Tracing *Vibrio parahaemolyticus* in oysters (*Tiostrea chilensis*) using a Green Fluorescent Protein tag

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Abstract

Oysters feed by removing particles from the water. This food is composed of complex mixtures of living microorganisms, detritus, and inorganic particles that widely range in size. It has been speculated that some marine heterotrophic microorganisms, such as *Vibrio parahaemolyticus*, could enter in this digestive process and persist in the oyster tissue. Since some strains of *V. parahaemolyticus* are pathogenic for humans, these bacteria are considered to be a constant menace for health and aquaculture. In order to improve the safety of marine products it is imperative to obtain more knowledge about *Tiostrea chilensis* and its interactions with *V. parahaemolyticus*. In this study *V. parahaemolyticus* ATCC 17802 was tagged using plasmid pKV111, which carries the *gfp* gene that codifies a Green Fluorescent Protein (GFP), thereby allowing these strains (VpGFP) to be detected under epifluorescence microscopy. Results obtained showed that *T. chilensis* can filter VpGFP directly from sea water and suggested that most of them were digested by oysters. However, in the postharvest stage, a small fraction can remain in oyster tissues after depuration and VpGFP can rapidly grow if the bivalves are stored at room temperature.

Keywords: Green Fluorescent Protein (GFP); Oyster; *Tiostrea chilensis*; *Vibrio parahaemolyticus*

1. Introduction

Oysters are a habitat for a large number of marine bacterial species. Some of them may cause severe gastroenteritis (Wright et al., 1996; Daniels et al., 2000; Volety et al., 2001) with serious consequences to humans consuming oysters. Understanding the bacterial ecology of these bivalves can help to improve both the management of hatcheries for higher productivity and the safety of oysters as food.

Bivalve mollusks filter large volumes of water to trap suspended particles and dissolved substances as a source of food. Pathogenic bacteria in seawater may be concentrated in bivalves. Because the entire body of shellfish, including its gastrointestinal tract is often

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consumed raw or slightly cooked, shellfish is generally classified as a high-risk food group by health authorities worldwide (Richards, 1991).

Vibrio is a genus found primarily in seawater and in association with aquatic animals, therefore, its presence is independent of anthropogenic pollution, but dependent on salinity, temperature and organic loads (Hjeltnes and Roberts, 2001). Some species of this genus, like *Vibrio parahaemolyticus* (Daniels et al., 2000) and *Vibrio vulnifus* (Rodrick, 1991; O'Neill et al., 1992; DePaola et al., 1994) can cause gastrointestinal diseases in humans. *V. parahaemolyticus* is a gram-negative, halophilic bacterium that naturally inhabits marine and estuarine environments.

Bivalves are filter-feeding animals that capture its feed by producing a current with its gill cilia and then sending particles to the gill filaments. Mucus secreted by the filaments capture many different microorganisms, detritus and inorganic particles, and carries them to the labial palps where they are sorted according to their physical and chemical characteristics (Winter, 1978; Shumway et al., 1985; Silvester, 1988; Ward et al., 1994; Newell and Langdon, 1996; Beninger and St-Jean, 1997; Ward and Shumway, 2004). Once in the stomach they are digested by enzymatic activity of the crystalline style (Ruppert and Barnes, 1994). The resulting particles are then carried to the digestive diverticula where they are absorbed. Non-absorbed particles pass to the intestine and are discarded through the anus. Some authors have reported that some microorganisms could be able to resist this digestive process (Cook, 1991; Paster et al., 1996).

According to Wright et al. (1982) and Charles et al. (1992), oysters are able to efficiently retain small particles (1 to 2 μm), but the capacity of bivalves to filter bacteria directly from the water is still in discussion (Charles et al., 1992). Le Gall et al. (1997) and Dupuy et al. (1999) proposed that oysters can retain bacteria only with a "trophic link" (i.e. a planktonic ciliate). On the other hand, Sakasaki (1969), Prieur et al. (1990), Olafsen et al. (1993) and Daniels et al. (2000) said that oysters can filter bacteria by themselves. Therefore, common marine bacteria, such as *Vibrio* sp. can be concentrated in bivalves.

