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High salinity relay as a post-harvest processing method for reducing *Vibrio vulnificus* levels in oysters (*Crassostrea virginica*)

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ABSTRACT

High salinity relay of Eastern oysters (*Crassostrea virginica*) was evaluated as a post-harvest processing (PHP) method for reducing *Vibrio vulnificus*. This approach relies on the exposure of oysters to natural high salinity waters and preserves a live product compared to previously approved PHPs. Although results of prior studies evaluating high salinity relay as a means to decrease *V. vulnificus* levels were promising, validation of this method as a PHP following approved guidelines is required. This study was designed to provide data for validation of this method following Food and Drug Administration (FDA) PHP validation guidelines. During each of 3 relay experiments, oysters cultured from 3 different Chesapeake Bay sites of contrasting salinities (10–21 psu) were relayed without acclimation to high salinity waters (31–33 psu) for up to 28 days. Densities of *V. vulnificus* and densities of total and pathogenic *Vibrio parahaemolyticus* (as *tdh* positive strains) were measured using an MPN-quantitative PCR approach. Overall, 9 lots of oysters were relayed with 6 exhibiting initial *V. vulnificus* > 10,000/g. As recommended by the FDA PHP validation guidelines, these lots reached both the 3.52 log reduction and the < 30 MPN/g densities requirements for *V. vulnificus* after 14 to 28 days of relay. Densities of total and pathogenic *V. parahaemolyticus* in relayed oysters were significantly lower than densities at the sites of origin suggesting an additional benefit associated with high salinity relay. While relay did not have a detrimental effect on oyster condition, oyster mortality levels ranged from 2 to 61% after 28 days of relay. Although the identification of the factors implicated in oyster mortality will require further examination, this study strongly supports the validation of high salinity relay as an effective PHP method to reduce levels of *V. vulnificus* in oysters to endpoint levels approved for human consumption.

1. Introduction

Oysters have historically been an important part of the diet of coastal communities and they are now supporting important aquaculture industries. In the United States, oyster aquaculture is a growing industry spanning the Pacific, Atlantic and Gulf coasts and producing > 27 million pounds of oysters annually for a value of $213 million in 2015 (FUS, 2015). The majority of these oysters are sold as raw product on the half-shell, a more profitable market compared to that of the cooked product. Because oysters are filter-feeders, they have the potential to accumulate human pathogens present in surrounding waters affecting their safety for human consumption, especially as a raw product. To minimize the risk of contamination from pathogens originating from human sewage, shellfish-growing waters are classified with regards to the potential presence and abundance of these allochthonous pathogens; however, for autochthonous pathogens such as *Vibrio* spp., which naturally occur in the marine and estuarine environment, different approaches need to be considered to ensure that oysters are safe for raw consumption.

In the United States, the two most concerning pathogenic *Vibrio* species associated with consumption of raw or undercooked shellfish, in particular oysters, are *Vibrio vulnificus* and *Vibrio parahaemolyticus*. The abundance of both species in the environment and in shellfish increases during the warm season (DePaola et al., 1990; DePaola et al., 2003; Johnson et al., 2012; Randa et al., 2004), and most illnesses associated with these species are reported from May through October when water temperatures are > 20 °C (Center for Disease Control and Prevention, 2017). Vibriosis caused by *V. vulnificus* occurs in a very limited portion of the population as it primarily affects immune-compromised individuals, such as those with liver disease or diabetes, or those on chemotherapy. Nonetheless, *V. vulnificus* is a major concern because it is a leading cause of seafood-borne mortality, with an ~30% fatality rate in the United States owing to the development of rapid systemic infection and acute septicemia (Jones and Oliver, 2009; Mead et al., 1999; Oliver, 2015; Scallan et al., 2011). In contrast, *V. parahaemolyticus* is mostly known for causing gastroenteritis with outbreaks that occasionally reach pandemic proportions (Drake et al., 2007;

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Martinez-Urtaza et al., 2017; Nair et al., 2007). In the United States, it is the leading cause of bacterial gastroenteritis with an estimated number of 45,000 cases per year (Center for Disease Control and Prevention, 2017).

Minimizing the risks of vibriosis associated with raw or undercooked oyster consumption relies on education, risk management, and the use of post-harvest processing methods (PHP) to reduce vibrio densities in shellfish (FAO/WHO, 2011; US FDA, 2015). Currently, four approved and validated PHPs are cool pasteurization, cryogenic individual quick freezing, high hydrostatic pressure and low-gamma dose irradiation (Muth et al., 2013). These PHPs are designed to reduce the density of *Vibrio* spp. in shellfish to safe levels (< 30/g of oyster tissue) and are effective in lowering the risk of illnesses associated with vibrios and other pathogens occurring in shellfish (US FDA, 2015). However, these methods present issues related to cost and to consumer perception since in most cases the oysters are killed during the process (Baker, 2016; Muth et al., 2013).

