Incidence, abundance, post-harvest processing and population diversity of pathogenic vibrios in oysters from the Great Bay Estuary

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INCIDENCE, ABUNDANCE, POSTHARVEST PROCESSING AND POPULATION DIVERSITY OF PATHOGENIC VIBRIOS IN OYSTERS FROM THE GREAT BAY ESTUARY

BY

JONG WHAN YU

B.S. University of New Hampshire, 2008

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In Partial Fulfillment of

The Requirement for the Degree of

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ABSTRACT

INCIDENCE, ABUNDANCE, POST-HARVEST PROCESSING AND POPULATION DIVERSITY OF PATHOGENIC VIBRIOS IN OYSTERS FROM THE GREAT BAY ESTUARY

By

Jong Whan Yu

University of New Hampshire, December, 2011

Shellfish-borne vibrio diseases have increased recently in the US, particularly in cooler, northern areas. Harvest area monitoring and post-harvest processing (PHP) have been implemented to reduce *Vibrio* disease risks in marketed shellfish, and rapid and reliable detection methods are needed to assess these risks. Both culture based and qPCR detection methods were useful for detecting *V. parahaemolyticus* (Vp) in oysters. QPCR is more rapid and less subjective, while the culture based method allowed detection of lower Vp concentrations in cold-water oysters where the Vp population diversity was more clonal. Depuration and relaying were assessed as PHP strategies for reducing Vp and *Vibrio vulnificus* (Vv) levels in live oysters. Relaying was significantly more effective, especially where higher salinity and indigenous microbial communities were factors. Both detection methods proved to be useful tools for detecting Vp and Vv, and relaying is a promising strategy for reducing *Vibrio* levels in harvested oysters.
INTRODUCTION

*Vibrio parahaemolyticus* and *Vibrio vulnificus* and Shellfish-Borne Diseases

*Vibrio parahaemolyticus* and *Vibrio vulnificus* are gram-negative, free-living, halophilic bacteria that are commonly found in estuarine environments in association with shellfish. *V. parahaemolyticus* and *V. vulnificus* are both threats to the general public through consumption of contaminated seafood and to the shellfish industry because of potential disastrous repercussions of outbreaks on shellfish consumption. *V. parahaemolyticus* can cause bacterial gastroenteritis following consumption of raw or undercooked shellfish. The clinical manifestation of the disease includes nausea, diarrhea, vomiting and headaches, and in rare cases can lead to septicemia (Levine et al., 1993; Drake et al., 2007). *V. parahaemolyticus* is now the leading cause of bacterial gastroenteritis in seafood worldwide (Altekruse et al., 2000). Different clinical strains with global distribution, and often containing thermostable direct hemolysin (*tdh*) and TDH-related hemolysin (*trh*) genes, rapidly induce inflammatory gastroenteritis (Shiraih et al., 1990; Honda and Iida 1993). *V. vulnificus* infections rarely cause gastroenteritis in healthy individuals; however, this pathogen is more typically associated with wound infections and septicemia among people who are immunocompromised or have underlying diseases that consequently increase the iron levels in the blood (Wright et al., 1996). *V. vulnificus* is a significant threat to the shellfish industry due to its 50% mortality rate to patients afflicted with *V. vulnificus* infections (Oliver 2005).
*V. parahaemolyticus* infection associated with seafood was first documented in Japan in 1953, where 272 patients became ill by consuming shirasu (Fujino). The first documented case of *V. parahaemolyticus* infection in the US was observed in Maryland, where 3 different outbreaks occurred where 425 patients were diagnosed with gastroenteritis after consuming undercooked crabs (Molenda *et al.*, 1972). Since then there have been documented reports of *V. parahaemolyticus* infections occurring throughout the US where outbreaks occurred between 1997 and 1998 in the Pacific Northwest (CDC 1998), Washington and Texas (DePaola *et al.*, 2000), and in Long Island Sound of New York (CDC 1999) through consumption of raw oysters. There was a *V. parahaemolyticus* outbreak on a cruise ship in 2004 that identified consumption of raw oysters from Alaska as being responsible for this outbreak (McLaughlin *et al.*, 2005). Presently, increasing incidence of Vibrio-related infections has been documented in New England from 2000 – 2008, where the increasing incidence of confirmed cases of non-cholera Vibrio infections have been noted in the past few years (Jones 2011).

The annual cost in dealing with Vibrio-related infections in the health care system is tremendously higher than any other seafood-borne illnesses. It is estimated that the cost from premature death from *V. vulnificus* infection is $232 million per year and followed by *V. parahaemolyticus* costing $21 million per year (Ralston *et al.*, 2011). The incidence rate of these *Vibrio* species are lower compared to the Norwalk virus, but the costs to deal with these bacterial pathogens is more costly than the Norwalk virus ($17.7 million per year), which has the highest incidence rate in regards to seafood-borne illnesses (Ralston *et al.*, 2011).
Incidence and Management of Vibrios in Estuarine Environments

The distribution and abundance of these *Vibrio* species have been monitored worldwide under a variety of environmental conditions (Alam et al., 2002; Martinez-Urtaza et al., 2008; O’Neill et al., 1992; Parveen et al., 2008; Paz et al., 2007; Sobrinho et al., 2010; Vezzulli et al., 2009; Zimmerman et al., 2007). The incidence and abundance of Vibrios detected in estuarine ecosystems vary with temperature, salinity, turbidity, dissolved oxygen, pH and chlorophyll a (Johnson et al., 2010; Jones et al., 2010; Jones and Summer-Brason, 1998; Parveen et al., 2008; Zimmerman et al., 2007). *V. parahaemolyticus* and *V. vulnificus* have different optimal growth conditions relative to water temperature and salinity (Drake et al., 2007; Motes et al., 1998; Parveen et al., 2008). The minimal temperature associated with *V. parahaemolyticus* infection is $>15^\circ C$ (McLaughlin et al., 2005) and $25^\circ C$ is the optimal temperature for growth. The optimal salinity for *V. parahaemolyticus* growth is 23 ppt (CFSAN 2005); however, it can tolerate a range of salinity from 5 – 34 ppt (Cook et al., 2002). The highest concentrations of *V. vulnificus* are detected when water temperatures are 20 - 30$^\circ C$ and salinity is at a range of 5 – 25 ppt (Drake et al., 2007; Motes et al., 1998). *V. parahaemolyticus* and *V. vulnificus* thrive in coastal water temperatures above 20$^\circ C$ but as the temperature decreases below 15$^\circ C$, the concentrations of viable Vibrios also decrease. Salinity also has a large influence on the abundance of these *Vibrio* species, with salinity higher than 30 ppt inhibiting Vibrio growth and decreasing concentrations in oysters (Audemard et al., 2011; Johnson et al., 2010; Motes et al., 1998). The abundance of pathogenic *Vibrio* species in oysters in the Great Bay Estuary (GBE) follows seasonal
temperature trends, as shown by earlier studies (Jones and Summer-Brason 1998; O’Neill et al., 1992).

The classification of shellfish growing waters is based mainly on levels of fecal coliforms in the water; however due to emerging outbreaks of Vibrio-related diseases in the US, concentrations of *V. parahaemolyticus* in oysters are monitored in areas where disease has occurred and shellfish are harvested, distributed, and processed (NSSP 2009). There are two main oyster beds and potential harvest sites in the GBE, Nannie Island and Oyster River, though they are categorized differently for shellfish harvesting. Nannie Island is classified as an approved area of shellfish growing waters allowing for recreational oyster harvesting while Oyster River is classified as prohibited waters due to the close proximity to the Durham wastewater treatment plant (NSSP 2009). The disinfected effluent from this wastewater treatment plant has the potential to cause high fecal coliform counts, instigating a Prohibited classification for shellfish harvesting in the Oyster River. Despite the fact that Nannie Island is considered as an Approved area of shellfish harvesting, the incidence of *Vibrios* is the same as what is found in Oyster River a is Prohibited area (Jones et al., 2010).

**Detection Methods for Vibrios in Shellfish**

There are two commonly used types of detection methods for detection and enumeration of Vibrios; the traditional culture-based method and real-time PCR (qPCR). The traditional culture-based method of detection has been established by the FDA Bacteriological Analytical Manual (BAM; Kaysner and DePaola 2004). Through phenotypic screens and colony multiplex PCR (Panicker et al., 2004) or colony
hybridization (Nordstrom et al., 2004), *Vibrio* species are detected and isolated from enriched oyster homogenates. However, this labor-intensive method takes 4 – 5 days to obtain confirmation of *V. parahaemolyticus* or *V. vulnificus* after harvesting the oysters (Su et al., 2007). Isolating Vibrios by phenotypic screening from thiosulfate citrate bile salts sucrose (TCBS) agar is currently used to differentiate *Vibrio* species based on sucrose fermentation (Kaysner and DePaola, 2004). This phenotypic screening becomes a subjective method because several *Vibrio* species exhibit same results in sucrose fermentation, such as *V. vulnificus, V. parahaemolyticus*, and *V. mimicus*, all of which yield sucrose-negative (green) colonies, creating problems for detecting specific *Vibrio* species (Chapela et al., 2010; Hara-Kudo et al., 2001; Jones et al., 2010). The FDA BAM suggests when isolating *V. parahaemolyticus* and *V. vulnificus* using TCBS, that sucrose-negative (green) colonies should be picked for confirmation (Kaysner and DePaola); however, putative sucrose-positive *V. parahaemolyticus* colonies on TCBS have been isolated (Hara-Kudo et al., 2001; Lam 1985) suggesting that all phenotypes should be screened to prevent underreporting of Vibrio concentrations in oysters. This would, however, defeat the purpose of using an isolation medium. Presently, there is a chromogenic agar that can be used to isolate and differentiate among *Vibrio* species. The frequency of *V. parahaemolyticus* detection was higher in chromogenic agar compared to TCBS because the color of the colonies depends on the reaction of bacterial beta-galactosidase to compounds in the media (Hara-Kudo et al., 2001). Another disadvantage of the culture-based method is the inability to detect viable but nonculturable (VBNC) Vibrios in unfavorable environmental and growth conditions
(Colwell et al., 2000; Wong et al., 2004). These VBNC Vibrios are not viable or culturable until the environment is suitable for growth and are not isolated on TCBS agar.

Presently, many researchers are using molecular based approaches that reduce the need for the culture based approaches though they still involve the use of enriched oyster homogenates as templates for real-time PCR (Blackstone et al., 2003; Campbell et al., 2003; Panicker et al., 2004; Randa et al., 2004; Zimmerman et al., 2007). Real-time PCR (qPCR) is a quicker method of detection because results can be obtained in a matter of hours or within 24 hours (Jones et al., 2009), it can avoid some of the more subjective steps involved in the culture-based approach (i.e., picking putative positive colonies from TCBS agar media) and also can be more sensitive method for detection than the culture-based method (Campbell and Wright 2003; Wright et al., 2007). One of the major disadvantages of using qPCR is interference by compounds that are present in oyster tissues and that inhibit qPCR (Chapela et al., 2011; Rizvi et al., 2006), preventing detection of Vibrios in minimally diluted oyster homogenate in the MPN enrichment scheme.

