Fishery Resource Grant Program

"Method and concept evaluation of relaying oysters from Chesapeake Bay to high salinity water as a post-harvest-process to reduce *Vibrio vulnificus* to undetectable levels"

Final Report

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Abstract

Post-harvest processing (PHP) methods for reducing vibrio levels that have been developed thus far are either capital intensive, have limited throughput, are not readily available, and/or are generally very expensive. In the summer of 2010 we initiated a small-scale evaluation to assess the effectiveness of relay to elevated salinity as a PHP strategy to reduce levels of *Vibrio vulnificus* in *Crassostrea virginica* oysters. This is potentially a relatively inexpensive PHP that could be used by local industry for shellfish harvested from Virginia waters. Industry members and VIMS scientists collaborated to conduct two experiments, one in August/September and the other in September/October, which is within the 2010 risk season as defined by US Food and Drug Administration (FDA). Three grow-out sites located in the Chesapeake Bay were chosen as the source of oysters to be relayed to the higher salinity; the Coan River (10-13 psu), the York River (18-22 psu) and Nassawadox Creek (20-25 psu). The relay site was Sandy Island located on the seaside of Virginia Eastern Shore (30-34 psu). Oysters were deployed in on-bottom cages and four to five replicate bags of 15 oysters/bag were collected for analyses at 0- (initial *V. vulnificus* baseline prior to deployment), 7-, and 14-day intervals after exposure to high salinities. Homogenates consisting of 10-12 oysters were prepared from each bag. A real-time quantitative polymerase chain reaction (qPCR) assay was used to detect *V. vulnificus* DNA in the positive alkaline peptone water (APW) tubes to determine the most probable number (MPN) for each sample. The results clearly showed that high salinity relay is a potentially viable method to reduce *V. vulnificus* in oysters grown and harvested in Virginia. The highest initial *V. vulnificus* density measured was a qPCR MPN of 750 g^{-1} (geometric mean of 160 g^{-1}). After 14 days the geometric mean *V. vulnificus* densities were all <1 g^{-1} oyster meats independent of site or experiment. In addition, the direct transfer to high salinity was associated with low mortality levels (< 5%) even for the Coan River oysters, which experienced the largest salinity differential (ca. 20 psu).

Project Background (Justification, Objectives and Methods Employed)

Last year FDA announced its intention to implement guidance that would require elimination of *Vibrio vulnificus* from raw shellfish harvested from Gulf of Mexico waters and sold during the months of April through October (http://www.fda.gov/NewsEvents/Speeches/ucm187014.htm). FDA asserts that post-harvest-processing (PHP) options now available have reached a level of maturity to facilitate this requirement and it intends to change the "Fish and Fishery Products

Hazards and Controls Guidance" such that PHPs will be required for the 2011 risk season for oysters harvested from Gulf waters.

Although shellfish from the Gulf of Mexico have been primarily associated with cases of *V. vulnificus,* it appears likely this change in FDA regulations will also eventually affect the shellfish industry in other regions. PHP methods thus far developed are either capital intensive, have limited throughput, are/or are not readily available. In addition, consumer responses to treated products are not always favorable. One relatively unexplored PHP that has not been fully evaluated is the controlled relay of shellfish to high salinity waters. Several investigators have shown through limited data that high salinities (ca. 35 psu) do not favor *V. vulnificus* persistence and its levels can drop to undetectable values in oysters relayed to high salinity waters (Motes and DePaola 1996). Motes and DePaola (1996) also reported relatively low mortalities associated with relay using an intermediate acclimation salinity before transfer to a high salinity offshore relay site. To the best of our knowledge there are no published data describing mortality rates associated with the salinity changes for the proposed PHP. High salinity relay for *V. vulnificus* reduction, moreover, has not been used or evaluated in Virginia and preliminary data are needed to support a full-scale validation of this process. In order to address the question of whether high salinity relay will be an effective and affordable PHP method for Virginia oysters, members of the Virginia shellfish industry (Gallivan, Erskine and Leggett) collaborated with VIMS scientists (Kator and Reece) to conduct two experiments during the 2010 risk season as defined by US Food and Drug Administration (FDA).

