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Research Note

High Salinity Relay as a Postharvest Processing Strategy To Reduce *Vibrio vulnificus* Levels in Chesapeake Bay Oysters (*Crassostrea virginica*)†

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ABSTRACT

In 2009 the U.S. Food and Drug Administration (FDA) announced its intention to implement postharvest processing (PHP) methods to eliminate *Vibrio vulnificus* from oysters intended for the raw, half-shell market that are harvested from the Gulf of Mexico during warmer months. FDA-approved PHP methods can be expensive and may be associated with unfavorable responses from some consumers. A relatively unexplored PHP method that uses relaying to high salinity waters could be an alternative strategy, considering that high salinities appear to negatively affect the survival of *V. vulnificus*. During relay, however, oysters may be exposed to rapid and large salinity increases that could cause increased mortality. In this study, the effectiveness of high salinity relay to reduce *V. vulnificus* to <30 most probable number (MPN) per g and the impact on oyster mortality were assessed in the lower Chesapeake Bay. Two relay experiments were performed during the summer and fall of 2010. Oysters collected from three grow-out sites, a low salinity site (14 to 15 practical salinity units [psu]) and two moderate salinity sites (22 to 25 psu), were relayed directly to a high salinity site (≥30 psu) on Virginia's Eastern Shore. Oysters were assayed for *V. vulnificus* and *Vibrio parahaemolyticus* (another *Vibrio* species of concern) densities at time 0 prior to relay and after 7 and 14 days of relay, using the FDA MPN enrichment method combined with detection by real-time PCR. After 14 days, both *V. vulnificus* and *V. parahaemolyticus* densities were ≤0.8 MPN/g, and decreases of 2 to 3 log in *V. vulnificus* densities were observed. Oyster mortalities were low (≤4%) even for oysters from the low salinity harvest site, which experienced a salinity increase of approximately 15 psu. Results, although preliminary and requiring formal validation and economic analysis, suggest that high salinity relay could be an effective PHP method.

Vibrio vulnificus is a naturally occurring bacterium in the Atlantic, Pacific, and Gulf of Mexico coastal waters of the United States. This bacterium is the leading cause of seafood-borne mortality and is associated with consumption of raw shellfish *(2, 10)*. Most cases are linked to raw oysters, *Crassostrea virginica,* harvested from the U.S. Gulf of Mexico, where *V. vulnificus* is more prevalent *(2, 19)*. In 2009 the U.S. Food and Drug Administration (FDA) announced its intention to implement new guidance requiring postharvest processing (PHP) to eliminate *V. vulnificus* from shellfish harvested during the months of April through October from Gulf of Mexico waters and destined for raw consumption *(23)*. Consequently, there is concern among shellfish growers along the East Coast that if mandatory PHP is implemented as proposed it will eventually be required in all regions, as the low reported prevalence of *V. vulnificus* infections in the mid-Atlantic could simply be a reflection of relatively lower production levels. Second, there has been one reported case of *V. vulnificus* associated with shellfish harvested from Virginia waters. A second case would trigger National Shellfish Sanitation Program guidelines that could require application of PHP *(15)*.

Approved PHP methods include cool pasteurization, cryogenic individual quick freezing, high hydrostatic pressure, or low-dose gamma irradiation. These technologies, which are not widely available in the Chesapeake Bay region, can be expensive and may be associated with unfavorable responses from consumers *(14)*. A relatively unexplored PHP method is the controlled relay of shellfish to high salinity waters. *V. vulnificus* is more abundant at moderate salinities, and, based on limited studies, exposure to high salinities (>30 practical salinity units [psu]) negatively affects its occurrence in oysters *(8, 9, 13)*. In a study in the Gulf of Mexico, Motes and DePaola *(12)* showed that relay of oysters, first to an intermediate acclimation salinity and then to a high salinity offshore site, resulted in a decrease in *V. vulnificus* abundance in oysters to <30 most probable number (MPN) per g, with

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mortalities <6%. This method, however, has not been evaluated in other shellfish-producing regions to assess its effectiveness and the rates of oyster mortality, nor has it been validated according to National Shellfish Sanitation Program PHP protocols *(15)*.