Although there are few publications about the bacterial microflora of these invertebrates (Prieur, 1987), several authors have contributed to the knowledge on this matter; these reports agree that *Vibrio* sp. is isolated frequently and reaches about 30% of the bacterial colony counts (Colwell and Liston, 1960; Kueh and Chan, 1985; Prieur, 1987; Olafsen et al., 1993). *V. parahaemolyticus* had been reported as the etiologic agent in two outbreaks of gastroenteritis associated to the consumption of raw shellfish in Chile (Córdova et al., 2002; González-Escalona et al., 2005).

After harvest, bivalves pass through several situations, i.e. transport from harvest location to oyster retailer while remaining dry and without refrigeration. During this time, the size and composition of the microbial community changes remarkably (Hoff et al., 1967; Cook, 1991; Romero et al., 2002); it represents spoilage of organoleptic properties and sanitary quality of mollusks (Jackson et al., 1997). In *Tiostrea chilensis*, it has been demonstrated that bacteria counts increase 50–100 fold with notable changes in the bacterial composition during storage (Romero et al., 2002). In *Crassostrea virginica* and *Crassostrea gigas*, it has been observed that *V. parahaemolyticus* and *Vibrio vulnificus* diminish in bivalves stored at temperatures below 10 °C, however, they can reach very high concentrations when the bivalves remain over 20 °C (Kaspar and Tamplin, 1993; Cook, 1994; DePaola et al., 1990). It has been observed that the amount of pathogenic vibrios, in bivalves during depuration, depends on water temperature (Murphree and Tamplin, 1995). DePaola et al. (1990) indicated that temperature was correlated with *V. parahaemolyticus* densities in either water or oysters (*C. virginica* and *C. gigas*).

In this study, the fate of *V. parahaemolyticus* once filtered by oysters and its capacity to proliferate in different post harvest conditions was defined, using a strain of *V. parahaemolyticus* with a plasmid that contains the *gfp* gene (VpGFP). This gene codifies for a fluorescent protein (GFP) that, when excited with UV light (470 nm), emits a wavelength of 502 nm (Stretton et al., 1998; Bloemberg et al., 2000; Lowder et al., 2000; Nyholm et al., 2000). Thus, these bacteria can easily be detected by their green coloration and be distinguished from other existent bacteria. This approach allows us to estimate the filtration of VpGFP by *T. chilensis* and trace it in several tissues after ingestion. The use of GFP to monitor the fate of microorganisms in invertebrate systems has been documented (Engelhard et al.,

1994; Barrett et al., 1998) and recently GFP has been used to monitor the development of the human parasites (Guevara et al., 2001). In this study, we incorporated a constitutive GFP construct into the pathogen *V. parahaemolyticus* ATCC17802 to monitor their fate during transit through oysters.

2. Materials and methods

2.1. Obtaining, maintenance and purification of oysters

Healthy oysters of commercial size (*T. chilensis*) from Calbuco (latitude 41 South, Chile) were used. Oysters were washed to eliminate epifauna, placed in a maintenance system for at least 15 h before beginning the experiments and fed with 10 $mg \cdot l^{-1}$ of *Isochrysis galbana*, what is equivalent to 8×10^4 $cel \cdot ml^{-1}$, according to adapted values of Newell and Langdon (1996). A system was built to maintain and purify oysters with controlled temperature, salinity, pH and turbidity, according to the norms stipulated in Scheduled Depuration Process (SDP) (FDA, 1992). Natural seawater was used, with a salinity of 34.5 PSU, filtered by 5.0 and 0.2 μm.

