-13.
60. **Lee, R., A. Lovatelli, and L. Ababouch.** 2008. Bivalve Depuration: Fundamental and Practical Aspects. FAO Fisheries Technical Paper **511:**139.
61. **Lin, M., and J. R. Schwarz.** 2003. Seasonal shifts in population structure of *Vibrio vulnificus* in an estuarine environment as revealed by partial 16S ribosomal DNA sequencing. FEMS Microbiology Ecology **45:**23-27.
62. **Litwin, C. M., T. W. Rayback, and J. Skinner.** 1996. Role of Catechol Siderophore Synthesis in *Vibrio vulnificus* Virulence. Infection and Immunity **64:**2834.
63. **Mahmoud, B. S. M.** 2009. Reduction of *Vibrio vulnificus* in Pure Culture, Half Shell and Whole Shell Oysters (*Crassostrea virginica*) by X-ray. International Journal of Food Microbiology **130:**135-139.
64. **Mahmoud, Z. H., S. B. Neogi, A. Kassu, B. T. M. Huong, I. K. Jahid, M. S. Islam, and F. Ota.** 2008. Occurrence, Seasonality and Genetic Diversity of *Vibrio vulnificus* in Coastal Seaweeds and Water Along the Kii Channel, Japan. FEMS Microbiology Ecology **64:**209-218.
65. **Mallett, J. C., L. E. Beghian, T. G. Metcalf, and J. D. Kaylor.** 1991. Potential of Irradiation Technology for Improved Shellfish Sanitation. Food Safety **11:**231-245.
66. **Massad, G., and J. D. Oliver.** 1987. New Selective and Differential Medium for *Vibrio cholerae* and *Vibrio vulnificus*. Applied and Environmental Microbiology **53:**2262-2264.

67. **Merkel, S., S. Alexander, E. Zufall, J. Oliver, and Y. Huet-Hudson.** 2001. Essential Role for Estrogen in Protection Against *Vibrio vulnificus*-Induced Endotoxic Shock. Infection and Immunity **69:**6119-6922.
68. **Motes, M. L., and A. DePaola.** 1996. Offshore suspension relaying to reduce levels of *Vibrio vulnificus* in oysters (*Crassostrea virginica*). Applied and Environmental Microbiology **62:**3875-3877.
69. **Motes, M. L., A. DePaola, D. W. Cook, J. E. Veazey, J. C. Hunsucker, W. E. Garthright, R. J. Blodgett, and S. J. Chirtel.** 1998. Influence of water temperature and salinity on *Vibrio vulnificus* in Northern Gulf and Atlantic coast oysters (*Crassostrea virginica*). Applied and Environmental Microbiology **64:**1459-1465.
70. **Musher, D. M., M. V. Hansen, A. Goree, F. Gyorkey, A. J. Chapman, and R. E. Baughn.** 1986. Emergence of Bacteriacidal and Opsonizing Antibody to *Vibrio vulnificus* Following Bacterial Infection. Clinical Microbiology **23:**411-415.
71. **Muth, K. M.** 2002. Effects of Post-Harvest Treatment Requirements on the Markets for Oysters. Agircultural and Resource Economics Review.
72. **Muth, M. K., S. A. Karns, D. W. Anderson, and B. C. Murray.** 2002. Effects of Post-Harvest Treatment Requirements on the Markets for Oysters. Agircultural and Resource Economics Review.
73. **Nelson, C.** 2009. FDA's Proposed Plan Could Kill the Business Along the Coast. The Press Register.
74. **Nilsson, W. B., R. N. Pranjype, A. DePaola, and M. S. Strom.** 2003. Sequence polymorphism of the 16S rRNA gene of *Vibrio vulnificus* is a possible indicator of strain virulence. Journal of Clinical Microbiology **41:**442-446.
75. **Novak, A. F., J. A. Liuzzo, R. M. Grodner, and R. T. Lovell.** 1966. Radiation Pasteurization of Gulf Oysters. Food Technology **20:**103-4.
76. **O'Neil, K. R., S. H. Jones, and D. J. Grimes.** 1992. Seasonal Incidence of *Vibrio vulnificus* in the Great bay Estuary of New Hampshire and Maine. Applied and Environmental Microbiology **58:**3257-62.
77. **Oliver, J. D.** 2006. *Vibrio vulnificus*, p. 349-366. *In* F. L. Thompson, B. Austin, and J. Swing (ed.), The Biology of Vibrios. ASM Press, Washington, DC.
78. **Otwell, W. S., G. E. Rodrick, and R. E. Martin.** 1991. Molluscan Shellfish Depuration. CRC Press, Inc., Boca Raton, FL.
79. **Panicker, G., M. L. Myers, and A. K. Bej.** 2004. Rapid Detection of *Vibrio vulnifucus* in Shellfish and Gulf of Mexico Water by Real-Time PCR. Applied and Environmental Microbiology **70:**498-507.
80. **Paranjpye, R. N., and M. S. Strom.** 2005. A *Vibrio vulnificus* Type IV Pilin Contributes to Biofilm Formation, Adherence to Epithelial Cells, and Virulence. Infection and Immunity **73:**1411-1422.
81. **Perkins, B. E.** 1997. Aquacultured Oysters. Alabama Cooperative Extension System **ANR-1030**.
82. **Pitcher, D. G., N. A. Saunders, and R. J. Owen.** 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Letters in Applied Microbiology **8:**151-156.
83. **Rhee, J. E., J. H. Rhee, P. Y. Ryu, and S. H. Choi.** 2002. Identification of the *cadBA* operon from *Vibrio vulnificus* and its Influence on Survival to Acid Stress. FEMS Microbiology Letters **208:**245-251.

