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Research Note

Refrigerated Seawater Depuration for Reducing *Vibrio parahaemolyticus* Contamination in Pacific Oyster (*Crassostrea gigas*)

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ABSTRACT

The efficacy of refrigerated-seawater depuration for reducing *Vibrio parahaemolyticus* levels in Pacific oyster (*Crassostrea gigas*) was investigated. Raw Pacific oysters were inoculated with a mixed culture of five clinical strains of *V. parahaemolyticus* (10^5 to 10^6 most probable number [MPN] per g) and depurated with refrigerated seawater (5°C) in a laboratory-scale recirculation system equipped with a 15-W gamma UV sterilizer. Depuration with refrigerated seawater for 96 h reduced *V. parahaemolyticus* populations by >3.0 log MPN/g in oysters harvested in the winter. However, 144 h of depuration at 5°C was required to achieve a 3-log reduction in oysters harvested in the summer. Depuration with refrigerated seawater at 5°C for up to 144 h caused no significant fatality in the Pacific oyster and could be applied as a postharvest treatment to reduce *V. parahaemolyticus* contamination in Pacific oysters. Further studies are needed to validate the efficacy of the depuration process for reducing naturally accumulated *V. parahaemolyticus* in oysters.

Vibrio parahaemolyticus is the leading cause of bacterial gastroenteritis associated with seafood consumption in the United States *(14)*. Consumption of raw or undercooked seafood, particularly shellfish, contaminated with *V. parahaemolyticus* may lead to the development of acute gastroenteritis characterized by diarrhea, headache, vomiting, nausea, abdominal cramps, and low fever *(15, 18, 20)*.

Numerous outbreaks of *V. parahaemolyticus* infection linked to raw oyster consumption have been documented in the United States *(3–5, 10, 17)*. The Centers for Disease Control and Prevention (CDC) *(5, 6)* reported a 78% increase in the incidence of *Vibrio*-associated infections in 2006 from the 1996 to 1998 baselines. The incidence of *Vibrio* infections had by then increased to the highest level since FoodNet began conducting surveys, despite efforts directed at seafood consumers (especially high-risk consumers) to warn them of the potential hazards of eating raw shellfish. The often perceived and occasionally very real threat of *V. parahaemolyticus* infection associated with consumption of raw or undercooked oysters is a concern for public health and may result in substantial economic loss to the shellfish industry.

V. parahaemolyticus can quickly multiply to an infectious dose in oysters that are stored at ambient temperatures before consumption *(12, 23)*. To minimize the risk of *V. parahaemolyticus* infections associated with shellfish consumption, shellfish harvesting areas in the United States that have been previously implicated in *V. parahaemolyticus* outbreaks are routinely monitored by state agencies to control transmission of this infection. However, an unexpected outbreak of 177 cases of *V. parahaemolyticus* infection (72 confirmed and 105 probable) linked to the consumption of raw oysters harvested in Washington state and British Columbia (Canada) occurred in New York City and the states of New York, Oregon, and Washington in 2006 *(5)*. Thus, the current routine shellfish monitoring program cannot totally prevent illness associated with raw oyster consumption. The CDC estimated that 4,500 cases of *V. parahaemolyticus* infection occur each year in the United States *(7)*.

Four processes, low-temperature pasteurization, high-pressure processing, irradiation, and frozen storage, have been reported as capable of reducing *V. parahaemolyticus* in oysters postharvest *(1, 16, 21)*. However, these methods either require a significant amount of initial investment in equipment or result in high operating costs, and oysters often are killed by freezing, pasteurization, and high-pressure processes. Cost-effective intervention strategies for reducing *V. parahaemolyticus* in oysters without significant adverse effects remain to be developed.

