
Effects of temperature and salinity on haemocyte activities of the Pacific oyster, *Crassostrea gigas* (Thunberg)

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Abstract: The Pacific oyster, *Crassostrea gigas*, is extensively cultivated and represents an important economic activity. Oysters are reared in estuarine areas, subjected to various biotic and abiotic factors. One of the limiting factors in aquaculture is mortality outbreaks, which may limit oyster production, and the causes of these outbreaks are not completely understood. In this context, the effects of temperature and salinity on Pacific oyster, *C. gigas*, haemocytes, were studied. Haemocytes are the invertebrate blood cells and thus have been shown to be involved in defence mechanisms. Flow cytometry was used for monitoring several haemocyte parameters. An increase of temperature induced an increase of haemocyte mortality, in both in vitro and in vivo experiments. Temperature modulated aminopeptidase activity. An in vitro decrease of salinity was associated with cell mortality. During the course of in vivo experiments, an increase of phagocytic activity was reported at 15‰ and 50‰. Environmental physical parameters may modulate haemocyte activities.

Keywords: Pacific oyster; *Crassostrea gigas*; Haemocyte; Temperature; Salinity; Flow cytometry; Cellular activity

Introduction

Shellfish farming represents an important economic activity around the world. Among shellfish, the Pacific oyster, *Crassostrea gigas*, is the most cultivated species. In France, *C. gigas* was introduced in the 1970s to replace the Portuguese oyster, *C. angulata* (1). France ranks fourth worldwide in the production of *C. gigas* with 150 000 tons produced annually. However, oyster production may be subjected to various limiting factors including mortality outbreaks. For several decades now, French Pacific oyster livestock have presented abnormal mortality outbreaks during the summer period. This phenomenon called summer mortality has also been reported in North America and in Japan since the 1940s (2-4). Authors hypothesised that summer mortality outbreaks are the result of multiple factors, including elevated temperatures, physiological stress associated with sexual maturation, aquaculture practices, pathogens or pollutants (5). The Pacific oyster, *C. gigas*, is mostly reared in estuaries which are continually contaminated by pollutants (6). Estuaries are also subjected to important variations of abiotic environmental factors, including temperature and salinity. *C. gigas* is an osmo- and thermo-conformer species (7). In oysters natural habitat, salinity fluctuates with tidal cycles, rainfall and with drainage from adjacent terrestrial sites (8). In summer period, temperature can reach high values. Oysters are sessile benthic animals and as such are continually exposed to physico-chemical modifications of the environment. Physical stress such as tidal exposure, which modify temperature and salinity, can affect marine invertebrate defence mechanisms (9).

Bivalve defence mechanisms involve circulating blood cells, the so-called haemocytes (10). In *C. gigas*, two types of haemocytes can be differentiated on the basis of morphological features: hyalinocytes and granulocytes (10). Haemocytes constitute one of the main line of defence against non-self particles. They are involved in phagocytosis and encapsulation of foreign material (10-12). They also contain hydrolytic enzymes and produce reactive oxygen

species (ROS), which play a key role in pathogen degradation (13-15). They have been used as immune capacity indicators in many bivalve species (16-19).

Studies have previously been conducted on the effects of temperature and salinity on bivalve haemocytes (20-23). Since bivalves are both osmo- and thermo-conformers, haemolymph readily acquires salinity and temperature of the external environment (7). In fact, haemocytes found in haemolymph and in tissue sinuses are exposed to temperature and salinity variations that occur in the environment. High water temperatures inhibit haemocyte spreading and locomotion in the eastern oyster, *C. virginica* (Gmelin) (24) while variations of temperature can also affect haemocyte counts and phagocytic activity in *Ostrea edulis* and *Ruditapes philippinarum* (25, 26). On the other hand, elevated salinity increased the time for spreading and reduced haemocytes locomotion towards target particles and may therefore pose an additional stress (27) and may also reduce oyster defence capacities and leave them more susceptible to parasites (27). Moreover, the susceptibility of *C. virginica* to the protozoan parasites *Perkinsus marinus* and *Haplosporidium nelsoni* is influenced by temperature and salinity (24, 28, 29).

In this study, the effects of temperature and salinity on Pacific oyster, *C. gigas*, haemocyte parameters were investigated. Haemocytes were subjected *in vitro* to a range of temperatures and salinity. *In vivo* experiments were also carried out by placing oysters in waters at defined salinities or in incubators at controlled temperatures. Haemocyte parameters were monitored using flow cytometry. This emerging tool has often been used in marine bivalve research to describe haemocyte population characteristics (30, 31) or changes associated with pathology or environmental stress (32, 33). Cell mortality, esterase, aminopeptidase activities and phagocytic activity were monitored.

