Test of Effectiveness of Relaying as a Post Harvest Process for reducing levels of *Vibrio vulnificus* and *V. parahaemolyticus* in Shellstock Oysters

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Introduction

Laboratory observations by Kaspar and Tamplin (1993) of pure cultures of *Vibrio vulnificus* suggested that elevated salinities of 25 ppt or higher reduced *V. vulnificus* numbers. Motes and DePaola (1996) showed that oysters relayed to high-salinity offshore waters (>32 ppt) showed a significant reduction in *V. vulnificus* numbers after two (2) weeks.

Less well known, though, is the amount of time necessary for shellstock oysters, harvested and transported under refrigeration and placed back into the environment (relay) to reestablish "ambient" levels of *V. vulnificus* and *V. parahaemolyticus* consistent with other oysters in the same area. Current regulatory requirements in the Gulf States require rapid refrigeration post harvest for any oysters to be sold live, in the shell, for raw consumption. Shellstock which have been harvested and rapidly refrigerated in compliance with a *Vibrio vulnificus* Control Plan, are referred to as "white tag" shellstock. Oysters refrigerated less quickly are given a different tag and must be shucked by a certified dealer or post-harvest processed. These are referred to as "non-matrix", "restricted use" or "green tag" shellstock.

The present time temperature requirements which are imposed during the summer months are very restrictive and severely limit the harvest time for many, if not most, oyster harvesters. Should relaying prove effective in reducing *V. vulnificus* and *V. parahaemolyticus* levels it could be considered as a viable control for reducing *V. vulnificus* illnesses.

Given the fact that green tag shellstock are expected to be more common than white tag shellstock under the newest requirements, any post-harvest process that would reduce levels of *V. vulnificus* and *V. parahaemolyticus* in green tag shellstock to levels equivalent to those of the white tag product could be useful to the Gulf oyster industry. An argument may be made that, although the relayed product cannot be labeled as having either of these organisms reduced to non-detectable levels, the re-harvested product should be allowed for sale as "white tag" shellstock as if it had been placed under refrigeration in accordance with a state's *Vibrio vulnificus* Control Plan when it was originally harvested. The objective of the proposed research, therefore, was to test the effectiveness of the relay of oysters (*Crassostrea virginica*) to a monitored, inshore site and to a remote, higher salinity site as a means of reducing *Vibrio* levels.

Approach and Methodology

To determine experimentally the minimum amount of time for *Vibrio* loads within green tag oysters to return to levels no higher than those detected in white tag oysters from the same original harvest area, we conducted a field study at the Point aux Pins Oyster Farm, in Sandy Bay, AL and at a remote location at the west end of Dauphin Island, AL with two independent replicate runs. For each run, three (3) time zero (t_0) samples of 15 oysters each were taken from shellstock oysters on board a harvest vessel in Moss Bay, LA (Area 17) immediately after harvest and refrigerated with gel ice until sampling on both August 14, 2011 and September 18, 2011. During the same harvesting trip, oysters from the same area were segregated into two additional

groups aboard the vessel: one group that was refrigerated within one hour so that the internal temperature of the oysters putatively reached 55ºC or less within 6 hours (referred to as 'white tag') and one to remain unrefrigerated on the boat deck for the duration of the harvesting trip (referred to as 'green tag'). A temperature recording device was placed with both groups of oysters to record the approximate internal temperature of the oysters. Surface water temperature and salinity data were also collected at the original harvest site using a refractometer and immersion thermometer. After the harvesting vessel returned to the dock, the t_0 samples and the white and green tag oysters were loaded onto a refrigerated conveyance and brought to Bon Secour Fisheries, Inc in Bon Secour, AL.

At Bon Secour Fisheries, three (3) samples of 15 oysters each were taken from each of the white (t_{0WT}) and green tag (t_{0GT}) groups. All time zero samples were shipped overnight to the Texas A&M University, Seafood Safety Laboratory in Galveston, TX for bacteriological analysis (9 samples total per run). Within 24 hrs of arrival at Bon Secour Fisheries, green tag oysters were deployed in floating shellfish aquaculture cages (OysterGro™) at each of two sites: the Point aux Pins Oyster Farm in Sandy Bay, AL (~30º 23.007 N, 88º 23.007' W) and the northern side of the west end of Dauphin Island, AL (both within conditionally approved areas for shellfish harvest). On day 2 of the first deployment on Dauphin Island, the site was relocated from the middle of Dauphin Island (~30º 15.171' N, 88º 10.135' W) to a site further west (~30º 14.135' N, 88º 17.247' W) to increase the likelihood of sustained high salinity water.

