Study of Atrazine Effects on Pacific Oyster, *Crassostrea gigas*, Haemocytes

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Abstract: Shellfish farming is an important economic activity around the world. This activity often takes place in areas subjected to various recurring pollutions. The recrudescent use of herbicides in agriculture including atrazine implies pollutant transfer towards aquatic environment in estuarine areas. Harmful effects of such substances on animals in marine environment, particularly on cultured bivalves, are poorly documented. Bivalve molluscs such as mussels and oysters have been postulated as ideal indicator organisms because of their way of life. They filter large volumes of seawater and may therefore accumulate and concentrate contaminants within their tissues. Moreover, development of techniques allowing effect analysis of such compounds on bivalve biology may lead to the development of diagnosis tools adapted to analyze pollutant transfer towards estuarine areas. In this context, influence of atrazine on defence mechanisms was analyzed in Pacific oysters, *Crassostrea gigas*. Atrazine was tested *in vitro* and *in vivo* on oyster haemocytes, and its effects were analyzed by flow cytometry. Haemocyte viability, cell cycle and cellular activities were monitored. Atrazine induced no significant effect in oyster under tested conditions except for peroxidase activity.

Key words: *Crassostrea gigas*; Haemocytes; Flow cytometry; Atrazine; Cellular activities; Toxicity.

INTRODUCTION

In the last decades shellfish culture became particularly important economic activity around the world. The most economically important bivalve species is the Pacific oyster, *Crassostrea gigas*, with about 95% of the production in the world. This species is primarily reared in estuarine areas, which are subject to various pollutants from agricultural or industrial wastes. Bivalve molluscs are considered as indicator organisms for assessing environmental pollution levels. They filter large volumes of seawater and thus may bioconcentrate environmental contaminants within their tissues. The vulnerability of aquatic species to chemical pollution depends of pollutant properties, pollutant concentrations entering ecosystems and capacity of ecosystems to resist to pollutants [1].

Our work focused on atrazine effects on *Crassostrea gigas* immune response. Atrazine, a selective herbicide, is widely used in agriculture even though real effects of this substance have been little defined. Studies on atrazine were essentially carried out on genotoxicity and physiological changes.

Bivalve molluscs have an open circulatory system which is continually exposed to contaminants. These pollutants may directly interact with haemocytes that are involved in oyster defence system (phagocytosis, production of hydrolytic enzymes and reactive oxygen metabolites). Atrazine was tested *in vitro* by direct contact with haemocytes freshly collected and *in vivo* by contact with adult oysters using different concentrations. Our study was carried out at cellular level using flow cytometry. This technology allows rapid analysis of morphological characteristics of free cell suspensions. Resulting data are acquired by autofluorescence light scattering parameters related to cell size and granularity. Cellular activities may also be quantified with corresponding fluorescent markers. Proportion of granulocytes and hyalinocytes, cell viability, cellular cycle, presence of hydrolytic enzymes, and phagocytosis activity were monitored.

MATERIALS AND METHODS

Experimental Animals

Two years-old Pacific oysters, *Crassostrea gigas*, 8-10 cm in shell length were purchased from a shellfish farm located in Marennes-Oleron Bay (La Tremblade, Charente-Maritime, France) on the French Atlantic coast from February to April 2001. All animals originated from the same resource. They were acclimated during two weeks in the Ifremer hatchery.

Circulating Haemocyte Collection

After opening oyster shells by cutting off the adductor muscle, haemolymph was withdrawn directly from the

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pericardial cavity by puncture with a 1-mL syringe equipped with a needle (0,9525 mm). For each oyster, 0.5-1 mL of haemolymph was withdrawn. Haemolymphs from 30 oysters were pooled to eliminate possible inter-individual variation and to provide haemocytes to fulfil requirements of pollutant exposure protocol assays.

