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Depuration dynamics of oysters (*Crassostrea gigas*) artificially contaminated by *Salmonella enterica* serovar Typhimurium

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1 Oyster Depuration

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3 **Depuration dynamics of oysters (*Crassostrea gigas*) artificially contaminated by**
4 ***Salmonella enterica* serovar Typhimurium**

5

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1 **Abstract**

2 The State of Santa Catarina produces the greatest quantity of edible mollusks in Brazil. To
3 guarantee sanitary quality, mollusk cultures should be monitored for contamination by
4 pathogenic microorganisms. A self-purification or “deuration” system that eliminates
5 *Salmonella enterica* serovar Typhimurium contamination from oysters has been developed
6 and evaluated. The deuration process occurred within a closed system, in which 1,000 L of
7 water was recirculated for 24h. The water was sterilized with ultraviolet (UV) light, chlorine,
8 or both together. Oysters (*Crassostrea gigas*) artificially contaminated with *S. Typhimurium*
9 were harvested every six hours. Samples of oyster tissue were excised and both the presence
10 and numbers of bacteria were determined. Combined UV light and chlorine treatments
11 resulted in total elimination of bacteria within 12h. Polymerase chain reaction detected
12 bacteria in water exposed to the three treatments. This pioneering study is the first of its kind
13 in Brazil and represents a major contribution to commercial mollusk culture in this country.

14 Key-words: Oysters *Crassostrea gigas*, deuration, *Salmonella enterica* serovar
15 Typhimurium, PCR

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1 **Introduction:**

2 In Brazil, large amounts of treated, and sometimes untreated, human wastewaters are
3 discharged into marine environments in highly populated coastal areas, resulting in localized
4 increases in pathogen concentrations, which in turn serve as sources for transmission of many
5 diseases (Lemarchand, Masson, & Brousseau, 2004). Filter-feeding mollusks accumulate and
6 concentrate microorganisms when grown in polluted waters, including human pathogenic
7 bacteria and viruses. Contaminated mollusks can represent significant health risks when
8 consumed raw or lightly cooked (Butt, Aldridge, & Sander, 2004; Lee, Panicker, & Bej,
9 2003). The enteric bacteria *Salmonella* can readily contaminate the fauna in marine
10 environments, especially molluscan shellfish, and for this reason, oysters and mussels are
11 associated with worldwide salmonellosis outbreaks (Lee, Panicker, & Bej, 2003; Martinez-
12 Urtaza, Saco, de Novoa, Perez-Pineiro, Peiteado, Lozano-leon *et al*, 2004, Vazquez-nouvelle,
13 Pazos, Abad, Sánchez, & Pérez-Paralee, 2005).

14 Controlled self-purification (“deuration”) of shellfish is a method that reduces the levels of
15 bacteria present in mollusk meat, thus decreasing the potential for infections associated with
16 shellfish consumption. Depuration is a commercial process where shellfish are placed in tanks
17 filled with clean seawater for several hours to purge contaminants from their tissues by
18 filtering (Croci, Suffredini, & Cozzi, 2002; Rodrick & Schneider, 2003; Marino, Lombardo,
19 & Fiorentino, 2005).

20 There are two general types of mollusk shellfish depuration plants: flow-through or
21 recirculating (closed) system. Both depuration processes are effective, both the flow-through
22 system requires a near-by, reliable, but consistently clean source of seawater. In the closed
23 depuration system, seawater is recirculated for at least 24h, and during this cycle, the water
24 must be chemically (chlorine and/or ozone) or physically (UV irradiation) treated to eliminate

1 microbial contamination. The advantage of the closed system is that it uses vastly less
2 amounts of water (Richards, 1988; Power & Collins, 1989; Dore & Lees, 1995).

