Biomarkers, March–April 2007; 12(2): 173–187

informa
healthcare

Immunological and cytotoxicological responses of the Asian clam, *Corbicula fluminea* (M.), experimentally exposed to cadmium

O CHAMPEAU[1], M AUFFRET[2], M P. CAJARAVILLE[3], A BASSÈRES[4], & J.-F NARBONNE[1]

[1]*Laboratoire de Physico-Toxico-Chimie des Systèmes Naturels, UMR 5472 CNRS, Université Bordeaux 1, Talence, France,* [2]*LEMAR, UMR 6539, Institut Européen de la Mer, Université de Bretagne Occidentale, Technopôle Brest-Iroise, Plouzané, France,* [3]*Laboratory of Cell Biology and Histology, Department of Zoology and Cell Biology, University of the Basque Country, Bilbao, Basque Country, Spain and* [4]*Pôle R&D Mont/Lacq, TOTAL, Service Environnement, Lacq, France*

Abstract

Bivalve molluscs, as filter-feeding organisms, are known to accumulate metals that can produce deleterious effects on organisms. The phagocytic activity of haemocytes and lysosomal alterations in the digestive gland cells were measured in the freshwater Asian clam exposed to cadmium, in order to assess the possible use of immunocompetence and lysosomal responses as biomarkers of freshwater quality. Clams were exposed in the laboratory to nominal concentrations of 3, 10, 21.4, 46.5 and 100 $\mu g\ l^{-1}$ of cadmium and sampled after 7, 15 and 30 days of exposure. The results show a decrease of phagocytic activity after only 7 days of exposure to 10 $\mu g\ l^{-1}$ of cadmium. This response was also observed as the exposure time was increased. Lysosomes in the digestive cells increased in size and number after 7 days of exposure as cadmium concentration increased. After 30 days of exposure, a decrease in size and number indicated a change in the response to the metal from concentrations of 46.5 $\mu g\ l^{-1}$ of cadmium. A dose and time response both in phagocytic activity of haemocytes and lysosomal structure demonstrated a possible use of these biomarkers in freshwater biomonitoring.

Keywords: *Bivalve, freshwater, haemocytes, phagocytosis, lysosomes*

(Received 7 April 2006; accepted 13 October 2006)

Introduction

Bivalve molluscs represent useful organisms for toxicological studies because they live in direct contact with contaminated aquatic sediments and are exposed to waterborne contaminants. As many are filter-feeding organisms, bivalves and particularly marine mussels *Mytilus galloprovincialis* and *M. edulis* are suitable bioindicators for heavy metal contamination (Amodio-Cocchieri et al. 1993, Etxeberria et al. 1994, Narbonne et al. 1999, Devier et al. 2003). In addition, the measurement of biological

Correspondence: J.-F. Narbonne, Laboratoire de Physico-ToxicoChimie des Systèmes Naturels, Avenue des Facultés, 33405 Talence Cedex, France. Fax: +33-5-40-00-87-19. E-mail: jf.narbonne@lptc.u-bordeaux1.fr

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.1080/13547500601068390

markers in such sentinel organisms allows detection of sub-lethal and early pollutant effects on aquatic ecosystems (Livingstone 1993). The need for such a bioindicator and sentinel organism in freshwater ecosystems has focused attention on the clam, *Corbicula fluminea* (Müller), over the past few years (Abaychi & Mustafa 1988, Labrot et al. 1999, Tran et al. 2001, Vidal et al. 2001, Baudrimont et al. 2003, Ciutat and Boudou 2003, Bassères et al. 2004).

Originating from Asia, this opportunist species has progressively colonised the USA, and more recently Europe (Britton & Morton 1977, Araujo et al. 1993), to become a major component of benthic communities. Being both a filter- and a deposit-feeding species, *C. fluminea* is able to bioaccumulate heavy metals (Labrot et al. 1999) including cadmium (Abaychi & Mustafa 1988, Winter 1996, Baudrimont et al. 1997, Tran et al. 2001, Fraysse et al. 2002, Ciutat & Boudou 2003). The digestive gland of molluscs is a target organ in environmental pollution assessment. It accumulates miscellaneous pollutants and actively participates in the detoxification process (Marigómez et al. 2002). Uptake and accumulation of a pollutant can provoke measurable changes in volume, surface, size and number of lysosomes (Lowe et al. 1981, Marigómez et al. 1989, Cajaraville et al. 1995b, Marigómez et al. 1996, Marigómez & Baybay-Villacorta 2003). Changes in these cellular organelles have therefore been used as biomarkers of stress (Cajaraville et al. 2000). More recently, the digestive lysosomal response in the zebra mussel, *Dreissena polymorpha,* has been proposed as a biomarker for the assessment of freshwater pollution (Giamberini & Cajaraville 2005).

In molluscs, mainly in bivalves, haemocytes represent the major component of their immune system. In addition, they are involved in various physiological functions including wound repair, shell formation and repair, nutrient digestion and transport and excretion (Cheng 1981, Glinski & Jarosz 1997). It has been established that the efficiency of haemocytes may be affected by environmental contaminants (Anderson et al. 1981, Renwrantz 1990, Canesi et al. 2003, Gómez-Mendikute & Cajaraville 2003). Phagocytosis by haemocytes is the major line of defence against invading foreign material. Indeed, the central role of phagocytosis in immune defence and the sensitivity of this biological function to environmental xenobiotics in several other species (Loose et al. 1981, Kollner et al. 2002, Hillyer et al. 2003) have made it a major function to be assessed for evaluating the immunosuppressive effects of pollutants.

A multitude of toxic compounds is released in the aquatic environment from point or diffuse sources. Among them, cadmium (Cd), a non-essential heavy metal with both natural and anthropogenic origins, is of long-standing environmental concern. Cadmium is found in some soil solutions, urban runoff waters, landfill leachates, polluted river and ground waters (Von Gunten et al. 1991, Holm et al. 1995). Because of its ubiquity and its toxicity, cadmium is considered as a priority pollutant.

The aim of this study was to assess the effects of cadmium on the phagocytic activity of haemocytes and lysosomes of the digestive gland cells in *Corbicula fluminea* and to set up and develop these biomarkers for further validation and applications.

Material and methods

Animal sampling and acclimatisation

One thousand five hundred adult *C. fluminea* (15–20 mm shell length) were collected from the banks of the non-polluted freshwater Cazaux-Sanguinet lake (Aquitaine,

France), in March 2003. The excellent water quality makes this lake a reference site in environmental studies (Baumard et al. 1997, Vidal et al. 2001, 2002). Clams were transported back to the laboratory in plastic tanks filled with lake water and subsequently transferred into glass aquariums for an 8-day acclimatisation period into de-chlorinated and aerated tap water.