Postharvest Processing to Remove Vibrios from Shellfish

The threat of disease for consumers of shellfish, mainly oysters, has brought efforts by the shellfish industry and the U.S. Food and Drug Administration to provide consumers with vibrio-free shellfish. The National Shellfish Sanitation Program has set forth strict treatment guidelines for post-harvest processing (PHP) of Vibrio contaminated oysters to ensure that treated oysters are below 30 MPN/g of oyster tissue before being distributed to the public (NSSP 2009). PHP of contaminated oysters is one option for
treatment in areas with elevated levels of *Vibrios* in oysters due to warm water (> 27°C) or where there has been frequent disease incidence or an outbreak (DePaola *et al.* 2003; NSSP 2009; Sobrinho *et al.*, 2010). Recent PHP methods include (but are not limited to) immersion of oysters into liquid nitrogen followed by an extended storage in the -20°C, and low temperature heat pasteurization (Andrews *et al.*, 2000; Wright *et al.*, 2007). These methods of eliminating *Vibrios* in oysters are effective in lowering the concentration of bacterial pathogens below 30 MPN/g; however, these methods kill the oysters and/or change the palatability of the oyster meat.

Depuration and relaying of contaminated oysters could be alternative ways to reduce the concentrations of *Vibrios* in oysters. According to the NSSP guidelines, depuration is a treatment process of reducing pathogens in shellstock in a controlled aquatic environment using sterilization and filtration to create an ideal environment to reduce the levels of fecal coliforms. Relaying treatment transfers contaminated oysters from restricted areas of shellfish growth to an approved area of shellfish growth to, again, reduce fecal coliform levels. Unlike the other PHP methods, depuration and relaying do not cause oyster mortality. These methods of treatment have been extensively used to reduce levels of fecal coliforms but the effects on *Vibrios* have not been established (NSSP 2009). A few studies have shown that high salinity relay reduced levels of *V. vulnificus* in oysters (Audemard *et al.*, 2011; Jones 1994; Jones *et al.*, 1995; Motes *et al.* 1996). A study done by Tamplin *et al.* (1992) however, reported that depuration flow-through was not effective in removing *V. vulnificus* in oysters. In contrast, studies that involved artificially inoculated *Vibrios* in oysters showed that depuration flow-through was successful in removing *Vibrios* in oysters (Chae *et al.*, 2009; Croci *et al.*, 2002;
Lewis et al., 2010 Su et al., 2010). In this study, the oysters were temperature abused at 28°C for 18 – 20 h to increase the concentration of Vibrios in oysters (Staley et al., 2011, Wright et al., 2007), because artificially contaminated oysters may be more susceptible to depuration effects compared to naturally occurring Vibrios (Richards et al. 1988; Tamplin et al., 1992).

 Goals and Objectives

Rapid and reliable detection methods are necessary tools for monitoring Vibrio levels in shellfish and verifying that PHP treated oysters have Vibrio concentrations that do not pose health risks to consumers. The traditional culture-based method takes 4 – 5 days to verify the presence of Vibrios in oysters. Alternatively, qPCR takes 2 – 3 days to verify that Vibrios are present in the oyster homogenate. This leads to the first part of study, where the goal was to determine if qPCR can be used as an alternative method to detect V. parahaemolyticus in oysters than using the culture-based method. Also to observe if there are differences between the concentrations of V. parahaemolyticus detected using the culture-based method and qPCR from two harvest sites under a range of different seasonal conditions, as well as comparing detection in freshly harvested and temperature abused oysters from both sites.

One challenge of studying Vibrios in shellfish from the cold waters of New Hampshire is detection of the low Vibrio concentrations. Aside from ecological questions about the population structure of V. parahaemolyticus in these shellfish, the effect of temperature on population structure also could have implications on choice of detection method and in conducting PHP testing. Another goal of this study was to determine if V.
Evaluating the population structure changes in freshly harvested and temperature abused oysters from different water temperatures to determine effects on detection and PHP effectiveness.

The final goal was to determine, the efficacy of depuration and relaying of oysters as PHP strategies to lower *V. vulnificus* and *V. parahaemolyticus* concentrations in oysters. This was addressed by testing the efficacy of four PHP treatments that ranged from highly controlled to natural conditions.

The specific objectives of this study were as follows:

1. Compare the culture-based method and qPCR in detecting *V. parahaemolyticus* in oysters harvested in variety of temperatures from the Great Bay Estuary.

2. Determine if water temperature and temperature abuse influence the population structure of *V. parahaemolyticus* in oysters.

3. Determine the efficacy of depuration and relaying for reducing *V. parahaemolyticus* and *V. vulnificus* in oysters.
CHAPTER II

DETECTION OF *VIBRIO PARAHAEOMOLYTICUS* IN OYSTERS
(*CRASSOSTREA VIRGINICA*) BY REAL TIME PCR AND CULTURE-BASED METHOD

Abstract

Traditional culture-based method has been used to detect Vibrios for over 30 years through phenotypic screening from selective and differential media. Recently, there has been a push to use real-time PCR after performing an enrichment process to rapidly detect and enumerate concentration of Vibrios in oysters. This study compared traditional culture-based method and qPCR in enumerating *Vibrio parahaemolyticus* in oysters collected in Great Bay Estuary as well as analyzing the population structure of *V. parahaemolyticus* in oysters. *V. parahaemolyticus* concentrations were enumerated by both methods in oysters collected from Nannie Island and Oyster River from April through November of 2010, using freshly harvested oysters and temperature abused oysters. Fifty-four *V. parahaemolyticus* collected from Nannie Island oysters collected during April through July were assessed for population diversity. The geometric means of *V. parahaemolyticus* concentration between the two methods of detection throughout the course of this study was not significantly different, but the culture-based frequently detected this pathogen more when the water temperatures were <15°C. The overall *V. parahaemolyticus* population from Nannie Island oysters was highly diverse, but a large clonal group composed of 14 isolates was found in April and May isolates. Real-time
PCR is a suitable method to rapidly screen for *V. parahaemolyticus* in oysters after the enrichment process while using the culture-based method to obtain isolates that tested positive for this bacterium by qPCR. The study also suggests that the population diversity of *V. parahaemolyticus* is influenced by water temperatures.

**Introduction**

*Vibrio parahaemolyticus* is an emerging bacterial pathogen associated with consumption of raw or undercooked oysters (Daniels *et al.*, 2000; FDA CFSAN 2005; Su *et al.*, 2007). This gram-negative, free-living, halophilic bacterium is found in estuarine environments and commonly associated with oysters. The clinical manifestation of this pathogen, gastroenteritis, is more prevalent in temperate climates (DePaola *et al.*, 1990). Although infections caused by *V. parahaemolyticus* are not as severe as *V. vulnificus* or *V. cholerae* infections, it is the cause of the greatest number of seafood borne illnesses in the US (Daniels *et al.*, 2000; Kasyner *et al.*, 2001). To reduce the risk of vibriosis to shellfish consumers, public health and industry need rapid, sensitive and accurate detection methods.

The MPN enrichment procedure is a critical step in Vibrio detection methods to differentially promote growth of Vibrios and thus allow their detection in shellfish tissue. After enrichment, two different methods can be used to help detect and enumerate concentration of Vibrios. The traditional "culture-based" method (Bartley and Slanetz, 1971; DePaola *et al.*, 1990; Jones and Summer-Brason 1998; Nordstrom *et al.*, 2004; O’Neill *et al.*, 1992) used for detection and enumeration of *V. parahaemolyticus* in oysters involves using phenotypic screening from selective and differential media as
described by the FDA Bacteriological Analytical Manual (Kaysner and DePaola, 2004). This method can result in inconsistent detection (Campbell and Wright, 2003; Jones and Summer-Brason 1998; O’Neill et al., 1992) and it is also laborious and time consuming. The culture method takes up to 4 days from processing the oyster tissue to culturing and identifying colonies for *Vibrio* species either using colony-based multiplex polymerase chain reaction (PCR) or DNA probe hybridization (Nordstrom et al., 2004; Panicker et al., 2004).

Researchers have more recently adapted the use of quantitative real-time PCR (qPCR) for detection and enumeration of *Vibrios* in oysters (Blackstone et al., 2003; Campbell and Wright, 2003; Nordstrom et al., 2009, Randa et al., 2004). The main advantages of using qPCR for detection of *Vibrios* in oysters are due to the sensitivity of detection, more rapid detection after the enrichment step (Blackstone et al., 2003; Campbell et al., 2003; Jones et al., 2009; Rizvi et al., 2010) and it allows for detection of viable but nonculturable Vibrio cells (Randa et al., 2004). A disadvantage for using qPCR is the presence of compounds in oyster tissues that can inhibit qPCR detection of Vibrios when they are present at high concentrations, i.e., minimally diluted tissue samples (Chapela et al., 2010). This inhibition can be overcome by diluting the tissue; however, this reduces the sensitivity of the method by reducing the initial sample size.

Comparison of the cultured-based and qPCR methods is important for optimizing procedures for monitoring shellfish for *V. parahaemolyticus* under wide ranges of environmental conditions, and for verifying low concentration end points for *V. parahaemolyticus* in shellfish treated through postharvest processing (PHP). In addition, the assessment of PHP strategies requires relatively high initial concentrations of *Vibrios*
to be present in the shellfish. If the concentration of Vibrios in shellfish is too low to assess the efficacy of PHP, the Interstate Shellfish Sanitation Conference (ISSC) suggests increasing concentrations by artificial inoculation or temperature abuse (ISSC 2003). Temperature abuse has been reported to be effective in increasing concentration of V. vulnificus in oysters (Staley et al., 2011; Wright et al., 2007), thus, it is also important to determine the best method for detecting V. parahaemolyticus in temperature abused oysters.

The abundance of Vibrios in oysters is correlated to environmental factors such as temperature, salinity, dissolved nutrients, suspended solids, and other factors (DePaola et al., 2003; Jones and Summer-Brason 1998; O'Neill et al., 1992; Sobrinho et al. 2009). In the Northeast US, water temperature is the primary environmental factor affecting Vibrio concentrations, where lower concentrations of V. parahaemolyticus in oysters are found during months with colder water temperatures and higher densities are found in warmer waters. Previous studies in the Great Bay Estuary of New Hampshire and Maine have shown that V. parahaemolyticus and V. vulnificus detected by the traditional culture based method show marked seasonality, with widely varying temperature and other environmental factors affecting their abundance and incidence in both oysters and water (Jones and Summer-Brason, 1998; O'Neill et al. 1992). Effective management of shellfish harvesting to prevent disease incidence requires detection methods that are reliable under all environmental conditions.

In this study, qPCR was evaluated as an alternative method to the traditional culture based method for detecting V. parahaemolyticus in oysters harvested from two sites in the Great Bay estuary, NH. Concentrations of V. parahaemolyticus detected by
each method were compared to determine the differences between the two methods under varying environmental and climatic conditions. The population diversity of \textit{V. parahaemolyticus} was observed using two housekeep genes, \textit{dnaE} and \textit{recA}, to look for diversity of \textit{V. parahaemolyticus} isolates gathered from: 1) different harvest sites, 2) seasonal temperatures, and 3) following temperature abuse. These results help to frame decisions on which detection method may be superior under different environmental conditions and biological factors to help optimize detection of these Vibrios.

