AN ABSTRACT OF THE THESIS OF

Hakan Calik for the degree of Master of Science in Food Science and Technology presented on October 2, 2001. Title: Effect of High Pressure Processing on the Survival of *Vibrio parahaemolyticus* Strains in Pure Culture and Pacific Oysters.

Abstract approved: _____
Michael T. Morrissey

Different strains of *Vibrio parahaemolyticus* (Vp) in broth cultures and Vp-inoculated live Pacific oysters (*Crassostrea gigas*) were subjected to high pressure processing (HPP) at 241, 276, 310, and 345 MPa. Results showed Vp numbers were reduced by HPP in both pure culture and whole oysters. Vp inactivation was dependent on time and pressure. Optimum conditions for reducing Vp in pure culture and oysters to non-detectable levels were achieved at 345 MPa for 30 and 90 s, respectively. Resistance variations were detected between Vp in pure culture and in oysters. HPP proved to be an efficient means of reducing Vp in oysters.

The decimal reduction rates of Vp O3:K6 strain was compared with clinical ATCC 17802 and environmental AST Vp strains in pure culture and Pacific oysters under high pressure processing (HPP) at 276 and 310 MPa for different treatment times based on Calik and others (2001) data. Results showed that O3:K6 strain had relatively higher decimal reduction rates when compared to two other strains. The O3:K6 strain in oyster had the highest D-value of 1.77 min under 276 MPa HPP

treatment. Reduction trends presented by Vp O3:K6 strain must be considered when making commercial HPP setting decisions for reduction of Vp.

Effect of High Pressure Processing on the Survival of *Vibrio parahaemolyticus* Strains in Pure Culture and Pacific Oysters

by

Hakan Calik

A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirement for the

degree of

Master of Science

Complete October 2, 2001

Commencement June 2002

Master of Science thesis of Hakan Calik presented on October 2, 2001

APPROVED:

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Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Hakan Calik, Author

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my major professors Dr. Michael T. Morrissey and Dr. Haejung An for their invaluable advice, guidance and support throughout my study here at the Oregon State University Seafood Laboratory. I would further like to thank Dr. Paul W. Reno and Dr Jae W. Park who were always helpful and willing to give advice and support. My fellow student friends, Okan, Jacek, Haian and Tana deserve many thanks for their academic advice and valuable friendship, too. My thankfulness is extended to the Seafood Lab staff -Nancy, Tami, Toni, Angee, and Roger- for their kindness, helpfulness and moral support. With you, it was truly fun to get educated in this lovely rainy town, Astoria.

I would like to express my deep appreciation to my family who always support me with their love. It is to them that my work is dedicated.

CONTRIBUTION OF AUTHORS

Dr. Michael T. Morrissey, Dr. Haejung An, and Dr. Paul W. Reno were involved in the design, analysis, and writing of each manuscript. Mr. Roger T. Adams guided the high hydrostatic pressure processing. Ms. Prudence Caswell-Reno assisted in data collection for the study.

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CHAPTER 1

INTRODUCTION

High pressure processing (HPP) technology has attracted increased attention as a means of non-thermal treatment of raw or fresh foods for reduction of bacterial loads without causing significant changes in appearance, flavor, texture and nutritional qualities (Styles and others 1991; Berlin and others 1999). The application of HPP in foods was first investigated by Hite and his co-workers in 1899 (Hite 1899). HPP is a 'heatless' process where foods in flexible packaging are subjected to hydrostatic pressures up to 1035 Mega Pascal (MPa) (Swientek 1992). A typical HPP system consists of a high pressure vessel and its closure, a pressure generation system, a temperature control device, and a material handling system (Mertens and others 1993). Treatment effects are achieved through a pressure-transferring medium, which is usually water. The pressure is transmitted in a uniform and instantaneous manner throughout the whole biological sample without the necessity of direct contact with the pressure medium. Several studies showed that HPP treatment reduced the microbiological load and enzymatic activity of foods at ambient or low temperatures without breaking covalent bonds, thereby retaining many of the natural qualities of foods such as, flavor, color, and nutritional constituents (Hoover and others 1989; Ohshima and others 1993;

Hayashi and others 1989; Berlin and others 1999; He and others 2001). HPP can be used to inactivate microorganisms by inducing changes to the morphology, biochemical reactions, genetic mechanisms, cell membranes and overall, to microorganisms (Hoover and others 1989; Smelt 1998). Intracellular vacuoles can collapse at quite a low pressure of about 0.6 MPa (Walsby 1973) and cell division is slowed with the application of pressure (Zobell 1964). Under pressure, membranes are compressed and permeability is altered, eventually causing cell death (Morita 1975). The process has shown good potential in reducing pathogens and spoilage bacteria in the food industry (Farr 1990). Research has shown that *Vibrio* sp. are very susceptible to inactivation by HPP technology (Styles and others 1991; Farkas 1993; Hoover 1993; Berlin and others 1999). Berlin and others (1999) showed that pathogenic *Vibrio* sp. are susceptible to HPP treatment at pressure levels between 200-300 MPa.

Numerous epidemics of severe illness and gastroenteritis have been associated with the consumption of raw oysters in the United States, with the majority being attributed to *Vibrio* sp. (CDC 1989). *Vibrio parahaemolyticus* and *Vibrio vulnificus* have been implicated in outbreaks of gastroenteritis in the Southeast and the Pacific Northwest (Klontz and others 1993; Kaysner 1998). *V. parahaemolyticus* is an actively motile, gram-negative, curved, rod-shaped bacteria naturally present in marine water with an extremely short generation time (Kaysner and others 1987). *V. parahaemolyticus* is the most widely distributed of all the human pathogenic *Vibrio* sp. in the marine environment (Hagen and others 1994).

In 1997, more than 200 cases of gastroenteritis were caused by consumption of *V. parahaemolyticus* infected raw oysters harvested from California, Oregon, Washington, and British Columbia (CDC 1998). One of the largest *V. parahaemolyticus* outbreaks reported occurred in Galveston Bay area (Texas) between May and July 1998 causing gastroenteritis in 416 persons, including 15 hospitalized victims (CDC 2001). Oysters are water-filtering organisms and can accumulate pathogenic microorganisms, which are difficult to remove by depuration but can be eliminated by high pressure. Recent work using HPP has shown significant reduction in total plate counts of aerobic and anaerobic bacteria in oysters while maintaining good sensory characteristics (Shiu 1999; Lopez-Caballero and others 2000; He and others 2001).

The objectives of this study were: (1) to determine the effect of HPP treatment on clinical and environmental Vp strains in pure culture and in inoculated Pacific whole oysters and establish optimum HPP conditions (time/pressure) for Vp inactivation, and (2) to determine if there is any pressure resistance variation among different strains of Vp in pure culture and oysters.

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CHAPTER 2

LITERATURE REVIEW

H. Calik

Vibrio Genus Description

The genus *Vibrio* belongs to the family *Vibrionaceae* and includes 23 non-pathogenic and 12 pathogenic species for humans, including *V. cholerae, V. parahaemolyticus, V. vulnificus, V. alginolyticus, V. cincinnatiensis, V. carchariae, V. damsela, V. fluvialis, V. furnissii, V. hollisae, V. metschnikovii, V. mimicus* (Kelly and others 1991). The members of the genus *Vibrio* are characterized as gram (-) negative straight or curved rods, and are 0.5 – 0.8 μm in width and 1.4 – 2.6 μm in length. They are motile in liquid media with a singular polar flagellum. They do not form endospores and are facultative anaerobes capable of respiratory and fermentative metabolism. Most produce catalase as well as oxidase and all are chemoorganotrophs; most ferment glucose without gas production (Hugh and Feeley 1972). With the exception of *V. cholerae* and *V. mimicus*, *Vibrio* species require 2 – 3% NaCl for growth, and are referred to as "halophilic" (salt-loving) bacteria (Baumann and others 1986).

Vibrio sp. are a major cause of food-related illness, especially seafood. Raw oyster consumption is the most common route for 95% cases of human infection associated with *Vibrio* sp. In the United States, during the years between 1983 and 1992, more than 50 *Vibrio* related outbreaks have occurred each year from fish and shellfish poisoning (Lipp and Rose 1997). Diarrhea, abdominal cramps, nausea, vomiting, headache, fever, and chills may be associated with infections caused by *Vibrio* sp. In the U.S., *V. parahaemolyticus* and *V. cholerae* (serogroup non-O1) were the most common species causing *Vibrio*-associated diarrhea (Janda and

others 1988). Additionally, *V. vulnificus* have been reported to be the leading cause of reported human death associated with consumption of aquacultured molluscan shellfish (FNB 1991). The bacteria were blamed for two fatalities in Florida in 1998 and for a total of 33 deaths in the US in 1996 (Hlady 1997). According to the FDA, between years 1989 through 2000, 275 reported illnesses resulting in 143 deaths were linked to consumption of shellfish contaminated with *V. vulnificus*. Table 2.1 shows the non-cholera *Vibrio* infections reported from Gulf Coast in 1999 (CDC 2001).

Vibrio parahaemolyticus

V. parahaemolyticus is a gram-negative, polymorphous, motile rod with a single polar flagellum, halophilic, and facultative anaerobe which grows readily at temperatures between 22 – 42 °C but fails to grow below 4 °C and above 45 °C (Kaneko and Colwell 1973; Morris and Black 1985). Short generation time growth occurs at pH values between 5 – 11 and at NaCl concentrations between 1 – 8% (Baumann and others 1986). The biochemical characteristics of *V. parahaemolyticus* are shown in Table 2.2 (Jay 1996).

Table 2.1 Non-cholera *Vibrio* infections reported from the Gulf Coast *Vibrio* Surveillance System[1], by syndrome and complications, 1999 (N= 164)

Vibrio species	Total #	Syndrome				Complications	
		Gastroenteritis # (%)	Septicemia # (%)	Wound Infection # (%)	Other/ Unknown[2] # (%)	Hospitalized # (%)	Deaths # (%)
V. alginolyticus	15	2 (13)	0	10 (67)	3 (20)	4/14 (29)	0
V. cholerae non-tox[3]	25	15 (60)	2 (8)	3 (12)	5 (20)	10/24 (42)	0
V. damsela	1	0	0	1 (100)	0	1 (100)	0
V. fluvialis	9	4 (45)	2 (22)	3 (33)	0	4 (44)	0
V. furnissii	1	1 (100)	0	0	0	0	0
V. hollisae	8	7 (88)	0	1 (12)	0	6 (75)	0
V.metschnikovii	1	0	0	0	1 (100)	1 (100)	0
V. mimicus	8	6 (75)	0	1 (12.5)	1 (12.5)	4 (50)	0
V. parahaemolyticus	29	16 (55)	0	13 (45)	0	13/27 (48)	1/26 (4)
V. vulnificus	55	4 (7)	23 (42)	21 (38)	7 (13)	49 (89)	21/53 (40)
Species not identified	7	2 (29)	1 (14)	3 (43)	1 (14)	1 (14)	0
Multiple species	5	2 (40)	0	3 (60)	0	3/4 (75)	1 (20)
Total	164	59 (36)	28 (17)	59 (36)	18 (11)	96/159 (60)	23/155 (15)

[1]Includes Alabama, Florida, Lousiana, Mississippi, and Texas

[2]Includes eye, otitis, gall bladder, peritonitis, urine, and unknown

[3]Includes *V.cholerae* non-O1 non-O139 (24 isolates), and *V. cholerae* O1 (1 isolate)

Table 2.2 Some characteristics of *Vibrio parahaemolyticus* (Jay 1996)

Specification	*Vibrio parahaemolyticus*
Gram reaction	Negative
Mobility with flagella	Positive
Shape	Straight rod
Growth in 4%, 6%, & 8% NaCl	Positive
Growth in 0% & 10% NaCl	Negative
Growth at 42 °C	Positive
Sucrose utilization	Negative
Cellobiose utilization	Negative
Color on TCBS agar	Green

V. parahaemolyticus was first isolated in 1950 as a causative bacterium of food poisoning in Japan (Fujino and others 1953) with 272 victims and 20 deaths in a 1951 outbreak (Sakazaki 1979). Since then, it has been isolated from many species of fish, shellfish, and crustaceans (Rodrick 1991). In the United States, the first outbreak, in which Vp was the etiological agent, occurred in 1971 and was traced to steamed crabs and crab salad; there were 425 victims (Molenda and others 1972). Since this first outbreak, *V. parahaemolyticus* has consistently been found to cause shellfish food poisoning. In the summer of 1997, this organism caused a large outbreak in the US Pacific Northwest with 209 cases and one death (Fyfe and others 1998). Later in 1998, 416 persons in 13 states fell ill after eating raw oysters traced to Galveston Bay, Texas resulting in the largest *V. parahaemolyticus* related outbreak reported yet (NFSD 2001). No fatality was linked to this outbreak.

The *V. parahaemolyticus* minimal infective dose is still uncertain although, FDA reports that greater than one million organisms are enough to cause the

disease (CFSAN 2001). The disease is caused when the organism attaches itself to individuals' small intestine and excretes a yet undefined cytolytic toxin, which destroys the endothelium of the gut. Pathogenic strains of *V. parahaemolyticus* are differentiated from non-pathogenic strains by the ability to produce a thermostable direct hemolysin (TDH), whose production is termed the Kanagawa phenomenon (Sakazaki and others 1968). Most of all virulent strains, which are Kanagawa-positive (KP+) produce TDH, encoded by the *tdh* gene. TDH production has been detected by the presence of beta-hemolysis around the colonies on Wagatsuma blood medium, a specific blood-agar medium containing human O erythrocytes on high-salt mannitol (Joseph and others 1982). Since production of TDH is responsible for the Kanagawa phenomenon, TDH has been considered a major virulence factor (Yamamoto and others 1992). On the other hand, some cases of *V. parahaemolyticus* associated gastroenteritis have been found to be due to Kanagawa-negative (KP–) strains (Honda and other 1988). Some clinically isolated KP– strains were observed to produce a TDH-related hemolysin, designated by TRH and encoded by the *trh* gene (Honda and other 1988). TRH is immunologically similar to TRH, but the two have different physicochemical characteristics and lytic activities for various erythrocytes (Honda and others 1988). Thus, TDH as well as TRH are now considered to be important virulence factors. However, recent data from British Columbia (Kelly 1999) suggests an association of urease positive strains with clinical isolates, which may or may not be TDH positive. Although *V. parahaemolyticus* does not generally produce urease,

urease-positive isolates are increasingly reported from clinical sources (Kelly and Stroh 1990). Urease is encoded by the *ure* gene and strains that hydrolyze urea are abbreviated as 'uh+' (Kaysner and others 1994). Recently research has been concentrated on determining the relationship between uh+ and virulent gene, *tdh* or *trh*. It was first suggested by Kaysner and others (1994) that uh+ is a marker for virulent strains because they found that all TDH producing strains were uh+ however, not all of the uh+ produced TDH. Later studies confirmed that uh+ is also related to the production of TRH (Osawa and others 1996; Okuda and others 1997). Iida and others (1997) showed that there was a genetic linkage between the *ure* and *trh* genes in *V. parahaemolyticus*. They demonstrated that all *trh*+ isolates contained *ure* gene and that *trh* and *ure* genes were closely positioned on the chromosomal DNA. Thus, they suggested that urease production is a marker for the presence of gene *trh*, which is a virulence factor.

V. parahaemolyticus is very sensitive to temperature and pressure. Delmore and Crisley (1979) found the species to have a thermal destruction value (D-value) of 0.14 min at 85 °C and 2.8 min at 65 °C. Berlin and others (1999) showed that pathogenic *Vibrio* sp. are very susceptible to pressure treatment at levels between 200-300 MPa.

