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Pollutant effects on Pacific oyster, Crassostrea gigas (Thunberg), hemocytes: Screening of 23 molecules using flow cytometry

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Abstract: The shellfish industry is an important economic activity in France, occurring mostly in estuarine zones subject to pollution due to anthropogenic activities. The harmful effects of pollutants on species inhabiting these estuarine zones are not well known. Among marine species, bivalve mollusks---particularly Pacific oyster, Crassostrea gigas---may serve a model of interest. The species is sedentary and filter-feeding, which favors bioaccumulation of pollutants in their tissues. Oysters may be suitable for studies on disturbance by pollutants of physiological activities, among which defense mechanisms are poorly documented in bivalves. In this study, effects of pollutants on hemocyte functions were monitored in Pacific oyster, C. gigas. Hemocytes were exposed in vitro to selected pollutants. The strategy for investigating the effects of pollutants on hemocyte functions is based on several biomarkers, which is more relevant than that of published papers based on single-endpoint experiments. Pollutants belonging to the most important groups of xenobiotics (PAHs, PCBs, and pesticides) were selected and their effect on hemocyte activities was analyzed using flow cytometry. Twenty-three pollutants were tested and eight of them showed significant modulation of hemocyte activities. PAHs and PCB 77 induced a decrease of hemocyte activity after an incubation periods of 4 and 24 h at 200 μmol/L. Three pesticides (2,4D, paraoxon, and chlorothalonil) modulated hemocyte activities. A mixture of eight pesticides also decreased phagocytotic activity. This study is one of the first to investigate the effects of so many pollutants on hemocyte functions at the same time and therefore allows a real comparison of different pollutant effects

Keywords: cellular activities - flow cytometry - hemocytes - Pacific oyster - Crassostrea gigas - PAHs - PCBs - pesticides - toxicity

Introduction

Shellfish farming is an ancestral activity in France. It has been developed over the last century in an intensive way and now represents a major economic activity. The Pacific oyster, *Crassostrea gigas*, is the most cultivated species, introduced in France in the 1970s after the decline of the Portuguese oyster, *C. angulata* (Grizel and Heral, 1991). This species is mostly reared in estuary zones, under the continual threat posed by contamination of pollutants. Natural and man-made foreign compounds (xenobiotics) enter and are dispersed in aquatic ecosystems by various routes including direct discharge, land run-off, atmospheric deposition, *in situ* production, abiotic and biotic movements and food-chain transfer (Livingstone, 1998). Xenobiotics include polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides and heavy metals. Many of these compounds come from anthropogenic activities including industry, fuel transport and agriculture.

Bivalve molluscs are often used as sentinel organisms in invertebrate immunotoxicology. Their world-wide distribution, their sedentary mode of life, and their filter-feeding behaviour susceptible to induce pollutant bioaccumulation, make them ideal species for the assessment of environmental pollution (Wade et al., 1998; Wootton et al., 2003). The study of modulation of immune system or immunomodulation in marine molluscs has become one of the privileged ways for evaluating the physiological effects of environmental factors (Oubella and Auffret, 1995). Physiological responses of bivalve molluscs to environmental and biological stresses are mediated, in part, by hemocytes circulating within the open vascular system and across all the epithelial boundaries (Cheng, 1981). Hemocytes are responsible for recognition, phagocytosis, and elimination by oxidation of non-self particles including virus, bacteria and parasites (Cheng, 1981). In bivalve molluscs, it has been well established that hemocytes can be affected by environmental factors such as stress (Lacoste et al., 2002) or pathogens (Anderson et al., 1995; Oubella et al., 1993). In the past decades, emerging diseases were reported in marine species and disease outbreaks have also increased considerably (Harvell et al., 1999). Anthropogenic compounds may be partly responsible by depressing defence capacities of host animals and therefore increasing susceptibility to infections, as proposed by several authors (Coles et al., 1994; Pipe and Coles, 1995). Hemocytes can be affected by environmental contaminants. Studies have demonstrated effects of PAHs (Coles et al., 1994; Pipe and Coles, 1995), PCBs (Canesi et al., 2003; Pipe et al., 1995), pesticides (Auffret and Oubella, 1997; Tripp, 1971), heavy metals (Auffret et al., 2002; Cheng et al., 1987; Gagnaire et al., 2004) and contaminated sediments (Oliver et al., 2001; Sami et al., 1993) on whole oysters or directly on hemocyte activities.

Flow cytometry is a routine tool in vertebrate biomedical research and it has been applied more recently to marine invertebrate research. This powerful tool has been used to describe hemocyte population characteristics in oysters (Goedken and De Guise, 2004; Xue et al., 2001) or changes associated with environmental contaminants (Fournier et al., 2001; Sauve et al., 2002b). In the present work, we investigated the *in vitro* effects of 23 xenobiotics on several hemocyte activities of Pacific oyster, *C. gigas*, using flow cytometry. This study is one of the first study which investigated so numerous pollutants on the hemocyte functions at the same time and therefore allows a real comparison of different pollutant effects. The strategy to look at effects of pollutants on hemocyte functions is based on several biomarkers which is of more impacts that the usual published papers based on single endpoint experiment. The first step of the study focused on optimisation of incubation conditions for the subsequent *in vitro* experiments. Toxic molecules were selected among the major groups of xenobiotics: 5 PAHs, 2 PCBs and 16 pesticides (7 herbicides, 6 insecticides, 2 fungicides and pentachlorophenol (PCP)). Concentrations ranging from 7 pM to 500 µM were used in order to observe a rapid effect of toxic compounds on oyster hemocytes. Resulting data were based on autofluorescence light scattering parameters related to size and internal complexity

(granularity). Cellular activities were monitored using appropriate fluorescent markers, with respect to cell mortality, esterase activity, production of Reactive Oxygen Species (ROS), lysosomes and phagocytosis.

Material and Methods

Oysters

Pacific oysters, *C. gigas*, with a shell length of 7-10 cm, were produced in the IFREMER hatchery of La Tremblade laboratory (Charente-Maritime, France) in February 2002. They were held at Bouin (IFREMER, Vendee, France). Analyses were performed from August to December 2003.

Hemocyte collection

Hemolymphs were collected from the posterior adductor muscle sinus, with the use of a 1-mL syringe equipped with a needle (0.9x25 mm) after breaching the shell using a pincers. For each oyster, 1-1.5 mL of hemolymph were collected. Hemolymph samples were filtered on a 60 µm mesh to eliminate debris and then maintained on ice to avoid aggregation. Hemocytes from ten oysters were pooled to reduce inter-individual variation and to provide enough hemocytes for the various exposure protocol. After pooling, hemocytes were counted on a Malassez cell.

