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Journal of Food Protection, Vol. 69, No. 8, 2006, Pages 1829–1834
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Effects of Electrolyzed Oxidizing Water Treatment on Reducing *Vibrio parahaemolyticus* and *Vibrio vulnificus* in Raw Oysters

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MS 05-637: Received 15 December 2005/Accepted 25 March 2006

ABSTRACT

Contamination of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in oysters is a food safety concern. This study investigated effects of electrolyzed oxidizing (EO) water treatment on reducing *V. parahaemolyticus* and *V. vulnificus* in laboratory-contaminated oysters. EO water exhibited strong antibacterial activity against *V. parahaemolyticus* and *V. vulnificus* in pure cultures. Populations of *V. parahaemolyticus* (8.74×10^7 CFU/ml) and *V. vulnificus* (8.69×10^7 CFU/ml) decreased quickly in EO water containing 0.5% NaCl to nondetectable levels (>6.6 log reductions) within 15 s. Freshly harvested Pacific oysters were inoculated with a five-strain cocktail of *V. parahaemolyticus* or *V. vulnificus* at levels of 10^4 and 10^6 most probable number (MPN)/g and treated with EO water (chlorine, 30 ppm; pH 2.82; oxidation-reduction potential, 1131 mV) containing 1% NaCl at room temperature. Reductions of *V. parahaemolyticus* and *V. vulnificus* in oysters were determined at 0 (before treatment), 2, 4, 6, and 8 h of treatment. Holding oysters inoculated with *V. parahaemolyticus* or *V. vulnificus* in the EO water containing 1% NaCl for 4 to 6 h resulted in significant ($P < 0.05$) reductions of *V. parahaemolyticus* and *V. vulnificus* by 1.13 and 1.05 log MPN/g, respectively. Extended exposure (>12 h) of oysters in EO water containing high levels of chlorine (>30 ppm) was found to be detrimental to oysters. EO water could be used as a postharvest treatment to reduce *Vibrio* contamination in oysters. However, treatment should be limited to 4 to 6 h to avoid death of oysters. Further studies are needed to determine effects of EO water treatment on sensory characteristics of oysters.

The United States produces more than 27 million pounds (ca. 12.3 million kilograms) of oysters each year, and most of them are sold and consumed raw without further processing *(11).* Oysters can be easily contaminated with spoilage and pathogenic bacteria such as *Vibrio parahaemolyticus* and *Vibrio vulnificus* through contaminated seawater. Growth of naturally occurring bacteria in oysters during storage and retail sale results in loss of quality, reduced shelf life, and potential human gastroenteritis.

V. parahaemolyticus and *V. vulnificus* occur naturally in the marine environment and are commonly found in shellfish *(3, 7, 8, 19).* These organisms are the leading causes of foodborne infections associated with seafood consumption in the United States *(2).* Recent outbreaks of *V. parahaemolyticus* infections associated with raw oyster consumption in several regions of the United States *(5, 6, 21)* indicate a need of developing effective postharvest processes for reducing these pathogens in oysters for safe consumption.

Several postharvest treatments including low-temperature pasteurization, rapid chilling, freezing, high-pressure processing, irradiation, and heat shock have been reported to be capable of achieving certain degrees of reductions of these pathogens *(1, 4, 12, 24, 25).* However, most of these processes require either significant amounts of initial investment or major effort on personnel training, and oysters are often killed during the process.

Depuration is a controlled process allowing shellfish to purge sand and grit from the gut into clean seawater. The process usually leads to a reduction of microbial contaminants in shellfish and therefore increases shelf life of refrigerated products. However, studies have shown that depuration with clean seawater was not effective in reducing certain persistent bacteria, including *Vibrio* spp., in shellfish because of the colonization of those bacteria in the intestinal tract *(9, 14).* Therefore, it limits the usage of conventional depuration as a means for eliminating *Vibrio* contamination in oysters. Replacing clean seawater with a solution exhibiting strong antimicrobial activities might improve the efficacy of the depuration process for reducing *Vibrio* contamination in oysters.

