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Running title: Risk analysis of oyster depuration

Analysis of *Vibrio vulnificus* infection risk when consuming depurated raw oysters

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ABSTRACT

A beta Poisson dose-response model for *Vibrio vulnificus* food poisoning cases leading to septicemia was used when evaluating the effect of 15°C depuration on the estimated risk of raw oyster consumption. Statistical variability sources included *V. vulnificus* load at harvest, time and temperature during harvest and transportation to processing plants, decimal reductions (SV) observed during experimental circulation depuration treatments, refrigerated storage time before consumption, oyster size, and number of oysters per consumption event. Although reaching non-detectable *V. vulnificus* levels (<30 MPN/g) throughout the year and a 3.52 SV were estimated not possible at 95% confidence, depuration for 1, 2, 3, and 4 d would reduce the warm (Jun-Sep) season risk from 2,669 cases to 558, 93, 38, and 47 cases per 100 million consumption events, respectively. At 95% confidence, 47 and 16 h depuration would reduce the warm and transition (Apr-May, Oct-Nov) season risk, respectively, to 100 cases per 100 million consumption events assumed to be an acceptable risk, while 1 case per 100 million events would be the risk when consuming untreated raw oysters in the cold (Dec-Mar) season.

Pathogens frequently present in oysters include *Vibrio* species and noroviruses *(6, 17)*. Among *Vibrio* species, 11 can cause human disease including *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* causing severe illnesses *(19)*. During warm seasons, these halophilic Gram-negative bacteria can reach high numbers in oyster harvesting areas with moderate salinity *(20)*. The CDC reports over 400 *Vibrio* illnesses each year including about 90 due to *V. vulnificus* occurring mostly during warm-weather months *(5, 25)*. Diseases caused by *V. vulnificus* are among the most severe food-borne infections and have the highest case-fatality rate in the USA *(23)*. A number of dose-response models have been used to predict the probability of illness when consumers are exposed to a given pathogen dose *(12, 27)*. Since human dose-response studies cannot be conducted, modelling of the *V. vulnificus* dose-response relationship is based on estimates of dose exposure per serving, number of servings in the susceptible population and the number of oyster-associated cases of *V. vulnificus* septicemia cases reported to the CDC *(1)*. The frequently used Beta-Poisson model *(18)* has been used to estimate the number of *V. vulnificus* cases likely to occur when consuming raw oysters harvested in the Gulf of Mexico *(1)* and was the model used in this study.

Depuration consists of placing live oysters in circulating seawater tanks. During treatment, the oysters' pumping activity expels *V. vulnificus* and other contaminants from their gills and intestinal tract *(9, 22)*. To allow its reuse, seawater is filtered and then disinfected by UV, ozone or chlorine *(21)*. A 15^{o}C treatment temperature has been recommended for the depuration of oysters

63 in circulating seawater *(9)*. A 44 h depuration time reducing *V. vulnificus* to non-detectable counts
64 determined using a 3-tube most probable number (MPN) procedure and 1:10 dilution, i.e., less
65 than 30 MPN/g is prescribed in the National Shellfish Sanitation Program *(*see p. 140, *3)*.
66 Increasingly high consumer expectations of quality and safety make it necessary to develop
67 depuration treatments that are effective for every raw oyster production lot. The design should
68 consider the variability of production and handling factors including harvest, transportation, post-
69 harvest processing, storage and other risk factors. This is not possible using deterministic
70 algorithms or experimental test runs in processing plants. In this study, a Monte Carlo procedure
71 was applied to estimate the risk of consuming raw oysters treated by depuration. Several recent
72 reports describe its application to evaluate the uncertainty of food safety, quality and shelf-life
73 estimations *(10, 11, 26, 29, 31, 32)*. In this study, procedures were developed to estimate the
74 number of septicemia infection cases per 100 million oyster consumption events, and to
75 determine depuration times that would reduce this risk to an acceptable level defined as 100
76 cases *(31)*. This study included risk factors beginning at harvest and ending when raw oysters are
77 consumed. The procedures used in this study estimated whether process objectives are met with a
78 confidence set at 95%, while considering the statistical variability of these multiple factors.

