

Evaluation of viral shellfish depuration in a semi-professional size tank.

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ABSTRACT

Depuration processes try to eliminate microorganisms using seawater to allow living, filter-feeding shellfish to naturally purge themselves from agents they accumulated from the environment. Until now the only parameter to evaluate depuration was the decrease in *E. coli* counts. Studies have shown that viruses can persist in the environment longer than *E. coli* and that shellfish meeting the end-standard (< 230 *E.coli*/100g) have been involved in viral outbreaks. Starting in February 2000, the European Commission has been funding (Fifth Framework Programme), a 3-year project entitled "Virus Safe Seafood". One of the specific objectives of this project is to innovate technology for shellfish depuration. For this purpose a depuration system was designed and built: a four-tank pilot was selected allowing different parameters (temperature, aeration, nutrition..) to be tested simultaneously and on the same shellfish group. The nominal capacity of each tank (960 kg of oysters) was a compromise between scientific experiments and producer needs. After tank calibration (hydraulic, water stability, water disinfection), assays were carried out with artificially-or naturally-contaminated oysters (*Crassostreas gigas*). The bacteriophage F+RNA specific was used as a viral indicator model. Eleven experiments, four with naturally-contaminated shellfish, have been performed in the pilot with continuous aeration, different water temperatures (from 8°C to 25°C) and, in two experiments, phytoplankton (*Chaetoceros calcitrans* and *Isochrysis affinis galbana*) feeding. The results indicate the major effect of temperature on shellfish depuration, while feeding seems to increase the decontamination only when active physiology is observed (22°C). Viral depuration rates (T90D : times for 90% i.e. 1 log₁₀ reduction) were found to vary between 3-4 days (T>20°C) to 7-11 days (T<16°C). The lower T90D -2 days- was found at 22°C with algae feeding. Mortality rate and oyster flesh weight recorded after each

experiment indicated no effect of any treatment (even high temperature) on these parameters. The same results were obtained on naturally-contaminated shellfish. These data show that the pilot provides all facilities to assess shellfish depuration according to the professional practices.

KEY WORDS: shellfish, depuration, bacteriophage FRNA.

INTRODUCTION

The consumer demand is more and more orientated towards seafood. Because this food represents fresh, safe and dietetic product direct from Nature, seafood demand is growing. However, "natural" has to be distinguished from "safe". Thus, raw, slightly or under cooked food can introduce another main risk associated to the presence of pathogenic microorganisms (Koopmans et al. 2002). For these reasons, the European market has to produce natural safe seafood to assure the safety of the consumers as well as the sustainable development of high quality resource and production. As opposed to many other food products, shellfish or fish quality mainly depend on hydric environment and water contamination. A recent review reports the links between the route of viral transmission by seafood and outbreaks (Lees 2000). Seafood are grown in coastal waters and thus, exposed to different sources of contaminants, among them pathogenic enteric microorganisms. The quality of the shellfish will mainly depend on the microbial quality areas where they are grown (Metcalf 1978, Miossec et al. 1988b). When contamination events occurred, i.e. during the rainfall season, in winter, depuration system could be used to eliminate potential contamination in shellfish (Richards 1988, Jackson & Ogburn 1996). Shellfishborne diseases (included hepatitis A) and gastro-enteritis have been reported after oyster consumption even with shellfish meeting EU bacteriological standards (Gill et al. 1983, Richards 1985, Dowell et al. 1995, Otsu 1999). A few data have been published in France on outbreaks of gastroenteritis related to shellfish consumption (Daurat 1992, Miossec et al. 1988a). Nevertheless, the presence of viruses in some harvesting areas (Le Guyader et al. 1998, 2000) justifies specific actions on viral depuration. Thus, this study investigated specific treatments based on specificity of viral contamination (seasonal occurrence). For this purpose a depuration pilot was designed and built. Here are reported the first results obtained to test the pilot and determine the main parameters that would have an effect on oyster viral depuration. To assess the efficiency of the pilot, MS2 strain was used as a viral model.

