

# Relationships between metal bioaccumulation and metallothionein levels in larvae of *Mytilus galloprovincialis* exposed to contaminated estuarine sediment elutriate

Alain Geffard<sup>1,\*</sup>, Olivier Geffard<sup>2</sup>, Edouard His<sup>2</sup>, Jean-Claude Amiard<sup>1</sup>

<sup>1</sup>Service d'Ecotoxicologie, SMAB, ISOMer, GDR CNRS 1117, Université de Nantes, 2 rue de la Houssinière, BP 92208, 44322 Nantes Cedex 3, France

<sup>2</sup>IFREMER, Quai du Commandant Silhouette, 33120 Arcachon, France

**ABSTRACT:** The bioavailability of Cd, Cu and Zn from a metal-contaminated sediment was studied using *Mytilus galloprovincialis* embryos and larvae exposed to sediment elutriates. The elutriate concentrations tested were always lower than the threshold at which abnormal larval development appears. The Cd, Cu and Zn contents of the sediment, of the elutriate and of the larvae, as well as the larval growth, the condition index (CI), and the induction of metallothionein (MT) in the larvae were measured. The CI was only affected after 96 h of exposure, principally at the highest elutriate concentration (25 %, corresponding to 0.1435 µg Cd l<sup>-1</sup> and 14 µg Zn l<sup>-1</sup>). Cd, Cu and Zn bioaccumulation was observed (48 and 96 h), in whole larvae as well as in the cytosolic fraction of their tissues. For the individual treatments (controls or different degrees of exposure), the lowest metal concentrations were observed in larvae after 96 h exposure, indicating biological dilution. Metallothionein induction in larvae was observed after 96 h exposure at the lowest elutriate concentrations tested, and was always strongly correlated with increasing metal contents in the cytosolic fraction. These relationships were significant for all 3 metals studied either individually or combined. The results indicate that these 3 metals are bioavailable to *M. galloprovincialis* larvae and that MT induction constitutes a more sensitive indicator of heavy metal pollution than embryotoxicity or larval growth experiments.

**KEY WORDS:** Sediment elutriate · *Mytilus galloprovincialis* larvae · Metal bioaccumulation · Metallothionein

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## INTRODUCTION

Micropollutants resulting from anthropogenic activities adsorb to fine particles in the water column. The sedimentation of these particles then creates deposits which constitute micropollutant reservoirs with pollutant concentrations 1000 to 5000 times higher than those in water (Livett 1988). The remobilization of sedimentary micropollutants, either by natural processes

(resuspension during tidal movements and periods of high water turbulence) or artificial means (management-related and dredging operations), has been demonstrated in numerous studies (e.g. reviews by Chapman & Long 1983, Burton 1992).

Concentrations of sediment-associated contaminants alone are inadequate to assess the potential threats to the marine and estuarine environment posed by contaminated sediments. The bioassay is an essential tool for environmental assessment, and embryo-larval tests with marine bivalves (oysters of the genus *Crassostrea* and mussels of the genus *Mytilus*) have proved partic-

\*E-mail: alain.geffard@isomer.univ-nantes.fr

ularly sensitive (His et al. 1999). This type of bioassay, first used by Woelke (1972) to evaluate industrial effluents, has also been proposed as a method of quantifying the toxicity of contaminated sediments (Cardwell et al. 1976, Chapman & Long 1983, Melzian 1990, Hill et al. 1993, Louens et al. 1995, Miller et al. 2000).

Metallothioneins (MT), biomarkers of metal exposure, have come into use recently. MT is a cytosolic protein of low molecular weight that has been shown, both in the laboratory and *in situ*, to be induced by metal contamination in numerous taxa, including mammals, fishes and marine invertebrates. It is generally recognised that the primary role of MT is the homeostasis of essential metals such as Cu and Zn. This protein is also involved in the detoxification of non-essential metals such as Ag, Cd and Hg (Roesijadi 1992, Amiard & Cosson 1997, Cosson & Amiard, 2000). The use of bivalve MT as a biomarker of metal exposure has been reviewed recently (Langston et al. 1998, Cosson 2000). MT in adult mussels has been proposed as a biomarker of exposure by George & Olsson (1994) and is used in international biomonitoring programs (UNEP/RAMOGÉ 1999).

An MT-like protein has also been detected in bivalve larvae. Pavicic et al. (1985) found an induction of protein of low molecular weight similar to the mammalian metallothionein in unfertilized eggs and D-stage larva of *Mytilus galloprovincialis* after exposure to 0.1 µg Cd ml<sup>-1</sup> in seawater. (In *M. galloprovincialis*, the D-stage larva is the first larval stage attained 48 h after egg fertilization.) The induction of MT in veliger larvae has been studied in *Crassostrea virginica* by Ringwood & Brouwer (1995) and Roesijadi et al. (1995, 1996, 1997), but these studies were based on exposures to experimental doses that were unrealistic compared to environmental concentrations, even in metal-rich areas.

The present study sought to demonstrate the bioavailability of sediment-bound metals to embryos and larvae of *Mytilus galloprovincialis*. Experiments were carried out using sediment from the Gironde estuary (Atlantic Coast, South of France), which is known to be contaminated by metals (Boutier et al. 1989, RNO 1995, Amiard-Triquet et al. 1998a). On the other hand, low levels of Polycyclic Aromatic Hydrocarbons (<1000 ng g<sup>-1</sup>; Budzinski et al. 1997), polychlorinated biphenyls (Pierard 1995), and organophosphorous and organonitrogenous compounds (Lartiges 1994) have been recorded in this area. This sediment induced 25% abnormality in *Crassostrea gigas* larvae exposed to sediment elutriate (Geffard 2001). In the present study, fertilized mussel eggs were exposed to diluted elutriates (in order to avoid abnormalities) for 48 and 96 h. The bioaccumulation of metals and MT levels was measured in each experimental series at the end of each exposure period.

## MATERIALS AND METHODS

**Sediment sampling and storage.** Sediment was collected in October 1997 at Le Cheyzin (Gironde estuary, Atlantic coast, South of France). Only oxidized (top 1 cm) sediment (about 1.5 kg) was removed for sampling, corresponding to the fraction most likely to be re-suspended during tidal movements. Desorption studies (Amiard-Triquet et al. 1998a) have shown significant mobility of the metals contained in this sediment.

Aliquots of well-homogenized sediment (500 mg) were immediately prepared for metal analysis, and the remainder was freeze-dried, homogenised and stored in hermetically sealed bottles until bioassays were performed in March 1999. Water content was determined by drying to constant weight at 50°C, and organic matter content by drying at 70°C for 48 h followed by incineration at 450°C for 48 h.