Recently, electrolyzed oxidizing (EO) water generated through electrolysis of a dilute salt solution was introduced as a new antimicrobial agent. Studies have shown that EO water exhibited strong bactericidal effects against many foodborne pathogens, including *Salmonella* Enteritidis, *Listeria monocytogenes, Campylobacter jejuni,* and *Escherichia coli* O157:H7 *(15, 16, 22, 23, 27).* Application of EO water as a disinfectant for reducing microbial contaminations has been reported for fresh fruits and vegetables *(13, 17, 18),* poultry carcasses *(10, 23),* and cutting boards *(28).* These results suggested that EO water might be used in oyster depuration to enhance reduction of *Vibrio* contamination. This study investigated the antimicrobial activity of EO water against *V. parahaemolyticus* and *V. vulnificus* and the potential application of EO water as a postharvest processing to reduce *Vibrio* contamination in oysters.

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MATERIALS AND METHODS

Bacterial cultures preparation. *V. parahaemolyticus* (10290, 10292, 10293, BE 98-2029, and 027-1c1) and *V. vulnificus* (93A3097, 93A4153, 96A6135, ATCC 27562, and DI27-3C) were used in this study. All strains, except *V. vulnificus* ATCC27562 and *V. vulnificus* DI27-3C (isolated from oyster), were clinical isolates obtained from the collection of the Food and Drug Administration Pacific Regional Laboratory Northwest (Bothell, Wash.). Each culture was grown individually in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, Md.) supplemented with 1.5% NaCl (TSB-salt) at 37°C for 18 to 24 h. The cultures were streaked onto individual plates of tryptic soy agar (TSA; Difco, Becton Dickinson) supplemented with 1.5% NaCl (TSA-salt) and incubated at 37°C for 18 to 24 h. A single colony was selected from the TSA-salt plate and enriched in TSB-salt at 37°C for 4 h. The enriched cultures of *V. parahaemolyticus* or *V. vulnificus* were pooled into a sterile centrifuge tube and centrifuged at 3,000 × *g* (Sorvall RC-5B, Kendro Laboratory Products, Newtown, Conn.) at 5°C for 15 min. Pelleted cells were resuspended in 50 ml of salt solution (1%) to produce a culture cocktail of approximately 8.7×10^8 CFU/ml.

EO water production. EO water containing chlorine contents ranging from 10 to 50 ppm ([1] chlorine: 10 ppm, pH 3.17, oxidation-reduction potential [ORP]: 1104 mV; [2] chlorine: 30 ppm, pH: 2.82, ORP: 1131 mV; and [3] chlorine: 50 ppm, pH 2.70, ORP: 1139 mV) was produced with an electrolyzed water generator (model V-500, Electric Aquagenics Unlimited, Inc., Lindon, Utah) according to manufacturer's instruction. EO water was produced on the day of experiments and used within 10 min after production.

Antibacterial activity of EO water against *V. parahaemolyticus* and *V. vulnificus*. The antibacterial activity of EO water against *V. parahaemolyticus* and *V. vulnificus* was determined by mixing 1 ml of the bacterial culture (approximately 8.7×10^8 CFU/ml) with 9 ml of EO water in a sterile tube. Survival of *V. parahaemolyticus* and *V. vulnificus* in EO water was determined at 15, 30, and 60 s after mixing by the pour plate method, using TSA plates with serial dilutions in Butterfield's phosphate buffer (pH 7.2 to 7.4). The plates were incubated at 37°C for 48 h, and colonies formed on plates were counted.

Effects of salt (NaCl) on antibacterial activity of EO water against *V. parahaemolyticus* and *V. vulnificus*. Effects of salt concentrations on antibacterial activity of EO water against *V. parahaemolyticus* and *V. vulnificus* were determined by mixing 1 ml of *Vibrio* culture cocktail with 9 ml of EO water (10, 30, or 50 ppm chlorine) containing various amounts of NaCl (0.5, 1, 1.5, and 2%). Survival of *V. parahaemolyticus* and *V. vulnificus* in EO water was determined at 15, 30, 60, 90, and 120 s with the pour plate method by using TSA-salt plates and incubation at 37°C for 48 h. Optimal EO water and salt combination on inactivating *V. parahaemolyticus* and *V. vulnificus* was selected for oyster treatments.