2.2. Obtaining and culturing the bacterial strain V. parahaemolyticus ATCC 17802 with the plasmid pKV111

To produce a strain of *V. parahaemolyticus* with the plasmid pKV111, triparental mating was performed (Stabb and Ruby, 2002). The plasmid pKV111 contains the *gfp* gene that codifies for the Green Fluorescent Protein (GFP) and also contains a gene that provides chloramphenicol and erythromycin resistance. When this protein is synthesized, the bacterium can be easily detected under UV light (470 nm) (Nyholm et al., 2000). Triparental mating was performed for 24 h at 37 °C in Luria Bertani culture media (LB) between *Escherichia coli* strain DH5α carrying pKV111, donated by Erick V. Stabb (Kewalo Marine Laboratories, University of Hawaii), and *V. parahaemolyticus* strain ATCC 17802. An *E. coli* helper strain, carrying the genes that code for conjugation and DNA transfer was also included. An aliquot of this culture was then seeded in SLB with 5 $\mu g \cdot ml^{-1}$ chloramphenicol at 37 °C (Nyholm et al., 2000) to allow only the proliferation of bacteria with the plasmid. After 24 h of culture, an aliquot was seeded in thiosulphate–citrate–bile salts agar (TCBS), a selective culture media for vibrios, and then incubated for 24 h at 37 °C (Wright et al., 1996). The Colony Forming Units (CFU) that grow in the TCBS plate were observed with epifluorescence microscopy with an excitation filter of 470–490 nm, then one fluorescent CFU was selected. These bacteria were cultivated in saline Luria Bertani media (SLB). The culture media SLB were always used with 5 $\mu g \cdot ml^{-1}$ of chloramphenicol at 37 °C (Nyholm et al., 2000). When the culture reached the exponential phase, it was washed with seawater and added to the container with the oysters, reaching a final concentration of 5×10^6 $cell \cdot ml^{-1}$. The determination of the VpGFP concentration in oysters and in the water of the aquarium was made counting the number of CFU in TCBS plates as described above. Only the fluorescent colonies observed under an epifluorescence microscope were counted.

2.3. Determination of the filtration activity of oysters

The filtration activity of the oysters was verified by feeding them with 5 $mg \cdot l^{-1}$ of *I. galbana* var. Tahiti, as described by Newell and Langdon (1996). The reduction in this concentration was observed using a Neubauer counting chamber. The laboratory culture of *I. galbana* was made from a stock donated by Bernardita Campos of the University of Valparaiso. The microalgae were cultivated in f/2 media of (Parrish et al., 1999), with a 12 h photoperiod in a culture camera at 16 °C. In order to determine the concentration of VpGFP in the soft body of the oysters, their tissues were homogenized with Tissue Tearor (model 985-370, Biospec Products) in sterile seawater at maximal velocity by 2 min in an ice bath (Tamplin and Capers, 1992; Romero and Espejo, 2001). Later, the samples were seeded in Petri dishes with TCBS and incubated at 37 °C. After 24 h, fluorescent CFU in the Petri dishes were counted.

2.4. Statistical analysis

Data are expressed as means ± S.E. Statistical comparisons were made using chi-square test, Stu-

dent's *t*-test for paired data and ANOVA test using the statistical package Origin 6.0. A probability (p value) < 0.05 was considered significant.

3. Results

3.1. Filtration and ingestion of VpGFP by T. chilensis

In order to determine if *T. chilensis* is able to retain and eat VpGFP, oysters were placed in a closed system for 24 h. During this time an 85% reduction in VpGFP concentration was observed measuring it at different times after the addition of VpGFP to the aquarium water containing oysters with verified activity. The initial VpGFP concentration measured in water samples was 8.8×10^6 fluorescent CFU $\cdot$ ml^{-1}. Eight hours later, the concentration increased to 3.3×10^7 fluorescent CFU $\cdot$ ml^{-1}. Twelve hours after the beginning of the experiment, the VpGFP concentration began to decrease, reaching 1.3×10^6 fluorescent CFU $\cdot$ ml^{-1} in 24 h, representing an 85% decrease compared to the initial concentration (Fig. 1A). In the control tank without oysters, VpGFP concentration remained relatively constant and a single increase was observed at 24 h (Fig. 1A). The variation in the VpGFP concentration between the experimental and control groups were significantly different ($p = 0.00874$). In order to determine the fate of VpGFP apparently ingested by the oysters, its presence in oysters was analyzed. But, of the total VpGFP apparently ingested, only 0.1% (4×10^7 fluorescent CFU per oyster) was recovered.

To verify that the reduction in VpGFP concentration in the water was due to the oysters' filtration activity and not to the colonization of exposed oyster surfaces, a similar situation was prepared simultaneously by using cooling to kill the oysters. An increase of VpGFP concentration in the aquarium water was observed from 6.8×10^7 fluorescent CFU $\cdot$ ml^{-1} up to 2.0×10^8 fluorescent CFU $\cdot$ ml^{-1} in 24 h (Fig. 1B). In the control without oysters, VpGFP concentration remained relatively constant and a single increase was observed at 9 h (Fig. 1B). The variation in the VpGFP concentration between dead oysters and the control group were significantly different ($p = 0.00837$). Oysters were analyzed in order to determine if VpGFP has the capacity to proliferate in the oyster tissues without active filtration. A concentration of 2.4×10^8 fluorescent CFU per oyster was found.