79 **MATERIALS AND METHODS**

Statistical distributions can describe: (i) *V. vulnificus* load at harvest as a function of season; (ii) time and temperature during transportation from harvest site to processing plants; (iii) kinetic *V. vulnificus* growth parameters during harvest and transportation; (iv) depuration parameters for models developed using published circulation depuration laboratory data; (v) oyster size; (vi) refrigerated storage time before consumption; (vii) *V. vulnificus* die-off during refrigerated storage; and, (9) oysters consumed per serving.

Motes et al. *(24)* quantified the *V. vulnificus* (*Log (No, MPN/g oyster)*) in oysters collected from northern Gulf and Atlantic Coast sites including sites implicated in major *V. vulnificus* infection outbreaks yielding values of 3.22±0.60, 2.01±1.12, and -0.29±0.51 for the warm (Jun-Sep), transition (Apr-May, Oct-Nov) and cold (Dec-Mar) season, respectively. The USFDA Gulf Coast Seafood Laboratory described the time for the unrefrigerated oyster harvest and transportation time to processing plants using beta-PERT distributions (Table 1) *(2)*. Data for Louisiana, Alabama, Texas and Florida (1000 values for each state) were generated using the Excel Add-in OpenPERT.xlam (https://code.google.com/p/openpert/downloads/list). These values were then grouped into cold, warm and transition season and used with the same Excel Add-in to find the *min*, *max* and *ml* values for each season in the four states. During harvest and transportation, oysters are exposed to air temperatures slightly higher than seawater. Since this difference

98 is small (1.6-3.3°C) *(see p. 34, 1)*, oysters were assumed to be at air temperature with
99 normal distribution values of 13.1±4.3, 23.3±4.1, 27.2±2.0 and 16.4±5.5°C for Winter,
100 Spring, Summer and Fall, respectively, reported in the National Oceanic and Atmospheric
101 Administration/National Data Buoy Center database *(1)*. As before, 1000 generated
102 temperature values were grouped to calculate the warm, transition and cold season
103 temperature values used in this study. Next, the *V. vulnificus* load for oysters arriving at the
104 processing plant (*Log (N_1, CFU/g)*) was estimated using Eq. (1) where $\mu_m(T)$ represents the
105 growth rate at a random temperature *T(°C)* obtained from the seasonal air temperature
106 distribution, *t(h)* the unrefrigerated oyster handling time at this temperature *T*, and *A*
107 (= *Log ($N_{1,max}$, CFU/g oyster*) = 6) the maximum *V. vulnificus* counts possible in raw oysters
108 *(1, 7)*. The temperature dependence of the growth rate $\mu_m(T)$ in Eq. (1) was described by
109 Eq. (2) where *T* is the seasonal air temperature, *k* (= 0.011 *Log (CFU)/(h °C)*) is the *V.*
110 *vulnificus* growth rate above T_0, and T_0 (= 13°C) a threshold temperature below which *V.*
111 *vulnificus* does not grow *(see p. 32, 1, 13)*.

$$Log\left(N_1, \frac{CFU}{g_{oyster}}\right) = \min\left(Log\left(N_0, \frac{CFU}{g_{oyster}}\right) + \mu_m(T)\cdot t,\ A\right) \quad (1)$$

$$\mu_m(T) = \max\left(0, k\left(T - T_o\right)\right) \quad (2)$$

112 Data on the *V. vulnificus* load reduction by depuration at 15°C obtained by Chae et al. *(9)* by

113 sampling inoculated oysters every 24 h (Table 2a) was used to generate 1000 random pathogen
114 load values at 0, 24, 48, 72 and 96 h. Quadratic models fitted to these values were used to estimate
115 the *V. vulnificus* load after a 0 to 96 h depuration time ($t_{depuration}$). The difference between the initial
116 *V. vulnificus* load and that after depuration was used to estimate decimal reduction (*SV*) values
117 achieved during that time for each of the 1,000 randomly generated datasets. *SV* values were then
118 used to estimate the microbial load after depuration (*Log (N_2, MPN/g oyster)*) (Eq. 3).

