Oyster processing strategies for eliminating *Vibrio parahaemolyticus* and *Vibrio vulnificus* from New Hampshire oysters

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Introduction

Vibrio parahaemolyticus (Vp) and *Vibrio vulnificus* (Vv) are human pathogens commonly associated with bivalve shellfish. These *Vibrios* cause infections to humans when patients consume raw or undercooked shellfish or when it's in free-living form in estuarine environments. There are three clinical manifestations of disease caused by Vp and Vv; most common for Vp is gastroenteritis and rarely causing wound infections and septicemia and Vv causes more wound infections and septicemia than gastroenteritis. In order to provide consumers with safe raw oysters, shellfish companies have implemented post-harvest strategies to remove *Vibrios* within the oysters. However, these methods to remove the *Vibrio* species from the oysters can be relatively expensive and typically kill the oysters. The main focus of this study was to investigate a range of depuration and relaying strategies for reducing Vp and Vv levels in oysters. The first objective was to adopt published real-time PCR, complemented with Most-Probable-Number (qPCR-MPN), methods to detect Vp and Vv from enriched oyster homogenate instead of using culture-based identification methods. The main focus was to conduct relay and depuration experiments from warm to cold seasons under controlled and natural environmental conditions. The final objective was detection of pathogenic strains of Vp, strains of Vp containing the thermostable direct hemolysin (*tdh*) and TDH-related hemolysin (*trh*) in pre-temperature abused oysters and temperature abused oysters.

Methods and Materials

The oysters were collected from the Piscataqua River in New Hampshire each month during July through November 2009. Before performing any treatments the oysters were subjected to temperature abuse at 28^{o}C for 18-20h to increase the concentration of Vp and Vv. The qPCR-MPN concentrations of Vp and Vv in pre- and post-temperature abused oysters were determined. The temperature-abused oysters were subjected to three depuration and relaying treatments, and freshly harvested oysters subjected to one relay treatment (Table 1), all performed in and in close proximity to the commercial shellfish depuration facility, Spinney Creek Shellfish Inc, Eliot, ME. Samples were retrieved after 2 and 5 days and Vp and Vv concentrations were determined on 3 batches of 12 oysters for each day and treatment. The detection of Vibrio concentrations with qPCR-MPN involved use of primers and modification of the protocols of Nordstrom *et al.* (2007) for Vp, and use of primers and modification of protocols from Wright *et al*. (2007) for Vv. Positive MPN tubes where *tlh* was detected (Vp+) were screened for the virulence genes *tdh* and *trh*. The PCR primers and modified protocols used to detect *tdh*+ and *trh*+ strains of Vp were from Panicker *et al*. (2004).

Table 1. Post-harvest processing treatment options for contaminated oysters

Treatments	Annotation	Time	Location	Description

Depuration recirculation	RC	July - November	In tanks on land	UV sterilized, filter sterilized, foam fractionation, recriculated creek water
Depuration flow-through	FT	July - November	In tanks on land	UV sterilized, flow-through of creek water into the tank
Creek relay	CR	August - November	Creek	Oyster bed in Spinney Creek, Eliot, ME (temperature abused)
Natural relay	NR	September - November	Creek	Direct relay from Piscataqua River to Spinney Creek Eliot, ME (pre-temperature abused)

Results and Discussion

Vp and Vv concentrations were consistently increased following temperature abuse by at least a two $\log_{10}$ difference for both species (Table 2). The freshly harvested oysters had Vp and Vv concentrations that were highest during the warmer summer months, and concentrations declined to low or undetectable levels as water temperatures fell through autumn into November. The higher concentrations provided a better starting point for evaluating the effects of depuration and relaying treatments.

Table 2. Concentration of Vp and Vv (MPN/g) in freshly harvested (Pre-TA) and temperature abused (Post TA) oysters from the Piscataqua River, NH.

Vp				Vv			
Month	**Pre-TA**	**Post TA**	**Δ (Log)**	**Month**	**Pre-TA**	**Post TA**	**Δ (Log)**
Jul-09	1.48	4.25	2.78	Jul-09	2.62	6.49	3.87
Aug-09	2.19	5.05	2.86	Aug-09	3.05	5.51	2.46
Sep-09	1.59	4.05	2.46	Sep-09	0.66	2.91	2.25
Nov-09	0.44	3.30	2.86	Nov-09	No detection	No detection	N/A

Vp and Vv showed varying responses to the different R&D treatments.