Depuration is a process of holding filter-feeding shellfish in clean seawater to allow shellfish to release sand and bacteria over time *(2)*. The process has a long history as a postharvest treatment for reducing total microbial

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populations in shellfish. However, depuration at ambient temperatures has been reported as ineffective for reducing *Vibrio* contamination in oysters *(8, 25)*. Many studies have reported that occurrence of *V. parahaemolyticus* in marine environments is correlated with water temperature, with *V. parahaemolyticus* rarely detected in oysters or environmental samples until water temperatures rose to 15°C or higher *(9, 11, 13)*. Therefore, reducing water temperature for depuration might increase the efficacy of the process for decontaminating shellfish harboring *V. parahaemolyticus*. This study was conducted to determine the efficacy of refrigerated seawater (5°C) depuration for reducing *V. parahaemolyticus* in Pacific oysters.

MATERIALS AND METHODS

Bacterial culture preparation. Five clinical strains of *V. parahaemolyticus* obtained from the culture collection of the Food and Drug Administration Pacific Regional Laboratory Northwest (Bothell, WA) were used in this study: 10290 (O4:K12, tdh^+ and trh^+), 10292 (O6:K18, tdh^+ and trh^+), 10293 (O1:K56, tdh^+ and trh^+), BE 98-2029 (O3:K6, tdh^+), and 027-1c1 (O5:K15, tdh^+ and trh^+). Each culture was grown in 10 ml of tryptic soy broth (Difco, Becton Dickinson, Sparks, MD) supplemented with 1.5% NaCl (TSB-salt) at 37°C for 18 to 24 h. Enriched cultures were streaked to individual plates of tryptic soy agar (Difco, Becton Dickinson) supplemented with 1.5% NaCl (TSA-salt) and incubated at 37°C for 18 to 24 h. A single colony from a TSA-salt plate was transferred to 10 ml of TSB-salt and incubated at 37°C for 4 h. The enriched cultures of *V. parahaemolyticus* were pooled into a sterile 50-ml centrifuge tube and harvested by centrifugation at 3,000 × *g* (Sorvall RC-5B, Kendro Laboratory Products, Newtown, CT) at 5°C for 15 min. Pelleted cells were resuspended in 50 ml of sterile salt solution (2%) to obtain a culture cocktail of 10^8 to 10^9 CFU/ml.

Oyster preparation. Raw Pacific oysters were obtained from Oregon Oyster Farms (Yaquina Bay, Newport, OR) and delivered in a cooler with ice to the laboratory on the day of harvest. Oysters were washed with tap water to remove mud on the shell and placed in a rectangular high-density polyethylene (HDPE) tank (46 by 30 by 30 cm; Nalgene, Rochester, NY) containing 30 liters of artificial seawater (ASW; salinity, 30 ± 2 ppt) at room temperature (20 to 22°C) for 2 to 4 h before being inoculated with *V. parahaemolyticus*. Marine microalgae concentrate (Shellfish Diet 1800, Reed Mariculture Inc., Campbell, CA) was added to the ASW according to the manufacturer's recommendations to help oysters regain normal activities before bacterial inoculation.

Accumulation of *V. parahaemolyticus* in oysters. For each depuration study, 60 oysters were transferred from the ASW containing marine microalgae to an identical HDPE tank containing 30 liters of fresh ASW containing the *V. parahaemolyticus* culture cocktail at approximately 10^4 to 10^5 CFU/ml. Inoculated oysters were kept at room temperature overnight (16 to 18 h) with water circulated at a rate of approximately 12 liters/h. Air was pumped into the solution to keep dissolved oxygen levels favorable for oyster pumping and uptake of *V. parahaemolyticus*.

Oyster depuration. Oysters inoculated with *V. parahaemolyticus* were depurated in 60 liters of filtered seawater in a laboratory-scale recirculation (25 liters/min) system equipped with a 15-W gamma UV sterilizer (Current-USA Inc., Vista, CA), a water chiller (Delta Star, Aqua Logic, Inc., San Diego, CA), and a temperature regulator capable of regulating water temperature at 5 ± 1°C. The depuration was conducted three times in the winter and two times in the summer. Populations of *V. parahaemolyticus* in oysters were analyzed immediately after inoculation (0 h) and every 24 h for up 96 h in the winter and up to 144 h in the summer. The recirculated seawater was analyzed for *V. parahaemolyticus* at each test time to ensure no *V. parahaemolyticus* survived the UV sterilization to become a source of recontamination during the process.

