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Sublethal effects of experimental exposure to mercury in European flat oyster *Ostrea edulis:* Cell alterations and quantitative analysis of metal

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Oysters display a diversity of uptake mechanisms for metallic elements and distribution in the target organs, namely gills and the digestive gland. Various tissues of the flat oyster, *Ostrea edulis*, were studied following experimental exposure to 0.025 μM (5 μg l^{-1}) of mercury, for up to 34 days. All animals survived the treatment. Data indicate Hg accumulation in gill tissue with a maximum concentration of 38.76 μg g^{-1} dry weight after 25 days of exposure. Hg levels were lower in remaining tissues, in which the maximum concentration (18.47 μg g^{-1} dry weight) was reached after 18 days of exposure. After these times, concentration in both tissues decreased. Results show that oysters can accumulate Hg from the environment, without their survival being affected during the experimental period. Structural alteration of epithelial tissues of gill and digestive gland of flat oyster was comparable with effects described for other metallic elements in bivalve molluscs. Interstitial tissue was disorganized in the digestive gland, and ultrastructural changes in intracellular endomembranes were detected in epithelial cells of the digestive gland after 18 days of treatment. After 25 days, absorptive epithelial cells of gills showed highly dilated, swollen microvilli. These intracellular alterations are parameters of the incipient response to the accumulation of mercury.

Keywords: bioaccumulation, cellular alterations, digestive gland, gills, Hg, *Ostrea edulis*

Introduction

Among the pollutants that can be found in continental and marine waters, mercury is widely distributed in several environmental compartments. The average Hg concentration in seawater is 0.4–2 ng l^{-1}. Mercury levels detected in Mediterranean seawater are 0.5–20 ng l^{-1}, and emissions in industrial areas can reach 100 ng l^{-1} (Copin-Montegut *et al.* 1984, Sorensen & Bjerregaard 1991, Von Burg & Greenwood 1991). Mercury levels detected in bivalve mollusc tissues may be several orders of magnitude greater than in the surrounding water. Studies on uptake mechanisms of Hg in bivalve molluscs have shown the significance of different species as indicator organisms for metal pollution (Fowler *et al.* 1975, Langston 1982, Chin & Chen 1993).

Oysters and mussels are widely distributed along the Mediterranean coast, and the interest of these bivalves as sensitive biological indicators of pollution is well known (Goldberg *et al.* 1978, Bayne 1989). Like other marine invertebrates, they have the ability to incorporate and concentrate several heavy metals, which are potentially toxic, without affecting their viability. Detoxification processes of metal elements (Cd, Cu, Zn, Ag and Pb) and tissue effects of their accumulation in target organs (kidney, mantle, gills and digestive gland) have been studied in several species of bivalve mollusc

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(Hemelraad *et al*. 1990, Metayer *et al*. 1990). There is great variability of response in bioaccumulation mechanisms, depending on specimens and sources of pollution (Pirie *et al*. 1984, Martoja *et al*. 1988). In different species of oysters from highly polluted environments, blood amoebocytes accumulate high concentrations of copper and zinc in intravesicular granules and lysosomes (Thomson *et al*. 1985). Target organ cells develop specific mechanisms for the uptake, storage and compartmentalization of intracellular metal elements, and thus the isolation of inorganic pollutants. However, the cell incorporation pathways and changes induced at cellular level are scarcely known.

Here we study the distribution and bioaccumulation of mercury by analysing specimens of European flat oyster, the tissues of which had been experimentally contaminated with low Hg doses; we also examine the intracellular alterations induced in oyster tissues.

Materials and methods

Experimental conditions

Experimental contamination was carried out during December 1993 and January 1994. *Oysters edulis* (L.) were obtained from a commercial farm at Cadaques Bay (Gerona, Spain). They were two years old and of a similar size shell length (6.7 cm, SD 1.7). The oysters were transported to the laboratory, where they were weighed, labelled and placed, in groups of 20 individuals, into four glass aquaria (two for Hg exposure and two for control) containing 20 l of natural, aerated seawater (salinity 37.2 ‰, SD 0.6).

