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Cytological response of hemocytes in the European flat oyster, *Ostrea edulis*, experimentally exposed to mercury

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Abstract

Molluscs bivalves have been widely used as bioindicators to monitor contamination levels in coastal waters. In addition, many studies have attempted to analyze bivalve organs, considered pollutant-targets, to understand the bio-accumulation process and to characterize the effects of pollutants on the organisms. Here we analyzed the effects of mercury exposure on flat oyster hemocytes. Optical and electronic microscope procedures were used to characterize hemocyte morphology. In addition, cell solutions treated with acridine orange were analyzed by flow cytometry and laser scanning cytometry in order to evaluate the variations of cytoplasmic granules (red fluorescence, ARF) and cell size (green fluorescence, AGF) of hemocyte populations over time. Light and electron microscopical studies enabled us to differentiate four hemocyte subpopulations, agranulocytes (Types I and II) and granulocytes (Types I and II). Slight morphological differences were observed between control and Hg-exposed cells only in granulocytes exposed to Hg for 30 days, where condensed chromatin and partially lysed cytoplasmic regions were detected. Flow and laser scanning cytometry studies allowed us to differentiate three hemocyte populations, agranulocytes (R1) and granulocytes (R2 and R3). The exposure time to Hg increased the average red fluorescence (ARF) of agranulocytes and small granulocytes, while there was no change in large granulocytes, which showed a loss of membrane integrity. In control oysters, the three hemocyte populations showed an increase of ARF after 19 days of exposure although initial values were restored after 30 days. The average green fluorescence (AGF) was more stable than the ARF throughout the experiment. In Hg-exposed oysters, the values of AGF of agranulocytes showed an increase at half Hg-exposure period while the AGF values of large granulocytes decreased throughout the experiment, confirming the instability of these types of cells. The relative percentage of small granulocytes and granulocytes showed time variations in both control and exposed oysters. However, the values of small granulocytes remained constant during the whole experiment. The fact that there were only changes in agranulocytes and large granulocytes suggested a possible relationship between these two types of cells. In a quantitative study, we found a significant linear relationship between the agranulocytes and large granulocytes.

Introduction

The ability of bivalve molluscs to accumulate the concentration of a pollutant agent relative to its level in the aquatic environment, is well known. Thus, xenobiotics may alter functional parameters such as viability, phagocytosis, and lysosomal membrane stability (Matozzo *et al.* 2001).

Oysters, like other bivalves molluscs have an open circulatory system, with hemolymph

osmolarity and temperature may fluctuate with the environment. The hemolymph cell population, hemocytes, play important roles in several functions, such as digestion, excretion and defence (Cheng 1988). Therefore, they are vulnerable to environmental and pathogenic changes, and phagocytic and microbial activities depend on their structural and functional integrity. Hemocytes in molluscs have been classified according to morphological criteria i.e. the size and shape of cells and the presence of cytoplasmic granules. However, different techniques for preparing hemolymph samples lead the great variability on the description of the hemolymph cells, (Friedl *et al*. 1988). Numbers and sizes of the different hemocyte types may be due to their individual metabolism or to exposure to polycylic aromatic hydrocarbons and heavy metals, (Sammi *et al*. 1992).

The new technology used to study blood vertebrate cells, such as flow cytometry which allowed us to analyze a large number of cells, has meant that hemocytes have become a good model to study the biological effects of pollutants. The subpopulations of *Crassostrea gigas* hemocytes, which were studied by cell sorting and microscopy, correlated well with those identified by flow cytometry (Ashton-Alcox & Ford 1998).

It is known that the hemocytes of bivalve molluscs can accumulate high levels of metals, mainly in lysosomes (Moore & Gelder 1985). A reduction in lysosome membrane stability has been reported in mussels and oysters exposed to heavy metals and has been proposed as an indicator of cell damage (Ringwood *et al*. 2002).

The present study used microscopy to compare morphological hemocyte features from oysters exposed to $HgCl_2$ and flow cytometry to identify hemocyte sub-populations. This study was carried out to evaluate the effects of exposure to Hg on changes in relative numbers of hemocyte types and variations on the endosomal-lysosomal compartment of hemocytes in exposed oysters.

Material and methods

Experimental conditions

Individual specimens of *Ostrea edulis* were obtained from a shellfish farm at Cambados (Pontevedra, Spain) on the Spanish Atlantic coast, and transferred to our laboratory, where they were placed in glass aquaria in groups of 20 individuals with a population density of one individual per litre, which was maintained during the whole experimental period. All animals were 2-years-old and were homogeneous in size (6.6×6.2 cm). They were maintained at 20 °C, at a salinity of 37.2‰ and were fed daily with a compound specific for marine invertebrates (Liquizell, Dohse Aquaristik, Bonn, Germany). After the first week, considered as an acclimatization period, the Hg exposure was started. Daily, a mercury solution was added to the aquaria to obtain a final concentration of 5μg Hg/l. During the 30 day exposure period, the oysters, both controls and exposed, were randomly selected at 7, 19 and 30 days. The selected oysters were weighed and processed as follows.

Hemocyte extraction

Five control oysters and seven Hg-exposed were individually analyzed at each time, (two oysters control and two exposed were used for the examination of hemocytes, by light and electron microscopy, while the hemolymph of the three controls and five Hg-exposed were analyzed using a flow cytometer). 0.5–1 ml of hemolymph was withdrawn, immediately before use, from the adductor muscle with a needle (0.6×25 mm) coupled to a disposable syringe which contained 50 μl of antiaggregant solution (Tris–HCl 0.5 M; 2% glucose, 2% ClNa, 0.5% EDTA, pH 7.3) and the contents were gently mixed by rotation (Friedl *et al*. 1988). After the hemolymph was collected, the solutions were processed by different methods, as discussed later.

Analysis of Hg

The Hg analysis was made on the same oysters used to obtain the hemolymph. After the extraction, the whole tissue of the oysters was frozen and stored at −20 °C until Hg measurement. The Hg amount in the soft tissues was assessed by cold vapor atomic absorption spectroscopy, using an absorption spectrometer, Phillips PV90 200 with a deuterium lamp for background correction (Bigas *et al*. 1997, 2001).

Light and electron microscopy

The light microscopy study was carried out on hemolymph and isolated hemocytes. After the

needle was removed from the syringe barrel, a drop of hemolymph solution (30 μl approximately) was placed on a pre-cleaned round glass coverslip (10 mm in diameter). Simultaneously we made two smear preparations per sample, both coverslips and glass slides were kept in a moist chamber for 30 min to allow the cells to settle. Two replicates were made for each sample. The remaining hemolymph solution was transferred to an Eppendorf tube and centrifuged at 300 g for 5 min. The supernatant was discarded and the cells re-suspended in 500 μl of the antiaggregant solution. The hemolymph solutions were prepared in the same way as the hemocyte solutions. Both, hemolymph and hemocytes samples were processed in the same way. One of replicates was stained with Hematoxylin–Eosin. The samples were studied through a Reichert Polyvar-2 light microscope.