Material and Methods

Oysters

Eighteen month-old Pacific oysters, *C. gigas*, 7-10 cm in shell length, were produced in the IFREMER hatchery in La Tremblade (Charente-Maritime, France). Temperature experiments were conducted in April and May 2002 and salinity experiments were undertaken in July 2004. For both experiments, oysters were held in tanks receiving a constant flow of external seawater.

Haemocyte collection

For temperature experiments, haemolymph was withdrawn from the pericardial cavity while for salinity experiments, haemolymph was collected from the posterior adductor muscle sinus. In both cases, a 1-mL syringe equipped with a needle (0.9x25 mm) was used. Haemolymph samples were filtered on a 60 µm mesh to eliminate debris and were maintained on ice to prevent aggregation. In order to reduce interindividual variation and to provide enough haemocytes for experiments, haemolymph samples were pooled.. Haemocytes counts were performed using a Malassez cell and the cell concentration was adjusted to 10^6 cells per mL with artificial seawater (ASW: 23.4 g NaCl, 1.5 g KCl, 1.2 g MgSO₄ 4 H₂O, 0.15 g CaCl₂ 2 H₂O, 0.1.5 g CaCl₂ anhydrous; H₂O qsp 1L).

Effect of temperature on haemocyte parameters

Before each experiments, the oysters were acclimated in tanks receiving external seawater (temperature and salinity were 11.6°C-17.7°C and 31.5 ‰-32.3 ‰).

In vitro experiments

After collection of haemocytes and resuspension in ASW, antibiotics (kanamycin, erythromycin, oxolinic acid, 0.1 mg.mL⁻¹) were added. Haemocytes were incubated for 2 h

and 4 h at varying temperatures (4°C, 11°C, 20°C, 25°C, 35°C, 40°C, 50°C and 60°C). Cell mortality, phagocytic activity, esterase and aminopeptidase activities were monitored by flow cytometry as described below. Experiments were carried out in triplicates.

In vivo experiments

Five oysters were emersed during 4 hours in incubators at different temperatures (4°C, 11°C, 20°C, 25°C, 35°C, 40°C, 50°C and 60°C). Haemolymphs were then withdrawn and pooled from five oysters without any treatment. Cell mortality, phagocytosis, esterase and aminopeptidase activities were analysed by flow cytometry as described below. Experiments were carried out in triplicates.

Effect of salinity on haemocyte parameters

Before each experiment, the oysters were acclimated in tanks receiving external seawater (temperature and salinity of external seawater were 18.7°C-19°C and 33.9 ‰-34.5 ‰).

In vitro experiments

After collection, haemocytes were divided into eight tubes, and centrifuged (10 min, 100 g, 4°C; Microfuge Beckman). The cells were resuspended in haemolymph only in tube 1. The cells from tubes 2 to 7, were resuspended in a haemolymph-distilled water mixture in order to obtain a range of decreasing salinity (tube 2: 29 ‰, tube 3: 25.5 ‰, tube 4: 22.5 ‰, tube 5: 16 ‰, tube 6: 6.5 ‰, tube 7: 3 ‰). The cells from tube 8 were resuspended in distilled water (0 ‰).

Cell mortality was monitored using flow cytometry after 2 h and 18 h at 15°C. Experiments were carried out four separate times.

In vivo experiments

In the first experiment, 40 oysters were placed in three tanks at 15 ‰ (hyposalinity), 35 ‰ (control) and 45 ‰ (hypersalinity), respectively. Hyposalinity was obtained by mixing 25 L of seawater and 15 L of freshwater. Hypersalinity was obtained by the addition of 516 g of aquarium sea salts (Instant Ocean Aquarium Systems, synthetic sea salts without nitrate and phosphate, Haurit, Saintes, France) in 40 L of seawater. Oysters were fed with *Chaetoceros gracialis* ($3 \cdot 10^9$ cells per tank). Water and food were provided every day. Temperature of external seawater was maintained between 18.7°C-19°C during the experiments which lasted 7 days. Sampling of ten oysters per tank were then performed at day 1, 3 and 7. Ten oysters were also analysed at the beginning of the experiment. At each time, the oysters were divided in three pools. Cell mortality and phagocytosis were analysed by flow cytometry as described below.

A second experiment was conducted using the same protocol as previously described with the following differences: oysters were maintained at 5 ‰ (hyposalinity), 35 ‰ (control) and 60 ‰ (hypersalinity). Hyposalinity was obtained by mixing 35 L of freshwater and 5 L of seawater. Hypersalinity was obtained by adding 1219 g of aquarium sea salts (Instant Ocean Aquarium Systems, synthetic sea salts without nitrate and phosphate, Haurit, Saintes, France) in 40 L of seawater.

