# 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.

Keywords: *Vibrio vulnificus*, *Vibrio parahaemolyticus,* oyster, post-harvest processing, salinity, relay

## 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 more than 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; 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

89 evaluating exposure to high salinity waters as an additional PHP to decrease *V. vulnificus*
90 in oysters. Two main approaches have been investigated, with one approach involving the
91 transfer –or relay- of oysters to sites exposed to high salinity waters (Audemard et al.,
92 2011; Motes and DePaola, 1996; Parveen et al. 2017), and another relying on
93 recirculating depuration land-based systems (Larsen et al., 2013; Larsen et al., 2015;
94 Parveen et al., 2017). The first relay experiment was conducted in the Gulf of Mexico and
95 involved a site of intermediate salinity for acclimation of oysters before relay to offshore
96 waters (Motes and DePaola, 1996). Subsequently, both Parveen et al. (2017) and our
97 preliminary study (Audemard et al., 2011) showed that direct relay without acclimation
98 did not affect oyster survival (mortalities < 7%), simplifying the relay process and
99 reducing potential costs for oyster growers. Similarly, high salinity depuration
100 experiments conducted without acclimation were associated with < 7% oyster mortality
101 (Larsen et al., 2013; Larsen et al., 2015; Parveen et al., 2017). Overall, results from both
102 the high salinity relays and the high salinity depuration trials were promising and showed
103 reductions in *V. vulnificus* levels and to a lesser extent in total *V. parahaemolyticus* levels
104 after 7 to 28 days of exposure. Nevertheless, the results obtained suggested that more
105 work was needed for high salinity relay or depuration to be validated as a PHP.
106 Demonstrating that a PHP can reliably be used to reduce vibrio densities to non-
107 detectable levels requires validation following guidelines established by FDA (US FDA,
108 2015). These guidelines specify, among other things, the initial vibrio density before the
109 process, the number of samples to be analyzed, the analytical methods to be used, and the
110 endpoint criteria to be reached for process validation (Table 1; US FDA, 2015). Parveen
111 et al. (2017) were the first to report results of relay trials based on this guidance.

112 Although some samples met the log reduction and end point densities criteria for *V.*
113 *vulnificus*, validation failed for all 5 trials. Because salinities at the relay sites ranged
114 from 29 to 33 psu in that study, and based on previous relay results (Audemard et al.,
115 2011; Motes and DePaola., 1996), we hypothesized that constant high salinity (>30 psu)
116 might be necessary to ensure reproducible decreases in *V. vulnificus* levels.
117 The objective of the present study was to provide validation data for high salinity
118 relays to be considered as an additional PHP for reducing *V. vulnificus* occurring in
119 oysters. To reach this goal, three consecutive relay experiments were conducted wherein
120 oysters originating from three sites located in the lower Chesapeake region (Virginia)
121 were relayed without acclimation to a high salinity site located in euhaline waters (30-35
122 psu) on the seaside of the Eastern Shore of Virginia. The study herein was conducted
123 based on FDA validation guidance (Table 1); however, because these guidelines are
124 designed for well-controlled "industrial" processes, they are not entirely applicable to a
125 process relying on the natural environment. In addition to *V. vulnificus* densities, we
126 measured oyster mortality, oyster condition index and levels of total *V. parahaemolyticus*
127 and pathogenic *V. parahaemolyticus*. i.e., strains possessing the thermostable direct
128 hemolysin gene (*tdh*) in oysters (Honda and Iida, 1993). Based on previous high salinity
129 relay and depuration studies, *V. parahaemolyticus* appears to be more tolerant to high
130 salinity exposure than *V. vulnificus*, (Audemard et al., 2011; Larsen et al., 2015; Parveen
131 et al., 2017). However, to the best of our knowledge, the influence of relay exposure to
132 high salinity water on naturally-occurring pathogenic *V. parahaemolyticus* in oysters has
133 never been investigated.
134

## 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 relayed, was exposed to euhaline waters (30-35 psu) and was located in a seaside bay of the Eastern Shore. Oysters, as well as access to grow-out and relay sites, were kindly provided by local growers interested in the potential use of this PHP method. Grow-out site locations were not disclosed to preserve anonymity of the growers. Sites were further characterized by water temperature data monitored using in situ loggers (HOBO, Onset Inc., Bourne, MA) and by salinity data measured upon collection of the samples using a calibrated refractometer.

All *C. virginica* oysters used in this study were polyploid and more specifically triploid. These oysters are often favored over diploid oysters by oyster growers because of faster growth and higher yield (Dégremont et al. 2012; Hudson, 2017). At the low salinity site, the oysters were grown either in off-bottom cages that remained submerged or directly on the river bottom. Oysters from the moderate salinity Site 1 were grown in bags placed on racks that were exposed to air periodically on extreme low-tides. The oysters from the moderate salinity Site 2 were grown in on-bottom cages that remained submerged. At the relay site, the oysters were deployed in off-bottom cages that remained submerged. Water depth at each site including the high salinity site ranged from 1.2 to 1.8 m.

The oysters collected during each trial to assess *Vibrio* spp. levels included a pre-relay sample to assess naturally-occurring *V. vulnificus* levels at each site of origin, a Time 0 sample (day of relay) and samples collected after 14, 21 and, for one relay trial, after 28 days of relay (see below). To facilitate sampling at each time point, the oysters were deployed into 3 cages with each cage containing oysters from all 3 sites to be sampled at one time point. The relayed oysters were bagged in mesh bags in groups of 15 and each bag was labeled with the name of the site of origin before they were placed in the cages. Oyster samples were kept chilled in insulated coolers and separated from direct contact with ice during transport. When holding was necessary prior to sample processing, oysters were kept in a cold room maintained at 10ºC as in Audemard et al. (2011). Additional samples were collected to assess oyster mortality and oyster condition during the relays as described below.

180 *2.2.* Vibrio *spp. densities in pre-relay samples*

181

182 Pre-relay samples were collected from each site of origin a week prior to each
183 relay to assess natural *Vibrio* spp. levels at these sites and to decide if a temperature
184 abuse would be necessary prior to relay to force densities of *V. vulnificus* to ≥ 10,000
185 MPN/g as required by FDA validation guidance (Table 1; US FDA, 2015). At each site,
186 the pre-relay sample consisted of 4 replicate samples of 10 oysters each. Each sample
187 was processed using a 3-tube most-probable number (MPN) approach with dilutions
188 ranging from $1 \times 10^{-1}$ to $1 \times 10^{-6}$ g equivalents followed by quantitative PCR (qPCR) as
189 described below.

190

191 *2.3.* Vibrio *spp. densities at time of relay*

192

193 Oysters collected from each site of origin were brought to the laboratory 1 to 3
194 days before deployment to the relay site. This time lag was necessary to accommodate
195 collection and transport of oysters from 3 different sites of origin to the laboratory, the
196 application of temperature abuse if needed, preparation of the oysters for deployment
197 and transport from the laboratory to the relay site. Oysters that needed to be temperature
198 abused based on the data from the pre-samples, were bagged in plastic bags, placed in
199 microbiological incubators and exposed to 30˚C for 3-8 hours (Table 3). Selection of the
200 oyster samples for temperature abuse and length of abuse time was based on *V. vulnificus*
201 densities measured in the pre-relay samples. Our intent was to reach densities of *V.*
202 *vulnificus* ≥ 10,000 MPN/g following FDA guidelines (US FDA, 2015) without

producing overly high values irrelevant in terms of naturally-occurring *V. vulnificus* concentrations. Unless the oysters were temperature abused, they were maintained at 10˚C as described above until the day of deployment (Time 0). On deployment day, 4 replicate samples of 10 oysters each were processed from each site using a 3-tube MPN with dilutions ranging from $1 \times 10^{-1}$ to $1 \times 10^{-6}$ g equivalent as suggested by FDA validation guidelines (Table 1; US FDA 2015) followed by qPCR as described above. These samples constituted the Time 0 samples to assess initial vibrio densities at time of deployment to the high salinity site. While processing was conducted at the laboratory, the oysters to be deployed were transported (2-3 hours) to the high salinity site in coolers and deployed as described above.