**Hypotheses**

The goal of this study was to determine if qPCR is a better method of detection for \textit{V. parahaemolyticus} in oysters compared to the culture-based method. Though some advantages and disadvantages with qPCR are known, its use in the Northeast US is not well documented for detecting \textit{V. parahaemolyticus} under the wide range of different environmental factors, especially water temperatures that are found in this region. The first hypothesis is that qPCR is more a rapid and specific method for the detection and enumeration of \textit{V. parahaemolyticus} in oysters compared to the tradition culture-based method. The second hypothesis is that the effects of environmental factors and population density and diversity on \textit{V. parahaemolyticus} detection are the same for both methods.
Materials and Methods

Oyster Collection, Processing and *V. parahaemolyticus* Detection by the Culture Based Method. Oysters (*Crassostrea virginica*) were collected from two oyster beds in the Great Bay estuary, New Hampshire, U.S.A. The oyster bed near Nannie Island is classified as Approved for shellfish harvesting and the oyster bed in the Oyster River is classified as Prohibited due to its proximity to the effluent discharge pipe from the Durham, N.H. wastewater treatment facility. At each site, 24 oysters were collected monthly during April – November, with bi-weekly collection during June – August of 2010. Environmental conditions were determined using *in situ* readings of salinity, water temperature, and dissolved oxygen recorded with a YSI 85 meter (Yellow Springs Inc., Yellow Springs, OH) with each sample collection. The harvested oysters were transported on ice to the University of New Hampshire/Jackson Estuarine Laboratory in Durham, NH. Half (12 out of 24) of the oysters from each site were cleaned and then subjected to temperature abuse (TA) for 18 – 20 h at 28°C (Wright *et al.*, 2007) to increase *V. parahaemolyticus* concentrations in oysters from relatively low natural levels.

The remaining freshly harvested oysters (FH) were cleaned, shucked, and processed for enumeration via 3-tube MPN enrichment method following the FDA Bacteriological Analytical Manual (BAM; Kaysner and DePaola, 2004), coupled with the culture-based and qPCR methods of detection that were used to confirm the presence of *V. parahaemolyticus*. The oysters were cleaned and shucked into a sterile beaker (liquor and meat), weighed and diluted 1:3 with buffered peptone water (BPW; 10 g Peptone, 5 g NaCl, 3.5 g disodium phosphate, 1.5 g monopotassium phosphate per L) and homogenized for 30 s on low and 60 s on high speed. Ten grams of homogenate was
added to three tubes containing 10 ml of alkaline peptone water (APW, pH 8.6, 1% NaCl), and 1 g of homogenate was added into each of three separate APW tubes and into a separate dilution tube containing 9 ml of BPW. A serial 10-fold dilution was done in BPW tubes to $10^{-4}$, 1 ml aliquots of diluted homogenate were added to 10 ml of APW in three tubes at each dilution and all tubes were incubated at 37°C overnight (18 - 20 h).

APW tubes that were turbid after incubation were scored as positive for growth. Aliquots (1 ml) from each positive APW tube were placed into microcentrifuge tubes, boiled for 10 min, centrifuged at 16,000 x g for 5 min and all contents were stored at -20°C for qPCR enumeration. For culture-based enumeration, each positive APW tube was streaked onto TCBS agar (BD, Franklin Lakes, NJ) and incubated at 37°C for 18 – 20 h. Sucrose negative colonies (that include *V. parahaemolyticus*) and sucrose positive colonies (typically not *V. parahaemolyticus*) exhibiting different phenotypes (size and opacity) from TCBS media were streaked onto tryptic soy agar (BD) and incubated at room temperature for 18 – 20 h. The colonies isolated from these TSA plates were used as templates for colony multiplex PCR for verification of *V. parahaemolyticus* isolates.

**Development of Colony PCR and of Standard Curves for qPCR.** A colony-based PCR method (Panicker et al., 2004) targeting the *tlh* gene was used to identify *V. parahaemolyticus* as the final step of the culture based detection method. PCR was performed on each suspected *V. parahaemolyticus* isolate from the APW enrichment that appeared as different phenotypes on TCBS. The cultures were transferred from the TCBS plates to TSA plates and these cultures were used as template for PCR. The mastermix was composed of 1X iQSupermix (Bio-Rad, Hercules, CA) containing dNTPs, 25 U/ml iTaq DNA polymerase, 3 mM MgCl₂ and then 125 nM of *tlh* primers (Nordstrom et al.).
2007) and nuclease free water to a total volume of 25 μl. The PCR conditions were as
follows: 3 min at 95°C followed by 30 cycles of the denaturation step at 95°C for 30 s,
the annealing step at 59°C for 30 s, and an elongation period at 72°C for 30 s then
followed by a final elongation step at 72°C for 1 min. The PCR amplicons were
visualized on 1.2% agarose gel with addition of Gel Red (Phenix Research Products,
Candler, NC) under UV light.

Preliminary assays using genomic DNA indicated that it was not a stable template
due to degradation and therefore we cloned the species-specific tlh (thermolabile
hemolysin) gene from V. parahaemolyticus F11-3A (an environmental tdh+ and trh+
isolate, DePaola et al., 2003) to generate qPCR standard curves. DNA was extracted
using chloroform:phenol extraction (Ausubel et al. 1990) from overnight cultures in heart
infusion broth (HI, Fluka, Buschs, Switzerland) at 37°C overnight, and tlh was amplified
with published primers (Nordstrom et al., 2007). The amplicons (208 bp) were cloned
into a TOPO vector following the manufacturer’s protocol (TOPO 2.1, Invitrogen, Grand
Island, NY). The tlh-TOPO plasmids were extracted using a plasmid mini-prep
following the manufacturer’s protocol (Qiagen, Valencia, CA) and identity of the
sequence was confirmed by sequencing the tlh gene in the TOPO plasmid (Hubbard
Center for Genomic Sequencing, Durham, NH).

The range of DNA used to determine the standard curve was from 100 pg to 100
fg of pDNA. The qPCR mastermix was composed of 1x iQSupermix (Bio-Rad)
including dNTPs, 25 U/ml iTaq DNA polymerase, 10 nM of SYBR Green I fluorescein,
and 3 mM MgCl₂. In addition, 2 mM MgCl₂ (Bio-Rad), 125 nM of tlh primers
(Nordstrom et al. 2007), ultra-pure water and 2 μl of V. parahaemolyticus plasmid DNA
were added to the mastermix to create a total reaction volume of 15 µl. The *tlh* qPCR was performed in an iCycle (Bio-Rad) and the PCR parameters started with a hot-start for 3 min at 95°C, then 40 cycles of 95°C for 10 s and 59°C for 15 s, and a melt curve provided by the manufacturer (Bio-Rad). The average PCR efficiency for the standard curve was 89.9%, which was within an acceptable range. The Ct values (± standard deviation) of the standard curve were 10.8 (± 1.5) for 100 pg, 14 (± 1.4) for 10 pg, 17 (± 1.7) for 1 pg and 21.1 (± 1.5) for 100 fg. The average $r^2$-value was at 0.992, meaning that the C$_T$ values were linear over the exponential pDNA concentration range. The lowest concentration of detection used in this assay was 100 fg and all ensuing qPCR analyses were considered below the detection limit if the Ct value was greater than 21.

Once the standard curve was validated, the detection method was applied to positive APW tubes to determine *V. parahaemolyticus* concentrations in oyster homogenate. Three µl of the APW enriched oyster homogenate was used as a template for qPCR. Tubes positive for *V. parahaemolyticus* by qPCR were used to calculate the MPN concentration (MPN 100/g) in the oyster homogenates from different temperature regimes (10 g, 1 g, 0.1 g for < 20°C and 1 g, 0.1g, and 0.01 g for waters > 20°C).

**Phylogenetic Analysis and Diversity Assessment.** Fifty-four *V. parahaemolyticus* isolates were obtained from freshly harvested and temperature abused oysters collected from Nannie Island during April through July 2010 to screen for any clonality of *V. parahaemolyticus* in oysters by sequencing two housekeeping genes. Two housekeeping genes, *dnaE* (Jolley *et al.*, 2004) and *recA* (Sawabe *et al.*, 2007), were amplified from genomic DNA extracted from the 54 *V. parahaemolyticus* isolates (Ausubel *et al.*, 1990). The primers and PCR parameters for *dnaE* were as reported by
Jolley et al. (2004) and the primers and PCR parameters for recA were as reported by Sawabe et al. (2007). The PCR amplicons of dnaE and recA were sequenced at the Hubbard Center for Genome Studies (Durham, NH) or by Functional Biosciences, Inc. (Madison, WI).

Diversity and evolutionary relationships were determined from concatenated sequence data for dnaE and recA genes generated from the 54 V. parahaemolyticus isolates obtained from this study and 77 V. parahaemolyticus isolates collected from oysters during 2007 – 2009 from Nannie Island and Oyster River oysters (Jones et al., 2010 ICMSS, Mahoney et al., 2010). All sequences were aligned with ClustalW (Tamura et al., 2011 In press) and then concatenated by MEGA version 5.0. Nucleotide distances were measured from the 2010 sequences and the combine sequences from 2007 – 2010 by performing an overall mean distance using Jukes-Cantor model with 1000 bootstrap replicates. Mean group distances were calculated in V. parahaemolyticus isolates from cold water temperatures (April and May) and warm water temperatures (June and July). A minimal-evolution phylogenetic tree was creating using the Jukes-Cantor method (Tamura et al., 2011 In press) with 1000 bootstrap replicates and observed for diversity or clonality of V. parahaemolyticus population in Great Bay Estuary.

**Data Analysis.** The MPN concentrations (100/g) of oysters of V. parahaemolyticus from Nannie Island and Oyster River were log_{10} transformed to normalize the data and to compare the differences between the culture based and qPCR methods. The log_{10} transformed MPN values were averaged and tested for significant differences by using a standard one-way student t-test and an analysis of variance
(ANOVA) using JMP 9 (SAS Institute Inc, Cary, NC). *V. parahaemolyticus* concentrations detected in oysters using the culture-based and qPCR methods were analyzed for significant differences between freshly harvested oysters and temperature abused oysters collected at Nannie Island and Oyster River. Samples where the *V. parahaemolyticus* levels were below detection limits were transformed to values 50% less than the detection limit to allow for statistical analyses (DePaola et al., 2003).

**Results**

**Comparison of the Culture-Based and qPCR Methods to Detect and Enumerate *V. parahaemolyticus* Levels in Oysters.** The FDA BAM (Kaysner and DePaola, 2004) suggests picking sucrose negative colonies (green colonies) only from the TCBS media to isolate *V. parahaemolyticus* strains from oysters, though yellow (sucrose-positive) colonies were also picked in this study. There were 245 positive *V. parahaemolyticus* isolates out of 564 suspected isolates collected from both freshly harvested and temperature abused oysters. 200 out of the 245 *V parahaemolyticus* isolates were sucrose negative and 45 positive strains were sucrose positive colonies. Including sucrose-positive isolates only slightly increased some of the *V. parahaemolyticus* concentrations in oysters (data not shown).

The water temperature ranged from 7.2°C to 24.7°C at Nannie Island and 8.1°C to 23.7°C at Oyster River (Table 1.1). This wide range of temperatures was a major influence on levels of *V. parahaemolyticus* detected in oysters from both sites (Figure 1.1). *V. parahaemolyticus* concentrations were very low or non-detectable using the culture-based and qPCR methods at both sampling sites when water temperatures were
<15°C. *V. parahaemolyticus* was always detected at relatively higher levels by both methods in freshly harvested oysters harvested from both sites when the water temperatures were >20°C.

