DEPURATION OF STRIPED VENUS CLAMS FOLLOWING ARTIFICIAL VIBRIONES CONTAMINATION

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ABSTRACT

Vibrio spp are widely distributed in the marine environments and are responsible for common illnesses in many countries. The main objective of this study was to investigate the depuration capacity of *Venus gallina* artificially contaminated with *Vibrio parahaemolyticus* and *Vibrio vulnificus*. Contamination experiments were used to assess the accumulation capacity of clams in poorly, moderate and highly contaminated waters (*Vibrio spp*10^3/ml, 10^4/ml, 10^6/ml). All bivalves were exposed to contamination for 72 h and 8 experiments were performed. Accumulation capacity varied with respect to initial level. Comparing experiments dataset with two vibrios species, clams showed a different specific accumulation: in particular, molluscs evidenced a scarce accumulation capacity of *V.vulnificus*. Depuration trials were performed in close-circuit seawater-disinfection system that uses filtration, Ultra violet (UV) and ozone. Bivalves samples were collected every 12h until 3 days of depuration. Most of the depuration trials with *V.parahaemolyticus* showed a decrease in initial bacterial loads (4times lower values) after36-48 hours, but in subsequent periods, the trend remained stationary. In *V. vulnificus* tests, clams showed a scarce depuration capacity instead. Future studies are still required to assess the efficacy of the depuration process in reducing pathogenic Vibrio strain naturally accumulated in clams and to prevent significant economic losses to stakeholders due to long depuration periods.

Keywords: food safety, depuration, clams, *V. parahaemolyticus,V.vulnificus*

1. INTRODUCTION

The exploitation of bivalve molluscan shellfish is of great social and economic importance in the coastal ecosystems of southern Europe (BERTHOU *et al.,* 2005). In particular, the landing of *Venus gallina* play an important economic role in the central and northern Adriatic coasts of Italy, where it has considerably increased in the last decades with the introduction of the hydraulic dredges (MORELLO *et al.,* 2005; MOSCHINO and MARIN, 2006). The venerid bivalve, *Venus gallina,* is a mollusc distributed throughout the Mediterranean and Black Sea (MOSCHINO and MARIN, 2006). The interest towards this clam increased also in relation to its nutritional characteristics. In fact, it has interesting dietetic properties due to the low lipid and cholesterol contents, presence of phytosterols and the high percentages of healthy polyunsaturated fatty acids (ORBAN*et al.,* 2006). Since bivalves are filter-feeding organisms, they can accumulate pathogenic microorganisms (for example, bacteria, human viruses and microalgae), having a significant health risk if consumed raw or lightly cooked (COOK, 1991; LEES *et al.,* 2010). Generally, bivalves' bacterial composition is dominated by Gram-negative bacteria like Vibrionaceae and Enterobacteriaceae (CAO *et al.,* 2009). Among the indigenous microbiota of coastal environments, the family Vibrionaceae, particularly *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae*, is targeted as a causative agent of human disease due to the consumption of shellfish (BUTT *et al.,* 2004; NORMANNO *et al.,* 2006; RIPABELLI *et al.,* 1999). The occurrence of potentially pathogenic *Vibrio spp*. in coastal waters and shellfish of European countries has already been documented in Italy, Spain and France (BARBIERI *et al.,* 1999; HERVIO-HEATH *et al.,* 2002; MARTINEZ-URTAZA *et al.,* 2008, ROQUE *et al.,* 2009). The number of *Vibrio spp*. in shellfish varies widely and depends on the geographical area, environmental conditions and local parameters. Salinity and temperature are in fact important parameters in the dynamics of vibrios in marine systems (HSIEH *et al.,* 2008; BLACKWELL and OLIVER, 2008; DEPAOLA, 2003; PFEFFER *et al.,* 2003; RANDA *et al.,* 2004). Some studies have shown that *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* enter a viable but non-culturable state when water temperature average is below 15°C (COLWELL and GRIMES, 2000; ROSZAK and COLWELL, 1987). On the other hand, temperatures above 20°C favor the growth of Vibrio spp. in seawater (BLACKWELL and OLIVER, 2008; DEPAOLA *et al.*, 2003).