The electron microscopy study of hemolymph cells was made with the same samples used for the light microscopy study. The hemolymph of two oyster controls and two Hg-exposed were withdrawn at every sample time, as described previously. After the first centrifugation, the cells were re-suspended with 100 μl of the fixative solution, (0.5% glutaraldehyde-3% paraformaldehyde buffered with the same antiaggregant solution). After a second centrifugation (300 g during 5 min) small drops of cells were embedded carefully into a thin Agar layer (2% with distilled water) disposed over a plastic bag. The agar fragments containing the cells were processed in the normal way to obtain resin blocs. They were fixed for 2 h at 4 °C by immersion in 1% glutaraldehyde in 0.1 M buffer Phosphate (pH 7.4). The osmolarity of the fixative solution was corrected to 1100 mOsm using NaCl. After fixation the samples were rinsed with the same buffer and post-fixed for 2 h at 4 °C in 1% buffered osmium tetraoxide. The samples were then dehydrated in a graded series of acetone and embedded in Spurr's resin. Semi-thin sections, 1μm, were mounted on glass slides, stained with toluidine blue and examined by light microscopy. Ultra-thin sections, 70 nm, were cut with a diamond knife on an ultra-microtome, collected on copper grids, double-stained with uranyl acetate and lead citrate (Reynolds 1963) and examined under an HITACHI-600 transmission electron microscope. In addition, fragments of gill excised from control and exposed oysters were fixed for 2 h at 4 °C by immersion in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The fixative solution osmolarity was corrected to 1100 mOsm using NaCl. After the first fixation, the samples were rinsed in the same buffer and post-fixed for 2 h at 4 °C in buffered osmium tetraoxide. The samples were then dehydrated in a graded series of acetone and then embedded in Spurr's resin. Ultra-thin sections, 70 nm, were collected on copper grids, doubled stained with uranyl acetate and lead citrate (Reynolds 1963) and examined under a HITACHI-600 transmission electron microscope. Semi-thin sections, 1μm, were stained with methylene blue and examined, for the morphological study, under a REICHERT POLYVAR-2 light microscope.

Electron probe X-ray microanalysis

Microanalysis study of hemocytes for metal detection was carried out on the gill samples from the same individuals processed and studied by TEM. Microanalysis by electron probe X-ray microanalysis (EPXMA) is a technique based on the effect of the impact of the incident electronic beam on samples sections, and orbital electrons from the atoms of the sample make transitions between different shells. This transition produces X-ray signal characteristics for each element and we can obtain a spectrum of X-ray energies. Orbitals are indicated by the letters K, L and M (from the internal to the more external layers) and the energy emitted by the transition depends of the element affected by possible changes of electron position, giving as a result, a set of characteristic signals for each element, which can be examined in the X-ray spectrum.

Semi-thin sections, 0.2 μm, of gill tissue were collected on titanium grids, covered by Formvar film and recovered by a carbon film to stabilize them and, at the same time, increase their conductivity. The elemental analysis was carried out, under an acceleration voltage of 100 kV, using a scanning transmission mode electron microscope (STEM) HITACHI 800 Mt. The X-ray emission was analyzed using a Kevex detector (Keves, California) with a window (10 mm^2) connected to a Kevex 800 analytical system with Quantex 6.13 software. The gain rate was adjusted to 1000–1500 counts seg^{-1} and an acquisition time of 100 s was used.

Flow cytometry

Three control oysters and five Hg-exposed were examined at each sample time. The hemolymph collection was made as described previously. After the first centrifugation and re-suspending the cells with the antiaggregant solution, one aliquot of each sample was used to count the cell concentration using a Neubauer chamber. Next, we made the necessary dilutions, with the same antiaggregant solution, in order to have the same number of cells in each sample. In order to study the cellular viability, the technique of Propidium Iodide (PI) exclusion was used. 0.5 μl of a 1 mg ml^{-1} stock solution of PI was added to 200 μl of cell suspension and flow cytometry of these suspensions was performed.

A volume of the metachromatic fluorescent probe acridine orange solution (AO, 2 μg/ml) was added to each hemocyte solution to obtain a final concentration of 1μg/l. This probe accumulates in the cytoplasmic granules or lysosomes of live cells as human granulocytes, where it acquires a red fluorescence, while the probe that remains in the cytoplasm or inside the nuclei joined to RNA or DNA shows a green fluorescence. Both, red and green fluorescence are detectable by flow cytometry (Darzynkiewick *et al*. 1976; Darzynkiewick & Kapucinski 1990; Melamed *et al*. 1974). The mixture was allowed to stand at room temperature before flow cytometry analysis. Hemocyte solution was analyzed using a COULTER EPICS XL cytometer. The argon laser was set at 488 nm and run at 15 mW. Calibration of the instrument was determined using Coulter Immunocheck beads. The distribution and the heterogeneity of the hemocyte populations were determined by their forward (FS) and right angle scatter log (Sslog) properties. The data were initially analyzed for two parameters: cell size (FS) and cell complexity or granularity (SSC). The data were collected and displayed on two-parameter histograms (FLS×SSlog). Electronic windows were made arbitrary around the three putative subpopulations that were present in all the samples analyzed. In addition, the green (525 nm) and red (675 nm) fluorescence emitted by the hemocytes of each subpopulation were recorded and displayed on mono-parameter histograms. Data was collected for the first 10,000 particles of each sample, stored as "list mode file" and then analyzed with a WinMidi program, 2.8, (Joseph Trotter Scrips. Research Institute).

After flow cytometer measurements, the samples were centrifuged to increase the cell concentration and resuspended in 25μl of antiaggregant solution, then 10μl of each new sample were observed by a Fluorescence Microscope Reichert Polyvar-2 and analyzed by Laser Scanning Cytometer, LSC (Compocyte). The use of LSC allowed discrimination of subpopulations on the basis of their FS values and the fluorescence emissions and, simultaneously, see the cells. Comparing these results with the flow cytometry analysis we were able to study the types of cells of each subpopulation.

Results

Analysis of Hg

Hg concentrations in the whole soft tissues of oysters exposed to Hg for 30 days increased at the same rate as was detected in previous studies (Bigas *et al*. 1997). From the 19th day, the means of Hg concentration increased very quickly, reaching 28μg Hg/g dry weight at the end of the experiment (Figure 1).