Vibrio vulnificus

Vibrio vulnificus, known as *Beneckea vulnificus* until 1979, is a lactose-postive, halophilic, gram-negative, opportunistic pathogen, found in estuarine environments and associated with various marine species such as plankton, shellfish (oysters, clams, and crabs), and finfish (Farmer 1979; Oliver 1989). *V. vulnificus*, as a mesophile, grows optimally at 37 °C at which its generation time is 22-30 min. The optimum NaCl concentration for the organism is 1 – 2%, however they can survive up to 6% salt concentration. Table 2.3 shows salt tolerance of some *Vibrio* species (Joseph 1982).

Table 2.3 Salt tolerance of some *Vibrio* species (Joseph 1982)

	Vibrio species				
Growth at	*V. parahaemolyticus*	*V. vulnificus*	*V. cholerae*	*V. alginolyticus*	*V. fluvialis*
0 % NaCl	–	–	+	–	–
1 % NaCl	+	+	+	+	+
6 % NaCl	+	+	+/–	+	+
7 % NaCl	+	–	–	+	+
10 % NaCl	–	–	–	+	+/–

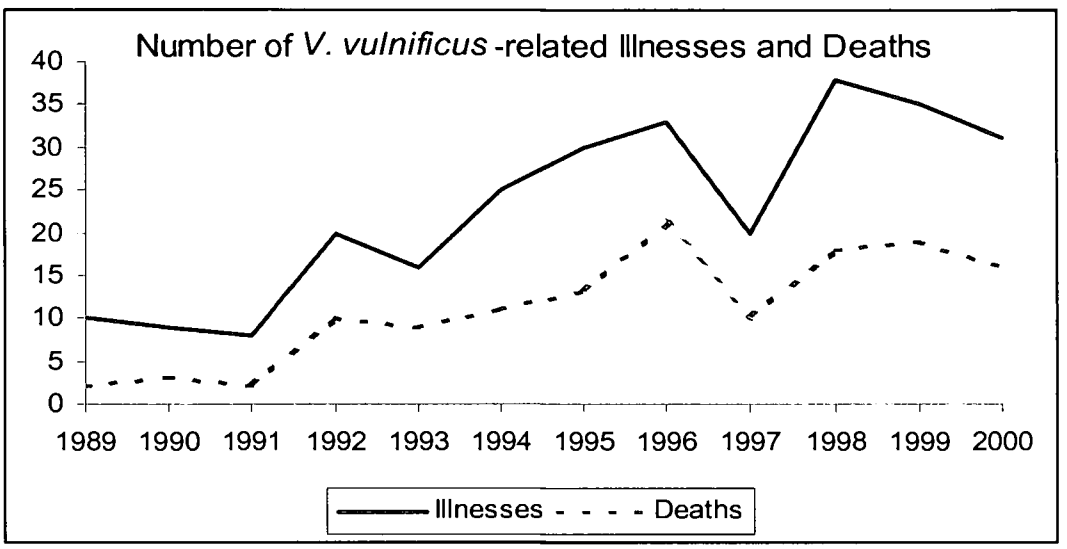
+ growth; – no growth; +/– varies depending on strain

One unique biochemical characteristics that differentiates *V. vulnificus* from other *Vibrio* species is its ability to ferment lactose (Oliver 1989). The organism also possesses the somewhat unique ability to enter the viable but non-culturable

state (VBNC). This was first reported when Oliver and Wanucha (1989) observed that *V. vulnificus* was not able to grow when incubated at temperatures lower than 10 °C, however this temperature was not lethal to all cells. The so-called VBNC state is described by a loss of platebility, a reduction in cell size, a reduction in macromolecular synthesis and characteristic cellular morphology changes (Oliver 1993).

V. vulnificus is ubiquitous in marine environments and has been isolated from diverse geographical locations, from Miami, FL to Portland, ME (Oliver and others 1983) and can accumulate to high numbers in seawater and shellfish, particularly in oysters (Tamplin and others 1982; Oliver and others 1983). The adverse human health effects of *V. vulnificus* were first reported in 1975 (Blake and others 1979). Since that time, *V. vulnificus* gained considerable attention due to lethal characteristic of the diseases it causes in humans. *V. vulnificus*, is a particularly invasive organism, which causes diseases including primary septicemia and gastroenteritis with a mortality exceeding 50% (Klontz and others 1993). These diseases are mostly associated with the consumption of raw shellfish or contact with seawater (Blake and others 1979; Fyfe 1998). In addition, *V. vulnificus* can cause skin infections when open wounds are exposed to warm seawater; these infections may lead to skin breakdown and ulceration (CDC 2001).

Figure 2.1 *V. vulnificus*-related illnesses and deaths from shellfish reported to FDA between 1989-2000 in US (GAO 2001)



Pathogenity of *V. vulnificus* was first investigated by Poole and Oliver (1978). It was proposed that once the bacteria contacted the intestines, they rapidly penetrate the intestinal wall and enter the bloodstream, resulting in septic shock and ultimately death, due to heart failure (Poole and Oliver 1978). Although the organism can be extremely virulent upon ingestion, mortality often is associated with certain types of health conditions. The fatality often involves susceptible individuals with a chronic underlying illness associated with elevated serum iron levels and/or immunosuppression who have eaten raw shellfish prior to onset of symptoms (Hlady and others 1993). Persons at high risk for infection include those with liver disease, cancer, AIDS, chronic kidney disease, diabetes, and inflammatory bowel disease. In fact, liver disease emerged as the primary risk

factor for a series of *V. vulnificus* cases reported during 1988–1996: 80% of the 120 fatal cases for whom data were available had liver disease, primarily cirrhosis or hepatitis (Shapiro 1998). The infectious dose of *V. vulnificus* in humans is yet unknown; however as few as 100 bacterial cells can cause fatal infections in susceptible individuals (Oliver and Kaper 1997; CSFAN 2001).

V. vulnificus was shown to be sensitive to mild heat treatment (Cook and Ruple 1992). Bacteria inoculated in oysters were easily destroyed ($D_{47\ °C}$ = 78 s; $D_{50\ °C}$ = 39.8 s). FDA approved, 'all-natural', Ameripure® process claims to inactivate *V. vulnificus* in un-shucked oysters by applying heat with a successive cool down. Mallett and others (1991) showed that *V. vulnificus* is very sensitive to radiation and that can be totally eliminated with low doses of Cobalt (^{60}Co). Recent studies show that high pressure is also a viable means for reduction of the organism (Berlin and others 1999).

Vibrio cholerae

Vibrio cholerae was first isolated in pure culture by Robert Koch in 1883, although it had been examined by other researchers including Pacini, who is known to be describing it the first time in Florence, Italy in 1854 (Morris and Black 1985). *V. cholerae* is the causative agent of the disease 'cholera'. Cholera is caused by a specific heat-sensitive enterotoxin produced by *V. cholera* (Morris and Black 1985). This species is not considered a halophilic *Vibrio* because its obligate requirement for Na^+ ion can be satisfied by the trace amounts present in most media

(Singleton and others 1982). The organism is characterized by various biochemical properties and antigenic types. Most *V. cholerae* strains recovered from cholera cases contain a somatic antigen in common and include serotype O group 1 (Gardner and Venkatraman 1935; Hugh and Feeley 1972). *V. cholerae* O:1 have several serotypes including Inaba, Ogawa, and Hikojima (Yamamoto and others 1983). *V. cholerae* strains that are identical to, or closely resemble, clinical strains in biological characteristics but fail to agglutinate in anti-O1 serum are referred to as *V. cholerae* non-O:1 (West and others 1986). Evidence indicates that non-O1 are sometimes involved in cholera-like diarrheal diseases (Hughes and others 1978; Blake and others 1980).

V. cholerae was proved to be a heat sensitive microorganism by Shultz and others in 1984. The study revealed a 7 $\log_{10}$ cycle reduction of *V. cholerae* O:1 in peptone water within 11.9 min at 49 °C ($D_{49\,°C}$ = 1.70) and 2.59 min at 63 °C ($D_{49\,°C}$ = 0.36). Moreover, the study showed that the crabmeat provided thermal protection for *V. cholerae* ($D_{49\,°C}$ = 8.15). Reily and Hackney (1985) examined the survival of *V. cholerae* in refrigerated and frozen storage in crab, oyster, and shrimp samples. The counts of inoculated cells declined under both storage conditions but some survivors were observed beyond three weeks.

Vibrio mimicus

Following a set of DNA homology studies, Center for Disease Control (CDC) demonstrated that sucrose-negative, biochemically atypical strains of *V.*

cholerae constituted a separate species. Therefore in 1981 the name "*Vibrio mimicus*" was proposed due to the similarity of these strains to *V. cholerae* (Davis and others 1981). Clinical and environmental strains of *V. mimicus* produce a heat-labile toxin that appears to be identical to cholerae toxin. Furthermore, positive strains and those that are negative for cholera toxin can produce an enterotoxin similar to that described for *V. cholerae* non O:1 (Nishibuchi and Seidler 1983). *V. mimicus*, however, can be distinguished from *V. cholerae* by its lack of sucrose fermentation; colonies appear green on thiosulfate citrate bile salts sucrose (TCBS) agar and would grow in most common media without added NaCl. *V. mimicus* has been isolated from a number of environmental source including oysters (Davis and others 1981) but is thought to be less ubiquitous than *V. cholerae* O:1 (Morris and Black 1985).

Vibrio hollisae

This halophilic *Vibrio* species has been classified and identified as an acute gastrointestinal pathogen associated with eating raw shellfish (Hickman and others 1982; Morris and Black 1985). This species appears to grow on less sensitive agars such as sheep blood agar and marine agar, thus presents isolation and identification difficulty since it cannot be cultured on selective agars such as TCBS or MacConkey agar (Hickman and others 1982). This microorganism exhibits a delayed motility pattern uncharacteristic of the other *Vibrio* species (Hickman and others 1982).

Other *Vibrio* Species

Other halophilic *Vibrio* species such as *V. alginolyticus*, *V. fluvialis* sp., and *V. metschnikovii*, have been recovered from brackish coastal waters, sediment, and sea life taken from the temperate estuarine environments (Blake and others 1980). These species appear to be normal components of that environment and have been reported to be associated with human illnesses.

Methods to Reduce Pathogens in Oysters

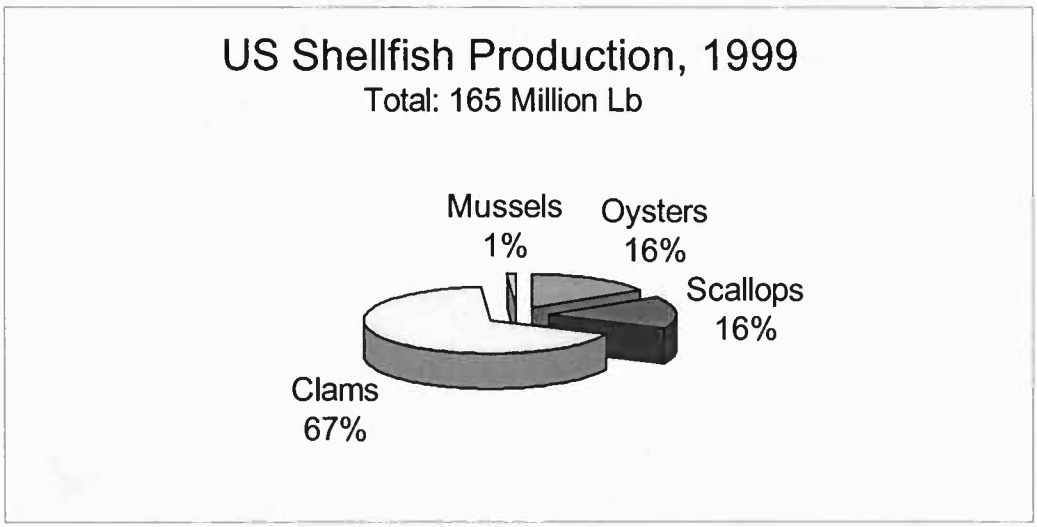
There had been numerous cases of severe illnesses and various forms of gastroenteritis associated with consumption of raw oysters in the US (Brown and Dorn 1977; Earampamoorty and Koff 1975; Gerba and Goyal 1978; Wood 1976; Kaysner 1998; Fyfe 1998). *V. vulnificus* and *V. parahaemolyticus* have been implicated in illness outbreaks in the Southeast and Pacific Northwest (Klontz and others 1993; Kaysner 1998). The largest *V. parahaemolyticus* related outbreak was reported in Galveston Bay area, Texas in 1998 with 416 cases (NFSD 2001). The outbreaks of *V. parahaemolyticus* infections associated with eating raw oysters were found in Pacific Northwest both in 1997 and 1998 with the highest frequency in summer months and with 209 confirmed cases of illness from California, Oregon, Washington and British Columbia (CDC 1998). The outbreaks of *V. parahaemolyticus* infections were also reported in 1998 in the Connecticut, New Jersey, and New York areas for the first time involving 23 culture-confirmed cases from July through September (CDC 1999). Records showed that bivalves implicated in the outbreaks were harvested from New York and the state of Washington, representing a wide geographic spread. Seafood-induced illness from 1977 to 1984 represented about 5% of all food-borne illness cases, and molluscan shellfish contributed about 53% of these cases (GAO 1988). A 1997 review of 333 case reports revealed that *V. vulnificus* was responsible for 80% of all *Vibrio*-related septic infections. Other species implicated in primary septicemia included *V. parahaemolyticus* (9%), *V. colerae* non-O1 (8%), and *V. hollisae* (3%) (Hlady

1997). Reported clinical symptoms included diarrhea, abdominal cramps, nausea, vomiting, fever, blood stools, headache, and myalgia for gastroenteritis patients (CDC 1999). Fatalities were associated with *V. vulnificus*.

Oysters are filter feeders that are farmed in bays and estuaries and they consequently take-up and accumulate the micro-flora prevalent in that particular water column. This flora eventually leads to spoilage of oysters (often limiting their post-harvest shelf-life to <2 weeks) and at times, overgrowth of pathogenic bacteria may pose a threat to public health (Andrews and others 1975; Kaneko and Colwell 1973; Vanderzant and Thompson 1973; Fyfe 1998). Pathogenic organisms such as *Salmonella, Shigella sp., Vibrio sp., Clostridium perfringens, Clostridium botulinum, Yersinia enterocolitica,* as well as numerous enteric viruses have been isolated from oysters (Andrews and others 1975; Barrow and Miller 1974; Wood 1976). *V. vulnificus* and *V. parahaemolyticus* are common inhabitants in estuarine waters and their numbers in oysters depend on several factors such as season, location, environmental conditions of harvest and possible temperature abuse after harvest (Cook 2001).

Oysters are important to US marine fisheries as indicated by total landings and value. In 1997, 37.1 million pounds were landed at a total estimated value of 92 million dollars (50% contributed by aquaculture). The total value of the oysters produced in the US represents a figure as high as 3% of the total value generated by both finfish and shellfish fisheries (NMFS 2000). Figure 2.2 shows US shellfish production by type of shellfish in 1999, a total of 165 million pounds (GAO 2001).

