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In vitro and in vivo effects of mercury on haemocytes of Pacific oyster, Crassostrea gigas (Thunberg) : development of techniques evaluating estuarine pollution

Etude *in vivo* et *in vitro* des effets du mercure sur la réponse hémocytaire de l'huître creuse, *Crassostrea gigas* : développement de techniques évaluant les impacts de la pollution estuarienne

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Abstract : In the last decades, shellfish industry represents an important economic activity in France and particularly in Charente-Maritime. Marennes-Oleron basin is the first French area of oyster production, especially Pacific oyster, *Crassostrea gigas*. But this area is also subjected to many pollution due to anthropic activities. Industrial wastes are sources of mercury pollution in the Charente river. The harmful effects of such contaminants on animals inhabiting these estuarine zones are poorly known, although many studies begin to be developed. Most of these studies use bivalve molluscs as biological indicators : these animals can bioaccumulate pollutants within their tissues by filtration. Moreover, they are sedentary so they cannot escape in the case of a chronic or sudden pollution.
In this context, influence of mercury on defence mechanisms developed by Pacific oyster, *Crassostrea gigas*, was studied. Mercury effects were analysed *in vitro* on haemocytes, immune cells in bivalves, and also *in vivo* in laboratory on adult oysters. Effects of this pollutant on haemocyte activities have been monitored. For all experiments, haemocytes were analysed by flow cytometry ; this technique allows to monitor different haemocyte characters in a short period. Viability and cellular activities have been analysed. Results indicate that mercury induces an *in vivo* oyster mortality and an *in vitro* high haemocyte mortality.
Key words : *Crassostrea gigas*, mercury, haemocytes, flow cytometry.

Résumé : Depuis plusieurs décennies, l'ostréiculture est une activité économique importante en France et particulièrement en Charente-Maritime. En effet, le bassin de Marennes-Oléron est le premier bassin français producteur d'huîtres, notamment l'huître creuse, *Crassostrea gigas*. Cependant, cette zone est soumise à de nombreuses pollutions de nature anthropique. Les industries rejettent du mercure dans la Charente. Les effets de ce polluant sur les animaux présents dans ces zones d'estuaire sont encore peu connus, bien que de nombreuses études soient en cours de développement. La majeure partie de ces études utilise les mollusques bivalves comme indicateurs biologiques : en effet, ces animaux peuvent accumuler les polluants dans leurs tissus par filtration de l'eau. De plus, leur sédentarité leur interdit toute possibilité de fuite face à une pollution chronique ou soudaine. Dans ce contexte, l'influence du mercure sur les mécanismes de défense développés par l'huître creuse, *Crassostrea gigas*, a été étudiée. Les effets du mercure ont été recherchés d'une part *in vitro* sur les hémocytes, cellules impliquées dans les mécanismes de défense chez les bivalves, et d'autre part *in vivo* en laboratoire sur des huîtres adultes. L'effet de ce contact sur les hémocytes a également été suivi. Pour toutes les expériences, les hémocytes ont été analysés en cytométrie de flux ; cette technique permet d'obtenir rapidement de nombreuses informations sur un grand nombre de cellules. Ainsi, la viabilité ainsi que différentes activités cellulaires des hémocytes d'huître creuse ont été analysées. Les résultats montrent que le mercure provoque à la fois une mortalité chez les huîtres adultes maintenues au contact de ce polluant et une mortalité élevée des hémocytes.
Mots-clés : *Crassostrea gigas*; mercure; hémocytes; cytométrie de flux

Introduction :

For several decades, shellfish culture has developed in a significant way around the world and particularly in France including the Pacific oyster, *Crassostrea gigas*, exploitation. Shellfish are frequently reared in disturbed ecosystems subjected to pollutants which affect the environmental quality of coastal waters including estuarine waters. Bivalve molluscs are ideal indicator organisms because they are ubiquitous, sedentary and filter-feeders inhabiting coastal and estuarine areas (1). They may therefore highly concentrate contaminants within their tissues. Molluscs possess a cellular defence system : the haemocytes, which have various activities including phagocytosis, intracellular degradation of pathogens by means of hydrolytic enzymes and production of reactive oxygen metabolites (2).

Our work studied the *in vitro* and *in vivo* effects of mercury on *Crassostrea gigas* haemocytes. Haemocytes supply the defence responses in bivalves by phagocytosis and release of hydrolytic enzymes, so they would be the first to be concern by pollutants. Substances selected were methylmercury and mercury, known for their effect in all the trophic chain (3). Effects on haemocytes were studied using flow cytometry . The flow cytometer allows rapid analyses of morphological and functional characteristics of free cell suspensions (4). Various cellular parameters were studied and different concentrations of pollutants were tested.