Morphological study

Gill filaments showed that branchial vessels at their basal region had not changed significantly during the experimental period. In this tissue,

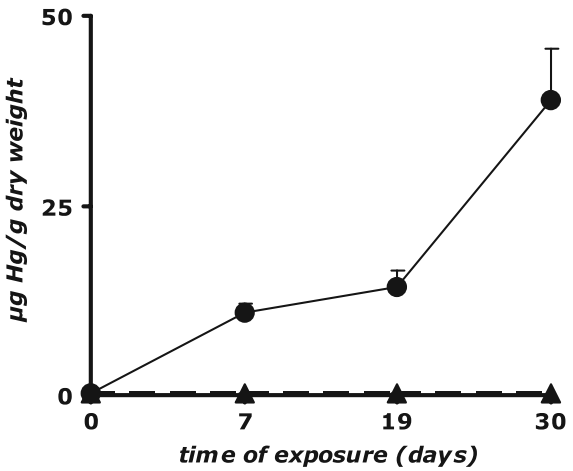

Figure 1. Results of the analysis of mercury concentrations in the soft tissues of oyster, *Ostrea edulis* exposed to5μg Hg l^{-1}, during 30 days of exposure.(●) exposed oysters, (▲) unexposed control oysters. Mean + SD; *n* control and *n* Hg = 5.

hemocytes were concentrated inside the vessel, located near the basal region of gill epithelium. The only significant difference between control and Hg-exposed oysters was that the number of hemocytes inside the branchial vessels was higher in Hg-exposed individuals (Figure 2).

As has been described in previous studies by different authors, according to morphological characteristics such as the size, the N/C ratio and cytoplasmic granules, two well differentiated hemocyte populations were detected: agranulocytes (hyalinocytes) and granulocytes (Figure 3).

In agranulocytes, the most abundant hemocytes, we could distinguish two different subpopulations. Agranulocytes type I (Figure 3B) were the smaller cells (5–7 μm), with high N/C ratio and mitochondria located around the central nucleus and agranulocytes type II (Figure 3D)(12μm) showed an eccentric and smaller nucleus. The contour of cells showed a few short and slender protrusions.

The common characteristic of the different subpopulations from granulocytes is the low N/C ratio, showing a variability of size between 11 and 14 μm and an eccentric nucleus with peripheral heterochromatin. Two kinds of subpopulations of granulocytes were detected in the electron microscope study, according to the morphology and the size of heterogeneous granules. In the type I subpopulation, granules (1–1.5 μm) have a low electron-dense core surrounded by an electron-dense material (Figure 3F,G,I). Some cisternae of rough endoplasmic reticulum and a scarcely developed Golgi complex were present in their cytoplasm. In the subpopulation of granulocytes type II, granules were scarcer and smaller with an heterogeneous and high electron-dense material inside (Figure 3H). Both kinds of granulocytes

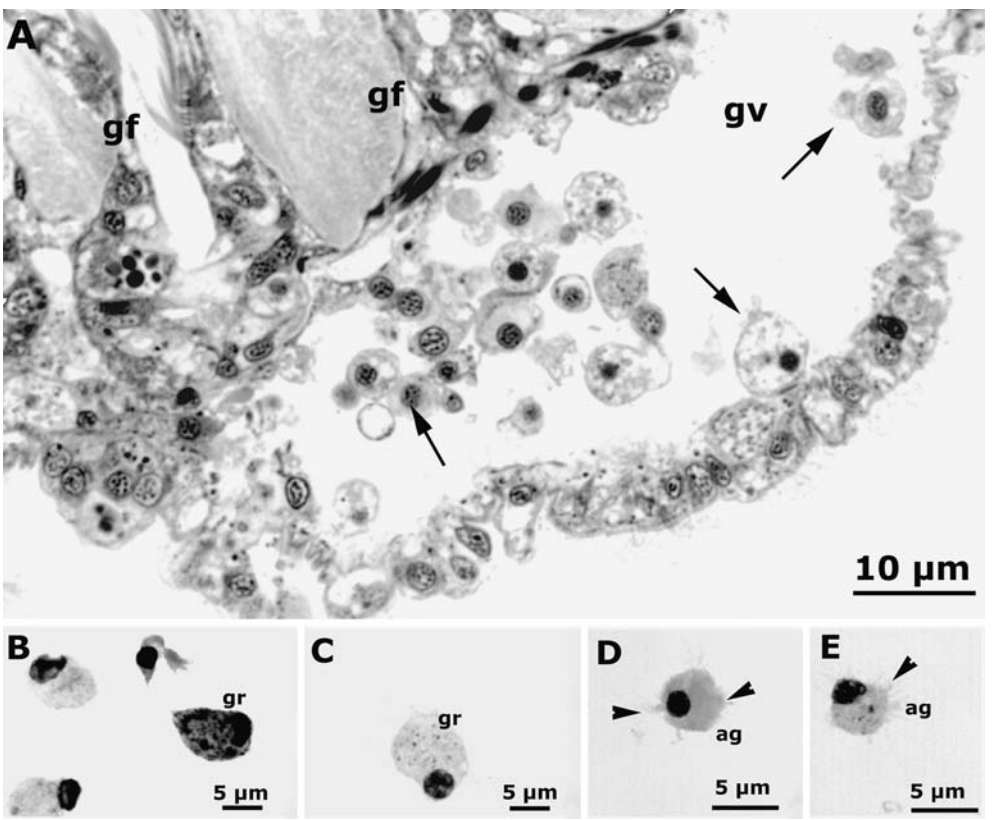

Figure 2. Light microscopy images of semi-thin section of the gill tissue from a Hg exposed oyster. (A) Different kind of hemocytes (arrows) are contained inside of the gill vessel (gv), which is located under gill filaments (gf). (B–E) Hemolymph smear from a control oyster. (B, C) granulocytes (gr) with different morphology of cytoplasmic granules (D, E) Agranulocytes (ag) with thin cytoplasmic protrusions (arrow heads).