Flow cytometry analysis

The protocols used in the present study were previously described (34). For each sample, 3 000 events were counted using an EPICS XL 4 (Beckman Coulter). Results were depicted as cell cytograms indicating cell size (FSC value) and cell complexity (SSC value) and the fluorescence channel(s) corresponding to the marker used. Recorded fluorescence depended on the monitored parameters: enzymatic activities and phagocytosis were measured using

green fluorescence while cell mortality was measured using red fluorescence. Mortality was quantified using 200 μL of haemocyte suspension. Haemocytes were incubated in the dark for 30 min at 4°C with 10 μL of propidium iodide (PI, 1.0 $\text{mg}\cdot\text{L}^{-1}$, Interchim). Esterase and aminopeptidase activities were evaluated with commercial kits (Cell Probe TM Reagents, Beckman Coulter). Each analysis required 200 μL of haemolymph and 20 μL of the corresponding kit (FDA•Esterase and A•Aminopeptidase M). Haemocytes were incubated in the dark at ambient temperature 15 min for esterases and 30 min for aminopeptidases. Phagocytosis was measured by ingestion of fluorescent beads. Two hundred μL of haemocyte suspension were incubated 1 h in the dark at ambient temperature with 10 μL of a 1/10 dilution of Fluorospheres[®] carboxylate-modified microspheres (diameter 1 μm , Interchim).

Statistical analysis

Results were expressed as percentage of positive cells. In order to detect an effect of tested conditions, an ANOVA was performed using Statgraphics[®] Plus version 5.1 software. To ensure respect of *a priori* assumptions for normality and homogeneity, values were converted into r angular arcsinus $\sqrt{\text{(\% of positive cells)}}$ before analysis and .in the case of rejection of H_0 , an *a posteriori* test was used. Significance was concluded at $p \leq 0.05$.

Results

Temperature effects

In vitro experiments

After a 2 h incubation period, cell mortality significantly increased at 40°C (Figure 1, $p < 0.001$), while after a 4 h incubation period, mortality was significantly higher at 50°C and 60°C (Figure 1, $p < 0.001$). Percentages of aminopeptidase positive cells were significantly lower for both incubation periods at 50°C and 60°C (Figure 2). Percentages of esterase positive cells were significantly lower after a 2h incubation period at 50°C and after a 4h incubation period at 50°C and 60°C (Figure 3).

Figure 1: Haemocyte mortality percentage of oysters evaluated by flow cytometry after an *in vitro* 2 h or 4 h incubation period at several temperatures (4°C, 11°C, 20°C, 25°C, 35°C, 40°C, 50°C and 60°C). Values are mean of three replicates. Bars represent standard deviation. ***: $p < 0.001$.

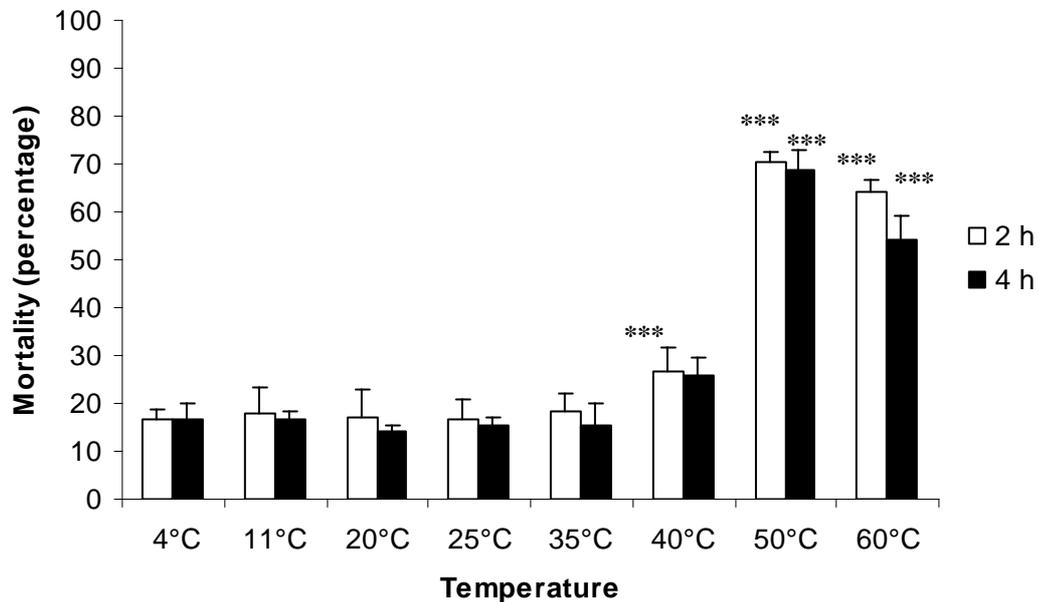


Figure 2: Percentage of positive cells for aminopeptidases of oysters evaluated by flow cytometry after an *in vitro* 2 h or 4 h incubation period at several temperatures (4°C, 11°C, 20°C, 25°C, 35°C, 40°C, 50°C and 60°C). Values are mean of three replicates. Bars represent standard deviation. *: $p < 0.05$, ***: $p < 0.001$.