$$Log\left(N_2, MPN/g_{oyster}\right) = Log\left(N_1, MPN/g_{oyster}\right) - SV_{depuration} \quad (3)$$

119 Cooling time to refrigeration temperature from depuration at 15°C was assumed short and
120 ignored in this study. Under refrigeration, the *V. vulnificus* die-off rate is 0.041 log CFU/day and
121 the refrigerated time before consumption follows a beta-PERT distribution with *min*, *ml* and *max*
122 values of 1, 6 and 21 d *(14)*. The lognormal distribution for oyster meat weight reported by the
123 Interstate Shellfish Sanitation Conference (ISSC)/FDA is *Log (W, g/oyster)* = 1.18±0.15 *(1, 2)*. A
124 published survey of metropolitan areas within 100 miles of Cedar Key in Florida encompassing 5
125 million residents generated data for 306 oyster consumption events *(15)*. Random sampling of this
126 non-parametric distribution (numbers (frequency) = 1, 2, 3(9x), 4(10x), 5(14x), 6(61x), 7, 8(11x),
127 10(15x), 12(95x), 13, 15(5x), 17, 18(8x), 20(8x), 24(37x), 25(5x), 30(3x), 36(7x), 40(3x), 45(2x),
128 48(4x), 50(3x), 60) was used in this study.

129 The shape and scale parameter α (= $9.3\text{x}10^{-6}$) and β (= $1.1\text{x}10^{5}$) of the Beta distribution dose-

response model (Eq. 4), frequently used to estimate the *V. vulnificus* septicemia risk probability (P_{ill}) for a population ingesting a given pathogen dose (D) per serving published by the World Health Organization *(*WHO, *1)*, incorporate pathogenicity heterogeneity, i.e., not every ingested microorganism survives to cause an infection.

$$P_{ill} = 1 - \left(1 + \frac{D}{\beta}\right)^{-\alpha} \quad (4)$$

Safety risk was expressed as the number of septicemia cases in 100 million oyster consumption events. Estimations were divided into 8 steps repeated 1,000 times to obtain the number of infection cases with 95% confidence *(31)*: (1) *V. vulnificus* oyster load at harvest (*Log (N_0, CFU/g)*); (2) *V. vulnificus* load for oysters arriving to processing plants (*Log (N_1, CFU/g)*); (3) *SV* value reached after a given depuration time ($SV_{depuration}$); (4) *V. vulnificus* load after depuration (*Log (N_2, CFU/g)*); (5) *V. vulnificus* load at consumption (*Log (N_3, CFU/g)*); (6) *V. vulnificus* dose per serving (*D, CFU/serving)*); (7) infection probability (P_{ill}); and, (8) number of infection cases per 100 million consumption events (*N, cases*). In each step, parameter values were randomly generated using their normal, lognormal or beta-PERT distribution or by random sampling (number of oysters per consumption event). In the depuration step, 1,000 sets of *V. vulnificus* load randomly generated using their lognormal distribution were fitted into quadratic expressions for SV values after depuration times between 0 and 96 h. *V. vulnificus* load as a function of

147 depuration time, season, and handling step was analyzed by ANOVA tests. As in previous
148 studies *(30)*, a health risk of 100 cases per 100 million oyster consumption events at 95%
149 confidence was used to recommend a depuration time.