Figure 2.2 US shellfish production by type of shellfish in 1999 (GAO 2001)



The US Food and Drug Administration has established a level of concern for total *V. parahaemolyticus* numbers and will consider enforcement action against the sale of molluscan shellfish with levels equal to or greater than a most probable number (MPN) count of 10,000/g (FDA 1997). FDA's compliance program has set a guidance level for *V. vulnificus* in ready to eat fishery products as "presence or absence of pathogenic organism showing mouse lethality" (Cook 2001). FDA has not yet set an "allowable level" of *V. vulnificus* in shellfish (Cook 2001). However, according to Interstate Shellfish Sanitation Conference (ISSC) *V. vulnificus* Risk Management Plan for oysters, it is suggested that the post harvest treatments should ensure the counts are fewer than 3 MPN/g when average monthly water temperature exceeds 24 °C (ISSC 2000). The ultimate goal of the risk management

plan is to reduce the rate of etiologically confirmed shellfish-borne *V. vulnificus* septicemia illnesses from the consumption of commercially harvested raw or undercooked oysters by 40% at the end of 2005 and by 60% by the end of 2007 (ISSC 2000). Due to serious public health concerns, there have been many areas of research concentrating on different treatment techniques to reduce pathogens in oysters down to acceptable count levels set by government authorities. The following section discusses oyster post harvest treatments to increase product quality and safety.

Depuration and Relaying

Oysters are filter feeders, trapping food particles in mucus on the gills, which is then drawn into the mouth; eventually, the particles are accumulated in the gut (Buisson and others 1981). Pathogenic bacteria or viruses can readily accumulate in the oyster digestive system (Buisson and others 1981). To ensure safe oysters for consumption, a means of oyster purification is necessary. Depuration is the process of in-plant purification of commercial sized shellfish raised in waters not approved for natural purification (FDA 1979). It is a dynamic process whereby the oysters are allowed to purge themselves of contaminants in tanks of sterilized or pathogen free seawater (Fleet 1978). When placed in an environment free from contamination, a polluted oyster continues to feed, taking new material into the gut and expelling digested contaminated material as faeces (Buisson and others 1981). Depuration generally takes two or three days and is

intended to remove coliform bacteria, which act as indicator organisms for presence of pathogens. The process is not performed to remove pollutants of other types including chemicals, petroleum, heavy metals, or the plankton-associated paralytic shellfish poisoning (FDA 1979). Depuration techniques vary considerably because several factors, such as shellfish species, likely contaminant level, and water temperature should be considered. Generally, ultraviolet disinfected circulating seawater tanks are used in depuration plants.

The technology of oyster depuration has been well studied and reviewed (Furfari 1976; Fleet 1978). Eyles and Davey (1984) studied the effectiveness of a commercial depuration plant, which purified oysters from a polluted estuary. Although depuration significantly reduced aerobic plate counts (APC) and counts of coliforms and *Escherichia coli*, study findings revealed that it had no effect on naturally occurring *V. parahaemolyticus*. The study findings by Tamplin and Capers (1992) revealed that close associations have evolved between endogenous *V. vulnificus* and shellfish in estuarine environments and artificially inoculated *Vibrio* sp. might respond differently than *Vibrio* sp. in the wild. In addition, it was reported that depuration at temperatures above 23 °C actually caused *V. vulnificus* numbers to increase in oysters. They concluded that *V. vulnificus* is persistent and unamenable to the uv-light depuration process.

Relaying is defined by FDA as the process of reducing pathogenic organisms that may be present in shellfish by transferring shellfish from a growing area classified as restricted to a growing area classified as approved ambient

environments (CSFAN 2000). There are few data available on this approach. Son and Fleet (1980) demonstrated a decrease in bacteria counts to fewer than 5 *V. parahaemolyticus*/g after 6 days. A series of investigations by Jones between 1989 and 1992 demonstrated that relaying oysters containing relatively high levels of *V. vulnificus* to waters with low or no detectable *V. vulnificus* can result in the removal of the organism from the shellfish. It was mentioned that the relay waters used in the described studies had relatively high salinities (about 25 ppt even at low tide) and low levels of fecal coliforms. This was in contrast to the harvest site where salinities were variable and relatively low at low tide because of fresh water input. It was concluded that up to 28 days of treatment duration, the salinity difference, and possibly other water quality differences experienced by relayed oysters, appears to be a means to control and reduce the levels of *V. vulnificus* in oysters (Jones 1994). Table 2.4 presents the results of the relaying study.

Table 2.4 *Vibrio vulnificus* in oysters relayed to relay lagoons (Jones 1994)

V. vulnificus/g oyster meat		
Freshly harvested	Relayed	*V. vulnificus*/100 ml water
930	24	< 3
4600	4.3	< 3
1500	< 3	< 3
150	< 3	< 3
4.3	< 3	< 3
< 3	< 3	< 3
< 3	< 3	< 3

A later study confirmed that relaying Gulf Coast oysters to offshore waters decreased *V. vulnificus* counts to fewer than 10 MPN/g in 7 to 17 days (Motes and DePaola 1996).

National Shellfish Sanitation Program (NSSP) requires oyster flesh to have a MPN of fewer than 230 fecal coliform per 100 g and a total aerobic plate count of fewer than 500,000 colonies per g (PHS 1946). Throughout studies, depuration and/or relaying is considered to be a successful means to meet these standards; however, complete elimination of *Vibrio* sp. by these processes is rarely achieved, indicating the need for better postharvest treatments to ensure for shellfish safety. Additionally, oyster industry close monitor programs run by FDA, revealed the necessity of design, development, and education concerned with depuration systems in plants.

Pasteurization

Pasteurization, by definition, implies the use of heat to reduce or eliminate pathogenic and spoilage organisms in certain food products (Jay 1996). Early work on pasteurization resulted in reduction of standard aerobic plate, coliform, and fecal coliform counts in oysters (Pringle 1961). Since then, pasteurization has been investigated to extend shelf lives of safe oysters. Generally, seafood products are not easily sterilized by conventional heat processing methods due to unfavorable changes in taste and texture (Learson and others 1969). However, according to sensory tests performed by Chai and others (1984), there was little difference in the

organoleptic characteristics of pasteurized and freshly shucked oysters. Additionally, with further study, Chai and others (1991) found out that a pasteurization process of 76 °C for 8 min produced oysters with the optimum physical and sensory quality. In both studies, there was a 2 – 3 $\log_{10}$ reduction of bacteria in pasteurized oysters, yet no tests were done to investigate *Vibrio* sp. reduction.

A study on the identification of bacterial flora surviving after pasteurization under refrigerated storage was conducted by Pace and others in 1988. According to the study, only gram-positive bacteria survived the treatment, *Bacillus* being the predominant aerobic bacterium during the entire 5-month storage. In addition, the facultative anaerobic plate count gradually increased during post-pasteurization refrigeration and *Clostridium, Corynebacterium, Listeria, Peptostreptococcus* and *Staphylococcus* were isolated from the samples. Experiments regarding survival of *Vibrio* sp. in oysters were conducted using mild heat treatments rather than pasteurization (Cook and Ruple 1992).

Heat-shock treatment is another effective way in reducing counts of *V. vulnificus* in oysters (Hesselman and others 1999). Heat-shock is a relatively simple method of heating the whole oysters in a heated water tank so that oyster meat reaches 50 °C for 1 to 4 min. This method is also used to facilitate the shucking of oysters. The heat-shock method was shown to reduce *V. vulnificus* counts from 1 to 4 $\log s_{10}$ in the finished product. Reports by Green (2001) verified heat-shocking as a viable method for reducing *V. vulnificus* counts in oysters. Tank design and

careful monitoring of the water temperature is important if the process will be used commercially.

Many outbreaks of diseases have been reported due to contaminated raw oyster consumption (CDC 1989; Fyfe 1998). Therefore, it is critical to develop a process that would extend shelf-life by destroying all naturally deteriorative enzymes and eliminate pathogens while maintaining the flavor and texture of fresh oysters. Considering that oyster texture and flavor are strongly affected by temperature and time of pasteurization (Chai and others 1991) and that most consumers prefer oysters raw (Jarosz and others 1989), it is a challenge for the oyster industry to choose and employ the best oyster post-harvest treatments that would satisfy all requirements.

Ameripure® Process

In 1998, Center for Science in the Public Interest (CSPI) reported that in the Gulf of Mexico area severe illnesses and deaths have been associated with consumption of raw oysters contaminated with *V. vulnificus*. This major concern triggered the oyster industry to seek new methods to reduce or completely eliminate *V. vulnificus*. The cool pasteurization process for oysters is a patented process developed by AmeriPure Oyster Companies of Empire, LA. The process has US patent numbers 5-679-392, 5-773-064, and 5-976-601, which were approved in 1997, 1998, and 1999, respectively (USPTO 2001). Ameripure® process is a mild thermal treatment of oysters in a warm water bath, followed by a

rapid cool down in ice-water slurry. The length of the mild thermal treatment depends on the estimated contaminant level, the season, temperature, and amount (or size) of the oysters (Andrews 1999). This FDA approved treatment raises the temperature of the oyster enough to destroy *V. vulnificus* but does not sterilize or cook the oyster.

The effect of mild heat treatment on the survival of *V. vulnificus* in both pure culture and oysters was first studied by Cook and Ruple (1992). Rapid death of *V. vulnificus* in broth cultures was observed with a decimal reduction value of 78 s at 47 °C. Furthermore, thermal inactivation experiments were performed with naturally-contaminated oysters, where a treatment of 50 °C for 10 min reduced *V. vulnificus* to non-detectable levels. Table 2.5 shows the effect of heating time and temperature on the numbers (MPN/g) of *V. vulnificus* from this study. Data to document non-detectable levels of *V. parahaemolyticus* have also been compiled. Results of these studies showed that the mild heating process did not impart a noticeable cooked appearance or taste.

Table 2.5 Effect of heating time and temperature on the numbers (MPN/g) of *V. vulnificus* in oyster meats (Cook and Ruple 1992)

Heating Time (min)	Heating Temperature		
	45 °C	47 °C	50 °C
0	59,000	59,000	59,000
5	29,000	14,000	220
10	870	4	<0.3

A similar study was conducted with Gulf Coast oysters to test the effects of mild thermal treatments. Chen and others (1996) reported that even though the flavor, texture and smell of the treated oysters were comparable to untreated ones, a slight lightening of meat color was noticeable. Furthermore, according to a recent study by Andrews and others (2000), artificially inoculated *V. vulnificus* and *V. parahaemolyticus* along with naturally contaminated live oysters were heat treated with low temperature pasteurization of 50 °C up to 15 min. Samples of processed and unprocessed oysters were enumerated for *Vibrio* sp. and aerobic bacteria for 14 days. Results of the study showed that low temperature pasteurization was effective in reducing these pathogens from more than 1,000,000 CFU/g to non-detectable levels in <10 min of processing. The spoilage bacteria were reduced 2 – 3 fold resulting in increased shelf-life up to 7 days beyond live unprocessed oysters. The Ameripure® process is often referred to as low-temperature pasteurization, however unlike pasteurization, it appears to be mainly effective on *Vibrio* sp. As an economical incentive, companies that make use of this technology claim that there are shucking yield increases up to 30% when compared with untreated oysters (Tesvich and Fahey 2000). Since the studies confirm that Ameripure® process does not cause a change in fresh oyster flavor and taste, FDA allowed companies that use this treatment to market their products as "raw oysters" (Nelson 1999). Oysters treated with this process need to be kept under refrigeration at or below 5 °C (Nelson 1999).

Cooling and Freezing Treatment

Consumers who enjoy raw shellfish create a challenge for the oyster industry in providing safe, pathogen-free, raw seafood. Although thermal processes inactivate *Vibrio* sp. and other pathogenic bacteria in oysters, they degrade the sensory qualities and prevent them from being marketed as raw. Consequently, cold temperatures such as refrigeration and freezing have been studied as an alternative method to process oysters while preserving the integrity and organoleptic quality of raw oysters.

V. vulnificus survival in oyster homogenates held at 4 °C were first investigated by Oliver (1981). His results indicated that *V. vulnificus* decreased rapidly when in oyster broth or in whole shell oysters 0.5 °C to 4 °C. He further showed that the organism diminished even more rapidly when inoculated into pre-cooled oyster broth than into whole oysters followed by storage on ice. The findings of these studies suggested the concept of quick chilling or freezing as a potential control strategy of *V. vulnificus* in oysters. However, studies conducted by Kaspar and Tamplin in 1993 indicated that storage of naturally contaminated oysters at 2 and 4 °C for 14 days caused only 1 $\log_{10}$ reduction and that storage at 30 °C resulted in increases in *V. vulnificus* counts in oysters. These findings were confirmed by Cook (1997) when he showed that *V. vulnificus* counts increased in un-chilled oysters at temperatures above 18 °C. These study findings strengthened the National Shellfish Sanitation Program (NSSP) recommendations of keeping oysters at temperatures below 8 °C during storage to suppress growth of *V.*

vulnificus. Cook (1997) continued his research to identify and set the optimal refrigeration conditions necessary to prevent growth of *V. vulnificus* in freshly harvested oysters. He observed that the period immediately after harvest (3 – 4 hours) caused the greatest increase. Based on his research results, both NSSP and ISSC suggested additional control plans to help reduce the risk of *V. vulnificus* infection by minimizing post-harvest bacteria growth. In general, the higher the temperature of harvest waters, the sooner the refrigeration is necessary to prevent growth. A study on commercial processing techniques revealed that there was no significant reduction of *V. vulnificus* when shucking, washing, draining, and packing oyster meat was done on ice (Ruple and Cook 1992).

A freeze inactivation storage study revealed the durability of *V. vulnificus* to cold temperatures; it was possible to culture *V. vulnificus* from oysters after 12 weeks at –20 °C (Cook and Ruple 1992). Even though there was 2 – 3 $\log_{10}$ reduction in numbers, complete elimination was not seen. A study by Parker and others (1994) revealed that even after 70 days of –20 °C frozen storage, the organism was viable, further confirming the strength of *V. vulnificus*. The authors indicated that due to economic aspects, along with unacceptable changes in quality and the inefficiency of the process to completely eliminate *V. vulnificus*, not many further studies were warranted. One recent study by Schwarz (2000) compared the rates of *V. vulnificus* reduction in oyster shellstock with conventional cooling to rapid chilling procedures. To achieve rapid chilling, the oysters were lowered down from 30 °C to <2 °C within an hour. The study results demonstrated that *V.*

vulnificus levels were reduced up to 99% following a rapid chilling treatment, whereas the conventionally cooled oysters required 4 days of cold room storage to achieve a similar reduction (Schwarz 2000). Cryogenic freezing with carbon dioxide (CO_2) eliminated almost all *V. vulnificus* in oysters while preserving quality and freshness in a study by Berne (1996). Individual quick-frozen (IQF) process at very low temperatures (–74 °C) demonstrates virtually no flavor loss in oyster meat and decreases bacteria counts dramatically, while traditional freezing methods cause cell damage and moisture loss, leaving the oyster meat mushy and flavorless (Berne 1996). In general, studies show that all *Vibrio* species show susceptibility to freezing treatments, however decline in counts depend on freezing rate and method (blast freezing or use of carbon dioxide (CO_2) and nitrogen (N_2) as source) (Cook 2001). Additionally, long term frozen storage helps reduce the *Vibrio* counts down to fewer than 3 MPN/g (Cook 2001).