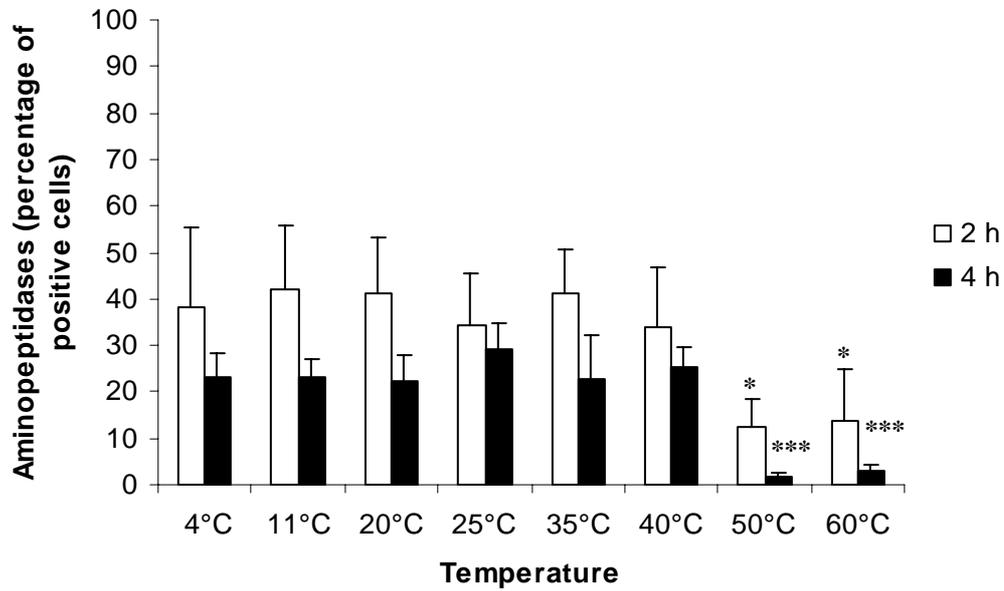
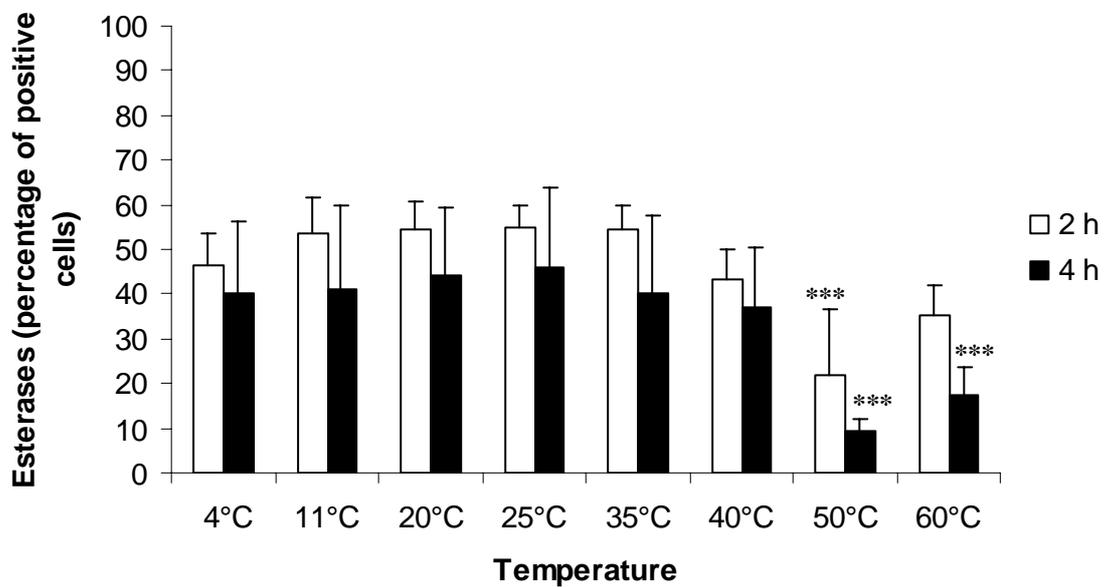