3 The State of Santa Catarina is a major producer of mollusks in Brazil due to excellent
4 geographical conditions for marine organism cultures, especially bivalve mollusks, such as
5 the oyster species *Crassostrea gigas* (Souza, 2003; Oliveira-Neto, 2005). In recent years,
6 however, increased contamination of mollusk culture habitats by sewage discharged from
7 coastal communities has lead to contamination of cultured mollusks by bacterial and viral
8 pathogens (Coelho, Heinert, Simões, & Barardi, 2003; Rigotto, Sincero, Simões, & Barardi,,
9 2005; Sincero, Levin, Simões, & Barardi, 2006). The increasing pollution of the coastal
10 waters of Santa Catarina requires monitoring of cultured bivalve mollusks to ensure health
11 safety standards. While depuration is not yet an established practice in Brazil, it is used in
12 many other countries, such as European countries and even in United States according to the
13 water quality. To expand commercial bivalve mollusk culture in Brazil, a safe product with
14 high commercial value must be guaranteed.

15 In this study, the efficacy of a commercial oyster depuration system was evaluated. The
16 system was designed by an aquaculture company (Blue Water Aquaculture, Florianopolis,
17 Santa Catarina State, Brazil). Oysters (*Crassostrea gigas*) artificially contaminated with
18 *Salmonella enterica* serovar Typhimurium were used, and the efficacy of the system was
19 evaluated through the detection of this pathogen by microbiological and molecular methods.

20 **Material and methods**

21 **Bacterial strain, oysters and seawater:** *S. Typhimurium* (ATCC 14028) was used to
22 contaminate oysters and was kindly provided by the Laboratory of Medical Microbiology of
23 the Universidade Federal de Santa Catarina. The bacterial strain was propagated in brain heart
24 infusion (BHI, Oxoid, Basingstoke, Hampshire, U.K.) broth and xylose-lysine-deoxycholate
25 agar (XLD, Oxoid). Liquid cultures were obtained by overnight growth at 37 °C. Bacterial

1 suspensions were quantified by direct plate counting. The Pacific oysters (*C. gigas*) were
2 harvested from an oyster culture farm located in the city of Florianopolis, Santa Catarina
3 (SC), Brazil. Seawater was collected from the same location.

4 **Depuration System:** The depuration (UV/Chlorine system) system was designed by the
5 company Blue Water Aquaculture (Florianopolis, SC). The depuration plant was placed in the
6 same oyster farm where the oysters were harvested. The depuration unit consisted of a 1,000
7 litre (L) water tank, with 200 dozen oysters, in 19 perforated plastic baskets, distributed in
8 four floors. The water was circulated by a pump at a rate of 2,200 L/h. The sterilizing system
9 consisted of one 25W UV (254 nm) low-pressure tube, a chlorine metering pump, a sand
10 filter, and a refrigeration system. The perforated plastic baskets were kept in a plastic basin
11 with a grid base, to avoid recontamination by particulate matter released from the oysters. The
12 temperature of the depuration system was maintained at 19°C during all experiments in order
13 to prevent the mollusks from spawning.

14 **Artificial Contamination:** Prior to the depuration experiment, oysters were placed in an
15 aquarium (55 x 45 x 30 cm) containing 30 L of seawater. A fresh, overnight culture of *S.*
16 *Typhimurium* was added to the aquarium to a final concentration of approximately 1,000
17 CFU/ml. The oysters were allowed to bio-accumulate the seeded bacteria for 2 h. After this
18 period, the oysters were removed from the aquarium and their shells were washed in potable
19 water plus 0.5% chlorine for 10 min. At this point, three oysters were retained as an
20 undepurated control. The remaining oysters were labeled with red ink and immersed in the
21 depuration tank together with uncontaminated oysters.