Oysters were stocked into replicate plastic mesh (12 mm) bags at ~150 oysters/bag and held in the oyster cages. Samples of oysters (n = 15) were collected from each of five randomly selected bags at each site at 2 d (t_{2d}), 7 d (t_{7d}), and 14 d (t_{14d}) after relay from each site (5 samples/site/time period x 2 site x 3 time periods, or 30 samples total). All oysters were immediately chilled on board and refrigerated prior to shipping. All samples were then shipped priority overnight to the Texas A&M Laboratory in Galveston, TX for bacteriological analysis. Water temperature and salinity were measured at each relay site with a handheld YSI at each sampling period.

Once received by the Seafood Safety Lab, oyster samples were immediately processed according to the procedures in Chapter 9 of the FDA Bacteriological Analytical Manual for enumeration using a DNA alkaline phosphatase-labeled gene probe. Each oyster sample consisted of 15 oysters: 3 used to check the internal meat temperature, and 12 shucked for enumeration of *V. vulnificus* and *V. p*. The 12 shucked oysters (meat and liquor) were blended with an equal weight of Alkaline Peptone Water (APW), which was then used to create a 6-dilution, 3- replicate serial dilution in APW. Following 18-24 hours incubation, positive dilutions were streaked onto modified cellobiose-Polymixin B-Colistin (mCPC) agar and thiosulfate-citrate-bile salts-sucrose (TCBS) agar, which are specific for the growth of *V. vulnificus* and *V. parahaemolyticus*, respectively.

Following 18-24 hours incubation of the agars, suspect colonies of *V. vulnificus* (yellow with a "fried egg" appearance on CPC) and *Vp* (dark green on TCBS) were isolated into 96-well culture plates, and allowed to resuscitate for 4 hours. At this point, the isolated colonies were replicated onto T_1N_3 (1% tryptone, 3% NaCl) and Vva (*Vibrio vulnificus* agar) agar plates. From there, the colonies were lysed onto Whatman #541 filters, and the DNA prepared with Proteinase K.

Finally, the filters were run through the AP-labeled gene probe procedure for *Vibrio vulnificus*, and the tlh(+)tdh(-)forms of *V. parahaemolyticus*. Positive results for all the gene probes appear as dark brown/purple colonies, as opposed to tan/yellow colonies indicating negative results. Upon comparing the positives to the original serial dilutions, results were determined using an MPN (Most Probable Number) per gram format.

Results

Upon initial harvest on Aug. 14, 2011 at 8:05 AM, the water temperature was 28.5º C and salinity was 20 psu. Upon initial harvest for the second run on Sep. 18, 2011, the water temperature was 25.6º C and salinity was 22 psu.

Temperature and salinity for the deployment sites of green tagged oysters (Table 1) were broadly similar across sites. Temperature dropped at the end of the second run at both sites. Though salinity was expected to be higher at the Dauphin Island sites, this was not consistently observed, though all salinities observed exceeded 21.2 psu.

Table 1. Observed water temperatures and salinities at deployment sites in Mississippi Sound (AL)

Day	Sandy Bay		Dauphin Island	
	Temp (ºC)	Salinity (PSU)	Temp (ºC)	Salinity (PSU)
August Run				
2	29.1	25.3	30.2	22.6
7	29.8	26.9	30.8	27.6
14	28.2	27.5	30.2	29.0
Sept. Run				
2	28.5	21.2	27.1	27.8
7	26.8	22.3	27.4	26.8
14	22.6	23.7	21.6	22.1

Though there appeared to be differences among the time zero samples in terms of *V. vulnificus* and *V. parahaemolyticus* abundances, neither of these effects was significant due to the high variation in initial numbers and low replication (ANOVA, df = 2, 3, $p = 0.18$ and $p = 0.41$, respectively). Still, there is a clear trend of the abundances being highest in the green tagged oysters (with corresponding increased variation), with little apparent difference between the oysters iced immediately after harvest and the white tagged oysters.