Experimental design

Forty animals were placed in each of 12 test aquaria. The aquaria contained 5 l of de-chlorinated tap water. The clams were not fed and were held under a natural light cycle. Aeration was provided continuously by air bubbling. Dead clams were removed to avoid water fouling. Physicochemical parameters of the water were monitored every day during the experiment with a Universal Pocket Meter Multiline P4 WTW instrument (Wissenschaftlich Technische Werkstätten, Weilheim, Germany). The mean temperature was 17.8°C with a range of 16–19°C, the mean pH was 8.5 with a range of 8.4–8.6, the mean dissolved O_2 was 8.7 mg l^{-1} with a range of 8.4–9.3 mg l^{-1}, NO_2^- was less than 0.005 mg l^{-1} and NH_4^+ less than 0.02 mg l^{-1}; water hardness was 9°F. Accordingly, the water quality was regarded as suitable for maintaining the test organisms.

Cadmium contamination

A stock solution of cadmium chloride ($CdCl_2 \cdot 2H_2O$, Fluka) was prepared in nitric acid-rinsed glassware and stored in polyethylene bottles. Amounts of stock solutions were added to water in order to obtain the equivalent of a logarithmic series of nominal cadmium concentrations: 0, 3, 10, 21.5, 46.4 and 100 μg l^{-1}. The water of each aquarium was renewed every 2 days with fresh, de-chlorinated and aerated tap water and cadmium added according to the treatment. Animals were sampled after 7, 15 and 30 days of exposure and dissected on ice.

Biomarker measurements

Phagocytosis assay. This assay (Blaise et al. 2002) was conducted on six animals per condition, with four measures for each animal. Haemolymph was collected from the posterior adductor muscle sinus with a 1-ml syringe fitted with a 26-gauge needle through a notch near the posterior margin of the clam shell. Samples were kept in an ice bath.

The phagocytosis assay was firstly improved with freshly harvested haemocytes of *C. fluminea*. The optimal adhesion time of haemocytes on 96-well microplate was optimised by incubating 100 μl of fresh haemolymph in wells for different periods at 20°C. After each incubation period the supernatant (serum) was removed by aspiration, and non-adherent cells in each well were counted under microscope at 400 × magnification with a Malassez cell. The time required for haemocytes to internalise particles was determined by varying the time of exposure of a fixed number of haemocytes in the presence of a known number of fluorescein isothiocyanate (FITC)-marked yeast (*Saccharomyces cerevisae*) in PBS for freshwater molluscs (KH_2PO_4 2.3 mM, Na_2HPO_4 3.8 mM, NaCl 8.5 mM, KCl 10 mM, pH 7.52, 66 mOsm). Remaining free yeasts were subsequently counted under microscope at 400 × magnification with a haemocytometer.

Phagocytic activity was assessed by incubating haemocytes with fluoresbrite yellow-green polystyrene microspheres (2 μm diameter) (Polysciences Europe, Eppelheim, Germany) in the proportion of 1: 50, haemocyte: beads, in PBS. Haemocytes were detached from the well bottom by adding 50 μl of trypsin (0.5 mg ml^{-1} final concentration) and incubated for 5 min at 4°C. Cells were then fixed with paraformaldehyde 1% and stored at 4°C until analysis.

The percentage of cells containing fluorescent spheres was determined by flow cytometry using a FACScan™ (Becton Dickinson, Le Pont de Claix, France) with an air-cooled argon laser, providing an excitation at 488 nm. Haemocyte cell populations were defined based on their forward (FSC) and right angle scatter (SSC) properties. Fluorescence emission was collected by the FL1 photomultiplier tube. A total of 10 000 events was acquired for each sample and stored in the list mode data format. The data were analysed, once displayed, as two-parameters, complexity (SSC) and cell size (FSC) (Figure 1). The distribution histogram of the haemocyte population corresponding to three and more ingested FITC beads was retained to estimate phagocytic activity. The whole cell population was considered to establish the number of phagocytising cells.

Lysosomal responses. Five visceral masses from freshly dissected animals were frozen in *n*-hexane at −70°C for 45 s (UNEP/RAMOGE 1999) and frozen in liquid nitrogen. Visceral masses were embedded in Jung's Tissue Freezing Medium. Sections (8 μm) were cut in a cryostat (Microm HM 500; Zeiss, Francheville, France) at a cabinet temperature of −20°C. Sections were then collected onto warm glass slides and stored at −70°C until required for staining.

The cytochemical reaction for β-glucuronidase was demonstrated according to Moore (1976) with modifications (Cajaraville et al. 1989). Osmolarity of the different

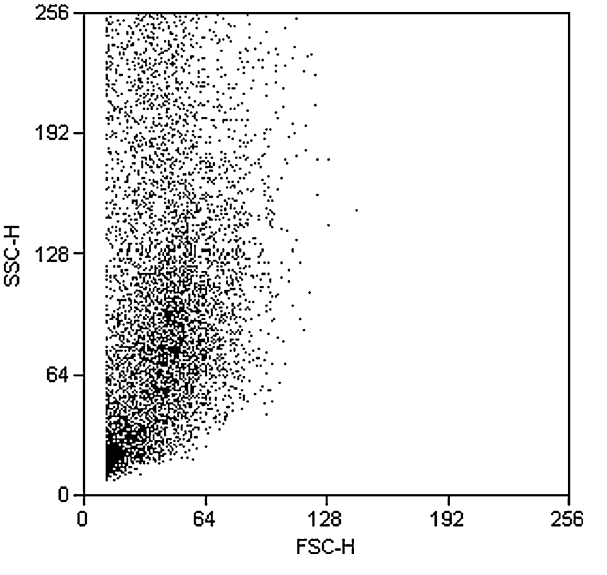

Figure 1. Flow cytometric analysis for size (FSC-H) versus complexity (FSC-H) of haemocytes from an untreated individual. The dot plot displays a single population of cells with very variable complexity, possibly due to intracellular organelles and inclusions. Debris are localised in the lower left corner. The number of events was set to 10 000.

experimental media and incubation time were adapted to freshwater organisms. Briefly, polyvinyl alcohol at a 20% (w/v) concentration was used as a colloid stabiliser; incubations were carried out at pH 4.5 and 37°C for 30 min. After post-coupling with Fast Garnet GBC and fixation in Baker's formol calcium, sections were counter-stained with a 0.1% aqueous solution of Fast Green FCF for 10 min.

A stereological procedure was applied in order to quantify the structure of the digestive cell lysosomes in the clams, with the aid of an automated image analysis system (Cajaraville et al. 1995b) allowing comparisons between organisms.