Preliminary experiments

Cell treatments

Collected hemocytes were subjected to three different treatments and cell concentration was adjusted to 10^6 cells.mL^{-1}. One pool received a volume of artificial seawater to adjust cell concentration (treatment 1); one pool was centrifuged (260 g, 15 min, 4°C, Microfuge 12, Beckman) and cells were re-suspended in artificial seawater (ASW, Lewis composition; for 1 L distilled water: 234 g NaCl, 15 g KCl, 12 g $MgSO_4$ 4 H_2O, 1.5 g $CaCl_2$ 2 H_2O, 1.5 g $CaCl_2$ anhydrous; use at dilution 1/10) (treatment 2); the third pool was centrifuged (260 g, 15 min, 4°C, Microfuge 12, Beckman); supernatant (serum) was filtered and the cell pellet was then re-suspended in the 0.22 µm filtered serum (treatment 3). Experiments were performed three times at 15°C or 20°C for 4 h and 24 h. Cell mortality was monitored using flow cytometry as described in the section "Flow cytometry analysis".

Effect of temperature and incubation time on hemocyte activities

For all these experiments, cell concentration was adjusted to 10^6 cells.mL^{-1} by addition of artificial seawater without centrifugation (treatment 1). Different temperatures (10°C, 15°C or 20°C) and incubation periods (4 h, 24 h, 48 h and 72 h) were tested. Experiments were performed three times. Cell mortality and esterase activity were monitored using flow cytometry as described in the section "Flow cytometry analysis".

Xenobiotics

Twenty-three xenobiotics were selected based on their immunotoxic potential described in literature of invertebrate models. They belong to 5 families of xenobiotics: PAHs (benzo(a)pyrene (BaP), phenanthrene, pyrene, anthracene, fluoranthene), PCBs (PCB 77, PCB 153), herbicides (metolachlor, alachlor, terbutylazine, glyphosate, diuron, atrazine, 2,4 dichlorophenoxy-acetic acid (2,4 D)), insecticides (DDT, carbofuran, chlordane, dieldrin, paraoxon, carbaryl) and fungicides (chlorothalonil, fosteyl aluminium). Pentachlorophenol (PCP) is a pesticide (herbicide, insecticide and fungicide). A mixture of 8 pesticides (carbaryl, fosetyl aluminium, alachlor, metolachlor, terbutylazine, atrazine, diuron and glyphosate) was

also tested. Solvents were used as recommended by manufacturers (Table 1) and final solvent concentration was 0.5 % in order to avoid a possible disturbance of hemocyte parameters.
Pyrene, anthracene, fluoranthene, PCB 77, PCB 153, PCP, 2,4 D, DDT, chlordane, dieldrin, paroxon and chlorothalonil were purchased from Fluka (Sigma-Aldrich). The 8 molecules composing the pesticide mixture were purchased from LGC Promochem. BaP, phenanthrene and chlordane were kindly provided by J. Faucet (IFREMER Nantes) and F. Quiniou (IFREMER Brest).

Exposure protocol
For all exposure experiments, hemocyte concentration was adjusted to 10^6 cells.mL^{-1} by addition of artificial seawater without centrifugation (treatment 1). Pollutants were added individually at 5 µL per mL of hemocyte suspension. In each experiment, a same volume of corresponding solvent was used as negative control. Six concentrations were tested for BaP, phenanthrene, PCP, metolachlor, alachlor, terbutylazine, glyphosate, diuron, atrazine, DDT, carbofuran, carbaryl, fosteyl aluminium and the pesticide mixture (Table 1), five for pyrene (Table 1) and four for PCB 153, chlordane and dieldrin (Table 1). In addition, three concentrations were tested for anthracene, fluoranthene, PCB 77, 2,4 D, paraoxon and chlorothalonil (Table 1). Hemocytes were incubated at 15°C. Analyses were carried out after a 4 h and 24 h incubation period, except for paraoxon and chlorothalonil for which analyses was only carried out after a 4 h incubation period. Cell mortality, granulocyte percentage, esterase activity, phagocytosis, production of superoxide anion and lysosome presence were analysed using flow cytometry as described below. Experiments were carried out three times for BaP, phenanthrene, DDT, pesticide mixture, PCP and pyrene, twice for 2,4 D, anthracene, chlordanee, chlorothalonil, dieldrin, fluoranthene, paraoxon, PCB 77 and PCB 153 and once for each of the 8 pesticides composing the mixture.

Flow cytometry analysis
Flow cytometry protocols were previously described (Gagnaire et al., 2003; Gagnaire et al., 2004). 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. A gate was defined on the basis of FSC value in order to eliminate cell debris. Recorded fluorescence depended on monitored parameters: enzymes, lysosomes and phagocytosis were measured using green fluorescence and cell mortality was measured using red fluorescence. Mortality was quantified using 200 µL of hemocyte suspension. Hemocytes were incubated in the dark for 30 minutes at 4°C with 10 µL of propidium iodide (PI, 1.0 mg.L^{-1}, Interchim). The EPICS XL 4 software allows differentiation between supposed populations of granulocytes and hyalinocytes based on their FSC and SSC values. Esterase activity was measured using the non-specific liposoluble substrate fluoresceine diacetate (FDA, Molecular Probes). One µL of a FDA solution (400 µM) was added to 200 µL of hemocyte suspension. Cells were incubated for 30 minutes in dark at room temperature and then the reaction was stopped on ice (5 minutes). Lysosome presence was measured using a commercial kit (LysoTracker ® Green DND-26, 1mM in DMSO, Molecular Probes). One µL of a LysoTracker aliquot was added to 200 µL of hemocyte suspension. Cells were incubated for 2 hours in dark at room temperature and then the reaction was stopped on ice (5 minutes). Production of ROS was measured using the dihydrorhodamine 123 (DHR123, Molecular Probes), specific of superoxide anion O_2^-. One µL of a DHR123 solution (145 µM) was added to 200 µL of hemocyte suspension. Cells were incubated for 30 minutes in dark at room temperature and the reaction was stopped on ice (5 minutes). Phagocytosis was measured by ingestion of fluorescent beads. Two hundred µL of hemocyte suspension were incubated for 1 h in dark at ambient temperature with 10 µL

of a 1/10 dilution of Fluorospheres ® carboxylate-modified microspheres (1 µm diameter, Interchim).
For esterase activity, ROS and lysosomes, gates were defined on the cytograms in order to distinguish different populations according to fluorescence intensity (Figure 1). Events were previously gated in order to analyse only cells and not bacteria or debris. Analysis were performed on the whole population of cells, live and dead. Gates obtained were similar for all cytograms. Three cell populations were defined: a population of negative cells, a population of moderately stained cells and a population of strongly stained cells. For these three parameters, further analysis were realised only on the population of stronlgy stained cells.