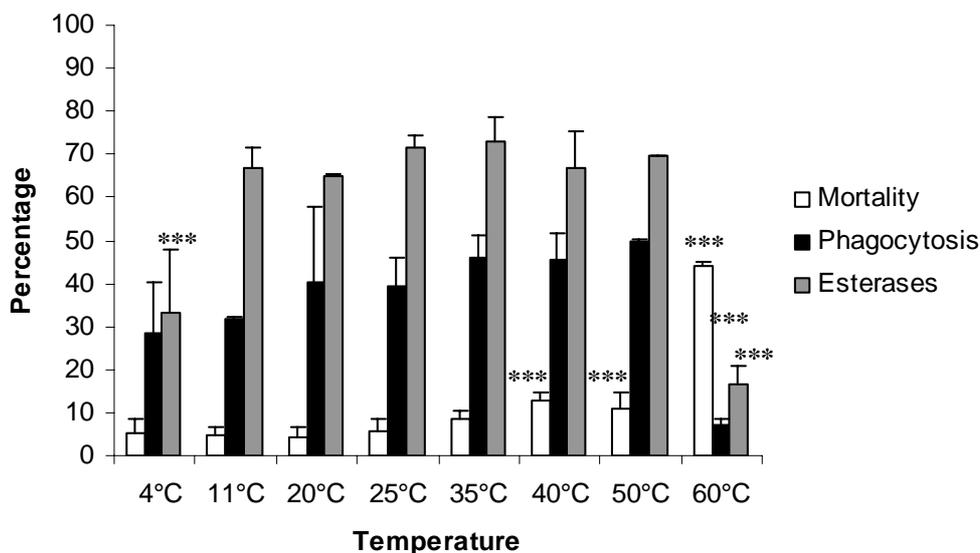
Figure 3: Percentage of positive cells for esterases of oysters evaluated by flow cytometry after an *in vitro* 2 h or 4 h incubation period at several temperatures (4°C, 11°C, 20°C, 25°C, 35°C, 40°C, 50°C and 60°C). Values are mean of three replicates. Bars represent standard deviation. *: $p < 0.05$, ***: $p < 0.001$.



In vivo experiments

Cell mortality significantly increased at 40°C, 50°C and 60°C (Figure 4, $p < 0.001$). The percentage of esterase positive cells was significantly lower at 4°C and 60°C compared to other temperatures (Figure 4, $p < 0.001$) and phagocytosis activity decreased at 60°C (Figure 4, $p < 0.001$).

Figure 4: Haemocyte mortality percentage, phagocytosis percentage and percentage of positive cells for esterases of oysters evaluated by flow cytometry after an *in vivo* 4 h incubation period at several temperatures (4°C, 11°C, 20°C, 25°C, 35°C, 40°C, 50°C and 60°C). Values are mean of three replicates. Bars represent standard deviation. **: $p < 0.01$, ***: $p < 0.001$.



Salinity

In vitro experiments

After a 2 h incubation period, cell mortality was significantly higher for lower salinities (6.5 ‰, 3 ‰ and 0 ‰) (Figure 5, $p < 0.001$) in contrast with the percentage of esterase positive cells which was significantly lower (Figure 6, $p < 0.001$). After a 18 h incubation period, mortality was significantly higher for 3 ‰ and 0 ‰ (Figure 5, $p < 0.001$) and esterase

percentage of positive cells was significantly lower for the same salinities (Figure 6, $p < 0.001$).

Figure 5: Haemocyte mortality percentage of oysters evaluated by flow cytometry after an *in vitro* 2 h or 18 h incubation period at different salinities (32 ‰, 29 ‰, 25.5 ‰, 22.5 ‰, 16 ‰, 6.5 ‰, 3 ‰ and 0 ‰). Values are mean of four replicates. Bars represent standard deviation. ***: $p < 0.001$.

