Effectiveness of superchilling storage for control of naturally occurring *Vibrio cholerae* and *Escherichia coli* in shellstock Eastern oysters (*Crassostrea virginica*) depurated with ozonated seawater.

V.T. Pardío[1]*, K.N. Waliszewski[2], S.R. Rodríguez[1], D. I. Martínez[1], D. Romero[1], K.M. López[1], I. Rivas[2], V. Suárez and R. Uscanga[1]

[1] Facultad de Medicina Veterinaria y Zootecnia, Universidad Veracruzana, Avenida Circunvalación esquina Yáñez S/N, Colonia Unidad Veracruzana, Veracruz, Ver. CP. 91710 (vpardio@uv.mx, vpardío@yahoo.com.mx)
[2] Unidad de Investigación y Desarrollo en Alimentos, Instituto Tecnológico de Veracruz, Avenida Miguel Ángel de Quevedo 2779, Colonia Formando Hogar, Veracruz, Ver. CP. 91870 (kw@itver.edu.mx)
* corresponding author

Introduction

According to Lizárraga-Partida and Quilici (2009) the Mexican Gulf Coast may act as an environmental reservoir of *Vibrio cholerae* O1. Being aquatic bacteria naturally present in coastal environments, *V. cholera* has been implicated in foodborne illness linked to consumption of undercooked or raw shellfish. Consumption of raw oyster in México accounted for 44,380 ton in 2009 (CONAPESCA, 2010). Our previous studies revealed a high prevalence of *V. cholerae* O1 and *E. coli* in oysters (*C. virginica*) harvested from estuarine lagoons of Veracruz State, Mexico, in the summer months, implicating a potential public health hazard (Castañeda *et al*., 2005; Pardío *et al*., 2008). Depuration with ozonated water is an effective postharvest process for shellfish moderately contaminated with faecal coliform bacteria, and its efficacy in reducing *Vibrio cholerae* in *C. virginica* harvested from highly contaminated waters has not been conclusively quantified. Moreover, unsuitable methods of preserving molluscs after depuration could create particularly favourable conditions for the proliferation of vibrios. Refrigeration has been the most commonly used method for preserving quality and extending shelf life of shellstock and shucked oysters (Andrews 2004), but the fact that *Vibrios*, *V. cholera* in particular, can survive under chilled and frozen conditions for up to 4 weeks on shellfish (Januario and Dykes, 2005), suggests that alternative methods for decontaminating oysters may need to be found. The aim of his study was to determine the antimicrobial efficacy of ozone depuration process followed by superchilling storage on *V. cholera* and *E. coli* loads of naturally polluted shellstock American raw oysters.

Materials and Methods

American oysters (*Crassostrea virginica*) (5 - 6 g, 9 cm long) were collected from producing banks of harvesting areas in Mandinga lagoon, Veracruz, México, immediately transported to the laboratory, washed by highpressure water spray to remove surface debris and attached algae, and damaged or gaping animals were discarded. The oysters were placed in closed pilot-scale recirculating seawater system (9 L/min, $27^0/_{00}$ salinity, 90% dissolved oxygen, 25.0°C, and pH 8.0) within 2 h of harvesting and depurated with clean seawater during 2-4 hours to promote oyster activity and to increase filtration rates. Afterwards, seawater was disinfected by ozone generated by corona discharge (Enaly ozonator model 1000BT-12) at 0.2, 0.4, and 0.6 mg/L. Dissolved ozone was measured by the indigo method (Bader and Hoigné, 1981). Each experimental trial consisted of 2 tanks with ozonated seawater and one control tank with no-ozonated seawater, each tank loaded with 250 oysters placed into plastic mesh baskets as single overlapping layer, and depuration started within 4-6 h of shellfish collection. Duplicate samples of 30 oysters from aleatory locations in each tank were collected at 0, 2, 4, and 6 h and all microbial analyses began within 2 h of

collection. Oysters were opened, and meats and liquors were pooled. Oyster samples were analyzed according to approved method for *E. coli* by the Mexican Ministry of Health (PROY-NOM-242-SSA1-2005) and data were expressed as MPN/100g. *V. cholerae* was performed according U.S. Food and Drug Administration methodologies (FDA, 2004) and expressed as percent isolation. Presumptive *V. cholerae* isolates were confirmed with polyvalent antisera Serobac (Difco Laboratories Ltd., Franklin Lakes, NJ, USA) to identify the O1 serogroup, and the serotype was identified using Inaba and Ogawa specific antisera. When there was no agglutination with the polyvalent antisera, the presence of *V. cholerae* non O1 was reported. Results from the most efficient depuration treatments were repeated and oysters depurated samples were placed in covered plastic bins to prevent drying, then stored under superchilling temperature chamber (-1ºC), and analyzed at 5, 9, and 14 day of storage. Results were analyzed for significant differences ($p<0.05$) using the statistical software Minitab 13.0.