In this note, we report results of a preliminary study to evaluate the effectiveness of high salinity relay in Virginia for reducing *V. vulnificus* levels in oysters (*Crassostrea virginica*) collected from low to moderate salinity grow-out areas of the lower Chesapeake Bay. For simplicity and to reduce cost we did not use an intermediate salinity relay site in this study. The effect of high salinity relay on *V. parahaemolyticus* densities in relayed oysters was also evaluated. *V. parahaemolyticus* occurs worldwide in temperate estuarine and tropical waters and is recognized as a leading cause of gastroenteritis transmitted by seafood in the United States *(3)*. Although a positive correlation between water temperature and *V. parahaemolyticus* occurrence has been established, its response to salinity appears less clear *(4–6, 11, 17),* and the effect of relay to high salinity water on its levels in oysters is not known.

MATERIALS AND METHODS

Study sites. This study was performed in the Virginia portion of Chesapeake Bay during the summer and fall of 2010, with two trials, one conducted from 18 August to 2 September and the second from 21 September to 5 October. Three approved grow-out sites, the Coan River, the York River, and Nassawadox Creek (Fig. 1), were chosen as the sources of oysters to be relayed to higher salinities. The relay site near Sandy Island is on the Atlantic Ocean side of the Virginia Eastern Shore (Fig. 1).

Environmental parameters. Salinities were measured at the grow-out areas during harvesting and at each relay sampling time point at Sandy Island using a calibrated conductivity-temperature-depth sonde (YSI Inc., Yellow Springs, OH). Temperature was continuously recorded at all sites using in situ temperature loggers (HOBO, Onset Inc., Bourne, MA). Care was taken during transport of samples from harvest and relay sites (~2 h) to avoid temperature abuse that could affect vibrio levels. Oysters and water samples were kept chilled in insulated coolers and separated from direct contact with ice. When holding was necessary prior to sample deployment or processing, oysters and water samples were kept in a cold room (10°C). Water samples were never held longer than 2 h prior to processing.

Trials 1 and 2. Oysters collected from each grow-out site were deployed in on-bottom cages approximately 0.15 m above bottom in a water depth of ~2 m. After 7 and 14 days of relay, oysters were retrieved and mortalities recorded. At each sampling time point, a minimum of four replicate samples from each harvest site were recovered and processed as described below. A replicate sample consisted of at least 12 oysters. Surface water samples (100 ml) were collected on day 0 at all sites and on days 7 and 14 at Sandy Island.

Sample processing. Homogenates consisting of 10 to 12 oysters and water samples were inoculated into an alkaline peptone water MPN series as described in Chapter 9, ''*Vibrio,*'' of the most recent online version of the FDA's *Bacteriological Analytical Manual (20)*. Briefly, samples and decimal dilutions thereof were inoculated into a three-tube MPN series containing 10 ml of alkaline peptone water per tube. In cases in which low vibrio concentrations were anticipated (i.e., for days 7 and 14), the equivalent of 1-g portions of oyster tissue were inoculated into three 100-ml volumes of alkaline peptone water. Inoculated samples were incubated at 35°C for 18 to 24 h.