GRAS Compounds

In search of different methods to provide safe raw oysters to consumers, Generally Recognized as Safe (GRAS) compounds were tested to determine their lethal effect on *V. vulnificus* in 1994. Particularly, diacetyl, lactic acid and butylated hydroxyanisole (BHA) were examined in these studies. According to Sun and Oliver (1994), only diacetyl proved lethal to *V. vulnificus* in naturally contaminated oysters, whereas lactic acid and BHA exhibited no reduction in counts and in some cases increased organism numbers. Authors reported that even though diacetyl

decreased *V. vulnificus* counts, treatment did not have any noticeable effect on total aerobic bacteria count, suggesting a selective anti-microbial activity of diacetyl against *V. vulnificus*.

Acidic Marinade

The effect of low pH on microorganisms has been well studied and it is well established that many grow best at values 6.6 – 7.5 and very few grow below 4.0. Bacteria tend to be more fastidious to pH changes than molds and yeasts; pathogens being the most sensitive. Table 2.6 shows the reported minimum pH levels for growth of food-borne pathogens (Jay 1996). Low pH affects the enzymatic functions and nutrient transportation eventually causing death of the cell. When microorganisms are placed in environments below or above neutrality, their ability to proliferate depends on their ability to bring the environmental pH to an ideal range; neutrality for most bacteria. When placed in acidic environments, the cells must either keep H^+ ions from entering or expel them as rapidly as they enter because important life sustaining key compounds such as DNA and ATP require neutrality (Jay 1996). Additionally, transportation of nutrients depends on electrical charge of the cell and the nutritive compounds; naturally non-ionized compounds can enter the negatively charged cells. In low pH environment the cell nutrient transportation equilibrium changes adversely (Jay 1996). Furthermore, the ionic character of side chain ionizable groups is affected in either basic or acidic environments, resulting in increasing denaturation of cell membrane and transport

enzymes (Jay 1996). The treatment of soaking oysters in vinegar for several hours before consumption is based on the sterilizing effects of low pH. Since the pH of vinegar is close to 2.0, most food-borne bacteria cannot survive such acidic conditions. Acidic marinade is a popular style of eating raw oysters in Japan.

It has been reported that the minimum pH value for the growth of *Vibrio* sp. is 4.8 and that acidic conditions would most likely eliminate these organisms. Preliminary test results showed that *V. parahaemolyticus* inoculated in Pacific oysters kept in seafood cocktail sauce (pH 3.8) were reduced 4 $\log_{10}$ cycles to non-detectable levels within 3 weeks under refrigeration (Calik and others 2001b).

Ahmed (1991) pointed out that even though it has not yet been confirmed, gastroenteritis could be caused not only by bacteria and/or viruses but also by lipid-soluble toxins. It has been well established that an increase in free polyunsaturated fatty acids (PUFA) in a product might be of concern as they are precursors of oxidation, which bring off-flavors, quality deterioration (Wilson and others 1976), and toxicity in animals (Kanazawa and Ashida 1991). Sajiki (1994) confirmed this phenomenon with his studies where oysters were marinaded in 4% acidic acid at 37 °C for 3 hours and increase in formation of PUFA and toxicity in treated oysters was observed.

Table 2.6 Reported minimum pH values for the growth of some food-borne bacteria (Jay 1996)

Bacteria	pH
Aeromonas hydrophila	ca. 6.0
Alicyclobacillus acidocaldarius	2.0
Bacillus sereus	4.9
Clostridium botulinum Group I	4.6
C. botulinum Group II	5.0
C. perfringens	5.0
Escherichia coli O157:H7	4.5
Gluconobacter sp.	3.6
Lactobacillus brevis	3.16
L. plantarum	3.34
Lactococcus lactis	4.3
Listeria monocytogens	4.1
Plesiomonas shigelloides	4.5
Pseudomonas fragi	ca. 5.0
Salmonella sp.	4.1
Shewanella putrefaciens	ca. 5.4
Shigella flexneri	ca. 5.5
S. sonnei	5.0
Staphylococcus aureus	4.0
Vibrio parahaemolyticus	4.8
Yersinia enterocolica	4.2

Irradiation Treatment

Food irradiation is a process for the treatment of food products to increase quality, enhance shelf-life, and improve microbial safety. Irradiation technology is not a new process. The chemistry, technology, and commercial aspects of food irradiation have been discussed in many articles and books over the years (WHO 1994). Electromagnetic radiations, namely, gamma and X-rays having short wavelength (<300 nm) and higher energy than visible light, can cause ionization by removing electrons from the outer-shell of atoms and molecules. In a typical irradiation process, ionizing energy released from a safe source is directly exposed to target foods. The effects of this process are proportionally dependent on irradiation dose and treatment duration (Giddings 1984). The unit for food irradiation dose is the Gray (Gy), which is equal to the absorption of the energy equivalent to one Joule per Kg of absorbing material (1 Gy = 1 JkG^{-1}) (Venugopal and others 1999). Generally, Cobalt-60 (^{60}Co) and Cesium-137 (^{137}Cs) are used for food preservation (Venugopal and others 1999). Most of the present day irradiators use ^{60}Co as a source of irradiation energy mostly due to its high penetration and easy availability. Low gamma rays emitted by ^{60}Co have been proven effective in reducing spoilage and pathogenic microorganisms in variety of seafood products. Thus, this technology has been recognized as a preservation method of foods since it can eliminate indigenous microflora in foods. The first use of irradiation on foods was approved by the FDA in 1963. Till then there had been advances in food irradiation. The electron beam accelerator (electron beam) technology is a

relatively new, flexible, and more effective source for food irradiation than the regular ^{60}Co source (Huang 1997). The electron beam is easy to adapt to different radiation processes, such as operating at different beam energy levels. No radioactivity is present when the accelerator is off, and therefore, no radioactive waste accumulates, whereas usually up to 21 years are required to dispose ^{60}Co (Huang 1997). The application of electron beam is limited to thin foods because of its low penetration, however studies indicate that it is very effective in decontamination or disinfestations (Blank and Corrigan 1995). Further studies need to be conducted to evaluate the effects of electron beam irradiation on seafood products.

According to the reports of Center for Disease Control and Prevention (CDC), *E. coli* 0157:H7 causes an average of 20,000 illnesses with 500 deaths annually (CDC 1990). To help combat this serious public health problem, FDA approved treating red meat products with a measured dose of irradiation (Henkel 1998). The ionizing irradiation was approved for application in ground meat on December 23, 1999 to reduce food-borne pathogens and to extend the shelf-life (USDA-FSIS 1999). In addition, earlier in 1990 FDA approved the use of this technology with poultry (dose range 1.5 – 3.0 kGy) to prevent frequent outbreaks of *Salmonellae* associated diseases (FDA 1990). A list of all FDA irradiation approved foods, the irradiation dose, and the purpose of irradiation is given on Table 2.7. Currently, FDA does not allow irradiation treatment of seafood and thus there are no approved irradiation dosages for seafood products (Hilderbrand 2001).

Table 2.7 List of all approved uses of radiation on foods to date, the purpose for irradiating and dose allowed

Food	Approved use	Dose
Spices and dry vegetable seasoning	Decontaminations and controls insects and microorganisms	30 kGy
Dry or dehydrated enzyme preparations	Controls insects and microorganisms	10 kGy
All foods	Controls insects	1 kGy
Fresh foods	Delays maturation	1 kGy
Poultry	Controls disease-causing microorganisms	3 kGy
Red meat (beef, lamb, and pork)	Controls spoilage and disease-causing microorganisms	4.5 kGy (fresh) 7 kGy (frozen)

The radiation sensitivity of aerobic and pathogenic organisms in shrimp was studied by Hau and others in 1992. The D-values for *V. cholerae, Staphylococcus aureus, E. coli,* and *S. enteritidis* were 0.11, 0.29, 0.39, and 0.48 kGy, respectively, when shrimp were treated at 10 kGy at 2 °C. Recently irradiation has been applied to mollusks, such as clams, mussels, and oysters, to reduce both spoilage and pathogenic bacterial counts. In a study conducted with hard-shelled clams (*Mercenaria mercenaria*), ^{60}Co-gamma irradiation was shown to effectively inactivate total coliforms, fecal coliforms, *E. coli*, *Clostridium perfringens* and F-coliphage. The average D-values were reported to be 1.32 kGy for total coliforms, 1.39 kGy for fecal coliforms, 1.54 kGy for *E. coli*, 2.71 kGy for *Clostridium perfringens* and 13.5 kGy for F-coliphage (Harewood and others 1994). These results showed that the pathogenic bacteria were similar in their susceptibility and were more susceptible to irradiation than the viruses. Table 2.8 shows the D-values of some pathogens in fish/shellfish medium. Irradiation studies on the oyster, *Crassostrea virginica*, have clearly demonstrated that *Vibrio* species are among the most radiation sensitive bacteria and that both *V. cholerae* and *V. vulnificus* can be eliminated with low doses of ^{60}Co-gamma radiation (Mallet and others 1991). Furthermore, results indicated that irradiated oysters did not exhibit any significant organoleptic changes according to a nine point hedonic scale. In a preliminary study conducted with Louisiana shellstock oysters, a similar effect on survival of the animals was noted (Kilgen and others 1987).

Table 2.8 D-values of some pathogens in fish/shellfish medium (Venugopal 1999)

Pathogen	D-value (kGy)
Staphylococcus aureus	0.29
Vibrio cholerae	0.11
V. parahaemolyticus	0.44
V. vulnificus	0.30
V. alginolyticus	0.19
Shigella flexneri	0.22
Streptococcus fecalis	5 – 7.5
Bacillus cereus	0.2 – 0.3
Listeria monocytogenes	0.15 – 0.25
Yersinia enterocoliticus	0.10 – 0.15
Hepatitis A virus	2.02

The investigators reported that doses <1 kGy were lethal to *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus*, although D-values were not reported. These oysters survived radiation exposures of <2.5 kGy. Based on these studies, it is clear that ionizing irradiation will deliver promising results in reducing *Vibrio* sp. in live oysters without killing the animals, which helps extend the shelf-life of oysters. Results of the study conducted by Novak and others in 1966 demonstrated that oysters treated with 2 kGy had a shelf-life of 23 days under refrigeration, whereas untreated oysters deteriorate within 7 days of storage.

Despite the encouraging technical and scientific assessment of irradiation as a safe alternative for preserving foods, there are still a substantial fraction of consumers who perceive it to be unsafe and undesirable. The effect of irradiation on the sensory quality and consumer acceptance of such products is still the major concern for seafood industries. The risk to workers and environmental issues are also other major concerns regarding irradiation (Chen and others 1996).

Effects on Microorganisms

The survival of microbes upon treatment with irradiation depends on several factors (Farkas 1989). These include the nature and the extent of direct damage produced inside the vital target, the number, nature, and durability of irradiation-induced reactive chemical species along with the ability of the cell to withstand these assaults and undergo repair. Resistance of the cell also depends on environmental conditions such as pH, temperature, and chemical composition of

the food in which the cell is suspended (Monk and others 1994). The resistance of microorganisms to irradiation was summarized by Ingram and Farkas in 1977. Vegetative bacteria were shown to be the most sensitive to irradiation. Bacterial spores are generally more resistant than yeasts and molds and viruses are the most radiation resistant. Gram-negative bacteria, including pathogens such as *Salmonellae* and *Shigella*, are generally more sensitive than gram-positive bacteria.

It is well recognized that when bacteria are exposed to irradiation, nucleic acids show the most significant change, resulting in damages and mutations and subsequent inhibitions of bacterial reproduction. Injury to cells is essentially due to scission of single or double strands of DNA, which is caused by the OH radical formed by radiolysis of water. The damage to DNA is enough to cause inactivation of a large fraction of the irradiated cells (Diehl 1995; Moseley and Gould 1989). A dose of 0.1 kGy can damage 2.8% of the DNA in bacterial cells, 0.14% of the enzymes, and 0.005% of amino acids (Moseley and Gould 1989). A major factor that influences the survival of cells during irradiation is temperature. Studies showed that organisms are considerably more resistant to irradiation at subfreezing temperatures than at ambient temperatures (Matsuyama and others 1964). Increased irradiation resistance of microorganisms at subfreezing temperatures has been attributed to a decrease in water activity (a_w). Burns and Maxcy (1979) compared the effects of temperature and cellular moisture content on the survival of highly irradiation resistant bacteria. Removal of water resulted in increase of resistance to irradiation, which was similar to resistance seen in cells at subfreezing

temperatures. On the other hand, subjecting foods to high or low temperatures before, during, or after irradiation has been reported to extend shelf-life (Kumta and others 1970). While the lethal effects of irradiation on microorganisms are more pronounced when treatment is administered at different temperatures, such treatments might have adverse effects on the sensory qualities of foods.

Effects on Sensory Quality and Acceptability

Sensory evaluation is of paramount importance in quality evaluation of fishery products (Codex 1996). It has been well established that fishery products have threshold irradiation doses above which organoleptic changes occur, influencing the taste, smell, texture, and hence overall acceptability of the treated products. Mainly, formation of radiation-induced volatile compounds due to degradation of sulfur containing amino acids causes sensory changes. Oxidation is also another degrading factor especially when irradiating fatty seafood (Venugopal and others 1999). Chen and others (1996) compared the microbial and sensory quality of irradiated (2 kGy or less) crab products (white lump, claw, and legs) through 14-day ice-storage. Results revealed that the irradiated samples had 3 days longer shelf-life than the untreated samples. Even though both treated and untreated samples had similar odor and flavors, the generation of off-flavors and odors developed earlier in untreated samples. As for sensory evaluations, irradiated crab samples got higher overall acceptability scores than control samples through the 14-day testing period. Resurrection and others (1994) studied the consumer

attitudes toward irradiated samples. According to authors, 72% of consumers were aware of irradiation, and among those, 87.5% indicated they are not familiar with the issues regarding irradiation. Over 30% of consumers believed irradiated foods are radioactive, which is a misperception and a concern to the food industry.

High Pressure Processing

High pressure processing (HPP), also described as high hydrostatic pressure (HHP), is a novel food processing technology which subjects liquid and solid foods, with or without packaging, to pressures between 100 and 800 MPa at or around room temperature (Mertens and Deplace 1993). The very first report of high pressure killing bacteria was by H. Roger in 1895; however, the most important work involving microbial inactivation was that by Bert Hite, published in June of 1899. Hite and his co-workers reported the reduction of microorganisms in milk and meat following pressure treatments (Hite 1899). Later, Hite, Giddings, and Weakley (1914) reported the effect of high pressure on food microorganisms. Larsen and others (1918) confirmed that high pressure treatments inhibit microbial growth and cause cells to die off. Few other reports on the application of pressurization for food preservation and microbial inactivation were published after these initial studies until 1980's. Renewed interest has been largely due to the successful development of commercialized high pressure processed jams, jellies, and beverages by the Japanese (Hoover 1993; Farkas 1993). High pressure process is a non-thermal process that subjects foods -sealed in flexible packaging- to

pressures up to more than 1000 MPa (Swientek 1992). A typical high pressure system consists of a pressure vessel of cylindrical design, two end closures, a means for restraining the end closures, a low pressure pump, an intensifier which uses liquid from the low pressure pump to generate high pressure process fluid for system compression, and necessary system controls and instrumentation. The pressure-transferring medium is normally water.

Pressures used in the process of foods appear to have little effect on covalent bonds (Tauscher 1998; 1999); thus, foods subjected to the treatment at or near room temperature do not undergo significant chemical changes. Only non-covalent bonds such as hydrogen, ionic, and hydrophobic bonds are destroyed or formed during the pressure treatment; this enables the foods to maintain their natural vitamin content, flavor and taste even after the treatment (Hayashi 1989). Knorr (1993) reported that high pressure treatment could be also used not only to cause microbial inactivation but also for enzyme inactivation or activation, gel formation, and textural modification in foods.