In Vivo Exposure Protocol

Oysters were exposed to water containing atrazine supplied by INRA (Saint-Laurent-de-la-Prée, Charente-Maritime, France). Two concentrations of atrazine were used (46.5 and 465 nM). For each concentration and for control, two batches were established. Oysters were acclimated six days in raceways. Then animals received pollutant and were maitained three weeks in raceways without seawater recirculation. Water samples from raceways were analyzed (CEMAGREF, Bordeaux, France) all experience long in order to control atrazine levels. Each raceway contained 157 L of seawater including atrazine. Water was changed every day. Raceways and biologic filters were cleared out and filled up with water maintained at 19.5°C+/-1°C. A volume of 157 mL of atrazine at 46.5 and 465µM diluted in 5 L of seawater was added in corresponding raceways. Oysters were daily fed with 8 L of *Isochrysis galbana* (6.10^6 cells per mL) and 3.5 L of *Tetracelmis suecica* ($1.5.10^6$ cells per mL) for each raceway.

Haemolymphs from four oysters were pooled in order to realise all the tests in flow cytometry.

In Vitro Exposure Protocol

Atrazine was tested using various concentrations (0-9.3-93-930µM respectively). Atrazine dilutions were prepared in dimethylsulfoxide (DMSO). Atrazine was added on

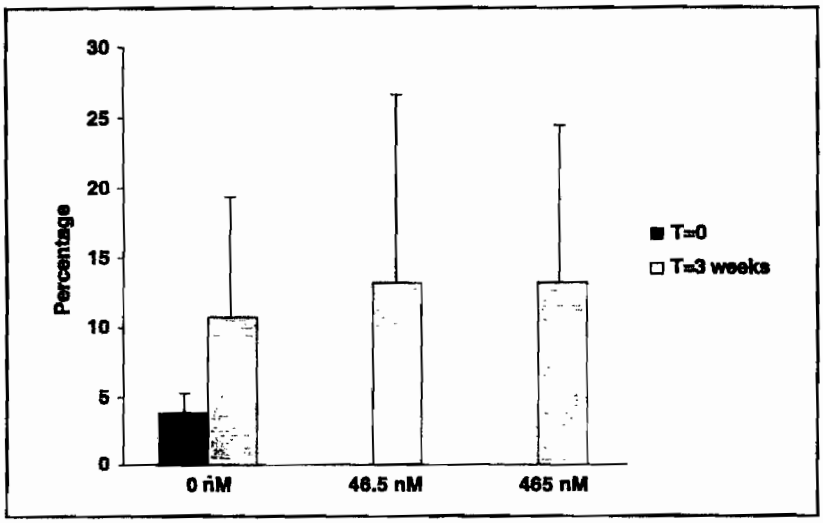

Fig. (1). Haemocyte mortality monitored by flow cytometry with increasing concentrations of atrazine *in vivo* (0-46.5-465 nM). Values are means of two replicates.

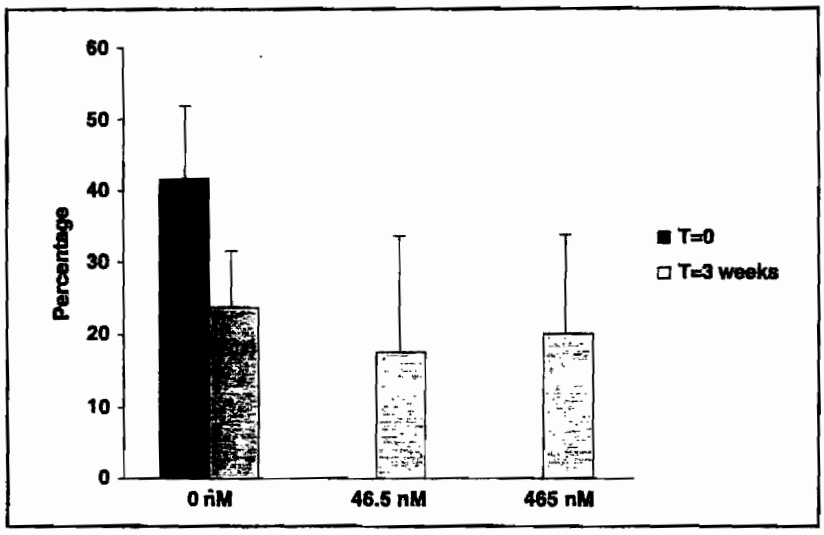

Fig. (2). Peroxidase activity monitored by flow cytometry with increasing concentrations of atrazine *in vivo* (0-46.5-465 nM). Values are means of two replicates.