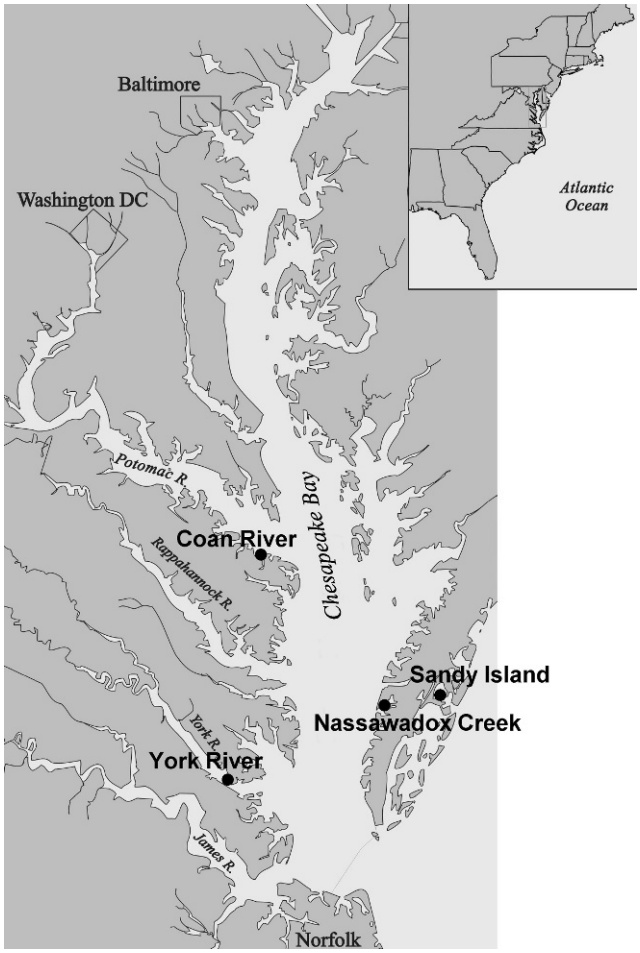

FIGURE 1. *Map showing the locations of the three grow-out sites and of the relay site.*

***V. vulnificus* detection.** A 1-ml volume was removed from the top centimeter of each alkaline peptone water enrichment tube showing turbidity and boiled for 10 min to lyse cells *(7),* and 1 μl of this lysate was used in each of the real-time PCR assays. Total *V. vulnificus* was detected using the primer pair and TaqMan probe described by Campbell and Wright *(1),* with the following modifications: bovine serum albumin was added at 0.4 μg/μl, final concentration, using TaqMan Fast Universal Master Mix (Applied Biosystems, Carlsbad, CA), and 1 μl of template was added in a 10-μl reaction volume. Real-time PCR reactions were run on a 7500 Fast Real-Time PCR machine (Applied Biosystems) using the published cycling conditions *(1)*. Results obtained on positive and negative control material using the original published conditions were compared with those obtained after alterations to optimize the assay. Each real-time PCR run included a positive control for which the template DNA was obtained from a *V. vulnificus* culture (ATCC 27562) and a negative control for which no template was added to the reaction tube. Resulting MPN values were calculated using approved MPN tables *(22),* and geometric means were determined for replicate samples at each site and time point. Box plots using replicate sample data were prepared with KaleidaGraph (Synergy Software, Reading, PA) for data evaluation and site comparisons.

TABLE 1. V. vulnificus *and* V. parahaemolyticus *geometric means in* C. virginica *samples harvested from Coan River (14 to 15 psu), York River (22 to 25 psu), and Nassawadox Creek (22 to 23 psu) growing areas and relayed to Sandy Island (30 to 32 psu) for 14 days*

Trial	Relay day	Site of origin	*V. vulnificus* (MPN/g)	*V. parahaemolyticus* (MPN/g)
1	0	Coan	224.5	17.0
		York	65.8	75.2
		Nassawadox	49.1	<1.9
	7	Coan	<2.7	<0.4
		York	<0.4	0.5
		Nassawadox	<0.3	1.9
	14	Coan	<0.4	<0.3
		York	<0.4	<0.5
		Nassawadox	<0.5	<0.4
2	0	Coan	176.0	<4.0
		York	29.2	<0.3
		Nassawadox	5.4	<0.5
	7	Coan	3.0	<1.3
		York	<0.3	<0.3
		Nassawadox	<0.4	<0.3
	14	Coan	<0.8	<0.3
		York	<0.3	<0.3
		Nassawadox	<0.3	<0.3