**Sediment treatment for analysis and bioassays.** Elutriate was obtained by a modification of the method of Melzian (1990). Of the well-homogenized freeze-dried sediment (initial sample ≈1.5 kg), several samples of 60 g were weighted, rehydrated, shaken mechanically (multi-wrist shaker, 500 rpm) in 240 ml of filtered seawater (FSW; 0.2 µm filter) and then allowed to settle for 8 h. A portion (100 ml) of the elutriate obtained by siphoning was recovered in an acid-washed polypropylene bottle (Berman et al. 1983) and acidified at 1:1000 by Suprapur hydrochloric acid (Carlo Erba). This portion was stored at 4°C in darkness for metal analysis. A second portion was diluted with FSW to test concentrations of 0 (control) 1, 5, 10, and 25%. These concentrations were chosen because previous experiments on the same lyophilised sediment indicated that they did not disturb the embryonic and larval development of *Crassostrea gigas* (Geffard 2001).

**Larval rearing.** Mature mussels *Mytilus galloprovincialis* were collected in the Bay of Arcachon on the day the experiment was performed. Egg-laying was induced by heat shock (alternate immersions in seawater at 16 and 20°C). Females in the laying process were isolated in 1 l of FSW, while spawning males were placed in a small amount (≈100 ml) of FSW to obtain a sperm-dense solution. The oocytes and sperm of different individuals were observed under an inverted microscope, and the best reproductive pair was selected (regularly shaped female gametes and very mobile spermatozooids) for the experiment. The oocytes were fertilized using 5 ml of the sperm-dense solution. Fifteen minutes after fertilization, the embryos were counted and placed in 2 l beakers (60 000 l<sup>-1</sup>) filled with the different media to be tested (6 replicates per treatment).

The embryos were incubated at 21 ± 1°C for 48 h in the presence of the different elutriate concentrations

tested (6 replicates per treatment) until D-stage larvae were obtained. After incubation, 1 portion (3 replicates) of the larvae was sacrificed for experimental purposes, and the remainder was maintained in rearing conditions for an additional 48 h. In both cases, the weight and mean shell height of veligers were determined according to the technique of His et al. (1983). Size was determined by measuring the length across the valves on photographs; 50 individuals were measured from each culture. The veligers maintained in rearing status were placed in 2 l beakers (10 000 larvae  $l^{-1}$ ; 3 replicates) and fed with cultures of *Isochrysis galbana* and *Chaetoceros calcitrans* (50 000 cells  $ml^{-1}$  of a mixture of each alga: Helm & Millican 1977. A condition index (CI) was estimated for each of the rearing categories as follows:

$$CI = \frac{\text{mean weight of a lyophilised larva } (\mu\text{g})}{\text{mean shell height of a larva } (\mu\text{m})} \times 100$$

After counting, the larvae were washed with 0.9% aqueous ammonium formate isotonic with seawater (elimination of NaCl: Holland & Hannant 1973), frozen, lyophilised and stored for chemical and biochemical analysis.

**Chemical analysis. Pretreatment of sediments:** Aliquots (0.5 g) of fresh sediment were taken from the well-homogenised total sample and placed in acid-washed glass tubes. These samples were then dried and weighted to determine metal concentration as a function of dry weight. Hot mineralization (95°C) was performed by addition of 5 ml  $HNO_3$  and 3 ml HCl. This process was conducted until dryness, and the residues were then resuspended in 10 ml of 1 N HCl for analysis of the metals (Charlou & Joanny 1983).

**Pretreatment of elutriates:** The extraction and concentration of metals (Cd, Cu, Zn) was performed in FSW (control) and raw elutriate according to the method of Boiteau & Métayer (1978) as modified by Amiard et al. (1991b). At pH 9, Cd, Cu and Zn were complexed by dithizone in a chloroform phase. However, at pH 2, Cd and Zn enter into acid phase, while Cu remains in the chloroform phase (Charlot 1966, Irving 1977). Eight ml of ammonium citrate buffer (which

increased the pH from 8.0 to 9.0) and 2 ml of dithizone in chloroform solution were added to 100 ml of elutriate or FSW (control) and shaken for 30 min. The FSW (control) or elutriate was then siphoned off, and the dithizone was washed away with deionized water before addition of 1 ml 1 N Suprapur hydrochloric acid. At this pH ( $\approx 2$ ), the Cd and Zn bound to dithizone entered into the acid phase and were analysed, whereas Cu was measured directly in the dithizonate phase.

**Extraction of metals and metallothionein from larvae:** The tissue compartmentalization of metals and the partial purification of MT were carried out according to the method described by Mouneyrac et al. (1998). Each replicate of lyophilised larvae was homogenized with a hand-held glass-grinder in 0.02 M, Tris-NaCl buffer, pH 8.6, at a ratio of 5 ml buffer  $g^{-1}$  of larvae (wet weight). Proteolytic reactions and the oxidation of MT molecules were avoided by working at 4°C with  $\beta$ -mercaptoethanol (10 mmol  $l^{-1}$ ) added to the Tris buffer. Cytosolic (S1) and insoluble (P1) fractions were separated by initial centrifugation (25 000  $\times g$ , 55 min at 4°C). MT was isolated from an aliquot of the S1 fraction by a second centrifugation (15 000  $\times g$ , 10 min at 4°C) after heating for 15 min at 75°C. This second (S2) supernatant containing the MT was frozen at -80°C until MT analysis. Before metal analysis, an acid digestion step at 60°C was required for the soluble (S1) and insoluble (P1) fractions. This step lasted 12 h and involved the addition of Suprapur nitric acid (Carlo Erba) at a ratio of 1:1 S1 supernatant and 1 ml nitric acid per 0.5 g larvae (P1). The solutions obtained were brought to a known volume (2 ml) with deionized water.