The aquaria were placed in an air conditioned room (19°C, SD 1). Before contamination, the animals were acclimatized to laboratory conditions for 10 days. After acclimatization, the Hg exposure phase began. The oysters were exposed to 5 $\mu g\ l^{-1}$ of mercury as $HgCl_2$ for 34 days. The seawater and pollutant were renewed every day throughout the experiment. Oysters were fed daily with a specific compound for marine invertebrates (Liquizell, Dohse Aquaristik. Bonn, Germany), which was added one hour after the water had been changed.

Three oysters were collected from each tank on days 0, 4, 10, 18, 25 and 34 in the exposure. The oysters were then weighed and the gills were excised from the rest of the body (remaining tissues). In order to have a constant water to oyster ratio throughout the experiment, the water volume was adjusted after each sampling operation.

Quantitative analyses

After removing fragments of the gills and the digestive gland of each oyster for the ultrastructure study, the rest of the gills and the remaining tissues were stored at –20°C until analysed. An aliquot of each sample (approximately 0.6 g) was digested in 2.5 ml of 65% nitric acid (Suprapur, E. Merck, Barcelona, Spain) for two hours at 150°C by using Parr acid digestion bombs (n-4746). Together with each series of ten samples, three blanks were processed. When the digested samples had cooled to room temperature, solutions were made up to 20 ml with deionized water. These solutions were each divided into two subsamples, one for Hg and the other for Zn, Cu and Fe quantification. The remaining tissues were used to calculate the ratio of wet weight to dry weight.

Hg concentrations were quantified by cold vapour atomic absorption spectroscopy (absorption spectrometer PHILLIPS PV9 200 with a deuterium lamp for background correction). Zn, Cu and Fe were quantified by atomic emission spectroscopy–inductively coupled plasma (AES–ICP) (THERMO-JARREL-ASH POLYSCAM 61EE).

Analyses of each sample were performed in triplicate, and samples were analysed in a random order. The analytical methods were validated previously using Canadian National Research Council Standards (DORM-1) (Table 1). To every two series of digestions, an aliquot of the same reference material was added as a control.

Statistical analysis

Data obtained from quantitative analyses were processed by ANOVA using the computer program STATGRAFIC.

Light and transmission electron microscopy

In order to monitor the morphological and histological state of oysters throughout the experiment, eight individuals (four control and four polluted) at 0, 10, 25 and 34 days of exposure were processed for light microscopical study. Whole oysters were fixed in 10% formaldehyde, dehydrated and embedded in paraffin. Sections of 7 μm were then stained with Hematoxiline-Eosine and examined in an OLYMPUS CH-2.

Table 1. Results of standard reference material analysis DORM-1 (National Research Council of Canada) (mean (SD), $n = 3$)

	Hg	Zn	Cu	Fe
Certified value	0.798(0.07)	21.3(1.0)	5.22(0.3)	63.6(5.3)
Value obtained in our laboratory	0.837(0.05)	20.9(5.6)	4.86(1.7)	59.6(7.6)

Fragments (1 mm) of gills and digestive gland, excised from the oysters processed for the quantitative analyses, were fixed for two hours at 4°C in 2.5% glutaraldehyde diluted in sodium cacodylate buffer 0.1 M (pH 7.4) and the osmolarity was corrected using NaCl (1090 mOsm). They were then postfixed for two hours in 1% osmium tetraoxide diluted in the same buffer (900 mOsm). After washing, the samples were dehydrated in acetone and then embedded in Spurr's resin. Ultrathin sections (70 nm) were sectioned on an Ultracut REICHERT Ultramicrotome, collected on copper grids and stained by uranyl acetate and lead citrate with the Reynolds method (Reynolds 1963). An electronic transmission microscope HITACHI 600 was used in these tests. Semithin sections of these samples were examined by light microscopy. All methods used in this study are summarized in Figure 1.