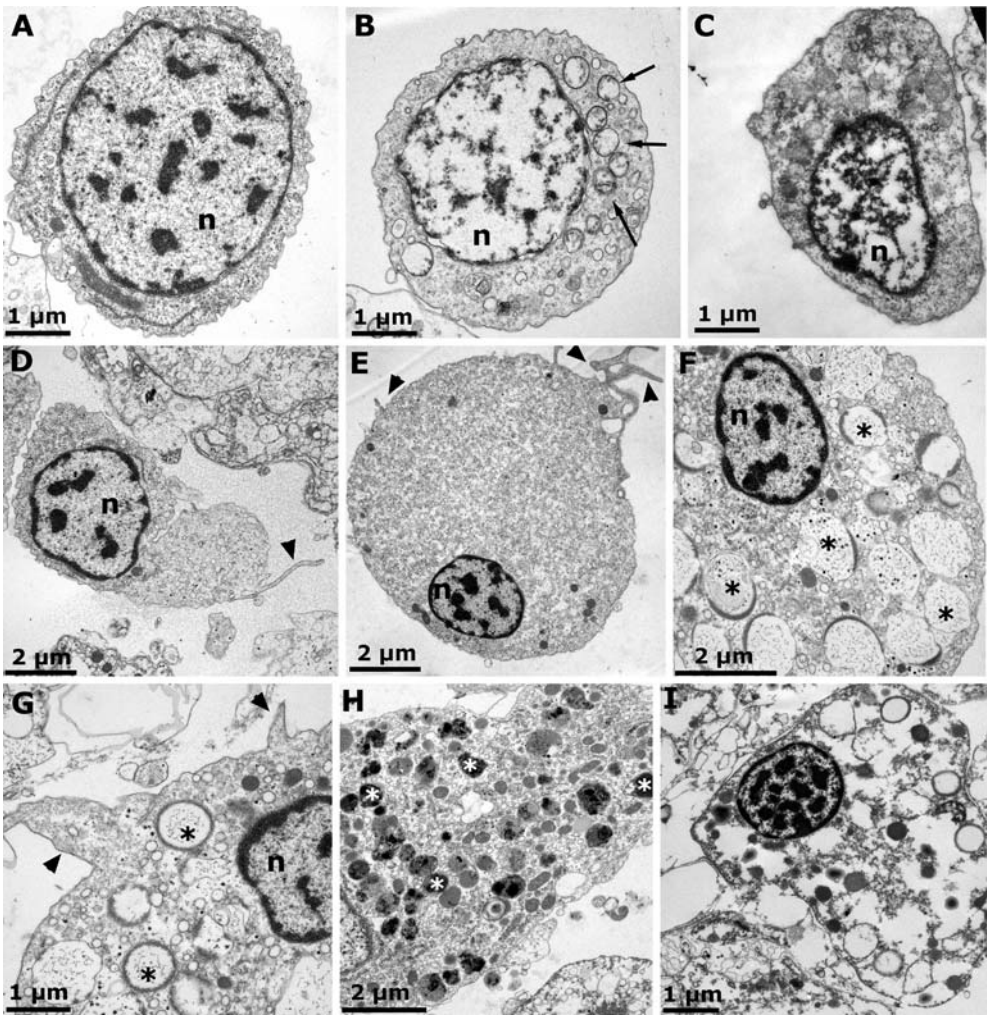

Figure 3. Transmission electron microscopy images of agranulocytes (A–E) and granulocytes (F–I). Different N/C ratios can be observed in agranulocytes. Some mitochondria are surrounding the nucleus (B, arrows). Thin cytoplasmic protrusions can be observed in D and E (arrow heads). Granulocytes show a low N/C ratio and different morphology of cytoplasmic granules (*) (F–H). Occasionaly granule content can be lysed (I). Cytoplasmic protrusions like pseudopodia are detected in granulocytes (G, arrow heads).

showed a rugged cell surface, with cytoplasmic protrusions, like pseudopodia.

There were very slight morphological differences between control hemocytes and the Hg exposed. Only in granulocytes from exposed oysters throughout 30 days were there, high condensed chromatin and partially lysed cytoplasmic regions, along with empty cytoplasmic granules, observed (Figure 3I).

Microanalytical study

By EPXMA study of hemocytes from gills, we observed electron-dense precipitates in cytoplasmic granules of granulocytes concentrated inside the branchial vessel. Among the hemocytes from Hg-exposed oysters, granulocytes showed greater electron-dense precipitates inside their lysosomes (Figure 4). The energy spectrum obtained from a spot analysis in electrodense precipitates inside lysosomes showed the presence of S, Ag, and Fe in hemocytes from both Hg-exposed and control. Also, we detected in granulocytes from control and exposed oysters, the presence of an Ag peak, with the different sublines $L\alpha, \beta$ very close, and a higher peak in cells from exposed oysters. Only lysosomic precipitates of granulocytes of Hg-exposed oysters showed a peak equivalent to $L\alpha_1$

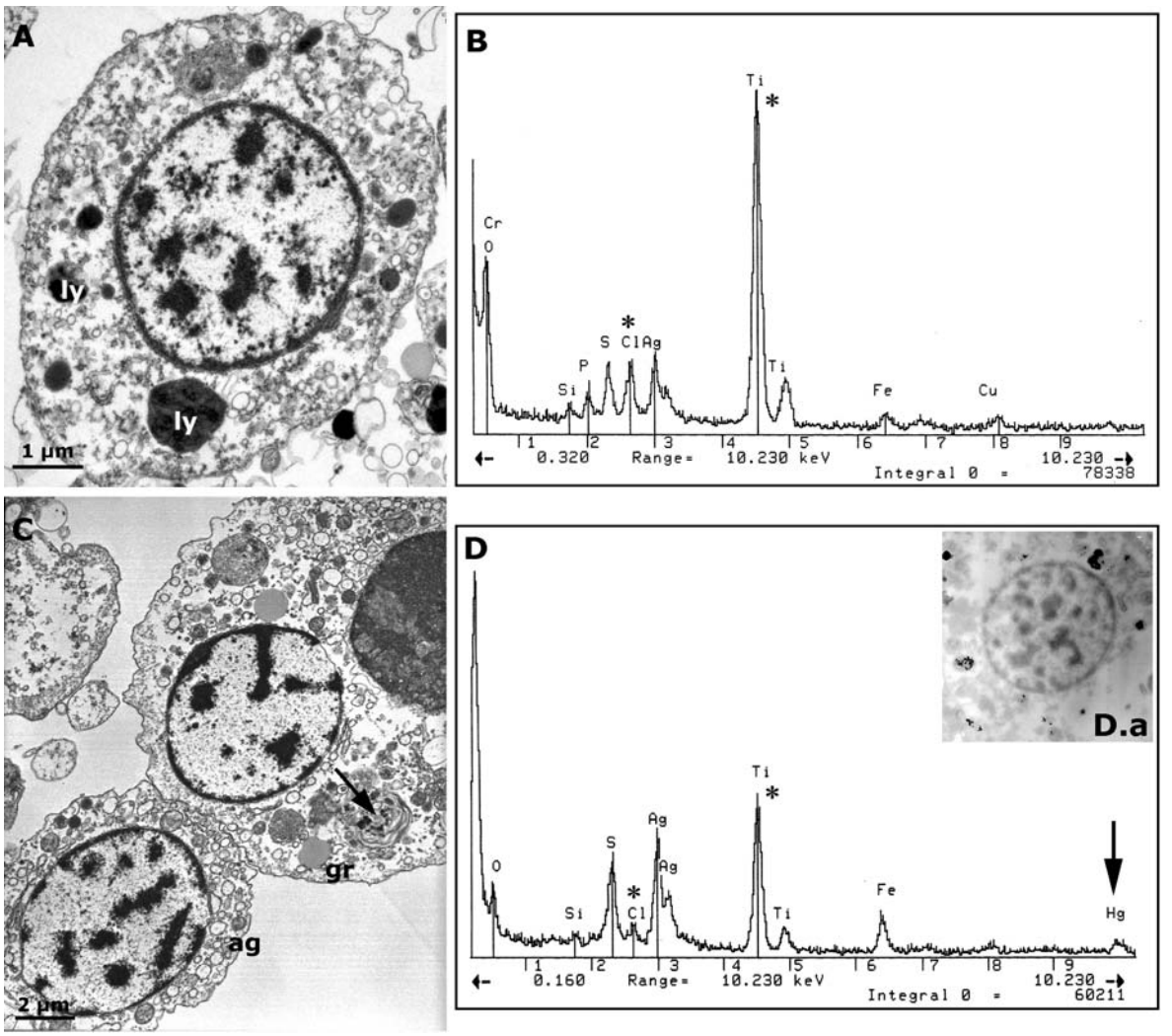

Figure 4. (A) TEM image of a granulocyte from gills of a control oyster. ly: lysosomes with electrodense precipitates inside. (B) X-ray energy spectrum of a single electrodense precipitate in a lysosome of a granulocyte. (C) TEM image of two hemocytes from an exposed oyster (ag: agranulocyte gr: granulocyte;) (D.a) Non contrasted semi-thin section (0.2 μm thick) of a granulocyte of gills of an exposed oyster. (D) X-ray energy spectrum of a cytoplasmic granule in the hemocyte shown. Ti and Cl signals (*) in both spectra are derived from the grid material and the inclusion resin used, respectively.

line Hg (9987 KeV). The increase of S peak in these cells could have covered the Mα line of Hg (2195 KeV), masking this element. In other analytical studies of epithelial cells from gills and digestive gland, no Hg peak was detected.