haemocytes in low quantities : two µL of pollutant per mL of pooled haemolymph. Four and 24 hour incubations were carried out at ambient temperature in the dark.

Cellular Activity Analysis by Flow Cytometry

Haemocytes were analyzed with a flow cytometer (Coulter EPICS XL 4). For each haemocyte samples, 10 000 events were counted. Results were expressed as cell cytograms indicating size (FSC value), complexity (SSC value) and fluorescence channel(s) corresponding to the marker used. Fluorescence depended of monitored activities : enzymatic activities and phagocytosis were measured using FL1 (green fluorescence) and mortality using FL3 (red fluorescence).

Esterase, aminopeptidase, cathepsin and peroxidase activities were evaluated with commercial kits (Cell Probe™ Reagents, Beckman Coulter). Each analysis required 200µL of haemolymph and 10µL of the corresponding kit (FDA•Esterase, TP•Cathepsin, A•Aminopeptidase M and DFCH, PMA•Oxidative Burst). Haemocytes were then incubated in the dark at ambient temperature 15 minutes.

Mortality was quantified using 200µL of haemolymph. Haemocytes were incubated in the dark 30 minutes at 4 °C with 5µL of propidium iodide (PI, 1.0 $mg.mL^{-1}$, Interchim).

Cell cycle was analyzed using 200µL of haemolymph. Haemocytes were incubated in the dark two hours at 4°C with 20µL of PI and 200µL of lysing buffer. Percentages of haploïd cells (gamets), diploïd cells (G_0G_1) and tetraploïd cells (G_2M) were determined.

Phagocytosis was measured by ingestion of fluorescent beads (Fluorospheres® carboxylate-modified microspheres, diameter 1µm, Interchim). Five hundred µL of haemolymph were incubated one hour in the dark at ambient temperature with 10µL of a 1/10 dilution of beads.

Morphological characteristics of haemocytes were also analyzed. Two hundred µL of haemolymph were analyzed without any additional product. Proportion of hyalinocytes and granulocytes were defined by FSC and SSC parameter analysis.

Statistical Analysis

All cellular parameters were analyzed three times after four and 24 hour incubations. Results were analyzed 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

Atrazine Effects on Haemocyte Viability and Cellular Activities *in Vivo*

Haemocyte viability and peroxidase activity were similar in control and treated cells after a three week-atrazine exposure (Figures 1 and 2). Statistical analysis confirmed that atrazine had no effect on haemocyte viability and peroxidase activity after treatment, whatever concentration used. However, both parameters were different in controls at time 0 and after three weeks. Haemocyte mortality increased in controls after a three week period (Figure 1), while percentages of peroxidase positive cells decreased (Figure 2).

Esterase positive cells and number of dividing cells increased with atrazine concentrations (Figures 3 and 4). However, statistical analysis indicated that increase of dividing cells and esterase positive cells was not significant. Furthermore, esterase positive cell percentages did not seem to be affected by a three week period in raceways (Figure 3),