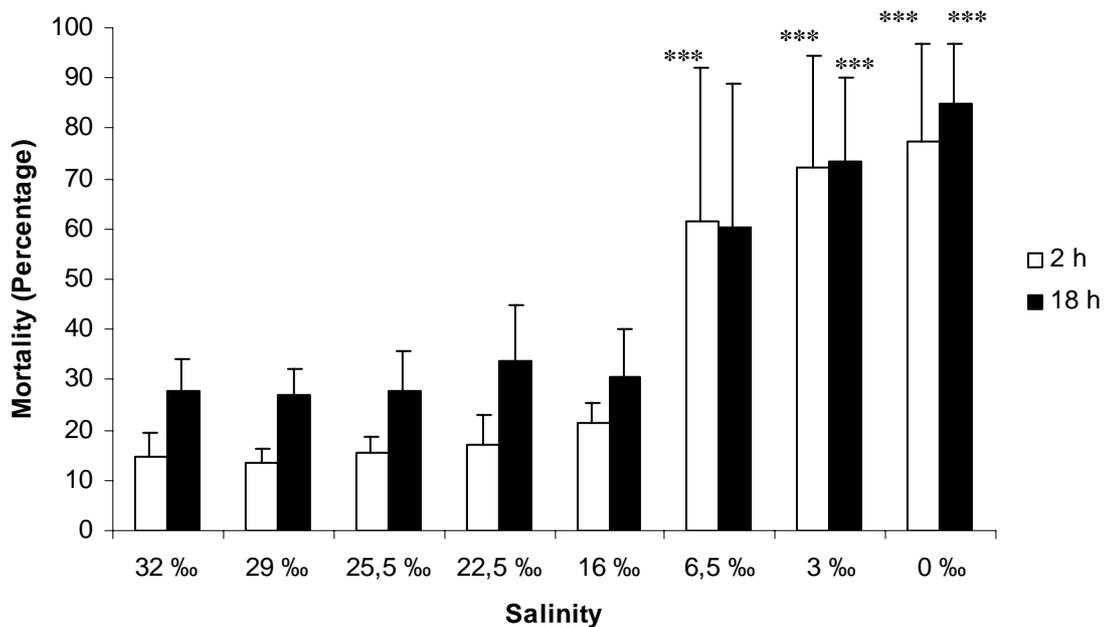
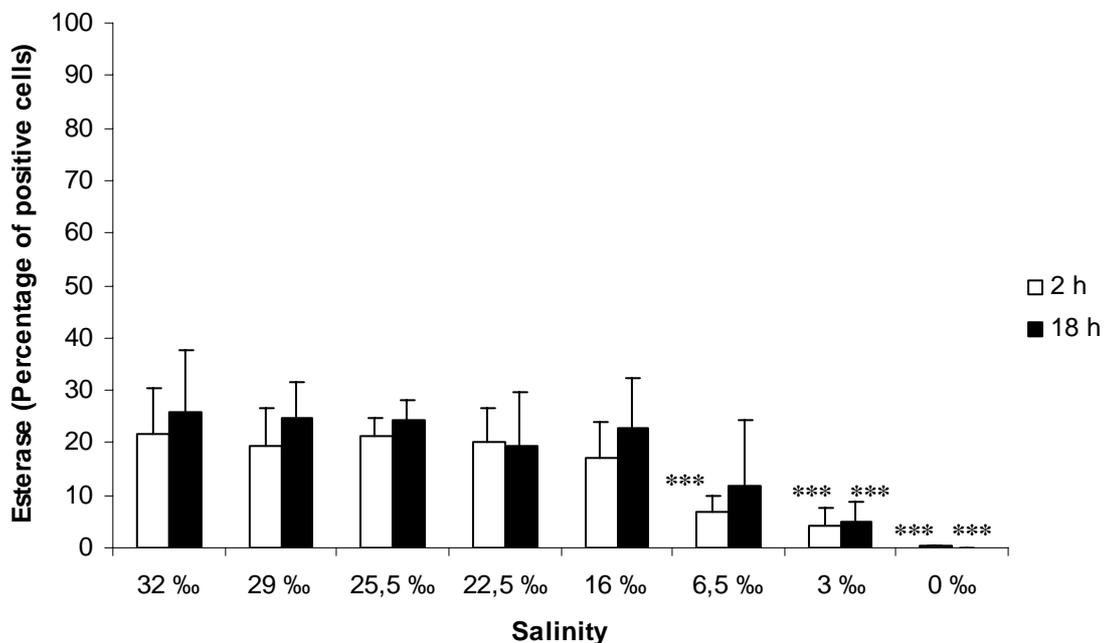


Figure 6: Percentage of positive cells for esterases of oysters evaluated by flow cytometry after an *in vitro* 2 h or 18 h incubation period at different salinities (32 ‰, 29 ‰, 25.5 ‰, 22.5 ‰, 16 ‰, 6.5 ‰, 3 ‰ and 0 ‰). Values are mean of four replicates. Bars represent standard deviation. ***: $p < 0.001$.



In vivo experiments

No mortality was noted during the first experiment. After one day, phagocytosis activity was significantly lower in oysters placed in hyposalinity compared to the two other conditions (Figure 7, $p < 0.05$). After three and seven days, phagocytosis activity was significantly lower in control oysters than in the two other conditions (Figure 7, $p < 0.01$). Two-way analysis of variance showed that phagocytosis activity was significantly lower in control oysters than in oysters placed in hypersalinity ($p < 0.05$). Cell mortality showed no variation in relation with salinity condition (data not showed).