**Metal assays:** Following the acid digestion phase, metals were analysed by flame atomic absorption spectrophotometry (AAS) for Cu and Zn, or electrothermal AAS with the Zeeman effect (Hitachi Z8200) for Cd. The analytical method has been described previously by Amiard et al. (1987). Standard addition analysis was performed in an isomedium and the concentrations of each element were +125, 250 and 500 ng  $ml^{-1}$  for Cu and Zn and +0.25, 0.5 and 1 ng

Table 1. Results of internal quality control (mean concentrations  $\pm$  SD in mg  $kg^{-1}$  dry wt) in mussels and sediment. BCR: Community Bureau of Reference

Source	Cadmium	Copper	Zinc
<b>Mussel tissue BCR Standard Reference Material 278 R, Sample Identification No. 622</b>			
Our values	0.349 ( $\pm$ 0.013)	9.58 ( $\pm$ 0.12)	76.2 ( $\pm$ 0.5)
Certified values	0.34 ( $\pm$ 0.002)	9.6 ( $\pm$ 0.16)	76 ( $\pm$ 2)
<b>Standard marine sediment homogenate SD-M-2/TM IAEA MONACO</b>			
Our values	0.127 ( $\pm$ 0.003)	28.7 ( $\pm$ 0.3)	71.8 ( $\pm$ 2.25)
Certified values	0.113 (0.108–0.149)	32.7 (31.7–34.2)	74.8 (72.0–78.3)

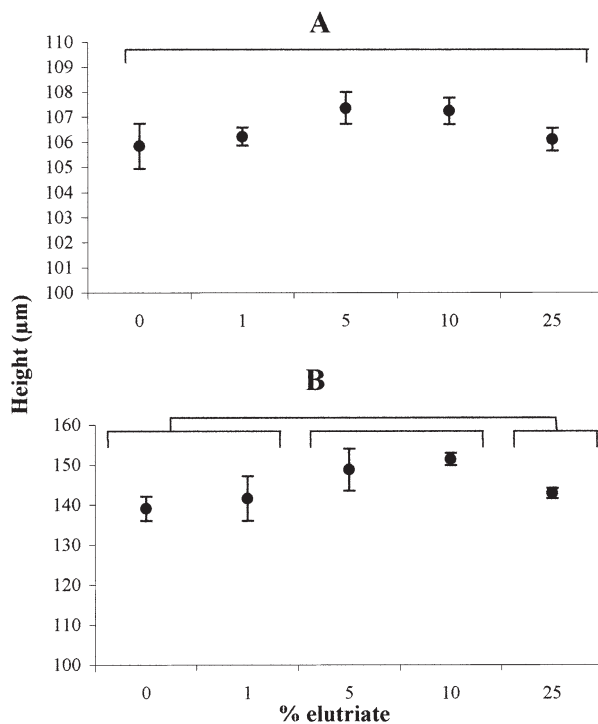


Fig. 1. *Mytilus galloprovincialis*. Height ( $\mu\text{m} \pm \text{SD}$ ) of larvae after exposure to a range of sediment elutriate percentages for 48 h (A) and 96 h (B). Values not significantly different from each other are grouped under a common overhead line (ANOVA,  $p < 0.05$ ; Tukey's HSD test). Each mean corresponds to 3 replicates of ca. 120 000 and 20 000 larvae at 48 and 96 h, respectively

$\text{ml}^{-1}$  for Cd. The assays were validated using certified sediment and mussel tissue samples (Table 1). The results are expressed as  $\text{mg kg}^{-1}$  dry weight. The total bioaccumulation of metals (Cd, Cu and Zn) in the larvae was calculated by summing up the amounts in the soluble (S1) and insoluble (P1) fractions.

**Assay of larval metallothionein:** The MT assay was performed in the S2 fraction by differential pulse polarography. The thiol groups (SH) were determined using Brdicka reagent (Brdicka 1933) according to the method described by Thompson & Cosson (1984). Measurements were performed at a constant temperature ( $4^{\circ}\text{C}$ ) on a polarograph with a PAR Model 174 analyser, a PAR/EG&G Model 303 electrode in SMDE mode and an RE0089 type X-Y recorder. The MT amounts measured were determined by a standard addition method using MT rabbit liver metallothionein standard (Sigma Chemical Co.) (no MT standard exists for mussels). The validity of this method has been confirmed by Olafson & Olsson (1991). The results are expressed in  $\text{mg kg}^{-1}$  dry weight.

**Statistical analysis.** For each series of results, the comparison of values was tested by Student's *t*-tests or 1-way ANOVA (Statistica software) after checking the homogeneity of variances (Cochran's test). Significant differences (at the 95 % level) were then determined by Tukey's HSD test. Linear regressions were performed and correlation coefficients (*r*) determined using Excel software.

## RESULTS

### Characterization of sediment, FSW and elutriate

The water content in the sediment tested was 51 %. The organic matter decreased from 7.12 % in raw sediment to 3.6 % after the preparation of the elutriate, indicating that about 50 % of the sediment organic matter had been remobilized as soluble compounds in the elutriate. Cd, Cu and Zn concentrations (expressed in  $\mu\text{g g}^{-1}$  dry weight) were 0.67, 33 and 218, respectively, for the sediment, and  $0.485 \mu\text{g Cd l}^{-1}$  and  $35 \mu\text{g Zn l}^{-1}$  for the elutriate (Table 2) (the samples for Cu determination of the elutriate were lost). Thus, dilutions of the raw elutri-

Table 2. *Mytilus galloprovincialis*. Cadmium, copper and zinc concentrations in the sediment ( $\mu\text{g g}^{-1}$  dry wt  $\pm$  SD), filtered seawater ( $\mu\text{g l}^{-1}$ , control used in this study), and concentrations at various estuarine and marine locations

	Cadmium	Copper	Zinc	Source
Sediment	0.67 (0.04)	33 (3)	218 (31)	This study
Filtered seawater (Arcachon)	0.015	0.4	7	This study
Raw elutriate	0.485	Lost	35	This study
Loire estuary water (mouth)	0.37 (0.05–1.43)	1.72 (0.6–2.3)	4.2 (0.7–10.6)	RNO (1984)
Loire estuary water (open sea)	0.02 (0.02–0.02)	1.67 (0.6–2.7)	3.85 (1.9–5.8)	RNO (1984)
Gironde estuary water (mouth)	0.67 (0.3–1.7)	5.92 (2–14)	12 (6–24)	RNO (1984)
Gironde estuary water (mouth)	0.380			Cossa & Lassus (1989)
Gironde estuary water (open sea)	0.25 (0.20–0.30)	3.6 (2.2–5)	10 (8–12)	RNO (1984)
Atlantic Ocean water (surface)	0.002–0.016			Boutier et al. (1989)