Statistical analysis
Results were expressed as percentage of positive cells. Values were converted into r angular arcsinus √ (% of positive cells) before analysis. After transformation, values were normal. ANOVA analysis was carried out using Statgraphics ® Plus version 5.1 software in order to detect any effect of the tested pollutants. In the case of rejection of H_0, an *a posteriori* test was used. Significance was set at $P \leq 0.05$.

Results

Maintaining hemocytes in vitro

Cell treatments before culture

Among the three treatments, treatment 2 caused the highest mortality (centrifugation and cells re-suspended in ASW, $P < 0.001$, Figure 2) for both temperatures tested after 4 h and 24 h incubation periods. No significant difference was found between treatments 1 and 3, nor for effect of incubation temperature on mortality percentage. Treatment 1 was chosen for xenobiotic exposure.

Effect of temperature and time of culture on hemocyte activities

Cell mortality significantly increased after 48 h and 72 h incubation periods at 15°C compared to 4 h and 24 h. At 20°C, mortality significantly increased after 24 h and 48 h incubation periods compared to 4 h ($P < 0.01$, Figure 3). However, no variation of mortality percentage was observed at 10°C (Figure 3).
For all temperatures, percentage of esterase positive cells was significantly lower after 48 h and 72 h incubation periods compared to 4 h and 24 h ($P < 0.01$, Figure 4). Cells incubated at 20°C also showed a significant decrease of positive cell percentage after a 24 h incubation period compared to 4 h ($P < 0.01$, Figure 4).
These results allowed to choose a temperature of 15°C and incubation periods of 4 h and 24 h for further xenobiotic exposures.

Modulations of hemocyte parameters by pollutants
Among the 23 pollutants tested, eight showed significant modulations of at least one hemocyte parameter (Table 2). These eight molecules belong to different families of xenobiotics: four PAH (BaP, phenanthrene, anthracene, fluoranthene), a PCB (PCB 77), an herbicide (2,4 D), an insecticide (paraoxon) and a fungicide (chlorothalonil). The pesticide mixture also showed significant effects. However, there was no significant effect of PCP on hemocyte parameters.

PAHs. BaP and phenanthrene had similar effects on hemocyte parameters. They significantly increased granulocyte percentage but significantly decreased cell mortality, esterase and lysosome positive cells for the highest concentration tested compared to the control (200 µM

for BaP and 300 µM for phenanthrene) after 4 h and 24 h incubation periods (Table 2). Anthracene at 180 µM induced similar effects to BaP and phenanthrene, except for a decrease of cell mortality after a 24 h incubation period ($P < 0.01$, Table 2) and of esterase and lysosome positive cell percentage after a 4 h incubation period ($P < 0.01$, Table 2). Fluoranthene only significantly decreased esterase positive cells at 500 µM after a 4 h incubation period compared to control ($P < 0.05$, Table 2). Values for the negative control for both BaP and phenanthrene were ranged between 66 % and 70 % after a 4 h incubation period and decreased significantly to 43.9 % with 200 µM of BaP and to 11.1 % with 300 µM of phenanthrene ($P < 0.01$, Figure 5). After a 24 h incubation period, values for controls for both pollutants were 27 % and decreased to 16.8 % with 200 µM of BaP and to 2 % with 300 µM of phenantren (Figure 5).

PCBs. PCB 77 significantly decreased lysosome positive cell percentage for 6 µM and 60 µM after a 4 h incubation period compared to control ($P < 0.05$, Table 2).

Herbicides. Presence of 2,4 D increased cell mortality at the highest concentration of 450 µM after a 4 h incubation period compared to control ($P < 0.05$, Table 2). Value was 7.8 % in the control and increased to 11.8 % with 450 µM of 2,4 D (Figure 6).

Insecticides. Paraoxon exposure decreased significantly the percentage of esterase positive cells after 4 h and 24 h incubation periods for 400 µM compared to control ($P < 0.01$, Table 2). A similar effect was found on percentage of lysosome positive cells for 40 µM and 400 µM but only after a 24 h incubation period ($P < 0.01$, Table 2). ROS positive cell percentage significantly increased for 400 µM after a 4 h than after a 24 h incubation period ($P < 0.05$, Table 2). Percentages of lysosome positive cells were 24 % in the negative control and decreased to 16.7 % at 400 µM after a 24 h incubation period (Figure 7). However, no significant effect was reported after a 4 h incubation period (Figure 7).

Fungicides. Presence of chlorothalonil significantly increased cell mortality at 2 µM ($P < 0.05$) and granulocyte percentage at 200 µM ($P < 0.01$) and significantly decreased esterase positive cell percentage at 200 µM ($P < 0.05$) after a 4 h incubation period (Table 2).

Pesticide mixture. Exposure to the pesticide mixture significantly increased phagocytosis activity after a 4 h incubation period for the highest concentration tested ($P < 0.05$, Tables 1 and 2). Values increased from 37.9 % in the negative control to 54.8 % with maximal concentration (Figure 8).

Discussion

In order to compare results, hemocyte concentrations must be adjusted (Brousseau et al., 2000; Fournier et al., 2001; Fournier et al., 2002). However, techniques for cell adjustment are poorly documented. Our results showed that cell re-suspension in ASW after centrifugation increased cell mortality. Cellular parameters including esterase activity were modified by centrifugation and dependently on the medium of cell re-suspension (data not shown). In immunotoxicity studies, modifications of cellular activities have to be due only with pollutant tested but not to culture conditions. We thus added ASW to adjust cell concentration. Under these conditions, no change in cell parameters was observed.
In experiments concerning the effects of temperature and time of incubation on hemocyte activities, esterase activities decreased as cell mortality increased. This has been previously reported for phagocytosis and cell viability (Brousseau et al., 2000). Protocols of flow

cytometry used in this study are therefore validated and allow also to validate further results of pollutant effects. Lysosomes have been described as a valid marker of cell viability (Lowe et al., 1995; Lowe and Fossato, 2000; Moore et al., 1978). A decrease of lysosome positive cells could also been related with an increase of cell mortality. However, in these experiments we only tested esterase activity.