In the second experiment, a high daily mortality was reported in hypo- and hypersalinity conditions (Figure 8). Oyster mortality appeared on day 3 for both conditions (15 % of mortality in hyposalinity, 25.9 % of mortality in hypersalinity, Figure 8). Highest mortality levels were observed on day 6 (55.5 % of mortality in hyposalinity, 66.6 % of mortality in hypersalinity, 0 % of mortality in the control, Figure 8). Cell mortality and phagocytosis activity showed no effect related to salinity conditions during the first three days of experiment (data not shown).

Figure 7: Phagocytic activity of oysters evaluated by flow cytometry after an *in vivo* exposure to 15 ‰ (hyposalinity), 35 ‰ or 45 ‰ (hypersalinity) during seven days. Values are mean of three pools. Bars represent standard deviation. *: $p < 0.05$; **: $p < 0.01$.

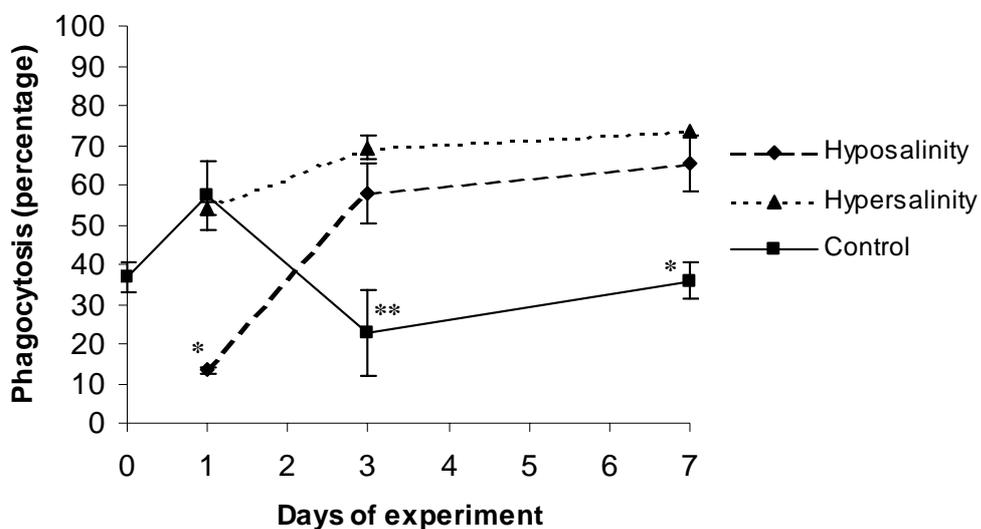
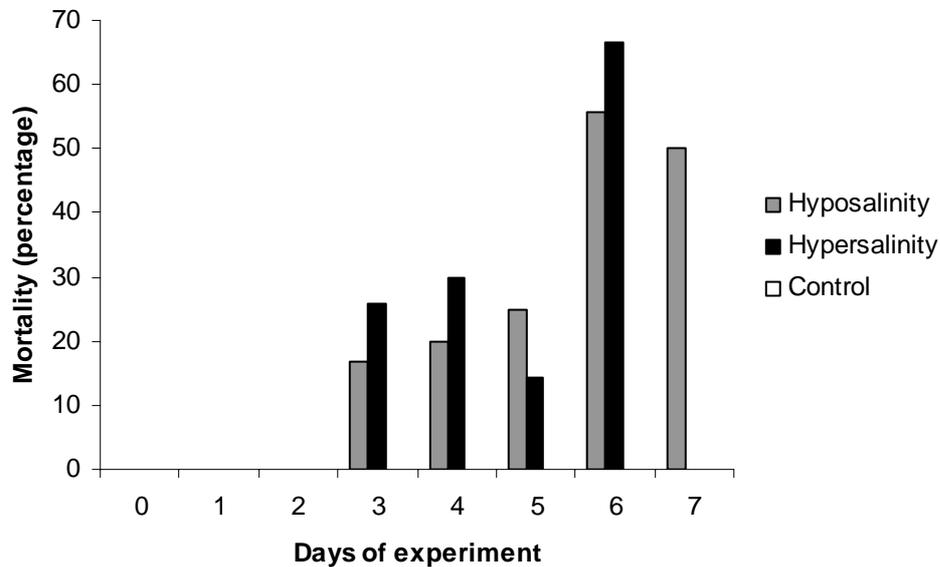


Figure 8: Daily mortality of oysters after an *in vivo* exposure to 5 ‰ (hyposalinity), 35 ‰ or 60 ‰ (hypersalinity) during seven days.



Discussion

In the present study, flow cytometry was used to demonstrate effects of two abiotic factors (temperature and salinity) on haemocyte parameters of the Pacific oyster, *C. gigas*. Flow cytometry has been shown to be an efficient tool for analysis of haemocytes in various mollusc species (31, 33-35). However, relatively few studies have used this tool for monitoring effect of temperature and salinity on haemocyte parameters (22, 36).