Effects on Enzymes

Enzymes are a special class of proteins in which biological activity arises from an active site, brought together by the three-dimensional configuration of the molecule. Even small changes in the active site can lead to a loss of enzyme activity (Tsou 1986). Since the protein denaturation caused by HPP is associated with conformation changes, it can change the functionality of the enzymes

(Hendrickx and others 1998). The process can either activate or inactivate enzyme activity. Thus, effects of high pressure on enzymes may be divided into two classes. Comparatively low pressures (~100 MPa) have been shown to activate some enzymes. This increase in activity is, however, only observed for monomeric enzymes. Much higher pressures, on the other hand, generally inactivate enzymes (Asaka and others 1994; Curl and Jansen 1950; Jollibert 1994). On the other hand, Farkas (1993) reported that pressure treatment had little effect on food spoilage, (browning and softening) enzyme activity. Recent studies showed that high pressurization treatments had significant effect on inhibition of undesired enzyme activities in oysters (Shiu 1999; He 2001).

Effects on Microorganisms

High hydrostatic pressure has been shown to cause changes on the morphology, biochemical reactions, generic mechanisms, and cell membrane/wall of microorganisms. Intracellular vacuoles can collapse at pressures as low as 0.6 MPa (Walsby 1973) and cell division is slowed with the application of pressure (Zobell 1964). The high pressurization process distorts bacterial cell membranes and denatures enzymes and nucleic materials necessary to carry on biological activities and reproduction, eventually leading to bacterial cell death. The cell membrane and its selective permeability play a very important role in cellular respiration. Phospholipids and proteins, maintained by hydrogen and hydrophobic bonds, are the main components of the cellular membrane. Under pressure, the

volume decrease of membrane bilayers occurs along with a reduction in the cross-sectional area per phospholipid molecule (Chong and Cossins 1983).

The cell wall completely covers and maintains the shape of the cell; however pressures of 40 MPa can cause the cell to lyse due to mechanical disruption of the stressed cell wall (Berger 1959). The gram-negative cell wall is a multi-layered structure composed of a peptidoglycan layer surrounded by an outer lipopolysaccharide and protein layer. The gram-positive cell wall, however, contains a thicker peptidoglycan layer (Brock and others 1984). Therefore, gram-negative bacteria, such as *Vibrio* species, appear to be more susceptible to the effects of pressure and/or heat than gram-positive bacteria, solely due to relatively brittle structure of gram-negative cell membrane. Carlez and others (1994), confirmed this phenomenon in a study on bacterial growth during chilled storage of pressure treated minced meat (lean beef muscle).

In 1974, Schwarz and Colwell examined the effect of hydrostatic pressure of 20 to 101 MPa on the growth and viability of three strains of *V. parahaemolyticus.* No sustained growth was observed at pressure of 40 MPa and above at 25 °C and with 20 MPa at 15 °C. The response of *V. parahaemolyticus* to high hydrostatic pressure was further studied by Styles and others (1991). A 10^6 colony forming unit (CFU)/ml population of *V. parahaemolyticus* was eliminated by 170 MPa for 10 min and 30 min in clam juice and phosphate buffer, respectively. Yukizaki and others found *V. parahaemolyticus*, *V. mimicus*, and *V. cholerae* non O:1 to be destroyed by 193, 294, and 486 MPa for 10 min in liquid

buffer at 0 °C, respectively. A recent work by Berlin and others (1999) has shown that pathogenic *Vibrio* species are very susceptible to pressure treatments at levels between 200 – 310 MPa. Another recent study with *V. vulnificus* by Kilgen (2000) demonstrated that the pathogen was reduced by 5 $\log_{10}$ cycles to non-detectable levels after pressure treatments between 200 – 345 MPa.

High Pressure Process as Post-Harvest Treatment of Oysters

In a survey conducted in a mid-western city, it was shown that 80% of the restaurants served oysters raw (Jarosz and others 1989). However, there is an increasing concern with safety of raw oyster consumption. Raw oyster consumption has led to several severe outbreaks in the US (CDC 1989). One of the methods the oyster industry utilizes to reduce the pathogens without altering oyster flavor is the high pressure process technology. This technology has been welcomed, in particular, for its remarkable effect on oysters that it can pop open shells and release the meat intact, thus lowering down the costs for the labor-intensive oyster shucking process. He (2001) showed that the optimum shucking pressures that cause minimum sensory changes to Pacific oysters were between the range 270 – 310 MPa for 1 – 2 min. An earlier study examining the chemical, enzymatic, and microbial changes in oysters showed that this technology could extend the shelf-life up to 4 weeks depending on the treatment settings (Shiu 1999). The oyster processing companies, which make use of this technology, claim yield increases up to 50% due to retained moisture in uncut, intact oyster meat and due to being able

to process (shuck) even the smallest size oyster (Voisin 1999, Nisbet 2001). Research has shown that pathogenic *Vibrio* sp. are very susceptible to inactivation by HPP technology (Styles and others 1991; Farkas 1993; Hoover 1993; Berlin and others 1999). All research and study results, along with positive remarks from the oyster industry, prove that high hydrostatic pressure process is a viable means to reduce pathogenic and spoilage bacteria and extend shelf-life while keeping oyster sensory qualities at its original level.

The oyster companies, which adopt high pressure process as a post-harvest treatment, develop their own processing design that best fits the companies' budget, feasibility, target and the current market conditions. However, for most companies the general process flow is similar. Oysters intended for the raw half-shell market are individually banded using a banding machine that shrink-wraps the band onto the oyster. Workers then load oysters into baskets, and a system of overhead rails conveys the baskets to the high pressure processor. The baskets are hoisted up and then lowered into the water-filled pressure chamber, which is then sealed and pressurized using an electrical pump. Following the treatments at appropriate settings, oysters are packed with their bands on. Pressure treated oysters must be kept refrigerated throughout their distribution process (ISSC 2000).

The primary factors affecting the feasibility of operating hydrostatic pressure process are the cost of process and the availability of financial sources. Table 2.9 summarizes the per-unit costs of treatment for a small, medium, and large hydrostatic pressure process. The treatment costs are based on the following

assumptions: the plant operates one 10-hour shift per day, 5 days per week, 50 weeks per year, and wages including benefits average $10 per hour. In addition based on the information provided by the industry, it is reasonable to assume shucker labor savings of 60% and increased shucked product yields of approximately 2 pounds per 100 pounds of shell-stock (Voisin 1999).

Table 2.9 Per-unit costs of treatment for a small, medium, and large hydrostatic pressure process (ISSC 2000)

Cost estimates	Small Process	Medium Process	Large Process
Per shucked oyster	- $0.025	- $0.029	- $0.030
Per raw halfshell oyster	$0.037	$0.033	$0.032
Per sack	$1.68	$0.51	$0.25
Throughput Assumptions			
Oysters per year	3,437,500	9,625,000	14,437,500
Shellweight pounds per year	1,250,000	3,500,000	5,250,000
Sack per year	12,500	35,000	52,500

Other factors affecting the feasibility of installing treatment equipment within an oyster plant include space requirements, the size of the plant relative to the treatment equipment capacity, and the management capabilities of the plant (ISSC 2000). According to the report prepared by the ISSC (2000), 85% of oyster plants in US do not have available floor space to install the treatment equipment in their facilities. Of those that do, only some (5%) have 2000 or more square feet available, which is sufficient for large size processes. Moreover, it is reported that, due to financial reasons, the smaller plants are less likely to spare funds for

equipment installation and that low volume product plants may not have the technical capabilities required to install and operate the treatment equipment.

Post harvest treatment of oysters affects the demand for shucked and raw half-shell oyster because it affects both the physical characteristics (sensory and safety) and the economical attributes (where/when it is sold and at what price) of each product. Furthermore, oyster post-harvest treatment technologies reduce processing costs, increase yield, eliminate pathogens and extend shelf-life. Ultimately, the effects of post-harvest treatment on oyster demand depend on whether consumers prefer or dislike the treated oysters.

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CHAPTER 3

EFFECT OF HIGH PRESSURE PROCESSING ON SURVIVAL OF *Vibrio parahaemolyticus* STRAINS IN PURE CULTURE AND PACIFIC OYSTERS

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Submitted to Journal of Food Science

Abstract

Different strains of *Vibrio parahaemolyticus* (Vp) in broth cultures and Vp-inoculated live Pacific oysters (*Crassostrea gigas*) were subjected to high pressure processing (HPP) at 241, 276, 310, and 345 MPa. Results showed Vp numbers were reduced by HPP in both pure culture and whole oysters. Vp inactivation was dependent on time and pressure. Optimum conditions for reducing Vp in pure culture and oysters to non-detectable levels were achieved at 345 MPa for 30 and 90 s, respectively. Resistance variations were detected between Vp in pure culture and in oysters. HPP proved to be an efficient means of reducing Vp in oysters.

Key Words: high pressure, oysters, *Vibrio parahaemolyticus*, microbial reduction

Introduction

High pressure processing (HPP) technology has attracted increased attention as a means of non-thermal treatment of raw or fresh foods for reduction of bacterial loads without causing significant changes in appearance, flavor, texture and nutritional qualities (Styles and others 1991; Berlin and others 1999). The application of HPP in foods was first investigated by Hite in 1899 (Hite 1899). HPP is a 'heatless' process where foods in flexible packaging are subjected to hydrostatic pressures up to 1035 MPa (Swientek 1992). Several studies showed that HPP treatment reduced the microbiological load and enzymatic activity of foods at ambient or low temperatures without breaking covalent bonds, thereby retaining many of the natural qualities such as, flavor, color, and nutritional constituents (Hoover and others 1989; Oshima and others 1993; Hayashi and others 1989; Berlin and others 1999; He and others 2001). HPP can be used to inactivate microorganisms by inducing changes to the morphology, biochemical reactions, genetic mechanisms, and cell membranes (Hoover and others 1989; Smelt 1998). The process has shown good potential in reducing pathogens and spoilage bacteria in the food industry (Farr 1990). Research has shown that *Vibrio* sp. are very susceptible to inactivation by HPP technology (Styles and others 1991; Farkas 1993; Hoover 1993; Berlin and others 1999). Berlin and others (1999) showed that pathogenic *Vibrio* sp. are susceptible to HPP treatment at pressure levels between 200-300 MPa.

Several recent epidemics of severe illness and gastroenteritis have been associated with the consumption of raw oysters in the United States, with the majority being attributed to *Vibrio* sp. (CDC 1989). *Vibrio parahaemolyticus* (Vp) and *Vibrio vulnificus* have been implicated in outbreaks of gastroenteritis in the Southeast and the Pacific Northwest (Klontz and others 1993; Kaysner 1998). In 1997, more than 200 cases were reported to be caused by consumption of Vp infected raw oysters harvested from California, Oregon, Washington, and British Columbia (CDC 1998). Vp is the most widely distributed of all human pathogenic *Vibrio* sp. in the marine environment (Hagen and others 1994). Oysters are water-filtering organisms and can accumulate pathogenic microorganisms, which are difficult to remove by depuration but can be eliminated by high pressure. Recent studies using HPP have shown significant reduction in total plate counts of aerobic and anaerobic bacteria in oysters while maintaining good sensory characteristics (Shiu 1999; Lopez-Caballero and others 2000; He and others 2001).

The objectives of this study were to determine the effect of HPP treatment on clinical and environmental Vp strains in pure culture and in inoculated whole Pacific oysters and to establish HPP parameters (time/pressure) for Vp inactivation.

Materials and Methods

Preparation of Oysters

The experimental design is presented in Figure 3.1. Live Pacific oysters (*Crassostrea gigas*) were obtained 1 to 2 days prior to exposure from Oregon Oyster Farm, Yaquina Bay, OR. Oysters were kept at 10 °C ± 1 during transportation to isolation laboratories at Hatfield Marine Science Center (Newport, OR). Oysters were cleaned and placed in an aerated circulating seawater tank maintained at 20 °C. Oysters were fed algae (*Isochrysis galbanea* and *Cheatoserous calcitrans*) daily and kept for a minimum of 24 hours prior to exposure for adjustment to the artificial environment.

Preparation of Bacteria

The clinical strain of Vp was obtained from American Type Culture Collection, Rockville, MD. (identification number: ATCC 17802). Another confirmed Vp strain (named AST) isolated from a local oyster company was used as the environmental Vp strain. The bacteria were cultured on trypticase soy agar (Difco, Detroit, MI) supplemented with 1% NaCl (TSAM) plates. The incubation time for culturing the strains was 24 hours at 35 °C. A single colony from a TSAM plate was transferred into 10 mL trypticase soy broth (Difco) supplemented with 1% NaCl (TSBM) which yielded a bacterial concentration of approximately 1 x 10^9 CFU/mL after an overnight (16 hours) incubation at 35 °C.

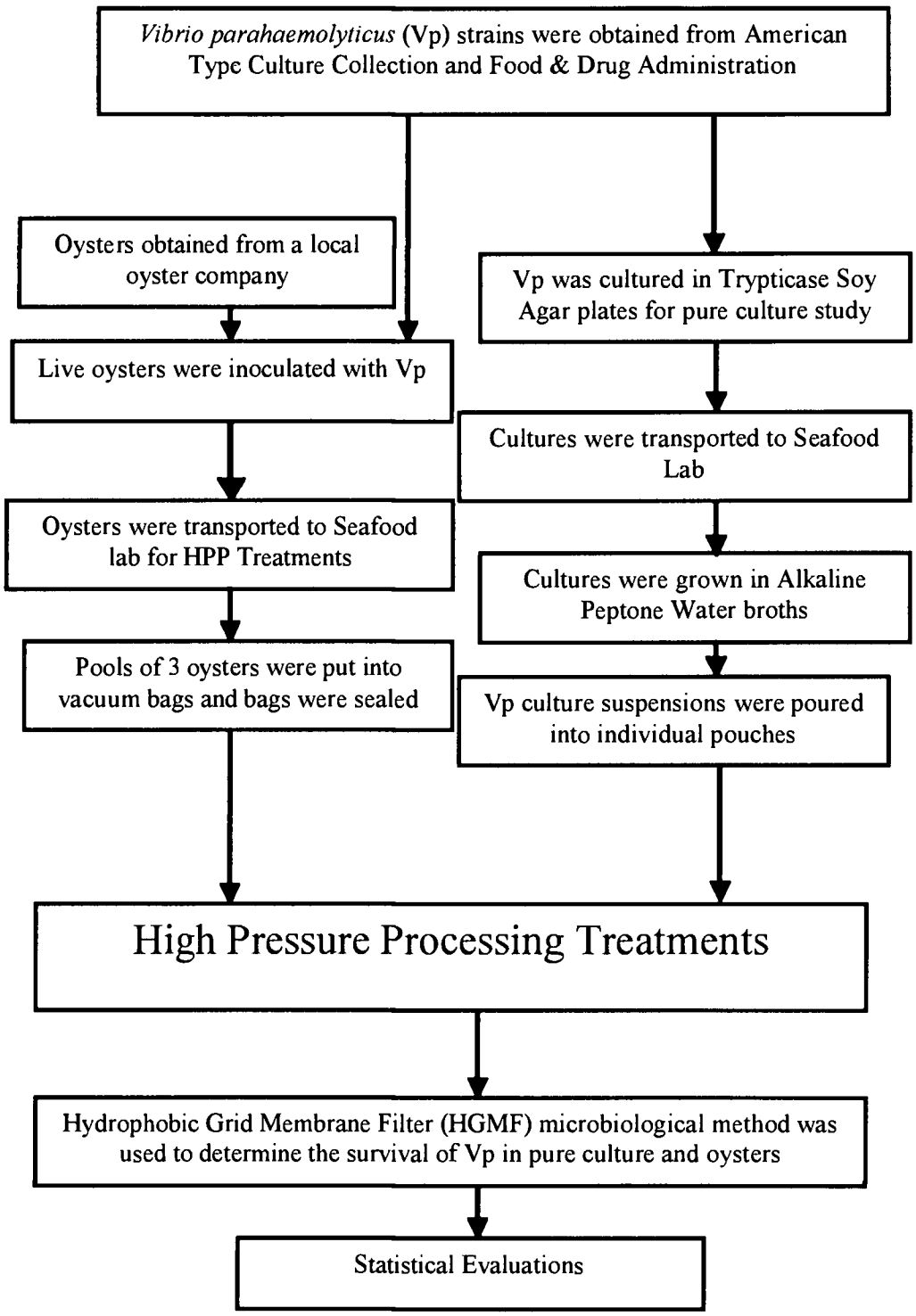

Figure 3.1 General outline showing the source, preparation, treatments, and post-treatment evaluations of Vp strains in pure culture and oysters

A 10 mL TSBM aliquot containing approximately 1 x 10^9 CFU/mL was poured into 100 mL TSBM working stock for another 1 day incubation at 35 °C. At the end of this incubation period, a 10 mL culture sample removed to measure the optical density (OD) at 525 nm and estimate the concentration of the bacteria in the working stock. An OD_{525} of 1.0 held approximately 1 x 10^9 CFU/mL Vp.