In vitro exposure of cells to pollutants may be an approach to evaluate pollutant toxicity although several mechanisms including mucus in the mantle cavity or alimentary may be involved in the pollutant toxicity in *in vivo* conditions (Fisher et al., 1987). *In vitro* conditions are frequently used for the assessment of pollutant toxicity in bivalve hemocytes (Alvarez and Friedl, 1992; Anderson et al., 1992; Baier-Anderson and Anderson, 2000; Brousseau et al., 2000; Gagnaire et al., 2004; Larson et al., 1989).

This study confirmed further significant effects of PAHs on hemocyte viability. Our results also revealed that PAHs significantly decreased lysosomes and esterases. This is in accordance with literature (Wootton et al., 2003). Lysosomes appeared to be one of the most important target of PAHs (Lowe et al., 1995; Moore et al., 1978). Literature reported that PAHs increased release of peroxidase and ROS production, both mechanisms occuring in oxidative burst (Coles et al., 1994; Gomez-Mendikute et al., 2002). Our results did not support this hypothesis. However, our detection method only permits quantification of superoxide anion O_2^- but not all components of the oxidative burst. Moreover, as PAHs significantly decreased lysosomes and esterases, we hypothesize that hydrolytic enzymes, which also take place in oxidative burst, may be affected by PAHs. Granulocyte percentages were increased in the presence of PAHs. We can hypothesize that percentage of hyalinocytes decreased because they were killed by pollutants. Granulocytes may be more resistant.

We found an effect of PCB 77 on lysosomes but not on ROS release or esterase activity. Some studies demonstrated that the effects of PCBs were congener-specific (Coteur et al., 2001). PCBs act in mussels on targets corresponding to those found in human neutrophils, i.e. signal transduction pathways involved in the innate immune response (Canesi et al., 2003). Moreover, PCBs could be more toxic in mixtures (Harper et al., 1995).

Lysosomes appear to be a more generalised target of toxic stress induced by PAHs and PCBs. Lysosomal enzymes are considered to play an important role in invertebrate defence reactions involving bacterial destruction (Moore and Gelder, 1985; Moore et al., 1978). Their functional impairment may affect host resistance to a pathogen infection (Anderson, 1981).

No effect was detected in this study with PCP. However, a previous study showed that PCP decreased ROS production in *C. virginica* (Baier-Anderson and Anderson, 1996). PCP is an uncoupler of oxidative phosphorylation and has been shown to inhibit NADPH, a component involved in ROS production (Anderson, 1981). The probe used in our study, DHR 123, is one for detection of superoxide anion. NADPH production was reported to take place earlier in oxidative burst mechanism (Anderson, 1981), and our probe may not be an effective indicator of NADPH production.

A previous study reported no effect of atrazine on hemocyte parameters in *C. gigas* (Gagnaire et al., 2003). Here, the range of herbicides tested showed no effects. Herbicides would act on photosynthesis. Studies on herbicide mostly showed a genotoxic potential of herbicides such as glyphosate (Lioi et al., 1998) and atrazine (Bouilly et al., 2003) in both vertebrates and invertebrates.

Organophosphorous compounds and carbamates including paraoxon and carbaryl are known to be acetylcholinesterase and carboxylesterase inhibitors in vertebrates (Cooreman et al., 1993) and in mussels (Galloway et al., 2002; Ozretic and Krajnovic-Ozretic, 1992). However, cholinesterases in *C. gigas* were found insensitive to insecticides (Bocquene et al., 1997). We reported a decrease of esterase and lysosome positive cell percentages with paraoxon. Esterases measured in this study were non specific esterases.
Dieldrin and chlordane induced a decrease of phagocytosis of *C. virginica* hemocytes (Larson et al., 1989). We reported no effect on phagocytosis at equivalent concentrations for both molecules. However, the previous authors used chemiluminescence to measure phagocytosis activity.

A few studies have explored effects of fungicides on oysters. Hemocytes from *C. virginica* exposed to chlorothalonil and triforine induced a decrease of ROS production and of cell viability and phagocytosis activity (Alvarez and Friedl, 1992; Baier-Anderson and Anderson, 2000). Our results showed effects on percentages of esterase and lysosome positive cells at similar concentrations. These molecules thus appear to modulate hemocyte activities.

Pesticide mixture (alachor, metolachlor, terbutylazine, glyphosate, diuron, atrazine, carbaryl and fosteyl aluminium) is representative of pollutants of the surface waters of the Marennes-Oleron Basin (Charente-Maritime, France). Concentrations of these molecules in the environment are between 0.25 nM and 4 nM (internal report). Interestingly, none of these eight molecules generated significant effects when tested individually on *C. gigas* hemocytes. However, as a mixture, the same molecules increased phagocytosis activity. The vast majority of available toxicity data is related to single substances, not to mixtures. However, in the environment molecules are usually presented concomitantly (Moore, 2002). Moreover, a study on joint toxicity of multi-components of triazine mixtures demonstrated that the toxic effects of the mixtures exceed that of each component alone (Faust et al., 2001). This synergistic effect (or concentration addition) may be enhanced by the fact that the different molecules possess a same specific target, but also by their additional effects on different sites (Faust et al., 2001).

Among the 23 pollutants (belonging to five groups of pollutants) tested, at least one molecule of each group showed effects on at least one hemocyte parameter. It may therefore be concluded that all groups of pollutant (PAHs, PCBs, herbicides, insecticides and fungicides) may be hazardous to bivalve defence system. However, PAHs appeared to be a group inducing more modulations due to that four of the five PAHs tested showed significant effects. This work is the first to study such large number of pollutants from different groups on Pacific oyster, *C. gigas*, hemocytes. However, only one mixture of pesticides was tested. This work needs to be continued by testing mixture of different pollutants by groups. Moreover, several of the xenobiotics selected in this study need to be bioactivated in order to be toxic. In this *in vitro* type of exposure, we can never be assured that hemocytes have the ability to perform this activation. Therefore it is difficult to compare the toxicity of these chemicals. We can hypothesise that in *in vivo* exposures, these xenobiotics could be more toxic.