Morphological parameters were obtained by image analysis. Slides were viewed under a 100 × lens light microscope, and five measurements were made in each section of five individuals per condition in order to calculate the stereological parameters (Lowe et al. 1981); the programme rapidly measures the areas, perimeters and numbers of the different structures and stores the data. This is then connected to the statistical package SPSS-PC (Redmon, WA, USA) for data treatment and quantification of the following four final parameters: lysosomal volume density (Vv), surface density (Sv) and numerical density (Nv), which are relative parameters and depend on the volume of the digestive cell cytoplasm, and surface to volume ratio (S/V), which is not referred to digestive cell cytoplasm and is generally interpreted as the inverse of lysosomal size.

The system, previously described in detail by Cajaraville et al. (1991), calculates the parameters: lysosomal volume density (Vv = VL/VC), lysosomal surface density (Sv = SL/VC), lysosomal surface to volume ratio (S/V = SL/VL) and lysosomal numerical density (Nv = NL/VC) where V, S, N, L and C are respectively volume, surface, number, lysosome and digestive cell cytoplasm (Lowe et al. 1981).

The sample size was determined based on previous analyses on the stabilisation of mean and standard deviation values of the four parameters (Etxeberria et al. 1995, Marigómez & Baybay-Villacorta 2003).

Statistical analysis. To allow statistical comparison, the experiment was performed twice, at the same time. All statistical evaluations were performed using the Statistica 6.0 computer software package (Statsoft Inc., Maison-Alfort, France). Data with normal distribution and homogeneity of variance were submitted to a multivariate analysis with contaminant concentration and time of exposure as the independent variables. Whenever a significant ($p \leq 0.05$) effect was established on a response, a Tukey HSD multiple comparison test was used to check significant ($p \leq 0.05$) differences between means. For the stereological parameters on lysosomal responses, significant differences between paired means were determined using Duncan's multiple range comparison data. Data for lysosomal Vv and Nv were logarithmically transformed before statistical analysis.

Results

Optimisation of phagocytosis assay

Phagocytosis measurements by flow cytometry have already been applied to crustaceans (Cardenas et al. 2000) and to freshwater and marine bivalves (Brousseau et al. 1999, Fournier et al. 2001, Auffret et al. 2002, Sauvé et al. 2002). In order to consider possible differences between species, time of adhesion of haemocytes and

phagocytising time had to be assessed. Adherence time of haemocytes to the microplate was assayed up to 120 min (Figure 2).

Although the maximum adherence time of haemocytes occurred after 120 min, we considered 90 min as the optimum time for cell adherence. The time allowed for cells to phagocytose particles was thus extended from 30 to 180 min (Figure 3).

The maximum ingestion response occurred after 120 min. However, as before, 90 min was deemed to be the optimal time (there were no significant differences between 90 min and 120 min). These optimal times for adherence and phagocytic activity were used for further phagocytosis assessments.

Effect of cadmium on phagocytosis

The two-way analysis of variance demonstrated a significant effect of dose and time exposure, separately, and a significant effect of the interaction of these two parameters on haemocyte phagocytosis activity (Table I).

The measurement of phagocytic activity (Figure 4) demonstrated significant responses of haemocytes as the dose increased. At the first sampling time, the number of phagocytising cells decreased significantly as the dose increased. Clams exposed to 100 $\mu g\, l^{-1}$ of cadmium had a significantly lower phagocytic activity than those exposed to 10 and 46.4 $\mu g\, l^{-1}$ of cadmium.

After 15 days of exposure, the activity of phagocytosis was lower than the controls for most animals exposed to cadmium, but not different from one another. Only animals exposed to 46.4 $\mu g\, l^{-1}$ had a higher phagocytic activity than those exposed to 21.5 $\mu g\, l^{-1}$ ($p < 0.05$). Apart from those exposed to cadmium at 10 $\mu g\, l^{-1}$, animals exposed for 30 days had a significantly different number of phagocytising cells compared with the control. It was reduced at the highest concentration (100 $\mu g\, l^{-1}$) by nearly 20%

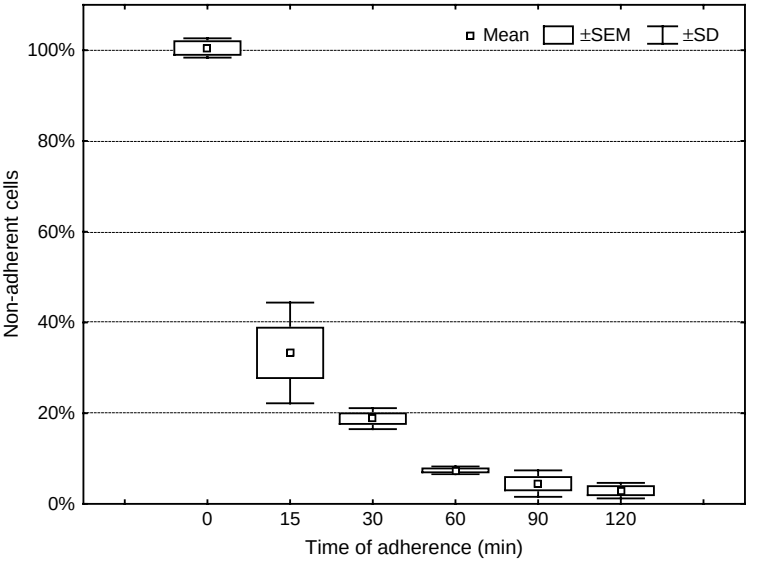

Figure 2. Time of adherence of haemocytes to microplate wells. A volume of 100 µl of haemolymph was added to the wells (in quadruplicate) and allowed to stand for different time periods. The haemolymph was collected for microscopic counting of non-adherent cells.

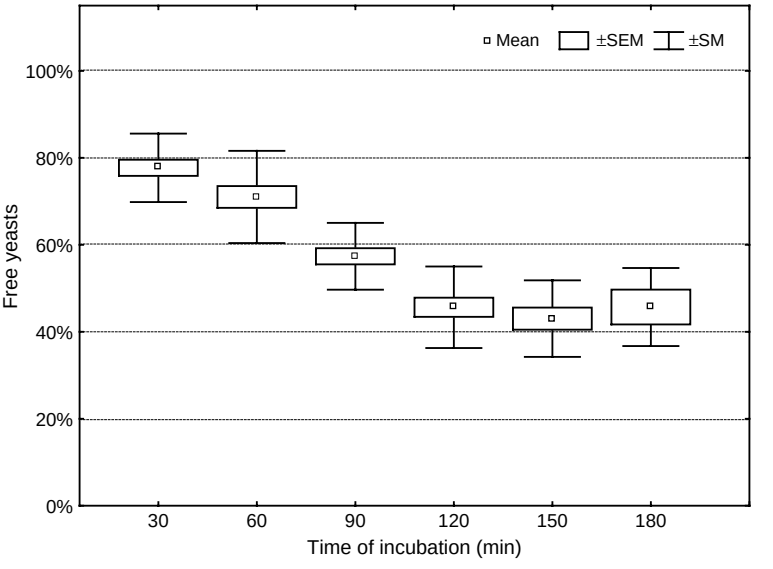

Figure 3. Phagocytosis activity of the Asian clam haemocytes compared to control. The quantity of free yeasts left in solution during the phagocytosis process was measured every 30 min. The data represent the mean, standard deviation and error of replicates ($n=4$).

compared with controls. The trend of decreased phagocytic activity with increase in dose was again observed after 7 days of exposure.