In order to reduce shellfish contamination and to minimize the inherent risks of shellfish consumption, legislation sets requirements for sample collection, wet storage, bivalve self-purification by depuration and/or relaying (tank construction and operation, packaging, labelling), shellfish processing, laboratory analytical methodologies and product distribution (Reg. N.853/2004/EC, Reg. N.854/2004/EC). The legislation employs a classification to the seafood harvesting areas according to bacterial indicators of sanitary quality (*E. coli*). This classification determines whether shellfish can be sent for direct consumption or must be treated prior to commercialisation (LEES, 2000). Furthermore, the current European legislation does not specify limits for *Vibrio spp*. (EC, 2001). Therefore, preventive measures should be implemented for *Vibrio spp*., taking into account that they are naturally found in seawater and normal constituents of mollusc flora (BARILE *et al.,* 2009) and some strains (such as *V. parahaemolyticus*) are the major cause of epidemics associated with the consumption of bivalves (MEAD *et al.,* 1999; WITTMANN and FLICK, 1995).

Relaying and depuration are common approaches to reducing bacterial loads in shellfish. In the relaying process, shellfish is transferred before harvest from polluted areas to an unpolluted waterway for natural biological purification. Depuration consists of a flow-through or recirculation system of chemically (chlorine, ozone, iodophores, and activated oxygen) or physically (UV irradiation) disinfected water to allow purification under

controlled conditions (LEES, 2000). Effectiveness of the depuration process depends on the diversity and physiology of shellfishes, initial loads of bacterial strains, environmental conditions (temperature, salinity, pH and so on.) and purification system (JOVEN *et al.*, 2011; SCHENEIDER *et al.*, 2009). Several studies have been carried out to evaluate the effect of depuration in physiological and microbiological aspects of some clams species, such as *Ruditapes decussatus*, *Venerupis senegalensis*, *Venus gallina* and *Mercenaria mercenaria* (EL-SCHENAWY, 2004; HOWARD *et al.*, 2003; MAFFEI *et al.*, 2009). In general, the depuration process is particularly efficient in the reduction of total viable counts and *E. coli* levels (ANACLETO *et al.*, 2013), but not with *Vibrio spp*. In fact, *Vibrio spp*. requires longer depuration periods than *E. coli* to become effective (COLAKOLU *et al.*, 2014; COZZI *et al.*, 2009; CROCI *et al.*, 2002; LOPEZ-JOVEN *et al.*, 2011). CROCI *et al.* (2002) indicated that 44 h of depuration process led to a decline in Vibrio by a factor of only 10, whereas, COZZI *et al.* (2009) describes that 72 h reduced Vibrio contamination to a level close or below the detection limit of the methods. Also, LOPEZ-JOVEN *et al.* (2011) reported that at least 10 days depuration at 20°C is effective in reducing Vibrio load.
In this context, the main objective of this study was to investigate the depuration capacity of *Venus gallina* artificially contaminated for three days with *Vibrio parahaemolyticus* and *Vibrio vulnificus* running tests.

2. MATERIALS AND METHODS

2.1. Collection of samples and treatments

Venus gallina specimens were taken at about 250-500 m from Molise coastline in classified areas. Clams were harvested with hydraulic dredges. The specimens (mean size: 25-32 mm) were taken to the laboratory in refrigerated containers (4°C) within 2 hours. Dead or damaged specimens were discarded and the remainder was divided into seven aliquots of 250 clams. Before the trial, specimens were acclimatised for 72h in aquariums with 100 litres of recirculated and artificial seawater (Instant Ocean Aquarium System salinity: 35‰). Temperature was fixed at 18°C.
After acclimatization, ten specimens for each aliquots underwent bacteriological analysis (*V. parahaemolyticus, V. vulnificus*) to evaluate the sanitary conditions and the initial load of each pathogen for determining the contamination levels.