This study showed that pollutant can increase or decrease hemocyte parameters. However, we cannot conclude whether an increase in an hemocyte activity is related to a positive or a negative effect on immunity. Hemocytes are involved in other physiological processes (Cheng, 1981; Fisher, 1986). Therefore, hemocyte activities are not only indicators of defense

activities. We can hypothesize that an increase or a decrease of hemocyte activities correspond to a perturbation of the immune system and, as other authors advanced, that pollutants induced modulation of the immune system due to toxic effects (Oubella and Auffret, 1995). Moreover, several studies have demonstrated that pollutants could have inhibitory or stimulating effects dependant on the dose used (Cheng and Sullivan, 1984; Sauve et al., 2002a). In vertebrates, an increase of phagocytosis activity may be related to an enhancement of cell sensibility and lead to the development of auto-immune diseases (Christin et al., 2004). An increase of hemocyte activity is not compulsorily related to a better defense capacity of the organism.

In order to demonstrate the link between hemocyte parameters and immune activities, experiments involving pollutant and pathogen exposure need to be conducted. Several authors have already demonstrated that alteration of hemocyte parameters in bivalves exposed to pollutants could be associated to an increase of disease susceptibility (Chu et al., 2002; Coles et al., 1994; Coles et al., 1995; Fournier et al., 1988; Pipe et al., 1999).

No bivalve cell lines are available. Experiments have to be conducted on cells maintained *in vitro* (Anderson, 1994; Brousseau et al., 2000; Sauve et al., 2002a). Hemocytes are of interest because of easy collection.

Among the hemocyte parameters tested, lysosomes and esterase activity appeared to be the most sensitive ones. They were modulated by 6 pollutants. As demonstrated previously, lysosomes are biomarkers of cellular viability (Lowe and Fossato, 2000; Moore et al., 1978; Moore, 2002). The probe used in this study allowed to measure the presence of lysosomes. We can hypothesise that a high number of lysosomes is correlated to a greater capacity of the cell to answer an aggression. A few studies reported evolution of esterase activity, many of them involving acetyl- and carboxylesterases (Cooreman et al., 1993).

Production of superoxide anion as measured by DHR123 was affected only by paraoxon. However, this parameter may also be modulated by PAHs (Gomez-Mendikute et al., 2002). Other studies used different methods for flow cytometric measurement of superoxide anion with DCFH-DA (Lambert et al., 2003). Further comparative studies on both of these components have to be realised.

Conclusion

This study is the first to analyse the *in vitro* effects of 23 xenobiotics on 6 oyster hemocyte parameters. All groups of pollutants could have an effect on one or more hemocyte parameters. These results need to be confirm by *in vivo* experiments in order to demonstrate effects of these potential immunomodulators in the whole animal.

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Table 1: Lists of the 23 pollutants tested individually and the pesticide mixture. Final concentrations of the pollutants in presence of hemocytes and the solvents used are presented. Solvents used were: DMSO: dimethylsulfoxide; CH: cyclohexane; TMP: trimethylpentan; AN: acetonitril; A80: ethanol 80 %; MeOH: methanol. For the pesticide mixture, numbers 1 to 6 correspond to different concentrations of the mixture, and concentrations of the eight components are indicated.

PAH					PCB	
BaP	Phenanthren	Pyren	Anthracen	Fluoranthen	PCB 77	PCB 153
DMSO	DMSO	CH	CH	CH	TMP	TMP
200 µM	300 µM	2,5 µM	180 µM	500 µM	60 µM	70 µM
20 µM	30 µM	250 nM	18 µM	50 µM	6 µM	7 µM
2 µM	3 µM	25 nM	1,8 µM	5 µM	600 nM	700 nM
200 nM	300 nM	2,5 nM				70 nM
20 nM	30 nM	250 pM				
2 nM	3 nM					

Pesticides	Herbicides						
PCP	Metolachlor	Alachlor	Terbutylazine	Glyphosate	Diuron	Atrazine	2,4 D
MeOH	A 80	A 80	A 80	H_2O	A 80	A 80	AN
2 µM	7 µM	7 µM	26 µM	12 µM	17 µM	30 µM	450 µM
200 nM	700 nM	700 nM	2,6 µm	1,2 µM	1,7 µM	3 µM	45 µM
20 nM	70 nM	70 nM	260 nM	120 nM	170 nM	300 nM	4,5 µM
2 nM	7 nM	7 nM	26 nM	12 nM	17 nM	30 nM	
200 pM	700 pM	700 pM	2,6 nM	1,2 nM	1,7 nM	3 nM	
20 pM	70 pM	70 pM	260 pM	120 pM	170 pM	300 pM	

Insecticides						Fungicides	
DDT	Carbofuran	Chlordan	Dieldrin	Paroxon	Carbaryl	Chlorothalonil	Fosteyl al
MeOH	A 80	AN	AN	CH	A 80	AN	H_2O
1,5 µM	120 µM	250 µM	1,3 µM	400 µM	1 µM	200 µM	700 nM
150 nM	12 µM	25 µM	130 nM	40 µM	100 nM	20 µM	70 nM
15 nM	1,2 µM	2,5 µM	13 nM	4 µM	10 nM	2 µM	7 nM
1,5 nM	120 nM	250 nM	1,3 nM		1 nM		700 pM
150 pM	12 nM				100 pM		70 pM
15 pM	1,2 nM				10pM		7pM

	Pesticide mixture							
	Carbaryl	Fosteyl al	Alachlor	Metolachlor	Terbutylazine	Atrazine	Diuron	Glyphosate
6	1 µM	700 nM	7 µM	7 µM	26 µM	30 µM	17 µM	12 µM
5	100 nM	70 nM	700 nM	700 nM	2,6 µm	3 µM	1,7 µM	1,2 µM
4	10 nM	7 nM	70 nM	70 nM	260 nM	300 nM	170 nM	120 nM
3	1 nM	700 pM	7 nM	7 nM	26 nM	30 nM	17 nM	12 nM
2	100 pM	70 pM	700 pM	700 pM	2,6 nM	3 nM	1,7 nM	1,2 nM
1	10pM	7pM	70 pM	70 pM	260 pM	300 pM	170 pM	120 pM

Table 2: Effects of pollutants on *C. gigas* hemocyte activities after an *in vitro* contact. For each pollutant, the concentration(s) and the period(s) of incubation for which an effect of the pollutant was observed are indicated. ▲ = increase, ▼ = decrease. * = $P < 0.05$, ** = $P < 0.01$.