3.2. Distribution of the surviving vibrios in oyster tissues and their variation in time

Oysters maintained in an aquarium with 3×10^6 VpGFP $\cdot$ ml^{-1} for 5 h, were transferred to an aquarium without VpGFP for 45 h, and the variation in VpGFP

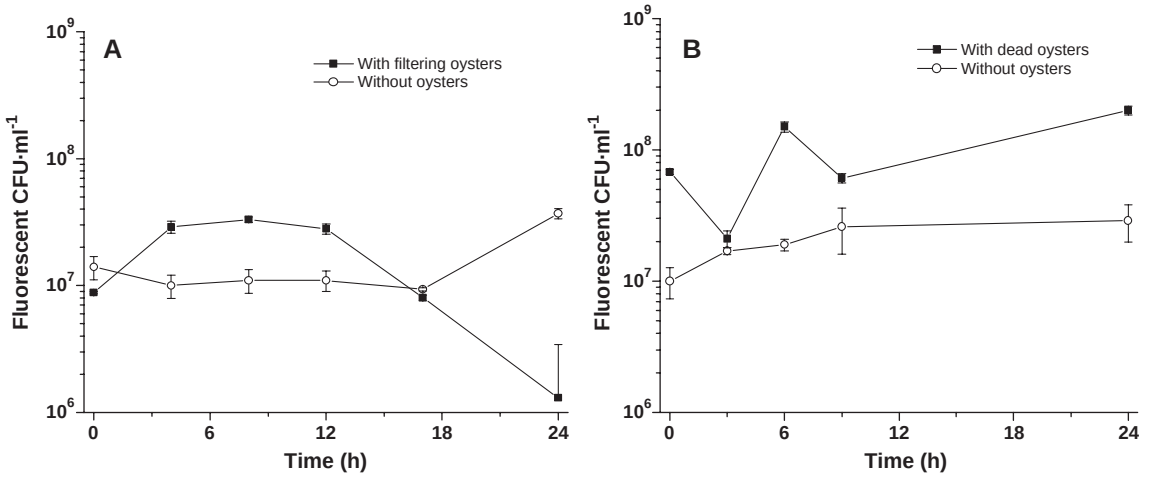

Fig. 1. VpGFP concentrations in seawater with different groups of *Tiostrea chilensis*, during exposure to 9×10^6 VpGFP ml^{-1}. (A) Variation in VpGFP concentrations in seawater with filtering oysters; (B) Variation in VpGFP concentrations in seawater with cooling to kill oysters. In both cases, control was seawater without oysters. Means $\pm$ S.E., $n = 3$.

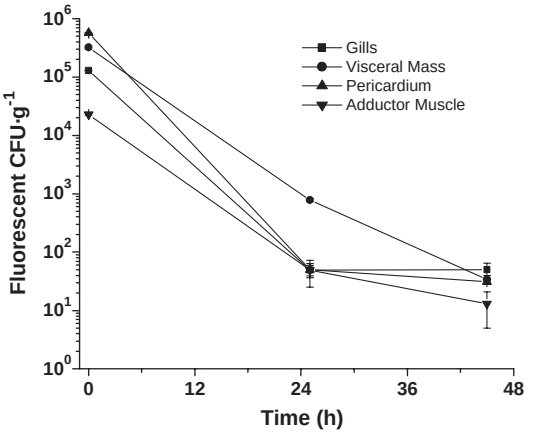

Fig. 2. Variation in VpGFP concentration in different tissues of depurating oysters formerly exposed to 3×10^6 VpGFP ml^{-1}. Means ± S.E., $n=4$.

concentration in their tissues was periodically measured. Because *T. chilensis* is not a large species, there were only analyzed gills, visceral mass, adductor muscle and pericardium. Gills, visceral mass and pericardium reached similar concentrations of VpGFP during the exposure. After 24 h of depuration, the VpGFP concentrations in these tissues diminished drastically in 99%; only 31 to 50 fluorescent CFU $\cdot g^{-1}$ could be found. These levels then remained constant throughout the remainder of the experiment (Fig. 2). The different VpGFP concentrations found in analyzed tissues were not significantly different ($p=0.68114$). In general terms, the VpGFP concentration in oysters reduced from 2.4×10^6 to 2.9×10^2 fluorescent CFU $\cdot g^{-1}$ in the 48 h following exposure (Fig. 2).