MATERIALS AND METHODS

Bench Scale experiments

Different parameters were evaluated in a small scale experiments in the laboratory at local water temperatures (< 17°C). Prior to evaluation in professional size tanks, small flow-through tanks of 30 l, with a water circulation of 3.5 l/min, were used. The shellfish load capacity was about 50 oysters. Different tests were done to study the effect of diets such as algae feeding, suspended matter (SM) on depuration speed. The absence of nutrient i.e. 48h storage in a dry area before

depuration or 0.50 μm filtered seawater used for depuration, were also investigated. All these experiments were done with a seawater salinity varying from 33 to 35‰ and local seawater temperature from 10°C to 17°C.

Depuration pilot

A four tank pilot was built in Prat ar Coum premises (Ets. Y Madec, Lannilis, France, Fig. 1). To obtain a compromise between scientific experimentation scale and producer needs, each tank had nominal capacity of approximately 960 kg of oysters (3.2m x 2.2m x 0.80m). The four tanks were independent from one and another and equipped with pumps providing effective aeration and seawater recycling (5 to 20 m^3/h). Water circulation was designed to provide a good aeration and limit recontamination. An overflow single layer was set up to flow through a weir into the tank to provide a maximum oxygenation when recycling. Plug located at the surface allowed elimination of surface film wastes. Heater/cooler equipment was set up in three of the four tanks to work at the same time with different constant temperatures. One tank was also equipped with sand filter and UV lights. Moreover, the pilot wastewater was disinfected through a large sand filter and UV.

One tank was used as the reference i.e. as the one in use at present by the professionals for shellfish depuration (aeration and natural seawater temperature). This temperature is dependent on the season and, in Brittany, varies from 8°C (min) to 12°C (max) from April to October.

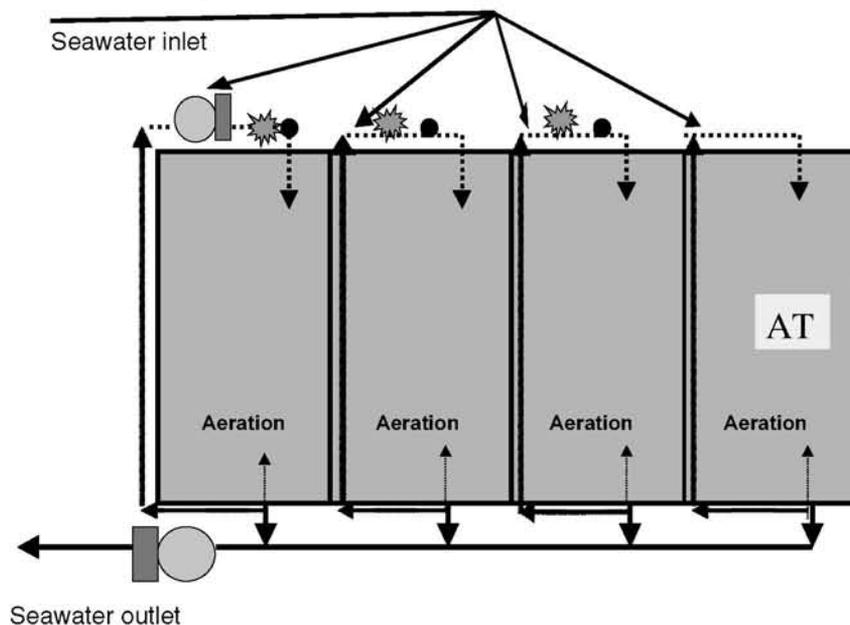


Fig. 1. Depuration pilot:

★ Cooler unit; ● Heater unit; █ Sand filter and U.V.; AT: aerated tank.

Calibration: Hydraulic and temperature calibrations were performed using rhodamine dye. The tank was calibrated with and without shellfish. Dye

concentration was measured using spectrofluorometer (Sequoia-Turner model 450). Circulation pattern was established from water sampling at different location or depth of the tank. Temperature stability was measured using Tomprobes.