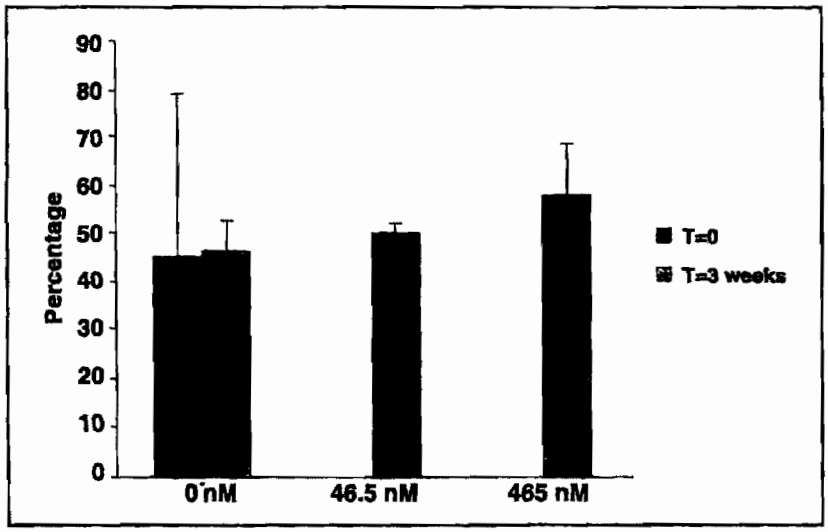

Fig. (3). Esterase activity monitored by flow cytometry with increasing concentrations of atrazine *in vivo* (0-46.5-465 nM). Values are means of two replicates.

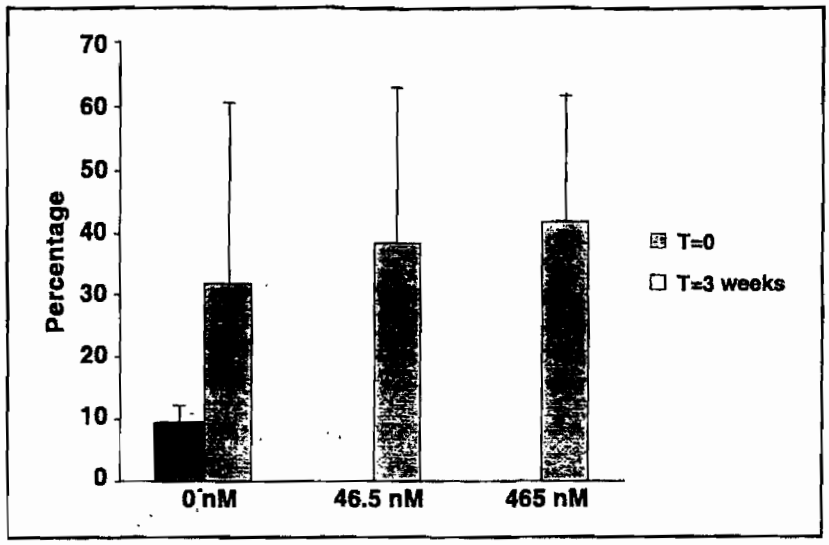

Fig. (4). Dividing cell number (G_2M) determined by flow cytometry with increasing concentrations of atrazine *in vivo* (0-46.5-465 nM). Values are means of two replicates.

while number of dividing cells was higher in controls after three weeks than in controls at time 0 (Figure 4).

No change was reported after atrazine treatment for all other tested parameters (cathepsins, aminopeptidases, phagocytosis activity and hyalinocyte percentage).

Atrazine Effects on Haemocyte Viability and Cellular Activities *in Vitro*

Esterase activity and haemocyte viability were similar in control and treated cells after a four hours incubation in presence of atrazine (Figures 5 and 6). Same results were reported after 24 hours incubation. Statistical analysis confirmed that atrazine had no effect on haemocyte viability and esterase activity after four and 24 hours incubations, whatever concentration used. However, no esterase activity was detected in control and treated cells after 24 hours incubation (Figure 5). Furthermore, haemocyte mortality was higher in controls after 24 hours incubation than in controls after a four hour incubation (Figure 6).