Results

Hg accumulation

The addition of Hg to the seawater induced increases in the Hg concentration in the oysters. The differences between polluted and control groups were significant both in gills and in remaining tissues, but although the behaviour was similar in both tissues, there were some important differences (Figure 2). The Hg accumulation pattern in gills was characterized by a fast uptake followed by a sharp fall, while in remaining tissues the Hg concentration decreased more slowly after the maximum had been reached.

The amount of Hg in gills of exposed oysters was higher than in remaining tissues at every point of analysis. Furthermore, the period of effective Hg accumulation in gills was longer (Figure 2). In gill epithelia, data showed a strong increase in accumulated Hg up to day 25 of exposure, the time of the highest concentration (38.76 $\mu g\ g^{-1}$ dry weight); after this point, at which the highest variability between specimens was recorded, the Hg in gills decreased rapidly. In remaining tissues the highest concentration was found after 18 days of exposure, a week before the maximum found in gills (Table 2).

Quantitative results for Zn, Cu and Fe are shown in Figure 3. Levels of Zn and Cu showed a similar response in gills: the amounts of these two metals were significantly higher in exposed oysters. Although the differences between the concentrations of Zn and Cu at the various sampling dates were not significant in exposed oysters, there was a slight decrease on day 25 of exposure, which coin-

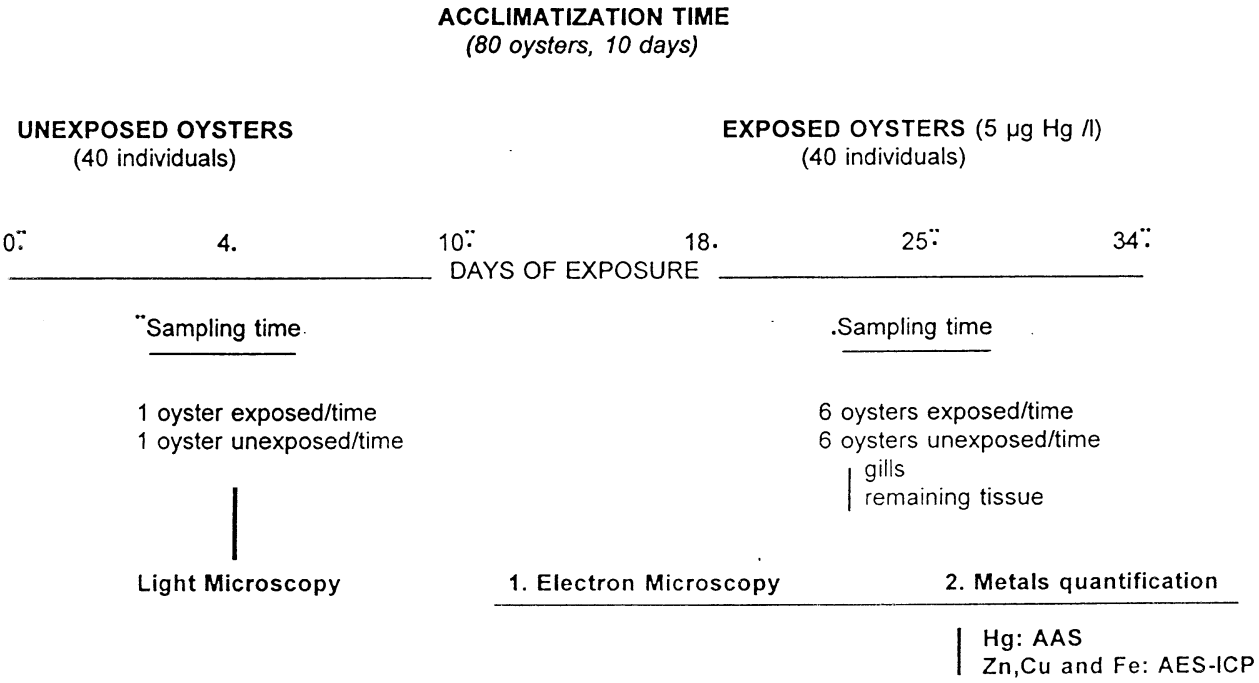

Figure 1. Models of sampling oysters at different exposure times.