2.2. Contamination experiment

Vibrio parahaemolyticus ATCC 17802 and *Vibrio vulnificus* ATCC 27562 were used for trials.
Bacterial contamination was conducted in 8 l (0.12 m^3) tanks filled with artificial seawater (Ocean Fish Marine Salts-Prodac). In this study, contamination experiments were conducted to assess the accumulation capacity of clams in poorly, moderate and highly contaminated waters (10^3/ml, 10^4/ml, 10^6/ml). Contamination levels were chosen from results of previous studies (BARILE *et al.* 2009; BARILE *et al.* unpublished). Details of each contamination trial are reported in Table 1.
To test the influence of temperature on *Vibrio vulnificus* accumulation in clams, two trials were conducted at different artificial conditions: 35‰salinity and 22°C temperature (test VV2), 30‰salinity and 25°C temperature (test VV3). Parameters were established considering results of previous studies (COLWELL and GRIMES, 2000; BLACKWELL and OLIVER, 2008).

All bivalves were exposed to contamination for 72 h. After contamination period, ten replicates for each test were analyzed to evaluate the variability of their accumulation capacity.

Table 1. Details of contamination trials.

Species	Bacterial load	Temperature	Salinity	Test
Vibrio parahaemolyticus	$2.30*10^3$	22°C	35‰	VP1
Vibrio parahaemolyticus	$3.75*10^4$	22°C	35‰	VP2
Vibrio parahaemolyticus	$9.37*10^4$	22°C	35‰	VP3
Vibrio parahaemolyticus	$1.12*10^6$	22°C	35‰	VP4
Vibrio vulnificus	$2.30*10^3$	22°C	35‰	VV1
Vibrio vulnificus	$7.5*10^4$	22°C	35‰	VV2
Vibrio vulnificus	$7.5*10^4$	25°C	30‰	VV3
Vibrio vulnificus	$1.12*10^6$	22°C	35‰	VV4

2.3. Depuration experiment

At the end of contamination, all clams were transferred to depuration system (Fig. 1), except for VV3 test. Specimens were placed in a single layer on a plastic grill to avoid contact with the bottom of the tank and thus, minimise recontamination.

Figure 1. Trial depuration system.

Depuration systems consisted of tanks fitted with a wool/Perlon prefilter and hyperactive carbon filter, an active biological filter using *Lithothamnium calcareum* algae and a UV sterilisation plant (Fig. 2), and filled with artificial seawater (Ocean Fish Marine Salts-Prodac). The pump takes water from the tank containing the biological filter (*L. calcareum*

algae) and pumps it into the UV sterilisation unit and then on, through the bubbler to reach the tank. It then crosses to the chamber fitted with the wool/Perlon and the carbon filters, through the serpentine cooling unit (in which ozonation also takes place) and back to the tank containing the biological filter. The water is then taken back up by the pump and recirculated. The water temperature is controlled by a thermostat (18°C).
The chemical, physical and microbiological parameters of water (temperature, salinity, dissolved oxygen, vibrios detection) and clams vitality were checked daily to control proper functioning of aquarium.
Bivalves samples were collected every 12h for 3 days. Samples were immediately processed using the methods reported below.

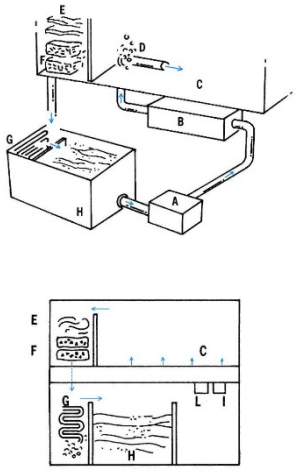

Figure 2. Depuration system:(A) Pump;B) UV sterilisation unit;C) Tank;D) Bubbler;E) Wool/Perlon filter;F) Carbon filter;G) Serpentine cooling unit;H) Biological filter;I) Ozonation air pump.

2.4. Enumeration of *Vibrio parahaemolyticus and Vibrio vulnificus*

For quantification of *Vibrio parahaemolyticus*, a MPN modified method was used. Clam meat was added to alkaline peptone water (APW). Serial ten-fold dilutions were prepared in APW, incubated at 37°C for 16-18h and subsequently plated on thiosulphate citrate bile salt sucrose agar (TCBS) and incubated at 37°C for 18-24h. Biochemical confirmation was performed with individual and miniaturised tests (API 20E: 4154106 for *V.parahaemolyticus*, 5346007 for *V. vulnificus*; API 20NE: 7077444 for *V. parahaemolyticus*, 7432055 for *V.vulnificus*). For the quantification of *Vibrio vulnificus*, analyses were conducted as described above, using the selective medium m-CPC (incubation at 40°C for 18-24h). Results are expressed in MPN/100g (Most probable Number).