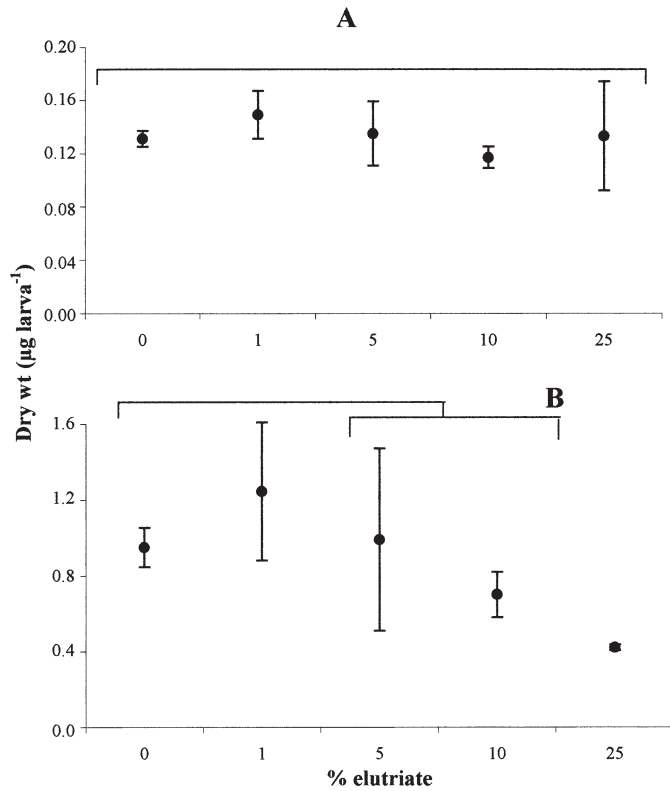


Fig. 2. *Mytilus galloprovincialis*. Dry weight ( $\mu\text{g} \pm \text{SD}$ ) per larva after exposure to a range of sediment elutriate percentages for (A) 48 h and (B) 96 h. Values not significantly different from each other are grouped under a common overhead line (ANOVA,  $p < 0.05$ ; Tukey's HSD test). Each mean corresponds to 3 replicates of ca. 120 000 and 20 000 larvae at 48 and 96 h, respectively

ate led to concentrations of 0.015, 0.0197, 0.0385, 0.062 and 0.14375  $\mu\text{g Cd l}^{-1}$  and 7, 7.28, 8.4, 9.8 and 14  $\mu\text{g Zn l}^{-1}$  in 0, 1, 5, 10 and 25% elutriate, respectively.

### Larval rearing

No larval deaths and abnormality occurred in the different rearing conditions. No significant differences in shell height were observed in any of the categories after 48 h (Fig. 1A). However, after 96 h (Fig. 1B), mean shell height had increased significantly (Tukey's HSD test,  $p = 0.013$ ) from 138  $\mu\text{m}$  for controls to 149 and 151  $\mu\text{m}$  for larvae exposed to 5 and 10% elutriate, respectively. Mean shell height was only 141  $\mu\text{m}$  for larvae exposed to 25% elutriate, a value not significantly different from that of controls. The mean weight of 1 larva was calculated for the different categories. No significant differences were apparent in any of the categories at 48 h (Fig. 2A). At 96 h, mean weight decreased significantly ( $p < 0.05$ ) when the percentage

of elutriate increased from 1 to 25% (Fig. 2B). The lowest value (0.422  $\mu\text{g}$ ) was obtained with 25% elutriate. After 48 h, the CI had not changed relative to the elutriate level (Fig. 3A). However, at 96 h (Fig. 3B) it decreased in larvae exposed to 5–25% elutriate, although the decrease only became significant ( $p < 0.05$ ) upon exposure to 10% elutriate.

### Bioaccumulation of metals by larvae

The concentration of total Cd (Fig. 4A) in veligers increased as a function of increasing elutriate percentage. The increase became significant at 48 h for concentrations above 5%, with values ranging from 0.15  $\text{mg kg}^{-1}$  (controls) to 0.45  $\text{mg kg}^{-1}$  (25% elutriate). After 96 h exposure, the increase was significant ( $p = 0.0001$ ) for 10% elutriate and above, and Cd concentrations ranged from 0.06  $\text{mg kg}^{-1}$  (controls) to 0.39  $\text{mg kg}^{-1}$  (25% elutriate).

The total concentration of Cu (Fig. 4B) also increased in larvae as a function of increasing elutriate percentage. This effect was noted with 5% elutriate and above after 48 h exposure and for 10% elutriate and above after 96 h. At 48 h, Cu concentrations ranged from 5.11  $\text{mg kg}^{-1}$  (controls) to a maximum value of 11.6  $\text{mg}$

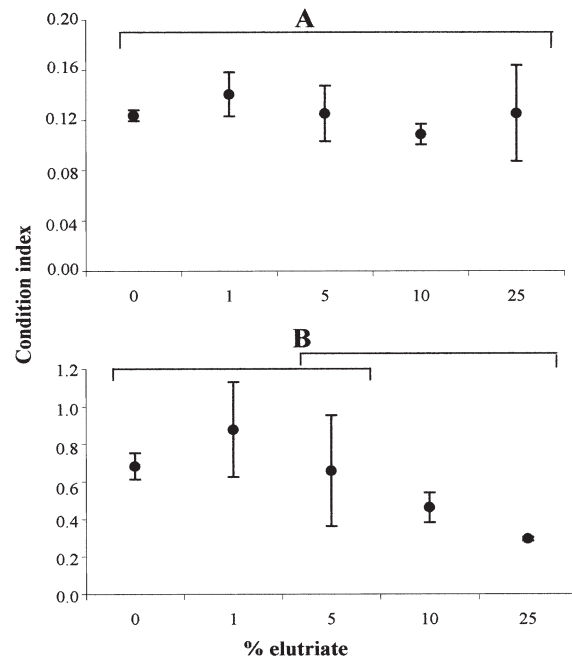


Fig. 3. *Mytilus galloprovincialis*. Condition index ( $\pm \text{SD}$ ) of larvae after exposure to a range of sediment elutriate percentages for (A) 48 h and (B) 96 h. Values not significantly different from each other are grouped under a common overhead line (ANOVA,  $p < 0.05$ ; Tukey's HSD test). Each mean corresponds to 3 replicates of ca. 120 000 and 20 000 larvae at 48 and 96 h, respectively

