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Use of metallothionein in gills from oysters (*Crassostrea gigas*) as a biomarker: seasonal and intersite fluctuations

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The use of oyster gills for the analytical determination of metallothionein (MT) concentration as a biomarker of metal exposure was investigated. Temporal variations in MT and metal concentrations (which can interfere with inter-site differences) were examined over a 7 month period (from spring to autumn) in Japanese oysters from a clean site (Bay of Bourgneuf, France) and a metal-rich site (Gironde estuary, France) as well as in individuals translocated from the clean to the contaminated area. The ratio between the annual average of MT concentrations in specimens from the clean and the metal-rich sites was 1.3. During the last 3 months of the experiment, significant differences were no longer registered between transplants and residents from the Gironde estuary. Metals concentrations in oyster gills differed consistently between the clean and the metal-rich sites (annual average ratios of 1.5, 2.7 and 9.8, respectively, for zinc, copper and cadmium) and a fast increase in metal concentrations (over a few months) was observed in transplants, mainly for cadmium. MT and soluble metal concentrations were found to be positively and significantly correlated over the period of the study. This relationship is a positive argument for a possible use of gill MT concentration as a biomarker of metal pollution in contrast to previous findings on the digestive gland, there being a smaller amount of seasonal variability in the weight of oyster gills.

Keywords: Metallothionein, biomarker, metallic pollution, oyster, translocation.

Introduction

Metallothioneins (MTs) are metalloproteins, the presence of which has been recognized in numerous phyla, including vertebrates, invertebrates, plants and micro-organisms. It is generally thought that these molecules play a role in the homeostasis of the essential metals zinc (Zn) and copper (Cu) and are involved in the detoxification of non-essential trace elements such as silver (Ag), cadmium (Cd) and mercury (Hg). The known induction of MT synthesis in aquatic organisms (fish, crustaceans and molluscs) by Cd, Cu and Zn has led to the suggestion that MT determinations could provide a suitable monitoring procedure for assessing metal contamination in the marine environment (George and Olsson 1994, Langston *et al*. 1998, Cosson 2000, Cosson and Amiard 2000).

MT induction was first demonstrated in organisms exposed to metals under laboratory conditions, generally in studies using short-term exposure to unrealistically high metal concentrations (Cosson *et al*. 1991). Contrary to the idea that using high doses would produce 'better MT induction', it appears that the acute toxicity of metals, particularly non-essential ones such as Cd, reinforces the negative effects of laboratory conditions, disturbing total protein metabolism

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and thus limiting MT synthesis. For example, in fish injected intraperitoneally with Cd at subtoxic doses, good correlation between metal and MT levels were obtained in the liver, whereas this relationship deviated from linearity at hepatotoxic concentrations (George and Olsson 1994). Similar findings were obtained in the crustacean *Tigriopus brevicornis* exposed to waterborne Hg (Barka 2000, Barka *et al*. 2001). In the gills of the oyster *Crassostrea gigas*, similar increases in MT levels were shown in specimens exposed in a Cd-rich estuary (Gironde, France) or in the laboratory at an experimental dose as high as 200 ng ml^{-1}, which induced an elevation of cytosolic Cd levels 40 times higher than in non-experimental specimens (Géret *et al*. 2000). This discrepancy may be attributed to the toxicity of Cd in this experiment, since 17% mortality was registered in oysters exposed for 21 days (Géret 2000). Such a phenomenon which has previously been termed 'spill-lover' (Brown and Parsons 1978) and has also been recognized in the field in freshwater bivalves (*Corbicula fluminea*) (Baudrimont *et al*. 1999).

It is generally agreed that, in vertebrates, metal binding to MT is the major pathway for metal detoxification, whereas in invertebrates, this process co-exists with biomineralization of metals into insoluble deposits (Brown 1982, George 1990, Mason and Jenkins 1995). Such deposits have been shown in oysters (George *et al*. 1978, Thomson *et al*. 1985). Due to the difference in importance of MT in metal detoxification in vertebrates and invertebrates, it has been suggested that fish probably provide a better biological matrix for the use of MT as a biomarker. However, from the point of view of biomonitoring, bivalves are generally recognized as the most suitable indicator species (NAS 1980). In 1994, George and Olsson concluded that *Mytilus* sp. were the only promising invertebrate candidate for metal monitoring based on MT determination. However, numerous studies, including field studies, have shown that MT concentrations fluctuate in a concentration-dependent manner according to changes in the ambient levels of contaminants in a number of different invertebrates, including sea urchins (Aspholm and Hylland 1998), crustaceans (Pedersen *et al*. 1997) and bivalves such as the freshwater species *Pyganodon grandis* (Couillard *et al*. 1995a, b, Wang *et al*. 1999), the oyster *C. gigas* (Mouneyrac *et al*. 1998), *Ruditapes decussatus* (Hamza-Chaffai *et al*. 1999) and *Macoma balthica* (Mouneyrac *et al*. 2000).