Phage behavior control: The fate of the phage in seawater was evaluated in tanks without shellfish: MS2 phage culture (1×10^{12} UFP) was diluted in the seawater (34‰). Sampling started after homogenisation of the phage suspension in the tank and then done twice a day for ten days. Water analyses were performed directly from the sample at the appropriate dilution and phage detection was performed as described for shellfish.

Artificially-contaminated oysters: A stock of calibrated oysters (*Crassostrea gigas*) was selected; measurements of size, weight and filling index were performed, in order to assess the homogeneity of the shellfish stock and limit the physiological discrepancies on depuration failures. These oysters were then artificially contaminated with MS2 phages or *E. coli*. Suspension of phages (1×10^{12} UFP) was diluted into 40 liters of seawater and then poured in a tank containing 6 m³ seawater. The contamination of oysters by *E. coli* was done in the same conditions: 10 ml of a stationary phase culture of *E. coli* (strain CIP 54117) was diluted in seawater (final concentration 10^5 CFU/ml) and added to the tank. In the both cases, oysters were immersed for two days (12°C), after a 20 min homogenisation of the phage or bacterial suspension in the tank.

Naturally-contaminated oysters: Shellfish were collected from B harvesting areas (classification according to EU regulation).

Depuration experiments: Naturally- or artificially-contaminated oysters were then immersed in the four tanks and exposed to different water temperatures (8°C, 16°C, 20°C...). In two experiments, the oysters were fed continuously with phytoplankton (*Chaetoceros calcitrans* and *Isochrysis affinis galbana*) at the rate of 2×10^9 cells/oyster/day. Every two days, oysters were sampled and analysed for phages and *E. coli*.

Phage and E coli analysis: For analyses, at least twelve oysters were collected. Each sample for each analysis was composed of at least 6 oysters and duplicate analyses were systematically performed on each sample. Prior to analyses, samples were kept at 4 °C. The six oysters were washed, shucked and weighed. The stomach and digestive diverticula were removed by dissection and then homogenised in a warring blender in 2 volumes of peptone water and analysed for MS2 phages according to the protocol ISO 10705-1, 1995. For *E. coli* analyses, tissue and liquor were homogenised in a warring blender with 1 volume of a 10 % (wt/vol) NaCl solution. *E. coli* contamination level was determined by conductance measurement (NF- VO8-106).

Statistical analyses

The variables of *E. coli* and FRNA phages were transformed by log (x+1) function for the statistical analyses. The regression model and logistic regression were applied. The significance of the regression lines was assessed by a student *t* test applied to the regression slope. The loss of culturability was calculated by applying the following law of logarithmic decrease:

$$N = N_0 \times 10^{-t/T90}$$

where N is the concentration at a t time in hour; N_0 , the initial concentration; $T90$ is the time required for a decimal reduction of the initial population. $T90$ designation is used for MS2 water behaviour, whereas $DT90$ is the time required for a decimal depuration of the initial population in shellfish.

RESULTS

1. Bench scale experiments

The effect of some parameters on depuration was evaluated in small scale experiments and at local water temperatures ($< 17^\circ\text{C}$), prior to *in-situ* experiments. The influence of diets such as algae or suspended mater was evaluated: the depuration efficiency was estimated to approximately one log of depuration in 6 days for the stock feeded with algae and in 12 days for the non-feeded stock. No difference was observed when contaminated oysters were kept in a dry storage for 2 days prior to immersion in the tank and depuration or when the water used for the depuration was filtered to eliminate all the suspended materials.

2. Water temperature and hydraulic calibration in the pilot

The water temperature was adjusted in each tank with a heater/cooler equipment. Twelve hours were needed to increase water temperature from 8 to 22–25°C. A good stability was achieved in the four tanks at different temperatures (from 8°C to 25°C) and was not influenced by the temperature of air premise or the water temperatures from other tanks (Fig. 2). The hydraulic assay performed with rhodamine dye, in the presence or absence of shell, in the tank, showed no influence of the shellfish load on water circulation. Using a recycling water speed of 20m³/h, an homogeneous dye concentration was obtained in the whole tank: rapid circulation and diffusion of the dye in the whole tank was observed after 10 min and 17 min, with and without oysters, respectively.