Percentages of peroxidase positive cells increased and hyalinocyte percentage decreased after 24 hours incubation in haemocytes treated with 930µM of atrazine (Figures 7 and

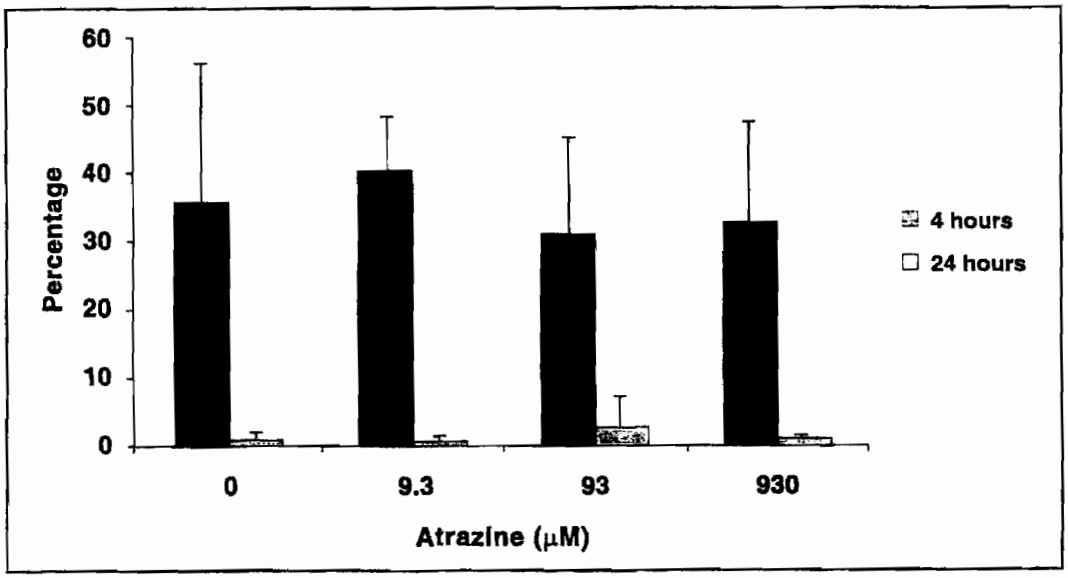

Fig. (5). Esterase activity monitored by flow cytometry with increasing concentrations of atrazine *in vitro* (0-9.3-93-930µM). Values are means of three replicates.

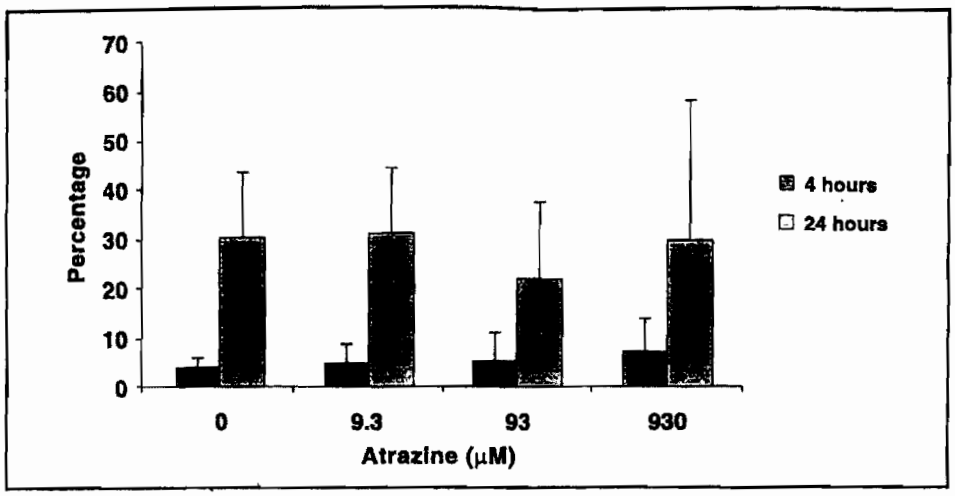

Fig. (6). Haemocyte mortality monitored by flow cytometry with increasing concentrations of atrazine *in vitro* (0-9.3-93-930µM). Values are means of three replicates.