Figure 1.1 Concentration (Log_{10} MPN/ g of oysters) of *V. parahaemolyticus* in freshly harvested oysters from Nannie Island and Oyster River oysters during 2010 detected by the culture-based method and qPCR.
Table 1.1 Concentration of *V. parahaemolyticus* in freshly harvested (FH) and temperature abused (TA) oysters from Nannie Island and Oyster River using the culture-based method and qPCR

<table>
<thead>
<tr>
<th>Date</th>
<th>Temperature (°C)</th>
<th>Nannie Island</th>
<th>Oyster River</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Culture based Method</td>
<td>qPCR</td>
</tr>
<tr>
<td>4/26/2010</td>
<td>10.6</td>
<td>1.03</td>
<td>2.11</td>
</tr>
<tr>
<td>5/16/2010</td>
<td>11.4</td>
<td>1.44</td>
<td>3.86</td>
</tr>
<tr>
<td>6/14/2010</td>
<td>17.7</td>
<td>1.78</td>
<td>4.52</td>
</tr>
<tr>
<td>6/30/2010</td>
<td>22.1</td>
<td>4.45</td>
<td>7.86</td>
</tr>
<tr>
<td>7/15/2010</td>
<td>24.7</td>
<td>4.45</td>
<td>6.52</td>
</tr>
<tr>
<td>8/10/2010</td>
<td>23.5</td>
<td>4.94</td>
<td>5.86</td>
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<td>8/24/2010</td>
<td>22.4</td>
<td>2.27</td>
<td>4.56</td>
</tr>
<tr>
<td>9/20/2010</td>
<td>17.2</td>
<td>2.03</td>
<td>5.45</td>
</tr>
<tr>
<td>10/20/2010</td>
<td>11.3</td>
<td>1.68</td>
<td>3.45</td>
</tr>
<tr>
<td>11/12/2010</td>
<td>7.2</td>
<td>1.44</td>
<td>0.18</td>
</tr>
</tbody>
</table>
The geometric mean concentrations of V. parahaemolyticus in freshly harvested oysters detected by culture-based method and qPCR were not significantly different \((P = 0.56)\). The qPCR detection rates for V. parahaemolyticus were 50% and 67%, of freshly harvested oyster samples from Nannie Island and Oyster River, respectively, while 100% of the oysters harvested from Nannie Island tested positive for V. parahaemolyticus and 78% oysters from Oyster River tested positive for V. parahaemolyticus using the culture-based method. These differences in detection occurred when the water temperatures were <15°C. The geometric mean concentrations of V. parahaemolyticus detected in water temperatures <15°C using the culture-based method was significantly higher than qPCR for Nannie Island \((P = 0.0001)\) and for Oyster River \((P < 0.05)\) oysters.

Temperature abusing oysters significantly increased V. parahaemolyticus levels from freshly harvested oysters (Table 1.1) collected from both sites as determined using qPCR \((P = 0.0005)\) and the culture-based method \((P = 0.0007)\). Despite consistent increases in V. parahaemolyticus concentrations resulting from the temperature abuse, oysters originally collected in water temperatures below 15°C resulted in V. parahaemolyticus concentrations that were generally lower (<4 log_{10} MPN/100g of oysters) than in oysters collected in water temperatures above 20°C (all >4 log_{10} MPN/100g of oysters) for both detection methods. The geometric mean concentration of V. parahaemolyticus for temperature abused oysters from water temperatures <15°C was significantly lower than that for temperature abused oysters from water temperatures >20°C for both qPCR and the culture-based method \((P < 0.0001\) and \(P = 0.0003\), respectively). The geometric mean concentrations of V. parahaemolyticus detected using
culture-based method and qPCR from temperature abused oysters were not significantly different in Nannie Island ($P - 0.93$) and Oyster River ($P - 0.95$) oysters.

**Phylogenetic Analysis of *V. parahaemolyticus* Isolates from Oysters.** The 54 *V. parahaemolyticus* isolates from Nannie Island collected throughout different seasonal water temperatures and from temperature abused oysters were analyzed for population diversity. The main question for the analysis of these isolates was whether environmental temperatures or temperature abuse affected the population structure of *V. parahaemolyticus* in oysters. The overall mean nucleotide distance for the 54 isolates indicates a high level of diversity (0.013); however, when broken down by water temperatures, isolates from colder temperatures (April and May; excluding May freshly harvested isolates) were less diverse and isolates from warmer temperatures (June and July) were more diverse (0.0 and 0.012, respectively).

The population structure of *V. parahaemolyticus* was more apparent in the phylogenetic analysis, where April and May isolates tended to form distinct clusters from the June and July isolates (Figure 1.2 box A). The exception are the two *V. parahaemolyticus* isolates from freshly harvested oysters in May that were more closely related to isolates from June and July than to isolates from April and May (Box B, Fig 1.2). When 77 *V. parahaemolyticus* isolates from Nannie Island and Oyster River oysters from 2007 – 2009 were combined with the 2010 strains, there was greater diversity of *V. parahaemolyticus* in oysters; however, the same clonal group from April and May of 2010 remained unique and distinct from all other isolates (data not shown) because no other strains from the 2007 – 2009 oysters grouped with this clonal complex.
Figure 1.2 Population structure of 54 *V. parahaemolyticus* strains collected from freshly harvested and temperature abused Nannie Island oysters constructed from concatenating 2 housekeep genes, *dnaE* and *recA*. The first 4 digits represent the isolate number, then designated with a month number, site, and freshly harvested (FH) or temperature abused (TA). Box A represents the 14 clonal isolates from April and May isolates while Box B shows two strains from May freshly harvested oysters that are closely related to isolates from June and July.
Discussion

The Northeast US (Jones 2011) and other regions of the US that have colder water temperatures such as Alaska (McLaughlin et al., 2005) and the Northwest US (CDC 1998 and DePaola et al., 2000) are facing emerging threats of increased incidence of V. parahaemolyticus reported infections. Monitoring of V. parahaemolyticus levels is underway in some of these areas as require by the NSSP (2009) to help inform management of this public health risk. Detection methods that give rapid detection of V. parahaemolyticus compared to the traditional culture-based methods are desirable to inform management and public health decisions in a more timely fashion. As these new methods emerge, there is a need to verify they are effective detection tools by vigorous testing in a wide range of environmental conditions, especially for water temperature. Testing in different areas of the US is essential to ensure that detection methods can be harmoniously applied for comparable results. Little such work has occurred in the Northeast US where disease incidence is only beginning to rise and less is known about the incidence of V. parahaemolyticus in this environment. Thus, the comparison of the culture-based and the qPCR methods for detecting V. parahaemolyticus is very important step. PHP strategy testing also requires consistent methods for detection of Vibrios to track rates of removal and to ensure that Vibrio levels in shellfish meet required endpoint concentrations.

Water temperature is a highly significant factor affecting the incidence and concentrations of V. parahaemolyticus in coastal waters and shellfish, especially in the Northeast US. The V. parahaemolyticus levels found in the oysters in this study were influenced by seasonal water temperatures as seen in other studies in GBE and other
areas (DePaola et al., 2003; Johnson et al., 2010; Jones and Summer-Brason, 1998; Motes et al., 1998; O’Neill et al., 1992; Oliver et al., 1995; Sobrinho et al., 2010). The abundance of this pathogen has a wide seasonal range from low levels in colder water temperatures to higher levels in warmer water temperatures but we also saw the persistence of *V. parahaemolyticus* in oyster tissues during the colder seasonal temperatures in waters <15°C (Jones and Summer-Brason, 1998; Martinez et al., 2010).

The two main methods currently in use for *V. parahaemolyticus* detection are variations of the traditional culture-based method and an MPN enrichment/qPCR approach. The variety of potential applications for detection methods may be met by use of one of these methods, if found to be superior to the other, or a combination of the two, depending on application. In this study, there were differences in the rate of detection and significant differences in concentrations between the two methods for *V. parahaemolyticus* detected in oyster tissue from cold water temperatures. *V. parahaemolyticus* concentrations are much lower, approaching detection limits, in cold water and this necessitates the use of larger initial amounts of oyster tissue (10 g of oyster homogenate) to enable detection of these low *V. parahaemolyticus* levels. The culture-based method resulted in detection of low levels of *V. parahaemolyticus* in the 10 g homogenate sample, as shown by others (Jones et al., 2010; Oliver et al., 1995; Wong et al., 2003). Using 10 g of oyster homogenate also increases the amount of potentially inhibiting compounds, suggesting that nondetection of *V. parahaemolyticus* by qPCR may have been due to these inhibitory agents in undiluted tissue (Chapela et al., 2010; Rivzi et al., 2006). Without using the internal amplification control (IAC), we could not determine if this non-detection of *V. parahaemolyticus* was due to compounds truly
inhibiting detection or due to *V. parahaemolyticus* concentrations being below the detection limit of the qPCR assay (Jones *et al.*, 2010; Nordstrom *et al.*, 2007). This study suggests that the inhibitory compounds interfered with qPCR in *V. parahaemolyticus* detection because *V. parahaemolyticus* was detected using the culture-based method from Nannie Island oysters when qPCR could not enumerate *V. parahaemolyticus* from these same oysters.

Aside from being a time-consuming procedure, there are also some subjective aspects of the culture-based method. The phenotypic screening of *V. parahaemolyticus* on TCBS poses a challenge because the varying colors of sucrose-negative (green) colonies due to pH dependent color (due to lack of sucrose fermentation) can change if the TCBS plate is incubated too long. When sucrose-positive (yellow) colonies are in close proximity to sucrose-negative colonies, the pH changes from the sucrose-positive colonies can change the color of sucrose-negative colonies to yellow (Hara-kudo *et al.*, 2001). In addition, other *Vibrio* species exhibit similar phenotypes as *V. parahaemolyticus*, and *V. vulnificus* or *V. mimicus* overgrowth on TCBS plates can interfere with *V. parahaemolyticus* detection (Jones *et al.*, 2009; Parveen *et al.*, 2008). Yellow colonies, like the 18% (45/245 isolates) of the *V. parahaemolyticus* confirmed colonies in this study, are assumed to be sucrose-positive. Mucoid, sucrose-positive *V. parahaemolyticus* colonies have been detected on TCBS from stool samples isolated from a patient in Singapore and this isolate showed similar biochemical properties as sucrose-negative *V. parahaemolyticus* (Lam 1985). This presence of sucrose-positive colonies may lead to underreporting of *V. parahaemolyticus* in any given samples if picking sucrose-negative colonies is the sole criteria for positive identification of *V. 
*V. parahaemolyticus* on TCBS by phenotypic screening. By picking all phenotypes, as done in this study, more accurate, higher *V. parahaemolyticus* counts in oysters could be detected as opposed to the limited approach of picking only green colonies as dictated by the FDA BAM (Kaysner and DePaola 2004). Screening every phenotype isolated from TCBS is not an ideal method for rapid detection of *V. parahaemolyticus*. Improvements in the culture-based methods, including the use of alternative media like CHROMagar (DRG-International Inc, Mountainside, NJ), are needed to help overcome these problems and improve *V. parahaemolyticus* detection by culture-based methods (Hara-kudo *et al.*, 2001).