22 **Depuration Assay:** Depuration assays were undertaken over a 24-h period. Oysters were
23 removed at 6, 12, 18 and 24 h for analysis. Water temperature was monitored routinely. Three
24 treatments were conducted: chlorine (1ppm) alone, UV irradiation (254 nm) alone, and
25 chlorine plus UV irradiation. Each treatment was repeated three times, and for each sample

1 period, one sample (three oysters artificially contaminated) per level was collected randomly.
2 After the collection, each sample was put into a plastic bag and stored at 4°C. At the end of
3 the depuration assay, the samples were transported to the laboratory for the analyses. In each
4 depuration assay, uncontaminated samples were collected before and after as negative
5 controls. At the end of each depuration assay, the water was discharged into the sewage
6 system. The experiment was devised to obtain depurated shellfish in the shortest period of
7 time and at the lowest cost to local producers.

8 **Analytical procedures:**

9 **Bacterial analysis:** Following artificial contamination and depuration, oyster's shells were
10 opened aseptically using a sterile shucking knife under aseptic conditions. The digestive
11 tissue was dissected and then processed in a homogenizer (Ultra-Turrax, model T25 basic,
12 IKA Labortechnik). The tissues of three oysters (one sample) were pooled and weighed. To
13 quantify the bacterial concentration, one gram of homogenate was diluted in 9 ml of non-
14 selective lactose broth (1:10 dilution). The time zero sample (undepurated tissue) was
15 subjected to subsequent decimal serial dilutions (1:100 and 1:1000) and the depurated
16 samples, were subjected to 1:10 dilution. Portions of each dilution (0.1mL) were spread onto
17 plates of selective XLD agar medium. The CFU/g of samples was counted after overnight
18 growth at 37 °C.

19 The first dilution of each sample was incubated overnight at 37 °C in selective XLD liquid
20 medium to confirm the presence of *S. Typhimurium*, when present in amounts below the plate
21 detection limit.

22 **DNA extraction from contaminated oyster tissues**

23 After incubation in liquid medium, a 1.5 ml aliquot was centrifuged at $1,400 \times g$ for 5 min in
24 a microcentrifuge (Eppendorf 5415 C, Hamburg, Germany). The pellets were suspended in
25 567 μL of TE buffer (10 mM Tris-HCl, pH 7.0, 1 mM EDTA), 30 μL of 10 % SDS (sodium

1 dodecyl sulfate) and 6 μL of proteinase K (20 U/mg) (Invitrogen, Carlsbad, CA, USA), and
2 incubated at 56 $^{\circ}\text{C}$ for 1 h. After that, 80 μL of 10 % CTAB (cetyltrimethylammonium
3 bromide) and 100 μL of 5 M NaCl were added, gently homogenized and incubated at 65 $^{\circ}\text{C}$
4 for 10 min. They were subsequently extracted with an equal volume of phenol-chloroform-
5 isoamyl alcohol (25:24:1). After centrifugation (12,000 \times g, 5 min, RT), the aqueous phase
6 was transferred to a fresh microtube and the same procedure was repeated with one volume of
7 chloroform-isoamyl alcohol (24:1). Isopropanol precipitation was carried out for 10 min at
8 RT. Resulting pellets were washed with chilled 75% (v/v) ethanol, suspended in 200 μL of TE
9 buffer and stored at -20°C until used (Sambrook & Russel, 2001). The pellets were allowed
10 to dry for 10 min, suspended in 100 μL of TE buffer, and stored at -20°C . DNA derived from
11 pure cultures of *S. Typhimurium* was used as positive control in all experiments.

12 **Oligonucleotides primers:** The primer set used in this study was previously described by
13 Cocolin, Manzano, Cantoni, & Comi (1998) (Salm3 5'-GCT GCG CGC GAA CGG CGA
14 AG-3' and Salm4 5'-TCC CGG CAG AGT TCC CAT T-3'), and amplified a 387 bp genome
15 region of *Salmonella* spp. These primers were derived from the *invA* gene sequence (Genbank
16 accession number M90846), essential for *Salmonella* invasiveness into epithelial cells (Galan,
17 Ginocchio, & Costeas, 1992).