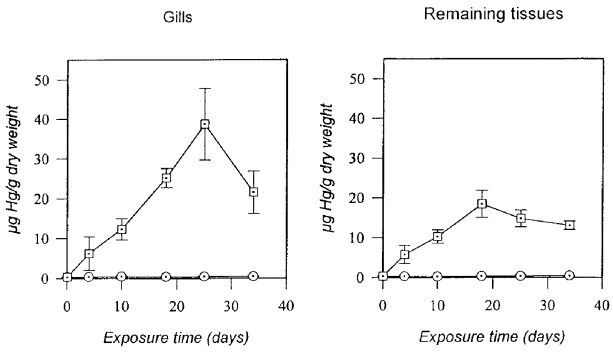

Figure 2. Mercury concentrations in the tissues of gills and remaining tissues of oyster, *Ostrea edulis*, exposed to 5 µg Hg^{-1} (□), or unexposed (○), over time. Mean ± SD for six specimens.

cided with the maximum concentration of Hg. In remaining tissues, the levels of Zn and Cu were similar in control and polluted oysters. The amounts of Fe in gills and remaining tissues of exposed oysters were not significantly different from controls.

Biological effects

With the low concentrations of Hg used in our experimental conditions, the levels of Hg and other metals detected in oyster tissues affected neither specimen survival nor growth. We quantified individual weight variations, and both controls and contaminated oysters showed the same behaviour. The differences in the variation in weight in exposed and control oysters are not significant. (Table 3)

Table 2. Hg concentration (µg g^{-1} dry weight) in soft tissues of oysters at different times of exposure ($n = 6$)

Mercury concentrations in µg g^{-1} dry weight (mean (SD))		
Days	Gills	Remaining tissue
Control		
0	0.350(0.05)^{a}	0.361(0.05)^{ab}
4	0.382(0.04)^{ab}	0.360(0.07)^{a}
10	0.464(0.09)^{bc}	0.303(0.05)^{a}
18	0.430(0.07)^{abc}	0.371(0.04)^{ab}
25	0.503(0.06)^{c}	0.368(0.05)^{ab}
34	0.502(0.02)^{c}	0.452(0.07)^{b}
5 µg Hg l^{-1}		
0	0.36(0.04)$^{a'}$	0.36(0.05)$^{a'}$
4	6.21(4.18)$^{a'b'}$	5.81(2.35)$^{b'}$
10	12.35(2.73)$^{b'}$	10.28(1.67)$^{c'}$
18	25.17(2.42)$^{c'}$	18.47(3.45)$^{e'}$
25	38.76(11.7)$^{d'}$	14.81(2.11)$^{d'}$
34	21.55(5.29)$^{c'}$	13.13(3.21)$^{c'd'}$

Values in the same column not showing a common superscript are significantly different at $P < 0.05$.