150 **RESULTS**

151 The estimated *V. vulnificus* growth rate during harvest and transportation to processing
152 plants was 0.159±0.022, 0.074±0.047, and 0.022±0.030 *Log (MPN/g oyster)/h* in the warm,
153 transition, and cold season, respectively, and combining these values with random harvest and
154 transportation time values, the estimated pathogen load increase was 1.21±0.28, 0.57±0.38, and
155 0.23±0.32 *Log (MPN/g oyster)*, respectively. Combining the latter values with the harvest load
156 variability yielded pathogen load values before depuration (*Log* N_1) of 4.41±0.28, 2.59±0.38
157 and -0.09±0.32 *Log (MPN/g oyster)*, respectively.

158 SV values achieved by 24, 48, 72 and 96h depuration at 15^{o}C using quadratic models
159 (average R^2 = 0.95) were 1.55±0.17, 2.56±0.24, 3.03±0.24, and 2.95±0.24 *Log (MPN/g oyster)*,
160 respectively, showing that after 72 h the pathogen load reduction is not significant (P-value>0.05).
161 Microbial load values after depuration are summarized in Table 2b.

162 *V. vulnificus* die-off during refrigerated handling time before consumption was estimated as
163 0.31±0.15 *Log (MPN/g oyster)* yielding pathogen loads for untreated oysters at consumption

of -0.38±0.6 and 4.14±0.7 *Log (MPN/g oyster)* in cold and warm seasons, respectively, and a significantly lower value for the transition season (2.26±1.2 *Log (MPN/g oyster)*) when compared to the warm season (P value < 0.05). The season effect is reflected also in *V. vulnificus* loads for raw oyster treated by depuration for up to 96 h (Table 2c). The pathogen dose consumed per oyster serving (*D*) reflects the variability in the number of oysters consumed per serving, oyster weight, and pathogen load after depuration. In the case of untreated oysters, the dose of (10.0±3.1) x 10^6 *MPN/serving* in the warm season was significantly higher (P value < 0.05) than the (9.7±0.4) x 10^5 and 283.1±61.7 *MPN/serving* in the transition and cold season, respectively. Dose values after depuration for 24 to 96 h are shown in Table 2d.

At 95% confidence, the estimated number of infection cases in 100 million consumption events for untreated oysters in the warm season was 2669 cases, while 24 and 48h depuration would reduce it to 558 and 93 cases, respectively (Table 3). The depuration time required to reach the safety target used in this study (100 cases per 100 million consumption events) was estimated to be 47h. The discrepancy between the estimated 93 and 100 cases after 48 and 47h depuration time, respectively, reflects the non-deterministic procedure used in this study. In the transition season, the number of predicted cases would be 491 for untreated oysters, reaching the 100 cases safety-target after 16h depuration, meaning that the 44h recommended depuration *(*see p. 126, *3)* could be considered over-processing. Only 35 cases would be observed after depuration for 24h,

while a 48h depuration is likely to reduce the number of infection cases to 4. The number of cases at 95% confidence that could be expected after the recommended 44h depuration time would be 135, 8 and 0, for oysters harvested in the warm, transition and cold season, respectively (Table 3).

DISCUSSION

The U.S. FDA requires that "the dealer must demonstrate that the process reduces the level of *Vibrio vulnificus* and/or *Vibrio parahaemolyticus* … to non-detectable (<30 MPN/gram) and that the process achieves a minimum 3.52 log reduction *(*see p. 140, *3)*." Furthermore, *V. vulnificus* and *V. parahaemolyticus* levels must be determined following the sampling protocol *(4)* and microbial enumeration *(*see p. 345, *3)* described by the National Shellfish Sanitation Program (NSSP). The USFDA/CFSAN and the ISSC state that treated oysters meeting these specified endpoint and decimal reduction levels can be labeled as "Processed to reduce *Vibrio vulnificus* to non-detectable levels" *(*see p. 187, *3)*. The 3.52 log reduction in these regulations is based on assuming extremely high *V. vulnificus* or *V. parahaemolyticus* loads observed sometimes in the Gulf Coast during summer months (100,000 MPN/g) being lowered by processing to reach non-detectable levels (<30 MPN/g) *(*see p. 172, *3)*. Although artificial seawater depuration tests showed that reduction in *V. vulnificus* counts leveled off after 48 h and that 96 h depuration cannot achieve 3.52 log reductions for *(9)*, use of this experimental data showed that depuration would achieve large

199 reductions in the *V. vulnificus* infection risk reaching values below 100 cases per 100 million
200 consumption events (Table 3).