Moreover, significant differences between times were observed. Clams treated with 3 μg l^{-1} of cadmium had a significantly ($p<0.05$) lower level of phagocytosis after 30 days of exposure than clams exposed at the same dose after 7 or 15 days of exposure. A transient increase ($p<0.05$) of phagocytic activity was observed after 15 days of exposure in animals exposed at 46.4 and 100 μg l^{-1}.

These results indicate a gradual sensitisation of haemocytes to cadmium with increase in dose and time of exposure. Reduced phagocytosis activity was an early and sensitive response to cadmium exposure, as a depression of the activity could be detected as early as 7 days of exposure to a concentration of 10 μg l^{-1} (3 μg l^{-1} after 30 days).

Structural changes in lysosomes

The two-ways analysis of variance indicated a significant effect of time and dose exposure for Vv (in log), Sv and S/V, whereas no effect of time was noted for Nv (in log) (Table II).

Table I. Two-way analysis of variance table showing the effects of exposure time and dose on phagocytosis activity.

Parameters	*df*	F	*p*
Time	2	11.253	<0.0001*
Dose	5	7.888	<0.0001*
Time and dose	10	4.498	<0.0001*

F, F ratio; *df*, degrees of freedom; *p*, probability of F.
*Statistically significant ($p<0.05$).

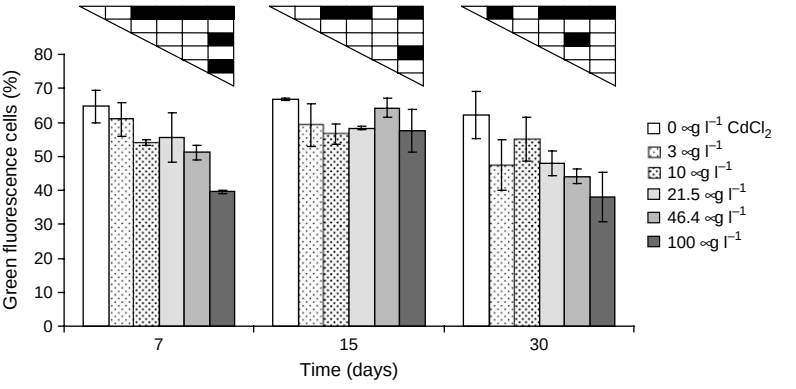

Figure 4. Mean and standard deviation of the phagocytic activity of haemocytes. Significant differences ($p < 0.05$) between pairs of means are indicated by black squares in the upper triangular matrix.

Variations of the stereological parameters (Figure 5) indicated reponses of the lysosomal compartment in the digestive gland.

After 7 days of exposure, the lysosomal compartment underwent a significant increase in volume (Figure 5a), surface (Figure 5b), size (Figure 5c) and number (Figure 5d) of lysosomes. The effects of cadmium at the highest concentrations (21.4, 46.4, 100 $\mu g\, l^{-1}$) were significant for all the stereological parameters (Vv, Sv, S/V and Nv). The lysosomal S/V (Figure 5c) decreased as expected, with increasing doses of cadmium. Although there was a significant difference between exposed and non-exposed animals, no differences were found among the exposed animals. Since S/V is inversely related to lysosomal size, this indicated that the lysosomes were enlarged and more numerous (increased Nv) in the digestive cells of exposed clams with increasing concentration of cadmium.

After 15 days of exposure, most of the stereological parameters were kept at levels similar to those recorded in the controls. Only clams exposed to 46.4 $\mu g\, l^{-1}$ had a Vv and Sv significantly different from the control. The Sv was, in addition, significantly different from one of the animals exposed to 3, 10 and 21.5 $\mu g\, l^{-1}$. The S/V indicated that, for this exposure time, the lysosomes were nearly the same size. The only significant differences occurred with the S/V of animals exposed to 46.4 $\mu g\ l^{-1}$ compared with animals exposed to 3 and 100 $\mu g\, l^{-1}$ of cadmium. In addition, the size of lysosomes in animals exposed for 15 days at this concentration was larger, without being more numerous, than 7 days earlier. Only animals exposed to the lowest

Table II. Two-way analysis of variance table showing the effect of exposure to different concentrations of cadmium on digestive lysosomal structure.

Parameters	F(T)	*df*(T)	*p*(T)	F(D)	*df*(D)	*p*(D)	F(I)	*df*(I)	*p*(I)
Log (Vv)	12.518	2	<0.0001*	9.598	5	<0.0001*	4.088	10	<0.0001*
Sv	9.861	2	<0.0001*	8.906	5	<0.0001*	2.773	10	<0.05*
S/V	13.390	2	<0.0001*	6.986	5	<0.0001*	3.651	10	<0.001*
Log (Nv)	1.257	2	0.287	4.228	5	0.001*	3.173	10	<0.001*

Vv, volume density; Sv, surface density; S/V, surface to volume ratio; Nv, numerical density;
F, F ratio; *df*, degrees of freedom; *p*, probability of F; T, exposure time; D, dose; I, interaction between time and dose.
*Statistically significant ($p < 0.05$).