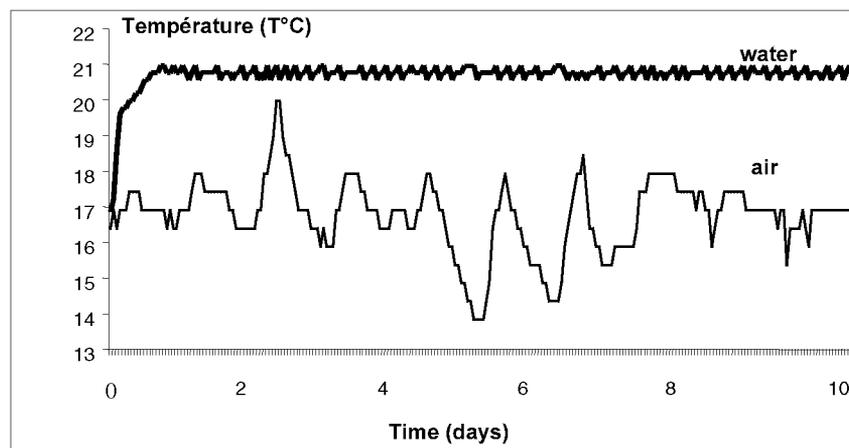


Fig. 2. Effect of the cooling system in the pilot: – air premise temperature; – water temperature (adjusted to $21 \pm 0.5^\circ\text{C}$).

3. Phage behaviour in the tank

Some experiments were done in the tanks without oysters to evaluate the temperature effect on the phage behaviour alone. At low temperature (12-13°C), in the aerated tank, MS2 decrease was low (1 log in 7 days), whereas, at 25°C, MS2 concentration was drastically reduced by 4 logs in 2 days. T90s was estimated to about 5.5 days at 13°C and to only 0.5 days at 25°C (Table 1, Fig.3). Assays were done with and without aeration, results demonstrated that this parameter did not affect phage behaviour. However, the re-cycling water speed in the tank was sufficient (20 m³/h) to have oxygen saturation in both conditions.

Table 1. Characteristics of the MS2 behaviour in seawater.

Experiment	Contamination	Seawater	Phage MS2 ¹	T90 (r ²)
		Temperature (°C)	UFP/ml	
I	artificial	25	9.0 10 ³	0.5 (0.97)
		20		1.5 (0.92)
		15		2.3 (0.95)
		13		5.5 (0.91)
II	artificial	25	2.2 10 ⁴	0.5 (0.99)
		20		1.5 (0.97)
		15		2.3 (0.95)
		13		5.5 (0.84)

1: initial concentration; T90: MS2 decay rate in shellfish; r²: regression coefficient.

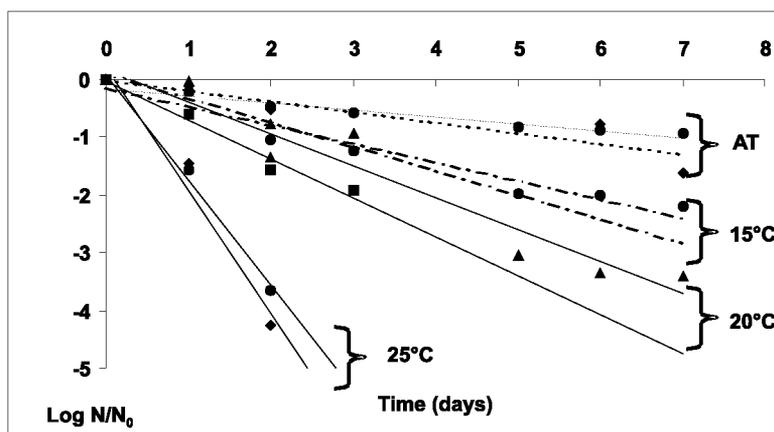


Fig. 3. MS2 behaviour in seawater in the pilot as a function of temperature. 15°C, 20°C, 25°C and the control: aerated tank (AT), 13°C.