Inoculation of Oysters with Vp

Samples of oysters collected from the source were tested for the presence of Vp, as was the incoming uv-irradiated seawater in which the oysters were housed. No Vp was isolated from these samples. Oysters were exposed to Vp by bath immersion 1 day prior to HPP treatment. An aliquot of TSBM solution containing sufficient bacteria to supply appropriate number of bacteria (between 1 x 10^3 and 1 x 10^6 CFU/mL for various experiments) of seawater was added to the tanks, which contained the oysters in 8 L of seawater. The circulation of seawater in the inoculation tank was suspended for 3 hours immediately after pouring in the TSBM solution. A 10 mL aliquot was taken to determine the initial bacteria count in the tank. At the end of the exposure period 3 oysters were removed and the bacterial counts in the oyster meats were determined as described below. The circulation of seawater, supplied with algae, was resumed for 24 hours until all the oysters were removed for HPP treatment. All oysters were transported at 10 °C ± 1 to Oregon State University Seafood Laboratory (Astoria, OR) where HPP treatments were carried out.

Preparation of Pure Vp Cultures

A single Vp colony, taken from a TSAM plate, was grown in 10 mL alkaline peptone water (APW; Difco) supplemented with 1% NaCl (pH 8.5) at 35 °C incubation for 16 h, 1 day prior to HPP treatment. Aliquots of bacteria in 10 mL APW suspension were aseptically transferred into 10 x 15 cm pouches (Kapak 402, Minneapolis, MN) and vacuum-sealed. The pouches were then inserted into 20 x 25 cm vacuum bags (Alpak Food Equipment, Portland, OR and sealed.

HPP Treatment

Both clinical and environmental Vp isolates in pure culture and in oysters were treated with HPP at the following settings: 1, 60, 120, 240, 360, 480, 600 s at 241 MPa; 1, 60, 120, 180, 240, 300 s at 276 MPa; 1, 30, 60, 90, 120, 180 s at 310 MPa; 1, 10, 20, 30, 40, 50, 60, 90, 120 s at 345 MPa. All treatments were performed twice for each strain. The treatment time 1 s refers to treatments in which the samples were brought to desired pressure levels followed by instantaneous decompression. Two pools of 3 oysters were used as controls and were not pressure treated. Another pool of 3 oysters was assigned as the 'secondary control' and treated at 7 MPa for 1 min. The secondary control was used to determine if there is an unexpected effect of the pressure medium on Vp strains. Pools of 3 oysters were aseptically placed into 20 x 25 cm vacuum bags (Alpak) for HPP. Individual bags were inserted into another vacuum bag and the outer bag was vacuum-sealed. The packaged samples were submerged in 2% hydrolubric oil-

water mixture (E.F. Houghton & Co., Valley Forge, PA), which acted as the hydrostatic pressurization medium in the processing chamber. All the treatments were done using an Autoclave Engineering Inc. (Erie, PA) isostatic pressure unit (model no. CPI32260-P2) with a cylindrical pressure chamber at room temperature (22 °C). After pressurization, bags were removed, rinsed with 97% ethanol, and opened with a sterile pair of scissors.

It was observed that the HPP unit did not reach the testing pressure levels instantaneously. Table 3.1 shows the come-up times for the HPP unit to attain the testing pressure levels. The come-up-time was longer for higher pressures; however all decompressions were < 1 s. The work of compression during HPP treatment increases the temperature of foods through adiabatic heating approximately 3 °C per 100 MPa, depending on the composition of the food. The effects of temperature during the process on bacteria inactivation, however, were negligible.

Table 3.1 The time spent to attain testing pressure levels with the high pressure unit used (decompressions < 1 s)

Hydrostatic Pressure Level (MPa)	Come-Up-Time (Seconds)
241	39
276	53
310	109
345	125

Microbiological Tests

The hydrophobic grid membrane filter (HGMF) method with the ISO-GRID membrane filtering system (0.45 mm pore size, 47 mm diameter) based on a technique introduced by Entis and Boleszczuk (1983) was used to determine Vp survival after treatment. Hydrophobic grid membrane filters, pre-filters and filtration units were manufactured and supplied by Millipore Corporation, Bedford, MA. Oysters treated with HPP were aseptically removed from the bags and meats were removed from the shells using a sterile oyster knife. All oyster meats (along with the fluid) were transferred into 100 mL stainless steel homogenizing jars (Virtis Company, Gardiner, NY) supplemented with an equal volume of APW to dilute the sample to a 1:2 ratio. After homogenizing for 60 s at high speed, the homogenates were serially diluted with APW and aliquots of dilutions were filtered using the filtration unit. After filtering each dilution, the membrane filters were aseptically placed on TSAM plates. The filters on TSAM plates were incubated for 4 to 6 hours at 35 °C to activate viable but non-culturable bacteria. The filters were then transferred to thiosulfate citrate bile salts (TCBS; Difco) agar plates for overnight incubation at 42 °C. After incubation, 25 to 250 green colonies greater than 1 mm diameteron the hydrophilic part of the filter were enumerated with the assistance of a colony counter (Redington, Windsor, CN) and recorded as presumptive Vp. On occasion, isolated colonies with the characteristic appearance of Vp were further characterized biochemically to confirm their identity as Vp. A similar microbiological procedure was conducted to observe the survival of Vp in

pure culture. After HPP treatment, 10 mL sample aliquots from each pouch were aseptically removed and serially diluted with APW. The procedure for filtration of dilutions, incubation of filters, and enumeration of bacteria was the same as described above.

Statistical Analyses

Data were analyzed for significant differences using multiple linear regression tests. All statistical analyses were performed using STATGRAPHICS Plus Version 3.1 (Statistical Graphics Corp, Rockville, MD).

Results and Discussion

Optimal HPP treatment pressure and time conditions for inactivation of Vp to non-detectable levels were determined for both pure Vp culture and inoculated whole oysters. Figure 3.2 shows different HPP treatment effects on environmental AST and clinical ATCC 17802 Vp strains in pure culture. Table 3.2 presents the reduction of Vp counts as $\log_{10}$ cycles recorded at each HPP treatment setting. The initial Vp population ranged between 7.6×10^6 and 5.5×10^8 CFU/mL. No Vp survivors were detected after treatments at higher pressure levels such as 310 and 345 MPa. The greatest Vp reduction in the shortest time was observed when the clinical ATCC 17802 Vp decreased 7.4 $\log_{10}$ cycles to non-detectable levels at HPP setting 345 MPa in 30 s. At the same pressure, the environmental AST strain required 20 additional s to be completely inactivated (an overall 8.2 $\log_{10}$-cycle

reduction after 50 s). This delay in lethality may be due to the differences in initial (control) counts for each strain as the initial Vp population of environmental AST strain was 1.3 $\log_{10}$-cycle more than that of clinical ATCC 17802 Vp strain. However, this difference in inactivation was not observed with 310 MPa treatment. The initial count of AST strain was 1.6 $\log_{10}$-cycle higher before the 310 MPa treatment and both strains dropped to non-detectable levels at the same treatment time (3 min at 310 MPa). This represents a 8.4 and 6.9 $\log_{10}$-cycle reduction for AST and ATCC 17802 Vp strains, respectively. Prior to treatment at 276 MPa, the initial counts of environmental AST and clinical ATCC 17802 strains were similar, 2.1 and 2.3 x 10^8 CFU/mL, respectively. Neither Vp strains were completely eliminated but they were reduced to non-detectable levels after 5 min at 276 MPa. At this setting, the counts for the environmental AST and clinical ATCC 17802 strains were 7.7 and 5.5 x 10^0 CFU/mL, respectively. Both strains showed a 7.5 $\log_{10}$-cycle reduction. Styles and others (1991) recorded complete inactivation (5 $\log_{10}$-cycle reduction) of Vp treated in phosphate-buffered saline at 170 MPa for 30 min. Berlin and others (1999) reported that HPP treatment of 250 MPa for 10 min at 25 °C achieved a reduction of *Vibrio vulnificus* and Vp to non-detectable levels. At a similar HPP treatment of 241 MPa for 10 min, our results showed a several $\log_{10}$ reduction of Vp. However, this setting did not reduce Vp to non-detectable levels. After the treatment of 241 MPa for 10 min, the counts were 3.8 and 2.7 x 10^1 CFU/mL for environmental AST and clinical ATCC 17802 strains, respectively.

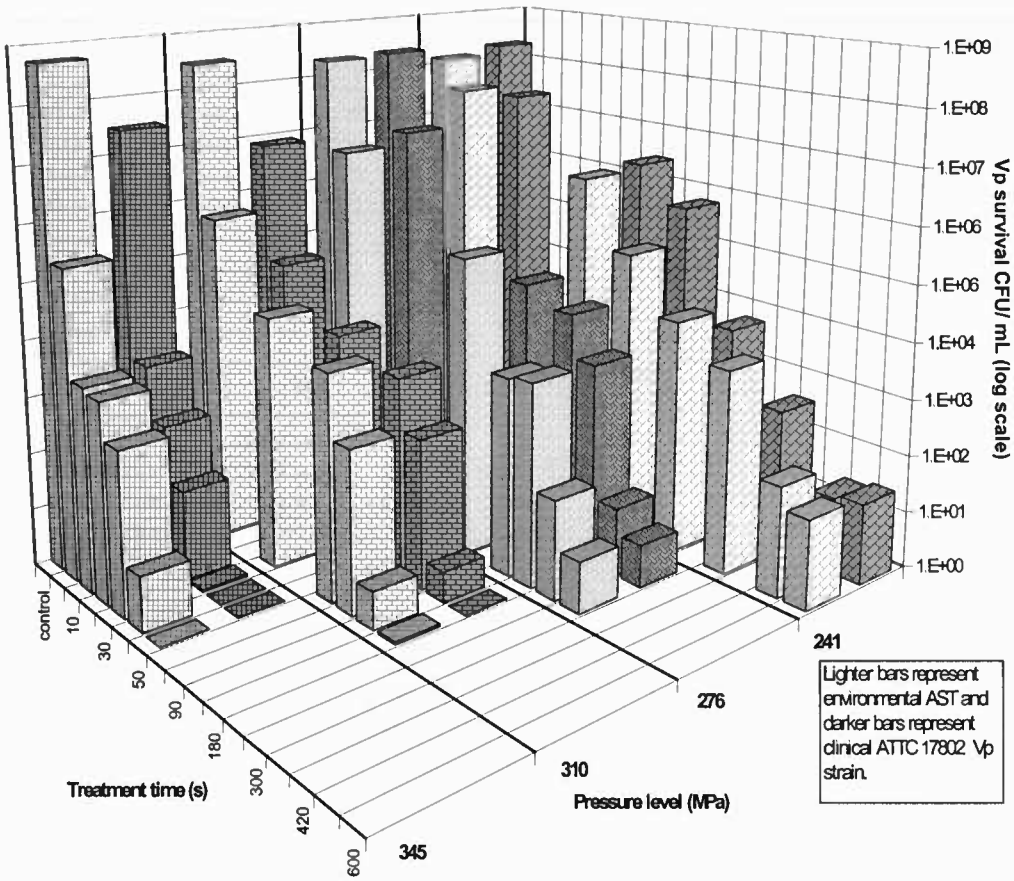

Figure 3.2 High pressure treatment effects at different pressure and time settings on environmental AST and clinical ATCC 17802 Vp strains as pure culture

At this pressure setting, the environmental AST Vp strain showed the least reduction among all strains and was the only pressure setting where the counts were not reduced to below detection levels (< 10 CFU/mL). In general, the environmental AST and clinical ATCC 17802 Vp strains responded similarly to high pressure treatments and there was statistically no significant difference between the resistance of the strains against pressure ($p>0.05$). Both intensity of pressure and time of exposure are important in determining the outcome of a treatment (Styles and others 1991). Figure 3.2 shows that a decrease in viable Vp count was directly proportional to increase in pressure and treatment time ($p<0.05$).

Table 3.2 Observed log cycle reductions after high pressure treatments for environmental AST and clinical ATCC 17802 Vp strains in pure culture and whole oysters

			Environmental AST	Clinical ATCC 17802
	Pressure (MPa)	Treatment Time (s)	Vp Reduction (log_{10} cycles)	
Pure Culture	241	600	6.6	6.9
	276	300	7.5	7.5
	310	180	8.4	6.9
	345	60	8.2	7.4
Oyster	241	600	5.2	3.5
	276	300	4.9	4.0
	310	180	4.8	5.1
	345	120	5.3	6.2

Figure 3.3 shows different HPP treatment effects on environmental AST and clinical ATCC 17802 Vp strains in whole oysters. Table 3.2 presents the reduction of Vp counts as log_{10} cycles recorded for each HPP treatment setting. No

differences were detected between the Vp counts in controls and secondary controls showing that the pressurization medium had no effect on Vp in oysters (data not shown). The initial Vp concentration in oysters (controls) ranged between 8.4×10^5 to 3.4×10^7 CFU/g. Even after the longest treatment time at each pressure setting, survivors of Vp were recovered in oysters. The highest pressure treatment of 345 MPa, however was the only treatment to reduce the Vp counts to non-detectable levels. The clinical ATCC 17802 Vp strain was diminished 6.2 $\log_{10}$ cycles to 2.2×10^0 CFU/g after 90 s treatment time at 345 MPa. The environmental AST Vp strain was reduced to 4.4×10^0 CFU/g after 2 min treatment time at the same pressure. After 310 MPa treatment, both Vp strains showed a reduction of approximately 5 $\log_{10}$ cycles with a treatment time of 3 min and final counts were 1.3×10^2 CFU/g for environmental AST and 6.8×10^1 CFU/g for clinical ATCC 17802 Vp strain. After 5 min at 276 MPa the environmental AST strain was reduced 4.9 $\log_{10}$ cycles to 4.6×10^2 CFU/g. The clinical ATCC 17802 strain was reduced to 4.2×10^3 CFU/g over the treatment time. At the lowest pressurization level of 241 MPa, environmental AST strain was reduced by 3.5 $\log_{10}$ cycles to 1.1×10^3 CFU/g, whereas the clinical ATCC 17802 strain was diminished by 5.2 $\log_{10}$ cycles to 3.8×10^1 CFU/g all after 10 min treatment time. There was no significant difference detected between the survival of environmental AST and clinical ATCC 17802 Vp strains in whole oysters at any pressure treatments ($p>0.05$). The pure culture tests showed a greater reduction in Vp in oysters as time and pressure were increased (Figure 3.3).