Tissue-related variations in MT levels have been reported in numerous species (Cosson 2000). The highest MT concentrations are generally found in the digestive gland of bivalves compared with the gills and other tissues (Bebianno *et al*. 1993, Géret *et al*. 1998, Mouneyrac *et al*. 1998). However, the low levels of significant *de novo* synthesis of MT in response to experimental Cd exposure reduces the value of the digestive gland for metal biomonitoring using MT as a biomarker, whereas gills appear to be a more suitable tissue in different species (*R. decussatus*, Bebianno *et al*. 1993; *C. fluminea*, Baudrimont *et al*. 1997). Despite its high level of MT, the digestive gland of the oyster *C. gigas* has limited value as a tissue for biomonitoring based on MT measurement (Geffard *et al*. 2001). In contrast, several authors have concluded that in mussels (*Mytilus edulis* or *Mytilus galloprovincialis*), analysis of the digestive gland seems more relevant than that of the gills (Amiard *et al*. 1998, Amiard-Triquet *et al*. 1998a, Raspor *et al*. 1999), whereas seasonal metabolic changes limit the efficiency of using MT as a biomarker at certain periods of the year.

These findings illustrate the influence of biotic and abiotic factors, which can interfere with MT synthesis in response to exposure to metallic contaminants. Body weight fluctuations associated with reproduction were probably responsible for the seasonal changes in MT concentrations observed in Baltic clams (Bordin *et al.* 1997). The sampling period, sex and size affected MT levels in a natural population of the clam *R. decussatus* (Hamza-Chaffai *et al.* 1999). In the oyster *C. gigas*, weight appeared to be an important factor in explaining variations in MT concentrations (Mouneyrac *et al.* 1998), whereas patterns of metal binding to MTs were significantly affected by temporal variation in *C. virginica* (Roesijadi 1994a).

Translocation experiments have been widely used (Couillard *et al.* 1995a, b, Baudrimont *et al.* 1999, Wang *et al.* 1999, Geffard *et al.* 2001) and caging is also a recommended procedure in biomonitoring programmes (UNEP/RAMOGE 1999). The length of translocation must allow translocated individuals to reach equilibrium, since the distribution of metals among cellular ligands, including MT, changes during exposure (Ishiguro *et al.* 1982, Köhler and Riisgard 1982, Nolan and Duke 1983, Evtushenko *et al.* 1986, Couillard *et al.* 1995b, Mouneyrac *et al.* 1999). If months of exposure are needed, seasonal variability is obviously not avoided by using translocation studies.

The present study aimed to evaluate MT as a biomarker of metal contamination based on the use of oyster gills as the preferred tissue for the analytical determination of MT levels. Temporal variations in MT and metal concentrations (which can interfere with intersite differences) were examined over a 7 month period (from spring to autumn) in Japanese oysters from a clean site (Bay of Bourgneuf, France) and a metal-rich site (Gironde estuary, France), as well as in individuals translocated from the clean to the contaminated area.

Materials and methods

Clean and contaminated near-shore coastal sites were selected from the data of the French biomonitoring programme based on chemical analysis in oysters and mussels (RNO 1995). Oysters (*C. gigas*) from a relatively clean coastal area devoted to oyster culture (Bay of Bourgneuf, France) were selected according to their size, caged in plastic bags identical to those used by oyster farmers, and translocated to the metal-rich Gironde estuary, 25 km upstream from the mouth of the river, in March 1997. Monthly sampling was then carried out until October 1997. Translocated oysters are referred to here as BG (originating from B for Bourgneuf, transferred to G for Gironde). Resident oysters from the Bay of Bourgneuf (group BB, originating from and remaining in B for Bourgneuf) were used as controls, and oysters growing naturally in the Gironde estuary (group GG, originating from and remaining in G for Gironde) were collected concomitantly. Specimens were transported to the laboratory in ice packs in an isothermic container at −20°C prior to their treatment. In each group, eight individuals were analysed monthly from 20 collected samples in order to select specimens similar in size. The controls and transplants, obtained from an oyster farm, had the same age and size. Due to their irregular forms, feral oysters sampled in the Gironde estuary were relatively difficult to select visually in the field according to their size.

The pretreatment of samples for metal analysis was carried out avoiding secondary contamination. All laboratory ware was soaked in 10% HCl, rinsed three times with deionized water and dried in a dessicator. Individual gills were homogenized in a buffer solution (20 mM TRIS, 150 mM NaCl, pH 8.6). Then cytosolic (S1) and insoluble (P1) fractions were separated by centrifugation (25 000*g* for 55 min at 4°C). An aliquot was retained for MT analysis. To determine metal concentrations in gill tissue fractions S1 or P1, it was necessary to carry out digestion of these samples using suprapure nitric acid (Carlo Erba) at 60°C for a minimum of 12 h. The volume was then adjusted to 5 ml with deionized water. Metals were determined in these acid solutions by flameless (Cd in gills of controls) or flame (all other determinations) atomic absorption spectrophotometry (AAS) using the Zeeman effect (Hitachi Z 8200) according to the analytical method described by Amiard *et al.* (1987). This methodology has been validated through international intercalibration exercises (Coquery and Horvat 1996, Campbell *et al.*

2000). Internal quality controls were based on the analysis of the metals of interest in standard reference materials (oyster tissue, US NBS/SRM 1566; mussel tissue, BCR 278 R).