Temperature abusing shellfish is a method to increase low levels of *V. parahaemolyticus*. The significant increases of *V. parahaemolyticus* in oysters from the Great Bay Estuary were consistent with findings of previous studies that reported increased levels of *V. vulnificus* following temperature abuse (Staley *et al.*, 2011; Wright *et al.*, 2007). Large increases of *V. parahaemolyticus* concentrations from temperature abused oysters may help PHP testing that requires a 3.52-log reduction for validating PHP methods (NSSP 2009). One complication for temperature abuse of shellfish is the potential for shifts in Vibrio population structure. The population structure of *V. parahaemolyticus* in temperature abused oysters were influenced by water temperatures, where clonal populations were observed in oysters harvested in colder water temperatures (April and May) and higher diversity was observed in oysters harvested in warmer waters (June and July). Similar trends were observed when temperature abused oysters collected in August in Louisiana revealed higher diversity of *V. vulnificus* after performing BOX-PCR (Staley *et al.*, 2011).
The consistency in detecting higher levels of *V. parahaemolyticus* with the culture-based method has been reported as a problem in previous studies (Jones *et al.*, 2010; Parveen *et al.*, 2008) due to other *Vibrio* species exhibiting the same phenotype as *V. parahaemolyticus* on TCBS, which can result in false positives and yielding in lower concentrations. Using species specific primers to detect *V. parahaemolyticus* by using qPCR bypasses the problem seen with culture-based method in higher *V. parahaemolyticus* counts, and can detect the *tlh* gene in the enriched oyster homogenate (Blackstone *et al.*, 2003; Nordstrom *et al.*, 2007). Although qPCR gives more specificity in detection of *V. parahaemolyticus*, no isolates can be obtained by this method (Parveen *et al.*, 2008). This suggests that qPCR can be a used as a rapid screening process to detect concentration of *V. parahaemolyticus* in oysters; the culture-based method could then be used on *tlh*-positive APW enrichment tubes to obtain *V. parahaemolyticus* isolates (Jones *et al.*, 2010). The results also show that qPCR would be an effective and rapid tool for evaluating PHP strategies that require use of high Vibrio concentrations.

This study suggests that the population structure of the 54 *V. parahaemolyticus* strains isolated from 2010 oysters were highly diverse. Two housekeeping genes were used as opposed to 7 (Ellis *et al.*, in press) to screen for any clonal populations of *V. parahaemolyticus* in temperature abused oysters collected from different water temperatures. The highest diversity of *V. parahaemolyticus* was observed during warmer water temperatures, where the highest concentrations of *V. parahaemolyticus* are found in both freshly harvested and temperature abused oysters (Jones *et al.*, 2010, Sobrinho *et al.*, 2010). In oysters from colder water temperatures where low *V. parahaemolyticus* levels are found, a more clonal population of *V. parahaemolyticus* was observed. In fact, this
clonal group composed of 14 *V. parahaemolyticus* strains collected from oysters in April and May suggests the same dominant *V. parahaemolyticus* strain from freshly harvested oysters in April was isolated again in temperature abused oysters in April and May. The study suggests that seasonal water temperatures may influence the population structure of *V. parahaemolyticus*. The population of Vibrios in temperature abused oysters may include strains that are resistant to PHP methods (Staley *et al.*, 2011), which is an important aspect to note when temperature abusing oysters to increase Vibrio concentrations in oysters for PHP testing.

There are disadvantages of using either the culture-based method or the qPCR method to detect *V. parahaemolyticus* in oysters; however, the rapid detection using qPCR, makes it a more desirable method while the laborious and time consuming culture-based method using subjective phenotypic screens is a less ideal methodology for detect *V. parahaemolyticus* in oysters. Using species-specific primers to detect *V. parahaemolyticus* from the enrichment tubes by qPCR is beneficial because it eliminates using phenotypic screens on selective media and resulting in faster detection of this pathogen in oysters. This study suggests that qPCR can be used as an alternative method to rapidly detect and monitor Vibrio levels in oysters found in harvest sites and to ensure that PHP treated oysters meets the standards set by the ISSC prior to being distributed to consumers. The culture-based method remains a useful method for detecting Vibrios present at low concentrations in cold water oysters until the effects of inhibitory compounds in shellfish tissue can be overcome for qPCR methods.
CHAPTER III

POSTHARVEST TREATMENT OF FRESHLY HARVESTED AND TEMPERATURE ABUSED OYSTERS (CRASSOSTREA VIRGINICA) TO REDUCE CONCENTRATIONS OF VIBRIO PARAEHAEMOLYTICUS AND VIBRIO VULNIFICUS

Abstract

The emergence of Vibrio parahaemolyticus and Vibrio vulnificus as human pathogens is a concern to the general public as well as the shellfish industry. These free-living halophilic, gram-negative bacteria cause gastroenteritis and septicemia in humans that consume raw or undercooked oysters. The goal of this study was to determine if depuration and relay strategies used to reduce or eliminate human fecal-borne pathogens in shellfish could be adapted for reducing Vibrios in oysters. Oysters were collected from July through November of 2009 from the Piscataqua River in NH, temperature abused at 28°C and brought to Spinney Creek Shellfish Inc., in Eliot, ME to perform the depuration and relay experiments. Postharvest processing (PHP) strategies using temperature abused oysters included depuration recirculation and flow-through performed in tanks at Spinney Creek Shellfish facility and oysters were relayed to an oyster bed in Spinney Creek (creek relay). Natural relay was performed on oysters immediately following harvest to an oyster bed in Spinney Creek. Each treatment was evaluated by measuring V. parahaemolyticus and V. vulnificus concentrations after 2 and 5 days using MPN-qPCR in triplicate. Creek relay was most effective in reducing concentration of V.
*parahaemolyticus* and *V. vulnificus* in oysters compared to the two depuration methods; however, all three treatments were not able to reduce these pathogens <30 MPN/g. *V. parahaemolyticus* strains harboring clinical markers (*tdh* and *trh* genes) were also detected in August temperature abused oysters at a concentration of 450 MPN/g of oysters. The findings suggest that the greater reduction of *Vibrios* in the relay oysters may involve biological displacement of *Vibrio* species by the endemic microbiota in the creek. Further studies using longer treatment periods are needed to validate relaying as a PHP method in reducing higher concentration *Vibrios* species in temperature abused oysters.

**Introduction**

There are a variety of bacterial pathogens that reside in bivalve shellfish, yet only some species pose significant threats to consumers of raw or undercooked shellfish (FDA CFSAN 2005; WHO 2005). *Vibrio parahaemolyticus* and *Vibrio vulnificus* are the top bacterial safety concerns in seafood for public health officials and the shellfish industry (ISSC 2003; NSSP 2009). *V. parahaemolyticus* is the leading cause of bacterial gastroenteritis from consuming raw or improperly cooked oysters in the United State (Su *et al.*, 2007). Although the clinical manifestation of *V. parahaemolyticus* is mild compared to *V. vulnificus*, patients are presented with acute gastroenteritis and suffer from vomiting, nausea, headache, fever, chills abdominal cramps and diarrhea (Barker and Gangarosa 1974; Kaysner and DePaola 2004; Levine *et al.*, 1993). *V. vulnificus* can cause life-threatening systemic infections from consumption of raw or undercooked shellfish and from wounds coming in contact with seawater or shellfish (Drake *et al.*, 33
The occurrence of this type of infection is increased when the patient has underlying conditions such as cirrhosis, hemochromatosis, and diabetes as well as when the patient is immunocompromised (WHO 2005). Though *V. vulnificus* infections are less common, the 50% fatality rate associated with infections makes this organism of great concern (Ralston *et al.*, 2011).

The National Shellfish Sanitation Program (NSSP) and the Interstate Shellfish Safety Conference (ISSC) have created guidelines to reduce microbial pathogen threats in shellfish and protect consumers from *Vibrio* infections. Post-harvest processing methods (PHP) are deemed effective if they are capable of reducing *Vibrio* levels in shellfish to <30 MPN Index/g prior to being sold to marketing (ISSC 2003; NSSP 2009). Different types of PHP methods are being tested and being implemented including ultra-low temperature quick-freeze (Wright *et al.*, 2007), high hydrostatic pressure treatment of oysters (Kural *et al.*, 2007), and hot water pasteurization treatment (Andrews *et al.*, 2000). These methods have all been found to be effective in reducing the vibrio populations to acceptable concentrations. These processes are, however, capital-intensive and end up killing processed shellfish, rendering them less desirable to consumers and less valuable for the shellfish industry.

Depuration and relaying are accepted strategies for reducing fecal-borne bacteria from shellfish, allowing shellfish harvesting from mildly contaminated areas. Depuration is a process of removing microbial contaminants in shellfish through a closed controlled aquatic environment, while relaying is a process of trans-locating shellfish from mildly contaminated growing waters to approved shellfish growing waters (NSSP). These shellfish treatment strategies reduce fecal coliform levels without killing the treated
shellfish, in contrast to the previously mentioned PHP methods. Previous studies have explored use of both of these strategies for *Vibrio* reduction and found that the levels of fecal coliforms decreased but this was not a good indicator organism for reduction of *Vibrios* in oysters (Croci et al., 2002; Jones et al., 1994). Depuration has been found to be generally ineffective in reducing *Vibrios* (Jones et al., 1991; Tamplin et al., 1992) but in contrast, several different relay approaches have resulted in significant reductions in *V. vulnificus* and *V. parahaemolyticus* levels (Jones 1994; Motes et al., 1996; Audemard et al. 2011).

This study compared the efficacy of depuration and relaying in removing *V. parahaemolyticus* and *V. vulnificus* from oysters in the Great Bay estuary (GBE) of New Hampshire and Maine. Environmental conditions in the GBE are spatially variable and seasonal climatic conditions (i.e., water temperatures) vary temporally with extremes of <0°C to ~30°C (Jones and Summer-Brason 1998; O’Neill et al., 1992; Schuster et al., 2011)), allowing rigorous testing of these shellfish treatment strategies. Quantitative real-time PCR (qPCR) was used for more rapid detection (Wright et al., 2007) of the two *Vibrio* species in treated oysters by a 3-tube most probable number (MPN) enrichment method (Kaysner et al., 2004).

**Hypotheses**

The first goal of this study was to analyze the efficacy of depuration flow-through, depuration recirculation, and creek relay in reducing *V. parahaemolyticus* and *V. vulnificus* in oysters. The second goal of this study was to determine which PHP method is the most effective in reducing *Vibrios* in oysters. There were two hypotheses
formulated for this study, first hypothesis is that relaying is more effective than the two depuration treatments for reducing *Vibrio parahaemolyticus* and *Vibrio vulnificus* concentrations in oysters. The second hypothesis is that relaying effectiveness is independent of water temperature but is most effective in higher salinity waters.

**Materials and Methods**

**Oyster Sample Collection and Pre-treatment Preparation.** Oysters (*Crassostrea virginica*) were collected from an oyster bed (43°10'08.49"/70°49'42.54") in the Piscataqua River, Dover, NH. Water temperature and salinity readings were measured using a YSI 85 meter (YSI, Yellow Springs, OH) at the time of collection and the harvested oysters were transported on ice to the UNH Jackson Estuarine Laboratory in Durham, NH. Oysters were scrubbed clean and either aseptically shucked for determining initial concentrations of *V. parahaemolyticus* and *V. vulnificus* using qPCR, or subject to temperature abuse at 28°C for 18 – 20 hrs (Wright *et al.*, 2007) to increase the concentration of *V. parahaemolyticus* and *V. vulnificus* in the oysters. Some of the temperature-abused oysters were shucked for determining initial concentrations of *V. parahaemolyticus* and *V. vulnificus* and the rest were transported to Spinney Creek Shellfish Inc. (SCS), Eliot ME on ice, to perform the after-harvest process treatments.