18 **PCR amplification and DNA analysis:** PCR amplifications were performed in a final
19 volume of 50 μl , containing 3 μl of DNA template, 1X Buffer (10 mM Tris-HCl pH 9.0; 50
20 mM KCl, 2 mM MgCl_2), 0,2 mM of each deoxynucleotide (Invitrogen Life Technologies,
21 Carlsbad, Ca, USA), 20 pmol of each primer and 0.5 units of Taq DNA polymerase (Biotools,
22 Madrid, Spain). Amplifications were carried out in a Thermocycler (Techne Flexigene®).
23 Initial denaturation was at 94 $^{\circ}\text{C}$ for 1 min, followed by 30 cycles of denaturation at 94 $^{\circ}\text{C}$ for
24 30 s, annealing at 62 $^{\circ}\text{C}$ for 30 s and extension at 72 $^{\circ}\text{C}$ for 2 min, with a final extension at 72
25 $^{\circ}\text{C}$ for 10 min. Negative (water) and positive controls (purified *S. Typhimurium* DNA) were

1 used in all experiments. Amplification products were separated by electrophoresis of 10 μ l of
2 the final reaction mixture on 1.5 % agarose gel, at 100 V. The agarose gel was visualized on
3 UV light after ethidium bromide (1 μ g/ml) staining.

4 **Statistics analysis:** The statistical analyses were conducted using GraphPad Prism 4.
5 Statistical differences in the numbers of bacteria per level of the depuration system across the
6 different treatments were determined by ANOVA and non-linear regression analyses. PCR
7 results were analyzed using the exact Fisher's Test with a p value of < 0.05 .

8 **Results and Discussion:**

9 Prior to the start of the purification process, *S. Typhimurium* counts in artificially
10 contaminated oysters ranged from 15,000 to 20,000 CFU/g of digestive tissue.
11 Samples collected before and after the purification period, and samples not artificially
12 contaminated were always negative for the presence of *S. Typhimurium*, indicating that cross
13 contamination due to contact with contaminated samples presented in the same basket did not
14 occur.

15 Ultraviolet irradiation (254 nm) and chlorine (1 ppm) treatments, separately, were effective in
16 reducing the numbers of *S. Typhimurium*, but did not totally eliminate the bacteria from all
17 four levels (data not shown). According to Son & Fleet (1980), UV irradiation treatment for
18 water depuration was effective in reducing high levels *S. Typhimurium* to low levels, on a
19 laboratory scale in glass tanks (20 L). The reduction in numbers of bacteria accomplished
20 here by UV and chlorine treatments was consistent with previously published studies on
21 oysters and mussels purification (Rowse & Fleet, 1984; Murphee & Tamplin, 1991; Doré &
22 Lees, 1995; Croci, Suffredini & Cozzi, 2002). Ultraviolet irradiation and chlorine treatment
23 together, however, effectively eliminated *S. Typhimurium* from all levels after 12 hrs (Figure
24 1). While plate counts showed that bacteria were eliminated from all levels by 12 hrs, *S.*
25 *Typhimurium* was detected up to 24 hrs (Table 1).

1 In general, the effectiveness of the three disinfection methods was similar for each of the four
2 levels of the depuration system. Linear regression analyses of each treatment revealed that the
3 decrease in the number of CFU of bacteria per gram of oyster tissue was closely associated
4 with purification time. The R^2 value was approximately 0.9 for all three treatments on all four
5 levels (Table 2).

6 The closed depuration system has a major advantage because it uses vastly less water than
7 flow-through system. The major disadvantage is that the water must be disinfected as it is
8 recirculated (Richard, 2001; Rodrick & Schneider, 2003). Treatment with chlorine is one of
9 the methods for disinfecting polluted waters. Although it is effective against enteric bacteria,
10 it is not very effective against bacterial spores, viruses and protozoa (Oppenheimer,
11 Jacangelo, Lay Ne, & Hoagland, 1997; Monarch, Feretti, Collivignarelli, Guzzella, Zerbini,
12 Bertanza *et al.*, 2000). Moreover, health risks due to mutagenic and/or carcinogenic agents
13 produced during chlorine treatment (Koivunem & Heinonem-Tanski, 2005) raise concerns
14 about the exclusive use of chlorine in depuration systems.