3.3. Determination of the VpGFP released from gills to the water (pseudofaeces) or from the alimentary canal (faeces)

The following experiment was performed in order to study whether the reduction in VpGFP concentration registered in oyster tissues (gills, visceral mass, pericardium and adductor muscle) in the previous experiment was due to the digestion of VpGFP by oysters or to its release into the water from gills in the pseudofaeces or from alimentary canal in the faeces. In this experiment VpGFP concentrations were measured in aquarium water containing oysters previously maintained by 5 h with 2×10^6 VpGFP $\cdot ml^{-1}$. The VpGFP concentrations were also measured in the oysters. The results showed that VpGFP release occurs mainly in the first 24 h. Within 100 h, 1.8×10^5 fluorescent CFU per oyster were released to water. This value corresponds to a minor fraction (4.8%) of the VpGFP that disappears from oysters during depuration (3.7×10^6 fluorescent CFU per oyster).

3.4. Proliferation of vibrios in oysters according to its maintenance in different storage conditions

In order to determine if VpGFP proliferation in oysters varies according to storage conditions, oysters, after having filtered this bacteria for 5 h, were stored in three different conditions—dry at room temperature, dry at 16 °C and in depuration at 16 °C. The initial concentration in oysters stored dry at room temperature was 5.0×10^3 fluorescent CFU $\cdot g^{-1}$, an increase of 150 times the initial VpGFP concentration was observed, reaching 7.5×10^5 fluorescent CFU $\cdot g^{-1}$ in 70 h. However, in oysters stored dry at 16 °C, VpGFP concentration slightly diminished, from 5.7×10^3 to 1.4×10^3 fluorescent CFU $\cdot g^{-1}$ in 100 h, showing a 40,4% reduction. Oysters in depuration at 16°C in seawater without bacteria decreased its VpGFP concentration drastically, from 3.3×10^4 to 6.1×10^2 fluorescent CFU $\cdot g^{-1}$ in 70 h (a 98.1% decrease), reaching 8.3 fluorescent CFU $\cdot g^{-1}$ in 100 h (Fig. 3). Significant differences were found between VpGFP concentrations in oysters in the three different storage conditions ($p=0.052$).

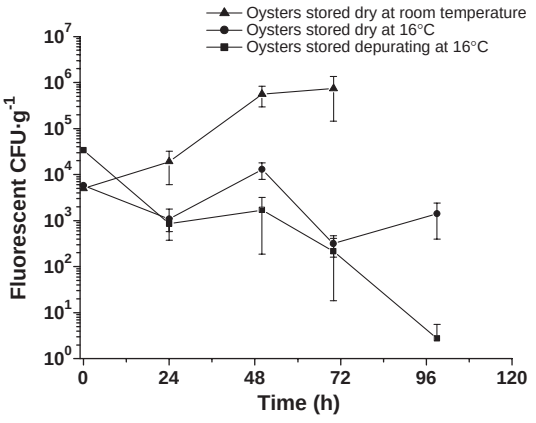

Fig. 3. Variation in VpGFP concentrations in oysters stored at different conditions, formerly exposed to 2×10^6 VpGFP ml^{-1} for 5 h. Means ± S.E., $n=3$ ($p<0.01$).

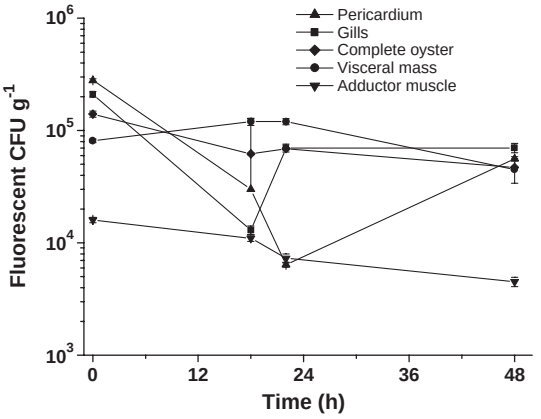

Fig. 4. Variation of VpGFP concentration in different oyster tissues in post-harvest conditions (dry at room temperature) previously exposed to 2×10^6 VpGFP ml^{-1}. Means ± S.E., $n = 3$.