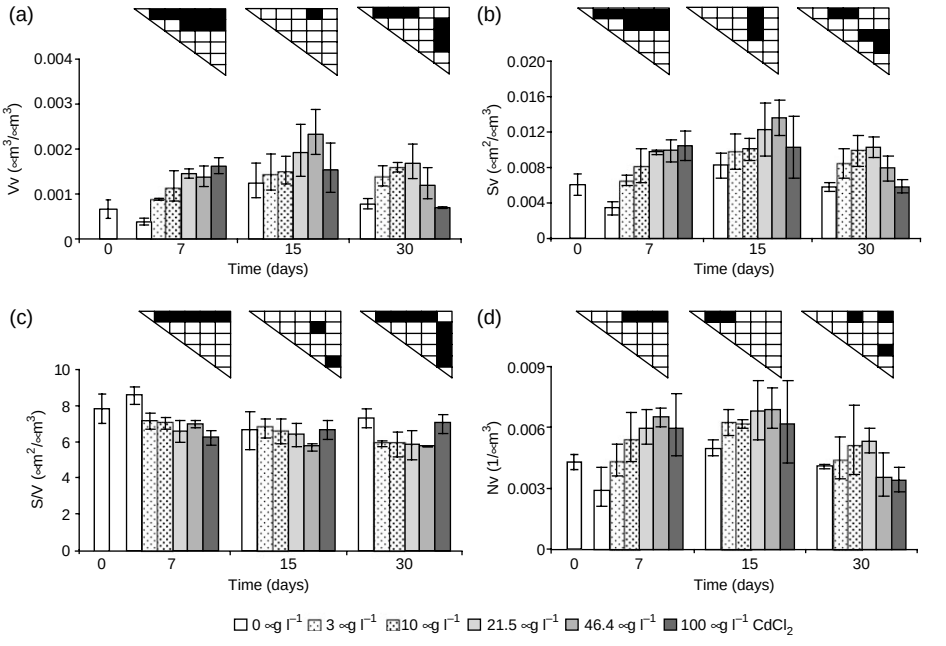

Figure 5. Means and standard deviations of the stereological parameters from the analysis of digestive cell lysosomes in clams exposed to cadmium for 7, 15 and 30 days. (a) Lysosomal volume density (Vv); (b) lysosomal surface density (Sv); (c) lysosomal surface to volume ratio (S/V); (d) lysosomal numerical density (Nv). Significant differences ($p < 0.05$) between pairs of mean values are indicated by black squares in the upper triangular matrix.

concentrations (3 and 10 μg l^{-1}) showed a larger number of lysosomes, but no change of size.

This increase of lysosomal volume, surface, size and number with increase in dose was observed again after 30 days of exposure. However, this increase was restricted to the lowest to medium concentrations (3 μg l^{-1} to 21.5 μg l^{-1}), while the endolysosomal compartment from animals exposed to the highest concentrations (46.5 and 100 μg l^{-1}) suffered a decrease in terms of volume, surface, size and number of lysosomes. This decrease was first observed in animals exposed for 15 days at 100 μg l^{-1} of cadmium. After 30 days of exposure, this decrease continued into the endolysosomal compartment of animals exposed to 100 μg l^{-1} of cadmium and was also observed for animals exposed to 46.5 μg l^{-1} of cadmium.

Discussion

Phagocytosis activity

In this study, we have shown a decrease in the phagocytic activity of haemocytes in *Corbicula fluminea* at the first (7 days) and last (30 days) sampling times. The same response has been observed in the crab, *Paratelphusa hydrodromous*, after exposure to sub-lethal cadmium chloride concentrations (Victor 1993). However, such clear inhibition was not observed after the second sampling time (15 days). As already observed, the toxicity of cadmium does not follow a linear pattern (Matozzo et al. 2001, Sauvé et al. 2002). Moreover, possible adaptative mechanisms could also

explain why the toxic effect of cadmium observed at 7 days is compensated for at 15 days. These mechanisms are no longer efficient at 30 days.

Once the metal enters the organism, one of the inner defence mechanisms of the mollusc includes the involvement of haemocytes. Haemocytes move around the body in haemolymph and may penetrate a number of tissues in the body by amoeboid movements (Simkiss & Mason 1983) and remove metals from the haemolymph in order to keep the concentration in the blood below toxic levels. They can remove metallothioneins and other metal complexes from the plasma by phagocytosis. However, metals can accumulate in cells and interact with their components either directly – lysosomes (Lekube et al. 2000) and cytoskeleton (L'Azou et al. 2002), or indirectly – oxidative stress (Brennan & Schiestl 1996).

Cell movements, displacement or phagocytosis, are supported by the cytoskeleton. Alterations to this cell compartment may explain the inhibition of phagocytosis observed after 7 and 30 days of exposure to cadmium (Cima et al. 1998, Gómez-Mendikute & Cajaraville 2003).

In mammalian species, such as the mouse, cadmium was found to exacerbate phagocytosis (Leffel et al. 2003). Cadmium was less toxic in bivalve molluscs such as *Crossostrea virginica* (Anderson et al. 1992) or *Tapes philippinarum* (Matozzo et al. 2001). In our study we thus demonstrated the sensitivity of the Asian clam immune system to cadmium exposure.

Lysosomal responses

Our study showed that the responses of the lysosomal compartment in the digestive gland cells of *C. fluminea* occurred by the 7th day of exposure at the lowest concentration of cadmium (3 $\mu g\, l^{-1}$). The increase of stereological parameters (Vv, Sv and Nv) was followed by a decrease at 30 days (with numerical density lower than in the controls after 30 days in clams exposed to 100 $\mu g\, l^{-1}$) with values close to those of the controls. This pattern suggests an elimination of digestive cell lysosomes exposed to high concentrations.

After transport into haemocytes, several tissues can be target sites for accumulation or detoxification of metals in molluscs as in the digestive gland (Marigómez et al. 2002). Metals are detoxified by sequestering them within digestive lysosomes, and their accumulation within lysosomes has been thoroughly reported (Jeantet et al. 1985, Marigómez et al. 1990, Soto et al. 1999, Kadar et al. 2001, Desouky et al. 2002, Marigómez et al. 2002).

Under laboratory conditions, metals such as Cu and Cd caused the enlargement of lysosomes in the digestive gland of *Mytilus edulis* (Lowe & Clarke 1989) and *Littorina littorea* (Marigómez et al. 1989). Mixtures of metals such as Fe, Mn, Cu and Zn, and particularly those rich in Pb, led to the same results in natural populations of the marine mussels *Mytilus galloprovincialis* and *M. edulis*. In these studies, an increase in lysosome size was concomitant with a decrease in their numbers. This suggests a process of smaller secondary lysosomes fusing to form large autolysosomes (Lowe et al. 1981). Our results are similar to those reported in other studies (Etxeberria et al. 1994, Giamberini & Pihan 1997, Giamberini & Cajaraville 2005) of an increase both in size and number of lysosomes in the digestive cells of *M. galloprovincialis* and *D. polymorpha* exposed to Zn, Cu, Cd and Pb under field and experimental conditions. The lysosomal alterations they observed did not always show a linear

dependence on metal concentrations. This may be related to the detoxification function of lysosomes. Digestive cell lysosomes are probably not accumulation sites but detoxification pouches and are very dynamic (Soto et al. 1996b). Metal sequestration in lysosomes might thus contribute to reducing their availability, at least temporarily, and is followed by excretion of the extruded metal-loaded lysosomes (Viarengo & Nott 1993, Cajaraville et al. 1995b, Marigómez et al. 2002) demonstrated by the presence of black-silver deposit in the cell debris in the lumen (Soto et al. 1996a). This could explain the different lysosomal responses observed; metal-loaded heterolysosomes accumulate within the digestive cell apex and are ejected later through the disintegration process. This process is known to provoke the breaking of the cellular apex with the exit of heterolysosomes and residual bodies into the tubule lumen (Cajaraville et al. 1995a) and seems to occur in *C. fluminea* exposed to a high dose of cadmium for 30 days.