Coincident with this study of the effectiveness of high salinity relay as a PHP method was the evaluation of applying a real-time quantitative polymerase chain reaction (qPCR) assay to the positive alkaline peptone water (APW) tubes in order to replace an older and now unavailable immunoassay-based most probable number (MPN) method (DePaola *et al.* 1997, Wright *et al.* 1993). Real-time qPCR is a technique that can be highly specific and rapid, enabling multiple analyses to be done in a relatively short time period. For this work we evaluated the primers and probe described by Campbell and Wright (2003).

The specific objectives of this study were:

1. To evaluate a molecular assay for rapid detection of *Vibrio vulnificus* that can be used with the ISSC/FDA PHP Validation/Verification MPN-based protocol (NSSP 2007).

2. To conduct two fact-finding relay experiments to address the absence of data describing the feasibility and effectiveness of a high salinity relay post-harvest processing method (PHP) for reducing *V. vulnificus* levels in oysters harvested from Virginia waters.

Methods, Data Collection and Analysis

Study sites. Three grow-out sites of varying salinities located in the Chesapeake Bay were chosen as the source of oysters to be relayed to the high salinity site. The low salinity site was in the Coan River and the York River and Nassawadox Creek sites were both moderate salinity sites. The relay site was in the Little Machipongo Inlet behind Sandy Island (37 deg 29' 27.17"N, 75 deg 41'27.69" W) located on the seaside of the Virginia Eastern Shore (Fig. 1).

Salinity was recorded at each sampling time point using a CTD sonde (YSI Inc). In addition, water temperature was continuously recorded at each site using a in situ temperature logger (HOBO, Onset, Inc.).

First Relay Experiment. The first relay experiment began on August 18, 2010. Approximately 200 oysters from each grow-out site were labeled and placed in mesh grow-out bags (15 oysters/bag). Coan and York River oysters were collected on August 17 and maintained at 10°C overnight and transported the following day to the relay site in insulated coolers. Nassawadox Creek oysters were collected, bagged, transported and deployed on the same day. All the oysters were deployed on 18 August at Sandy Island in on-bottom cages (Fig. 2). Four to five replicate bags of oysters from each of the salinity sites were collected for analyses at 0- (initial *V. vulnificus* baseline prior to deployment), 7-, and 14-day intervals after exposure to high salinities. Mortalities in each bag sampled were recorded on days 7 and 14.