Oyster preparation. Freshly harvested Pacific oysters were obtained from Oregon Oyster Farm (Yaquina Bay, Newport, Oreg.) and delivered immediately in a cooler with ice gels to the laboratory for experiments. The oysters were washed briefly with tap water to remove mud on the shell and placed in a rectangular high-density polyethylene tank (18 by 12 by 12 in.; Nalge, Rochester, N.Y.) containing artificial seawater ([ASW] salinity: 29.6 ppt) at room temperature for 3 to 4 h before being inoculated with *Vibrio* spp. The ASW was prepared by dissolving Instant Ocean Salts (Aquatic Eco-System, Inc., Apopka, Fla.) in deionized water according to manufacturer's instructions.

Inoculation of oysters with *Vibrio* spp. Forty oysters were transferred from the ASW to a similar high-density polyethylene tank of fresh ASW containing *V. parahaemolyticus* or *V. vulnificus* culture cocktail at a level of approximately 10^4 CFU/ml. The inoculation was conducted at room temperature overnight (12 to 14 h), with water being circulated at a flow rate of 11 liters/h. Air was pumped into the solution to facilitate colonization of *Vibrio* in oysters. A higher level of inoculation was conducted in ASW containing *V. parahaemolyticus* or *V. vulnificus* at a level of approximately 10^6 CFU/ml. Oysters and ASW were analyzed for *V. parahaemolyticus* or *V. vulnificus* contamination with a three-tube most-probable-number (MPN) method before the inoculation.

EO water treatment. Inoculated oysters were placed in a tank of EO water (30 or 50 ppm chlorine) containing 1% NaCl and transferred to freshly generated EO water every hour. The ratio of EO water to oyster was maintained at 1 liter of EO water for every four oysters. Total chlorine in EO water was determined immediately after being generated with a commercial chlorine detection kit (HACH Company, Loveland, Colo.). The pH and ORP of EO water were measured with a pH meter (model 420A, Orion Research, Inc., Boston, Mass.) and an ORP meter (CheckmateII Systems with Redox Sensor, Corning, Inc., Corning, N.Y.), respectively. Inoculated oysters held in ASW were used as controls.

Microbiological tests. Populations of *V. parahaemolyticus* and *V. vulnificus* in inoculated oysters held in EO water were analyzed before the treatment and at 2, 4, 6, and 8 h with a three-tube MPN method described in the U.S. Food and Drug Administration's *Bacteriological Analytical Manual (26),* using thiosulfate–citrate–bile salts–sucrose agar (TCBS) for *V. parahaemolyticus* or modified cellobiose polymyxin colistin agar (mCPC) for *V. vulnificus* determination. At each testing time, three oysters were randomly removed from the EO water tank and shucked with a sterile shucking knife in a sterile stainless tray. Each shucked oyster meat was placed in a sterile Whirl-Pak filter bag (Nasco, Modesto, Calif.), followed by addition of nine volumes of sterile alkaline peptone water. The oyster samples were homogenized with a stomacher (Seward Stomacher 400, Brinkmann, Westbury, N.Y.) at 230 rpm for 1 min to prepare a 1:10 sample suspension. Two additional 10-fold dilutions of each oyster sample were prepared with sterile alkaline peptone water. 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) of enriched APS from the top 1 cm of a turbid tube was streaked onto individual TCBS for *V. parahaemolyticus* detection, or onto mCPC for *V. vulnificus* detection. All plates were incubated at 35 to 37°C for 18 to 24 h. Formation of colonies that are round (2- to 3-mm diameter) and green or bluish on TCBS or colonies that are round (1- to 2-mm diameter), flat, and yellow on mCPC are considered positive for *V. parahaemolyticus* or *V. vulnificus,* respectively. Total populations of *V. parahaemolyticus* or *V. vulnificus* in oysters were determined by converting numbers of APS tubes that were positive for *V. parahaemolyticus* or *V. vulnificus* to MPN/g, using an MPN table. Results were reported as means of triplicate determinations.