### *2.4.* Vibrio *spp. densities during relay*

*Vibrio* spp. densities were measured in the oysters collected after 14 and 21 days of relay to high salinity waters. Samples were also collected after 28 days of relay during the first relay experiment and for oysters originating from the low salinity site because the low salinity oysters did not reach endpoint validation criteria at 21 days of relay, i.e., *V. vulnificus* levels remained > 30 MPN/g (Table 1; US FDA, 2015). At each time point, the objective was to collect 10 samples of 10 oysters for each site of origin or each lot with lot defined as oysters 'harvested from a particular area during a single day's harvest' as suggested by FDA guidelines. *Vibrio* spp. densities in the relayed oysters were determined using a 5-tube MPN with dilutions ranging from $1 \times 10^{-1}$ to $1 \times 10^{-3}$ g equivalent. This enumeration range differed from the FDA guidelines, which specifies a

226 5-tube MPN with only one dilution to be tested, either 1 $\times 10^{-1}$ or 1 $\times 10^{-2}$, depending on
227 the initial density to demonstrate validation of a process and assess if samples pass or fail
228 validation criteria (Table 1; US FDA, 2015).
229
230 *2.5. Sample processing using an MPN approach*
231
232 Densities of total *V. vulnificus*, total *V. parahaemolyticus* and pathogenic *V.*
233 *parahaemolyticus* (*tdh* gene) were determined using the MPN approach followed by
234 qPCR. For each replicate sample, a tissue homogenate was prepared by pooling the
235 tissues and liquor of 10 oysters. Homogenates were prepared using a Waring stainless
236 steel blender (Eberbach Labtools, Michigan) and were inoculated into an alkaline peptone
237 water (APW) MPN series as described in the FDA Bacteriological Analytical Manual
238 (US FDA, 2010). Inoculated samples were incubated at 35°C for 18-24 h. A 1 ml volume
239 was removed from the top cm of each APW enrichment tube showing turbidity and was
240 boiled for 10 min to lyse cells (Jones et al., 2009). This lysate was subsequently used as
241 the source of template DNA in each of the qPCR assays described below. Results of the
242 qPCRs were used to calculate MPN densities using approved tables (US FDA, 2010). For
243 all *Vibrio* spp. abundance data, the means were adjusted geometric means (AGM) of
244 replicate samples, which were calculated by multiplying the geometric mean by 1.3 as
245 recommended in the FDA guidelines (US FDA, 2015).
246
247 *2.6. Detection of* Vibrio *spp. in enrichments by qPCR*
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249 Detection of total *V. vulnificus* in APW enrichment lysates was performed by
250 targeting the hemolysin/cytolysin gene (*vvhA*) using the Taqman® assay designed by
251 Campbell and Wright (2003) as described in Audemard et al. (2011) except that the
252 Applied Biosystems® TaqMan® Fast Advanced Master Mix (Thermo Fisher Scientific,
253 Waltham, MA) and the fast cycling conditions recommended with this mix (20 s at 95˚C
254 followed by 40 cycles with each cycle consisting in 3 s at 95˚C and 30 s at 60˚C) were
255 used. Detection of total *V. parahaemolyticus* in the lysate was accomplished by targeting
256 the thermolabile hemolysin gene (*tlh*) in a multiplex qPCR assay during which both *V.*
257 *parahaemolyticus tlh* and *V. parahaemolyticus tdh* strains were targeted using the primers
258 and probes designed by Nordstrom et al. (2007). Modifications to the published protocols
259 were as described in Audemard et al. (2011) with the master mix and cycling conditions
260 as described above for the *V. vulnificus* assay. All qPCR reactions were run on an
261 Applied Biosystems® 7500 Fast Real-Time PCR system (Thermo Fisher Scientific,
262 Waltham, MA).
263
264 *2.7. Oyster mortality*
265
266 The potential effect of relaying oysters to a high salinity site on oyster mortality
267 was assessed and compared to mortality levels recorded in oysters left at their site of
268 origin as controls. For the relayed oysters, the percentage of dead oysters was assessed
269 for each of the mesh bags (15 oysters/bag) that were brought back to the laboratory at
270 each sampling time point (10 bags/site of origin or lot of oysters). For the controls left at
271 the site of origin, three bags containing 15 oysters each were collected at the end of each

trial (28 days of relay) to assess the percentage of dead oysters at each site. Using the oyster bags as replicates, means and standard deviations were calculated.

### *2.8. Oyster condition*

Oysters that had been relayed for 28 days were also analyzed to assess their condition and compared to oysters at their site of origin at Time 0 and 28 days later. Three additional bags of oysters (15 oysters/bag) were relayed for this purpose and sampled at the 28-day sampling time point. Controls consisted of 3 bags of oysters that remained at their site of origin until the 28-day sampling time point. For condition analysis, 25 oysters were sampled at each site (origin and relay site) and at each time point (Time 0 and 28 days). For each oyster, the dry meat weight and the dry shell weight were measured and the condition indices were calculated based on the formula from Rainer and Mann (1992): Condition = dry meat weight × 100/dry shell weight. A higher value indicated a higher condition of the oyster. Means and standard deviations were calculated for each site and time point.

### *2.9. Data analysis*

Densities of *V. vulnificus*, *V. parahaemolyticus* and *V. parahaemolyticus (tdh)* strains measured during the relay experiments were analyzed using a three-way analysis of variance (ANOVA) with relay experiment (Relay 1, 2 and 3), site of origin (low salinity site, moderate salinity Site 1 and moderate salinity Site 2) and treatment (pre-

295 relay, Time 0, 14 days and 21 days of relay) as independent class variables. The data
296 collected at the 28-day relay time point during Relay 1 were excluded from the ANOVA
297 because these data were not collected in the other relay trials. *Vibrio* spp. densities were
298 $\log_{10}$-transformed prior to the analysis in order to meet the assumptions of normality and
299 homogeneity of variance.
300 Similarly, three-way ANOVAs with relay experiment, site of origin and relay
301 treatment as independent class variables were used to analyze mortality and condition
302 index data. For the mortality data, the relay treatment variable included the data collected
303 at 14, 21, and 28 days of relay, as well as the data collected in the controls left at the site
304 of origin. Mortality data were arcsine transformed prior to analysis. For the oyster
305 condition index data, the relay treatment variable included the Time 0 at site of origin, 28
306 days at the site of origin and 28 days at the relay site. Owing to mortalities in one
307 treatment, there was missing data relative to the relayed oysters during Relay 1.
308 Therefore, the ANOVA was run on the data collected during Relays 2 and 3. The
309 condition index data were square root transformed prior to the analysis to meet
310 assumptions of normality and homogeneity of variance. For each analysis, a Tukey's
311 HSD post-hoc test was run to determine significant differences among means. All
312 statistical analyses were conducted using SPSS software (IBM SPSS Statistics 20; IBM,
313 Armonk, NY). An alpha level of 0.05 was considered statistically significant.
314
315
316 **3. Results**
317

318 *3.1. Environmental parameters*

319

320 Salinities measured at the sites of origin ranged from 10 to 12 psu at the low
321 salinity site, from 19 to 20 psu at moderate salinity Site 1 and from 17 to 21 psu at
322 moderate salinity Site 2. Salinities at the relay site ranged from 31 to 33 psu and water
323 temperatures were 20˚C in early June, increasing to 25-27˚C from July through early
324 September and decreasing to 20˚C by mid-September when the study was completed
325 (Table 2).