15 Ultraviolet irradiation is an effective disinfectant and it is used widely in water purification
16 systems (Caslake, Connolly, Menon, Duncanson, Rojas, & Tavakoli, 2004; Mamane-Grawetz
17 & Linden, 2004). According to Koivunem & Heinonem-Tanski (2005), UV irradiation
18 eliminates enteric bacteria, viruses, bacterial and protozoan spores in water, without the
19 production of toxic products or other chemical residues. The disadvantage of UV irradiation is
20 that, under certain conditions, some microorganisms inactivated by UV may be able to repair
21 DNA damage and continue to grow (Lubello, Caretti, & Gori, 2002). In some organisms,
22 particularly bacteria, the mutagenic effects of short wavelength UV can be repaired after
23 exposure to long wavelength light, especially visible light (320-400 nm) (Upadhyaya, Curry,
24 Nichols, Clevenger, & McDonald, 2004). Purification systems that use UV lamps for
25 disinfection must filter water through sand to eliminate suspended material, because large

1 suspended particles in the water limit the penetration of UV rays, and thus reduce the
2 effectiveness of this treatment (Richards, 2001).

3 The combination of chlorine and UV irradiation both increased the efficiency of disinfection
4 and decreased the time required to complete the depuration process. The two methods had a
5 synergistic effect because they inflict different types of damage in microorganisms
6 (Koivunem & Heinonem-Tanski, 2005). Chlorine treatment damage cell walls and
7 membranes, as well as enzymatic and transporting systems, while UV irradiation causes DNA
8 damages, and with repair mechanisms overloaded, the microorganisms die (Lubello, Caretti,
9 & Gori, 2002).

10 In this study, *S. Typhimurium* was detected in oyster tissues by PCR, even though no bacteria
11 were detected by plate counts. Detection of DNA by PCR in the samples that did not contain
12 viable bacteria was most probably due to the fact that bacteria that have been inactivated or
13 killed by chlorine and/or UV irradiation may be re-ingested and bioaccumulated by mollusks.
14 If DNA from these dead bacteria is not completely degraded, molecular analyses, such as
15 PCR, may still react with this DNA, resulting in false positive results. Therefore,
16 quantification of bacteria using the plate count method is essential to detect the viability of the
17 microorganisms present in the oyster's tissues. In a study that was similar to this one, Croci,
18 Suffredini & Cozzi (2002) evaluated depuration of mussels that had been artificially
19 contaminated with *E. coli*, *V. cholerae* O1, and *V. parahaemolyticus*. The effectiveness of the
20 depuration process was monitored using the Most Probable Number (MPN) methodology, and
21 PCR was used only as a confirmatory test for the *Vibrio* positive samples.

22 The results obtained in the present study are the first data in Brazil regarding the depuration
23 dynamics of *S. Typhimurium* in oysters using a closed system. Most countries that produce
24 bivalve mollusks for export markets have strict regulations for monitoring depuration systems
25 and operating procedures to ensure quality control of the purification process. Monitoring

1 temperature and salinity of the water during the purification cycle is also recommended
2 (European Communities, 1991; National Shellfish Sanitation Program, 2005). The Brazilian
3 legislation does not regulate depuration systems, and only the presence of fecal coliforms in
4 culture waters and the presence of *Salmonella* spp. in the mollusk meat are currently regulated
5 (ANVISA, 2001; CONAMA, 2005).