Effects of exposure on hemocyte populations

In the flow cytometry study we previously analyzed the hemolymph of 10 oysters by using the Acridine Orange probe individually, in order to evaluate the common patterns of oyster hemocytes. The relationship between the granularity (parameter related to Sslog value) and size of cells (parameter related to FS value) allowed us to establish three electronic windows, where most of the cells were located, and which characterized the three different cell populations (R1, R2 and R3) (Figure 5). The three regions were used to evaluate all samples studied in the present study and only three samples, one control and two exposed to Hg, presented with a different two-parametric scattergram.

Comparing the results of FS values and red and green fluorescence obtained with the flow cytometer

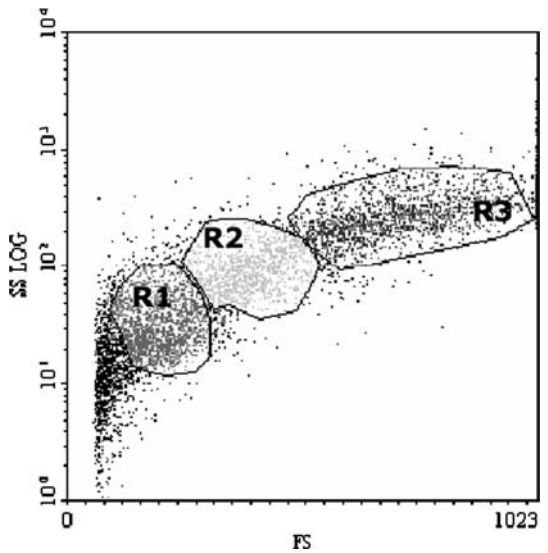

Figure 5. Dot-plot scattergram of hemocyte suspension analyzed by flow cytometry, at the beginning of the experimental time (0 days of exposure). Three sub-populations of hemocytes (R1 agranulocytes, R2 and R3 granulocytes) are outlined in the two parameter plots: forward light scatter (FS) and side scatter logarithmic (SSlog).

and the results obtained with the Laser Scanning cytometer, enabled us to relate different cellular morphology to each of regions detected by flow cytometry (Figure 6). Hemocytes with the lowest FS and SSlog values corresponded, mainly to agranulocytes (R1), which were round, small and only showed green fluorescence which meant that they did not have cytoplasmic granules. The two regions, R2 and R3, consisted of granulocytes that differed from each other by the number of cytoplasmic granules. The cells with the highest FS and SSlog values and also with the highest red fluorescence (R3) corresponded to granulocytes with a significant number of cytoplasmic granules, large granulocytes, which gave an intense yellow-red fluorescence, while the region with intermediate values (R2) consisted of granulocytes with a smaller number of granules, small granulocytes.

We evaluated the variations of red and green fluorescence, for each region, during the time of the experiment. The red fluorescence could be considered as an expression of total granules volume and as a parameter of membrane integrity. In turn, the green fluorescence could be considered as an indicator of cellular volume. The red fluorescence of agranulocytes was significantly lower than the red fluorescence observed in the granulocytes, both the small granulocytes, R2, and especially, the granulocytes of R3 (Figure 7). These results suggested that agranulocytes were mainly present in this region, while the granulocytes, where the acridine orange molecules were accumulated into their granules, were distributed between R2 and R3.

In the analysis of variations of average red fluorescence (ARF) and the average green fluorescence (AGF) during the time of the experiment (Figure 7), we detected that both, Hg-exposure and exposure-time had a significant effect over the hemocytes included in R1 and R2 ($P < 0.01$, both factors). On the other hand, the ARF of granulocytes included in R3 increase throughout the time in control oysters, but there was no differences in hemocytes of exposed samples. The agranulocytes of exposed oysters showed higher ARF values than agranulocytes of control oysters from the 19th day of exposure. The ARF of granulocytes R2 of exposed oysters showed an increase throughout the time, while in the R3 granulocytes of exposed oysters the ARF values were the same throughout the experiment. However, when we analyzed the cell samples of oysters exposed to Hg during the 30 days by LSC, the majority of large hemocytes quickly lost the red fluorescence and the red or yellow-granules changed to black (Figure 6). This could suggest a loss of membrane integrity and also could explain why the average red fluorescence of large granulocytes from exposed oysters did not increase throughout the experiment, as with the other hemocyte types. In the control oysters there was the same pattern in each region. There was an increase of ARF after 19 days of exposure, but in the last sample time it returned to the initial value.

The green fluorescence values showed greater stability than the red fluorescence throughout the exposure time. In the exposed oysters the green fluorescence values of agranulocytes showed a significant increase after 19 days of exposure. These results coincided with the red fluorescence results. The cells belonging to R2 showed constant values throughout the exposure time in both, control and exposed oysters, as did the cells of R3 of the control oysters, while the average of the green fluorescence of cells-R3 of exposed oysters decreased throughout the time, suggesting a possible destabilising of this type of cell.