326

327 *3.2.* V. vulnificus *densities*

328

329 Oysters collected from the sites of origin as pre-relay samples were associated
330 with *V. vulnificus* levels ranging from 140 MPN/g to 58,000 MPN/g (Table 3; Fig. 1). As
331 an attempt to increase densities to at least 10,000 MPN/g required by FDA validation
332 guidelines (Table 1; US FDA, 2015), all oyster lots except for the low salinity oysters
333 during Relay 2 were temperature abused. At Time 0, 6 of the 9 lots relayed were
334 associated with *V. vulnificus* density ≥ 10,000 MPN/g so 3 lots did not meet the FDA
335 initial density requirement (Table 3).

336 Relays to high salinity waters resulted in decreases in *V. vulnificus* densities
337 compared to Time 0 with log reductions ranging from 2.72 to 4.59 after 14 days of relay,
338 and from 2.85 to 5.20 after 21 days (Table 4; Fig. 1). Focusing on the 6 lots that had
339 AGMs ≥ 10,000 MPN/g at Time 0, log reductions ranged from 3.35 to 4.59 at 14 days

340 and from 3.70 to 5.20 at 21 days so after 21 days of relay all reductions were ≥ 3.52 logs
341 as required by FDA for PHP validation (Table 1; US FDA, 2015).
342 Densities of *V. vulnificus* were < 30 MPN/g for 7 out of 9 lots after 14 days of
343 relay and after 21 days of relay, all the samples were associated with AGMs < 30 MPN/g
344 except for oysters from the low salinity site during Relay 1 (39 MPN/g). The latter
345 required an additional 7 days to reach a density of < 30 MPN/g (Table 4). Oysters from
346 this site were associated with the second highest *V. vulnificus* AGM at Time 0 (976,956
347 MPN/g), which may explain the additional length of time needed to reach densities < 30
348 MPN/g.
349 Densities of *V. vulnificus* were significantly different between relay trials, sites of
350 origin and length of relay ($p < 0.0005$) (Table 5). Densities of *V. vulnificus* during Relay
351 3 were significantly lower (Tukey's HSD, $p < 0.001$) than during Relays 1 and 2.
352 Densities in oysters from the low salinity site were significantly higher (Tukey's HSD, $p$
353 < 0.001) than those at the two moderate salinity sites. As expected, densities at both day
354 14 and day 21 were significantly lower than at Time 0, and densities at day 21 were
355 significantly lower than those at day 14 (Tukey's HSD, $p < 0.001$).
356 For oyster lots associated with initial levels meeting the minimum ≥ 10,000
357 MPN/g requirement, 56 samples (total of 560 oysters divided into samples of 10 oysters
358 each) were analyzed after 14 days of relay and another 56 samples after 21 days of relay
359 (Table 3). Following FDA validation guidelines, for a process to be validated, no more
360 than 3 samples out of 30 (or no more than 10% of the samples) may fail (US FDA, 2015).
361 At day 14, 14 samples failed out of 56 (25%) and at day 21, 7 samples failed out of 56
362 (12.5%). When excluding the single oyster lot (low salinity site Trial 1) that required 28

363 days of relay to reach *V. vulnificus* densities < 30 MPN/g, only 4 samples out of 50 (8%)
364 failed after 21 days of relay (Table 4).
365
366 *3.3.* V. parahaemolyticus *densities*
367
368 Natural levels of *V. parahaemolyticus* at the sites of origin (pre-relay samples)
369 ranged from 94 MPN/g to 9,900 MPN/g and levels at Time 0 ranged from 880 MPN/g to
370 36,000 MPN/g (Fig. 2). Natural levels of *V. parahaemolyticus tdh* strains in pre-relay
371 samples were < 9 MPN/g (Fig. 2). At Time 0, as a result of temperature abuse, densities
372 of these strains increased up to 1,900 MPN/g in oysters originating from the moderate
373 salinity Site 2 during Relay 1. During Relays 2 and 3, levels of the *tdh* gene showed no or
374 only slight increases at Time 0 compared to the pre-relay values, with levels ≤ 16 MPN/g
375 at Time 0.
376 As with *V. vulnificus*, levels of *V. parahaemolyticus* varied significantly among
377 relay trials, site of origin and length of relay (Table 6); however the observed decrease in
378 *V. parahaemolyticus* after 14 days of relay compared to Time 0 was significant (Tukey's
379 HSD, $p < 0.001$) and consistent among relay trials (Fig. 2). Densities measured at day 14
380 and day 21 were not significantly different from each other and ranged from 65 to 1,600
381 MPN/g. Levels at these 2 relay time points were also significantly lower than the natural
382 levels (pre-relay) measured at each site of origin. A similar trend was observed for levels
383 of pathogenic *V. parahaemolyticus tdh* strains with levels at day 14 and day 21 of relay
384 not significantly different from each other, but significantly lower than the natural levels
385 measured at the site of origin prior to relay (Table 7). Overall, levels of *V.*

386 *parahaemolyticus tdh* strains observed during relay ranged between 2.3 MPN/g and 4.9
387 MPN/g (Fig. 2). When assessing the effect of site of origin, there was no significant
388 difference among sites on total *V. parahaemolyticus* levels but densities of *V.*
389 *parahaemolyticus tdh* strains were significantly higher at the moderate salinity Site 2 than
390 at the two other sites (Tukey's HSD, $p < 0.001$ for low salinity site; $p = 0.014$ for the
391 moderate salinity Site 1).
392
393 *3.4. Mortality*
394
395 Mortality levels were highly variable and varied significantly in relation to relay
396 trial, site of origin and length of relay (Table 8; Fig. 3). After 28 days of relay, mortality
397 levels in the oysters relayed ranged from 2 to 61% and were significantly higher (Tukey's
398 HSD, $p < 0.005$) than mortality levels observed in the controls left at the sites of origin
399 for the same length of time (0 to 22%). Mortality levels during Relay 2 were significantly
400 higher ($p < 0.006$) than those measured during relays 1 and 3. Mortality in Relay 3 was
401 also significantly lower than during Relay 1 (Tukey's HSD, $p = 0.009$). It was noted that
402 anoxic conditions were observed during Relay 2 owing to the cages being partially buried
403 in sediment as suggested by the presence of mud on top of the oysters at time of
404 collection. Finally, overall mortality levels in oysters originating from the moderate
405 salinity Site 1 were significantly lower ($p < 0.001$) than those measured in oysters
406 originating from the other two sites.
407
408 *3.5. Condition index*

409

410 Condition of the oysters was not assessed for the relayed oysters originating from
411 the low salinity site during Relay 1 due to high oyster mortality. The surviving oysters
412 were processed for *Vibrio* spp. densities as this was the main focus of this study. As
413 stated above, statistical analyses were conducted on data collected during Relay 2 and 3.
414 As observed with the bacterial densities and mortality, the oyster condition index varied
415 significantly in relation to relay trial, site of origin and length of relay (Table 9; Fig. 4).
416 The condition of the oysters that were relayed for 28 days trended slightly higher than
417 those that remained at their site of origin for the same length of time (Tukey's HSD, $p$ =
418 0.055); however, both of these treatments were associated with higher condition indices
419 than the Time 0 sample.

420

421

422 **4. Discussion**

423

424 The three relay trials conducted during this study demonstrated the effectiveness
425 of high salinity relay in reducing *V. vulnificus* levels in oysters to < 30 MPN/g in 14 to 28
426 days of exposure. Furthermore, the 3.52 log density reduction required by FDA PHP
427 validation guidelines (US FDA, 2015) was achieved for *V. vulnificus* in 21 days of
428 exposure for all lots associated with initial densities ≥ 10,000 MPN/g. The origin of the
429 oysters relayed, both in terms of grow-out method, salinity regime, or timeframe of the
430 relay (early, mid or late summer) did not affect the outcome of endpoints for *V. vulnificus*
431 densities, indicating that this method would be efficient in reducing *V. vulnificus* to safe

levels throughout the summer, a period associated with higher levels of vibriosis (Center for Disease Control and Prevention, 2017). Relay to high salinity waters was associated with a concomitant reduction in total and pathogenic levels of *V. parahaemolyticus* compared to the Time 0 controls, with levels ≤ 1,000 MPN/g and < 10 MPN/g, respectively, after 21 days of relay. The decrease in total *V. parahaemolyticus* was not as pronounced as for *V. vulnificus* similar to what was observed in previous studies (Audemard et al., 2011; Larsen et al., 2015; Parveen et al., 2017). Nonetheless, these levels, as well as those of pathogenic *V. parahaemolyticus tdh* strains, were significantly lower than the levels observed naturally in the oysters at the site of origin suggesting that high salinity relay can also reduce risks associated with *V. parahaemolyticus* in contaminated oysters.