Material and methods :

Pacific oysters, *Crassostrea gigas*, were purchased from a shellfish farm located in Marennes-Oleron Bay (Charente-Maritime, France). Haemolymph was withdrawn from the pericardial cavity. Haemocytes were analysed with a flow cytometer (Coulter EPICS XL 4, Beckman Coulter) after collection. Results were expressed as cell cytograms indicating the size (FSC value), the cellular complexity (SSC value) and the fluorescence channel(s) corresponding to the marker used.

Methylmercury (CH_3HgCl) and mercury chloride ($HgCl_2$) were tested *in vitro* on the haemocytes of twenty pooled oysters. Haemolymphs were supplemented with antibiotics (kanamycin, oxolonic acid, erythromycin, 0.1 mg/cm^3) at a concentration of 10^6 cells per cm^3. Concentration ranges of pollutants were 0; 2.10^{-11}; 2.10^{-10}; 4.10^{-10} 2.10^{-9}; 2.10^{-8}; 2.10^{-7} 4.10^{-7}; 2.10^{-6} M for methylmercury, 0; 2.10^{-7}; 2.10^{-6}; 2.10^{-5} M for mercury chloride. Mortality of haemocytes was evaluated after 4 and 24 hours of incubation at different temperatures : 20°C and 25°C for methylmercury, 15°C and 22°C for mercury. Mortality was quantified using propidium iodide (1.0 mg/cm^3, Interchim).

Mercury chloride was also tested *in vivo* on adult oysters. Oysters received water contaminated by mercury during 42 days at three concentrations : 0, 2.10^{-10} and 9.10^{-10} M. Water was changed every three days and mercury solutions were added. Twenty animals per raceway were removed at each time of analysis (0, 4, 7, 14, 21 and 42 days after the addition of mercury) in order to obtain four replicates of five animals for each raceway. Haemolymphs were withdrawn and pooled for each replicate and cell parameters were evaluated : mortality was quantified using propidium iodide, which enters cells with an injured membrane and marks DNA. Enzymatic activities (esterase, aminopeptidase, peroxidase) were quantified using commercial kits (« Cell Probe ™ Reagents », Beckman Coulter). Phagocytosis was quantified by the absorption of fluorospheres (fluorospheres ® carboxylate-modified yellow-green fluorescent (505/515 nm), 2 % solid, 1µm diameter, Interchim).

Results were analysed using a non-parametric Kruskall-Wallis test for independent samples. In the case of the reject of H_0, an a posteriori Student-Newman-Keuls (SNK) test was used.

Results :

In vitro

Methylmercury induced a high mortality of haemocytes at 25°C (Figure 1). Mortality increased only with the highest concentration of the pollutant (2.10^{-6} M). The same evolution of mortality was reported at 20°C (Figure 1).

Figure 1 : Evolution of haemocyte mortality in *in vitro* contact with different concentrations of methylmercury, incubated for four and 24 hours at 20°C and 25°C. Values are means of two replicates. Mortality increased with incubation time but temperature had no effect.

Figure 1 : Evolution de la mortalité des hémocytes mis en contact *in vitro* avec différentes concentrations de méthylmercure incubés pendant 4 et 24 heures à 20°C et 25°C. Les valeurs sont des

moyennes de deux réplicats. La mortalité augmente avec le temps d'incubation mais la température n'a pas d'effet.

Mercury chloride induced a significant dose-dependent response, mostly remarkable after 24 hours of incubation at 15°C (Figure 2). Increasing mortality was also reported at 22°C (Table 1).
Figure 2 : Evolution of haemocyte mortality in *in vitro* contact with different concentrations of mercury chloride incubated for four and 24 hours at 15°C. Values are means of three replicates. Π = significant difference between mortality percentage obtained in control and with mercury chloride.
Figure 2 : Evolution de la mortalité des hémocytes mis en contact *in vitro* avec différentes concentrations de chlorure de mercure incubés pendant 4 et 24 heures à 15°C. Les valeurs sont des moyennes de trois réplicats. Π = différence significative entre les pourcentages de mortalité obtenus dans le témoin et avec le chlorure de mercure.

Table 1 : Percentages of haemocyte mortality (mean and standard deviation) in control and in presence of mercury chloride (2.10^{-7}, 2.10^{-6}, 2.10^{-5} and 2.10^{-4} M) after 4 and 24 hours of incubation at 15°C and 22°C.
Tableau 1 : Pourcentages de mortalité hémocytaire (moyennes et écart-types) obtenus dans les témoins et en présence de chlorure de mercure (2.10^{-7}, 2.10^{-6}, 2.10^{-5} et 2.10^{-4} M) après 4 et 24 heures d'incubation) 15°C et 22°C.