3. RESULTS AND DISCUSSION

3.1. Clams mortality during trials

Mortality ranged from zero to five specimens during all trials. It should be stressed that, in this study, depuration experiments were carried out in a closed system, with a periodic control of water parameters. In addition, clams were placed in depuration systems at a

lower density than those commonly contained in bins of depuration centers. These excellent conditions could influence positively clams mortality rates.

3.2. Depuration system parameters

Chemical and physical parameters of water showed minimal variations (lower than 5%). No pathogen was detected in water samples.

3.3. Contamination experiment

Vibrio parahaemolyticus accumulation values were in one lower order of magnitude than water concentrations.
In fact, specimens of *Venus gallina* showed vibrios median values of 430MPN/100g, 9200 MPN/100g, 1100MPN/100g and15000MPN/100g respectively, at water levels of $2{,}3*10^{3}$/ml; $3{,}75*10^{4}$/ml; $9{,}37*10^{4}$/ml; and $1{,}12*10^{6}$/ml.
Considering experimental results, microbial loads changed markedly among different replicates in each trial (Table 2).

Table 2. *Vibrio parahaemolyticus* values after 72h contamination at controlled conditions: temperature 22°C and salinity 35%.

	TEST VP1 MPN/100 g	TEST VP2 MPN/100 g	TEST VP3 MPN/100 g	TEST VP4 MPN/100 g
Min	92	6.800	740	1.500
Max	930	17.000	2.200	360.000
average value	435	10.200	1.237	51.680
median value	430	9.200	1.100	15.000

Vibrio vulnificus accumulation values did not show a clear pattern regarding water concentrations (Table 3). This data was particularly evidenced in test VV4, where high contamination levels in water corresponded to a very low *Vibriovulnificus* loads in clams. Moreover, tests comparison between two vibrios species showed that shellfish accumulated fewer *Vibrio vulnificus* than *Vibrio parahaemolyticus*.

Table 3. *Vibrio vulnificus* values after 72h contamination at controlled conditions: temperature 22°C and salinity 35%.

	TEST VV1 MPN/100 g	TEST VV2 MPN/100 g	TEST VV4 MPN/100 g
Min	36	74	74
Max	230	15000	430
average value	125	4110	222
median value	92	1215	230

Besides, bivalve's inter- and intra-specie variability is well known in studies on microbiological contamination. In fact, the amount of water filtered is between twenty and one hundred liters per day, independent of the environmental conditions (RICHARDS,

1988; ROBERTSON, 2007). Bivalve molluscs feeding physiology determine the accumulation of pathogenic microorganisms filtered from the overlaying water (BURKHARDT and CALCI, 2000; HO and TAM, 2000). These phenomena may also partially explain seasonal and geographical differences in microbial content of bivalves (HERNROTH *et al.,* 2002).
In contamination experiments with *Vibrio vulnificus* conducted under different environmental conditions (test VV2 and test VV3), values were not comparable (Table 4).
In particular, at salinity of 30‰ and temperature of 25°C, most of the recorded values ranged from<30 to 350MPN/100g: it may indicate a lower accumulation capacity of clams oralower capacity of pathogen to survive and multiply under these conditions. These facts supported reports from other studies. KASPAR and TAMPLIN (1993) described that the greatest accumulation of microorganisms in hard-shelled clams occurred during certain periods in the spring, at temperatures ranging from 11.5 to 21.5°C. BURKHARDT *et al.* (1992) showed that temperatures outside the range of 13-22°C and salinities greater than 25 ppt reduced the survival of *V. vulnificus* in seawater.
Moreover, OLIVEIRA *et al.* (2011) reported that annual variation in water temperature and salinity influence shellfish's physiological state and therefore, affects the capacity of siphoning and accumulate microbial species.

Table 4. *Vibrio vulnificus* values after 72h contamination levels under different conditions: salinity of 35‰ and temperatures of 22°C (test VV2), salinity of 30‰ and temperatures of 25°C (test VV3).