6 **Conclusion:**

7 The bivalve mollusk culture in the State of Santa Catarina is growing rapidly. Countries that
8 produce bivalve mollusks for export markets have strict regulations for bacterial
9 contamination to ensure a safe, high quality product. The purification system designed by
10 Blue Water Aquaculture (Florianopolis, SC, Brazil) was effective for the purification of
11 oysters artificially contaminated by *S. Typhimurium*. The combination of UV irradiation and
12 chlorine treatments eliminated all bacteria by 12 hrs, as determined by plate counts.
13 Quantification of bacteria by plate counts proved to be a more reliable method of evaluating
14 the effectiveness of the depuration treatments, as PCR detected the presence of bacterial DNA
15 (but not viable bacteria) in oysters in all three treatments up to 24 hrs. The depuration can
16 increase the oyster commercial value for the producer, from around US\$ 2,00 (actual market
17 price) to US\$ 4,00. Actually, the final cost of the depuration system is around US\$ 7.000,00, and
18 the small and average farmers can afford it.

19
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1 **Figures legends**

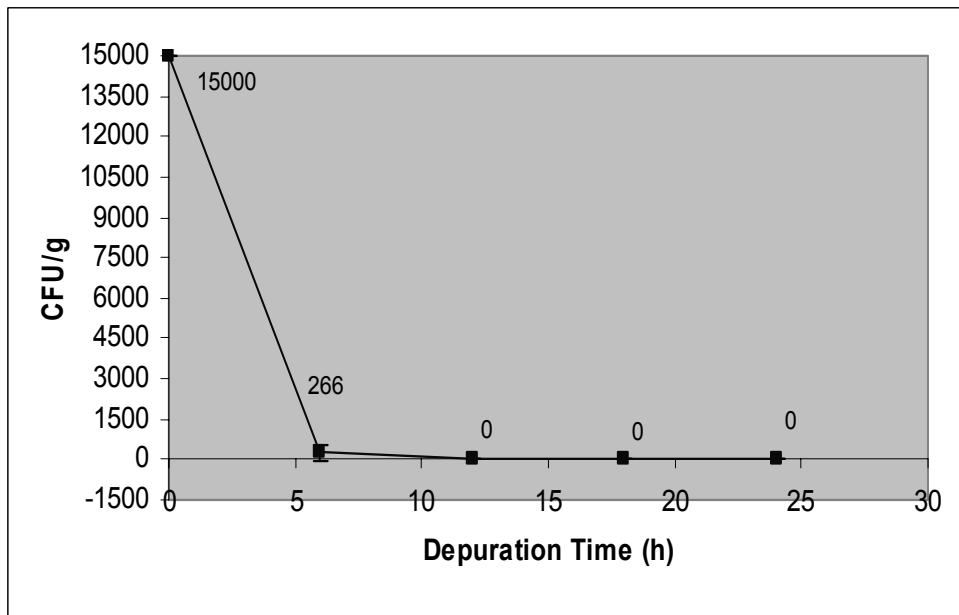
2 Figure 1. Elimination of *S. Typhimurium* from oysters after treatment with UV irradiation and
3 chlorine from **A)** samples located in baskets on the first level (top of the depuration tank); **B)**
4 samples located in baskets on the second level of the depuration tank; **C)** samples located in
5 baskets on the third level of the depuration tank; and **D)** samples located in baskets on the
6 fourth level (bottom of the depuration tank). The data represent the mean of three analyses, \pm
7 standard deviation.