84. **Schardt, S., and S. Schmidt.** 1996. Fishing for Safe Seafood. Nutrition Action Healthletter **23:**1-5.
85. **Schill, W. B., S. R. Phelps, and S. W. Pyle.** 1984. Multilocus eletrophoretic assesment of the genetic structure and diversity of *Yersinia ruckeri*. Appl Environ Microbiol **48:**975-979.
86. **Schwarz, J. R.** 1999. Validation of Individual Quick Freezing (IQF) of Oysters as a Post-Harvest Treatment Process. Texas A&M University at Galveston.
87. **Shin, S. H., D. Y. Shin, P. Y. Rhu, S. S. Chung, and J. H. Rhee.** 2002. Pro-infalmmatory Cytokine Profile in *Vibrio vulnificus* Septicemic Patients' Sera. FEMS Immunology and Medical Microbiology **33:**133-138.
88. **Simpson, L. M., V. K. White, S. F. Zane, and J. D. Oliver.** 1987. Correlation Between Virulence and Colony Morphology in *Vibrio vulnificus*. Infection and Immunity **55:**269-272.
89. **Smith, F. L. J.** 1993. Method of and Apparatus for Use in the Depuration of Bivalves. United States.
90. **Stabb, E. V., and E. G. Ruby.** 2003. Contribution of *pilA* to Competitive Colonization of the Squid *Euprymna scolopes* by *Vibrio fischeri*. Applied and Environmental Microbiology **69:**820-826.
91. **Strom, M. S., and R. N. Paranjpye.** 2000. Epidemiology and pathogenesis of *Vibrio vulnificus*. Microbes and infection **2:**177-188.
92. **Tacker, C. O., F. Brenner, and P. A. Blake.** 1984. Clinical Features and An Epidemiological Study of *Vibrio vulnificus* Infections. Infectious Diseases **149:**558-.
93. **Tamplin, M., A. Martin, A. Ruple, W. Cook, and C. Kaspar.** 1991. Enzyme Immuno Assay for Identification of *Vibrio vulnificus* in Seawater, Sediment, and Oysters. Applied and Environmental Microbiology **57:**1235-1240.
94. **Tamplin, M., G. E. Rodrick, N. J. Blake, and T. Cuba.** 1982. Isolation and Characteriziation of *Vibrio vulnificus* from Two Florida Estuaries. Applied and Environmental Microbiology **44:**1466-70.
95. **Tamplin, M. L., and G. M. Capers.** 1992. Persistance of *Vibrio vulnificus* in tissues of Gulf Coast oysters, *Crassostrea virginica,* exposed to seawater deisinfected with UV light. Applied and Environmental Microbiology **58:**1506-1510.
96. **Vanoy, R. W., M. L. Tamplin, and J. R. Schwarz.** 1992. Ecology of *Vibrio vulnificus* in Galveston Bay Oysters, Suspended Particulate Matter, Sediment and Seawater: Detection by Monoclonal Antibody-Imminoassay-Most Probable Number Procedures. Industrial Microbiology **9:**219-23.
97. **Vartain, C. V., and E. J. Septimus.** 1990. Osteomyelitis Caused by *Vibrio vulnificus*. Infectious Diseases **161:**363-.
98. **Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. Van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuper, and M. Zabeau.** 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. **23:**4407-4414.
99. **Wallace, R. K.** 2001. Cultivating the Eastern Oyster, *Crassostrea virginica*. Southern Regional Aquaculture Center **432**.
100. **Wang, R., W. Cao, and C. Cerniglia.** 1997. A Universal Protocol for PCR Detection of 13 Species of Foodborne Pathogens in Foods. Applied Microbiology **83:**727-736.

101. **Warner, J. M., and J. D. Oliver.** 1999. Randomly Amplified Polymorphic DNA Analysis of Clinical and Environmental Isolates of *Vibrio vulnificus* and other *Vibrio* species. Applied and Environmental Microbiology **65:**1141.
102. **WDSU.** 2009. New Process Could Hurt LA Oyster Industry. WDSU News.
103. **Weise, E.** 2009. FDA Plan Limits Live-Oyster Sales from Gulf in Warm Months. USA Today.
104. **Wright, A. C., R. T. Hill, J. A. Johnson, M.-C. Roghman, R. R. Colwell, and G. J. J. Morris.** 1996. Distribution of *Vibrio vulnificus* in the Chesapake Bay. Applied and Environmental Microbiology **62:**717-724.
105. **Wright, A. C., G. A. Miceli, W. L. Landry, J. B. Christy, W. D. Watkins, and J. G. Morris.** 1993. Rapid identification of *Vibrio vulnificus* on nonselective media with an alkaline phosphatase-labeled oligonucleotide probe. Applied and Environmental Microbiology **59:**541-546.
106. **Zuppardo, A. B., A. DePaola, J. C. Bowers, K. L. Schully, J. A. Gooch, and R. J. Siebeling.** 2001. Heterogeneity of environmental, retail, and clinical isolates of *Vibrio vulnificus* as determined by lipopolysaccharide-specific monoclonal antibodies. J Food Prot **64:**1172-7.