2,5, d

treatment

The oyster processing methods reduced levels of Vv and Vp in temperature-abused oysters after 5 days with a few exceptions (Table 3). The RC, FT and CR treatments all showed significant reductions in Vp concentrations during August, but only the CR treatment showed significant reductions in all three months it was tested (Sept. – Nov.). In September and November, the day 5 Vp concentration in the CR oysters was significantly lower than the concentrations in the RC and FT oysters. There were >1 log differences in Vp concentrations in both months where oysters were treated by natural relay, but the reductions were not significant. These results suggest that, despite some differences during different times of year and environmental conditions, the relay of oysters to the creek appears to be the most effective treatment amongst those tested for reducing Vp levels in oysters.

Like Vp results, the Vv concentrations in oysters from all three treatments in August showed significant reductions after 5 days (Table 3). Unlike the Vp results, there were no other significant

reductions in Vv concentrations by any treatment for July and September, and Vv was below detection limits in November. There was, however, a significant increase in Vv levels in the RC treatment in September after both 2 and 5 days. These results suggest different responses to depuration and relaying by the two vibrio species. The RC treatment also seems to be the least effective treatment for reducing vibrios in oysters.

The public health significance of these findings was further analyzed by assaying oyster homogenate enrichments for Vp virulence genes. Vp+ *tdh*$^+$/*trh*$^+$ were detected only in the August temperature abused oysters at a concentration of 450 MPN index per g of oyster. In addition, *tdh*$^+$/*trh*$^+$ strains were detected in August day 2 and day 5 RC treated oysters at a level of 3.6 MPN index / g. No virulence genes were detected in pre-temperature abused oysters or in other treatments and in other months.

The experiments in this study represent a range of conditions for treating shellfish from traditional depuration to natural relay. Previous studies have shown traditional depuration to be ineffective at reducing levels of pathogenic vibrios, including studies at the same study site (Jones *et al*. 1991, Tamplin and Capers 1992, Croci *et al.* 2002). There have been only a few studies on relaying to reduce vibrio concentrations in shellfish, yet these have shown promising results (Jones 1994; Jones *et al.* 1995; Motes and DePaola 1996). The suggestion that exposure to increased salinity levels in these studies helps to reduce vibrio levels is a valid observation. The mechanism underlying observed reductions in vibrio levels is probably more complicated given the relatively poor performance under depuration (RC) conditions using the same salinity, temperature and length of treatment in this study. The FT treatment represents conditions in between the RC and CR treatments where oysters were exposed to flow through creek water, yet the creek water was disinfected prior to entering the FT tanks. This suggests a biological factor may be involved in vibrio reductions. Further investigation of what mechanism(s) is involved in the reduction of vibrios under the tested oyster processing conditions is currently underway.

Table 3. Effects of depuration and relay treatments for reducing Vp and Vv concentrations (log_{10} MPN/g) in oysters. Statistically significant concentrations compared to Day 0 are designated by * ($p < 0.05$).

Treatment	Sample day	July		August		September		November	
		Vp	Vv	Vp	Vv	Vp	Vv	Vp	Vp
RC	0	4.25	6.49	5.05	5.51	4.05	2.91	3.30	BD
	2	3.61	4.81	4.57	4.82	4.26	4.05*	3.23	-
	5	4.14	3.72	3.50*	3.41*	4.23	3.75*	2.34	-
FT	2	4.33	5.34	4.61	5.36	4.36	3.36	1.62*	-
	5	3.66	3.63	3.96*	3.75*	3.58	2.20	2.31	-
CR	5	N/A	N/A	3.48*	3.05*	3.17*	2.36	0.46*	-
NR	0					1.59	0.66	0.44	BD
	5					0.17	0.59	-0.35	-

Conclusions

The results from this study suggest that, despite some differences during different times of year and environmental conditions, relaying oysters to the creek appears to be the most effective treatment amongst those tested for reducing Vp levels in oysters. The RC treatment seems to be the least effective treatment for reducing vibrios in oysters, with the intermediate FT treatment giving intermediate results. Temperature abuse of oysters was an effective way to increase concentrations to allow for observation of significant effects, especially the desired reductions. Comparable results were observed using natural relaying, but the concentrations were so low that finding significant differences after 5 days was not

possible. The results also suggest that the mechanism(s) involved in reducing vibrios under processing treatments may be more complex than simply increasing the salinity. Further research on this and other questions are currently underway.

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