Statistical analysis. Results of microbiological tests were transformed into log values for statistical analyses. Bacterial populations in oysters at different treatment times were analyzed with two-sample *t* test (S-Plus, Insightful Corp., Seattle, Wash.). Sig-

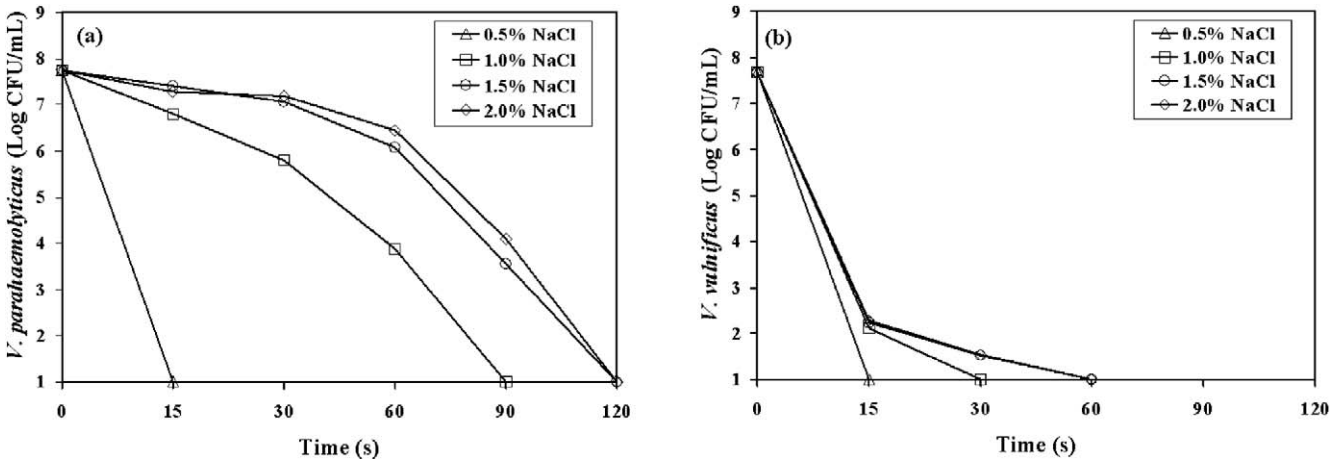

FIGURE 1. *Survival of (a)* V. parahaemolyticus *and (b)* V. vulnificus *in EO water (chlorine, 10 ppm; pH 3.17; ORP, 1104 mV) containing sodium chloride. Data are means of three determinations.*

nificant differences between means of treatments were established at a level of $P = 0.05$.

RESULTS AND DISCUSSION

Antibacterial activity of EO water against *V. parahaemolyticus* and *V. vulnificus* cultures. EO water exhibited strong antibacterial activity against pure cultures of *V. parahaemolyticus* and *V. vulnificus.* Populations of *V. parahaemolyticus* (7.74 log CFU/ml) and *V. vulnificus* (7.69 log CFU/ml) decreased very quickly in EO water containing ≥10 ppm of total chlorine. No culturable *V. parahaemolyticus* or *V. vulnificus* was detected after 15 s of mixing with EO water (>6.6-log reduction) (data not shown). The antibacterial activities of EO water against *V. parahaemolyticus* and *V. vulnificus* were found similar to those against other foodborne pathogens such as *L. monocytogenes, E. coli* O157:H7, and *C. jejuni.* Kim et al. *(15)* reported that cells of *L. monocytogenes* and *E. coli* O157:H7 were both reduced by >8 log CFU/ml after being added to EO water containing 10 ppm chlorine at 24°C for 10 s. A study conducted by Park et al. *(23)* showed that populations of *C. jejuni* decreased rapidly by >7 log CFU/ml within 10 s in EO water containing 25 ppm chlorine at 23°C.