3.5. VpGFP proliferation and determination of the tissue where this mainly occurs during storage

In order to determine which of the oyster tissues presents the major proliferation of VpGFP during storage, once oysters were maintained by 5 h with 2×10^6 VpGFP ml^{-1}, they were harvested and placed in the dry at room temperature storage condition, given that this condition showed the greatest proliferation in the previous experiment. Only the concentration of tissues during the first 48 h of storage was determined, due to the spoilage of oysters. The results showed that the adductor muscle is the tissue that always presents the lowest VpGFP concentration. The highest values were obtained in the visceral mass, which were only exceeded in the initial phase by gills and pericardium (Fig. 4). The VpGFP concentration in whole oysters remained constant during the 48 h of experimentation. There were no significant differences in VpGFP concentrations found in different tissues maintained dry at room temperature, except when the tissue with the greatest VpGFP concentration (visceral mass) was compared with that of the lowest concentration (adductor muscle) (Table 1).

4. Discussion

Numerous authors have discussed the capacity of oysters to filter bacteria directly from the water, especially marine bacteria that are pathogenic for humans (McHenery and Birkbeck, 1985; Prieur, 1987; Prieur et al., 1990; Charles et al., 1992; Olafsen et al., 1993; Murphree and Tamplin, 1995).

McHenery and Birkbeck (1986), Birkbeck (1987) and Prieur (1987) tested several strains of *Vibrio* in mytilids; their results indicate that some of these bacteria would inhibit the filtration of these bivalves. Le Gall et al. (1997) and Dupuy et al. (1999) hypothesized that oysters could not retain bacteria directly, but rather they filter a planktonic ciliate that feed on the bacteria and would act as a trophic link. These data are opposed to the results obtained in this study. The reduction observed in VpGFP concentration in the water (85%) would be due to the presence of filtering oysters, given that in the experiment made with dead oysters an increase of 195% was observed in the concentration during the same time period. The total increase in VpGFP concentration in seawater with dead oysters (195%), and during the first 4 h with filtering oysters, is due to the organic matter contributed by these bivalves, since growth was not registered when these bacteria were placed in filtered water without oysters.

Our results explain why *Vibrio* is present in tissues of wild *T. chilensis* (Romero and Espejo, 2001), agreeing with Sakasaki (1969), Prieur et al. (1990), Olafsen et al. (1993) and Daniels et al. (2000) that affirm that *V. parahaemolyticus* is a marine bacterium that can be concentrated by bivalves.

The concentration found in the analysis of live oysters, equivalent to 0.1% of VpGFP apparently ingested, would indicate that most VpGFP filtered by oysters is quickly digested, according to what was reported by Birkbeck and McHenery (1982) and by Prieur (1987). Volety et al. (2001) indicated that filtered microorganisms can be ingested by oysters,

Table 1
Probability (p values) obtained from one-way ANOVA tests comparing VpGFP concentrations in tissues of oysters exposed to these bacteria and then stored for 48 h dry at room temperature

Tissues	p value
Pericardium—gills	0.97627
Pericardium—visceral mass	0.98134
Pericardium—adductor muscle	0.23486
Gills—visceral mass	0.98743
Gills—adductor muscle	0.10209
Visceral mass—adductor muscle	0.00412[a]

[a] Significantly different ($p < 0.01$).

leading to its final destruction in the digestive tract or to its elimination in the faeces, although, some microorganisms can persist in the oysters by adhesion or invasion of the tissue. The low VpGFP concentration found in live oysters suggested that this bacterium were quickly digested (Prieur, 1987). This idea takes force when the analysis of tissues is considered, in which VpGFP does not show a preference for any tissue and is also eliminated from all tissues within a very short period of time. This is not in agreement with Cabelli and Heffernan (1970), who found that the organ where the accumulation of bacteria takes place is the digestive gland. Farley (1977) and Stein et al. (1987) reported that, unlike mammals, healthy invertebrates can have bacteria in their fluids and tissues. No evidence was found that indicate some kind of settlement or growing of the bacteria in the oysters. According to Tripp (1960), bacteria are rapidly removed from hemolymph of *C. virginica* by haemocytes within 1 or 2 days, but are able to persist in tissues for many more days. Similar data in hemolymph are reported by Feng (1966), Cheng and Cali (1974), Cheng and Rudo (1976), Hartland and Timoney (1979) and Birkbeck and McHenery (1982).