	TEST VV2	**TEST VV3**
	MPN/100 g	**MPN/100 g**
min	74	36
max	15000	9200
average value	4110	1437
median value	1215	92

At the end, contamination experiment conducted in this study evidenced a variability infiltration capacity; the presence of such inhomogeneous results provide useful suggestions for planning future tests in terms of the number of organisms to be tested and number of organisms and replicas to be examined at different times. The remark of this variability, although, it led to greater difficulty in data processing, was a crucial and essential factor in the discussion of depuration tests.

3.4. Depuration experiment

In depuration trials, VP1-VP3 (Fig. 3), bacterial load showed a decrease after 48 hours with values four times lower than the initial contamination levels. Instead, in tests VP2 and VP4, after 36 hours of depuration, recorded values were four times lower than those at the start. Observed decreases remained fairly constant in the subsequent hours of depuration during all tests. This finding suggests that the initial phase of pathogen's elimination was followed by a "plateau" phase.
Concerning *V.vulnificus* levels during the first depuration trial (VV1), a not clear trend was found over time (Fig. 4). After 48 hours of depuration, lower values were recorded with respect to the initial load, and at the end of experiment (after 72 hours of depuration), values were in one lower order of magnitude with respect to the initial load.

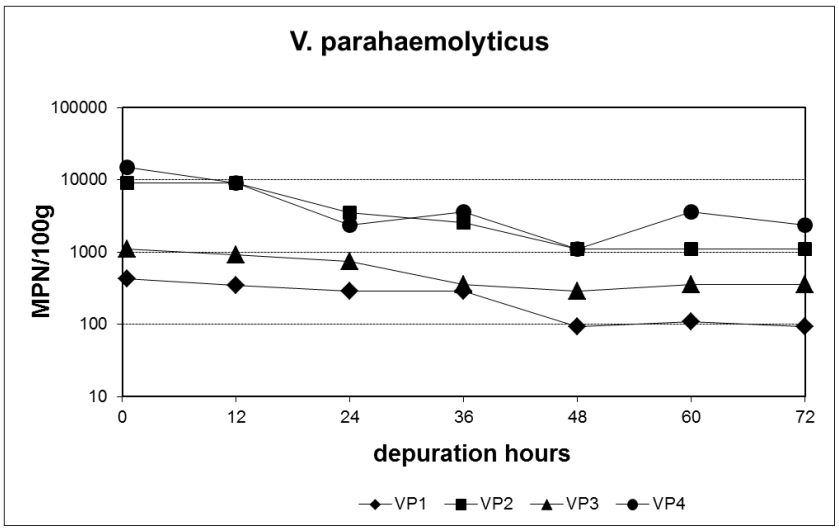

Figure 3. Temporal changes in *Vibrio parahaemolyticus* levels during depuration tests.

In tests VV2 and VV3 (Fig. 4), with low initial values (92 and 230MPN/100g), depuration levels lower than 30MPN/100g occurred after 36 hours. In test 2, this trend has remained constant, while in test 3, higher values (92MPN/100g) were detected after 48 hours. In all depuration treatments with *V. vulnificus*, recorded values were within the range defined by the initial contamination levels, then the apparent decrease may be associated with the variability in contaminated organisms and not to an effective depuration treatment.

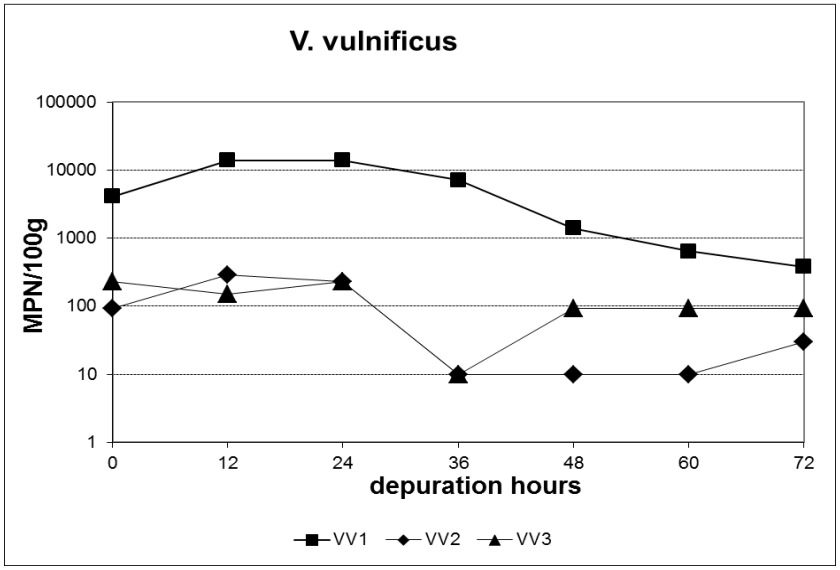

Figure 4. Temporal changes in *Vibrio vulnificus* levels during depuration tests.