Effects of salt concentrations on antibacterial activity of EO water against *V. parahaemolyticus* and *V. vulnificus*. Addition of at least 1% salt to EO water containing low level of chlorine (10 ppm) enhanced the survival of both *V. parahaemolyticus and V. vulnificus* in the water (Fig. 1). Although populations of *V. parahaemolyticus* and *V. vulnificus* also decreased to nondetectable levels in EO water containing 0.5% NaCl within 15 s, cells of both species were detected in EO water containing ≥1% salt after 15 s. *V. parahaemolyticus* were detected in EO water containing 1% NaCl after 60 s and in EO water containing ≥1.5% NaCl after 90 s. However, no culturable *V. parahaemolyticus* was detected in EO water containing 1.5 or 2% NaCl after 120 s. Similar results were obtained for *V. vulnificus.* Cells of *V. vulnificus* were detected in EO water containing 1.5 or 2% after 30 s. However, no culturable *V. vulnificus* was detected in any of the EO water after 60 s. These results indicated that *V. parahaemolyticus* was more resistant than *V. vulnificus* was to EO water, and addition of salt to EO water could decrease antibacterial activity of EO water against both *V. parahaemolyticus* and *V. vulnificus.* A study conducted by Andrews et al. *(1)* also found that *V. vulnificus* was more sensitive than *V. parahaemolyticus* was to irradiation. Irradiations at doses of 1.5 and 2 kGy, respectively, were required to reduce populations of *V. vulnificus* (MO-624) and *V. parahaemolyticus* O3:K6 (TX2103) in pure broth cultures from 10^7 cells per ml to nondetectable levels.

It was not clear if the reduced antibacterial activity of salt-containing EO water was caused by interactions between NaCl and antimicrobial components in EO water. Studies conducted by Liu et al. *(20)* demonstrated that the bactericidal activity of EO water against bacterial cells was mainly related to its chlorine contents. However, no apparent changes of chlorine contents in EO water were found in this study after salt addition (data not shown). Another possibility is that the addition of salt to EO water enhanced the survival of both *V. parahaemolyticus* and *V. vulnificus* in the water. Both species are halophilic and require certain amounts of salt to grow.

The reduced antibacterial activity of EO water against *Vibrio* cells caused by addition of salt was not observed when the chlorine contents in EO water increased to 30 or 50 ppm. No culturable cells of *V. parahaemolyticus* or *V. vulnificus* were detected in the EO water containing 0.5 to 2% NaCl after 15 s (data not shown). To minimize effects of salt on the antibacterial activity of EO water against *V. parahaemolyticus* and *V. vulnificus,* EO water containing chlorine concentrations of 30 and 50 ppm was used for oyster treatments.

Effects of EO water treatment on reducing *V. parahaemolyticus* and *V. vulnificus* in oysters. One concern of utilizing EO water as a postharvest treatment for reducing *Vibrio* contamination in oysters is that oysters might not survive in an acidic and chlorine-containing environment. Our initial studies of holding oysters in EO water containing 50 ppm chlorine found that exposure of oysters

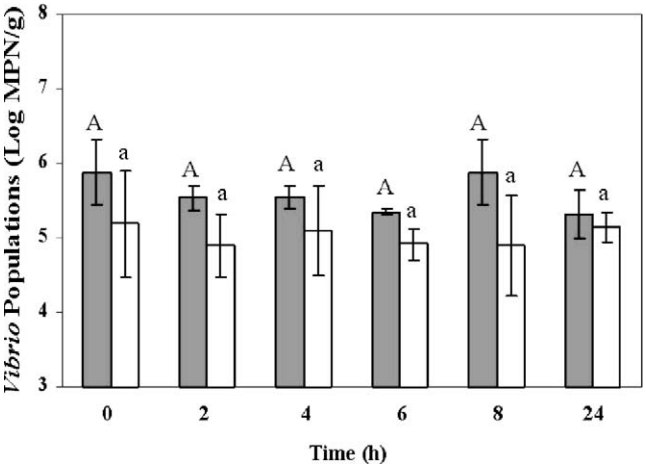

FIGURE 2. *Effects of artificial seawater (salinity: 29.6 ppt) treatments on oysters inoculated with* V. parahaemolyticus *(solid bars) and* V. vulnificus *(hollow bars). Data are means of three determinations ± standard deviations. Means with the same letter are not significantly different (*P > 0.05*).*