***V. parahaemolyticus* detection.** Simultaneous detection of the *V. parahaemolyticus* species-specific thermolabile hemolysin gene (*tlh*) and of the pathogenicity marker thermostable direct hemolysin gene (*tdh*) was performed using the multiplex real-time PCR assay designed by Nordstrom et al. *(16)* without the primers and probe targeting the thermostable-related hemolysin (*trh*) gene or the internal control. Attempts to amplify the *trh* gene using published methods, as well as efforts using slight variations while trying to optimize conditions, were not successful. Concentrations of the *tlh* and *tdh* primers and probes used were those published with modifications to the protocol as described above for the *V. vulnificus* assay. Each real-time PCR run included a negative control and two positive controls: DNA from the *V. parahaemolyticus* strain FIHES98 (*tdh*-negative and *trh*-negative) and from the TX2103 strain (*tdh*-positive and *trh*-negative). Cycling conditions were as published and the data were processed as described above.

RESULTS

Environmental parameters. The lowest salinities (14 to 15 psu) occurred at the Coan River site, with moderate salinities recorded at both the York River (22 to 25 psu) and Nassawadox Creek (22 to 23 psu) grow-out sites. Salinities measured at the Sandy Island relay site were always ≥30 psu (30 to 32 psu). Prior to the start of trial 1, the average water temperature over 14 days was 29, 28, and 25°C at Coan River, York River, and Nassawadox Creek, respectively. Prior to trial 2, the average temperature over a 14-day period before deployment was 24 to 25°C for all grow-out sites. At the relay site near Sandy Island, the average temperature during the 14 days of trial 1 was 26°C and was 23°C during trial 2.

***V. vulnificus*.** During both trials, Coan River oysters exhibited the highest initial *V. vulnificus* concentrations before relay, with geometric means of 224.5 and 176.0 MPN/g for trials 1 and 2, respectively (Table 1 and Fig. 2). The highest concentration among replicate oyster samples was 750 MPN/g. Initial York River and Nassawadox Creek oyster MPN geometric means were less than 65.8 MPN/g during trial 1 and 29.2 MPN/g during trial 2 (Table 1). Independent of grow-out site or trial, after 7 and 14 days of relay, MPN geometric means of *V. vulnificus* concentrations were ≤3.0 and ≤0.8 MPN/g, respectively.

V. vulnificus MPN values in water samples were highest at the Coan River grow-out site, ≤29 and ≤2.3 MPN/ml during trials 1 and 2, respectively. MPN values in water samples were much lower at the York River and Nassawadox Creek sites during trial 1 (<4.3 MPN/ml) and below the detection limit during trial 2. The bacterium was not detected in water at the Sandy Island site.

***V. parahaemolyticus*.** During trial 1, the highest geometric mean *V. parahaemolyticus* density (75.2 MPN/g of oyster) occurred in York River oysters on day 0. At that site the highest *V. parahaemolyticus* density observed in an individual replicate oyster sample was 430 MPN/g. At the same time point, geometric mean *V. parahaemolyticus* concentrations were 17.0 MPN/g for Coan River oysters and ≤1.9 MPN/g for Nassawadox Creek oysters. After 7 and 14 days of exposure, levels dropped to values ≤1.9 MPN/g regardless of site. During trial 2, *V. parahaemolyticus* levels were low in the oyster samples from all grow-out sites, with all values ≤4.0 MPN/g at time 0 (Table 1 and Fig. 2). *V. parahaemolyticus* values were low in water samples at the grow-out sites during both trials (≤2.3 MPN/ml), and none was detected in water samples from the Sandy Island relay site. Finally, only the *V. parahaemolyticus tlh* gene, not the *tdh* gene, was detected in oyster and water samples during this study.

Oyster mortalities. During both trials, the highest mortalities occurred in oysters harvested from the Coan River. However, total mortality was not higher than 4% (6 of 150) after 14 days of relay (Table 2). Mortalities over the 14-day relay were 2.2 and 1.6% in oysters from the York River and Nassawadox Creek, respectively.