Lysosomal enlargement in the mollusc's digestive gland can be induced not only by metals but also by various organic xenobiotics such as PAHs or PCBs (Lowe 1988, Lowe et al. 1995), oil-derived hydrocarbons (Lowe et al. 1981, Cajaraville et al. 1995a, Marigómez & Baybay-Villacorta 2003) and l-naphthol (Cajaraville et al. 1989). These studies concluded that lysosomal enlargement represents a good indicator for general environmental stress (Cajaraville et al. 1995b, 2000).

It should be noted that Asian clams may detect metals at low concentrations and avoid exposure (Tran et al. 2003) by reducing the volume of water used for their filtration activity (Barfield et al. 2001). Moreover, as the animals were not fed, starvation could have affected the results after 30 days of exposure. These responses may be relevant, as animals may encounter both types of stress together in the field (cadmium contamination and starvation). Nevertheless, as the controls were not significantly different during the exposure times, it can be assumed that the observed effects are mainly due to cadmium exposure. The possibility that interactions between starvation and contaminants might have occurred cannot, however, be completely excluded (Liess et al. 2001). Finally, it is relevant to mention the high levels of coincidence between the results of the phagocytosis assays with haemocytes and the results of the lysosomal responses in digestive gland cells. A transient 'recovery' of both biomarkers was observed at the second sampling time period. Both biomarkers showed changes as early as 7 days after exposure, whereas lysosomal changes occurred at a slightly lower dose than suppression of phagocytosis.

Cadmium is a heavy metal and its negative effect has been known for a long time. Biomonitoring in general would be a helpful tool for obtaining information on water quality. However, the reasons for the changes in immune parameters must be clarified in other ways and, because these changes are mostly multifactorial, biomonitoring should always be complemented by other toxicological diagnostic methods.

The present experimental study showed that the phagocytic activity of haemocytes and the structural changes in the digestive lysosomal system reflected changes in the exposure time and in concentrations of metals. Therefore, immunocompetence and digestive lysosomal responses in *Corbicula fluminea* could be used as markers for the assessment of freshwater contamination.

Acknowledgements

This study was supported by a PhD grant from the Environment Department of the Petroleum Society TOTAL. Investigations in the laboratory of M. P. Cajaraville were supported by a grant to consolidated research groups of the University of the Basque Country.

References

Abaychi JK, Mustafa YZ. 1988. The asiatic clam, *Corbicula fluminea*: An indicator of trace metal pollution in the Shatt al-Arab River, Iraq. Environmental Pollution 54:109–122.

Amodio-Cocchieri R, Del Prete U, Arnese A, Giuliano M, Roncioni A. 1993. Heavy metals and polycyclic aromatic hydrocarbons (PAHs) in marine organisms from the Ionian sea (Italy). Bulletin of Environmental Contamination and Toxicology 50:618–625.

Anderson RS, Giam CS, Ray LE, Tripp MR. 1981. Effects of environmental pollutants on immunological competency of the clam *Mercenaria mercenaria*: impaired bacterial clearance. Aquatic Toxicology 1:187–195.

Anderson RS, Oliver LM, Jacobs D. 1992. Immunotoxicity of cadmium for the eastern oyster (*Crassostrea virginica*). Journal of Shellfish Research 14:317–326.

Araujo R, Moreno D, Ramos MA. 1993. The Asiatic clam *Corbicula fluminea* (Müller, 1774) (Bivalvia: Corbiculidae) in Europe. American Malacological Bulletin 10:39–49.

Auffret M, Mujdzic N, Corporeau C, Moraga D. 2002. Xenobiotic-induced immunomodulation in the European flat oyster, *Ostrea edulis*. Marine Environmental Research 54:585–589.

Barfield ML, Farris JL, Black MC. 2001. Biomarker and bioaccumulation responses of Asian clams exposed to aqueous cadmium. Journal of Toxicology and Environmental Health, Part A 63:495–510.

Bassères A, Simonet F, Lafont M, Coste M, Narbonne J-F. 2004. Validation of biomarkers for impact evaluation of aqueous industrial waste in mesocosms. Water Science and Technology 49:123–130.

Baudrimont M, Andres S, Durrieu G, Boudou A. 2003. The key role of metallothioneins in the bivalve *Corbicula fluminea* during the depuration phase, after *in situ* exposure to Cd and Zn. Aquatic Toxicology 63:89–102.

Baudrimont M, Métivaud J, Maury-Brachet R, Ribeyre F, Boudou A. 1997. Bioaccumulation and metallothionein response in the Asiatic clam (*Corbicula fluminea*) after experimental exposure to cadmium and inorganic mercury. Environmental Toxicology and Chemistry 16:2096–2105.

Baumard P, Budzinski H, Garrigues P. 1997. Analytical procedure for the analysis of PAHs in biological tissues by gas chromatography coupled to mass spectrometry: application to mussels. Fresenius Journal of Analytical Chemistry 359:502–509.

Blaise C, Trottier S, Gagné F, Lallement C, Hansen PD. 2002. Immunocompetence of bivalve hemocytes as evaluated by miniaturized phagocytosis assay. Environmental Toxicology 17:160–169.

Brennan RJ, Schiestl RH. 1996. Cadmium is an inducer of oxidative stress in yeast. Mutation Research 356:171–178.

Britton JC, Morton B. 1977. Corbicula in North America: the evidence reviewed and evaluated. Proceedings of the First International Corbicula Symposium, Fort Worth, Texas. Texas Christian University Research Foundation Publication. p. 249–287.

Brousseau P, Pellerin J, Morin Y, Cyr D, Blakley B, Boermans H, Fournier M. 1999. Flow cytometry as a tool to monitor the disturbance of phagocytosis in the clam *Mya arenaria* hemocytes following in vitro exposure to heavy metals. Toxicology 142:145–156.

Cajaraville MP, Abascal I, Etxeberria M, Marigómez I. 1995a. Lysosomes as cellular markers of environmental pollution: time- and dose-dependent responses of the digestive lysosomal system of mussels after petroleum hydrocarbon exposure. Environmental Toxicology and Water Quality: An International Journal 10:1–8.

Cajaraville MP, Bebianno MJ, Blasco J, Porte C, Sarasquete C, Viarengo A. 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. The Science of the Total Environment 247:295–311.