		Mortality	Granulocytes (percentage)	Esterases	Lysosomes	ROS	Phagocytosis
PAH	BaP	▼ 200 µM 4 h and 24 h **	▲ 200 µM 4 h and 24 h **	▼ 200 µM 4 h and 24 h *	▼ 200 µM 4 h and 24 h *		
	Phenanthren	▼ 300 µM 4 h and 24 h *	▲ 300 µM 4 h and 24 h *	▼ 300 µM 4 h and 24 h *	▼ 300 µM 4 h and 24 h **		
	Anthracen	▼ 180 µM 24 h *	▲ 180 µM 4 h and 24 h **	▼ 180 µM 4 h **	▼ 180 µM 4 h **		
	Fluoranthen			▼ 500 µM 4 h *			
PCB	PCB 77				▼ 6 µM and 60 µM 4 h *		
Herbicides	2,4 D	▲ 450 µM 4 h *					
Insecticides	Paraoxon			▼ 400 µM 4 h and 24 h **	▼ 40 µM and 400 µM 24 h **	▲ 400 µM 4 h *	
Fungicides	Chlorotalonil	▲ 2 µM 4 h *	▲ 200 µM 4 h **	▼ 200 µM 4 h *			
Pesticides	Pesticide mixture						▲ cc=6 4 h *

Figure 1: Flow cytometry cytogram of cells stained with FDA. X-axis: intensity of fluorescence; Y-axis: number of cells. 1: population of negative cells; 2: population of moderately stained cells; 3: population of strongly stained cells.

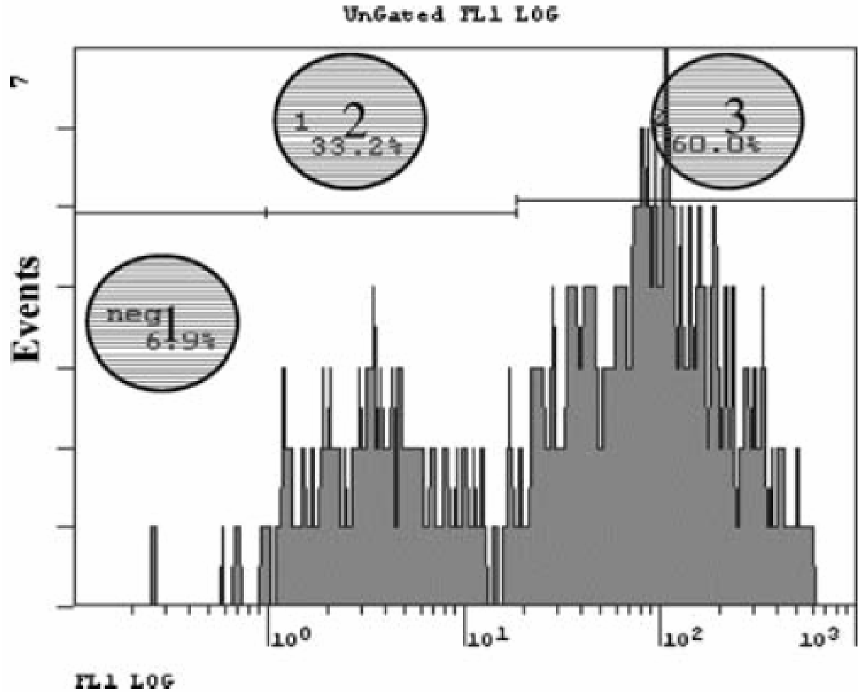

Figure 2: Cell mortality percentage after a 4 h incubation period at 15°C or 20°C. 1: cells adjusted to 10^6 cells.mL^{-1} by addition of ASW; 2: cells adjusted to 10^6 cells.mL^{-1} by re-suspension in ASW after centrifugation; 3: cells adjusted to 10^6 cells.mL^{-1} by re-suspension in hemolymph after centrifugation. Values are means of 3 replicates. Bars represent standard error. Stars indicate a significant difference (increase) between the three treatments for one given condition (temperature and incubation time). *** = $P < 0.001$.

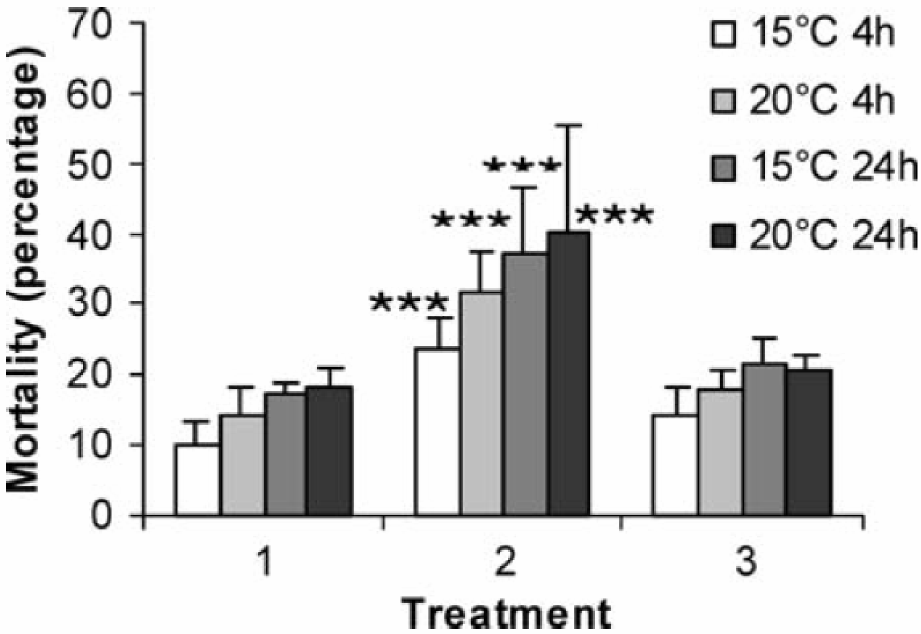

Figure 3: Cell mortality percentage after different incubation periods at 10°C, 15°C or 20°C. Values are means of 3 replicates. Bars represent standard error. Stars indicate a significant difference (increase) between the four incubation periods for one given temperature. ** = $P < 0.01$.