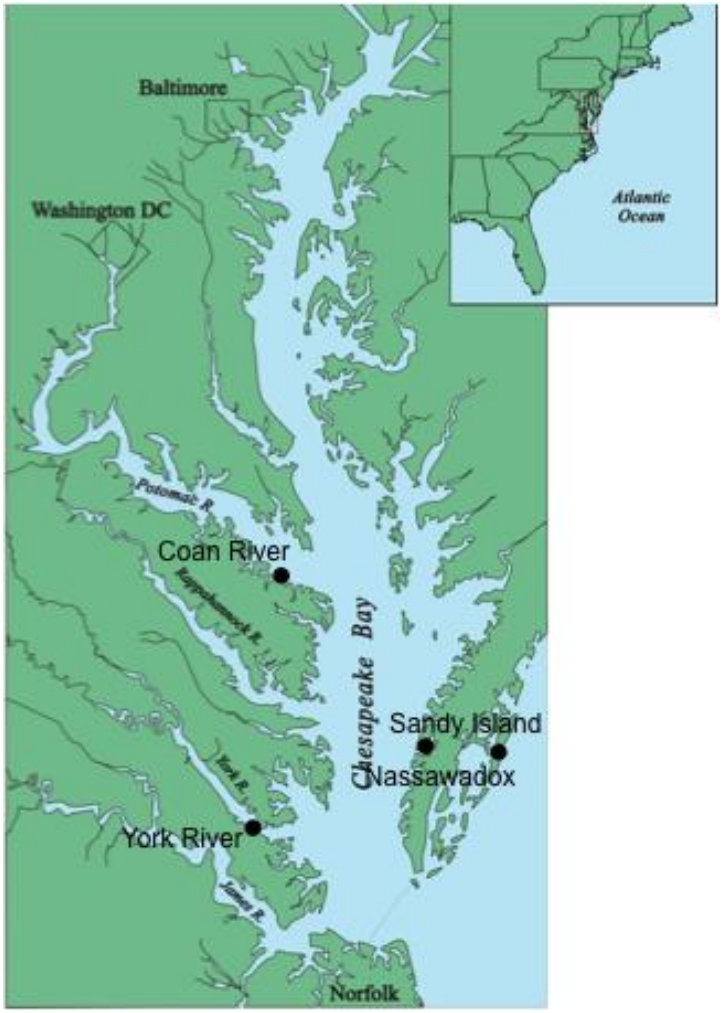

Figure 1: Location of the study sites.

Figure 2: On-bottom containers.

Sample processing. Oysters were transported from the relay site in insulated coolers and maintained at 10°C until processing. Homogenates consisting of 10-12 oysters were prepared following approved procedures (APHA 1970) and enriched as described in Chapter 9, "Vibrio" of the most recent online version of the FDA Bacteriological Analytical Manual (BAM)

(http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/UCM070830). All presumptive positive MPN tubes showing growth at zero time and during relay were assayed using qPCR (Campbell and Wright, 2003). In addition, samples from the surface of 30 randomly selected positive tubes were streaked on CC agar and yellow colonies were sub-cultured for future biochemical confirmation as *V. vulnificus* and testing by qPCR with sequence verification.

Second Relay Experiment. During the second experiment, our intent was to expose some oysters overnight from each site to warm room temperatures to increase initial *V. vulnificus* burdens prior to relay. For the Coan and York River sites, a group of oysters were exposed to 21°C (room temperature) while the rest were kept at 10°C overnight. For Nassawadox Creek, some of the oysters were left at air temperature overnight on a dock while the rest were kept in water on site. Oysters were then deployed on 21 September at Sandy Island as for the first experiment. At time 0 and at 14 days, 3 bags of the temperature abused and 2 bags of the non-abused were sampled for each oyster origin. At day 7, 2 bags of each treatment from each origin site were sampled.

Results

Site Salinities. Salinity was the lowest at the Coan River site (14-15 psu), while both York River and Nassawadox sites were moderate salinity sites (22-25 psu). Salinities measured at Sandy Island remained higher than 30 psu (30-32 psu) during the course of this study

Water temperatures at the grow-out sites prior to relay. Prior to the first trial, average temperatures over 14 days were 29°C at the Coan River, 28°C at the York River and 25°C at Nassawadox Creek (Fig 3). Daily variation in temperature was more pronounced at Nassawadox than at the two other sites.

For the second trial, temperatures were more uniform among the grow-out sites prior to relay. Average temperature over a 14 day period prior to deployment was 24°C at the Coan River, 25°C at the York River and 24°C at Nassawadox. Daily temperature variation observed at Nassawadox was less pronounced than prior to the first trial (Fig. 3).

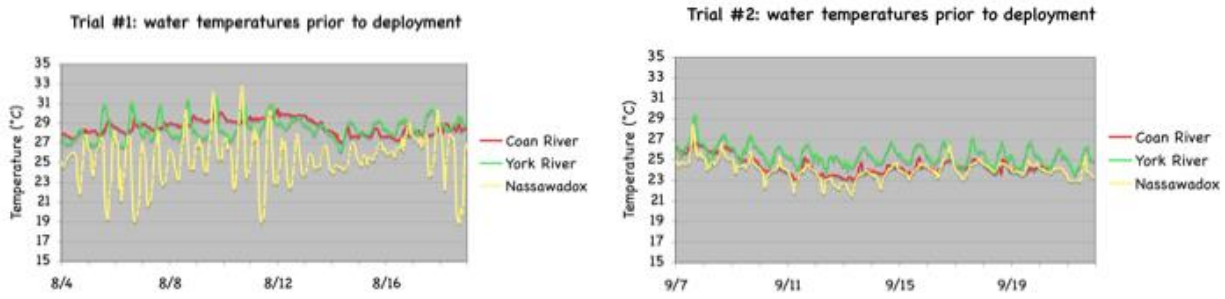

Figure 3: Water temperatures recorded over 14 days at each grow-out site prior to relay to the high salinity site.