Oyster condition during relay did not decrease compared to the oysters held at the site of origin suggesting that the quality and appearance of tissues of relayed oyster would not be negatively affected by relay. Such information is particularly relevant for oysters being sold raw as a half-shell product where such factors are key for marketing the product. Another key consideration is the potential for oyster mortality associated with relaying to high salinity sites. Mortality levels measured during this study ranged from 2 to 61% after 28 days of relay, but no consistent trends were observed between sites of origin or between relay experiments. The oysters were relayed directly to the high salinity site without the use of an acclimation site, so the influence of osmotic shock from the salinity shift cannot be excluded. Such wide ranges in mortality levels were not anticipated because of the low mortality seen in previous high salinity relays and high salinity depuration studies (Audemard et al., 2011; Larsen et al. 2013; Larsen et al., 2015;

455 Parveen et al. 2017). In those studies, oysters that were relayed or depurated in high
456 salinity waters without acclimation encountered similar salinity shifts as in the present
457 study (~15 psu shift), yet their mortality levels were low (< 7%), so we hypothesize that
458 other or additional interacting factors may be implicated in the observed mortality. First,
459 the time lag between the oyster collection from their site of origin and the deployment at
460 the relay site may account for some of the stress experienced by the oysters. Logistical
461 constrains were associated with collection of the oysters from 3 sites, transport to the
462 laboratory, temperature abuse, bagging, processing and transport to the relay site, limiting
463 our ability to collect and deploy the oysters on the same day. Second, the relay
464 conditions, such as the relay gear used and the relay site may not have been optimal
465 based on the sediment observed on top of oysters, especially during the second relay trial.
466 Third, the timeframe of the relay possibly played a role, particularly in relation to the
467 physiological condition of the oysters. Mortality in the early summer (Relays 1 and 2)
468 was significantly higher than in late summer to early fall (Relay 3). The summer period is
469 the season during which oysters undergo gonadal maturation and spawning while late
470 summer and early fall is a period during which spawning is completed and gonads are
471 resorbing. Although triploid oysters used in this study are partially sterile, they still
472 undergo some gonadogenesis (Allen and Downing, 1990) and may be more susceptible to
473 stress associated with relay during the summer than during the fall. Finally, triploid
474 oysters have been associated with mortalities of unknown causes in Virginia waters
475 during the summer (Matt and Allen, 2015) and our observations may also be part of this
476 larger unresolved issue surrounding triploid oysters. An accurate assessment of mortality
477 levels associated with high salinity relay will require studies conducted at different times

478 during the warm season using oysters that have not been temperature abused and using
479 optimal relay gear and siting.
480 Compared to previous studies assessing the effectiveness of high salinity relay,
481 this study was specifically designed to follow FDA guidelines regarding PHP validation
482 (Table 1; US FDA, 2015) and resulted in the analysis of a total of 36 samples (360
483 oysters total divided in groups of 10 oysters/sample) for initial assessment of *Vibrio* spp.
484 densities and 178 samples (1780 oysters total) during the actual relays. In some instances,
485 however, our approach differed from the FDA guidelines (Table 1; US FDA, 2015). First,
486 FDA guidelines recommend that processed samples be collected throughout the
487 processing day, which is appropriate for a controlled processing-plant approach. This
488 was, however, impractical during field relay trials, and the samples were collected all at
489 once. Second, FDA guidelines recommend that the initial vibrio densities be ≥ 10,000
490 MPN/g so a log reduction of 3.52 and non-detectable (< 30 MPN/g) levels following
491 processing can be demonstrated. Although most of the deployed oyster lots were
492 temperature abused to reach this value, surprisingly, for 3 out of 6 lots, initial densities
493 remained < 10,000 MPN/g even after the abuse. Based on FDA guidelines, these lots
494 could not be used to validate high salinity relay; however, as demonstrated in this study,
495 they still constitute additional data supporting the effectiveness of high salinity relay in
496 reducing *V. vulnificus* densities in oysters to < 30 MPN/g. Third, the analytical method
497 recommended by ISSC for detecting total *V. vulnificus* is a SYBR Green I qPCR assay
498 (ISSC, 2009; Wright et al., 2007). The primers used in this study were the same as those
499 used for the recommended method and they were used in combination with a Taqman
500 probe as described by Campbell and Wright (2003). The recommended SYBR Green I

501 assay and the Taqman assay used in this study were previously shown to perform
502 similarly both in terms of specificity and in terms of Ct values (i.e. the number of PCR
503 cycles necessary to reach threshold) associated with positive samples of *V. vulnificus*
504 (Wright et al., 2007). This suggests that the *V. vulnificus* results obtained during this
505 study using the Taqman assay should be similar to results that would be obtained using
506 the ISSC SYBR Green I assay. However, without a formal validation of the Taqman
507 assay under the same reaction conditions and with the same thermocycler used in this
508 study, our method did not technically meet FDA requirements. Finally, based on FDA
509 guidance, a process can be validated when no more than 3 processed samples out of 30 (<
510 10%) fail based on the initial AGM value and using the number of positive APW tubes
511 within either 0.1 g or 0.01 g per tube inocula (US FDA, 2015). When all 56 samples were
512 taken into account either after 14 or 21 days of relay, the number of failing samples
513 reached 12.5%, indicating that the relay time should be extended to 28 days.
514 High salinity relay differs from previously approved PHPs in that it is not a
515 controlled process. High salinity relay relies on the exposure of oysters to natural high
516 salinity waters for several weeks and, in this context, the identification of appropriate
517 relay sites would require additional studies to assess natural *Vibrio* spp. levels at each of
518 the potential relay sites. High and relatively stable salinities may be a key component in
519 selection of an appropriate high salinity relay site. In contrast to the relay site used in this
520 study which experienced salinities ranging from 31 to 33 psu, the relay sites used by
521 Parveen et al. (2017) had more variable salinity regimes with salinities ranging from 28.5
522 to 32.5 psu and from 24.5 to 30.7 psu, which may explain why validation following FDA
523 guidelines failed during their study. Although high salinity exposure appears to be

524 fundamental in decreasing *V. vulnificus* levels in oysters, the exact mode of action of high
525 salinity relay on *V. vulnificus* persistence still needs to be defined. Richards et al. (2012)
526 hypothesized that *Vibrio* predatory bacteria could play a major role in reducing *V.*
527 *vulnificus* abundance in oysters under elevated salinities. In this context, biological
528 factors influenced by high salinity and not high salinity per se may be implicated, which
529 may explain the lack of consistent results obtained during depuration experiments that
530 rely on artificial seawater (Larsen et al., 2013; Larsen et al., 2015; Parveen et al., 2017).
531 Further studies are needed to test these hypotheses in the context of in situ high salinity
532 relay.
533
534
535 **5. Conclusion**
536
537 The large number of oyster samples examined as well as the use of oysters of
538 different origins collected at different time points during the summer, contributed to a
539 more thorough investigation of high salinity relay as a PHP compared to previous studies
540 (Audemard et al., 2011; Motes et al., 1996; Parveen et al., 2017). Results of this study
541 suggested that high salinity relay should be considered as an additional method for
542 reducing *V. vulnificus* to levels safe for human consumption. Concomitant decrease in
543 total and pathogenic *V. parahaemolyticus* densities in relayed oysters was observed
544 suggesting additional benefit of such approach. Although oyster condition was preserved
545 during relays, the identification of the factors affecting oyster survival during high
546 salinity relay need to be examined. Future studies conducted in a more realistic setting for

547 an oyster grower, i.e. using oysters that were not abused and using optimal gear and site
548 selection for relays may be needed to fully address the potential of high salinity relay as
549 an economically viable PHP method.
550
551
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560
561
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**Figure legends**

Figure 1: Loads (AGM) of *V. vulnificus* (Vv) measured in lots of oysters originating from the low salinity site (Low), from the moderate salinity Site 1 (Mod.1) and from the moderate salinity Site 2 (Mod.2) prior to relay (Pre-relay), at Time 0 of relay (Day 0), and after 14 and 21 days of relay, as well as at 28 days of relay for the oysters originating from the Low salinity site during Relay 1. The 10,000 MPN/g and the 30 MPN/g levels are indicated by a dashed and dotted line, respectively. Error bars are standard deviations.