**Depuration and Relaying Treatment Processes.** The temperature-abused oysters were subjected to three different depuration and relaying treatments and freshly harvested oysters were subjected to one relay treatment (Table 2.1), all performed on site and in close proximity to SCS. The two depuration treatments used in this study were depuration recirculation and depuration flow-through. These were evaluated in two
separate tanks located indoors at the SCS facility under controlled conditions. Water from Spinney Creek was pumped into the tanks with the intake pipe drawing from an area near an oyster bed at the inlet side of the creek and running 20 feet below the surface to the facility where the water was sterilized by exposure to high intensity UV light. The water temperatures were adjusted for the recirculation treatment to match the ambient waters from Spinney Creek.

Table 2.1 Depuration and relaying treatments for contaminated oysters

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Time</th>
<th>Location</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depuration recirculation</td>
<td>July - November</td>
<td>On land</td>
<td>UV sterilized, filter sterilized, foam fractionation, recirculated water back to the tank</td>
</tr>
<tr>
<td>Depuration flow-through</td>
<td>July - November</td>
<td>On land</td>
<td>UV sterilized, flow-through of creek water into the tank, discharge water to creek</td>
</tr>
<tr>
<td>Creek relay</td>
<td>August - November</td>
<td>Creek</td>
<td>Oyster bed in Spinney Creek, Eliot, ME (temperature abused)</td>
</tr>
<tr>
<td>Natural relay</td>
<td>September - November</td>
<td>Creek</td>
<td>Direct relay from Piscataqua River to Spinney Creek Eliot, ME (freshly harvested)</td>
</tr>
</tbody>
</table>
Oyster Processing and Enumeration of *V. parahaemolyticus* and *V. vulnificus*.

Triplicate samples of 12 freshly harvested oysters collected from the Piscataqua River were tested from each collection and treatment day. The concentrations of *V. parahaemolyticus* and *V. vulnificus* in freshly harvested and in temperature-abused oysters were determined to provide initial concentrations before the oysters were placed in treatment systems. The oysters subjected to the depuration treatments were collected on day 2 and 5, while the relaying treatments were only collected after 5 days of treatment. Oyster survival and quality were evaluated at each sample event by observing foul smells and alteration to the oyster meat after the oysters were shucked. Water samples collected with each oyster sample were also tested for both *Vibrio* species by qPCR. All the oysters were enumerated by following an enrichment protocol described by the FDA Bacteriological Analytical Manual (Kaysner *et al.*, 2004) with some modifications. Triplicate samples of twelve oysters from each treatment were scrubbed, shucked and all contents (animal and liquor) were weighed, and diluted 1:3 (w:v) by adding buffered peptone water (BPW, pH 7.2). The oyster tissue was homogenized using a sterile blender at low speed for 30 s and then at high speed for 60 s. Aliquots of decimally diluted oyster homogenate were added into three enrichment tubes containing 9 ml of alkaline peptone water (APW, pH 8.6, 1% NaCl, 1% peptone) starting with 1ml of oyster homogenate in the first set of 3 APW tubes. Oyster homogenate was further diluted using 1 ml aliquots into 9 ml BPW tubes and 1ml diluted homogenate was added to further sets of 3 APW tubes with dilutions to $10^{-7}$ according to the seasonally changing *Vibrio* concentrations. The APW enrichment tubes were incubated overnight at 37°C and turbid tubes were scored as positive for growth. From each positive enrichment tube, 1
ml was aliquoted into a microcentrifuge tube, boiled for 10 min, centrifuged at 16,000 x g for 5 mins and stored at -20°C for qPCR assays.

*V. parahaemolyticus* and *V. vulnificus* qPCR assays used the same mastermix, comprised of 1x iQSupermix (Bio-Rad, Hercules, CA) including dNTPs, 25 U/ml iTaq DNA polymerase, 10 nM of SYBR Green I fluorescein, and 5 mM MgCl₂. Additionally, 125 nM of *tlh* primers (for *V. parahaemolyticus* reactions) or 125 nM of *vvh* primers (for *V. vulnificus* reactions), ultrapure water, and 2 μl of template (3 μl for oyster homogenate) were added to make a total reaction volume of 15 μl. Both *Vibrio* qPCR assays were performed in an iCycle (Bio-Rad) and the *tlh* qPCR parameters had an initial hot-start step for 3 min at 95°C, then 40 cycles of 95°C for 10 s and 59°C for 15 s, and a melt curve provided by the manufacturer (Bio-Rad). The *vvh* qPCR parameters also started with a hot-start step for 3 min at 95°C, then 40 cycles of 95°C for 15 s and 60°C for 15 s, and a melt curve provided by the manufacturer (Bio-Rad). An oyster homogenate positive for *V. parahaemolyticus* and *V. vulnificus* from a surveillance study done in the Great Bay, was used as a positive control for qPCR and ultrapure water was used for a negative control. The APW enrichment tubes positive for each *Vibrio* species were used to calculate the original concentration in the oyster homogenate using an MPN Index table.

Oyster homogenates were also analyzed for the presence of the *V. parahaemolyticus* clinical markers, thermostable direct hemolysin (*tdh*) and thermostable related hemolysin (*trh*) genes. Traditional PCR was performed on all oyster homogenate using the primers reported by Panicker (2004) (Table 2.2). An iQSuperMix (Bio-Rad)
Table 2.2  PCR primers used to detect *V. parahaemolyticus* and *V. vulnificus* in oyster homogenates

<table>
<thead>
<tr>
<th>Organism</th>
<th>Primer</th>
<th>Primer Sequence (5’ to 3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. parahaemolyticus</em></td>
<td><em>tlh</em> F</td>
<td>5’-ACT CAA CAC AAG AAG AGA TCG ACA A-3’</td>
<td>Nordstrom et al</td>
</tr>
<tr>
<td></td>
<td><em>tlh</em> R</td>
<td>5’-GAT GAG CGG TTG ATG TCC AA-3’</td>
<td>Nordstrom et al</td>
</tr>
<tr>
<td></td>
<td><em>tdh</em> F</td>
<td>5’-GTA AAG GTC TCT GAC TTT TGG AC-3’</td>
<td>Panicker et al</td>
</tr>
<tr>
<td></td>
<td><em>tdh</em> R</td>
<td>5’-TGG AAT AGA ACC TTC ATC TTC CAC C-3’</td>
<td>Panicker et al</td>
</tr>
<tr>
<td></td>
<td><em>trh</em> F</td>
<td>5’-TTG GCT TCG ATA TTT TCA GTA TCT-3’</td>
<td>Panicker et al</td>
</tr>
<tr>
<td></td>
<td><em>trh</em> R</td>
<td>5’-CAT AAC AAA CAT ATG CCC ATT TCC G-3’</td>
<td>Panicker et al</td>
</tr>
<tr>
<td><em>V. vulnificus</em></td>
<td><em>vvhA</em> F</td>
<td>5’-TGT TTA TGG TGA GAA CGG TGA CA-3’</td>
<td>Campbell et al</td>
</tr>
<tr>
<td></td>
<td><em>vvhA</em> R</td>
<td>5’-TTC TTT ATC TAG GCC CCA CCA AAC TTG-3’</td>
<td>Campbell et al</td>
</tr>
</tbody>
</table>

was used containing a 1x mix of dNTPs, 25 U/ml iTaq DNA polymerase, 3 mM MgCl₂, 100 nM of *tdh* and *trh* primers, ultrapure water, and 3 µl of oyster homogenate for a total reaction volume of 25 µl. The PCR conditions began with a hot-start for 3 min at 95°C then 30 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 5 min. The PCR amplicons were visualized on 1.2% agarose gel (Lonza, Rockland, ME) with addition of Gel Red (Phenix Research Products) under an UV light.
Data Analysis. MPN values from triplicate sample analyses were used to calculate average Vibrio concentrations for each sample date and treatment using an MPN table (USDA). All MPN data were analyzed using JMP 9 statistical software (SAS Institute Inc, Cary, NC) to determine statistical significance (F-ratio, df, and $P$ - value) by a one-way ANOVA analyzing for any significant reductions of *V. parahaemolyticus* and *V. vulnificus* after 5 days in each treatment and comparing for any significant reduction levels between both depuration treatments and creek relay.

Results

Temperature and Environmental Effects on Vibrio Concentrations. Water temperature and salinity at the harvest site (Piscataqua River) were compared with water conditions at the relay site (Spinney Creek). Water temperatures were similar, ranging from 8.8 to 22°C and 9.2 to 19.8°C at the harvest and relay sites, respectively, whereas the salinities were different, ranging from 20 to 28 ppt and 27 to 32 ppt at the harvest and relay sites, respectively (Table 2.3). Initial concentrations of *V. parahaemolyticus* and *V. vulnificus* in oysters from the Piscataqua River were highest during the warmest month (August) when the water temperature was 22°C and the concentrations were 2.19 and 3.05 log$_{10}$ MPN/g, respectively. The lowest concentration was observed in the coldest month (November) when the water temperature was 8.8°C and the concentrations of *V. parahaemolyticus* and *V. vulnificus* was 0.44 log$_{10}$ MPN/g and below the detection limit, respectively.
Table 2.3 Temperature and salinity levels of the harvest site in Piscataqua River and at the relay site in Spinney Creek

<table>
<thead>
<tr>
<th>Month</th>
<th>Harvest site</th>
<th>Relay site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Temperature (°C)</td>
<td>Salinity (ppt)</td>
</tr>
<tr>
<td>July</td>
<td>19.4</td>
<td>22</td>
</tr>
<tr>
<td>August</td>
<td>22.4</td>
<td>24</td>
</tr>
<tr>
<td>September</td>
<td>16.2</td>
<td>28</td>
</tr>
<tr>
<td>November</td>
<td>8.8</td>
<td>20</td>
</tr>
</tbody>
</table>

Concentrations of *V. vulnificus* were higher than *V. parahaemolyticus* concentrations during July and August; however, *V. parahaemolyticus* concentrations were higher than *V. vulnificus* concentrations during the colder months of September and November (Table 2.3). Temperature abusing oysters significantly increased (p-value < 0.05) the concentration *V. parahaemolyticus* and *V. vulnificus* from freshly harvested oysters in each month of this study (Table 2.4). No oyster mortality was observed when the post temperature abused oysters were analyzed for *V. parahaemolyticus* and *V. vulnificus* concentrations.
Table 2.4 Concentration ($\log_{10}$ MPN/g) of $V. \text{parahaemolyticus}$ and $V. \text{vulnificus}$ in freshly harvested (FH) and temperature abused (TA) oysters

<table>
<thead>
<tr>
<th></th>
<th>$V. \text{parahaemolyticus}$</th>
<th>$V. \text{vulnificus}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FH</td>
<td>TA</td>
</tr>
<tr>
<td>July</td>
<td>1.8</td>
<td>4.42</td>
</tr>
<tr>
<td>August</td>
<td>2.46</td>
<td>5.15</td>
</tr>
<tr>
<td>September</td>
<td>2.39</td>
<td>4.06</td>
</tr>
<tr>
<td>November</td>
<td>0.55</td>
<td>3.39</td>
</tr>
</tbody>
</table>

Depuration and Relaying of Oysters. Temperature and salinity in Spinney Creek were consistent due to the enclosed nature of the creek. Spinney Creek is not affected by the tides, so the water temperature and salinity remained essentially constant throughout the course of each five day experiment for all three treatments. Significant reductions of $V. \text{parahaemolyticus}$ concentrations from depurated oysters occurred only in August (Table 2.5) with a 1.09 $\log_{10}$ MPN/g reduction in the flow-through treatment ($F(1,4) = 28.43; P = 0.0076$) and a 1.5 $\log_{10}$ MPN/g reduction for the recirculation treatment ($F(1,4) = 14.692; P = 0.0186$) after 5 days of treatment. No significant reductions were observed after 5 days of treatment for both depuration methods in July, September and November. A significant differences in reduction when comparing flow-through and recirculation after 5 days was observed only in September ($F(1,4) = 7.7634; P = 0.0495$).