Water temperatures at the relay site. Average water temperature at the Sandy Island site during the 14 days of the first experiment was 26°C while it was 23°C for the second experiment. A seasonal decrease in temperature was clearly observed during the second experiment with temperatures reaching 17°C at the end of the 14 day period (Fig. 4).

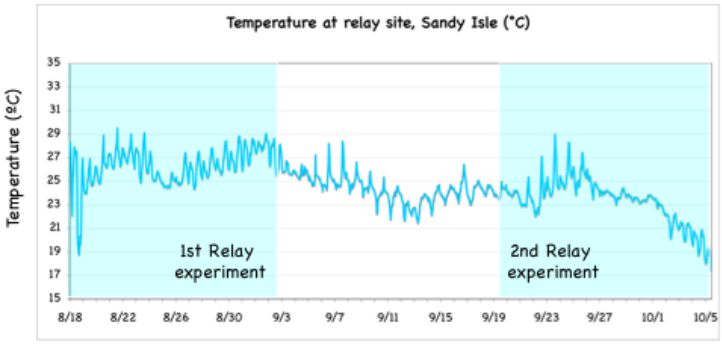

Figure 4: Water temperatures recorded at the relay site, Sandy Island.

***Vibrio vulnificus* densities.** One goal during the second relay experiment was to be able to test whether we could achieve a 3.52 log reduction in *V. vulnificus* densities to a final MPN of <30 *V. vulnificus* g^{-1} as recommended by ISSC PHP guidance (NSSP 2007). However, we were unable to achieve a sufficiently high initial density in order to test this level of reduction. Even the overnight exposure to room temperature of a subset of oysters from each growing area during the second experiment was insufficient to significantly increase initial *V. vulnificus* concentrations. The highest initial *V. vulnificus* density measured was a qPCR MPN of 750 g^{-1} (geometric mean of 160 g^{-1}) observed in Coan River oysters during the first experiment. Initial densities observed for the two other sites during the first experiment and at all sites during the second experiment were lower. After 14 days geometric mean *V. vulnificus* densities were all <1 qPCR MPN g^{-1} oyster meats independent of site or experiment (Table 1; Fig. 5).

Oyster mortalities. Mortalities were low overall for both experiments with the highest mortality rate being only 4% (6/150) for the Coan River oysters after 14 days of exposure to the high salinity during trial #1 (Fig. 5).

Table 1. *Vibrio vulnificus* densities measured in *Crassostrea virginica* samples harvested from Coan River (14 psu), York River (22 psu), and Nassawadox (23 psu) growing areas and relayed to a site on the Virginia eastern shore (Sandy Island, 32 psu) for 14 days. Results are shown for two trial experiments that occurred from 8-17-10 through 9-1-10 and 9-20-10 through 10-5-10. Average water temperatures at the relay site for these experiments were 26°C and 23°C, respectively. Each MPN value is from an independent sample of 10-12 oysters.