If VpGFP could survive the digestive process, they would be able to colonize in oysters by either growing or just staying in them (Volety et al., 2001); or they could be eliminated alive in faeces or pseudofaeces, according to Prieur et al. (1990) and Allam et al. (2001). There are two ways by which bacteria can get out of the oysters and into the water. One is by being released in the faeces (Volety et al., 2001), which would indicate that the bacteria were ingested. The other way is by being released in the pseudofaeces (Allam et al., 2001), which would indicate that the bacteria were not ingested, but instead were discarded in the labial palps (Beninger and St-Jean, 1997). Due to the fact that only 4.8% of the total VpGFP initially present in oysters were found in the water (in 100 h), the two possible ways of releasing bacteria described were discarded. The settlement hypothesis was also discarded when the remaining amount of VpGFP in the oysters in the same period (0.02%) was considered. All these results suggest that most of the initially present VpGFP in oysters were digested.

Hoff et al. (1967), Kaspar and Tamplin (1993), and Cook (1994) reported that growth of *V. vulnificus* in harvested oysters depends on temperature. In *T. chilensis*, the influence of temperature on the growth of VpGFP was noticed when the two groups of oysters maintained dry were compared. The oysters stored at room temperature had the highest VpGFP concentrations, but on the other hand, oysters maintained at 16 °C presented a 10-fold decrease in the initial concentration of VpGFP. Richards (1991) indicated that with the exception of vibrios, most bacterial indicators and bacterial pathogens can be adequately depurated within 72 h. This is opposite from our results, which verified the importance of depuration in the maintenance of VpGFP in low amounts, when the decrease of 98.1% in VpGFP concentration within 70 h in depurating oysters is compared with the variation of the same bacterium concentration in oysters kept dry at the same temperature (40.4%).

In the oysters stored dry at room temperatures, the highest VpGFP concentrations were found were in visceral mass, gills and pericardium. Such high concentrations in visceral mass and gills was most likely due to the fact that they provided an entrance for bacteria into filtering oysters. Probably the oysters' circulatory system that allows the pass of hemolymph through the membranous surfaces towards the tissues (Cheng, 1981) permitted VpGFP to reach the hemolymph (pericardium) with such high concentrations. None of the analyzed tissues showed a variation in their VpGFP concentration within 48 h. Tripp (1960) demonstrated that in living oysters, bacteria were rapidly removed from hemolymph by the haemocytes within 24 to 48 h, but they were capable of persisting in the tissues for a longer period of time. The same was observed, and also related to haemocytes phagocytosis, by Feng (1966), Cheng and Cali (1974), Cheng and Rudo (1976) and Hartland and Timoney (1979). If haemocytes are capable of phagocyting bacteria in such an efficient way, they must have stopped their activity since oysters were harvested and exposed to air at room temperature. It is likely due to the closure of the valves and the consequent decrease in the cardiac rate, oxygen partial pressure in the tissues and pH. If all this happened, VpGFP concentration should increase in time. But this did not occur within the 48 h of experimentation, probably because the reduction in haemocyte activity is gradual. According to that previously stated, VpGFP

would not present any kind of chemotaxis or preference for any specific oyster tissue, and would be phagocyted in each tissue with the same strength.

5. Conclusion

Results from our study suggest that VpGFP are obtained directly from seawater by *T. chilensis* and most of them are digested by oysters; only a small fraction is able to persist in the oyster tissues without showing preference for any one tissue in particular. The few non-digested bacteria that remain in the tissues grow according to storage conditions; only oysters stored at room temperature present VpGFP growth. Depuration eliminates nearly all VpGFP from oysters within 70 h.

Acknowledgments

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