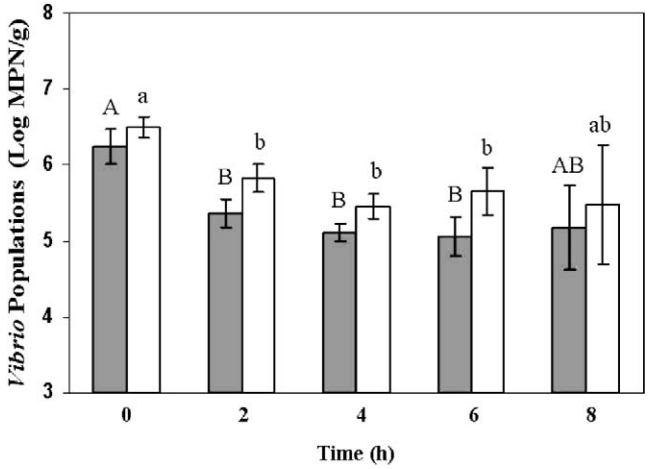

FIGURE 3. *Effects of EO water (chlorine, 30 ppm; pH 2.82; ORP, 1131 mV) treatment on reducing* V. parahaemolyticus *(solid bars) and* V. vulnificus *(hollow bars) in laboratory-inoculated oysters. Initial populations in oysters: V.* parahaemolyticus *(log 6.24 MPN/g),* V. vulnificus *(log 6.50 MPN/g). Data are means of three determinations ± standard deviations. Means with the same letter are not significantly different (*P > 0.05*).*

to EO water for an extended period was harmful to oysters. Many oysters died in EO water (50 ppm chlorine) after 12 h. However, oysters were able to survive in EO water containing 30 ppm chlorine for more than 12 h. Therefore, EO water containing 30 ppm chlorine was selected for decontamination of oysters inoculated with *V. parahaemolyticus* and *V. vulnificus.*

Another big challenge of applying EO water to oyster processing is that the water needs to be circulated through the digestive tract of oysters in order to inactivate colonized pathogens. Because oysters are grown in estuaries and marine environments, the presence of salt in EO water might promote the filtration activity of oysters and allow circulation of EO water through the digestive tract. However, addition of salt to EO water was found to decrease the bactericidal effects of EO water on both *V. parahaemolyticus* and *V. vulnificus.* To minimize the negative salt effects, salt concentration in EO water (30 ppm chlorine) was set at 1% for our study.

Holding laboratory-contaminated oysters in ASW free of *Vibrio* for 24 h did not yield apparent reductions of *V. parahaemolyticus* or *V. vulnificus* in the oysters (Fig. 2). This agrees with previous reports that depuration with clean seawater was ineffective in reducing *Vibrio* contamination in shellfish. Eyles and Davey *(9)* found no significant difference in mean counts of naturally occurring *V. parahaemolyticus* between depurated and nondepurated oysters. Kelly and Dinuzzo *(14)* reported that oysters required 16 days to depurate laboratory-contaminated *V. vulnificus* to nondetectable levels. However, holding contaminated oysters in EO water (30 ppm chlorine) containing 1% NaCl for a few hours resulted in significant reductions of *V. parahaemolyticus* and *V. vulnificus* in oysters (Fig. 3). Populations of *V. parahaemolyticus* were significantly reduced by 0.87 log MPN/g after 2 h of EO water treatment. The reduction increased to 1.13 log MPN/g after 4 h and remained at a similar level through 8 h. Similar results were observed when oysters were inoculated with *V. vulnificus* and held in EO water containing 30 ppm chlorine and 1% NaCl. Populations of *V. vulnificus* in oysters were significantly reduced by 0.68 log MPN/g after 2 h. The reduction increased to the highest level (1.05 log MPN/g) after 4 h and remained at a similar level through 8 h.

Similar results were observed when oysters were inoculated with a lower level of *V. parahaemolyticus* (log 4.47 MPN/g) and *V. vulnificus* (log 4.00 MPN/g). The greatest reductions of *V. parahaemolyticus* (log 1.58 MPN/g) and *V. vulnificus* (log 0.83 MPN/g) in oysters were observed after 4 and 8 h in EO water (30 ppm chlorine and 1% NaCl), respectively (Fig. 4). It was not clear why the EO water treatment did not yield a significant reduction of