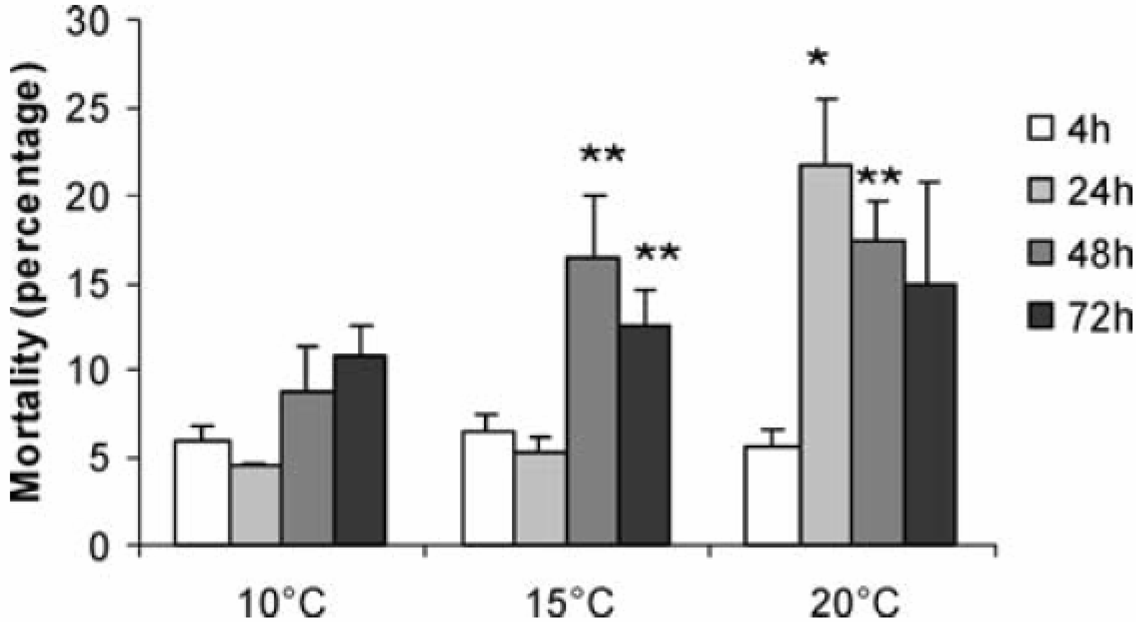

Figure 4: Percentage of esterase positive cells after different incubation periods at 10°C, 15°C or 20°C. Values are means of 3 replicates. Bars represent standard error. Stars indicate a significant difference between the four incubation periods for one given temperature. ** = $P < 0.01$.

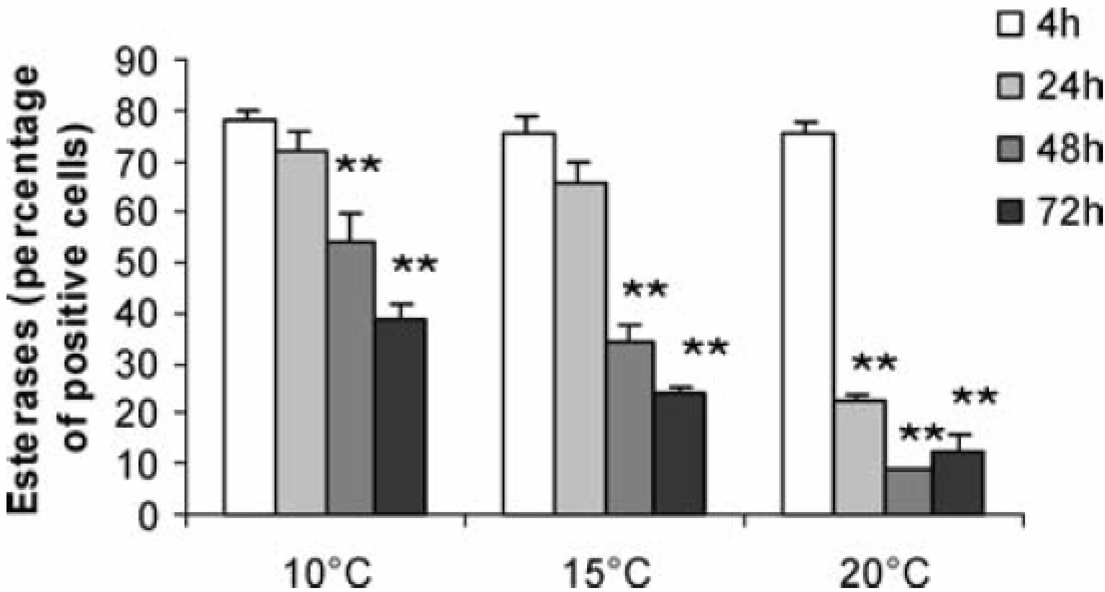

Figure 5: Percentage of esterase positive cells after 4 h and 24 h incubation periods with increasing concentrations of BaP or phenanthrene (Phe). BaP: 1 = 2 nM, 2 = 20 nM, 3 = 200 nM, 4 = 2 µM, 5 = 20 µM, 6 = 200 µM. Phe: 1 = 3 nM, 2 = 30 nM, 3 = 300 nM, 4 = 3 µM, 5 = 30 µM, 6 = 300 µM. Values are means of 3 replicates. Bars represent standard error. ** = $P < 0.01$.