4. Depuration experiments

The variation of salinity (from 33 to 34.5 ‰), recorded during the experiments was equivalent to the seawater salinity in the estuary; oxygen was up to 90 % of saturation in any conditions tested and even at the end of the experiments or when the water temperatures were elevated.

Artificially-contaminated oysters

Between March 2001 and January 2002, seven experiments (Table 2, from A to G) were performed using oysters artificially-contaminated with MS2 phages. Oyster contamination was about 3.9×10^4 PFU/g of digestive tissues for the first four experiments and 2.6×10^3 PFU/g of digestive tissues for the 3 other ones. For the first four experiments with high concentration of phage, 3 experiments (A and B) were done at 18°C-19°C and the time necessary to observe a one log decrease of the phage concentration, DT90, was about 6 days (from 5.6 to 7.2 days - Table 2). At 8°C, this time (DT90) increased to 8.6 days whereas at 22°C, only 3.8 days were needed to observe the same depuration rate (exp. C). One experiment done at 22°C with and without use of UV (exp. D), did not change the depuration time (Table 2). To further evaluate the temperature effect on depuration, three more experiments (E, F, G) were performed using oyster contaminated with approximately from 2×10^3 to 3×10^3 PFU/g of digestive tissues, at a water temperature of 25°C. In these conditions only 2.8 to 3.7 days were needed to observe a one log decrease of the phage concentration. No difference was recorded when the water temperature was increased from 12°C to 25°C in the presence of the oysters (E, F). To compare the slope independently from the initial concentration, N/N_0 was calculated as a function of the time (expressed in day) and regression lines were calculated for all experiments (Fig. 4). At low temperature, i.e. less than 20°C, 10 days were needed to get a one log MS2 decrease. However, a water temperature over 20°C improved the depuration efficiency: a one log decrease was observed in 2.3 days (Fig. 4).

In two experiments (D), the oysters were fed with phytoplankton (*Chaetoceros calcitrans* and *Isochrysis affinis galbana*). About 2×10^9 cells/oyster/day were added to seawater during a 10-day period, with a temperature of 22°C. This was efficient, as only two days were needed to obtain a one log decrease in the phage concentration.

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Table 2. Results of the depuration experiments with artificially- and naturally-contaminated oysters

Experiment	Contamination	Water	Phage MS2	<i>E. coli</i>	DT90 (r^2)
		Temperature (°C)	PFU/gdg ^a	UFC/100g ^b	
A	artificial	19	3.7 10 ⁴		6.6 (0.96)
		18.2			7.2 (0.85)
B	artificial	18.2	5.5 10 ⁴		5.6 (0.97)
C	artificial	22	2 10 ⁴	5 10 ⁴	3.8 (0.99)
		8		4.5 10 ³	8.6 (0.89)
D ^d	artificial	22	4.4 10 ⁴		2.0 (0.99)
		22 + UV			2.6 (0.96)
E	artificial	25 ^e	2.0 10 ³		3.7 (0.98)
		25			4.2 (0.99)
		16			6.7 (0.97)
		AT or 12			6.7 (0.83)
F	artificial	25 ^e	3.0 10 ³		2.8 (0.99)
		25			3.3 (0.98)
		16			10 (0.85)
		AT (12)			10 (0.97)
G	artificial	25	3.0 10 ³		3.1 (0.95)
		12			5.5 (0.91)
H	natural	20	4.1 10 ¹	DL ^c	6.2 (0.72)
I	natural	20	DL	ND ^f	
J	natural	25	4.0 10 ¹	1.8 10 ²	5.5 (0.79)
		12-13			20 (0.98)
K	natural	25	5.5 10 ¹	4.5 10 ¹	4.7 (0.85)
		12-13			20 (0.65)

a. dg: digestive gland; b:100g of shellfish flesh; c: DL: detection limit; d: oysters were fed with phytoplankton; e water temperature was increased from 12 C to 25 C, ND: not determined.