Cajaraville MP, Marigómez JA, Angulo E. 1989. A stereological survey of lysosomal structure alterations in *Littorina littorea* exposed to 1-naphthol. Comparative Biochemistry and Physiology 93C:231–237.

Cajaraville MP, Marigómez JA, Angulo E. 1991. Automated measurement of lysosomal structure alterations in oocytes of mussels exposed to petroleum hydrocarbons. Archives of Environmental Contamination and Toxicology 21:395–400.

Cajaraville MP, Robledo Y, Etxeberria M, Marigómez I. 1995b. Cellular biomarkers as useful tools in the biological monitoring of environmental pollution: molluscan digestive lysosomes. In: Cajaraville MP, editor. Cell Biology in Environmental Toxicology. Bilbao: University of the Basque Country. p. 29–55.

Canesi L, Ciacci C, Betti M, Scarpato A, Citterio B, Pruzzo C, Gallo G. 2003. Effects of PCB congeners on the immune function of *Mytilus* hemocytes: alterations of tyrosine kinase-mediated cell signaling. Aquatic Toxicology 63:293–306.

Cardenas W, Jenkins JA, Dankert JR. 2000. A flow cytometric approach to the study of crustacean cellular immunity. Journal of Invertebrate Pathology 76:112–119.

Cheng TC. 1981. Bivalves. In: Ratcliffe NA, Rowley AF, editors. Invertebrates blood cells – 1 – General aspects, animals without true circulatory systems to cephalopods. London; New York; Toronto; Syndey; San Francisco: Academic Press. p. 233–300.

Cima F, Ballarin L, Bressa G, Burighel P. 1998. Cytoskeleton alterations by tributyltin (TBT) in tunicate phagocytes. Ecotoxicology and Environmental Safety 40:160–165.

Ciutat A, Boudou A. 2003. Bioturbation effects on cadmium and zinc transfers from a contaminated sediment and on metal bioavailability to benthic bivalves. Environmental Toxicology and Chemistry 22:1574–1581.

Desouky M, Jugdaohsingh R, McCrohan CR, White KN, Powell JJ. 2002. Aluminum-dependent regulation of intracellular silicon in the aquatic invertebrate *Lymnaea stagnalis*. Proceedings of the National Academy of Sciences of the USA 99:3394–3399.

Devier MH, Augagneur S, Budzinski H, Mora P, Narbonne JF, Garrigues P. 2003. Microcosm tributyltin bioaccumulation and multibiomarker assessment in the blue mussel *Mytilus edulis*. Environmental Toxicology and Chemistry 22:2679–2687.

Etxeberria M, Cajaraville MP, Marigómez I. 1995. Changes in digestive cell lysosomal structure in mussels as biomarkers of environmental stress in the Urdaibai estuary (Biscay coast, Iberian peninsula). Marine Pollution Bulletin 30:599–603.

Etxeberria M, Sastre I, Cajaraville MP, Marigómez I. 1994. Digestive lysosome enlargement induced by experimental exposure to metals (Cu, Cd and Zn) in mussels collected from zinc-polluted site. Archives of Environmental Contamination and Toxicology 27:338–345.

Fournier M, Pellerin J, Clermont Y, Morin Y, Brousseau P. 2001. Effects of in vivo exposure of *Mya arenaria* to organic and inorganic mercury on phagocytic activity of hemocytes. Toxicology 161:201–211.

Fraysse B, Baudin J-P, Garnier-Laplace J, Adam C, Boudou A. 2002. Effects of Cd and Zn waterborne exposure on the uptake and depuration of 57Co, 110mAg and 134Cs by the Asiatic clam (*Corbicula fluminea*) and the zebra mussel (*Dreissena polymorpha*) – whole organism study. Environmental Pollution 118:297–306.

Giamberini L, Cajaraville MP. 2005. Lysosomal responses in the digestive gland of the freshwater mussel, *Dreissena polymorpha*, experimentally exposed to cadmium. Environmental Research 98:210–214.

Giamberini L, Pihan JC. 1997. Lysosomal changes in the hemocytes of the freshwater mussel *Dreissena polymorpha* experimentally exposed to lead and zinc. Diseases of Aquatic Organisms 28:221–227.

Glinski Z, Jarosz J. 1997. Molluscan immune defenses. Archivum Immunologiae et Therapiae Experimentalis (Warsz) 45:149–155.

Gómez-Mendikute A, Cajaraville MP. 2003. Comparative effects of cadmium, copper, paraquat and benzo[a]pyrene on the actin cytoskeleton and production of reactive oxygen species (ROS) in mussel haemocytes. Toxicology in Vitro 17:539–546.

Hillyer JF, Schmidt SL, Christensen BM. 2003. Hemocyte-mediated phagocytosis and melanization in the mosquito *Armigeres subalbatus* following immune challenge by bacteria. Cell Tissue Res 313:117–127.

Holm PE, Andersen S, Christensen TH. 1995. Speciation of dissolved cadmium: Interpretation of dialysis, ion exchange and computer (GEOCHEM) methods. Water Research 29:803–809.

Jeantet AY, Ballan-Dufrancais C, Martin JL. 1985. Mechanisms of detoxication of cadmium by the oyster *Crassostrea gigas* (Mollusca, Bivalve). II. Intracellular sites of accumulation of the metal in absorbant and excretory organs]. Compte-Rendu de l'Académie des Sciences III 301:177–182.

Kadar E, Salanki J, Jugdaohsingh R, Powell JJ, McCrohan CR, White KN. 2001. Avoidance responses to aluminium in the freshwater bivalve *Anodonta cygnea*. Aquatic Toxicology 55:137–148.

Kollner B, Wasserrab B, Kotterba G, Fischer U. 2002. Evaluation of immune functions of rainbow trout (*Oncorhynchus mykiss*) – how can environmental influences be detected? Toxicology Letters 131:83–95.

Labrot F, Narbonne J-F, Ville P, Saint Denis M, Ribera D. 1999. Acute toxicity, toxicokinetics, and tissue target of lead and uranium in the clam *Corbicula fluminea* and the worm *Eisenia fetida*: comparison with the fish *Brachydanio rerio*. Archives of Environmental Contamination and Toxicology 36:167–178.

L'Azou B, Dubus I, Ohayon-Courtes C, Labouyrie J-P, Perez L, Pouvreau C, Juvet L, Cambar J. 2002. Cadmium induces direct morphological changes in mesangial cell culture. Toxicology 179:233–245.

Leffel EK, Wolf C, Poklis A, White J, Kimber L. 2003. Drinking water exposure to cadmium, an environmental contaminant, results in the exacerbation of autoimmune disease in the murine model. Toxicology 188:233–250.