Significant reductions were also observed for $V. \text{vulnificus}$ during July and August for both flow-through ($F(1,4) = 36.33; P = 0.0038$ and $F(1,4) = 83.446; P = 0.0008$, respectively) and recirculation ($F(1,4) = 532.48; P < 0.0001$ and $F(1,4) = 37.4838; P = \ldots$)
0.0036, respectively) treatments. No significant reductions were observed in September for depuration flow-through; however, a significant increase of *V. vulnificus* concentration occurred in oysters that were treated through depuration recirculation in Day 2 ($F(1,4) = 20.97; P = 0.0102$). Low concentrations of *V. parahaemolyticus* (0.11 log$_{10}$ MPN/g) and *V. vulnificus* (below detection limit) were present in the water sample collected from flow through and recirculation tanks.

Reduction of *V. parahaemolyticus* concentrations occurred more frequently in creek relay oysters compared to the depurated oysters (Table 2.5). The concentrations of *V. parahaemolyticus* were significantly reduced in August ($F(1,4) = 30.1304; P = 0.0054$), September ($F(1,4) = 45.6106; P = 0.0025$), and November ($F(1,4) = 47.2106; P = 0.0024$). *V. vulnificus* concentrations were significantly reduced by relaying only in August ($F(1,4) = 116.4765; P = 0.0004$) with no significant reductions observed during September and November. Low concentrations of *V. parahaemolyticus* (0.11 log$_{10}$ MPN/g) and *V. vulnificus* (below detection limit) were present in the water at the relay site were very low.

The natural relay oysters harbored initially low levels of *V. parahaemolyticus* and *V. vulnificus* compared to the temperature abused oysters in September and November. There were large, non-significant reductions of *V. parahaemolyticus* in naturally relayed oysters during September and November (Table 2.5). A small, non-significant reduction in *V. vulnificus* concentration was observed in September and *V. vulnificus* concentrations were not detected during November.
Table 2.5 Concentration of *V. parahaemolyticus* and *V. vulnificus* in oysters after postharvest processing

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration of <em>V. parahaemolyticus</em> (Log_{10} MPN Index/g)</th>
<th>July</th>
<th>August</th>
<th>September</th>
<th>November</th>
</tr>
</thead>
<tbody>
<tr>
<td>FH&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>1.48 (± 0.84)</td>
<td>2.19 (± 0.58)</td>
<td>1.59 (± 1.11)</td>
<td>0.44 (± 0.40)</td>
</tr>
<tr>
<td>TA&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>4.25 (± 0.46)</td>
<td>5.05 (± 0.34)</td>
<td>4.05 (± 0.16)</td>
<td>3.3 (± 0.38)</td>
</tr>
<tr>
<td>Day 2 flow-through</td>
<td></td>
<td>4.32 (± 0.33)</td>
<td>4.61 (± 0.43)</td>
<td>4.36 (± 0.43)</td>
<td>1.62 (± 1.08)*</td>
</tr>
<tr>
<td>Day 5 flow-through</td>
<td></td>
<td>3.66 (± 0.24)</td>
<td>3.96 (± 0.17)*</td>
<td>3.58 (± 0.24)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2.31 (± 0.30)</td>
</tr>
<tr>
<td>Day 2 recirculation</td>
<td></td>
<td>3.61 (± 0.25)</td>
<td>4.57 (± 0.52)</td>
<td>4.26 (±0.20)</td>
<td>3.23 (± 1.23)</td>
</tr>
<tr>
<td>Day 5 recirculation</td>
<td></td>
<td>4.14 (± 1.0)</td>
<td>3.50 (± 0.61)*</td>
<td>4.23 (±0.50)</td>
<td>2.34 (± 0.19)</td>
</tr>
<tr>
<td>Day 5 creek relay</td>
<td>N/A</td>
<td>3.48 (± 0.36)*</td>
<td>3.17 (± 0.16)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.46 (± 0.61)&lt;sup&gt;*&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Day 5 natural relay</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>0.17 (± 0.24)</td>
<td>-0.35 (± 0.65)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration of <em>V. vulnificus</em> (Log_{10} MPN Index/g)</th>
<th>July</th>
<th>August</th>
<th>September</th>
<th>November</th>
</tr>
</thead>
<tbody>
<tr>
<td>FH&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>2.62 (± 0.25)</td>
<td>3.05 (± 0.16)</td>
<td>0.66 (± 0.77)</td>
<td>BDL</td>
</tr>
<tr>
<td>TA&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>6.49 (± 0.17)</td>
<td>5.51 (± 0.36)</td>
<td>2.91 (± 0.40)</td>
<td>BDL</td>
</tr>
<tr>
<td>Day 2 flow-through</td>
<td></td>
<td>5.34 (± 0.45)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>5.36 (± 0.38)</td>
<td>3.36 (± 0.38)</td>
<td>BDL</td>
</tr>
<tr>
<td>Day 5 flow-through</td>
<td></td>
<td>3.63 (± 0.51)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>3.75 (± 0.36)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>2.20 (± 0.41)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>BDL</td>
</tr>
<tr>
<td>Day 2 recirculation</td>
<td></td>
<td>4.81 (± 0.35)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.82 (± 0.28)</td>
<td>4.05 (± 0.16)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>BDL</td>
</tr>
<tr>
<td>Day 5 recirculation</td>
<td></td>
<td>3.72 (± 0.12)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>3.41 (± 0.47)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>3.75 (± 0.36)</td>
<td>BDL</td>
</tr>
<tr>
<td>Day 5 creek relay</td>
<td>N/A</td>
<td>3.05 (± 0.16)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>2.36 (± 0.43)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>BDL</td>
<td></td>
</tr>
<tr>
<td>Day 5 natural relay</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>0.59 (± 0.49)</td>
<td>BDL</td>
</tr>
</tbody>
</table>

<sup>a</sup> Concentration from freshly harvested oysters  
<sup>b</sup> Concentration from temperature abused oysters  
<sup>1</sup> Significantly different with Day 5 FT and RC (p-value < 0.05)  
<sup>2</sup> Significantly different with Day 5 FT (p-value < 0.05)  
<sup>3</sup> Significantly different with Day 5 RC (p-value < 0.05)  
* Significantly different from Day 0 (p-value < 0.05)

Relaying was more consistent in reducing *Vibrio* concentrations in oysters compared to both depuration treatments. In September, the reduction of *V. parahaemolyticus* varied significantly comparing the three treatment methods ($F(2,6) = 13.2658; P = 0.0063$) and the post-hoc comparison revealed significant difference
between creek relay and recirculation (Tuky-Kramer HSD test, \( P = 0.0053 \)) and flow-through and recirculation \( (P = 0.0474) \). In November, the reduction of \( V. \) parahaemolyticus varied significantly with treatment methods \( (F(2,6) = 17.0475; \ P = 0.0034) \). Post-hoc comparisons revealed a significant difference between creek relay and depuration flow-through (Tukey-Kramer HSD test, \( P = 0.0058 \)) and depuration recirculation \( (P = 0.0054) \). The reduction of \( V. \) vulnificus concentration found in oysters from the recirculation treatment in September were significantly lower after 5 days compared to the flow through treatment \( (F(1, 4) = 20.6976; \ P = 0.0104) \) and relaying \( (F(1, 4) = 22.7346; \ P = 0.0089) \). The concentrations of \( V. \) parahaemolyticus and \( V. \) vulnificus in relayed oysters were lower than the flow through treatment in August, but the differences between the two treatments were non-significant. Oysters survived best and were in the highest quality condition under relay treatment conditions. Oysters subject to 5 days of recirculation were in poor condition based on objectionable odors and poor visible condition, and yielded the highest mortality rate (10%) among all treatments. The condition of the oysters after the flow through treatment was better than the recirculation treatment, with a mortality rate of 5%; no mortality was observed in the relayed oysters.

\( V. \) parahaemolyticus clinical markers, \( tdh \) and \( trh \) genes were not detected in any of the freshly harvested or temperature abused oysters except for temperature abused oysters in August at a concentration of 450 MPN/g. These markers were not detected in the August freshly harvested oysters, yet they were detected again at a concentration of 10.8 MPN/g of oyster on day 2 and 5 in depuration recirculation treated oysters.


Discussion

The emerging threats of Vibrio-associated illness in the Northeast US (Jones 2010) and other regions with cold water temperatures (CDC 1998; DePaola et al., 2000) shows that implementing PHP treatments is an important consideration, and eventually may be a requirement, to prevent outbreaks of Vibrios in these areas. This study is unique because it focused on the efficacy of both depuration methods in a commercial facility and relaying in natural waters for removing *V. parahaemolyticus* and *V. vulnificus* from oysters. The study period spanned a wide range of seasonally variable temperatures where both *Vibrio* species were present at their highest concentrations in the warmest summer months and at low or undetectable levels in colder November conditions (Jones and Summer-Brason, 1998, O’Neill et al. 1992).

Even though both species of *Vibrios* are present at their highest concentrations during warm summer months in oysters collected from the Great Bay Estuary (Jones and Summer-Brason 1998, O’Neill et al. 1992); the concentrations of *V. parahaemolyticus* and *V. vulnificus* are less than what is observed in warmer coastal areas like the Gulf of Mexico (Motes et al. 1998; Zimmerman et al., 2007. The NSSP guideline for PHP states that effective and acceptable postharvest processing treatments must decrease Vibrio concentrations in oysters by 3.52-log below 30 MPN/g after treatment (ISSC 2003, Kaysner and DePaola 2004). Temperature abusing oysters increases initial Vibrio concentrations (Staley et al., 2010, Wright et al., 2007), to a level where the 3.52 log reduction can be observed (NSSP 2009). Although temperature abuse increases the initial concentration of oysters, it is ineffective in increasing Vibrio concentrations if no viable *Vibrios* are present in oysters due to low water temperatures (Baker-Austin et al.,
2010; Drake et al., 2005; Motes et al., 1998; Tamplin et al., 1992). Obviously, if the concentrations are already below the 30 MPN/g endpoint concentration, there is no real need for PHP assuming the potential presence of viable but non-culturable Vibrios do not pose a public health threat.

Temperature abuse was used to increase Vibrio concentrations in oysters instead of artificial inoculation of Vibrios into oysters (Lewis et al., 2010; Chae et al., 2010; Su et al., 2010) prior to depuration and relaying treatments. Naturally occurring V. vulnificus exhibit different survival patterns in depurated oysters compared to laboratory grown strains of V. vulnificus (Tamplin et al. 1992). Several studies show that oysters that are artificially contaminated with Vibrios can be more easily depurated compared to shellfish containing naturally occurring Vibrios (Richards 1988; Tamplin et al., 1992). Previous studies involving temperature abuse of oysters to increase Vibrio populations have not indicated problems for the oysters (Staley et al., 2010; Wright et al., 2007), although it is a potentially problematic strategy that could affect or alter their ability to purge Vibrios.