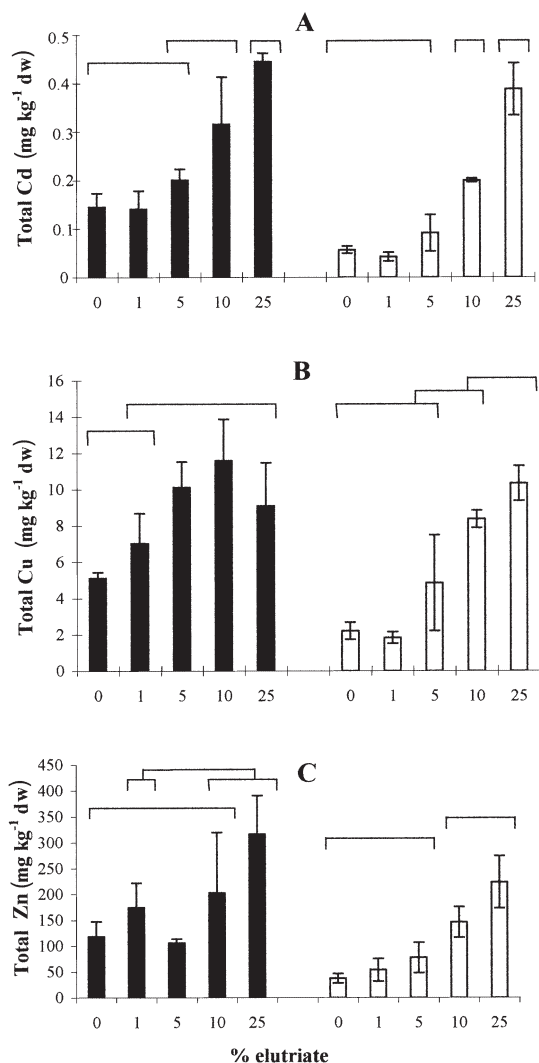


Fig. 4. *Mytilus galloprovincialis*. Total metal (soluble + insoluble; mean  $\pm$  SD) concentrations (A: Cd; B: Cu; C: Zn) in larvae after exposure to a range of sediment elutriate percentages for 48 h (solid bars) and 96 h (open bars). Values not significantly different from each other are grouped under a common overhead line (ANOVA,  $p < 0.05$ ; Tukey's HSD test). Each mean corresponds to 3 replicates of ca. 120 000 and 20 000 larvae at 48 and 96 h, respectively

kg<sup>-1</sup> (10% elutriate), and after 96 h, Cu concentrations ranged from 2.21 mg kg<sup>-1</sup> for controls to 10.35 mg kg<sup>-1</sup> for individuals exposed to 25% elutriate.

For Zn (Fig. 4C), a significant increase after 48 h was noted only at the highest elutriate percentage tested, with values of 119 mg kg<sup>-1</sup> in controls and 316 mg kg<sup>-1</sup> with 25% elutriate. After 96 h, the increase was significant for 10% elutriate and above, with values of 38 mg kg<sup>-1</sup> for controls and 224 mg kg<sup>-1</sup> with 25% elutriate.

For each percentage of elutriate tested, the metal concentration was generally lower in specimens exposed for 96 h than in those exposed for 48 h (except in the case of Cu with larvae exposed to 25% elutriate). However, for all 3 metals studied, these differences were significant ( $0.0009 < p < 0.0392$ ) only in control and in larvae exposed to 1 and 5% elutriates. For each metal and condition, the total metal quantities (S1 + P1) per individual were always greater ( $0.0001 < p < 0.0255$ ) after 96 h exposure (not shown), indicating that despite a lowering of concentrations, metals were incorporated in larvae throughout the exposure.

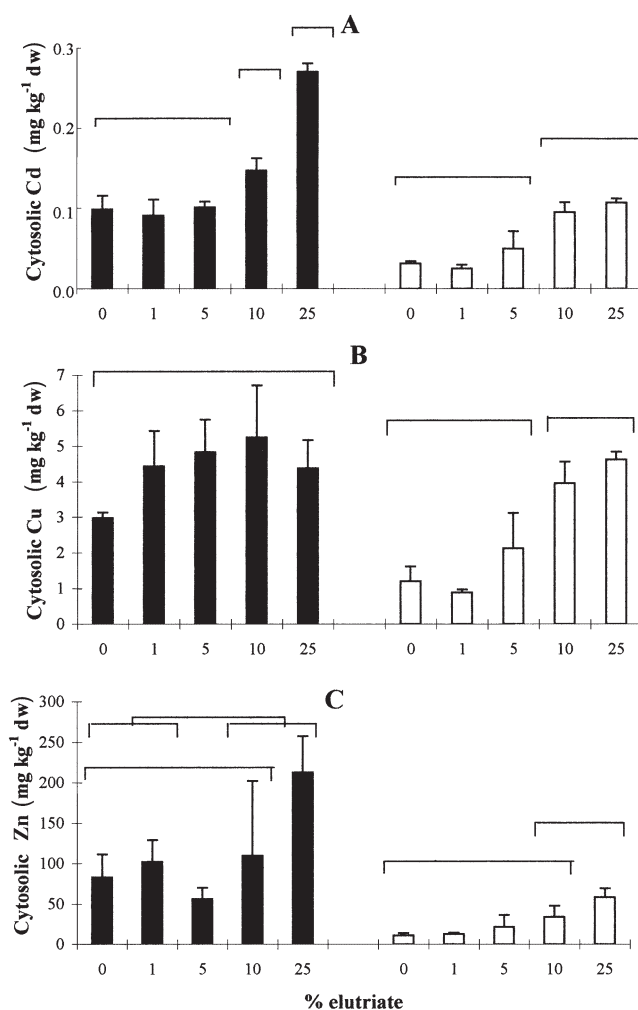


Fig. 5. *Mytilus galloprovincialis*. Cytosolic (S1) metal (mean  $\pm$  SD) concentrations (A: Cd; B: Cu; C: Zn) in larvae after exposure to a range of sediment elutriate percentages for 48 h (solid bars) and 96 h (open bars). Values not significantly different from each other are grouped under a common overhead line (ANOVA,  $p < 0.05$ ; Tukey's HSD test). Each mean corresponding to 3 replicates of ca. 120 000 and 20 000 larvae at 48 and 96 h, respectively

For the cytosolic fraction (Fig. 5), metal concentrations generally increased significantly with increasing degree of exposure (percentage of elutriate). The concentrations of cytosolic Cd (Fig. 5A) increased significantly ( $p < 0.05$ ) after 48 h at the highest percentages of elutriate tested (10 and 25%), with values of  $0.10 \text{ mg kg}^{-1}$  for controls and  $0.27 \text{ mg kg}^{-1}$  with 25% elutriate. At 96 h, this rise was significant for 10% elutriate and above, with values of  $0.03 \text{ mg kg}^{-1}$  for controls and a maximum value of  $0.11 \text{ mg kg}^{-1}$  with 25% elutriate.