ACCEPTED MANUSCRIPT

1 Figure 1:

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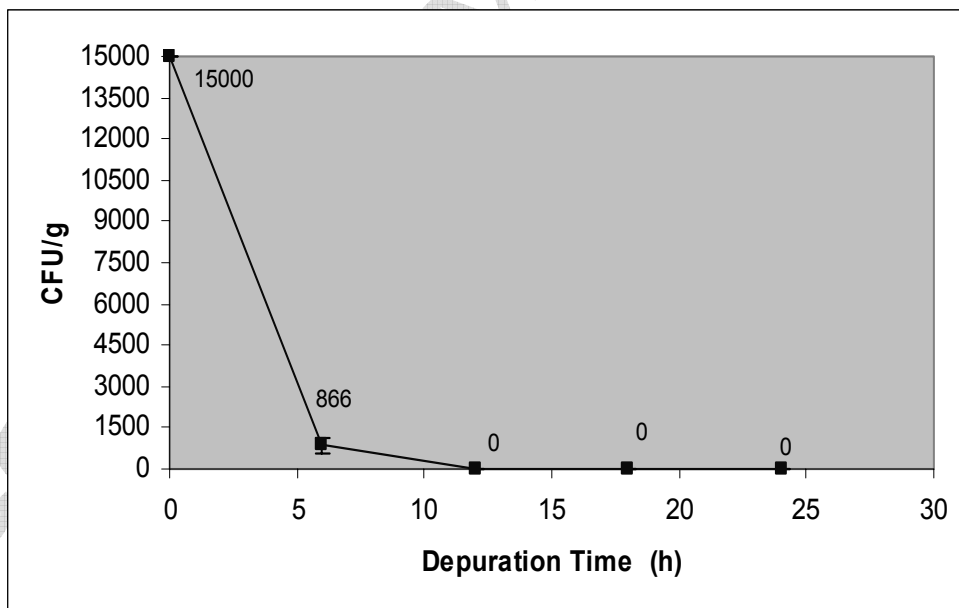
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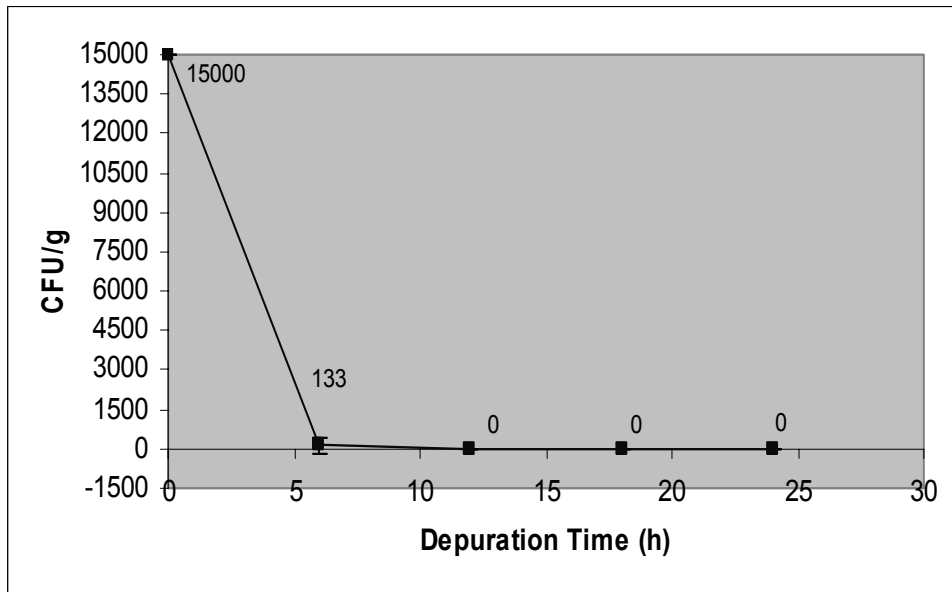
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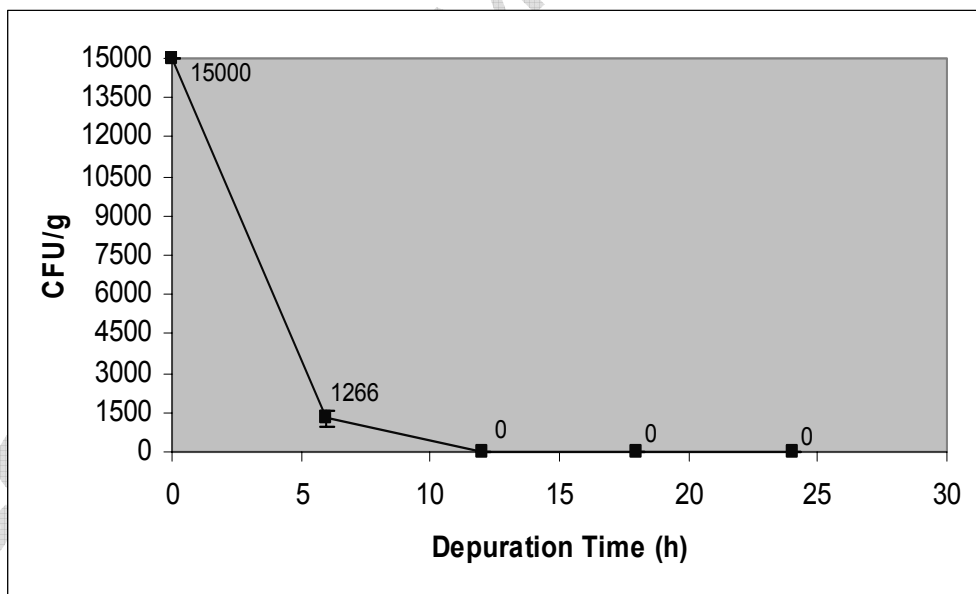
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1 Table 1: Detection of *S. Typhimurium* by PCR in oysters after treatment with UV irradiation,
 2 Chlorine, or combined UV/Chlorine treatment. Fractions indicate the number of PCR positive
 3 samples per 3 samples tested.