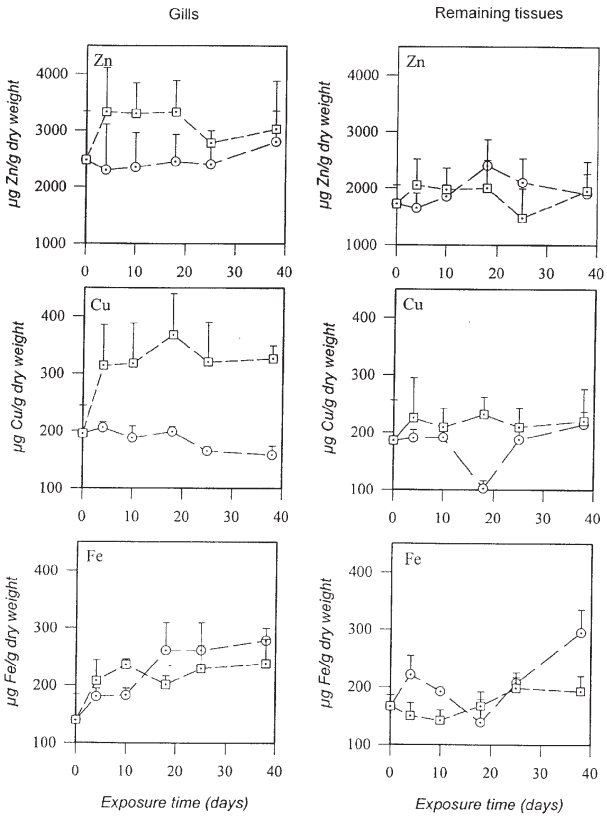

Figure 3. Metal concentrations in gills and remaining tissues of oyster, *Ostrea edulis*, exposed to 5 µg $Hg\ l^{-1}$ (□), or unexposed (○), over time. Mean ± SD for six specimens.

Tissue and cellular alterations

Light microscopy study of whole individuals, digestive gland and gills revealed well preserved cellular structures in all control individuals. The epithelia of both digestive gland and gill cells showed regular volume and shape and preserved the basal lamina structure (Figure 4A,C). The structure of the interstitial tissue of digestive gland was well preserved and the storage products (lipids) were maintained throughout the experiment (Figure 4A). Digestive gland tubules have mainly two kinds of cell: digestive and basophilic. At the ultrastructural level both types of cell showed regular shaped mitochondria, widely distributed rough endoplasmic reticulum cisternae and well preserved and developed Golgi complex (Figure 5A,B,C).

Table 3. Variation in oyster weight at different times of exposure

Variation with respect to the initial weight (23.3 g, SD 0.08; $n = 72$) (mean (SD))		
Days	Control ($n = 6$)	Contaminated ($n = 6$)
0	0.19(0.4)	0.03(0.2)
4	–0.20(0.2)	–0.80(0.4)
10	–0.41(0.2)	0.74(0.3)
18	–0.20(0.2)	0.28(0.2)
25	0.09(0.3)	0.47(0.2)
34	–0.06(0.2)	0.45(0.3)