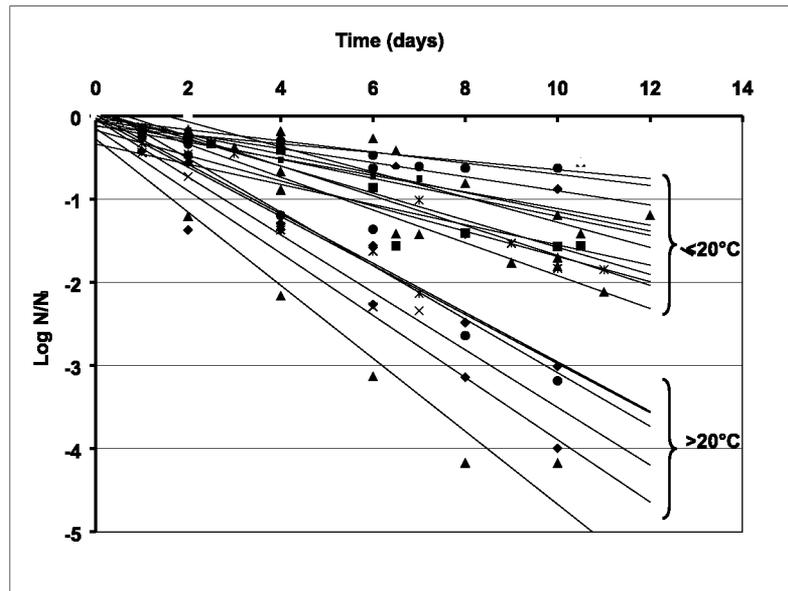


Fig. 4. MS2 depuration kinetics for oysters in pilot; effect of seawater temperatures.

Naturally-contaminated oysters

Four samples of 50 kg of oysters were collected in B harvesting areas (EU classification) were used in different depuration experiments (H, I, J, K - Table 2). One (I) done with very lightly contaminated-oysters showed a very fast depuration rate and the concentration of phage in the digestive gland was rapidly under the detection limit.

An experiment (K) was conducted using a batch of oysters with an initial concentration of *E. coli*, of about 45 CFU/100g of shellfish meat. Fig: 5 showed that, 2.5 days at 13°C, and 12 hours at 25°C, respectively, were needed to decrease the concentration *E. coli* under the detection limit (<12 CFU/100g). For phages, the initial concentration of 55 PFU/g of digestive gland, was stable at 13°C, and decreased to less than 5 PFU/g after 6.5 days at 25°C. In the aerated tank (AT), the water temperature varied between 12-13°C during our experiments. This tank, as a professional tank would be, was not equipped with a heating/cooling system and was used as a control.

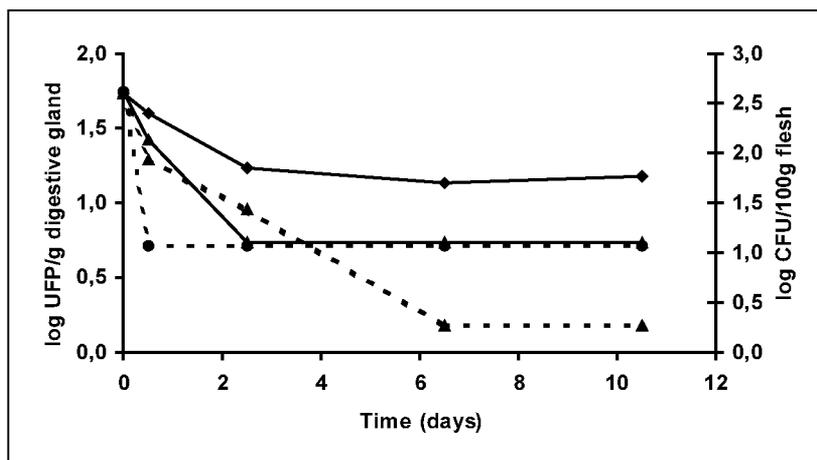


Fig. 5. Naturally-contaminated shellfish deputation kinetic in the pilot.