Table 2. Time zero abundances of *Vibrio vulnificus* and *V. parahaemolyticus* in MPN/g

	***V. vulnificus* (± SD)**	***V. parahaemolyticus* (± SD)**
Immediately Iced (t_0)	11,240 (± 6,562)	318 (± 308)
White Tagged (t_{0WT})	11,730 (± 10,734)	1,066 (± 1,391)
Green Tagged (t_{0GT})	67,600 (± 42,426)	3,761 (± 3,785)

Interestingly, there was a strong interaction between deployment site and deployment day for *V. vulnificus* abundance (ANOVA, df = 3,8, $p < 0.001$). Despite the interaction (Fig. 1), the pattern was consistent within each site, even using the most conservative estimate of time zero abundance of estimates from the immediately iced oysters as the time zero reference; abundance on day 2 were significantly higher than any other day (Tukey's HSD, $p < 0.001$) with no significant differences among any of the other days within a site (Tukey's HSD, $p \geq 0.965$). Additionally, the abundances on day 2 at Sandy Bay were significantly greater than the day 2 abundances of *V. vulnificus* at the Dauphin Island site (Tukey's HSD, $p < 0.001$).

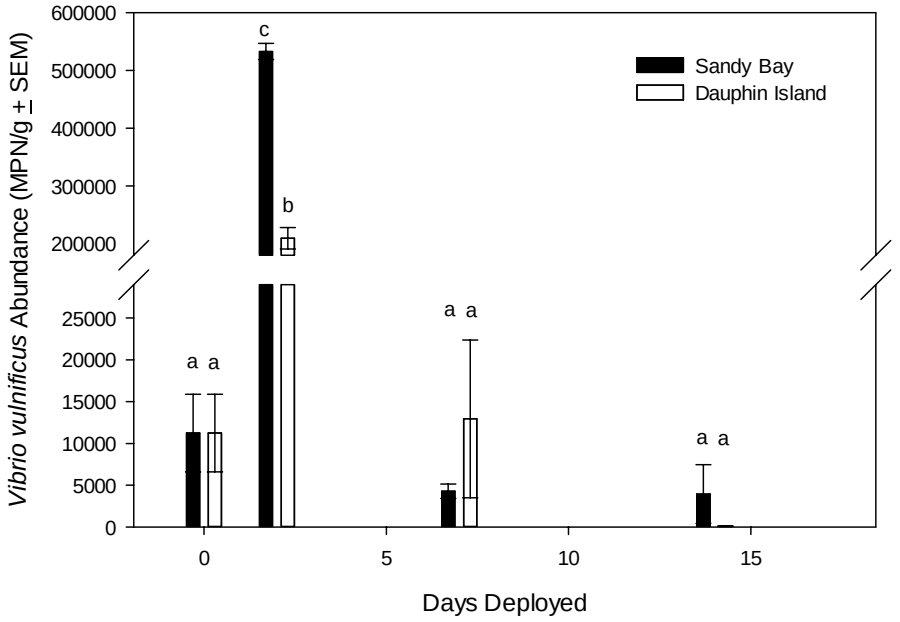

Figure 1. Relationship between days deployed and the abundance of *Vibrio vulnificus* (MPN/g) in green-tagged oysters deployed at two sites in coastal Alabama, Sandy Bay and Dauphin Island in 2011. Different letters indicate significant pair-wise differences.

For *V. parahaemolyticus*, data were rank-transformed to meet the assumptions of normality. In this case (Fig. 2), there was only a significant effect of day (ANOVA, df = 3, 8, $p < 0.001$), with no significant effect of sites or day*site interactions. The abundance of *V. parahaemolyticus* was greatest on day 2, but was not statistically greater than abundance on day 7 (Tukey's HSD, $p = 0.13$). Day 7, turn, was more abundant (but not significantly) than on day 14 (Tukey's HSD, $p = 0.19$). The abundance was lowest at time zero (using estimates again from immediately iced oysters as the time zero reference), but this did not differ significantly from abundance on day 14 (Tukey's HSD, $p = 0.53$).