Detection of *V. parahaemolyticus* in oysters. Populations of *V. parahaemolyticus* in oysters were quantified using the three-tube most-probable-number (MPN) methods described in the U.S. Food and Drug Administration *Bacteriological Analytical Manual (22)*. At each test time, five oysters were randomly picked from the depuration tank and shucked with a sterile shucking knife in a sterile stainless steel tray. Each shucked oyster was placed in a sterile blender jar and blended with nine volumes of sterile alkaline peptone water (APW) at high speed for 1 min in a two-speed laboratory blender (Waring Laboratory, Torrington, CT) to prepare a 1:10 sample dilution. Additional 10-fold dilutions of each oyster sample were prepared with sterile APW. All sample dilutions were individually inoculated into three tubes of alkaline peptone salt broth (APS). Inoculated APS tubes were incubated at 35 to 37°C for 16 to 18 h, and one loopful (3-mm inoculating loop) of enriched APS from a turbid tube was streaked onto individual thiosulfate–citrate–bile salts–sucrose agar plates. These plates were incubated at 35 to 37°C for 18 to 24 h. Colonies that were round (2 to 3 mm in diameter) and green or bluish on the plates were considered *V. parahaemolyticus*. Total populations of *V. parahaemolyticus* in oysters were determined by converting the numbers of APS tubes that were positive for *V. parahaemolyticus* to MPN per gram using the MPN table. Results were reported as the mean value obtained from five oysters.

TABLE 1. *Efficacy of refrigerated seawater (5°C) depuration for reducing* Vibrio parahaemolyticus *in Pacific oysters harvested in the winter*[a]

Time (h)	*V. parahaemolyticus* population (log MPN/g)[b]		
	Study 1	Study 2	Study 3
0	5.13 ± 0.47 A	4.87 ± 0.47 A	5.79 ± 0.55 A
24	3.70 ± 0.65 B (1.43)	3.39 ± 1.39 AB (1.48)	4.54 ± 1.29 AB (1.25)
48	2.86 ± 0.71 B (2.28)	2.29 ± 1.15 BC (2.57)	3.24 ± 0.77 C (2.55)
72	2.59 ± 1.05 BC (2.54)	1.51 ± 0.37 C (3.35)	2.61 ± 0.84 C (3.18)
96	2.05 ± 0.44 C (3.09)	1.38 ± 0.52 C (3.49)	2.61 ± 0.24 C (3.18)

[a] Studies 1 and 2 were conducted in February, and study 3 was conducted in March.

[b] Values are means ± standard deviation of five determinations. Within the same column, means with the same letter are not significantly different ($P > 0.05$). Values in parentheses are the reduction of *V. parahaemolyticus* after treatments.

TABLE 2. *Efficacy of refrigerated seawater (5°C) depuration for reducing* Vibrio parahaemolyticus *in Pacific oysters harvested in the summer*[a]

Time (h)	*V. parahaemolyticus* population (log MPN/g)[b]	
	Study 1	Study 2
0	6.09 ± 0.39 A	5.92 ± 0.38 A
24	4.79 ± 0.37 B (1.31)	4.88 ± 0.61 B (1.04)
48	4.69 ± 0.85 B (1.40)	3.90 ± 0.55 C (2.02)
72	4.44 ± 0.19 B (1.65)	3.21 ± 0.39 CD (2.71)
96	3.52 ± 0.49 C (2.57)	3.15 ± 0.34 D (2.77)
120	3.34 ± 0.44 CD (2.75)	3.08 ± 0.41 D (2.84)
144	2.87 ± 0.38 D (3.22)	2.92 ± 0.31 D (3.00)

[a] Study 1 was conducted in July, and study 2 was conducted in August.