In this study, although, it is known that the initial contamination levels affect the depuration efficiency and higher contaminated shellfish require longer purification periods, comparing trials with medium-high initial loads (10^3 to 10^5MPN/100g) showed similar trends. This evidence could be explained by a different response to purification processes by vibrios species. On the other hand, the presence of different pathogens could

affect clams filtration capacity differently. Such observations are in fact recorded for other bivalves: C*rassostrea virginica* oysters showed longer depuration times for *Vibrio spp*. than *E. coli* and *Salmonella tallahassee* (MURPHREE and TAMPLIN, 1991). Results of this research are in agreement with a previous study on depuration of *Venus gallina* by *E. coli*, *Salmonella tiphymurium*and *Vibrio parahaemolyticus* (BARILE *et al.*, 2009).
In addition, in regard to purification methods, previous studies reported that *V. parahaemolyticus* is sensitive to ultraviolet irradiation and chlorine dioxide (HAMAMOTO *et al.*, 2007, WANG *et al.*, 2010). REN and SU (2006) examined the effects of electrolyzed oxidizing (EO) water depuration in reducing *V. parahaemolyticus* and *V. vulnificus* in laboratory-contaminated oysters and found that both species could only be reduced by approximately 1.0-log unit after 8h at room temperature. Considering that adopted purification system was fitted with a UV sterilisation and ozonation plant, the data obtained confirmed those reported in literature. Concerning depuration experiments involving *V. vulnificus*, clams showed a scarce depuration capacity, although, further studies would be needed to give more strength to this hypothesis.
Clams purification capacity may have been influencedby temperature and salinityconditions of depuration processes (18°Cand 35‰).Some studies indicated the effects of water temperature on depuration of *Vibrio spp.* in other molluscan species, especially oysters. Limited reductions in *V. parahaemolyticus* (1.2 log MPN/g) and *V. vulnificus* (2.0 log MPN/g) were observed in oysters after depuration with a UV sterilizer at 22°C for 48 h (CHAE *et al.*, 2009). On the contrary, TAMPLIN and CAPERS (1992) reported that levels of *V. vulnificus* accumulated naturally in Gulf oysters increased by 5 log MPN/g after depuration in UV-sterilized water at 23°C for 48 h.
In this study, at temperatures of 18°C, reductions in one order of magnitude were recorded in only two tests with *V. Parahaemolyticus*. Specific studies are required to determine the optimal conditions for shellfish microbial depuration.

4. CONCLUSIONS

Shellfish production is done globally and their nutritional and economic value is well-known. However, filter-feeding bivalves is an efficient transmitter of seafood-born disease. In fact, the emergence of *Vibrio spp* as human pathogen is of particular concern to shellfish producers. Very few data are available on the number of pathogenic Vibrios and more information is needed to improve the quantitative risk assessment concerning the presence of Vibrios in shellfish (CANTET *et al.*, 2013; WHO, 2011). Bacterial indicators used for shellfish health evaluation were reported as inadequate predictors of the presence of autochthonous bacterial human enteric viruses. Over a long period of time, the high-risk nature of this product and underestimation factors have been well documented in many investigative reports and international agencies.
This study emphasizes a limited capacity of *Venus gallina* to release vibrios, in fact, clams with *V. Parahaemolyticus* showed a depuration capacity only in initial phases, while clams with *V. vulnificus* showed a scarce depuration capacity. Bivalves quality is reduced with time due to lack of feed in the depuration process. Stakeholders experience significant economic losses when depuration periods above 48h are implemented. More sensitive and reliable depuration procedures must be developed and a better knowledge of the parameters affecting the kinetics of the processes of depuration is still needed.

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