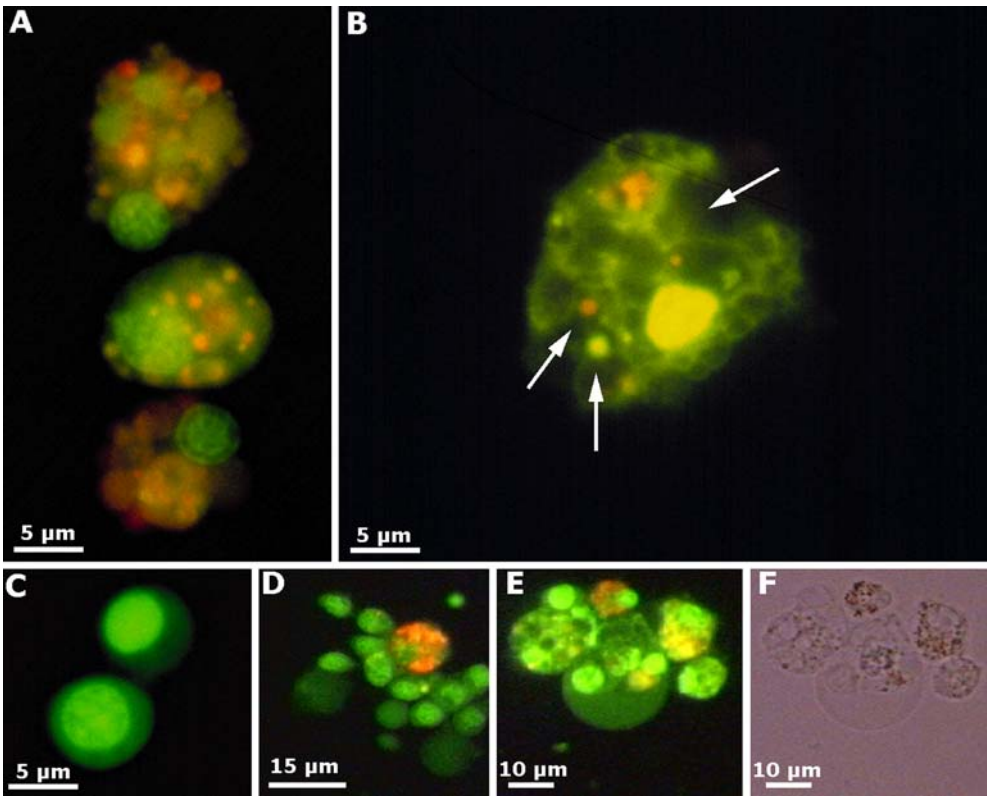

Figure 6. (A–C) Fluorescence microscopy images of hemocytes incubated with acridine orange probe. (A) granulocytes of a control oyster. Eccentric nuclei show a green fluorescence and granules corresponding to lysosomes show red fluorescence. (B) Granulocyte from an oyster exposed to Hg over 30 days. Changes in cell integrity and variations in the fluorescence of granules can be observed (arrows). (C) Agranulocytes from an exposed oyster. Green fluorescence can be observed in large nuclei and scarce cytoplasm. (D–F) Images obtained by the Laser Scanning Cytometer (LSC) of hemocyte samples analyzed by flow cytometry (D) hemocytes from an exposed oyster (7 days to Hg): a granulocyte with fluorescent granules is surrounded by some agranulocytes. (E) granulocytes of an exposed oyster (30 days). Their granules do not show the red fluorescence because of alterations in their membranes. (F) light field image of the same cells in E.

Effects of exposure on cell number populations

Parallel to population characterization, we were interested to determine if exposure time and/or mercury had an effect on the total number of hemocytes and on the relative percentage of cells in each population. These analyses were made with the same samples used to study the population characterization and using the same fluorescent probe (AO).

Prior to flow cytometer analysis, an aliquot of cell solution was counted using a Neubauer chamber. Four counts per sample were made. The results (Table 1), showed that there were no differences in the numbers of cells at any of the sample times between control and exposed oysters.

To study the effects of mercury and exposure time on the cell number in each population, we used the statistical data obtained from the flow cytometer. Analysing the three regions on dot-plot scattergrams, we normalized the data so that the cells included in the three regions were 100%. (In all the samples analyzed more than 76% of cells were included in one of the three regions, the rest could be considered as cell fragments). In all the samples analyzed the agranulocytes were the main type of cell, which confirmed the optical micros-

Table 1. Hemocyte concentration. Cell number/ml (average, DS).

t (days)	Control oysters	Hg exposed oysters
7	$1.10\ 10^6$ ($3.1\ 10^5$)	$1.30\ 10^6$ ($6.8\ 10^5$)
19	$1.20\ 10^6$ ($3.9\ 10^5$)	$1.30\ 10^6$ ($4{,}1\ 10^5$)
30	$1.06\ 10^6$ ($6.2\ 10^5$)	$1.41\ 10^6$ ($4{,}7\ 10^5$)

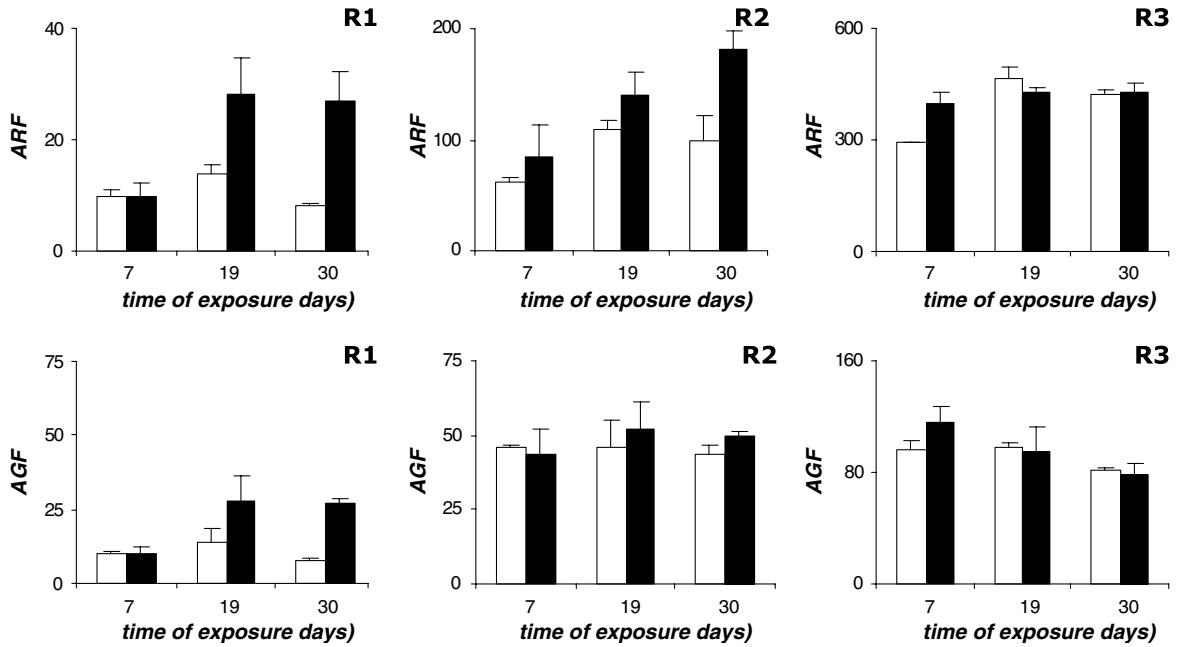

Figure 7. Red (*ARF*) and green (*AGF*) average fluorescence variations throughout the experimental period of exposure in each of the hemocyte sub-populations studied: R1 agranulocytes, R2 and R3 granulocytes.) (□) Control oyster hemocytes; (■) Exposed oysters hemocytes (Mean ± SD; *n* control = 3, *n* Hg = 3).

copy observations. The granulocytes of R2 and R3 showed similar percentages.

We found that the relative percentage of hemocytes of R1, agranulocytes, and R3, granulocytes, showed time variations on both control and exposed oysters, while the percentage of granulocytes of R2 remained constant throughout the time (Figure 8). On the other hand, the variations observed on agranulocytes showed the opposite pattern to the variations observed on large granulocytes, R3, and also there were differences when comparing controls with exposed oysters.