[b] Values are means ± standard deviation of five determinations. Within the same column, means with the same letter are not significantly different ($P > 0.05$). Values in parentheses are the reduction of *V. parahaemolyticus* after treatments.

Survival of oysters at room and refrigeration temperatures after refrigerated seawater depuration. Oysters depurated in refrigerated seawater for 96 to 144 h were transferred to fresh ASW containing marine microalgae and held at room temperature for 2 days. Oysters with shells that were open in the water and did not close upon touch were considered dead. Oysters that had been depurated in refrigerated seawater for 144 h and nondepurated oysters were stored in a cold room (5°C) for up to 17 days to study their ability to survive refrigeration. Oyster mortality was checked daily by knocking each oyster on its shell. Oysters whose shells opened upon knocking were considered dead.

Statistical analysis. Results of microbiological tests were log transformed for statistical analyses. Bacterial populations in oysters at different sampling times were analyzed with a two-sample *t* test (S-plus, Insightful Corp., Seattle, WA). Differences between treatment means were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Efficacy of refrigerated seawater depuration for reducing *V. parahaemolyticus* in Pacific oysters. The efficacy of refrigerated seawater (5°C) depuration treatment for reducing *V. parahaemolyticus* in Pacific oysters harvested in the winter is reported in Table 1. *V. parahaemolyticus* levels in oysters were reduced by 1.25 to 1.48 log MPN/g after 24 h of depuration. Reductions of *V. parahaemolyticus* in oysters increased to 2.28 to 2.57 log MPN/g after 48 h and to 3.09 to 3.49 log MPN/g after 96 h. These results indicate that depuration of Pacific oysters in refrigerated seawater (5°C) for 96 h can reduce *V. parahaemolyticus* by >3.0 log MPN/g in oysters harvested in the winter. However, the process was less effective for reducing *V. parahaemolyticus* in oysters harvested in the summer (Table 2). Populations of *V. parahaemolyticus* in Pacific oysters harvested in the summer were reduced by 1.04 to 1.31 log MPN/g after 24 h of depuration, and reductions of *V. parahaemolyticus* in these oysters gradually increased to 2.57 to 2.77 log MPN/g after 96 h. Extending the process to 144 h was required to achieve ≥3.0 (3.00 to 3.22)–log reductions of *V. parahaemolyticus* in summer oysters. These results suggest that depuration with refrigerated seawater could be utilized as a postharvest treatment to reduce levels of *V. parahaemolyticus* in Pacific oysters. However, the reductions were slightly smaller than the 3.52-log reduction recommended by the National Shellfish Sanitation Program *Guide for the Control of Molluscan Shellfish* for validation and/or verification of postharvest processing for *V. parahaemolyticus* and *Vibrio vulnificus* *(24)*.

The present study was conducted with bacteria grown in a laboratory. Richards *(19)* suggested that naturally occurring *V. parahaemolyticus* in oysters might be more resistant to the effects of depuration than those grown in a laboratory. Therefore, further studies are needed to identify an optimal depuration condition capable of reducing *V. parahaemolyticus* in oysters to <30 MPN/g with >3.52-log reductions.

Efficacy of UV light treatment for inactivating *V. parahaemolyticus* in recirculated water. In a recirculating depuration system, the water must be sterilize to prevent any *V. parahaemolyticus* released from the oysters into the water during the depuration process from becoming a source of recontamination of the depurated oysters. Analysis of water samples collected at each test time during depuration revealed small amounts of *V. parahaemolyticus* (generally ≤23 MPN/ml by the five-tube MPN method) in the water within the first 24 h of depuration. However, no *V. parahaemolyticus* (<1.8 MPN/g) was detected in any water samples analyzed after 24 h of the depuration process. This finding indicates that the UV treatment was able to

TABLE 3. *Survival of oysters during refrigerated seawater (5°C) depuration (96 to 144 h) and during room temperature storage postdepuration*[a]