201 The probability that depuration would reduce the *V. vulnificus* load to the non-detectable
202 endpoint (<30 MPN/g) was also determined (Table 4). A 95% confidence level is typically used in
203 process design calculations *(26, 33)*. Oysters harvested in the cold season would meet this
204 requirement at 95% confidence in less than 1h while in the transition and warm season it cannot
205 be met even after 96h depuration. If pathogen loads after depuration (N_2) are lower than the non-
206 detectable 30 MPN/g, the estimated infection risk at 95% confidence per 100 million consumption
207 events estimated using Eq. (4) would be less than 27 cases per year (calculations not shown).

208 An analysis of the effectiveness of the recommended depuration time (44 h) showed that
209 only 23% and 50% of oysters harvested in the warm and transition seasons, respectively, would
210 reach the non-detectable *V. vulnificus* level (i.e., below 30 MPN/g), while 100% would reach it in
211 the cold season (Table 4). At 95% confidence, the use of the recommended 44 h depuration time
212 would result in 135, 8, and 0 cases in the warm, transition, and cold season, respectively (Table 3).
213 Thus, in the summer season it would be too short while for other seasons it would be too long. The
214 microbial risk analysis of HPP-treated oysters completed by Serment-Moreno et al. *(31)* showed
215 that 4 cases of *V. vulnificus* infection cases (95% confidence) per 100 million consumption events
216 could be expected from the consumption of oysters harvested in the warm season if treated at 250

217 MPa for 2 min at 1°C with no cases expected in other seasons.

218 The number of oysters consumed per serving was obtained from central and north-central
219 Florida surveys *(15)* where the number consumed per oyster serving could be higher than in
220 inland states. Population inference errors may also occur when consumers recall consumption
221 events *(15)* and consumers eating oysters more frequently may tolerate higher pathogen loads
222 *(28)*. Moreover, *V. vulnificus* in oysters were assumed to correspond to equally virulent strains
223 *(16)*. Although samples below detectable level were assigned a half-way value between 0 and the
224 detection value, this can be ignored since the number of oyster samples below detection level was
225 reported to be small *(*i.e., 2 of 24 samples, *24)*.

226 This study used reported experimental data *(8)* focusing on *V. vulnificus* oyster depuration.
227 The same procedure could be applied to reduce the risks of other pathogens by depuration, or to
228 analyze other oyster treatment technologies by modifying only the SV estimation. The procedure
229 would be most effective when using data for individual processors or at least individual harvest
230 regions. This would reduce statistical variability and lower the treatment intensity recommended
231 (depuration time/temperature, pressure level/holding time, etc.). The positive effect of reducing
232 statistical variability has been previously shown *(10, 29)*.

233 In conclusion, the *V. vulnificus* infection risk associated with raw oyster consumption was
234 quantified to estimate a recommended depuration time. The 44h depuration set independently of

oyster harvest season, non-detectable endpoint (<30 MPN/g), and 3.52 *V. vulnificus* decimal reduction, were analyzed using a Monte Carlo protocol. The analysis included the variability of the seasonal oyster pathogen load at the point of harvest, the time and local temperature during oyster harvest and transportation to oyster processing facilities, time that oysters are kept refrigerated after processing and before consumption, and the size and number of oysters consumed per serving. For untreated oysters, the *V. vulnificus* infection risk at 95% confidence is exceedingly low in the cold season but unacceptably high in the warm and transition season. An acceptable risk, defined in this study as 100 cases per 100 million consumption events, could be achieved with 95% confidence by oyster depuration for 47 and 16 h in the warm and transition season, respectively.