Figure 2: Loads (AGM) of total *V. parahaemolyticus* (Vp) and *tdh*-positive strains (Vp tdh) measured in lots of oysters originating from the low salinity site (Low), from the moderate salinity Site 1 (Mod.1) and from the moderate salinity Site 2 (Mod.2) prior to relay (Pre-relay), at Time 0 of relay (Day 0), and after 14 and 21 days of relay, as well as at 28 days of relay for the oysters originating from Low salinity site during Relay 1. The 10,000 MPN/g and the 30 MPN/g levels are indicated by a dashed and dotted line, respectively. Error bars are standard deviations.

Figure 3: Oyster mortality (mean and standard deviations) in relayed oysters in relation to oysters left at site of origin (Controls).

Figure 4: Oyster condition indices (mean and standard deviations) in relayed oysters in relation to oysters left at site of origin (Controls). Note that condition was not assessed for relayed oysters originating from the low salinity site in Relay 1, owing to high mortality.

Figure 1

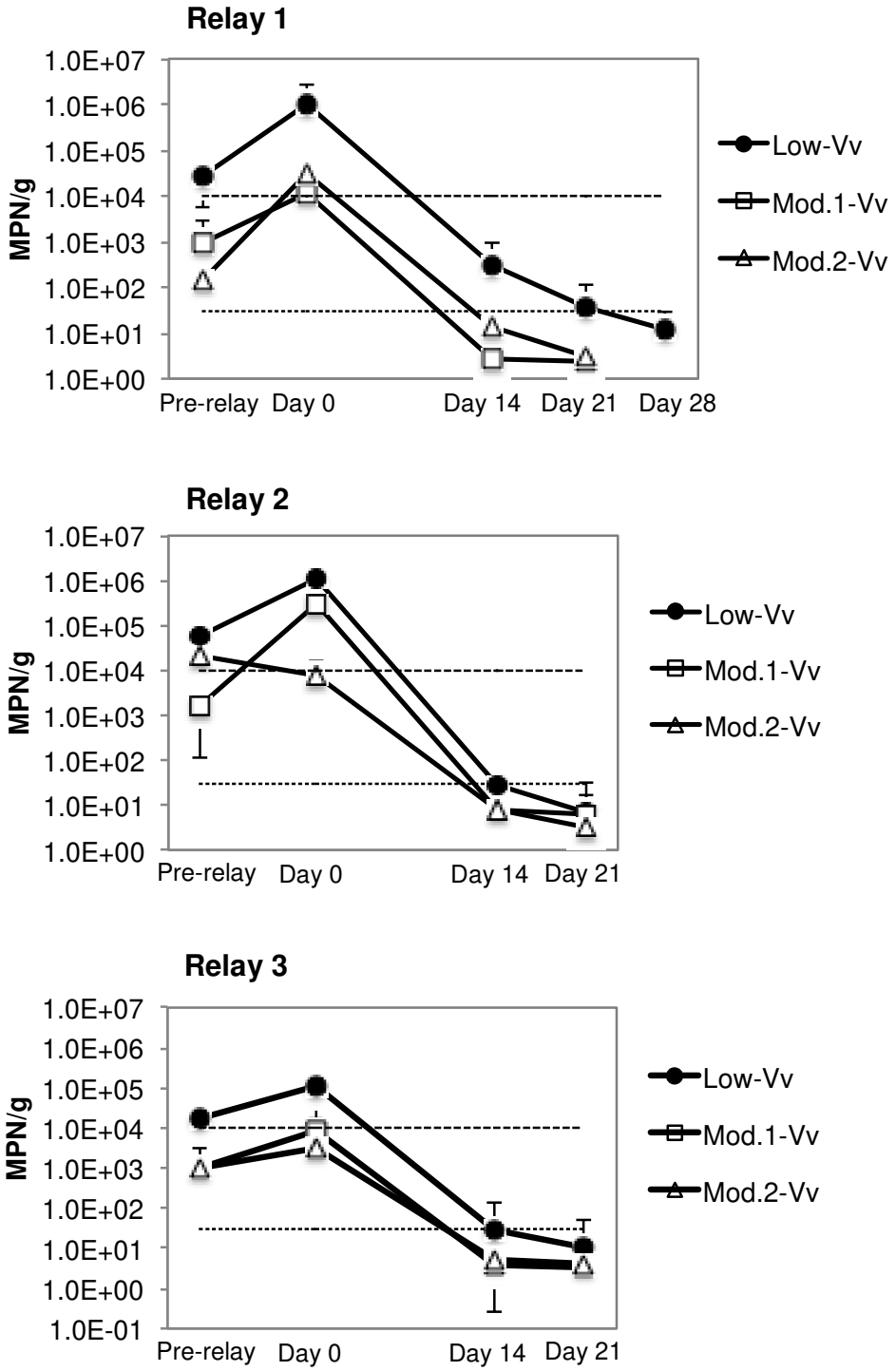
Relay 1
MPN/g
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
Pre-relay
Day 0
Day 14
Day 21
Day 28
Low-Vv
Mod.1-Vv
Mod.2-Vv
Relay 2
MPN/g
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
Pre-relay
Day 0
Day 14
Day 21
Low-Vv
Mod.1-Vv
Mod.2-Vv
Relay 3
MPN/g
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
1.0E-01
Pre-relay
Day 0
Day 14
Day 21
Low-Vv
Mod.1-Vv
Mod.2-Vv