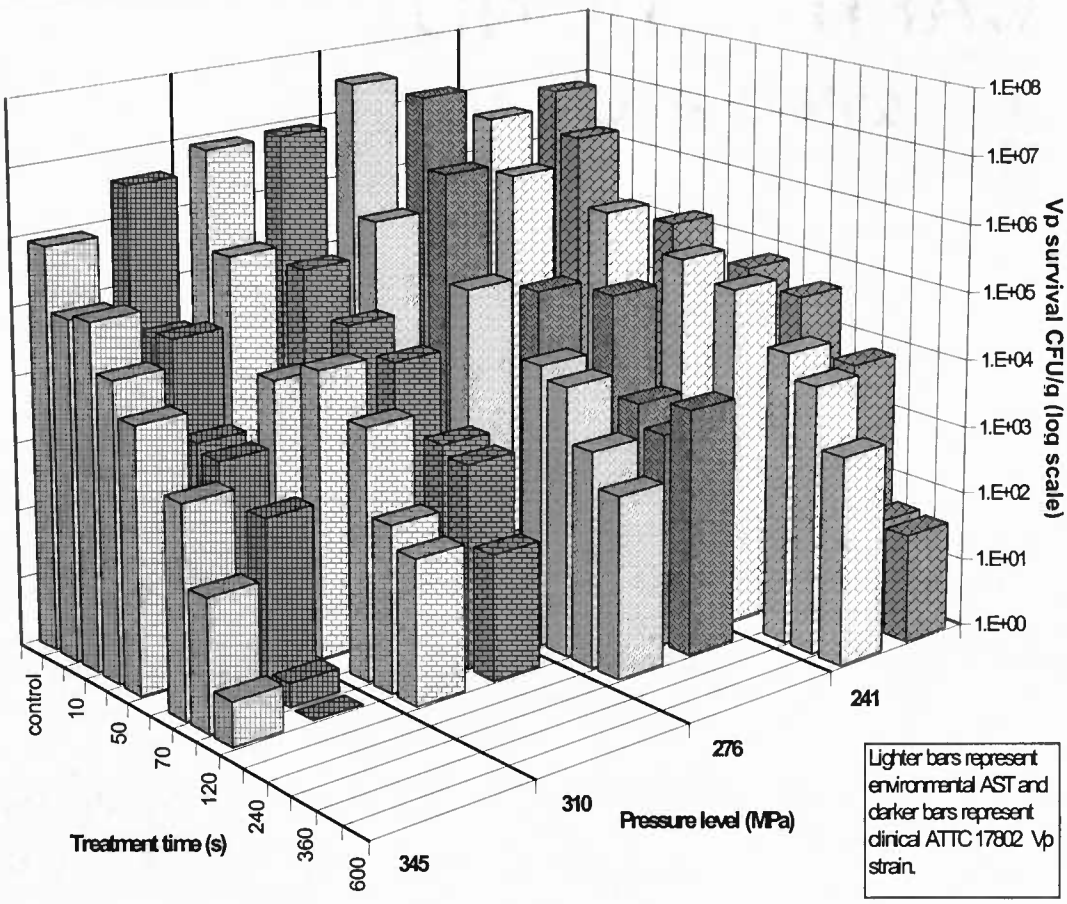

Figure 3.3 High pressure treatment effects at different pressure and time settings on environmental AST and clinical ATCC 17802 Vp strains inoculated in Pacific oysters

Table 3.3 presents the statistical evaluation of the effects of high pressure processing treatments on Vp strains in pure culture and in oysters. The table shows the results of fitting a multiple linear regression model to describe the relationship between survival of bacteria and affecting variables. In general, it required longer treatment times to reduce Vp counts in whole oysters than in pure culture. Both Vp strains appeared to be more barotolerant in oysters than in APW broth as pure culture ($p<0.05$). Result showed that there might be a protective effect when the microorganism is within the oyster, especially at the higher pressure levels. For instance, at 345 MPa treatment, it took 30 s to eliminate the clinical strain in pure culture (a 7.4 $\log_{10}$ cycle reduction), whereas 90 s were needed to reduce counts below detection levels in whole oysters (a 6.2 $\log_{10}$ cycle reduction). Likewise the environmental pure culture strain was reduced 8.5 $\log_{10}$ cycles to no counts in 50 s at 345 MPa while it took 120 s in whole oysters to reduce the counts 5.3 $\log_{10}$ cycles down under detection levels. At lower pressures there was not as dramatic a difference between Vp reduction in pure culture and that in whole oysters. However, there was not complete removal of Vp in whole oysters at 241, 276, or 310 MPa. Treatments at 310 MPa eliminated all Vp in pure cultures after 180 s while a Vp count of 10^2 CFU/g remained in whole oysters after the same time period. Similar results were found for the other lower pressure treatments. Although high pressure treatment, theoretically, is equal throughout the testing medium, a complex biological organism such as an oyster may provide some protective effects. Hoover and others (1989) reported that an enriched medium is

more protective against high pressure treatments since amino acids and other compounds are available to stressed cells. Styles and others (1991) discussed the reasons for possible differences in Vp resistance in tests in clam juice and attributed them to differences in NaCl levels and pH in the testing mediums. Swientek (1992) stated that effect of high hydrostatic pressure depends on product pH, water activity as well as salt concentration.

Table 3.3 High pressure treatments analyzed statistically for significant differences by multiple linear regression analysis

	Estimate	Standard Error	T Statistic	P-Value
Constant	0.470705	0.128024	3.67669	0.0003
Pressure	– 0.107593	0.0268774	– 4.00312	0.0001
Time	– 0.016511	0.0017786	– 9.28289	0.0001
Strain	0.0418617	0.036183	1.15694	0.2487
Medium	0.123534	0.0592806	2.08389	0.0385
Replicate	0.00307023	0.0288867	0.106285	0.9155

The equation of the fitted model is:
Survival = 0.470705 + (0.123534 x Medium) – (0.107593 x Pressure) + (0.00307023 x Replicate) + 0.0418617 x Strain – (0.0165106 x Time)

Vp is a human pathogen that is halotolerant and survives well in estuarine environments where shellfish is grown and harvested. The presence of Vp in shellfish is more prevalent in summer months when water temperatures are above 20 °C (Cook 2001). Recent surveys of oysters at retail outlets show that Vp levels can reach as high as 10^6 CFU/g at critical times of the year. The current United States Food and Drug Administration (FDA) action level for Vp in oysters is set at

10^4 CFU/g of oyster meat (ISSC & FDA 1997). In response to the recent outbreaks attributed to Vp in oysters in the United States, the FDA is undertaking a risk assessment (RA) on the public health impact of Vp, which may result in changes in policies and regulations of *Vibrio* sp. in shellfish (RA Task Force 2000). This has lead to an increased interest at both the federal and industry level for investigating post-harvest shellfish processing to reduce the levels of potential pathogens. The results of this research show that HPP is an effective post-harvest treatment for reducing Vp below present FDA action levels. Results also indicate the need for testing pathogens in their food hosts rather than pure culture to determine the rate of lethality for different treatment parameters.

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CHAPTER 4

DECIMAL REDUCTION COMPARISON OF *Vibrio parahaemolyticus* STRAINS BY HIGH PRESSURE PROCESSING

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Submitted to Journal of Food Science

Abstract

The decimal reduction rates of *Vibrio parahaemolyticus* (Vp) O3:K6 strain was compared with clinical ATCC 17802 and environmental AST Vp strains in pure culture and Pacific oysters (*Crassostrea gigas*) under high pressure processing (HPP) at 276 and 310 MPa for different treatment times based on Calik and others (2001) data. Results showed that O3:K6 strain had relatively higher decimal reduction rates when compared to two other strains. The O3:K6 strain in oysters had the highest D-value of 1.77 min under 276 MPa HPP treatment. Reduction trends presented by O3:K6 Vp strain must be considered when making commercial HPP setting decisions for reduction of Vp.

Key Words: high pressure, *Vibrio parahaemolyticus*, decimal reduction time, oysters

Introduction

Since its discovery in Japan in 1950 by Fujino and others (1953), the enteric pathogen *Vibrio parahaemolyticus* (Vp) has been shown to be indigenous to estuaries and oysters throughout the world (Baross and Liston 1970; El-Sahn and others 1982). In the United States, Vp was reported to be an important causative agent of gastroenteritis and intestinal infection outbreaks primarily due to consumption of raw or mishandled seafood (Barker 1974; Fyfe 1998). Raw oyster consumption is the most common route for human infection associated with *Vibrio* sp. (Rippey 1994). Oysters are filter feeders and they consequently accumulate the micro-flora of the water column in which they reside. This flora eventually leads to spoilage of oysters and at times, if oysters are mishandled, post-harvest growth of possible contaminated and/or natural pathogenic bacteria could result as a threat to public health (Andrews and others 1975; Kaneko and Colwell 1973; Vanderzant and Thompson 1973; Hlady 1997; Fyfe 1998). According to Center for Disease Control and Prevention (CDC) reports (1998), there had been over 200 cases of Vp associated illnesses during 1997. The largest Vp related outbreak was reported in 1998, causing illness in 416 persons in 13 states after ingesting raw oysters traced to Galveston Bay, Texas (NFSD 2001). In the 1998 outbreak a serotype previously reported only in Asia, O3:K6, emerged as the principal cause of illness in the United States for the first time. This particular strain was first detected in Calcutta, India in 1996 and thereafter in other countries. The strain demonstrates an unusual potential to spread and an enhanced propensity to cause infections. Therefore, it

introduced uncertainty about the effectiveness of previous FDA guidance indicating that no more than 10,000 Vp per g should be present in shellfish (CSFAN 2001). High pressure processing (HPP) has attracted increased attention as a means of non-thermal treatment of raw or fresh foods without causing significant changes in appearance, flavor, texture and nutritional qualities (Styles and others 1991; Berlin and others 1999). HPP has also been shown to have potential in reducing pathogens and spoilage bacteria in the food industry (Farr 1990). Researchers have shown that *Vibrio* sp. are very susceptible to inactivation by HPP technology (Styles and others 1991; Farkas 1993; Hoover 1993; Berlin and others 1999). Calik and others (2001) reported that environmental and clinical strains of Vp in pure culture and in inoculated oysters were reduced by HPP between the pressure ranges of 241 and 345 MPa.

Decimal reduction time (D-value), the time required to destroy 90% (or one $\log_{10}$ cycle) of a specific organism in a specific medium under constant stress, reflects the resistance of the organism to that particular treatment (Jay 1996). Mathematically, D-value is equal to the reciprocal of the survivor curve slope and is a measure in min for the death rate of the organism (Jay 1996). In this study, decimal reduction rates of clinical and environmental Vp strains in artificially inoculated live Pacific oysters (*Crassostrea gigas*) from the study by Calik and others (2001) were compared with the O3:K6 Vp strain under different high pressure treatments.

Materials and Methods

Oyster Preparation

Live Pacific oysters (*Crassostrea gigas*) were obtained 1 to 2 day prior to exposure from a local oyster company, Oregon Oyster Farm, Yaquina Bay, OR. Oysters were kept at 10 °C ± 1 during transportation to isolation laboratories at Hatfield Marine Science Center (Newport, OR). Oysters were cleaned and placed in an aerated circulating seawater tank maintained at 20 °C. Oysters were fed algae (*Isochrysis galbanea* and *Cheatoserous calcitrans*) daily and kept for a minimum of 24 hours prior to exposure for adjustment to the artificial environment.

Preparation of Bacteria

The O3:K6 strain was obtained from Food and Drug Administration, Seattle, WA. Bacteria were cultured on trypticase soy agar (Difco, Detroit, MI) supplemented with 1% NaCl (TSAM) plates. The incubation time for culturing the strains was 24 hours at 35 °C. A single colony from a TSAM plate was transferred into 10 mL trypticase soy broth (Difco) supplemented with 1% NaCl (TSBM) which yielded a bacterial concentration of approximately 1 x 10^9 CFU/mL after an overnight (16 hours) incubation at 35 °C. A 10 mL TSBM aliquot containing approximately 1 x 10^9 CFU/mL was poured into 100 mL TSBM working stock for another 1 day incubation at 35 °C. At the end of the incubation period, a 10 mL culture sample was removed and the optical density (OD) at 525 nm was measured

to estimate the concentration of the bacteria in the working stock. An OD_{525} of 1.0 held approximately 1 x 10^9 CFU/mL Vp.

Inoculation of Oysters with Vp

Samples of oysters were tested for the presence of Vp, as was the incoming uv-irradiated seawater in which the oysters were housed. No Vp was isolated from these samples. Oysters were exposed to Vp by bath immersion 1 day prior to HPP treatment. An aliquot of TSBM solution containing sufficient bacteria to supply appropriate number of bacteria (between 1 x 10^3 and 1 x 10^6 CFU/mL for various experiments) of seawater was added to the tanks, which contained oysters in 8 L of seawater. The circulation of seawater in the inoculation tank was suspended for 3 hours immediately after pouring in the TSBM solution. A 10 mL aliquot was taken to determine the initial bacteria count in the tank. At the end of the exposure period 3 oysters were removed and the bacterial counts in the oyster meats were determined as described below. The circulation of seawater, supplied with algae, was resumed for 24 hours until all the oysters were removed for HPP treatment. All oysters were transported at 10 °C ± 1 to Oregon State University Seafood Laboratory (Astoria, OR) where HPP treatments were carried out.

Preparation of Pure Vp Cultures

A single O3:K6 strain Vp colony, taken from a TSAM plate, was grown in 10 mL alkaline peptone water (APW; Difco) supplemented with 1% NaCl (pH 8.5) at 35 °C incubation for 16 h, 1 day prior to HPP treatment. Aliquots of bacteria in 10 mL APW suspension were aseptically transferred into 10 x 15 cm pouches (Kapak 402, Minneapolis, MN) and vacuum-sealed. The pouches were then inserted into 20 x 25 cm vacuum bags (Alpak Food Equipment, Portland, OR) and sealed.

HPP Treatment

The clinical O3:K6 Vp isolate in pure culture and in oysters was pressure treated for 1, 2, 3, 4, and 5 min at 276 and 310 MPa. All treatments were performed twice. Two pools of 3 oysters were used as controls and were not pressure treated. Another pool of 3 oysters was assigned as the 'secondary control' and treated at 7 MPa for 1 min. The secondary control was used to determine if there is an unexpected effect of the pressure medium on Vp strains. Pools of 3 oysters were aseptically placed into 20 x 25 cm vacuum bags (Alpak) for HPP. Individual bags were inserted into another vacuum bag and the outer bag was vacuum-sealed. The packaged samples were submerged in 2% hydrolubric oil-water mixture (E.F. Houghton & Co., Valley Forge, PA), which acted as the hydrostatic pressurization medium in the processing chamber. All treatments were done using an Autoclave Engineering Inc. (Erie, PA) isostatic pressure unit (model no. CPI32260-P2) with a

cylindrical pressure chamber at room temperature (22 °C). After pressurization, bags were removed, rinsed with 97% ethanol, and opened with a sterile pair of scissors.