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Table 1. Harvest and transportation time (hours) to processing plants in the U.S. Gulf Coast

Location		Winter (Jan-Mar)	Spring (Apr-Jun)	Summer (Jul-Sept)	Fall (Oct-Dec)
Louisiana	*max* =	13	11	11	13
	min =	7	5	5	7
	ml =	12	9	9	12
Alabama, Texas, and Florida	*max* =	11	10	10	10
	min =	2	3	3	3
	ml =	8	7	7	7

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Table 2. *Vibrio vulnificus* load and consumption dose of untreated and depurated oyster

Sample	Untreated*	Depuration time 24 h	48 h	72 h	96 h
a) Reduction during depuration test at 15°C**					
Inoculated	5.52 ± 0.16	3.49 ± 0.23	2.76 ± 0.19	2.67 ± 0.29	2.23 ± 0.20
b) Load after depuration for t = 0 (untreated) to 96 h, *Log ($N_{2,t}$ MPN/g oyster)*					
Warm	4.41±0.28[1]	2.89±0.7	1.87±0.7	1.41±0.7	1.48±0.7
Transition	2.59±0.38[1]	0.95±1.2	-0.07±1.2	-0.54±1.3	-0.46±1.2
Cold	-0.09±0.32[1]	-1.63±0.6	-2.65±0.6	-3.11±0.6	-3.03±0.62
c) Load after depuration and refrigerated storage, *Log (N_3, MPN/g oyster*					
Warm	4.14±0.7	2.59±0.7	1.58±0.7	1.12±0.7	1.19±0.7
Transition	2.26±1.2	0.71±1.2	-0.31 ±1.2	-0.77±1.2	-0.69±1.2
Cold	-0.38±0.6	-1.95±0.6	-2.96±0.6	-3.43±0.6	-3.35±0.6
d) Dose when consuming raw oysters, *D, MPN/serving*					
Warm	$10.0\pm3.1\times10^{6}$	$3.0\pm0.29\times10^{5}$	$3.1\pm0.53\times10^{4}$	$1.1\pm0.43\times10^{4}$	$1.4\pm 0.16\times10^{4}$
Transition	$9.7\pm0.4\times10^{5}$	$3.3\pm0.04\times10^{4}$	$3.6\pm 0.07\times10^{3}$	$1.1\pm0.06\times10^{3}$	$1.3\pm0.02\times10^{3}$
Cold	283.1±61.7	8.0±0.57	0.83±0.11	0.29±0.09	0.37±0.03

(*) Untreated load corresponds to the Log N1 value obtained in the previous calculation step
(**) Data obtained from Chae et al. *(9)*

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Table 3. Probable number of septicemia infection cases by consuming raw oysters treated by depuration for t = 0 (untreated) to 96 h and recommended depuration time ($t_{depuration}$)[1]

Season	Untreated	Depuration time					$t_{depuration}$ (h)
		24 h	48 h	72 h	96 h	44 h[2]	
Warm	2669	558	93	38	47	135	47
Transition	491	35	4	1	2	8	16
Cold	1	0	0	0	0	0	0[3]

(1) depuration time to reach N = 100 *cases per 10^8 consumption events*
(2) # of cases at 95% confidence expected after the recommended 44h depuration time
(3) depuration treatment is not recommended for cold season

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Table 4. Probability that the *V. vulnificus* load in raw oysters is reduced to <30 *MPN/g* by depuration for t = 0 to 96 h and recommended depuration time ($t_{30MPN/g}$) to reach this load with 95% confidence

Season	Untreated	Depuration time					$t_{30MPN/g}$
		24 h	48 h	72 h	96 h	44 h[1]	
Warm	0%	3%	31%	55%	50%	23%	>96 h
Transition	17%	66%	88%	95%	94%	50%	72 h
Cold	99%	100%	100%	100%	100%	100%	0 h

(1) Currently recommended depuration time

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