8). No effect was detected in haemocytes treated with both other atrazine concentrations. Although statistical analysis indicated that decrease of hyalinocyte percentage was not significant, increase of peroxidase positive cell percentages was statistically significant ($\alpha = 0.75$).

No change was reported after atrazine treatment for all other tested parameters (cathepsins, aminopeptidases, phagocytosis activity, cell cycle and hyalinocyte percentage).

DISCUSSION

Atrazine is a herbicide belonging to the triazine family, which is one of the most used around the world [2]. Atrazine inhibits photosynthesis. It interacts with photosystem II, which is an essential link of the electron transport. Atrazine enters into plants by leaves and roots and penetrates also through unicellular algal membranes [3]. In marine ecosystems, atrazine may affect phytoplancton. Furthermore, atrazine is classified as a potential human carcinogen by the International Agency for Research on Cancer (IARC, 1991) and the United States Environmental Protection Agency (USEPA, 1991). However, studies on atrazine effects (ecotoxicology, genotoxicity) provided contradictory results. Atrazine is not a real inducer of aneuploïdy in human cells [4] and no mutagenic effect was also reported [5]. Furthermore, a study carried out on chronically exposed populations revealed no increased incidence of benign or malignant disease attributable to atrazine [6]. On the

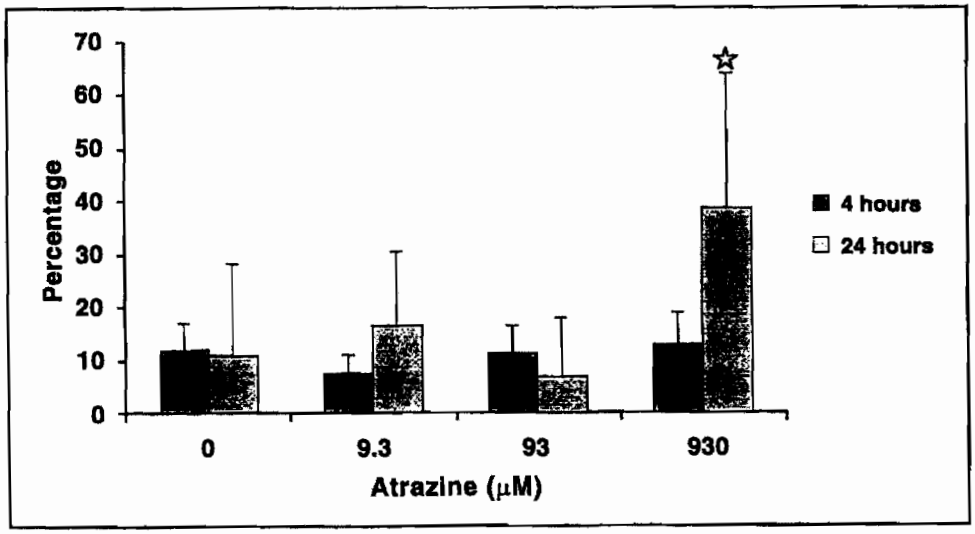

Fig. (7). Peroxidase activity monitored by flow cytometry with increasing concentrations of atrazine *in vitro* (0-9.3-93-930µM). Values are means of three replicates.
☆ = statitical difference for α=0.75.