Trial 1	*Vibrio vulnificus* qPCR MPN g^{-1}		
Relay Day	Coan	York	Nassawadox
0	240	24	23
	64	93	38
	240	240	290
	750	23	23
GeoMean	**229.3**	**59.2**	**49.1**
7	<0.3	0.92	0.36
	4.3	<0.3	<0.3
	4.3	<0.3	<0.3
	9.3	0.36	0.36
GeoMean	**<2.7**	**<0.4**	**<0.3**
14	0.92	0.74	1.5
	<0.3	0.3	<0.3
	<0.3	<0.3	<0.3
	<0.3	<0.3	0.36
GeoMean	**<0.4**	**<0.4**	**<0.5**

Trial 2	*Vibrio vulnificus* qPCR MPN g^{-1}		
Relay Day	Coan	York	Nassawadox
0	75	23	4.3
	460	38	4.3
	750	43	1.1
	43	75	9.3
	93	4.3	23
GeoMean	**159.6**	**26.1**	**5.3**
7	1.5	<0.3	<0.3
	1.1	0.36	0.36
	24	<0.3	<0.3
	2.1	<0.3	0.74
GeoMean	**3.0**	**<0.3**	**<0.4**
14	0.3	<0.3	<0.3
	0.92	<0.3	<0.3
	<0.3	0.36	<0.3
	4.3	<0.3	<0.3
	<0.3	<0.3	<0.3
GeoMean	**<0.6**	**<0.3**	**<0.3**

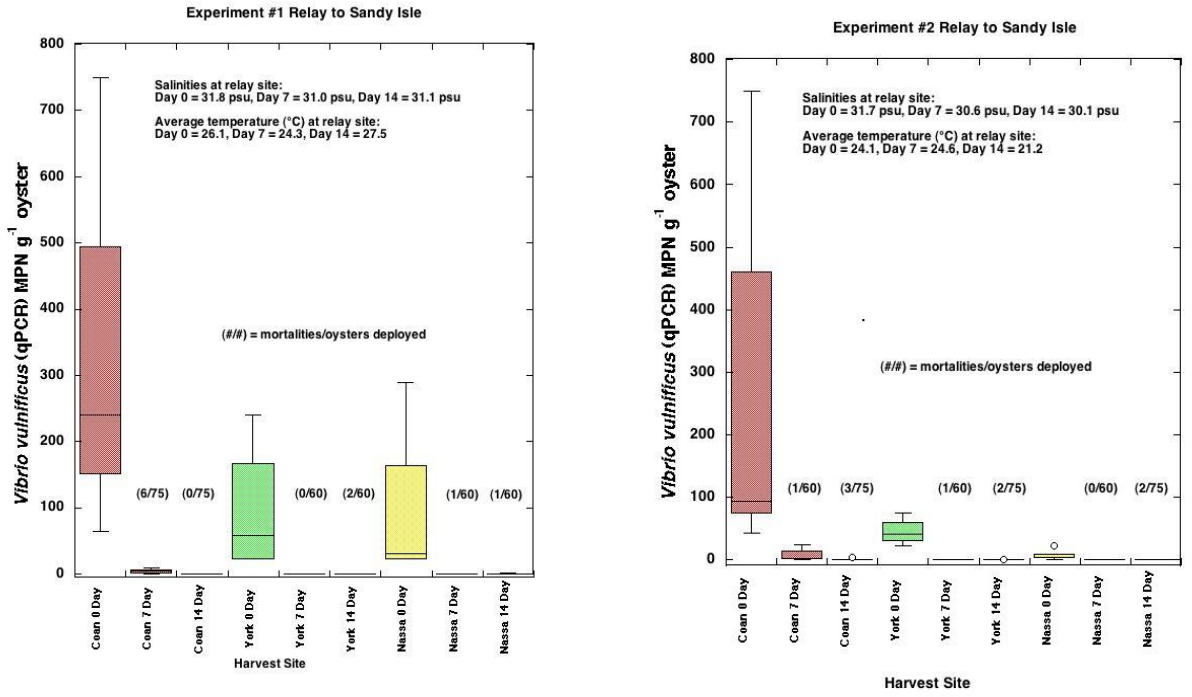

Figure 5: Box plots of *Vibrio vulnificus* qPCR MPN g^{-1} values from data shown in Table 1. The solid line in the center of each box represents the median value while the 25th and 75th percentile values are the lower and upper margins, respectively. Box plots are an easy way to compare *V. vulnificus* burdens as a function of growing area. The lowest salinity growing area consistently exhibited the highest MPN values.