In vivo
Mercury induced mortality of adult oysters : at the end of the experiment, mortality was of 13.5% for the highest concentration of mercury, and 4% for control (data not shown).
Haemocyte parameters have changed during the time of experiment : peroxydase and esterase positive cell percentage started at a very low level, then increased at 4 days, decreased at 7 and 14 days, and finally increased again. Variations seem not to be related to mercury (Figure 3).
Phagocytosis activity presented the same type of variation, with an increase, a decrease at 7 days, and another increase until the end of experiment. Variations are not related to mercury (Figure 4).

Figure 3 : Evolution of peroxydase positive cell percentage in function of time during the *in vivo* contact with different concentrations of mercury chloride (42 days). Values are means of four replicates.
Figure 3 : Evolution du pourcentage de cellules peroxydases positives en fonction du temps pendant la mise en contact *in vivo* avec différentes concentrations de chlorure de mercure (42 jours). Les valeurs sont des moyennes de quatre réplicats.
Figure 4 : Evolution of phagocytosis activity in function of time during the *in vivo* contact with different concentrations of mercury chloride (42 days). Values are means of four replicates.
Figure 4 : Evolution de l'activité de phagocytose en fonction du temps pendant la mise en contact *in vivo* avec différentes concentrations de chlorure de mercure (42 jours). Les valeurs sont des moyennes de quatre réplicats.

Discussion- Conclusion :
High pollutant concentrations were used *in vitro* in order to investigate acute toxicity on oyster haemocytes and to be close to those existing in animals because of bioaccumulation of pollutant present in water (1).
Methylmercury lead to mortality, although mortality was not statistically significant. Mercury chloride caused significant cell death at concentrations that can be found into the animals (5). No effect of temperature was shown, but oysters can tolerate great variations of temperature *in situ* between winter and summer, particularly at low tide. These results agree with literature where no mortality was found on haemocytes of *Mya arenaria* until 10^{-4} M for methylmercury and 10^{-3} for mercury (6). This study allows to prove the killer potential of heavy metals on cells : with dying cells, the defence system may be damaged and the effect on animals can be lethal.
Mercury chloride also lead to mortality of adults animals in *in vivo* contacts. The impact of this pollutant on the cellular parameters is not very clear : the variations of the parameters did not seem to

be related to mercury. This experiment enabled to accumulate informations on evolution of cellular parameters of animals maintained in laboratory conditions, which is poorly documented.
Mercury is well known for its toxicity because of its methylation realised by microorganisms, so its penetration in cells is facilitated (7). Studies on this pollutant have to be continued. Flow cytometry appears to be an efficient tool to study oyster haemocyte mortality and immunotoxicity.

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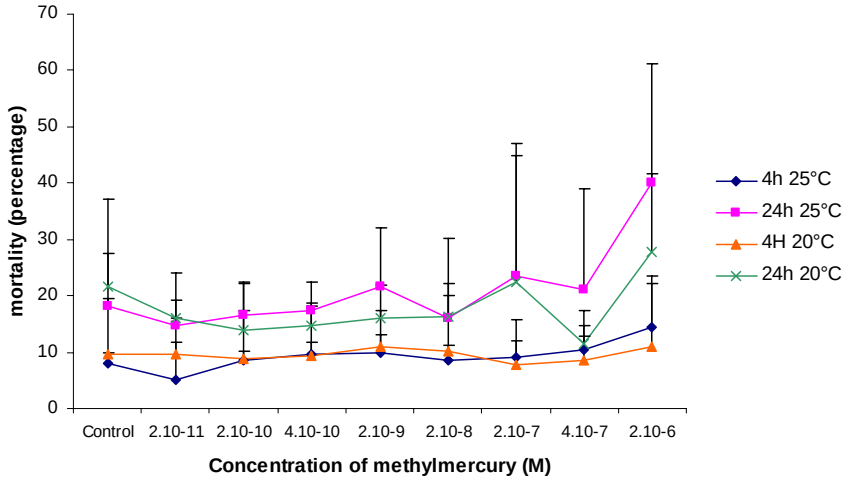