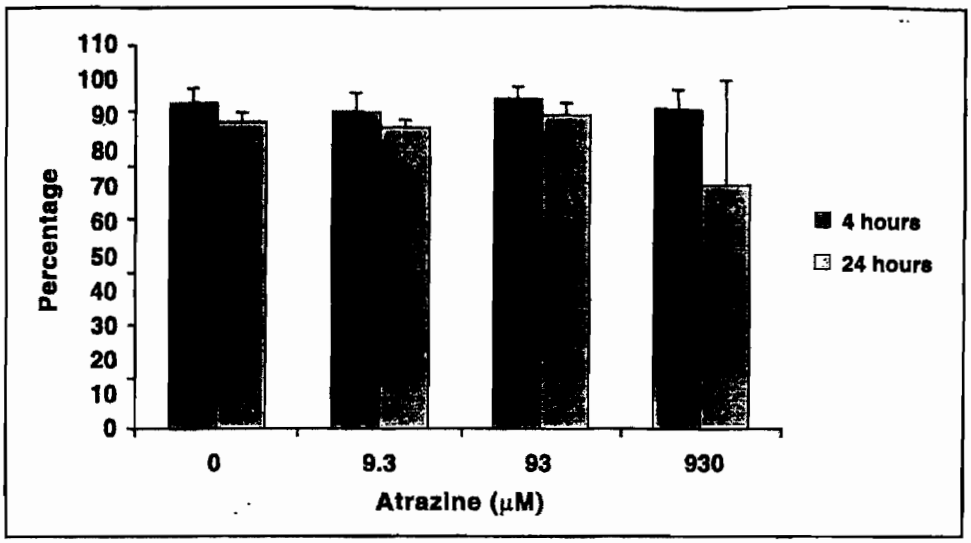

Fig. (8). Proportion of hyalinocytes defined by flow cytometry with increasing concentrations of atrazine *in vitro* (0-9.3-93-930µM). Values are means of three replicates.

contrary, a study [7] showed that atrazine induced sister chromatid exchanges and chromosome aberrations in human lymphocyte cultures exposed to 5 to 51µM of atrazine during 72 hours. However, gliphosate and vinclozolin showed a higher cytogenetic effect with increase of structural aberrations, sister chromatide exchanges and G6PD activity in exposed bovine lymphocyte cultures [8].

No significant effect of atrazine on most of the selected cellular parameters (phagocytosis activity, cell viability, cell cycle, enzymatic activities, hyalinocyte proportion) was detected in *Crassostrea gigas*. However, high concentrations of atrazine induced an increase of peroxidase positive haemocyte percentages *in vitro*.

Most studies about atrazine effects in *C. gigas* concerned physiology, ecotoxicology or genetic. A study [9] showed atrazine toxicity on growth of *C. gigas* larvae and two feeder-algae, *Isochrysis galbana* and *Chaetoceros calcitrans*. At 2.3µM, 5 % of larvae were abnormal and at 46.5µM, 75 % of larvae died in one week. Moreover, atrazine caused 60 to 70 % of mortality of *C. gigas* adults after two month *in vivo* exposure at 465 and 930nM [10]. Atrazine can also induce moderate changes of clotting of *C. gigas* haemocytes at 46.5 and 465nM [11]. Some works are in progress at the LGP laboratory on aneuploïdy induced by atrazine (Bouilly, personnal communication). However atrazine is not currently detected in bivalve tissues[12].

Effect of atrazine has also been studied in freshwater molluscs, which inhabit polluted areas. Atrazine induced a behavioural change of freshwater molluscs, *Physa acuta* and *Ancylus fluviatilis*, after a 18 day exposure at 70nM. Cellular lysis appeared after a 10 day exposure at 465 nM without significant effect on mortality and biomass [13]. Some studies [14] showed an increasing number of circulating haemocytes in *Lymnaea palustris* after atrazine exposure. They reported also a decrease of phagocytosis and oxidative burst metabolites in treated haemocytes.

In our experiments, atrazine induced an increase of peroxidase positive haemocyte percentages *in vitro* when high concentrations are used. Thus, atrazine is not a highly immunotoxic pollutant in *C .gigas* oysters. However, in *in vitro* experiments haemocytes were undoubtedly weaken after 24 hours incubation. *In vitro* maintenance of haemocytes is poorly documented and substances which may help it can have unknown effects on cellular activities. Furthermore, for *in vivo* experiments, results show that animal haemocytes had adapted to maturation conditions: cellular activities were different in controls after three weeks of maturation.

These results show the interest of using flow cytometry. This technique allows to analyze haemocyte viability and cellular functions after four and 24 hours *in vitro* incubations. It also permits to show how oysters react at cellular level after a maturation maintenance.

ACKNOWLEDGEMENTS

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