4

| Depuration Time | Level | UV | Chlorine | UV/ Chlorine |
|--|------------|-----|----------|--------------|
| 6h | 1st | 1/3 | 2/3 | 1/3 |
| | 2nd | 1/3 | 2/3 | 2/3 |
| | 3rd | 3/3 | 3/3 | 3/3 |
| | 4th | 2/3 | 2/3 | 2/3 |
| 12h | 1st | 1/3 | 3/3 | 2/3 |
| | 2nd | 1/3 | 2/3 | 1/3 |
| | 3rd | 1/3 | 3/3 | 2/3 |
| | 4th | 3/3 | 2/3 | 1/3 |
| 18h | 1st | 3/3 | 2/3 | 1/3 |
| | 2nd | 2/3 | 2/3 | 1/3 |
| | 3rd | 0/3 | 3/3 | 2/3 |
| | 4th | 2/3 | 3/3 | 1/3 |
| 24h | 1st | 1/3 | 3/3 | 1/3 |
| | 2nd | 3/3 | 2/3 | 1/3 |
| | 3rd | 2/3 | 3/3 | 0/3 |
| | 4th | 3/3 | 2/3 | 1/3 |
| Bioaccumulated Samples | 3/3 | | | |
| Uncontaminated samples after depuration | 0/3 | | | |

5

1 Table 2: Correlation between decontamination treatment and time for all locations within the
 2 depuration system. Decreases in the numbers of CFU/g oyster were correlated with time of
 3 treatment for each location within the depuration system. R^2 values from each linear
 4 regression, location and treatment indicate a significant correlation between decontamination
 5 and time for all locations and treatments.

6

| Water Treatment | Level | R^2 |
|-------------------------|--------------|-------------------------|
| Chlorine 1ppm | 1st | 0.8710 |
| | 2nd | 0.8562 |
| | 3rd | 0.8565 |
| | 4th | 0.8610 |
| UV | 1st | 0.8515 |
| | 2nd | 0.8724 |
| | 3rd | 0.8562 |
| | 4th | 0.8822 |
| Chlorine 1ppm/UV | 1st | 0.8669 |
| | 2nd | 0.8829 |
| | 3rd | 0.8622 |
| | 4th | 0.8885 |

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