Individuals exposed to elutriate for 48 h (Fig. 5B) had Cu concentrations greater than those of controls, although the difference was not significant. Cu concentrations varied from  $2.99 \text{ mg kg}^{-1}$  in controls to  $5.24 \text{ mg kg}^{-1}$  with 10% elutriate. At 96 h, the increase was significant ( $p = 0.0001$ ) for 10% and above, and cytosolic Cu concentrations ranged between  $0.88 \text{ mg kg}^{-1}$  with 1% elutriate and  $4.63 \text{ mg kg}^{-1}$  with 25%.

Cytosolic Zn (Fig. 5C) increased significantly after 48 h exposure to 25% elutriate compared to individuals exposed to 5% elutriate, with values between  $56 \text{ mg kg}^{-1}$  (5% elutriate) and  $212 \text{ mg kg}^{-1}$  (25% elutriate). After 96 h, the increase was significant for 10% elutriate and above, with values of  $11 \text{ mg kg}^{-1}$  in controls and  $58 \text{ mg kg}^{-1}$  with 25% elutriate.

Specimens exposed for 96 h exhibited lower metal concentrations than those exposed for 48 h, except in the case of Cu when specimens were exposed to 25% elutriate. These differences were generally significant except for Cu and Zn in specimens exposed to 10% elutriate ( $p = 0.3412$  and  $p = 0.35$ , respectively). As mentioned above for total metal quantities (S1 + P1), the quantities of cytosolic metals were significantly ( $0.0001 < p < 0.0414$ ) greater in individuals exposed for the longer period, except for Cd with 25% elutriate ( $p = 0.2051$ ) and Zn for all exposure levels ( $p > 0.0767$ ) (not shown).

The distribution of Cd among cytosolic and insoluble tissue fractions remained the same, regardless of the incorporation level after 48 h (Fig. 6A). However, at 96 h, Cd became predominant in the insoluble fraction when incorporation increased (Fig. 6B), reaching 75% of total Cd in larvae exposed to 25% elutriate. Cu was distributed equally between the 2 tissue fractions and for both time periods (data not shown). For 48 h old larvae, cytosolic Zn increased as a function of the degree of contamination, reaching 60 to 70% in the most contaminated individuals (Fig. 7A). After 96 h, Zn was found mainly in the insoluble fraction, with a maximum value of 80% for individuals exposed to 25% elutriate (Fig. 7B).

### Metallothionein

MT concentrations are shown in Fig. 8. During the first 48 h of exposure, MT concentration was not signif-

icantly affected by exposure of larvae to different doses of elutriates in their medium ( $p = 0.932$ ). In larvae exposed for 96 h, a significant ( $p = 0.0008$ ) increase in MT concentration was observed in specimens exposed to doses of 10% elutriate and above. As shown previously for the metals, MT concentrations were significantly lower ( $0.0001 < p < 0.0458$ ) after 96 h exposure than after 48 h, except for larvae exposed to the highest dose of elutriate ( $p = 0.9908$ ). In contrast to the results for the MT concentrations, at 96 h the amount of MT did not increase with increasing metal accumulation (not shown): the amount of MT was significantly ( $p = 0.001$ ) higher in larvae exposed to any elutriate concentration ( $0.58$  to  $0.69 \text{ ng larvae}^{-1}$ ) than in controls ( $0.42 \text{ ng}$ ), but was similar in all specimens exposed to the various concentrations (1 to 25%) of elutriates.

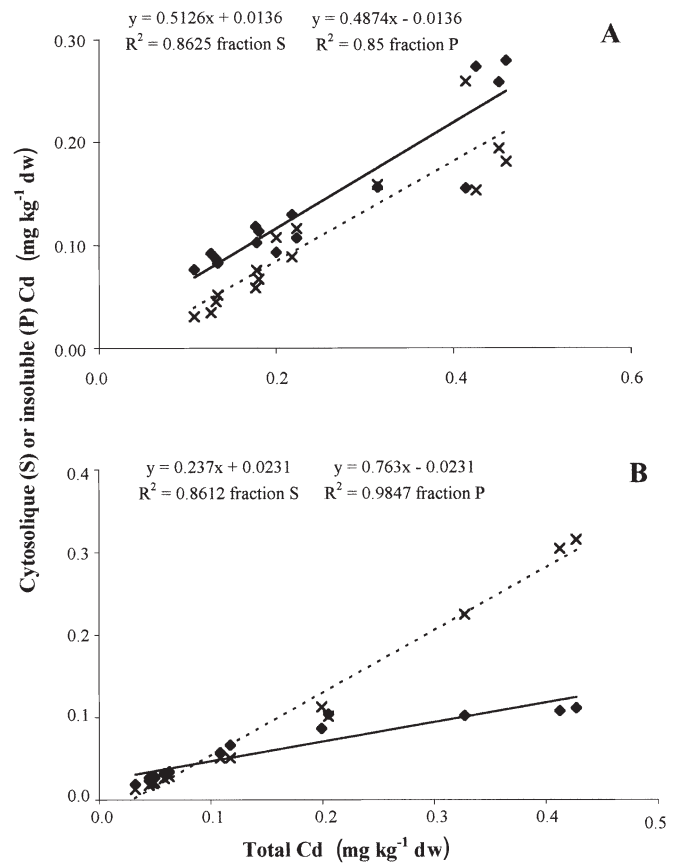


Fig. 6. *Mytilus galloprovincialis*. Relationships between total and cytosolic (solid lines) or insoluble (dotted lines) Cd in larvae after exposure to a range of sediment elutriate percentages for (A) 48 h or (B) 96 h. Equations and correlation coefficients were determined for each fraction. Each replicate contained ca. 120 000 and 20 000 larvae at 48 and 96 h, respectively