Trial	Study 1	Study 2	Study 3	Study 4	Study 5	Study 6	Study 7	Study 8	Study 9
Depuration time (h)	96	96	96	96	96	144	144	144	144
No. of oysters used for depuration	60	60	60	60	60	55	55	55	55
No. of oysters that died during depuration	0	0	0	0	0	6[b]	0	0	0
No. of oysters used for survival study	15	15	15	15	15	14	15	15	15
No. of oysters that survived for 2 days	15	15	15	15	15	14	15	15	15

[a] Studies 1 through 5 were conducted between November and March, and studies 6 through 9 were conducted between June and August.

[b] All six oysters died between 120 and 144 h of depuration.

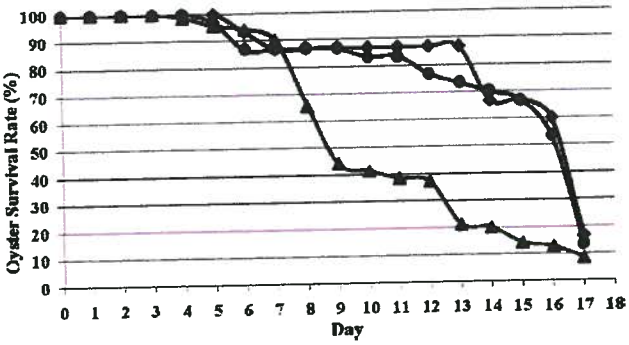

FIGURE 1. *Survival of oysters depurated in refrigerated seawater (5°C) for 144 h and stored at 5°C (◆, study 1; ●, study 2). Nondepurated oysters (▲) were used as controls.*

inactivate *V. parahaemolyticus* released from oysters into the water during the process.

Survival of oysters during refrigerated seawater depuration and holding at room or refrigeration temperature. A total of nine studies (55 to 60 oysters per study) were conducted to assess the ability of oysters to survive the refrigerated seawater depuration and storage at room or refrigeration temperature after the depuration process. All oysters survived 96 h of depuration, and only six oysters died between 120 and 144 h in one of four studies that lasted for 144 h (Table 3). When oysters surviving depuration were transferred to ASW containing microalgae at room temperature, all oysters were able to filter water for nutrients, as indicating by opening their shells (and closing upon touch), and remained alive for at least 2 days. It was not clear why six oysters died after 120 h of depuration at 5°C in one study. One possible explanation is that those oysters had other health problems or severe nutritional deficiency, which made them more sensitive to the low temperature. Nevertheless, nearly 99% (514 of 520) of the oysters studied were able to survive in refrigerated seawater (5°C) for 4 to 6 days.

Oysters that had been depurated in refrigerated seawater for 144 h survived better at the refrigeration temperature (5°C) than did those oysters that did not undergo the depuration process (Fig. 1). Both depurated and nondepurated oysters had similar survival rates during the first 7 days of storage at 5°C. However, a sudden decrease in the survival rate was observed for nondepurated oysters, from 90% after 7 days to 44% after 9 days of storage, whereas the survival rate of depurated oysters remained at >80% for 11 days. The survival rates of depurated and nondepurated oysters dropped to 67 and 14%, respectively, after 15 days of storage at 5°C. The rapid decline in the survival rate of depurated oysters after 15 days of storage at 5°C might be related to severe starvation. These results indicated that the refrigerated seawater depuration could enhance the oyster's ability to survive in a low-temperature environment.

In conclusion, depurations with refrigerated seawater (5°C) for 96 and 144 h achieved ≥3.0-log reductions of *V. parahaemolyticus* populations in Pacific oysters harvested in the winter and summer, respectively. Refrigerated seawater depuration could be applied as a postharvest treatment to reduce *V. parahaemolyticus* contamination in Pacific oysters without noticeable adverse effects. Further studies are needed to validate the efficacy of the depuration process for reducing naturally accumulated *V. parahaemolyticus* in Pacific oysters.

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