BIOLOG testing. Approximately 20 yellow colonies from CC agar obtained from randomly selected growth positive MPN-tubes were purified and tested using the BIOLOG^{TM} GEN III Microbial Identification System following the manufacturer's protocols. The results are shown for those isolates that either yielded a positive ID or for which the software suggested a "closest species." "Closest species" is not an ID but is differentiated from "no ID" where the former can have some validity. Results are shown in Table 2. Three *V. vulnificus* cultures identified as such by BIOLOG^{TM} were also "confirmed" by qPCR. None of the 5 isolates identified by BIOLOG^{TM} as other vibrio species were positive by qPCR. Two isolates that were not identified by BIOLOG^{TM} were positive by qPCR and one of these was a *V.vulnificus* "closest species." Although, these results are based on a very small data set they suggest false positives by qPCR were not a problem for isolates definitively identified by BIOLOG^{TM}. Although the BIOLOG^{TM} database is large, environmental strains of marine bacteria may not be definitively identified. Results also suggest that the "yellow" colony criterion may not always be indicative of *V. vulnificus* occurrence on CC agar.

Table 2. Comparison of BIOLOG™ and qPCR analyses of selected isolates from growth-positive MPN tubes.

Isolate number	Source	Date	Time	Tube dilution	Isolate Code	Growth on blood agar	Biolog ID	Biolog "closest species"	qPCR result
6	Coan	9/2/10	14 day	(-1)		heavy	***V. alginolyticus***		–
7	Coan	9/2/10	14 day	(-1)		heavy	***V. parahaemolyticus***		–
9	Nassawadox	8/19/10	0 day	(-1)		heavy	***V. alginolyticus***		–
14	York	8/19/10	0 day	(-6)		heavy	***V. alginolyticus***		–
19	York	9/21/10	0 day	(-1)	147	heavy	***V. vulnificus***		+
20	Nassawadox	9/21/10	0 day	(-1)	114	heavy	***Vibrio* spp.**	*V. vulnificus*	–
26	Coan	10/5/10	14 day	(-1)	258	poor	***V. alginolyticus***		–
30	Coan	9/21/10	0 day	(-1)	84	heavy	***V. vulnificus***		+
28	ATCC					heavy	***V. vulnificus*** (ATCC 27562	Not run	+
16	York	8/19/10	0 day	(0)		heavy	***Vibrio* spp.**	*V. harveyi*	+
10	Nassawadox	8/19/10	0 day	(-3)		heavy	No ID	*V. vulnificus*	+

Continuing work. We are currently running qPCR assays on the same samples that were used for this study to examine the effect of high salinity relay on *V. parahaemolyticus* densities in oysters.

Conclusions

Results clearly showed that high salinity relay is a potentially viable method to reduce *V. vulnificus* in oysters grown and harvested in Virginia. In addition, the direct transfer to high salinity was associated with low mortality levels even for the Coan River oysters which experienced the largest salinity differential (ca. 20 psu). The qPCR analysis of DNA isolated from the positive APW tubes worked well. Although our dataset is very small, isolates identified as *V. vulnificus* by BIOLOG™ were positive by qPCR, and conversely, isolates identified by BIOLOG™ as other vibrio species were negative by qPCR.

Recommendations

A full-scale validation study of the effectiveness of this process still needs to be conducted. Much higher initial *V. vulnificus* densities are needed to meet ISSC PHP guidance requiring a 3.52 log reduction to a final MPN of <30 *V. vulnificus* g^{-1}. Therefore, future work will require identifying a time/temperature abuse method that will lead to the required *V. vulnificus* initial densities while not physiologically stressing the shellfish. In addition, much larger sample sizes will be required for a full-scale validation. In addition, we will employ an in situ CT logger to record temperature and salinity at the relay site during future validation studies. A continuous record of temperature and salinity will be important to assess the efficacy of this method over the proposed PHP period.

References

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