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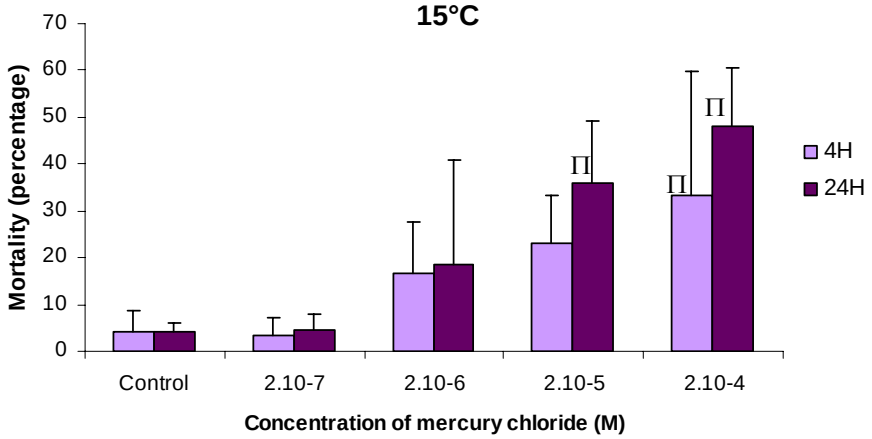

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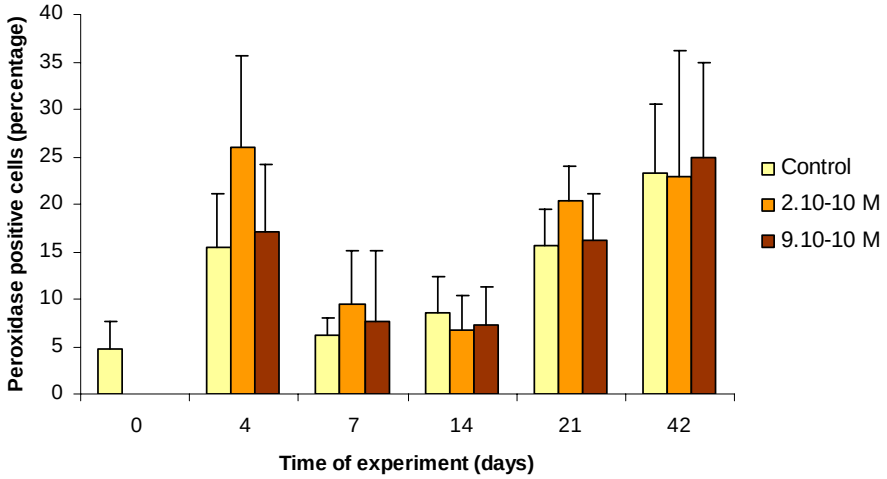

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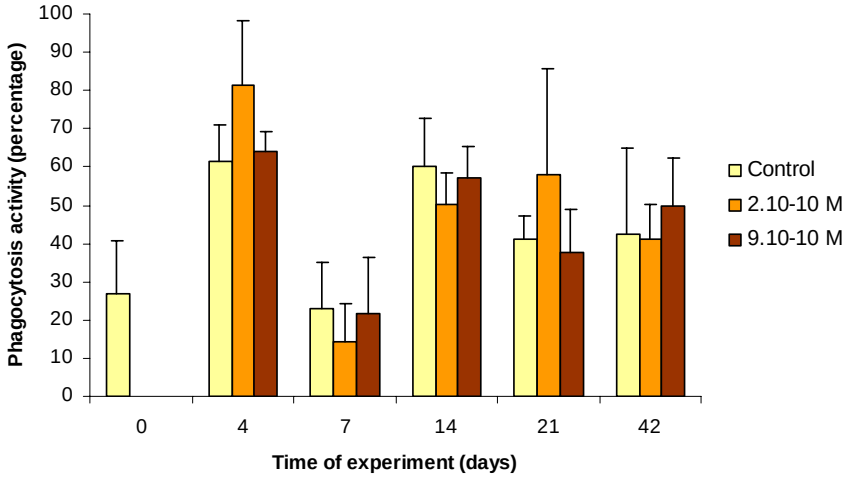

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		Control	2.10-7 M	2.10-6 M	2.10-5 M	2.10-4 M
4H	**22°C**					
	mean	2,57	2,30	2,87	21,70	40,40
	sd	1,30	1,51	2,31	4,78	19,97
	15°C					
	mean	4,23	3,57	16,57	23,07	33,33
	sd	4,46	3,76	11,11	10,25	26,49
24 H	**22°C**					
	mean	5,87	6,50	7,67	52,23	52,03
	sd	3,53	3,45	4,83	8,60	16,83
	15°C					
	mean	4,00	4,70	18,60	35,83	47,90
	sd	1,87	3,39	22,37	13,45	12,65

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