The fact that the the differences only occurred in cells belonging to R1 and R3 of the controls and exposed oysters could indicate that a relationship exists between the cells of these two sub-populations. In order to confirm this relationship, we compared the cell percentages of the different regions, without taking into account the time factor (Table 2). Regression analysis showed a significant linear relationship between the cells of R1 and R3, while there was none between R1–R2 and R2–R3.

Discussion

General morphology features of *Ostrea edulis* hemocytes show definable characteristics under

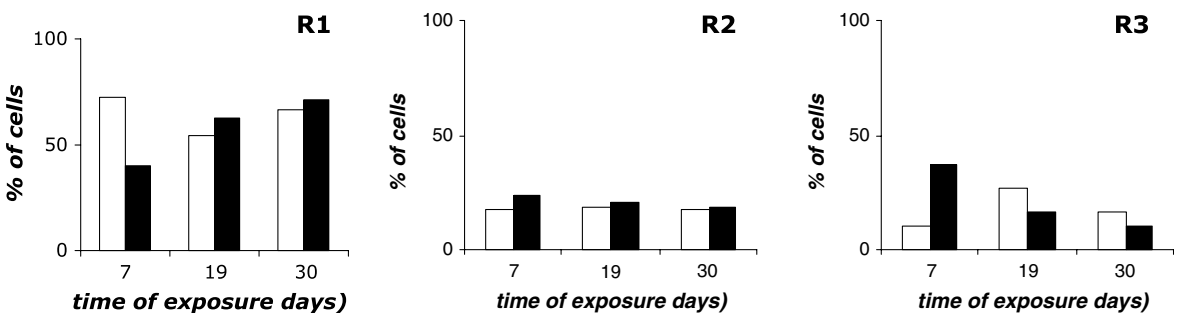

Figure 8. Variations in relative percentages of hemocytes from each sub-population throughout the experimental period. (□) Control oyster hemocytes; (■) Exposed oysters hemocytes.

Table 2. Regression analysis between the cell percentages of each two regions.

	Control (n = 8)		Hg (n = 14)	
	r	p	r	p
R1-R2	−0.27	0.420	−0.57	0.067
R1-R3	−0.99	0.000	−0.98	0.000
R2-R3	0.13	0.708	0.38	0.239

both light and electron microscopy. In this study, we could identify two main types of hemocytes: agranulocytes, which were in the majority in all the analyzed samples and two populations of granulocytes (large and small granulocytes) which were easily identified, as they showed granules inside the cytoplasm.

The electron microscopy study also revealed the presence of two principal types of hemocytes and allowed us to distinguish the different subtypes (I and II), although we were not able to establish a precise correspondence between the light microscopy results and those of the electronic microscopy. These results could indicate that both the Hg and the exposure time did not alter hemocyte morphology. In addition, no morphological differences were detected between isolated hemocytes and those cells from the hemolymph samples.

The results obtained in our study agreed with previous studies on the characterization of oyster hemocytes (Renwrantz *et al*. 1979; Cheng 1981, 1984; Pirie *et al*. 1984; Rasmunsen *et al*. 1985; McCormick-Ray *et al*. 1991).

Auffret (1989), described, at ultrastructural level, two types of granulocytes in *Crassostrea gigas*, which corresponded morphologically and in granule characteristics with the granulocytes observed in the present study, although he was only able to find one kind of granulocyte in *O*. *edulis*.

There are some factors which make it difficult to obtain a general bivalve hemocyte classification: the species variability and the different technical procedures for the hemocyte collection and their study and characterization. The only common point is the presence of two easily distinguishable types of hemocytes, granulocytes and agranulocytes (Cheng 1981; Hine 1999), described in most bivalve species: *Mya arenaria* (Hoffman & Tripp 1982), *Mytilus edulis* (Pipe 1990), *Mytilus galloprovincialis* (Cajaraville & Pal 1995; Caraball *et al*. 1997a, b); *Mercenaria mercenaria* (Tripp 1992); *Crassostrea virginica* (Ford *et al*. 1994); *Ruditapes decussatus* (López *et al*. 1997); *Tapes philippinarum* (Cima *et al*. 2000). The lack of information on hemocyte differentiation is another factor which makes it difficult to classify the bivalve hemocyte. It is not clear if the different subpopulations of hemocytes identified in bivalves are different cellular lines or are different stages of the same cell type. There were some attempts to establish the ontogeny of hemocytes (Mix 1976; Balouet & Poder 1979; Cheng 1981; Auffret 1988–1989), but it still remains to be resolved.

The variability in the different populations of mollusk hemocytes has generally been attributed to individual metabolic conditions and environmental stress factors or pathogenic conditions. Thus many studies have confirmed their role as biomarkers. It has been demonstrated that they are sensitive to organic and inorganic environmental pollutants and pathological conditions. The presence of pollutants could modify some hemocyte functional activities related to their defensive role, such as phagocyte activity (Cheng *et al*. 1988; McCormik-Ray & Howard 1991; Lowe *et al*. 1995). On the other hand, the activity of different lysosomal enzymes has been demonstrated both in agranulocytes and granulocytes and pollutants could modify the enzyme activities. Positive reactions to histochemical markers showed the development of lysosomes in hemocytes of different species of mollusks (Hoffman & Tripp 1982; More & Gelder 1985; Pipe 1990; Cajaraville & Pal 1995; López *et al*. 1997). Morphological results in our TEM study and the positive reaction to markers (data not shown) of characteristic lysosomal enzymes found in granulocytes allowed us to conclude that the granules belong to the lysosomal system of granulocytes.

Using microanalysis technique by TEM, we could determine the specific role of granulocytes. EPXMA has been proven to be a useful tool in detecting metals inside membranous compartments of cells. In the studied oysters, hemocytes, like other cell types of different tissues showed Fe and Cu inside the lysosomes. In granulocytes from Hg-exposed oysters we detected a higher accumulation of S and Ag. The presence of Ag only had been detected in electron-dense precipitates of the basal lamina of digestive epithelia (results not shown).

The detection of a higher content of metals (Fe, Ag) and Higher S peaks in the spectrum of electrodense precipitates, which were detected in lysosomes from Hg-exposed oysters, could be related to an increase of cysteine residues, due to the internalization of metallothioneins in the lysosomal compartment, according to Chassard Bouchaud *et al*. (1992), who described the mechanism of metals accumulation inside lysosomes as a final destination of metals previously bound to cytosolic proteins as a detoxification mechanism.

Also EPXMA has helped to define the specific role of hemolymph cells of bivalves on the transport of the essential metals, Cu and Zn. Hemocytes of bivalve mollusks, moving around body tissues in hemolymph, can keep the concentration of metals in hemolymph below toxic levels, by removing metals from it (Thompson *et al*. 1985). This mechanism of detoxification completes the role performed by the excretory organs (Ballan-Dufrançais *et al*. 1985).