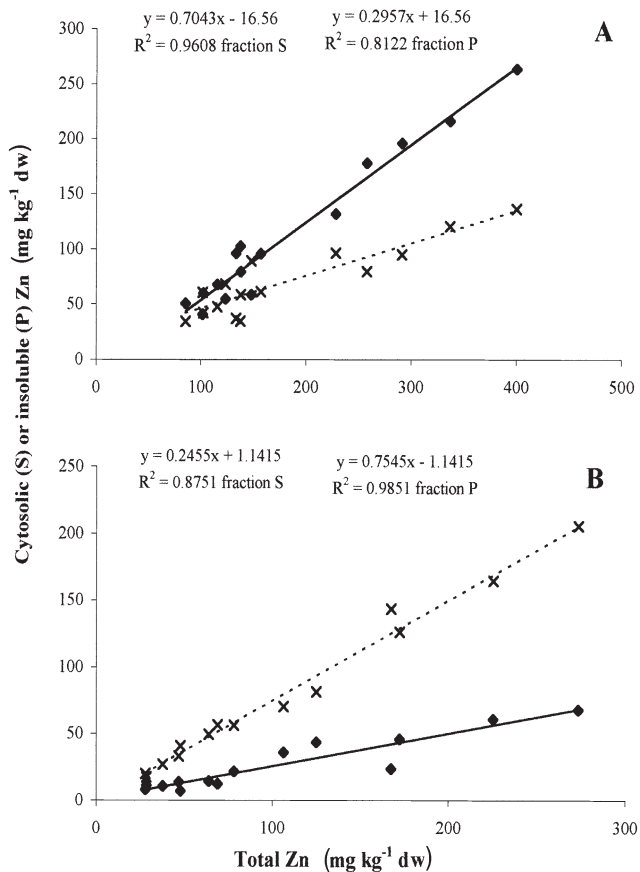


Fig. 7. *Mytilus galloprovincialis*. Relationships between total and cytosolic (solid lines) or insoluble (dotted lines) Zn in larvae after exposure to a range of sediment elutriate percentages for (A) 48 h or (B) 96 h. Equations and correlations coefficient were determined for each fraction. Each replicate contained ca. 120 000 and 20 000 larvae at 48 and 96 h, respectively

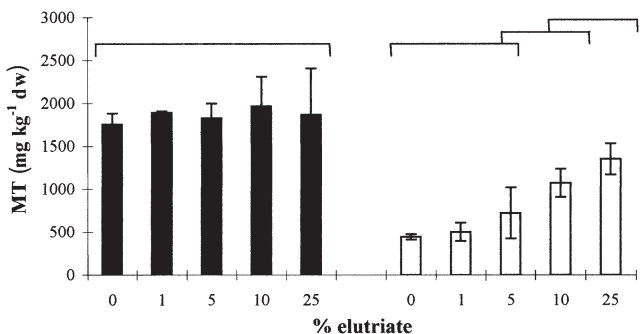


Fig. 8. *Mytilus galloprovincialis*. Metallothionein concentrations (means  $\pm$  SD) in larvae after exposure to a range of sediment elutriate percentages for 48 h (solid bars) or 96 h (open bars). Values not significantly different from each other are grouped under a common overhead line (ANOVA,  $p < 0.05$ ; Tukey's HSD test). Each mean corresponds to 3 replicates of ca. 120 000 and 20 000 larvae at 48 and 96 h, respectively

Table 3. *Mytilus galloprovincialis*. Equations showing relationship between cytosolic Cd, Cu, Zn or Cd+Cu+Zn and metallothionein in larvae after 96 h exposure to a range of sediment elutriate concentrations. n = number of samples (3 for each elutriate concentration tested);  $r^2$  = correlation coefficient; \*\*relationship significantly correlated at 99%

Metal	Equation	n	$r^2$
Cd	$y = 10\,166x + 205.5$	15	0.888**
Cu	$y = 228.53x + 240.03$	15	0.8829**
Zn	$y = 16.043x + 380.01$	15	0.7168**
Cd+Cu+Zn	$y = 1015.9x + 341.36$	15	0.7458**

**Relationships between metals and metallothionein**

As all 3 elements studied (Cd, Cu and Zn) bind to MT and might therefore contribute concomitantly to MT induction, the relationship between MT and metal levels was examined taking into account the metals individually or combined (mg at.  $kg^{-1}$  dry wt)

No significant correlation was observed between concentration of MT and that of cytosolic Cd, Cu, Zn or a mixture of these 3 metals during the first period of exposure (48 h; results not shown). In larvae exposed for 96 h, strongly significant positive relationships were always observed (Table 3), whether metals were considered individually or combined.

**DISCUSSION**

The sediment from the Gironde estuary tested in the present study was a fine mud with relatively high levels of organic matter. Desorption tests have shown that 15, 35 and 55% of sediment-bound Cd were extractable at pH 7.1, 5.5 and 4.1, respectively (Geffard 2001). Similar results were obtained in sediments collected previously in the same estuary (Amiard-Triquet et al. 1998a). Moreover, bioassays carried out with the sediment used in the present study showed that the main metal contaminants (Cd, Cu and Zn) were bioavailable, as demonstrated by the large number of larval abnormalities found at high concentrations of elutriate (100%: Geffard 2001). The seawater used in the present study was obtained from the Bay of Arcachon, which is extensively used for oyster farming based on Pacific oysters *Crassostrea gigas* and was thus assumed to have good 'biological quality' (His et al. 1999). (For instance, as shown in Table 2, metal concentrations were consistently lower in the Bay of Arcachon than in the Loire and Gironde estuaries.) From the Cd and Zn concentrations in the raw elutriate and seawater, and the percentages of this elutriate tested in the bioassays herein, it is estimated that larvae were



exposed to doses varying from 0.019 to 0.122  $\mu\text{g Cd l}^{-1}$  and from 7.3 to 14  $\mu\text{g Zn l}^{-1}$ , concentrations representative of those found in the Gironde estuary waters (Table 2). According to Boutier et al. (1989), seawater must be considered contaminated if Cd concentrations exceed 0.03  $\mu\text{g l}^{-1}$ . Nevertheless, the concentrations of both Cd and Zn were lower than those affecting embryogenesis in *Mytilus galloprovincialis* ( $\text{EC}_{50}$  of 4000  $\mu\text{g l}^{-1}$  for Cd and 145  $\mu\text{g l}^{-1}$  for Zn; see review by His et al. 1999). The  $\text{EC}_{50}$  for Cu were comparatively very low, from 3.2 to 10  $\mu\text{g l}^{-1}$  (His et al. 1999), that is to say, in the same order of magnitude as Cu concentrations at the mouth of the Gironde (Table 2). The speciation of these metals in natural waters of the Gironde estuary as well as in the elutriate, and thus their potential toxicity, presumably differed from those of the metal salts used in other bioassays. It has been shown that a significant fraction of the organic matter initially present in the sediment was indeed remobilized concomitantly with metals during elutriation.