Lekube X, Cajaraville MP, Marigómez I. 2000. Use of polyclonal antibodies for the detection of changes induced by cadmium in lysosomes of aquatic organisms. The Science of the Total Environment 247:201–212.

Liess M, Champeau O, Riddle M, Schulz R, Duquesne S. 2001. Combined effects of ultraviolet-B radiation and food shortage on the sensitivity of the Antarctic amphipod *Paramoera walkeri* to copper. Environmental Toxicology and Chemistry 20:2088–2092.

Livingstone DR. 1993. Biotechnology and pollution monitoring: use of molecular biomarkers in the aquatic environment. Journal of Chemical Technology and Biotechnology 57:195–211.

Loose LD, Silkworth JB, Charbonneau T, Blumenstock F. 1981. Environmental chemical-induced macrophage dysfunction. Environmental Health Perspectives 39:79–91.

Lowe DM. 1988. Alterations in cellular structure of *Mytilus edulis* resulting from exposure to environmental contaminants under field and experimental conditions. Marine Ecology Progress Series 46:345–358.

Lowe DM, Clarke KR. 1989. Contaminant-induced changes in the structure of the digestive epithelium of *Mytilus edulis*. Aquatic Toxicology 15:345–358.

Lowe DM, Moore MN, Clarke KR. 1981. Effects of oil on digestive cells in mussels: quantitative alterations in cellular and lysosomal structure. Aquatic Toxicology 1:213–226.

Lowe DM, Soverchia C, Moore MN. 1995. Lysosomal membrane responses in the blood and digestive cells of mussels experimentally exposed to fluoranthene. Aquatic Toxicology 33:105–112.

Marigómez I, Baybay-Villacorta L. 2003. Pollutant-specific and general lysosomal responses in digestive cells of mussels exposed to model organic chemicals. Aquatic Toxicology 64:235–257.

Marigómez I, Orbea A, Olabarrieta I, Etxeberria M, Cajaraville MP. 1996. Structural changes in the digestive lysosomal system of sentinel mussels as biomarkers of environmental stress in Mussel-Watch Programmes. Comparative Biochemistry and Physiology 113C:291–297.

Marigómez I, Soto M, Cajaraville MP, Angulo E, Giamberini L. 2002. Cellular and subcellular distribution of metals in molluscs. Microscopy Research and Technique 56:358–392.

Marigómez JA, Cajaraville MP, Angulo E. 1990. Cellular cadmium distribution in the common winkle, *Littorina littorea* (L.) determined by X-ray microprobe analysis and histochemistry. Histochemistry 94:191–199.

Marigómez JA, Vega MM, Cajaraville MP, Angulo E. 1989. Quantitative responses of the digestive-lysosomal system of winkles to sublethal concentrations of cadmium. Cellular and Molecular Biology 35:555–562.

Matozzo V, Ballarin L, Pampanin DM, Marin MG. 2001. Effects of copper and cadmium exposure on functional responses of hemocytes in the clam, *Tapes philippinarum*. Archives of Environmental Contamination and Toxicology 41:163–170.

Moore MN. 1976. Cytochemical demonstration of latency of lysosomal hydrolases in digestive cells of the common mussel, *Mytilus edulis*, and changes induced by thermal stress. Cell Tissue Research 175:279–287.

Narbonne J-F, Daubèze M, Clérandeau C, Guarrigues P. 1999. Scale of classification based on biochemical markers in mussels: application to pollution monitoring in Europeans coasts. Biomarkers 4:415–424.

Renwrantz L. 1990. Internal defence system of *Mytilus edulis*. In: Stefano GB, editor. Studies in Neurosciences, Neurobiology of *Mytilus edulis*. Manchester: Manchester University Press. p. 256–275.

Sauvé S, Brousseau P, Pellerin J, Morin Y, Senecal L, Goudreau P, Fournier M. 2002. Phagocytic activity of marine and freshwater bivalves: *in vitro* exposure of hemocytes to metals (Ag, Cd, Hg and Zn). Aquatic Toxicology 58:189–200.

Simkiss K, Mason AZ. 1983. Metals ions: metabolic and toxic effects. In: Hochachke, P, editor. The Mollusca. pp 101–114. New York: Academic Press.

Soto M, Cajaraville MP, Angulo E, Marigómez I. 1996a. Autometallographic localization of protein-bound copper and zinc in the common winkle, *Littorina littorea*: a light microscopical study. Histochemical Journal 28:689–701.

Soto M, Cajaraville MP, Marigómez I. 1996b. Tissue and cell distribution of copper, zinc and cadmium in the mussel, *Mytilus galloprovincialis*, determined by autometallography. Tissue & Cell 28:557–568.

Soto M, Lekube X, Marigómez I. 1999. Autometallographical localisation of Cu and Zn within target cell compartments of winkles following exposure to Cu&Zn mixtures. European Journal of Histochemistry 43:323–334.

Tran D, Boudou A, Massabuau JC. 2001. How water oxygenation level influences cadmium accumulation pattern in the Asiatic clam *Corbicula fluminea*: a laboratory and field study. Environmental Toxicology and Chemistry 20:2073–2080.

Tran D, Ciret P, Ciutat A, Durrieu G, Massabuau JC. 2003. Estimation of potential and limits of bivalve closure response to detect contaminants: application to cadmium. Environmental Toxicology and Chemistry 22:914–920.

UNEP/RAMOGE 1999. Manual on the Biomarkers Recommended for the MED POL Biomonitoring Programme. Athens : UNEP

Viarengo A, Nott JA. 1993. Mechanisms of heavy metal cation homeostasis in marine invertebrates. Comparative Biochemistry and Physiology 104C:355–372.

Victor B. 1993. Responses of hemocytes and gill tissues to sublethal cadmium chloride poisoning in the crab *Paratelphusa hydrodromous* (Herbst). Archives of Environmental Contamination and Toxicology 24:432–439.

Vidal ML, Bassères A, Narbonne J-F. 2001. Potential biomarkers of trichloroethylene and toluene exposure in *Corbicula fluminea*. Environmental Toxicology and Pharmacology 9:87–97.

Vidal ML, Bassères A, Narbonne J-F. 2002. Seasonal variations of pollution biomarkers in two populations of *Corbicula fluminea* (Müller). Comparative Biochemistry and Physiology 131C:133–151.

Von Gunten HR, Karametaxas G, Krahenbuhl U, Kuslys M, Giovanoli R, Hoehn E, Keil R. 1991. Seasonal biogeochemical cycles in riverborne groundwater. Geochimica et Cosmochimica Acta 55:3597–3609.

Winter S. 1996. Cadmium uptake kinetics by freshwater mollusc soft body under hard and soft water conditions. Chemosphere 32:1937–1948.