The increase in metal accumulation and mercury detected in granulocytes of Hg-exposed oyster in the present study, could be due to the wide distribution of these cells in the whole organism and to their phagocytic function during the experiment, which could increase the internalization ability of metals in these cells. Effects on the hemocyte defensive role related to the presence of pollutants have been reported (Grundy *et al*. 1996; Auffret & Oubella 1997; Brousseau *et al*. 2000)). A significant reduction of phagocytic activity was noted in bivalves samples of *Mya arenaria* exposed to high concentrations of metals and prolonged exposures, whereas short-term exposure to low concentrations of toxic substances stimulated phagocytic activity (Fournier *et al*. 2002).

The flow cytometry analysis of hemocytes stained with acridine orange, and the separate analysis of hemocyte samples confirmed the identity of three hemocyte populations. Moreover, we were able to analyze the variations which occurred in each one of them over the experimental period.

We analyzed the oyster hemocytes without using processes that could injure the membrane system of the cells. This guarantee that the red fluorescence observed was due to the acridine orange trapped inside granules, while the green fluorescence shown by nuclei was the consequence of acridine orange monomers linked to DNA (Traganos & Darzynkievicz 1994).

Otherwise, the analysis of the same samples by the flow cytometer and the Laser Scanning Cytometer, and the comparison of *forward scatter* and average values of both green and red fluorescence, allowed us to associate one cell model to each one of regions determined by the flow cytometer. We proposed that region 1 (R1) included the agranulocytes and that the granulocytes were located in regions 2 and 3. These results agreed with hemocyte subpopulations detected in *O. edulis* by flow cytometric analysis, using a monoclonal antibody specific for granulocytes (Renault *et al*. 2001). Nevertheless, in the study by flow cytometry of pooled hemocytes obtained from fifteen specimens of *Mya arenaria* (Brousseau *et al*. 2000; Fournier *et al*. 2001), only two populations were identified on the dot-plot histograms, which were opposited of the position shown in the present study. Our results partially agreed with the results obtained by Ashton-Alcox and Ford (1994), who analyzed, by flow cytometer, the *C. gigas* hemocytes. These authors confirmed the existence of three hemocyte populations: large granulocytes, small granulocytes and a heterogeneous population which included agranulocytes and granulocytes with a reduced number of cytoplasmic granules. The position of each population on dot-plot scattergrams was the same as those found in the present study, thus we could associate this heterogenous population to the R3 region showed in *O. edulis*. The results of these studies revealed the existence of variations between species and also individual variations in the cell population composition in a same species, depending on the experimental treatments and the use of different technical procedures (Hégaret *et al*. 2003).

In our study, the analysis of red fluorescence variations of each hemocyte population in oysters exposed to Hg, showed that both time and mercury exposure have effects on granule volume and membrane stability on all hemocyte types. The results found in the large granulocytes and some altered agranulocytes (R3) of Hg-exposed oysters suggested that the mercury could be responsible for lysosomes membrane destabilization, which resulted in the loss of fluorescence in the samples in the last exposure times. In hemocytes of mussels exposed to polycyclic aromatic hydrocarbons (Grundy *et al*. 1996) and in oysters exposed to metals, a significant decrease in lysosomal

membrane stability was observed (Ringwood *et al.* 2002).

More difficult to explain was why this effect could only be seen in large granulocytes (R3) and why the small granulocytes (R2), not only were not affected, but showed an increase in red fluorescence over the experimental period, which could suggest an increase in the lysosomal system volume. One possible explanation is that the large granulocytes (R3) were a population consisting of mature and aged cells, which could include some agranulocytes, and therefore more sensitive to Hg. On the other hand the loss of green fluorescence observed in this cell type over the 30 days in Hg exposed samples seems to confirm that the cell volume decreases. We also believe that the large granulocytes were the only ones in which the Hg was accumulated. But, although we confirmed the presence of Hg inside hemocytes by X-Ray microanalysis, we were not able to distinguish if Hg was accumulated inside all hemocytes or only in one type.

The statistical analysis of the results showed that, although the total number of hemocytes was not affected by the mercury, the relative number of cells in each of the populations showed significant differences over the experimental period and between control and Hg-exposed oysters. Agranulocytes were the major population in all samples analyzed, while granulocytes of R2 and R3 had a similar number of cells. These results confirmed the observation of hemolymph samples by optical microscope and partially agree with the quantitative results found by Ashton-Alcox and Ford (1998). The authors also found that agranulocytes are the major cell type in the *C. virginica* hemolymph, but they found that the number of small granulocytes was lower than the number of large granulocytes. In the same species, *C. virginica*, McCormik-Ray and Howart (1991), using the light microscope found that the large granulocytes were the major hemolymph cells, although they also described differences in relative cell numbers, over the year. Although a significant decrease in the number of hemocytes was described in specimens of *M. arenaria* and *Mactromeris polynima* from marine sediments polluted with PAHs and PCBs (Fournier *et al.* 2002), a decrease in the number of circulating hemocytes of bivalves have only been observed under high exposure levels of copper (Suresh & Mohandas 1990).

The statistical analysis in the relative cell numbers revealed that in control and Hg-exposed oysters the agranulocytes (R1) and large granulocytes (R3) showed significant differences over the experimental period, although the pattern observed in control oysters was the opposite to that seen in Hg-exposed animals. On the other hand, the number of small granulocytes (R2) was constant throughout the experimental period. The fact that the differences were only observed on R1 and R3, and that they were the opposite to that seen between control and Hg-exposed oysters, suggested that the mercury modified a process that occurs naturally. In addition, we found a high negative correlation between agranulocytes and large granulocytes, a correlation not found between agranulocytes or between large granulocytes and small granulocytes. Ashton-Alcox and Ford (1998), in a second study of *C. virginica* hemocytes, described a positive correlation between the total number of hemocytes and the percentage granulocytes and a negative correlation between the total number of hemocytes and the agranulocyte percentage. Results obtained in the present study on control oysters suggested that the relative number of agranulocytes and large granulocytes were modified during the first days of the experiment, but later they showed the same values that as at the beginning. In the exposed oysters, the results suggested that during the first days of exposure there could have been a maturation of agranulocytes into granulocytes. The reduction of agranulocytes observed after 7 days of exposure and the decrease in large granulocytes caused by mercury could have triggered the appearance of new agranulocytes, which could explain the increase of this type of cell on the 19th day of exposure. If this process actually occurs, it could support the hypothesis that agranulocytes are the immature precursors of granulocytes, described by Mix *et al.* (1976).

In conclusion, it was found that the hemocyte populations studied by flow cytometry contributed complementary and functional data to the morphological characterization of hemolymph cells by microscopy techniques. According to the present

results we suggest that the inverse relationship of the relative numbers of agranulocytes and large granulocytes could activate proliferation processes in agranulocytes population. To confirm this point, new studies need to be carried out on these specific cell lines. On the other hand, the use of flow cytometry allowed us to detect an increase of lysosomal compartment in agranulocytes and small granulocytes and, at the same time, a disruption of lysosomal integrity in granulocytes type I under the exposure conditions to Hg. Thus, the responses identified in hemocyte populations studied could be used as valuable biomarkers of environmental stress.

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