Figure 2

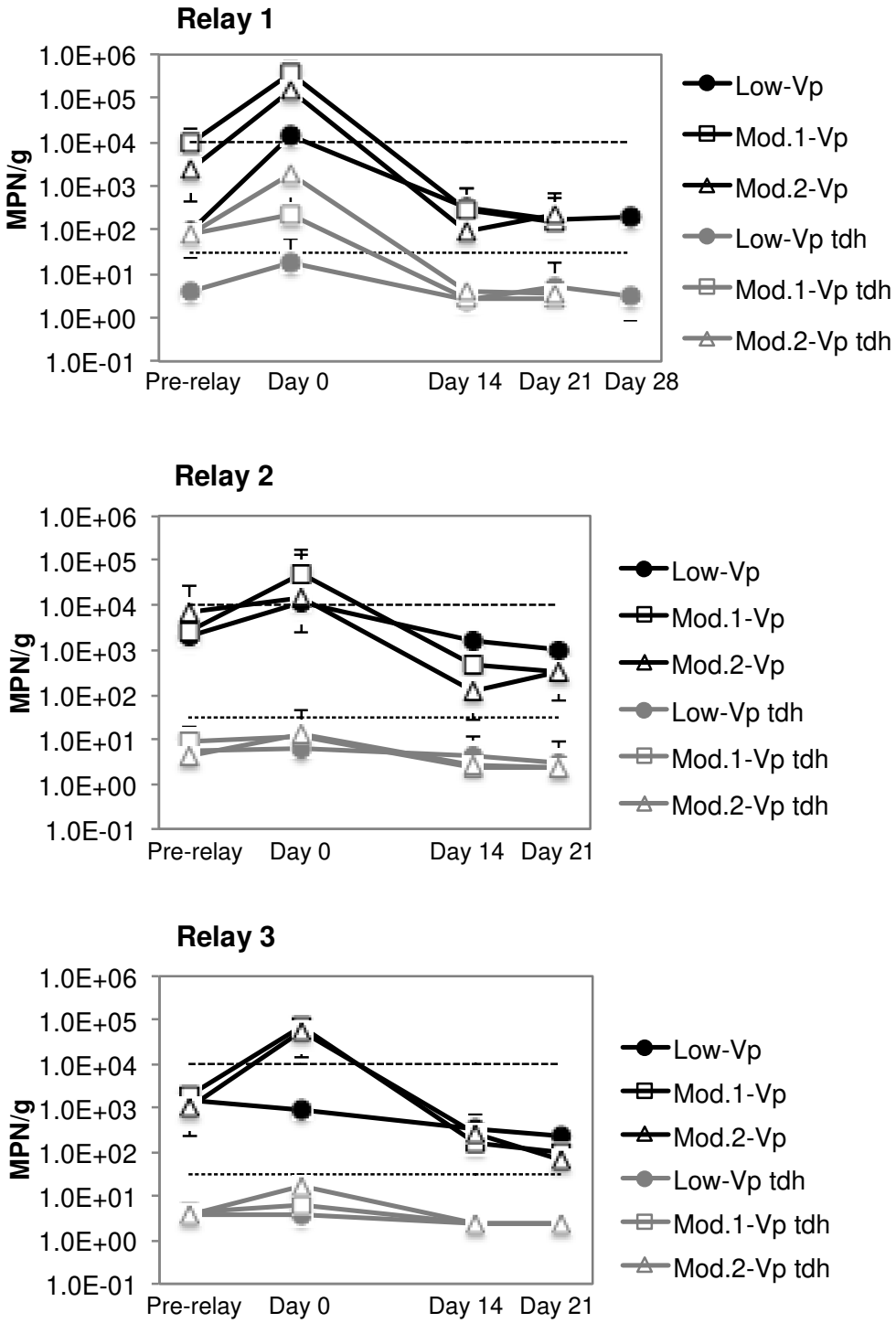
Relay 1
MPN/g
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
1.0E-01
Pre-relay
Day 0
Day 14
Day 21
Day 28
Low-Vp
Mod.1-Vp
Mod.2-Vp
Low-Vp tdh
Mod.1-Vp tdh
Mod.2-Vp tdh
Relay 2
MPN/g
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
1.0E-01
Pre-relay
Day 0
Day 14
Day 21
Low-Vp
Mod.1-Vp
Mod.2-Vp
Low-Vp tdh
Mod.1-Vp tdh
Mod.2-Vp tdh
Relay 3
MPN/g
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
1.0E-01
Pre-relay
Day 0
Day 14
Day 21
Low-Vp
Mod.1-Vp
Mod.2-Vp
Low-Vp tdh
Mod.1-Vp tdh
Mod.2-Vp tdh

Figure 3

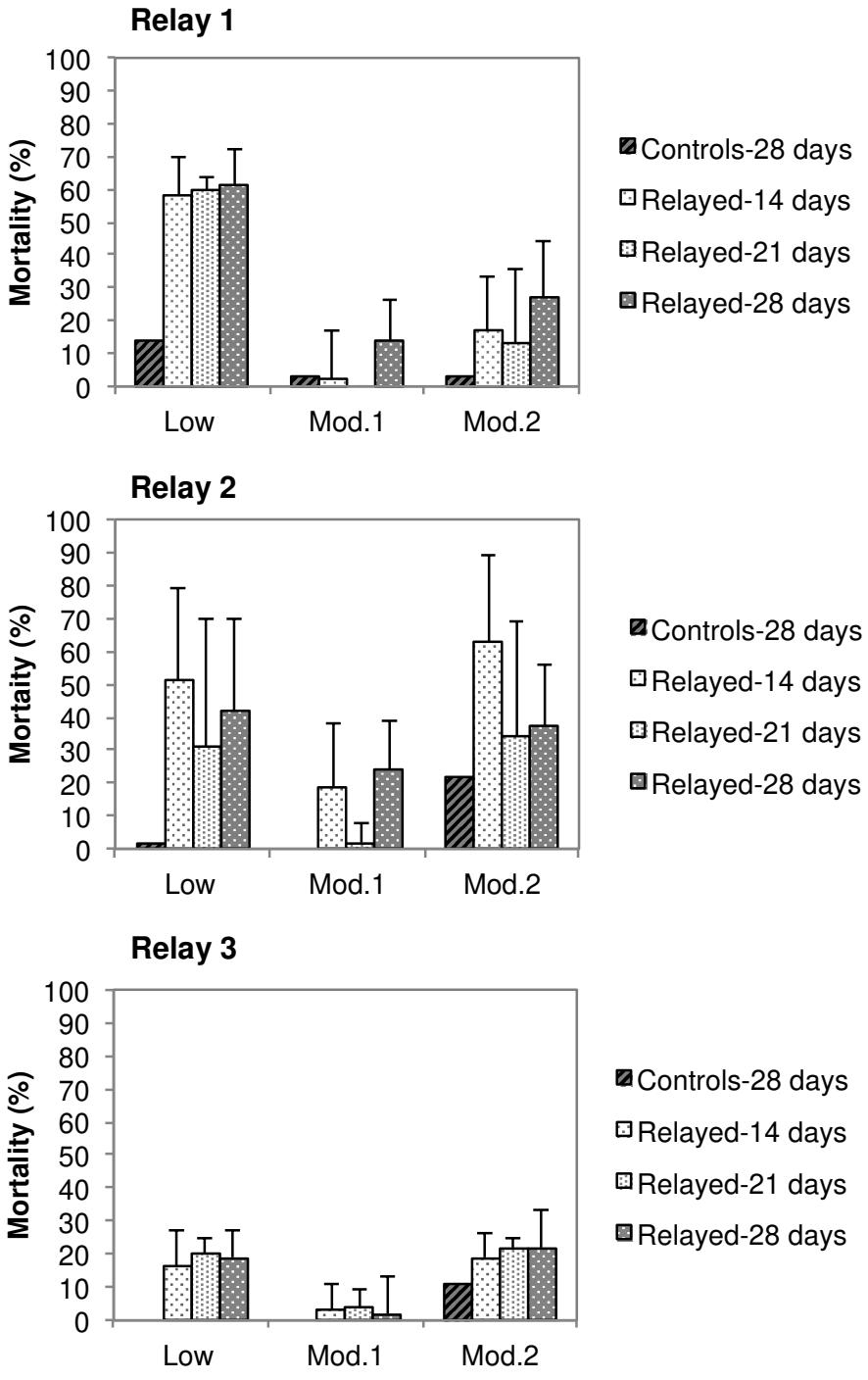
Relay 1
Mortality (%)
100
90
80
70
60
50
40
30
20
10
0
Low
Mod.1
Mod.2
Controls-28 days
Relayed-14 days
Relayed-21 days
Relayed-28 days
Relay 2
Mortaity (%)
Relay 3
Mortality (%)