◆ FRNA (13°C); ▲ FRNA (25°C); ◻ *E. coli* (13°C); ◻ *E. coli* (25°C) *E. coli* is expressed in CFU/100g of shellfish flesh, FRNA phage concentration in PFU/g digestive gland.

After deputation experiments (naturally- or artificially-contaminated oysters), mortality and filling index were recorded. Mortality rates were lower than 5% whatever the experimental conditions were, even at 25°C. This demonstrated that the water circulation and aeration set up in the tank were favourable to the well-being of the oysters. Moreover, mortality was not observed after a week storage of these oysters in the fridge in a basket ready to sale.

On Fig. 6, the relationships between MS2 die-off rate (T90) in seawater and shellfish (DT90) versus temperature were calculated ($T90 = -0.14T + 4.54$, $n = 8$, $r^2 = 0.92$; $DT90 = -0.39T + 13.45$, $n = 20$, $r^2 = 0.63$). Despite the point dispersion, especially for DT90/T, the relations between these parameters were quite good. MS2 decay-rate in shellfish was about 3 times longer than the one in seawater.

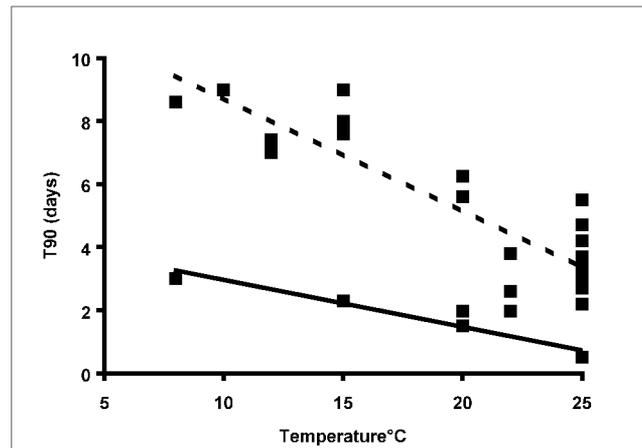


Fig. 6. Relationship between MS2 decay rate in water (■ T90), MS2 shellfish depuration rate (■ DT90) and temperature in the experimental depuration pilot.

DISCUSSION

It is now clear that shellfish meeting bacterial indicator could be implicated in viral outbreak. The lack of correlation between bacterial and viral contamination make necessary to develop a sensitive method to control virus contamination on a routine basis or an efficient method for shellfish depuration. Since a few years, different systems (small or large) have been proposed to depurate shellfish but few report data on naturally contaminated oysters depuration (Durgin et al. 1981, Boher et al. 1993, Doré et al. 1995, 1998, Kator & Rhodes 2001).

The two main interests of our work were the use of the semi-professional size depuration tanks for our experiments and the validation of the system using naturally-contaminated shellfish. The large size of the tank used in our study and the professional equipment allowed a good stability, reliability and reproducibility of the different parameters tested (aeration, UV, speed of water recycling). By setting up the parameters properly, the well-being of the oysters was taken into account and thus, excessive mortality avoided as it was suggested by Canzonier (1982).

Depuration process is a complex biological process and individual species respond differently to various combinations of operational criteria including water turbidity, salinity, temperature, depth of shellfish immersion in the water, oxygenation (Roderick & Schneider 1994). These factors along with shellfish stress, can alter the filtration rates (Haven et al. 1978, Metcalf 1978, Canzonier 1982). In most of commercial depuration processes, shellfish are placed in tanks with aerated and UV light (or ozone, chlorination) treated flowing marine water, for a minimum of 48 hours. The efficiency is based on the fact that, as the water contains little food, at the end of two days, the shellfish purge themselves of gut content which may include pathogens (Di Girolamo et al. 1975, Meinhold & Sobsey 1982, Cook et al. 1986). However, as virus accumulation could be linked to mucus content (corresponding to the glycogen content of the gonad and connective tissue development) by ionic

binding, this could explain why viruses and bacteriophages are eliminated slower than *E. coli* would be during the depuration of shellfish (Galstoff, 1964, DiGirolamo 1977, Burkhardt et al. 2000, Lees 2000, Muniain-Mujika et al. 2002).