Results and Discussion

Table 1 shows the results of the depuration experiments. *E. coli* levels declined in the first 2 h of depuration, and after 4 h the MPN decreased 88, 50, and 64% of its original concentration, and 88, 50 and 82% after 6 h of depuration when 0.2, 0.4, and 0.6 mg/L of ozone were applied, respectively. In contrast, *E. coli* in oyster control samples decreased to 33.3% during 4 h of depuration, and increased to 650 MPN/100g at 6 h. Meanwhile, *Vibrio cholerae* non O1 was not detected after 4 and 6 h of depuration with 0.2 and 0.4 mg/L of ozone, respectively, and after 4 h of depuration with 0.6 mg/L, reaching the legally bacteriological limit of No detection of *V. cholerae* non O1 in 50 g of oyster flesh (PROY-NOM-242-SSA1-2005).

Table 1. Effect of ozone depuration on American oyster naturally contaminated with *Escherichia coli* and *Vibrio cholerae*

	E. coli (MPN/100 g)				*V. cholerae* non O1 (% isolation)			
Time (hour)	Control	0.2 (mg O_3/L)	0.4 (mg O_3/L)	0.6 (mg O_3/L)	Control	0.2 (mg O_3/L)	0.4 (mg O_3/L)	0.6 (mg O_3/L)
0	600^a	340^b	40^c	110^c	100	100	100	100
2	200^a	60^b	20^c	40^c	100	100	100	100
4	200^a	40^b	20^c	70^b	100	0	100	0
6	650^a	40^b	20^c	20^c	100	0	0	0

Means with different letter are significantly different ($P < 0.05$) among columns.

As it can be observed, oysters released *E. coli* rapidly after 2 h of depuration and reached the legally bacteriological limits of *E. coli* < 230 MPN/100 g (PROY-NOM-242-SSA1-2005), indicating that *E. coli* was more sensitive than *V. cholera* non O1. Croci *et al.* (2002) depurated blue mussels (*Mytilus galloprovincialis*) with 50 mg/h of ozone and found that MPN of *E. coli* fell to 42% of its original concentration after 5 h of depuration, meanwhile *V. cholerae* was reduced by 1 log after 24 h of depuration, and this level remained almost constant even for 44 h. This faster reduction of *E. coli* would induce to adopt short depuration times although *V. cholerae* non O1 would be present, representing a potential health hazard.

The most effective depuration treatments were stored at -1ºC and results are presented in Table 2. This superchilling temperature significantly reduced and controlled the levels of *E. coli* in depurated oysters at both ozone concentrations throughout the time of storage when compared to control oysters, but *V. cholerae* non O1 was still possible to be isolated from oysters depurated with 0.4 mg/L at 14 day, and at 9 and

14 days from oysters depurated with 0.6 mg/L of ozone. Cava *et al*. (2001) inoculated ice samples with populations of 10^9 ufc/ml of *V. cholerae* O1, and found that the inoculated population decreased by six to seven log units after 21 days and non viable cells were recovered after 30 days, being freezing at -5ºC the most damaging process to *V. cholerae* O1 cells. Hood *et al*. (1983) reported that *V. cholerae* O1 levels in shellstock oyster were significantly higher after 7 days at 2ºC.

Table 2. Changes of *Escherichia coli* and *Vibrio cholerae* in naturally contaminated oyster depurated with ozonated seawater and stored at -1ºC

	E. coli (MPN/100 g)			*V. cholerae* non O1 (% isolation)		
Time (days)	Control	0.4 (mg O_3/L)	0.6 (mg O_3/L)	Control	0.4 (mg O_3/L)	0.6 (mg O_3/L)
0	60 ± 14^a	60 ± 28^a	65 ± 35^a	100	100	0
5	80 ± 14^a	30 ± 14^a	30 ± 14^a	100	0	0
9	330 ± 46^a	20 ± 0^b	80 ± 14^b	100	0	100
14	110 ± 28^a	95 ± 0^b	70 ± 7^b	100	100	100

Means with different letter are significantly different ($P < 0.05$) among columns.

Conclusions

Oysters depurated with 0.4 and 0.6 mg O_3/L during 6h may be safe for human consumption during 5 days of storage at -1ºC. These findings are of importance as *V. cholerae* non O1 populations were decreased to non-culturable levels by the combination of ozone depuration and the superchilling storage (-1ºC). Nevertheless, a long term survival (≥9 days) of *V. cholera* non O1 in cold-stored oysters would afford a significant public health risk to raw oyster consumers.

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