No mortality occurred during the experiments and no abnormal larvae were observed. No significant differences were registered in regard to shell height, weight or the condition index of larvae exposed to sediment elutriates for 48 h. At 96 h, mean shell height was slightly greater in 5 and 10% elutriates, either as a result of hormesis phenomena (Stebbing 1982) or the additional supply of small amounts of essential elements or soluble organic matter which may have enhanced larval growth. However, at 25% elutriate, larval height was no longer different from that of controls. The 96 h old larvae reared at elutriate concentrations higher than 10% had a lower weight than controls, with a correspondingly lower condition index (CI), which may thus represent an early indicator of stress. Determination of the CI in veligers exposed to micropollutants may therefore constitute a more sensitive toxicity endpoint than larval growth based on the determination of height, which is the criterion commonly used to assess sublethal toxicity (His et al. 1999).

Metal levels in the whole larvae as well as in their cytosol (particularly Cd), increased with increasing degree of contamination in the experimental medium. The potential bioavailability of sediment-bound metals was also observed by Fichet et al. (1998) in the larvae of several invertebrate species. At similar doses of contaminants in the experimental medium, metal levels in larvae were lower at 96 h than at 48 h. Such a phenomenon, termed 'biological dilution', corresponds to a tissue growth which is faster than metal incorporation, leading to a decrease in the concentration. Similar negative relationships between metal concentration and weight (and/or age and/or size) have been observed in adults from different species (Langston et al. 1998), including mussels and oysters (Amiard et al.

1986, Amiard & Berthet 1996) and particularly the spat of clams *Ruditapes philippinarum* (Amiard et al. 1991a) and oysters (Amiard et al. 1994). Moreover, in the bivalve *Isognomon californicum*, Ringwood (1991) observed a higher accumulation rate of Cd during embryogenesis than in veligers and pediveligers, with highest concentrations in embryos. As for metals, MT concentrations were lower in 96 h old larvae. The general growth of larval tissues was more important than the induction rate of this particular protein. Similarly, negative relationships between tissue weight and MT concentrations have been observed previously in adults belonging to different bivalve species, such as *Crassostrea gigas* (Mouneyrac et al. 1998) or *Macoma balthica* (Amiard-Triquet et al. 1998b).

No MT induction was observed during embryogenesis (48 h) in larvae exposed to different doses of elutriates. This may be due to the fact that little uptake occurred in the soluble fraction, except at the highest doses of Cd and Zn. Pavicic et al. (1994) observed MT induction during embryogenesis, but in specimens exposed to high doses of Cd (2.75  $\text{mg l}^{-1}$ ). At the lower doses tested in our study, the amount of MT in oocytes (termed 'maternal MT' by Roesijadi et al. 1996 in *Crassostrea virginica*) could be sufficient to regulate the essential needs of the larvae, particularly with respect to Zn, as well as to handle excessive amounts of Cd. In addition, in regard to MT concentrations, the important decrease in 96 h old specimens compared to 48 h old is striking. It must be noted that the tissue distribution of Cd and Zn is strongly modified during this period of the life cycle, with the highest concentrations of soluble metals and the highest MT concentrations being recorded in 48 h old specimens. If larvae have to cope with metals after this period of embryogenesis, their native MT is no longer sufficient to detoxify additional metals, particularly Cd and Cu, which have the greatest affinity for this protein (Viarengo & Nott 1993). Therefore, between the age of 2 and 4 d, MT induction enables the larvae to detoxify Cd and other essential elements present in excessive amounts. Indeed, in the 96 h old larvae, the MT concentration increase paralleled increasing doses of sediment elutriate in the medium. However, in these 96 h old larvae, MT amounts were higher in exposed larvae than in controls, but no significant differences were revealed between elutriate treatments (1 to 25% elutriate). The similarity of MT amounts in 96 h old larvae exposed to a range of contaminant doses as well as their relatively poor physiological state (CIs lower than controls) could evoke a situation which has previously been termed 'spillover' (Brown & Parsons 1978). This phenomenon has been described in field experiments with the freshwater bivalve *Corbicula fluminea* translocated along a polymetallic (Cd, Zn) pollution gradient: at the most

polluted station, where mortality occurred, MT levels did not increase despite a very important metal accumulation, whereas at lesser impacted sites, positive correlations were registered between metal and MT levels (Baudrimont et al. 1999).

## CONCLUSIONS

No assessment of potential noxious effects on living organisms can be derived from chemical analysis carried out to determine the pollution of sediments. The complex actions and the bioavailability of contaminants depend on their speciation (Kersten & Förstner 1989, Luoma 1995), and accurate assessment requires bioassays (Chapman & Long 1983, Burton 1992, Miller et al. 2000).

By determining veliger contamination and MT induction, bioassays with D-stage larva of *Mytilus galloprovincialis* in the present study showed that contaminated sediment from the Gironde estuary releases metal micropollutants into the water column. As indicated by Bayne (1985): 'the molecules that probably offer the greatest potential for monitoring of biological effects ... are enzymes, and the functional proteins such as metallothioneins'. Bivalve embryos and larvae are the marine organisms of choice for bioassays. In a review of the literature, His et al. (1999) classified the various larval stages by their sensitivity to micropollutants as: growth > embryogenesis > D-stage larva > umboned larva > pediveliger. In agreement with the conclusions of Bayne (1985) and Pavicic et al. (1994), the present study confirms the greater sensitivity of MT induction compared to larval growth (based on larvae height) and even the CI in veligers.

With regard to regulations for the protection of the coastal environment, the assessment of sediment quality, which is essential to any management activity, is mainly based today on tests with various marine organisms. The 'sediment quality triad' of Chapman et al. (1987) is one of the most sophisticated methods for this purpose. If measures are to be taken to protect the economically important aquaculture sector, it is necessary to use the most sensitive instruments available, such as an early indicator of stress. This is particularly true for enclosed bodies of water in which the natural reproduction of bivalves constitutes the basis for an entire area of the economy. A striking example of this is tributyltin (TBT) in antifouling paints, which caused reproduction failure in *Crassostrea gigas* over a period of about 5 yr and almost led to the disappearance of oyster culture in the Bay of Arcachon (His 1996).

In closed bays with extensive reproduction, it is possible to monitor cohorts of larvae in the natural environment, both for their growth and biochemical com-

position (His & Maurer 1988). However, except in special cases, it is difficult to identify the possible causes of reproductive failure (i.e. poor larval growth, with important size differences within the same cohort) that may result from various environmental factors and/or 1 or more micropollutants in the environment (e.g. His et al. 1999). As it is possible to isolate bivalve larvae in sufficient numbers for biochemical analysis, it should be easy to identify unfavourable causes due to metal micropollutant effects by measuring MT induction in veligers.

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