This could also explain why, feeding or starving conditions had no major effect at low temperatures (10°C-17°C). Shellfish at low temperature seem to be in dormancy, and are not re-activated by the tested parameters (algae feeding, suspended matter (SM), feeding, absence of nutrient i.e. 48h storage in a dry area, or 0.50 µm filtered seawater used for depuration). In our experiments at least one week was needed to obtain a one-log decrease of the phage concentration in the digestive gland. This result, previously described *in the field* could also be related to the mucus binding of the virus (Kator & Rhodes 2001). Pathogens, among them viruses, could be firmly attached to the gut walls (Power & Collins 1990) and thus, be depleted very slowly at low temperature (Richards 1985). However, temperature could affect extracellular digestion (enzyme activities, ciliary tract, phagocytosis..) during digestive and excretory processes occurring in shellfish (Metcalf 1978). However, apparently our comparison of phage behavior in water and shellfish, showing a concentration of a thousand times higher in shellfish than in water, demonstrated that shellfish gut could act as a protector for phage particles. In water alone, the phage decay rates are significantly correlated with temperature, low temperature favouring viral survival in natural water (Bosch et al. 1997, Wait & Sobsey 2001). In water, phage T90 was found to be 3 times faster than depuration time in shellfish (DT90). This difference could be due to the fact that freshly cultured MS2, directly poured into seawater, are sensitive to marine aggressions (among them temperature), whereas, in digestive tract, adherence to the walls can play a protective role.

The temperature increase proved to be the most efficient parameter, (up to 20°C) on depuration. Our experiments confirmed those already described in the literature (Sobsey & Jaykus 1991, Lees 2000, Doré et al. 2000), but we further demonstrated that feeding oyster at 22°C, is even better. A few years ago, Metcalf (1978) made these hypotheses but this was never confirmed at that scale.

Studies on oyster physiology demonstrate that *Crassostrea gigas* breathing follows an exponential curve according to the temperature, and its food consumption follows a bell-shaped curve with an optimum to 18 °C (Bougrier et al. 1995). Above 18°C, for a constant ratio of food, the scope for growth becomes negative and the animal exhausts its reserves – among them attached microorganisms - to compensate for this energy uptake (Maurer et al. 1986), as demonstrated in bioenergetic model of *Crassostrea gigas* (Barillé et al. 1997). The exhaustion of the reserves is done by the use of the glycogen, especially the one stored in the cells of the digestive diverticula. Thus, up to 21°C, it was demonstrated that these cells are autolysed by the oyster to use its reserves and thus, we can assume that, indirectly, attached microorganisms could be eliminated.

Naturally-contaminated oysters tested in our experiments were depurated in same delay and this was consistent with results reported by Doré *et al* (1998). Durgin et al. (1981) also demonstrated an effective viral depuration on weakly-contaminated shellfish either naturally or artificially using a flow-through depuration tank. Further experiments are necessary to confirm these first results.

CONCLUSIONS

The pilot of shellfish depuration set in professional premises on the site of Prat ar Coum makes it possible to test various conditions of depuration i.e. the algae supply, the oxygenation of water, the speed of water circulation, the disinfection of wastewater, temperature, on the effectiveness of the elimination of faecal microorganisms from the shellfish. Equipment was evaluated to approximately 23000 Euros of investment for each tank. The system is easy to maintain, hydraulic and water temperatures are well established. Moreover, it is easy to use these tanks as a structure as they can hold up to in a professional corresponds to 960 kg of treated shellfish. Results obtained on depuration efficiency demonstrated the main role of temperature on bacteriophage depuration. However, experiments are still in hand to validate the results with enteric viruses elimination, in order to have a good adequacy between the physiological and microbiological quality of the end product.

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