DISCUSSION

Relay of Chesapeake Bay oysters during August through October to a high salinity site without an intermediate acclimation step was effective in reducing *V. vulnificus* densities with low mortality (≤4%). Decreases of up to three orders of magnitude in *V. vulnificus* densities were observed after 14 days, with final values ≤0.8 MPN/g of oyster meat. Oysters harvested from the lowest salinity site, the Coan River, which experienced a salinity increase of approximately 15 psu, exhibited ≤4% mortality. This suggests that exposure to an intermediate adaptation salinity, as was done in a previous study that reported 6% mortality *(12),* may not be necessary for oysters harvested from the Virginia sites that we tested, reducing costs and simplifying use of high salinity relay as a PHP method. Moreover, relay to high salinity was also associated with a decrease of *V. parahaemolyticus* levels in the oysters, which was particularly evident with York River oysters during trial

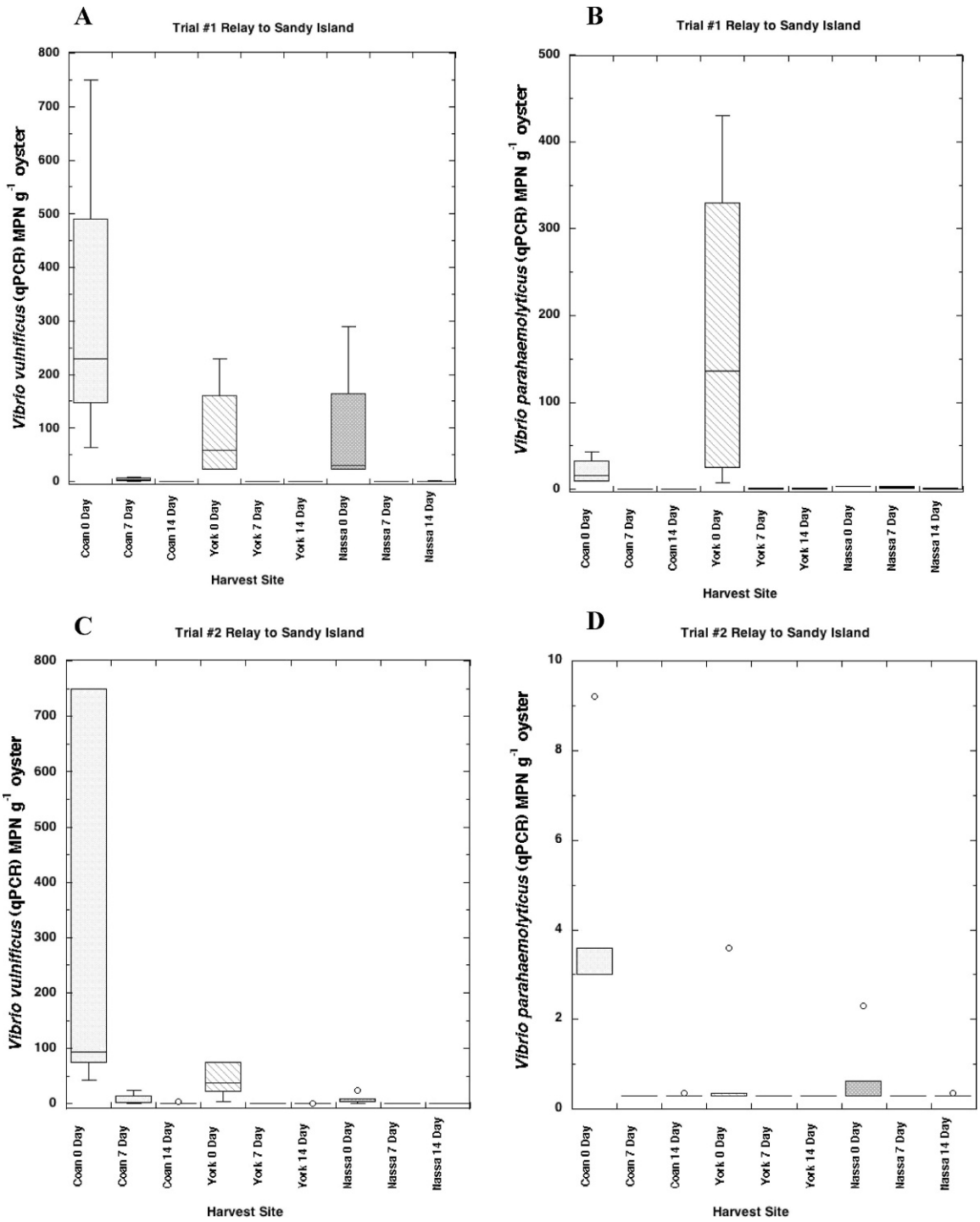

FIGURE 2. *Box plots of* V. vulnificus *and* V. parahaemolyticus *MPN per gram values in oysters measured during trials 1 and 2. The solid line in the center of each box represents the median value, while the 25th and 75th percentile values are the lower and upper margins, respectively. Circles represent outliers. (A)* V. vulnificus *densities in oysters from trial 1; (B)* V. parahaemolyticus *from trial 1; (C)* V. vulnificus *from trial 2; and (D)* V. parahaemolyticus *from trial 2.*

1. Except for York River oysters during trial 1, however, measured initial levels of total *V. parahaemolyticus* were well under the FDA level of concern of 50 *V. parahaemolyticus* cells per g of oyster (or 10,000 cells per serving) *(21)*.

Differences in initial *V. vulnificus* concentrations between grow-out sites, with the highest *V. vulnificus* densities at the low salinity site (14 to 15 psu), were consistent with observations that salinities in the 10 to 15 psu range are optimal for this organism and the general acceptance of an inverse relationship between cell densities and salinities above this range *(18)*. The highest *V. vulnificus* density we observed in oysters (i.e., 7.5×10^2 MPN/g) was lower than high values reported for oysters from a slightly lower salinity site in the Maryland portion of Chesapeake Bay *(24)*. In that study using colony lift probing, concentrations exceeding 10^4 cells per g were recorded during July and September. We never detected *V.*