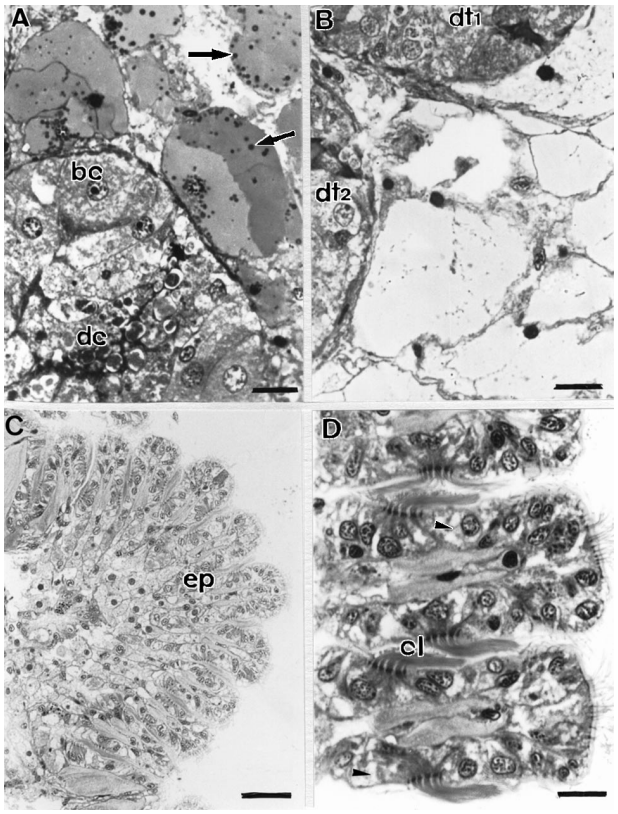

Figure 4. Photomicrographs of semithin sections of digestive gland and gill epithelium of oysters exposed to $HgCl_2$, and control oysters, after 18 days of experiment. (A) Epithelium in a digestive tubule of digestive gland in a control specimen, with a digestive cell (dc) and large basophilic cells (bc). The large cells of interstitial tissue (IT) show storage lipids (arrows). Scale bar: 15 μm. (B) Disruption of interstitial tissue between two digestive tubules (dt1,dt2) of an exposed oyster. Scale bar: 15 μm. (C) Different filaments of a well preserved epithelium (ep) of a gill lamella of a control specimen. Scale bar: 40 μm. (D) Detail of gill ciliate epithelium from an exposed individual (ep) of three filaments, with distinct and ordered cilia (cl). Note some dilated spaces between cells (arrowhead). Scale bar: 20 μm.

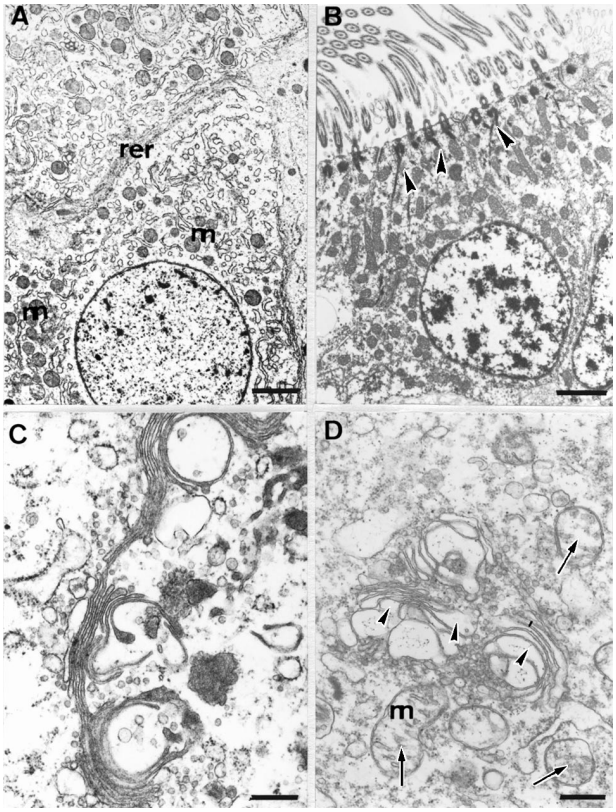

Figure 5. Transmission electron micrographs of ultrathin sections of different epithelial cells of control and exposed oysters. (A) Basophilic cell of the digestive gland of a control oyster, with mitochondria (m) and rough endoplasmic reticulum (rer) vesicles at the periphery of the nucleus. Scale bar: 2 μm. (B) Epithelial gill cell of a control individual, which shows a high number of mitochondria beneath ciliar roots (arrowheads). Scale bar: 1.5 μm. (C) Golgi complex in epithelial cell of digestive gland in a control oyster. Scale bar: 0.5 μm. (D) Region of a Golgi complex of an exposed specimen after 25 days of exposure. The *trans* faces cisternae (arrowheads) are dilated and show an increase in vesicles and dilated mitochondria (arrows:m). Scale bar: 0.5 μm.

Digestive epithelia of exposed individuals did not reveal histopathological alterations, and parasitosis or other infectious alterations were not observed in the tissue of any individual studied. The only structural alteration was observed in the digestive gland of exposed specimens, with a breakdown of the interstitial connective tissue, and the vessels it contains (Figure 4B). At the ultrastructural level, basophilic cells in digestive tubules showed marked alterations only after 25 days of exposure in the region of the Golgi complex: dilation of cisternae in the *trans* face and compression of those in the *cis* face. In addition, exposed individuals later showed

swollen mitochondria, not detected in control cells (Figure 5D). In the same period, occasional cell lysis and a rise in the lysosomic system and residual bodies in digestive cells were detected.