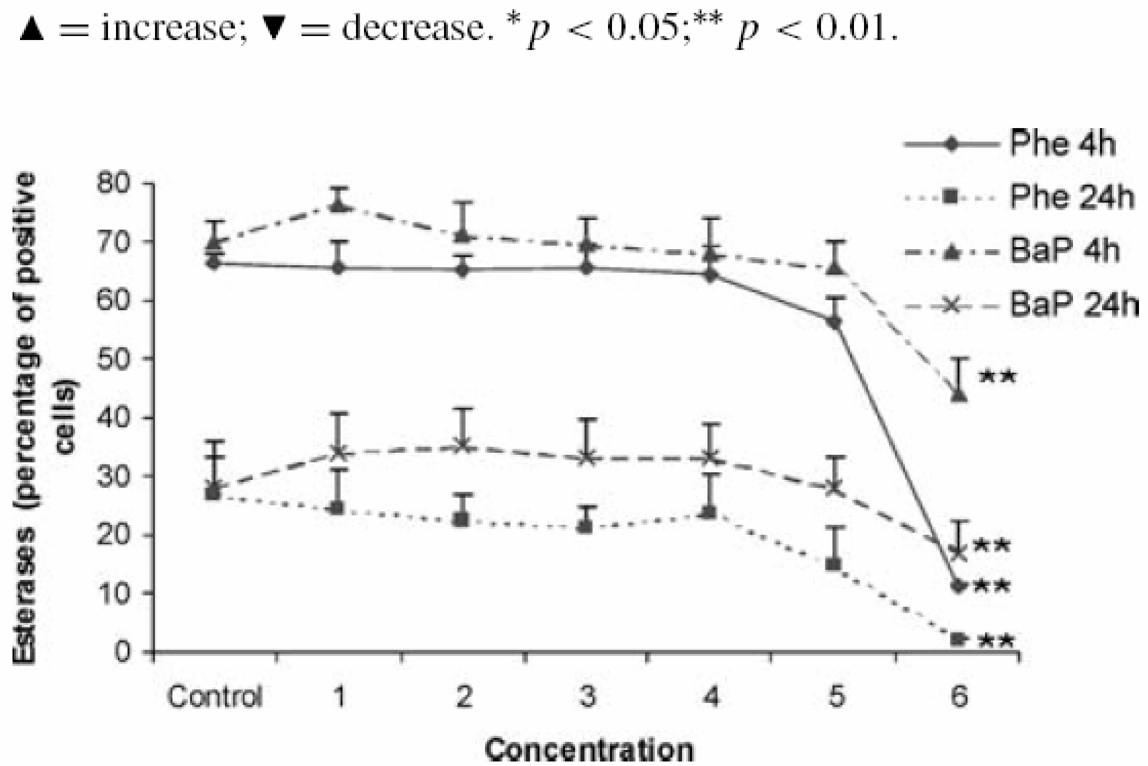

Figure 6: Cell mortality percentage after a 4 h incubation period with increasing concentrations of 2,4 D. Values are means of 2 replicates. Bars represent standard error. * = $P < 0.05$.

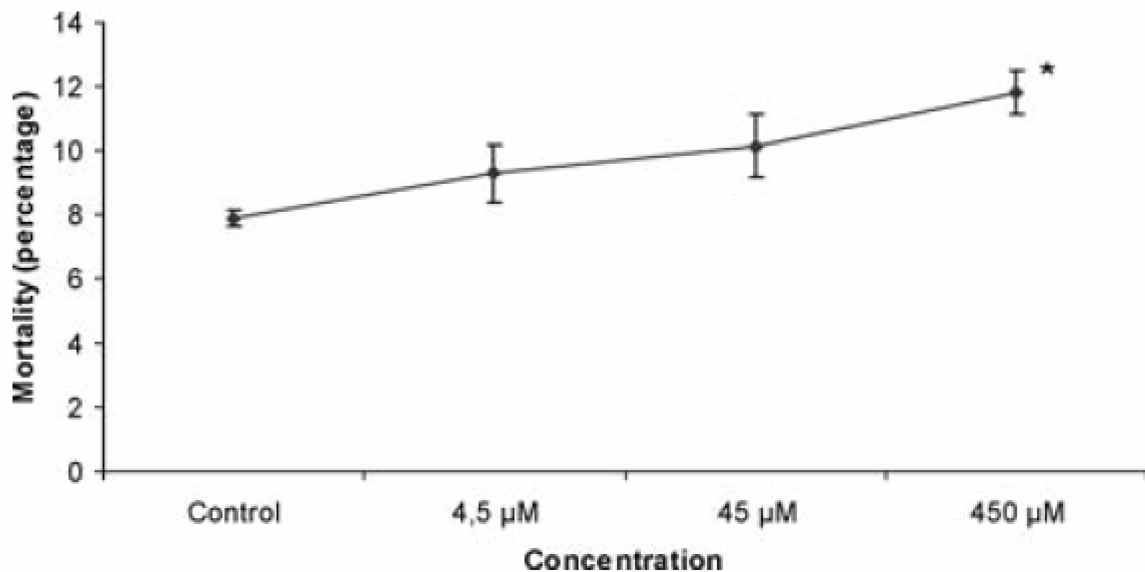

Figure 7: Percentage of lysosome positive cells for after 4 h and 24 h incubation periods with increasing concentrations of paraoxon. Values are means of 2 replicates. Bars represent standard error. ** = $P < 0.01$.

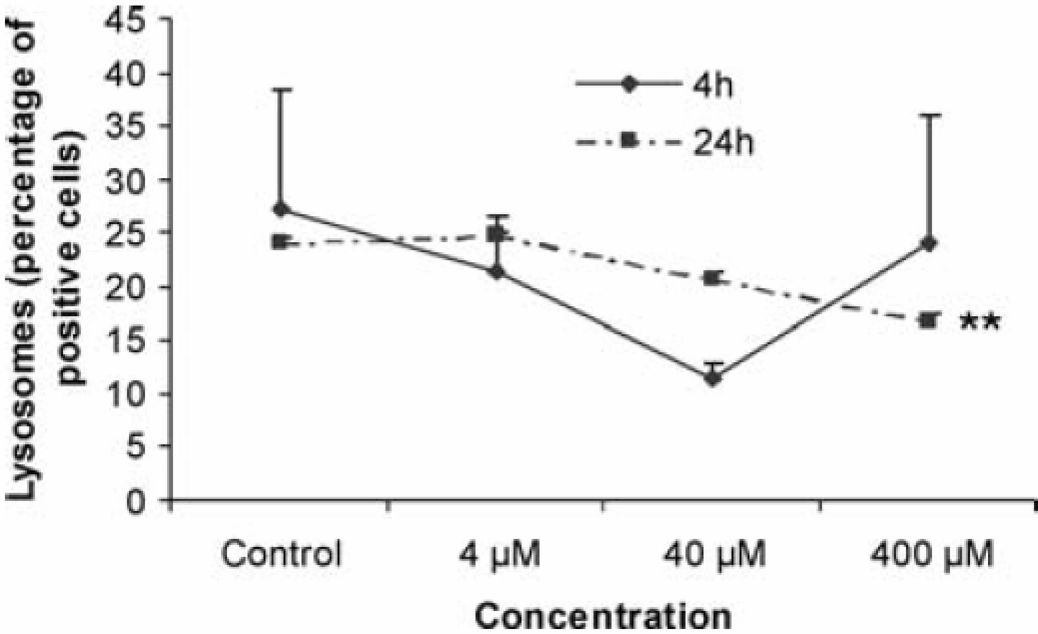

Figure 8: Phagocytosis percentage after a 4 h incubation period with increasing concentrations of the pesticide mixture. See table 1 for the different concentrations of the eight pesticides in the mixture. Values are means of 3 replicates. Bars represent standard error. * = $P < 0.05$.