An observed reduction of *V. vulnificus* densities in oysters held at salinities > 25 psu (Kaspar and Tamplin, 1993; Kelly, 1982; Motes et al., 1998) laid the foundation for evaluating exposure to high salinity waters as an additional PHP to decrease *V. vulnificus* in oysters. Two main approaches have been investigated, with one approach involving the transfer –or relay- of oysters to sites exposed to high salinity waters (Audemard et al., 2011; Motes and DePaola, 1996; Parveen et al., 2017), and another relying on recirculating depuration land-based systems (Larsen et al., 2013; Larsen et al., 2015; Parveen et al., 2017). The first relay experiment was conducted in the Gulf of Mexico and involved a site of intermediate salinity for acclimation of oysters before relay to offshore waters (Motes and DePaola, 1996). Subsequently, both Parveen et al. (2017) and our preliminary study (Audemard et al., 2011) showed that direct relay without acclimation did not affect oyster survival (mortalities < 7%), simplifying the relay process and reducing potential costs for oyster growers. Similarly, high salinity depuration experiments conducted without acclimation were associated with < 7% oyster mortality (Larsen et al., 2013; Larsen et al., 2015; Parveen et al., 2017). Overall, results from both the high salinity relays and the high salinity depuration trials were promising and showed reductions in *V. vulnificus* levels and to a lesser extent in total *V. parahaemolyticus* levels after 7 to 28 days of exposure. Nevertheless, the results obtained suggested that more work was needed for high salinity relay or depuration to be validated as a PHP.

Demonstrating that a PHP can reliably be used to reduce vibrio densities to non-detectable levels requires validation following guidelines established by FDA (US FDA, 2015). These guidelines specify, among other things, the initial vibrio density before the process, the number of samples to be analyzed, the analytical methods to be used, and the endpoint criteria to be reached for process validation (Table 1; US FDA, 2015). Parveen et al. (2017) were the first to report results of relay trials based on this guidance. Although some samples met the log reduction and end point densities criteria for *V. vulnificus*, validation failed for all 5 trials. Because salinities at the relay sites ranged from 29 to 33 psu in that study, and based on previous relay results (Audemard et al., 2011; Motes and DePaola, 1996), we hypothesized that constant high salinity (> 30 psu) might be necessary to ensure reproducible decreases in *V. vulnificus* levels.

The objective of the present study was to provide validation data for high salinity relays to be considered as an additional PHP for reducing *V. vulnificus* occurring in oysters. To reach this goal, three consecutive relay experiments were conducted wherein oysters originating from three sites located in the lower Chesapeake region (Virginia) were relayed without acclimation to a high salinity site located in euhaline waters (30–35 psu) on the seaside of the Eastern Shore of Virginia. The study herein was conducted based on FDA validation guidance (Table 1); however, because these guidelines are designed for well-controlled "industrial" processes, they are not entirely applicable to a process relying on the natural environment. In addition to *V. vulnificus* densities, we measured oyster mortality, oyster condition index and levels of total *V. parahaemolyticus* and pathogenic *V. parahaemolyticus*. i.e., strains possessing the thermostable direct hemolysin gene (*tdh*) in oysters (Honda and Iida, 1993). Based on previous high salinity relay and depuration studies, *V. parahaemolyticus* appears to be more tolerant to high salinity exposure than *V. vulnificus*, (Audemard et al., 2011; Larsen et al., 2015; Parveen et al., 2017). However, to the best of our knowledge, the influence of relay exposure to high salinity water on naturally-occurring pathogenic *V. parahaemolyticus* in oysters has never been investigated.

2. Materials and methods

2.1. High salinity relay study design

Three consecutive high salinity trials were conducted in the Chesapeake Bay region, USA, with the first relay starting in early June 2013 and the last one completed by late-September 2013 (Table 2). During each trial, eastern oysters, *Crassostrea virginica*, originating from three different grow-out sites were relayed to a high salinity site and were collected after 14, 21 and in some instances 28 days of relay. Two of the grow-out sites were located within estuarine systems along the western shore of Chesapeake Bay, with one site located in the mesohaline zone (low salinity site) and the other in the polyhaline zone (moderate salinity Site 1). The third site also was located within the polyhaline zone of Chesapeake Bay, but within a creek on the bayside of the VA Eastern Shore (i.e., the Delmarva Peninsula) (moderate salinity Site 2). The high salinity site (> 30 psu) where the oysters were

Table 1
Study design assessment in comparison to US FDA guidelines for validation of a post-harvest relay process for *Vibrio* spp. (US FDA, 2015). A checkmark indicates that the study met the guidance whereas no checkmark indicates that the study design deviated from the guidance. See the discussion for details and explanation.

	US FDA guidance for PHP validation	Present study
General methodology	• A sample consists of 10–12 oysters	✓
	• Means are adjusted geometric means (AGM)	✓
	• Analytical methods: official ISSC methods	Used Taqman assay instead of official SYBR Green assay
Initial load testing	• 4 samples	✓
	• 3-tube MPN (1×10^{-1} to 1×10^{-6} MPN/g)	✓
	• Initial vibrio loads ≥10,000 MPN/g	Reached for 6 out of 9 lots
Processed samples	• 10 processed samples distributed throughout processing day	Samples collected at individual time points
	• 3 processing days (total 30 samples)	3 relay trials and 2 to 3 sampling time points during relay
	• Samples originating from same lot on each processing day	3 lots or site of origin tested
	• Single dilution 5-tube MPN (1×10^{-2} or 1×10^{-1} g)	Three dilutions 5-tube MPN (1×10^{-1} to 1×10^{-3} MPN/g)
Endpoint criteria	• Vibrio load < 30 MPN/g and demonstrating ≥3.52 log reduction	Reached for all lots associated with initial loads ≥10,000 MPN/g after 21 or 28 days of relay
	• No > 3 samples out of 30 may fail based on initial load and number of positive enrichment tubes (see US FDA, 2015 for more details)	See Results and Discussion