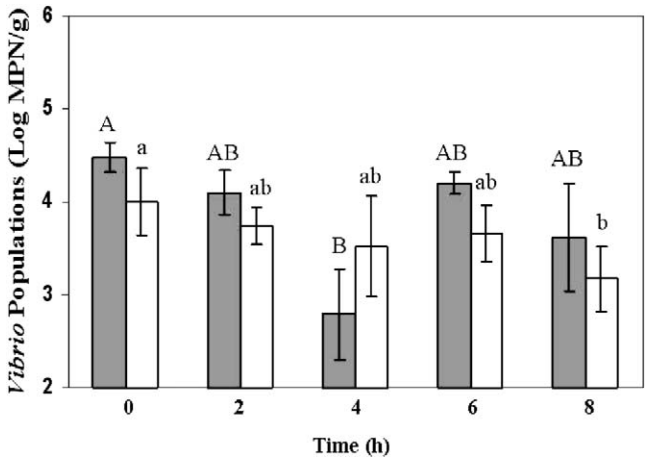

FIGURE 4. *Effects of EO water (chlorine, 30 ppm; pH 2.82; ORP, 1131 mV) treatment on reducing* V. parahaemolyticus *(solid bars) and* V. vulnificus *(hollow bars) in laboratory-inoculated oysters. Initial populations in oysters:* V. parahaemolyticus *(log 4.47 MPN/g),* V. vulnificus *(log 4.00 MPN/g). Data are means of three determinations ± standard deviations. Means with the same letter are not significantly different (*P > 0.05*).*

V. vulnificus in oysters until 8 h. A hypothesis is that this group of oysters was less active in filtering water and, therefore, required a longer time to allow the treatment to yield a significant reduction of *Vibrio* in oysters.

It is believed that the acidity and chlorine of EO water create an unfavorable growth environment for oysters. Therefore, oysters could be forced to stop the water-filtering activity after a few hours of exposure to EO water. This may explain why the reduction of *Vibrio* cells in oysters reached the highest level after 4 h in EO water and no further reductions were observed afterward. Whereas a short-term (<8 h) treatment with EO water (30 ppm chlorine and 1% NaCl) was found to be capable of reducing *Vibrio* contamination in oysters, exposure of oysters to the EO water for an extended period should be avoided. Our studies also found that holding oysters in the EO water (30 ppm chlorine) for 24 h could result in deaths of oysters.

It was not clear if the detrimental effect was related to the acidity or chlorine of EO water. The chlorine contents in EO water decreased from 30 ppm to less than 10 ppm after 1 h, whereas the pH value increased only slightly by 0.2 to 0.3 units. There was no apparent change in ORP value during the treatment. Because chlorine is one of the major components contributing to EO water's antimicrobial activity, it is critical to keep the chlorine content in EO water at a level that is high enough to allow a reduction of *Vibrio* cells in oysters, without killing oysters during the treatments. This study demonstrated that *V. parahaemolyticus* and *V. vulnificus* in oysters could be reduced by treatment with EO water containing 30 ppm chlorine and 1% NaCl. Although the treatment resulted in only about 1 log MPN/g of reduction of *V. parahaemolyticus* or *V. vulnificus* in oysters, such a reduction would decrease the possibility of *Vibrio* infections associated with raw oyster consumption.

In conclusion, contamination of *V. parahaemolyticus* and *V. vulnificus* in raw oysters could be reduced by holding oysters in EO water containing 30 ppm chlorine and 1% NaCl in 4 to 6 h. However, treatment of oysters with EO water should be limited to <8 h to avoid the death of oysters. Further studies are needed to improve the efficacy of EO water treatment on reducing *Vibrio* in oysters and to determine effects of EO water treatments on sensory characteristics of oysters.

ACKNOWLEDGMENTS

This study was supported by the National Sea Grant Gulf Industry Program of National Oceanic and Atmospheric Administration (NOAA), grant no. NA04OAR4170032, and the Interstate Shellfish Sanitation Conference (ISSC) Project "Laboratory assessment of the relation between water temperature, immersion time and exposure to bactericidal oxidation products on *Vibrio parahaemolyticus* levels in Pacific oysters," awarded in 2006. The authors are sincerely grateful to PRLNW of FDA (Bothell, Wash.) for providing bacterial cultures.

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