Microbiological Tests

The hydrophobic grid membrane filter (HGMF) method with the ISO-GRID membrane filtering system (0.45 mm pore size, 47 mm dia) based on a technique introduced by Entis and Boleszczuk (1983) was used to determine Vp survival after treatment. Hydrophobic grid membrane filters, pre-filters and filtration units were manufactured and supplied by Millipore Corporation, Bedford, MA. Oysters treated with HPP were aseptically removed from the bags and meats were removed from the shells using a sterile oyster knife. All oyster meats (along with the fluid) were transferred into 100 mL stainless steel homogenizing jars (Virtis Company, Gardiner, NY) supplemented with an equal volume of APW to dilute the sample to a 1:2 ratio. After homogenizing for 60 s at high speed, the homogenates were serially diluted with APW and aliquots of dilutions were filtered using the filtration unit. After filtering each dilution, the membrane filters were aseptically placed on TSAM plates. The filters on TSAM plates were incubated for 4 to 6 hours at 35 °C to activate viable but non-culturable bacteria. The filters were then transferred to thiosulfate citrate bile salts (TCBS; Difco) agar plates for overnight incubation at 42 °C. After incubation, 25 to 250 green colonies greater than 1 mm diameter on the hydrophilic part of the filter were enumerated with the

assistance of a colony counter (Redington, Windsor, CN) and recorded as presumptive Vp. On occasion, isolated colonies with the characteristic appearance of Vp were further characterized biochemically to confirm their identity as Vp. A similar microbiological procedure was conducted to observe the survival of Vp in pure culture. After HPP treatment, 10 mL sample aliquots from each pouch were aseptically removed and serially diluted with APW. The procedure for filtration of dilutions, incubation of filters, and enumeration of bacteria was the same as described above.

Statistical Analysis

All treatments were done in duplicate. Data were analyzed for significant differences using multiple linear regression analysis. All statistical evaluations were performed using STATGRAPHICS Plus Version 3.1 (Statistical Graphics, Rockville, MD)

Results and Discussion

Comparisons of decimal reduction times were made between the O3:K6 Vp strain and the clinical ATCC 17802 and environmental AST strains inoculated in oysters based on data by Calik and others (2001). Observed D-values were determined by plotting the $\log_{10}$ of the number of survivors against time for each treatment. The effects of the time necessary to reach the desired pressure (come-up time) were excluded when calculating D-values. Table 4.1 shows the slopes and R^2

values of best-fit regression lines, observed D-values, and the log reductions of each strain in pure culture under high pressure treatment. None of the treatment settings completely eliminated O3:K6 Vp strain in pure culture. At 276 MPa the O3:K6 Vp strain in pure culture showed a 6.17 $\log_{10}$ cycle reduction as counts decreased from 2.4 x 10^7 to 1.6 x 10^1 CFU/mL after 300 s of treatment time. Likewise, clinical and environmental Vp strains at 276 MPa showed similar 6.14 and 5.77 $\log_{10}$ cycle reduction, respectively. At 276 MPa, the observed D-values for all strains were within a close range (Table 4.1). At 310 MPa pressure treatment a 5.76 $\log_{10}$ cycle reduction was seen with the environmental Vp strain, when counts dropped from 6.4 x 10^5 to 1.1 x 10^0 CFU/mL. A $\log_{10}$ reduction of 3.84 cycles and a D-value of 0.79 min belonged to O3:K6 Vp strain after 310 MPa (Table 4.1). At this treatment, the clinical and environmental Vp strains had D-values as 0.59 and 0.50 min, respectively. There was no significant difference in resistance to pressure among all three strains in pure culture ($p>0.05$). Although statistically not significant, the O3:K6 Vp strain as pure culture in APW broths exhibited relatively higher resistance to pressure at 310 MPa. However the strain's higher resistance to pressure was not that apparent during 276 MPa treatments. These findings confirmed the results of Cook (2001) who reported a relatively higher resistance to pressure of 03:K6 Vp strain in pure culture when compared with other Vp strains. He reported a D-value of 0.9 min for O3:K6 Vp strain in phosphate saline buffered solution (PBS) treated with pressure at 248 MPa, whereas treatments with other environmental strains gave D-values in the range of

0.4 min. The variation of reported D-values for the O3:K6 Vp strain from these two studies is caused by the different treatment pressure levels used. Additionally, culturing media differences (PBS opposed to APW) might have led Vp to respond differently.

Table 4.1 Observed D-values, slope and R^2 values of best-fit regression lines of *V. parahaemolyticus* in pure culture high pressure treatments

Pressure (MPa)	Vp Strains In Pure Culture	D-values (min)	Slope	R^2 Value
276	O3:K6	0.82	-0.0203	0.9695
	Clinical	0.84	-0.0198	0.9458
	Environmental	0.89	-0.0188	0.9639
310	O3:K6	0.79	-0.0210	0.9416
	Clinical	0.59	-0.0283	0.9340
	Environmental	0.50	-0.0336	0.9464

Table 4.2 shows the slopes and R^2 values of best-fit regression lines, observed D-values, and the log reductions of each strain in Pacific oysters under high pressure treatment. At 276 MPa the O3:K6 Vp strain in oysters showed a 4.41 $\log_{10}$ cycle reduction when counts decreased from 2.5 x 10^4 to no CFU/mL after 420 s of treatment time. The clinical and environmental Vp strains showed 4.01 and 3.54 $\log_{10}$ cycle reduction after 300 s under 276 MPa. At the 276 MPa treatments, the highest observed D-value of 1.77 min was seen with O3:K6 Vp strain, followed

by the environmental strain (1.52 min) and the clinical strain (1.34 min) (Table 4.2). At 310 MPa for 240 s treatment a reduction was seen when counts dropped from 2.5 x 10^4 to 7.4 x 10^0 CFU/mL with a 3.54 $\log_{10}$ cycle decrease for the O3:K6 Vp strain. At 310 MPa, environmental Vp strain showed 3.31 $\log_{10}$ reduction and 3.16 $\log_{10}$ cycles reduction was seen with clinical Vp strain. The highest D-value of 1.11 min was seen with O3:K6 strain, followed by clinical Vp strain (0.96 min) and environmental Vp strain (0.84 min). There was no significant difference in resistance to pressure among all three strains in Pacific oysters ($p>0.05$). Although statistically not significant the O3:K6 Vp strain in oysters exhibited relatively higher resistance to pressure than other Vp strains (Table 4.2). Generally, both in pure culture and in oysters the O3:K6 Vp strain exhibited relatively higher D-values than the clinical and environmental Vp strains. At both pressure levels, the D-values were higher for all strains in oysters than in pure culture. Although high pressure treatment, theoretically, is equal throughout the testing medium, a complex biological organism such as an oyster may provide some protective effects. Hoover and others (1989) reported that an enriched medium is more protective against high pressure treatments since amino acids and other compounds are available to stressed cells.

Table 4.2 Observed D-values, slope and R^2 values of best-fit regression lines of *V. parahaemolyticus* in oysters high pressure treatments

Pressure (MPa)	Vp Strains In Oysters	D-values (min)	Slope	R^2 Value
276	O3:K6	1.77	-0.0094	0.9340
	Clinical	1.34	-0.0124	0.9357
	Environmental	1.52	-0.0110	0.9603
310	O3:K6	1.11	-0.0150	0.9650
	Clinical	0.96	-0.0174	0.9831
	Environmental	0.84	-0.0198	0.9496

The HPP unit used in these experiments did not establish the desired pressure levels instantaneously. The tested Vp strains were being exposed to lower hydrostatic pressure during the time spent to attain the desired pressure level and were affected. Table 4.3 shows the come-up times for the unit used. Naturally, the come-up-time was longer when establishing higher pressures. The results show that come-up-time had a significant contribution to Vp reduction and that the bacteria were affected, even before the actual holding time (treatment) started (data not shown). Therefore, the come-up time effects were excluded when calculating D-values for each strain at each treatment. A powerful industrial HPP machine could launch the desired pressure levels immediately, preventing additional come-up-time effects that would alter the actual treatment time.

Table 4.3 Time spent to attain testing pressure levels with the high pressure unit used (decompressions were < 1 s)

Hydrostatic Pressure Level (MPa)	Come-Up-Time (Seconds)
276	53
310	109

Vp has been shown to be a pressure-sensitive food pathogen (Metrick and others 1989; Styles and others 1991). Results showed that O3:K6 Vp strain is susceptible to pressure treatment in oysters, however the strain exhibited a higher resistance to pressure than clinical ATCC 17802 and environmental AST strains. The US Food and Drug Administration action level for Vp is set at 10^4 CFU/g of oyster meat, above which oyster harvests are prohibited (ISSC & FDA 1997). In this study, HPP treatments with O3:K6 strain showed that relatively longer treatment times are necessary to reduce counts of O3:K6 below FDA acceptable levels. Therefore, the HPP process setting decisions must be made according to reduction trends presented by this particular relatively resistant and virulent Vp strain to comply with FDA regulations.

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CONCLUSION

The study comparing the high hydrostatic pressure processing (HPP) effects on environmental and clinical *Vibrio parahaemolyticus* (Vp) strains in broth culture and in artificially inoculated whole Pacific oysters (*Crassostrea gigas*) showed that both strains were susceptible to effects of pressure in broth culture and in oysters. Results showed that Vp inactivation was dependent on treatment time and pressure. There were not any significant differences between the resistance of clinical and environmental strains against pressure ($p>0.05$). Both Vp strains in oysters required longer times for similar reduction when compared with pure culture broths especially at higher pressure levels (310 and 345 MPa). The oysters provided a protective environment against pressure for Vp strains. More than 10 min of treatment time was necessary to reduce Vp counts in pure culture broth and in oysters to non-detectable levels at 276 MPa. Shortest treatment times for reducing Vp in pure culture broth and oysters to non-detectable levels were achieved at 345 MPa for 30 and 90 s, respectively. The study proved that HPP is a viable means to reduce Vp counts in pure culture broth and oysters.

The study comparing the clinical and environmental Vp strains with the O3:K6 Vp strain (the isolate from the largest Vp outbreak, 1998 Texas) under HPP at 276 and 310 MPa showed that O3:K6 Vp strain required relatively longer treatment times to be reduced to non-detectable levels as opposed to clinical and environmental Vp strains. At both treatment pressures O3:K6 Vp strain both in

pure culture and Pacific oysters had the highest decimal reduction times (D-value). The highest D-value of 1.77 min belonged to 276 MPa HPP treatment on O3:K6 strain in oysters. The lowest D-value of 0.50 min was observed with the environmental strain in pure culture at 310 MPa after 180 s of treatment time. There were no statistically significant differences in resistance to pressure among all Vp strains tested both in pure culture and Pacific oysters ($p>0.05$). The US FDA permits no more than 10,000 colony forming units of Vp per g of oyster meat. Therefore, decision on HPP operating parameters must be made according to the relatively resistant and virulent O3:K6 Vp strain to comply with FDA regulations.

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APPENDIX

EFFECTS OF INDUSTRIAL HIGH PRESSURE PROCESSING ON *Vibrio parahaemolyticus* IN LIVE PACIFIC OYSTERS

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Introduction

There is increasing concern with the safety of oyster consumption. Numerous epidemics of severe illness have been associated with the consumption of raw oysters in the United States, with the majority being attributed to *Vibrio* sp. (CDC 1989). Several efforts have been made to purify oysters and render them safe for human consumption. The newest treatment technologies currently being used in the oyster industry are individual quick freezing (IQF), cool pasteurization (Ameripure process), and high hydrostatic pressure processing (HPP) (Muth and others 2000). Research has shown that *Vibrio* sp. are very susceptible to inactivation by HPP technology (Styles and others 1991; Farkas 1993; Hoover 1993; Berlin and others 1999). Berlin and others (1999) showed that pathogenic *Vibrio* sp. are susceptible to HPP treatment at pressure levels between 200-300 MPa. Calik and others (2001) reported that environmental and clinical strains of *Vibrio parahaemolyticus* (Vp) in pure culture and in inoculated oysters were reduced by HPP between the pressure ranges of 241 and 345 MPa. The objectives of this study were to determine the effects of industrial HPP on Vp strains in live Pacific oysters and to verify the results of the study by Calik and others (2001).

Materials and Methods

Preparation of live Pacific oysters (*Crassostrea gigas*) and the O3:K6 Vp strain as well as inoculation of oysters with bacteria were all done following the procedures outlined in the study by Calik and others (2001). Industrial high pressurization of oysters were performed at Nisbet Oyster Co., Inc. (Bay Center, WA) using a two barrel 90 L HPP unit by Flow International Corporation. Table A.1 shows the treatment settings of inoculated live Pacific oysters using the industrial HPP unit. For each setting, pools of 3 oysters were wrapped in sealed vacuum bags and treated. All treatments were performed in duplicate. After treatments the oysters, kept at 10 °C, were transported to microbiology laboratories at OSU Seafood Laboratory (Astoria, OR). The microbiological testing was conducted exactly as described in the study by Calik and others (2001). Data were analyzed for significant differences using multiple linear regression tests using STATGRAPHICS Plus Version 3.1 (Statistical Graphics Corp, Rockville, MD).

Table A.1 Industrial HPP treatment settings of inoculated Pacific oysters

Pressure Level	Treatment Time
241 MPa	2 min
	4 min
256 MPa	2 min
	4 min
276 MPa	2 min
	4 min
310 MPa	2 min
	4 min

Results and Discussion

Table A.2 presents the effect of industrial HPP treatment on Vp in oysters. Vp count reduction increased significantly as the treatment pressure and time were increased ($p<0.05$). The treatments at highest pressure level of 310 MPa for both 2 and 4 min were effective enough to reduce Vp counts to zero presenting a 4.1 $\log_{10}$ reduction. Calik and others (2001) reported a similar reduction trend for Vp in oysters treated at 310 MPa. According to their results, at 310 MPa there was a 5 $\log_{10}$ cycle reduction after 3 min. Treatments at 276 MPa could not reduce the counts to zero. A total of 1.1×10^2 and 4.4×10^1 colony forming units (CFU)/g Vp remained in oysters after 2 and 4 min of treatment time at 276 MPa showing 2.1 and 2.4 $\log_{10}$ reductions, respectively. Similarly, in the study by Calik and others (2001) it was shown that a treatment at the same pressure level for 4 min brought 2.3 $\log_{10}$ reduction. Treatments at 256 MPa were less effective in reducing Vp counts. Even though treatment for 4 min lowered the counts to detection level, after 2 min 1.9×10^2 CFU/g Vp remained viable. The results showed 1.8 and 3.0 $\log_{10}$ reduction after 2 and 4 min treatment times, respectively. The least effective treatments were at the lowest pressure level, 241 MPa. 1.3 and 1.8 $\log_{10}$ reduction was observed at treatment times 2 and 4 min, respectively. Likewise, Calik and others (2001) showed only a 3.5 $\log_{10}$ reduction in Vp counts after 10 min of treatment time.

Table A.2 Effect of industrial HPP treatment on Vp in oysters

Pressure Level (MPa)	Treatment Time (min)	CFU/g		
		Duplicate 1	Duplicate 2	Average
	Control (no treatment)	10300	14100	12200
241	2	510	840	675
	4	110	240	175
256	2	220	170	195
	4	22	0	11
276	2	44	170	107
	4	44	44	44
310	2	0	0	0
	4	0	0	0

Conclusion

Results showed that Vp reduction in oysters was dependent on HPP treatment pressure and time. Results obtained using an industrial HPP unit verified the results of the study by Calik and others (2001).

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