The increase in Vibrio levels in some of the treatments in this study after 2 days is similar to the findings of Jones et al. (1991) and could be an indication of problems for the oysters resulting from the harvest process and transfer to experimental conditions, as well as temperature abuse. This is in contrast to the effective depuration of fecal coliforms within 48 hours of depuration (Jones et al., 1991), but also remains a factor to consider for future studies on depuration and relaying at SCS and in New Hampshire and Maine waters. Given the unpredictable response of Vibrios in oysters after two days
suggests it may require more than five days for effective reduction of *Vibrios* in oysters to occur.

Temperature abusing oysters was a successful way to increase the initially low to higher concentrations of *Vibrios* in oysters. Higher *Vibrio* concentrations during summer months in the Great Bay are associated with more diverse populations of *Vibrios* in oysters (Ellis *et al.*, 2011, In review). The diverse populations of *Vibrios* in oysters may differ in resilience to PHP methods, with some more capable of persisting through the PHP treatment (Staley *et al.*, 2011). The finding from this study that *V. parahaemolyticus* strains harboring *tdh* and *trh* genes persisted in oyster tissues after depuration recirculation is further evidence that temperature abuse affects the strain composition in oysters. The detection of clinical markers in *V. parahaemolyticus* strains only after oysters are subject to temperature abuse suggests that this strategy may not be a suitable way to increase Vibrios levels in oysters to validate PHP methods because of potentially significant differences in population diversity compared to untreated oysters.

There were major differences between depuration flow-through and depuration recirculation that suggests depuration recirculation is the least effective treatment for removing *Vibrios*. The recirculation treatment had intake of UV treated water from Spinney Creek that is foam fractionated, filtered, and had a second round of UV treatment, and then the water is circulated back into the tank. There were no suspended particles present in this sterile, filtered environment for maintaining oyster health, a probable significant factor affecting their capacity to purge *Vibrios*. The recirculation treatment also had the highest mortality rate for oysters, further illustration of the poor conditions for oyster survival; the presence of dead oysters in the treatment system was a
factor of unknown, but probably negative, effect. For any recirculation treatment to be effective, the oysters would probably require food to be introduced into the system, especially if the treatment time exceeds 2 days. The flow-through treatment had constant intake of UV sterilized creek water but the water was not filtered or foam-fractionated allowing inclusion of food particles for the oysters, and yielding only 5% mortality after treatment. This constant flow-through of food particles from Spinney Creek may be a contributing factor to the greater reduction of *V. parahaemolyticus* and *V. vulnificus* under depuration flow-through compared to depuration recirculation treatment.

The length of each PHP treatment may have affected the efficacy of reduction of *V. parahaemolyticus* and *V. vulnificus* in oysters. As previously suggested, it is possible that 5 days may not have been long enough to reduce the elevated *Vibrio* levels down to the acceptable levels of < 30 MPN/g in temperature abused oysters (ISSC, 2005). Staley *et al.* (2010) reported similar difficulties in lowering *V. vulnificus* levels in oysters subject to quick freeze for 5 days, where the same treatment for 21 days effectively reduced levels to < 30 MPN/g (Wright *et al.*, 2010). Audemard *et al.* (2011) used freshly harvested oysters with relatively low initial *Vibrio* concentrations to study the effects of high salinity relay and observed that *V. vulnificus* and *V. parahaemolyticus* concentrations were reduced below 30 MPN/g after 7 days of treatments. In this study, oysters that were naturally relayed in September and November also reduced initially low *V. parahaemolyticus* concentrations to < 30 MPN/g after 5 days. This suggests that high concentrations of Vibrios in oysters, and potentially the process of increasing concentrations by temperature abuse, may result in the requirement of a longer treatment period to meet the NSSP guideline for PHP validation.
The normal depuration process for removing fecal coliforms in shellfish is two days; however, the removal of fecal coliforms does not correlate with removal of Vibrios in oysters (Croci et al. 2002). Even after 5 days of treatment, both depuration treatments in this study showed inconsistent reductions of both Vibrio species, similar to the findings of Jones et al. (1991) where concentrations of V. vulnificus were both increased and decreased after 48 hours of depuration at SCS. The persistence of Vibrio species in oysters was also reported by Tamplin and Capers (1992), where depuration with UV disinfected seawater did not remove V. vulnificus in oysters after 3 days of treatment. In future studies, a longer treatment period may be necessary to see the full treatment effect of depuration and relaying on reducing V. parahaemolyticus and V. vulnificus levels to below 30 MPN/g. Because of the potential for shifts in population diversity to strains that may differ in their capacity to persist under PHP conditions, the effects of temperature abuse to increase concentrations also needs further scrutiny.

When comparing all three (with temperature abused oysters) treatments, relaying was more consistent in reducing concentrations of V. parahaemolyticus and V. vulnificus, with September as an exception, and holds promise as an effective treatment strategy. The relatively minimal reductions in concentrations for both Vibrio species observed in relayed oysters during September occurred simultaneously with increases for V. vulnificus and V. parahaemolyticus concentrations in oysters treated by the recirculation treatment. The higher water temperature at the treatment site compared to the harvest site, and the relatively small difference in salinities between sites probably contributed to the increase of Vibrio concentrations in the depuration treatment and the ineffectiveness of the relaying treatment.
The results of this study suggest that there may be biological and environmental factors that contribute to the reduction of *Vibrios* in contaminated oysters during the PHP treatments. As seen in other studies, relaying to higher salinity waters is a contributing factor in the reduction of *V. parahaemolyticus* and *V. vulnificus* (Audemard *et al.*, 2011; Jones 1994; Jones *et al.*, 1995; Motes *et al.* 1996); however, all treatments were subjected to the same water salinity, and the creek water near the relay site was used to fill the depuration tanks. More consistent reductions for both *Vibrios* in the relay systems suggest a more complex explanation of why reductions occur.

The major difference between relaying and depuration approaches was the lack of any sterilization process in relaying which suggests that the microbial community in the water may play a significant role in displacing the *Vibrios* in the oysters (Jones 1994; Tamplin *et al.*, 1992). Several biological mechanisms could be involved in reducing Vibrio levels during relaying (Tepliski *et al.*, 2009). In addition, temperature, salinity, suspended particles, phytoplankton and other conditions in natural estuarine ecosystems may be important factors that are crucial in changing microbial communities in shellfish and the water column with the potential for a decrease in the presence of pathogenic Vibrios in oysters. This hypothesis is suggested because if one of these factors was offset (such as small salinity changes between the harvest site and relay site) the reduction of *Vibrios* may be smaller, as seen in September for both *V. parahaemolyticus* and *V. vulnificus*, as well as the fact that many of these factors have been found to be significant factors affecting Vibrio abundance in previous GBE studies (Jones and Summer-Brason, 1998).
Relaying oysters holds promise in reducing *Vibrio* concentrations in contaminated oysters. This postharvest process was consistent in reducing *V. parahaemolyticus* and *V. vulnificus* concentrations in oysters throughout the course of this study. The factors that were taken into consideration in this study that may be important in reduction of *Vibrios* were temperature, salinity, oyster condition and the microbial community interacting with the oysters from the water. In addition, the different strains of *V. parahaemolyticus* and *V. vulnificus* that are found in the oysters and other biological factors present in the natural relay conditions may also contribute to how effective these PHP methods are for displacing *Vibrios* in oysters. The results from this study show that there is a need for further controlled experiments to take into account more environmental factors and to study potential biological factors that may affect the efficacy of these after-harvest treatments.
OPTIMIZATION AND EVALUATION OF DIFFERENT REAL-TIME PCR METHODS

Optimization of qPCR

The qPCR protocol (primers, probes, and qPCR parameters) used to detect *V. parahaemolyticus* in oysters in this study was based on the Nordstrom et al. (2007) procedure; however, the qPCR protocol in this study was optimized for an iCycler (Bio-Rad) platform instead of the SmartCycler platform used by Nordstrom et al. (2007). The *tlh* primers and *tlh* TaqMan probes were synthesized by Integrated DNA Technologies (Coralville, IA) and working stocks of 10 nM of primers and probes were created. Genomic DNA extracted from *V. parahaemolyticus* cells was diluted to a working stock of 50 ng/μl. The working stock of gDNA was diluted 10-fold from $10^{-1}$ to extinction and this dilution series was used to create the standard curve and establish the limit of detection for gDNA. The limit of detection was 100 fg. To calculate the concentration of *V. parahaemolyticus* cells in relation to the limit of detection, $1.0 \times 10^8$ CFU/ml of *V. parahaemolyticus* cells were serially diluted to $10^3$, then the cells were boiled and the supernatant used as a template for qPCR. The detection limit for qPCR on these pure cultures was 1000 CFU/ml, corresponding to 100 fg of DNA.

Detection of *V. parahaemolyticus* in unenriched and minimally-diluted (1:3; w:v) oyster homogenate by qPCR was unsuccessful due to compounds in the oyster tissue that inhibited the qPCR reactions. This necessitated an enrichment step as part of the qPCR
assay for use with oyster tissue. A 3-tube MPN enrichment method was used following a modified FDA BAM (Kaysner and DePaola 2004) for increasing concentrations of *Vibrios*, and supernatants from positive APW tubes were used to detect and enumerate *V. parahaemolyticus* concentrations in oysters by qPCR.

**Evaluation of TaqMan-based qPCR and SYBR Green I qPCR**

When the qPCR assay was being optimized, initial testing of a TaqMan-based qPCR procedure gave problems because the TaqMan probes degraded rapidly during optimization, resulting in no *V. parahaemolyticus* detection in oyster homogenates as well as the standard curve for pure DNA. A possible alternative procedure was suggested in a previous study that used SYBR Green I assay to detect *V. vulnificus* in oysters (Wright *et al.*, 2007). In that study, no differences in *V. vulnificus* detection was observed between the TaqMan-based and SYBR Green I methods, as both assays were 100% sensitive and species specific in detecting *V. vulnificus* (Wright *et al.* 2007). The results of the SYBR Green optimization in this study yielded the same limit of detection (100 fg) with genomic DNA as found for the TaqMan-based qPCR. The advantages of using SYBR Green I for detection of *Vibrios* in oysters and confirming the amplicons via melt curve were that it was a more time-efficient and cost-effective method compared to the TaqMan-based qPCR and, among other advantages (Table A1), probes were not rapidly degraded as they were with the TaqMan-based qPCR method.
<table>
<thead>
<tr>
<th>SYBR Green I qPCR</th>
<th>TaqMan qPCR</th>
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<tbody>
<tr>
<td>No addition of probes*</td>
<td>Need to add TaqMan probes</td>
</tr>
<tr>
<td>Verify PCR amplicons by using a melt curve</td>
<td>Verify PCR amplicons by running a gel from each reaction</td>
</tr>
<tr>
<td>Longer assay</td>
<td>Shorter assay (unless running a gel to verify PCR amplicons)</td>
</tr>
<tr>
<td>Not specific; chemistry based on dye binding to dsDNA</td>
<td>More specific; binds to specific sequence of target organism DNA</td>
</tr>
<tr>
<td>Costs less</td>
<td>Expensive to synthesize probes</td>
</tr>
<tr>
<td>SYBR Green doesn't degrade rapidly</td>
<td>Higher chances for contamination by adding more reagents to the master mix</td>
</tr>
<tr>
<td></td>
<td>Probes degrade rapidly</td>
</tr>
</tbody>
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*iQSupermix SYBR Green I already contains the intercalating dye*
LIST OF REFERENCES