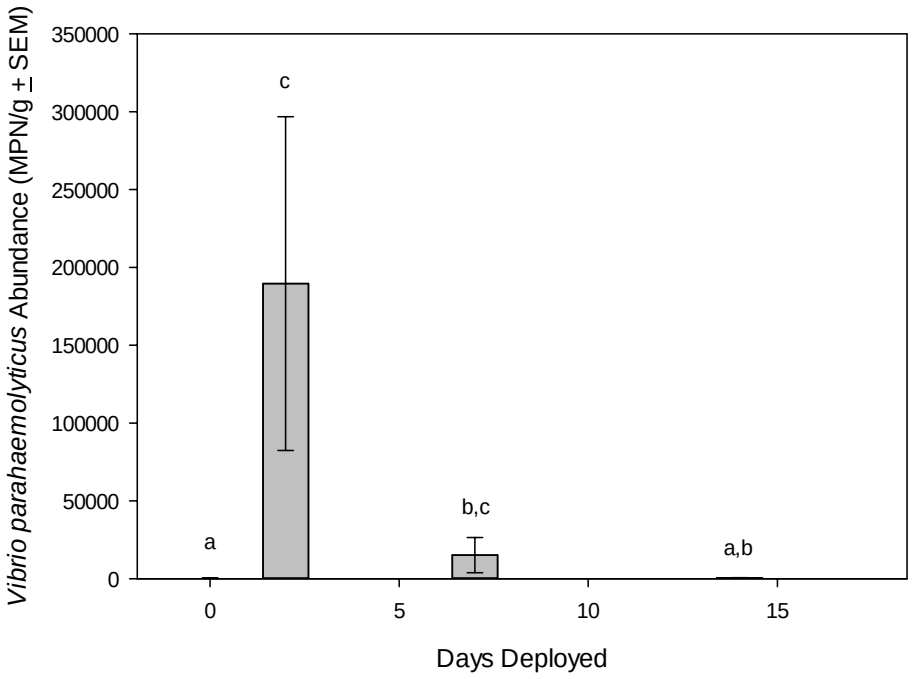

Figure 2. Relationship between days deployed and the abundance of *Vibrio parahaemolyticus* (MPN/g) in green-tagged oysters deployed in coastal Alabama in 2011. Different letters indicate significant pair-wise differences.

There was noticeable mortality across the deployments, with an average survival of 83.4% (± 3.13 SD). There was no difference observed between the two sites (ANOVA, df = 1, 10, $p < 0.18$).

Conclusions

Based on this preliminary study, relaying of green tagged oysters to relatively high salinity waters (approved for harvest) shows some promise as a means of reducing both *V. vulnificus* and *parahaemolyticus* abundance. Using the most conservative estimate of initial (time zero) abundances (within oysters immediately iced upon harvest), deployment was able to reduce *V. vulnificus* abundance to levels equivalent to those upon harvest within 7 days. Similarly, again using the most conservative estimate of initial abundances, deployment was able to reduce *V. parahaemolyticus* abundance levels equivalent to those upon harvest within 14 days at the two tested sites.

There are several important caveats to note. First, deployment of green tagged oysters actually led to very pronounced spikes in the abundances of both species of *Vibrio* by day 2. While deployment may be able to reduce abundances over time, it is not a linear decrease and no assumptions should be made about the dynamics in abundance during the first days of deployment of green tagged oysters.

Second, over the deployments, mortality approached 20%. These losses need to considered in any evaluation of the feasibility of this approach as a means of 're-claiming' green tagged oysters by deploying them in the field.

Third, the intent of the research was to determine if the risk associated with 'green tagged' oysters could be reduced back to that of oysters upon harvest, allowing a subsequent 'second harvest' of the oysters with the opportunity to maintain them as 'white tagged' oysters. Therefore, oysters treated in this manner would not be considered in any way free of all *Vibrios*.

Finally, this study was undertaken as an initial proof of concept and was minimally replicated. Any conclusions need to consider the very low power of the test. The results, however, suggest that this approach shows promise as an effective means of re-claiming green tag oysters.

References

Kaspar, C. W., & Tamplin, M. L. (1993). Effects of temperature and salinity on the survival of *Vibrio vulnificus* in seawater and shellfish. *Applied and Environmental Microbiology,* 59, 2425-2429.

Motes, M.L. & DePaola, A. (1996) Offshore suspension relaying to reduce levels of *Vibrio vulnificus* in oysters (*Crassostrea virginica*). *Applied and Environmental Microbiology,* 62**,** 3875-3877.