After 25 days of exposure, gill epithelial cells showed dilation of intercellular spaces and occasional disruption of cells (Figure 4D). By contrast with the dilated intercellular spaces between gill epithelial cells in exposed oysters, specialized adhering junctions were preserved between epithelial cells (Figure 6A). At the ultrastructural level, alterations were found in microvilli in this epithelium after 18 days of exposure (Figure 6B,C). A marked increase in dilated and forked microvilli was detected (Figure 6B,C), with the formation of membrane blebs and swelling microvilli only after 25 days of exposure. All these alterations were detected up to the end of the exposure period (Table 4), even when the Hg levels in the tissues studied decreased.

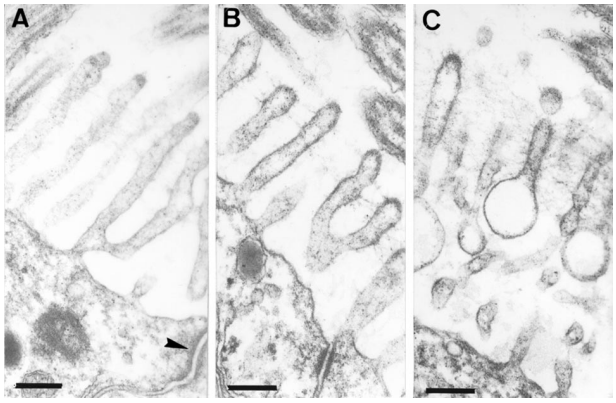

Figure 6. Transmission electron micrographs of microvilli from the apical region of a gill epithelial cell of oyster exposed to $HgCl_2$, at different times in the experiment. (A) Simple and bifurcated microvilli from the epithelium of a control oyster. Note the well preserved adhering junction (arrow). Scale bar = 0.30 μm. (B) Short and slightly dilated microvilli after 18 days of exposure. Scale bar: 0.25 μm. (C) Swelled and irregular shaped microvilli after 25 days of exposure. Scale bar: 0.25 μm.

Discussion

Results obtained in this experiment reveal that the oysters increase tissue concentrations of elements foreign to their metabolism, without viability being affected. From different concentrations of Hg tested in previous experiments (Poquet *et al.* 1993), we selected the concentration of 5 μg l^{-1} because it produced and maintained higher body loads of Hg with minimal cell damage during time of the experiments. On the other hand, it is difficult to determine sublethal doses of Hg with minimal risk, because of the differences in the bioavailability of this metal, depending on its chemical form and the organisms studied. The resistance to the levels of Hg used in this study and the demonstrated bioaccumulation ability of oysters agrees with the significance of *Ostrea edulis* as an important pollutant vector within the trophic chain. The shape of the curve of Hg incorporation in gills, and the higher accumulation level in this tissue, are partly due to a direct input of pollutant during gill filtration. Dose versus mercury incorporation curves differ from those obtained for silver in the closely related species *Crassostrea gigas*: following a maximum, reached after two weeks of exposure, stability is achieved, which is represented by a plateau

Table 4 Many of the cellular changes detected in oyster tissues following experimental exposure to Hg

	Exposure time to $HgCl_2$ (days)					
Alterations	0	4	10	18	25	34
Gill epithelial cells					► Rarely cellular lysis	
				► Irregular shape and high swelling of microvilli		
					► Generalized and strong swelling of microvilli	
					► Increase in residual bodies	
Epithelial cells of digestive gland				► Irregular cell shape		
					► Rarely cellular lysis	
					► Increased lysosomes and residual bodies	
					► Mitochondrial swelling and reduction of cristae	
					► Dilation of *trans* face cisternal and increased vesicles	
Interstitial tissue in the digestive gland				► Tissue fragmentation and disorganization		
				► Decrease in storage products: lipids and glycogen		

(Metayer *et al.* 1990, Amiard-Triquet *et al.* 1991). Results obtained for Hg concentration in remaining tissue of oysters in this experiment differ from those obtained for Hg accumulation in the bivalve *Meretrix lusoria*, in which a maximum pollutant concentration was reached after about two weeks of exposure, followed by a marked decrease in Hg levels in several tissues (Chin & Chen 1993). By contrast, in our experiment, oysters showed a longer period of accumulation of Hg for gill tissue (up to 25 days).