TABLE 2. *Mortalities among oysters from each grow-out site during each trial*

Trial	Relay interval (days)	No. of mortalities/no. of oysters sampled (%)		
		Coan	York	Nassawadox
1	0–7	6/75 (8)	0/60 (0)	1/60 (1.6)
	7–14	0/75 (0)	2/60 (3.3)	1/60 (1.6)
	0–14	6/150 (4)	2/120 (1.6)	2/120 (1.6)
2	0–7	1/60 (1.6)	1/60 (1.6)	0/60 (0)
	7–14	3/75 (4)	2/75 (2.6)	2/75 (2.6)
	0–14	4/135 (2.9)	3/135 (2.2)	2/135 (1.5)

vulnificus in water samples at the relay site, but these results represent a very small sample and do not adequately account for variability in environmental conditions.

This preliminary study indicates that high salinity relay could provide an effective PHP method for the shellfish industry in Virginia and should be further evaluated to encompass the range of environmental conditions encountered over the proposed *V. vulnificus* PHP period (May to October). Full-scale validation of this method, however, will necessitate much higher initial *V. vulnificus* densities than we encountered to meet the National Shellfish Sanitation Program PHP guidance *(15),* which requires a 3.52-log reduction to a final MPN of <30 *V. vulnificus* per g, using 10 replicate samples. It could be argued that this validation protocol, which was developed for oysters from the Gulf of Mexico region, was designed for vibrio concentrations that cannot be reached under reasonable harvest and transport activities in the Chesapeake Bay, and that reduction from what constitutes ''abused oysters'' in the mid-Atlantic to undetectable levels should be a regional criterion. Unless the guidance is modified, our future studies will focus on developing a reproducible abuse protocol (one that is as physiologically benign as possible) to increase *V. vulnificus* densities in freshly harvested oysters. Studies will involve testing oysters from several other grow-out sites in Virginia and will be aimed at understanding the potential effects of environmental conditions at the relay site on *V. vulnificus* reduction. Finally, it will be important to evaluate whether high salinity relay is not only effective, but also economically sound for Virginia shellfish growers. Therefore, the costs of relaying to mitigate *V. vulnificus* will need to be characterized.

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