Figure 4

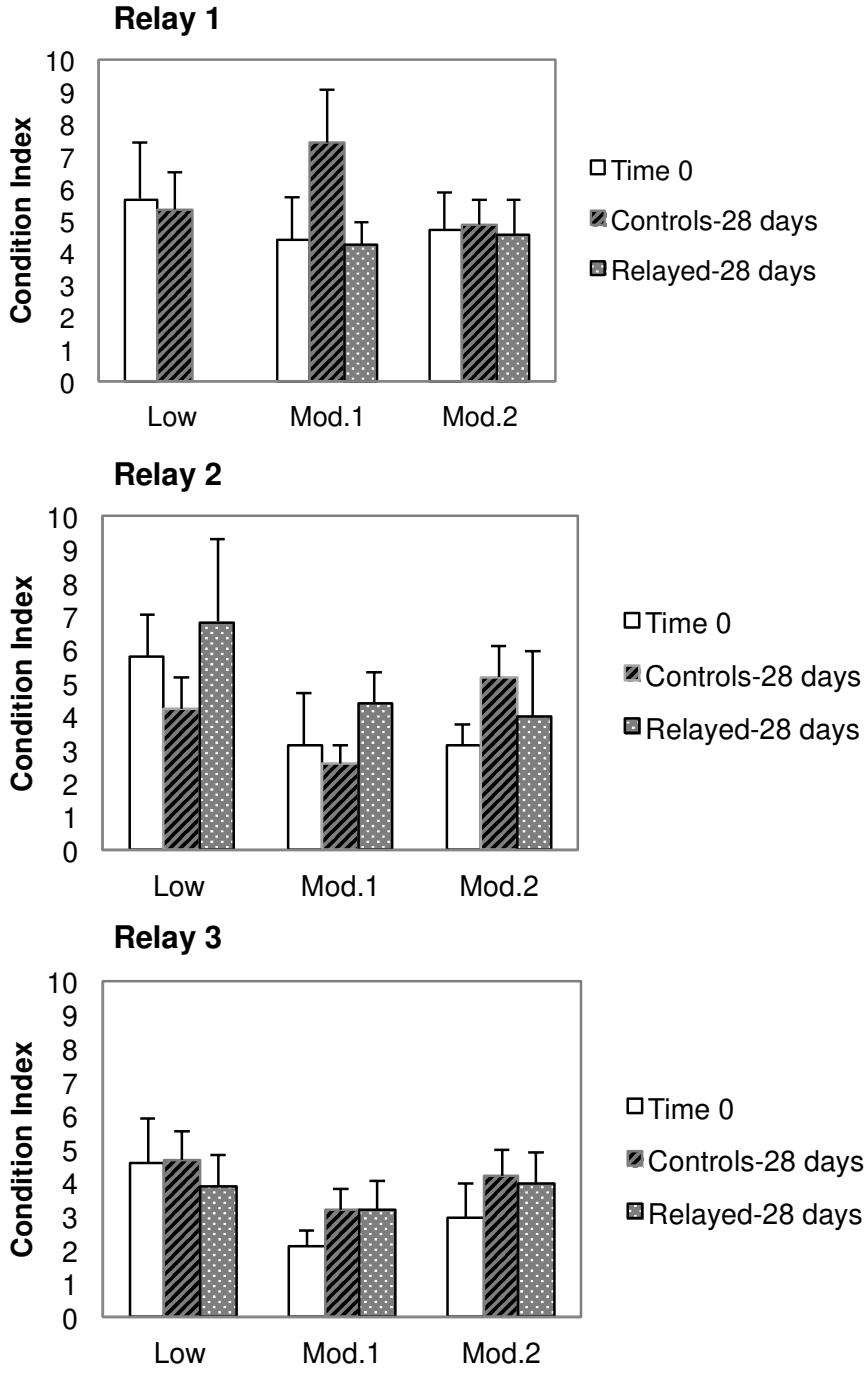
Relay 1
Condition Index
10
9
8
7
6
5
4
3
2
1
0
Low
Mod.1
Mod.2
Time 0
Controls-28 days
Relayed-28 days
Relay 2
Condition Index
10
9
8
7
6
5
4
3
2
1
0
Low
Mod.1
Mod.2
Time 0
Controls-28 days
Relayed-28 days
Relay 3
Condition Index
10
9
8
7
6
5
4
3
2
1
0
Low
Mod.1
Mod.2
Time 0
Controls-28 days
Relayed-28 days

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 ($1\times10^{-1}$ to $1\times10^{-6}$ 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 ($1\times10^{-2}$ or $1\times10^{-1}$ g) | Three dilutions 5-tube MPN ($1\times10^{-1}$ to $1\times10^{-3}$ 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 more than 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 |

Table 2: Sampling dates and environmental parameters recorded at the relay site for each relay trial.

| Trial # | Time Point | Date | Temperature (˚C) | Salinity (psu) |
|---|---|---|---|---|
| 1 | Day 0 | 6/5/13 | 18 | 32 |
| | Day 14 | 6/19/13 | 21 | 32 |
| | Day 21 | 6/26/13 | 26 | 32 |
| | Day 28 | 7/2/13 | 24 | 33 |
| 2 | Day 0 | 7/17/13 | 26 | 32 |
| | Day 14 | 7/31/13 | 26 | 33 |
| | Day 21 | 8/7/13 | 25 | No data |
| | Day 28 | 8/14/13 | No data | 32 |
| 3 | Day 0 | 8/28/13 | 26 | 31 |
| | Day 14 | 9/11/13 | 27 | 32 |
| | Day 21 | 9/18/13 | 21 | 32 |
| | Day 28 | 9/25/13 | 20 | 31 |

Table 3: *Vibrio vulnificus* initial loads and number of relay samples failing US FDA validation criteria (US FDA, 2015).

| Trial | Site of origin | Pre-relay sample | | Temperature abuse (hrs) | AGM at Day 0 (MPN/g) | # of samples failing/# of samples analyzed at each relay time point | | |
|---|---|---|---|---|---|---|---|---|
| | | Collection date | AGM (MPN/g) | | | Day 14 | Day 21 | Day 28[a] |
| 1 | Low[b] | 5/28/13 | 27,000 | 5 | 976,956 | 5/6 | 4/6 | 0/6 |
| | Mod. - 1 | 5/27/13 | 930 | 8 | 12,055 | 0/10 | 0/10 | nd |
| | Mod. - 2 | 5/28/13 | 140 | 8 | 31,419 | 3/10 | 0/10 | nd |
| 2 | Low | 7/8/13 | 58,000 | 0 | 1,149,946 | 0/10 | 1/10 | nd |
| | Mod. - 1 | 7/8/13 | 1600 | 4.5 | 303,482 | 4/10 | 2/10 | nd |
| | Mod. - 2 | 7/8/13 | 20,000 | 4.5 | 7,855 | na | na | nd |
| 3 | Low | 8/16/13 | 18,000 | 3 | 104,196 | 2/10 | 1/10 | nd |
| | Mod. - 1 | 8/16/13 | 1,000 | 6 | 8,355 | na | na | nd |
| | Mod. - 2 | 8/16/13 | 1,000 | 6 | 2,930 | na | na | nd |
| Overall # of samples failing/# samples analyzed (% failure) | | | | | | 14/56 (25%) | 7/56 (12.5%) | Assume 0% |

[a] In addition to measurement of *V. vulnificus* loads at Day 14 and 21 of relay, *V. vulnificus* loads were also measured at Day 28 of relay for the oysters originating from the low salinity site during Trial 1.

[b] For the oysters originating from the low salinity site during Trial 1, mortalities allowed for only 6 samples to be analyzed at each time point.

'na' stands for 'not applicable' in cases where the AGM at Time 0 was < 10,000 MPN/g.

'nd' stands for 'no *Vibrio* spp. data' in cases where the samples were not analyzed for *Vibrio* spp. abundance, i.e., for all the samples collected at Week 4, except for the Low salinity site oysters during Trial 1.