The decrease in mercury levels in both gills and visceral mass may be due to several factors, including a weight increase or a greater effectiveness in detoxification processes, or cellular alterations. The weight factor may be ruled out, since the variations found throughout the bioassay were insignificant (Table 3). The decrease in tissue Hg levels could result from a reduction in Hg uptake in altered tissue, and from specific detoxification processes lead by mercury-binding proteins (metallothioneins). Mechanisms involved in heavy metal cation homeostasis have been described in marine invertebrate cells (Roesijadi 1982, Viarengo & Nott 1993).

In this experiment we have obtained quantitative data for Fe, Cu and Zn from different specimens of oysters, control and exposed to $HgCl_2$. Concentration levels of Fe in gills and remaining tissues did not show variations after exposure to Hg. On the other hand, Cu and Zn levels of gill samples showed an increase in exposed oysters. These elements could thus be involved in bioaccumulation mechanisms, with interaction in detoxification processes. These results agree with those reported by Thomson *et al.* (1985) on oyster amoebocytes from a highly metalliferous environment; levels of Zn and Cu were higher than in oyster amoebocytes from a relatively metal-free area. Amoebocytes could contribute to a defensive response, highly important in gill tissues because of their function in direct uptake of metals, whereas Fe may not be involved in this mechanism.

The low Hg concentrations used resulted in neither the digestive gland nor the gill epithelium showing severe damage at any sampling time. No extensive necrotic epithelial regions or whole gland ducts were observed, only lysis of single cells, which could be related to normal epithelial renewal mechanisms. In bivalve mollusc tissue, effects such as a decrease in epithelial height and different forms of necrosis have been reported under higher concentrations of metal exposure than those used in the present study (Sunila 1988). A relationship between histological changes and experimental pollution by metals was found in quantitative studies of the lysosome system in digestive cells of winkles exposed to sublethal concentrations of Cd and in mussels polluted with oil derivatives (Simkis & Masson 1984, Berthon *et al.* 1987, Gardner & Yevich 1988, Marigomez *et al.* 1989, Cajaraville *et al.* 1992). Although no serious tissue or cellular damage was found in the present experiment, a disturbance to the structure and storage of glycogen and lipids was shown by the interstitial tissue; this we have described under similar experimental conditions (Poquet *et al.* 1993). Similar changes in glycogen metabolism were observed in *Crassostrea gigas*, after exposure to silver (Martoja *et al.* 1988). Other ultrastructural alterations in endomembrane systems have been detected in contaminated oysters, especially in Golgi complex dilations. As for alterations shown in membrane systems, the stability provided by osmolarity control during the fixing process is shown by the good preservation of the intermembrane spaces in mitochondria of control specimens. These therefore represent the initial stages of cell degeneration with ultrastructural changes maintained throughout the exposure. Similar alterations in cell compartments have been described in several gastropod cell types which have suffered different molluscicide concentrations (Triebskorn & Köhler 1992). Differences in alterations detected in the different cell types of *O. edulis* analysed (absorptive and basophilic epithelial cells) show the extent to which the cell reaction to low Hg doses is specialized. These altered states of membrane systems and microvilli of epithelial cells constitute an incipient response to stress caused by Hg doses supplied to the oyster specimens in the present study. Alterations to the membrane could reduce the ability of the cell to take up Hg, with the subsequent decrease in metal concentration in the tissues. Although forked microvilli appeared in gill cells of contaminated individuals, this was not necessarily induced by Hg (Durfort *et al.* 1990). The decrease in Hg concentration after 25 days of contamination may be explained by more marked alterations found in gill and digestive gland epithelia after the same period.

In conclusion, Hg concentrations in tissues after experimental exposure confirm the ability of the flat oyster to accumulate Hg, which provides a reflection of the environmental concentrations of the metal. The low Hg doses used did not induce mortality and the cell alterations observed constitute parameters by which to evaluate initial tissue specific responses to accumulation of Hg.

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