Pacific oysters were exposed to varying regimes of temperature and salinities in order to assess their sensitivity to abiotic factors including temperature and salinity. In *in vitro* experiments, we have demonstrated that while high temperatures induced hemocyte mortality, hemocytes can tolerate a temperature of 35°C without presenting any mortality. In contrast, a 4 h *in vivo* incubation period at 40°C, 50°C and 60°C increased cell mortality of oysters. Esterase activity was decreased at 4°C and 60°C and phagocytosis was decreased only at 60°C. This was consistent with the decreased phagocytosis activity of *Ostrea edulis* haemocytes (25) and of *C. virginica* haemocytes (22, 36) as well as with the increased cell mortality of *C. virginica* haemocytes (22, 36) following temperature elevation. However, the

decrease of enzymatic activities was predictable, as hemocytes presented mortality which is a consequence of morbidity. In Marennes-Oleron Bay (Charente-Maritime, France), 4 hours correspond to the time of exondation between low tide and high tide. The temperature in the field may often reach 40°C during summer period (37). Since oysters often encountered these extreme conditions, they may have become tolerant and thus the effect of high temperature may have been masked. In addition, oysters are thermo-conformers and our results confirm that oyster hemocytes can adapt to elevated temperatures.

In vitro decrease of salinity also induced an increase of cell mortality. However, as mentioned before, the decrease of enzymatic activities was predictable, as hemocytes presented mortality. Salinity variations also reduced haemocyte activities of *C. virginica* haemocytes in other studies (24, 27). However, oyster haemocytes are able to synthesize osmotic shock protein therefore protecting themselves from acute salinity variations (8). The relationships between *in vitro* measurements of haemocyte activities and the ability of marine bivalves to develop accurate defence in the field have not been established. *In vitro* measurements do not represent *in vivo* or *in situ* conditions (25).

In vivo experiments involving salinity were also conducted. In the first experiment, phagocytosis activity was enhanced at high both salinity (45 ‰) and low salinity (15 ‰). Another study reported a decrease of phagocytosis in *Ruditapes philippinarum* associated with an increase of salinity (38). These results indicate that salinity may modulate phagocytic activity. In the second experiment, oysters were reared at 5 ‰ and 60 ‰, and both conditions induced high mortality. However, those values are distant from the range of salinity reported in Marennes-Oleron Bay (21-34 ‰), where *C. gigas* are reared (37). Oysters may not be able to acclimate to high salinities. However, a part of oysters are reared in “claires” (oyster pounds), a confined zone, where salinity may decrease to 15 ‰ after rainfalls (Soletchnik, personal communication).

C. gigas have been successfully introduced in many countries over the world since the 1950s (1, 39). We can therefore conclude that this species is naturally subjected to important variations of environmental conditions and can acclimate to them. Moreover, our results clearly demonstrated that only extreme values of temperature and salinity can modify haemocyte activities of *C. gigas*. Haemocytes are apparently resistant cells because only high values of temperature and salinity kill them. This phenomenon has already been observed with pollutant exposure: only high concentrations of mercury chloride were able to kill haemocytes after 4 h of *in vitro* contact (40).

These results could lead us to study possible interactions between the effects of abiotic factors and pollutants or susceptibility to infections. Most of the studies conducted on abiotic factors including temperature and salinity on bivalve defence functions pointed on to the relationships between abiotic factors and diseases. The virulence of infectious agents in the field has been correlated with high salinities and temperatures (41). Prevalence and intensity of *Perkinsus marinus* in *C. virginica* is positively correlated to salinity (29) and to temperature (42), suggesting that parasite virulence may be increased or oyster resistance may be decreased at high salinities (43) and high temperatures.

Viral infection may also be influenced by temperature and salinity. In the hard clam, *Meretrix lusoria*, birnavirus has proved to be more pathogen to young stages when a rapid increase of water temperature occurs (44). Moreover, herpes virus affecting the Pacific oyster, *C. gigas*, may exist as a latent form or low productive infection and temperature elevation can declare the disease into the whole organism for larvae and spat (45, 46).

In vivo acute variations of temperature and salinity increase could temporarily affect the ability of shellfish haemocytes to resist foreign invasion (25). Temperature and salinity appear to be key factors modulating the host immune defence in invertebrates and influences the

severity of disease in several bivalve species, particularly during young stages (47). Their role in massive mortalities affecting different invertebrate species of economic interest must be taken into account. The interactions between temperature, salinity, pollutants and pathogens, added with all other environmental factors (pH, dissolved oxygen) could represent scraps of explanation of summer mortality phenomenon in *C. gigas*. In this context, experiments studying relationships between modulation of abiotic factors and infectious agents (including bacteria or OsHV-1) could be conducted in the future.

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