Table 4: Change in AGM for *Vibrio vulnificus* levels during the 3 relay trials. AGM levels < 30 MPN/g and log reductions ≥ 3.52 required for PHP validation are in **bold**.

| Trial | Site of origin | AGM (MPN/g) | | | Log reduction | | |
|---|---|---|---|---|---|---|---|
| | | Day 14 | Day 21 | Day 28 | Day 14 | Day 21 | Day 28 |
| 1 | Low | 330.0 | 39 | **12** | 3.47 | **4.40** | **4.91** |
| 1 | Mod. - 1 | **3** | **2** | nd | **3.62** | **3.70** | nd |
| 1 | Mod. - 2 | **14** | **3** | nd | 3.35 | **4.01** | nd |
| 2 | Low | **28** | **7** | nd | **4.59** | **5.20** | nd |
| 2 | Mod. - 1 | **8** | **6** | nd | **4.55** | **4.67** | nd |
| 2 | Mod. - 2 [a] | **8** | **3** | nd | 3.02 | 3.39 | nd |
| 3 | Low | 31 | **10** | nd | **3.51** | **4.00** | nd |
| 3 | Mod. - 1 [a] | **4** | **3** | nd | 3.33 | 3.41 | nd |
| 3 | Mod. - 2 [a] | **6** | **4** | nd | 2.72 | 2.85 | nd |

[a] These oyster lots were associated with initial *V. vulnificus* at time of relay < 10,000 MPN/g.

'nd' stands for not determined indicating that samples were not processed for that particular time point.

Table 5: ANOVA for *Vibrio vulnificus* load in relation to relay trial, site of origin, and length of relay. Data collected during Day 28 of relay for Trial 1 were excluded from the analysis. In this case, the independent variable 'Treatment' included the Pre-relay, Day 0, Day 14 and Day 21 of relay.

| Source | Sum of Squares | Degree of freedom | Mean Square | F | *p* value |
|---|---|---|---|---|---|
| Corrected Model | 647.240 | 35 | 18.493 | 97.757 | <0.001 |
| Intercept | 1167.501 | 1 | 1167.501 | 6171.735 | <0.001 |
| Relay trial | 7.144 | 2 | 3.572 | 18.883 | <0.001 |
| Site of origin | 54.264 | 2 | 27.132 | 143.428 | <0.001 |
| Treatment | 540.122 | 3 | 180.041 | 951.745 | <0.001 |
| Relay trial * Site of origin | 5.943 | 4 | 1.486 | 7.854 | <0.001 |
| Relay trial * Treatment | 10.270 | 6 | 1.712 | 9.048 | <0.001 |
| Site of origin * Treatment | 8.496 | 6 | 1.416 | 7.486 | <0.001 |
| Relay trial * Site of origin * Treatment | 9.119 | 12 | 0.760 | 4.017 | <0.001 |
| Error | 38.401 | 203 | 0.189 | | |
| Total | 1421.349 | 239 | | | |

Table 6: ANOVA for *Vibrio parahaemolyticus* load in relation to relay trial, site of origin, and treatment period. The data collected during Day 28 of relay during Trial 1 were excluded from the analysis. In this case, the independent variable 'Treatment' included the Pre-relay, Day 0, Day 14 and Day 21 of relay.

| Source | Sum of Squares | Degree of freedom | Mean Square | F | *p* value |
|---|---|---|---|---|---|
| Corrected Model | 187.025 | 35 | 5.344 | 28.479 | <0.001 |
| Intercept | 1802.544 | 1 | 1802.544 | 9606.919 | <0.001 |
| Relay trial | 4.187 | 2 | 2.094 | 11.159 | <0.001 |
| Site of origin | 4.452 | 2 | 2.226 | 11.864 | <0.001 |
| Treatment | 138.580 | 3 | 46.193 | 246.194 | <0.001 |
| Relay trial * Site of origin | 5.008 | 4 | 1.252 | 6.672 | <0.001 |
| Relay trial * Treatment | 6.906 | 6 | 1.151 | 6.134 | <0.001 |
| Site of origin * Treatment | 20.017 | 6 | 3.336 | 17.780 | <0.001 |
| Relay trial * Site of origin * Treatment | 8.305 | 12 | 0.692 | 3.688 | <0.001 |
| Error | 38.089 | 203 | 0.188 | | |
| Total | 1998.334 | 239 | | | |

Table 7: ANOVA for *Vibrio parahaemolyticus tdh* strains in relation to relay trial, site of origin, and treatment period. The data collected during Day 28 of relay during Trial 1 were excluded from the analysis. In this case, the independent variable 'Treatment' included the Pre-relay, Day 0, Day 14 and Day 21 of relay.

| Source | Sum of Squares | Degree of freedom | Mean Square | F | *p* value |
|---|---|---|---|---|---|
| Corrected Model | 64.867 | 35 | 1.853 | 29.855 | <0.001 |
| Intercept | 93.496 | 1 | 93.496 | 1506.096 | <0.001 |
| Relay trial | 14.878 | 2 | 7.439 | 119.831 | <0.001 |
| Site of origin | 3.628 | 2 | 1.814 | 29.220 | <0.001 |
| Treatment | 28.753 | 3 | 9.584 | 154.390 | <0.001 |
| Relay trial * Site of origin | 4.853 | 4 | 1.213 | 19.544 | <0.001 |
| Relay trial * Treatment | 11.266 | 6 | 1.878 | 30.248 | <0.001 |
| Site of origin * Treatment | 6.791 | 6 | 1.132 | 18.234 | <0.001 |
| Relay trial * Site of origin * Treatment | 4.535 | 12 | 0.378 | 6.087 | <0.001 |
| Error | 12.602 | 203 | 0.062 | | |
| Total | 147.293 | 239 | | | |

Table 8: ANOVA for oyster mortality in relation to relay trial, site of origin, and treatment period. The independent variable 'Treatment' included the 14, 21, 28 days of relay and the controls left at the site of origin.

| Source | Sum of Squares | Degree of freedom | Mean Square | F | *p* value |
|---|---|---|---|---|---|
| Corrected Model | 24.662 | 35 | 0.705 | 13.447 | <0.001 |
| Intercept | 41.851 | 1 | 41.851 | 798.636 | <0.001 |
| Relay trial | 0.944 | 2 | 0.472 | 9.010 | <0.001 |
| Site of origin | 10.456 | 2 | 5.228 | 99.767 | <0.001 |
| Treatment | 2.414 | 3 | 0.805 | 15.354 | <0.001 |
| Relay trial * Site of origin | 2.787 | 4 | 0.697 | 13.298 | <0.001 |
| Relay trial * Treatment | 1.432 | 6 | 0.239 | 4.555 | <0.001 |
| Site of origin * Treatment | 0.584 | 6 | 0.097 | 1.857 | <0.001 |
| Relay trial * Site of origin * Treatment | 1.177 | 12 | 0.098 | 1.871 | <0.001 |
| Error | 15.878 | 303 | 0.052 | | |
| Total | 101.458 | 339 | | | |

Table 9: ANOVA for oyster condition index in relation to relay trial (Trials 2 and 3), site of origin and treatment period. Data collected during Trial 1 were excluded from the analysis due to high mortality in this particular relay trial. The independent variable 'Treatment' included the Day 0 at the site of origin prior to relay, 28 days at the site of origin (control) and 28 days at the relay site.

| Source | Sum of Squares | Degree of freedom | Mean Square | F | *p* value |
|---|---|---|---|---|---|
| Corrected Model | 34.652 | 17 | 2.038 | 26.605 | <0.001 |
| Intercept | 1728.932 | 1 | 1728.932 | 22566.254 | <0.001 |
| Relay trial | 3.033 | 1 | 3.033 | 39.583 | <0.001 |
| Site of origin | 16.938 | 2 | 8.469 | 110.540 | <0.001 |
| Treatment | 2.872 | 2 | 1.436 | 18.745 | <0.001 |
| Relay trial * Site of origin | 0.530 | 2 | 0.265 | 3.461 | <0.001 |
| Relay trial * Treatment | 2.086 | 2 | 1.043 | 13.611 | <0.001 |
| Site of origin * Treatment | 5.594 | 4 | 1.399 | 18.254 | <0.001 |
| Relay trial * Site of origin * Treatment | 3.527 | 4 | 0.882 | 11.509 | <0.001 |
| Error | 33.021 | 431 | 0.077 | | |
| Total | 1796.260 | 449 | | | |