The cytosolic heat-stable compounds (MTs) were isolated by centrifugation of the soluble fraction aliquots after heat treatment (for method, see Geffard *et al.* 2001). The amount of MT was determined in the heat-denaturated cytosol (S2) by differential pulse polarography, a technique based on –SH compound determination according to the Brdicka reaction (Brdicka 1933), as described by Thompson and Cosson (1984).

Differences between concentrations in different groups of oysters were evaluated using paired *t*-tests or by analysis of variance (ANOVA), and *post hoc* comparisons were assessed by the multiple range test of Scheffé, using a standard statistical package (StatView^{TM}SE + Graphics). Linear regressions and correlation coefficients were determined using Excel97.

Results

Biometric data

Gill weights are shown in table 1 for the different groups of oysters collected monthly. Temporal fluctuations within each group were examined using ANOVA. No significant temporal fluctuations were shown in controls (BB) from the Bay of Bourgneuf ($p = 0.113$), whereas some heterogeneity was demonstrated in residents (GG) of the Gironde estuary ($p = 0.0001$) and in specimens transplanted (BG) from the clean to the contaminated site ($p = 0.0054$). However, when differences were examined using Scheffé contrasts, no differences between the monthly samples were revealed in transplants (BG) and only a limited number of significant differences were shown in residents from the Gironde (GG) (March and July > April and October).

Within each month, intergroup differences were also examined. The gill weights were always comparable for transplants BG and residents GG from the Gironde estuary. In contrast, except in March (ANOVA, $p = 0.7234$) and July ($p = 0.064$), gill weights of controls BB were generally significantly higher than those of both other groups.

Table 1. Wet weight of gills of oysters originating from the Bay of Bourgneuf (BB), from the Gironde estuary (GG) or translocated from the first to the second site (BG)

	BB		GG		BG	
	Mean (g)	SD (g)	Mean (g)	SD (g)	Mean (g)	SD (g)
March	0.89	0.19	0.96[c]	0.26	—	—
April	0.86[a]	0.16	0.44[c,d]	0.19	0.62	0.16
May	0.98[a]	0.18	0.75	0.20	0.67	0.10
June	1.08[a]	0.25	0.71	0.22	0.80	0.13
July	1.02	0.17	1.02[d]	0.22	0.84	0.10
August	1.02[a]	0.20	0.69	0.20	0.78	0.12
September	1.10[a]	0.32	0.73	0.19	0.69	0.18
October	0.85[b]	0.16	0.53[c,d]	0.07	0.62	0.25

[a] Mean for the specimens from the clean site BB higher than that for specimens from the metal-rich site (resident GG or translocated BG).

[b] BB > GG.

[c] Mean in March significantly higher than in April and October.

[d] July > April and October (Scheffé's test).

Variations in MT and metal concentrations

At the beginning of the experiment, determined MT concentrations were higher on average in specimens from the Gironde estuary (GG) than in those originating from the clean site (BB), but the difference was not statistically significant (figure 1, which includes the results of the statistical study of intersite differences). During the first months of the study, MT concentrations remained relatively steady in all the groups of oysters, followed by a noticeable increase in September and October. In July and during the following months, MT concentrations became significantly higher in the gills of specimens from the Gironde estuary (resident GG and/or translocated BG) than in oysters from the clean site (BB). During the last 3 months of the experiment, no significant differences were registered between the residents of the Gironde estuary (GG) and translocated oysters (BG). The ratio between the annual average MT concentrations in specimens from the clean and the metal-rich sites was 1.3.

Variations in metal concentrations are depicted in figure 2 (together with the results of the statistical study of intersite differences). For Cd, the most striking features are the differences observed between controls BB and residents of the Gironde estuary GG, as well as the fast increase in Cd concentrations in the transplanted specimens. Within 3 months of their transplantation, no significant differences were observed between the transplants BG and the residents GG. In oysters from the clean site BB, Cd concentrations decreased during the first 4 months of the study, reaching the lowest value in July, and then increased again. In specimens from the Gironde estuary (GG), the seasonal tendency was not so pronounced, with maxima in May and August and the lowest values in October. The rapid increase in metals in the transplants BG as well as the intersite difference (BB versus GG) also occurred with Cu and Zn (figure 2), although the ratios between the maximum and minimum values were considerably smaller.

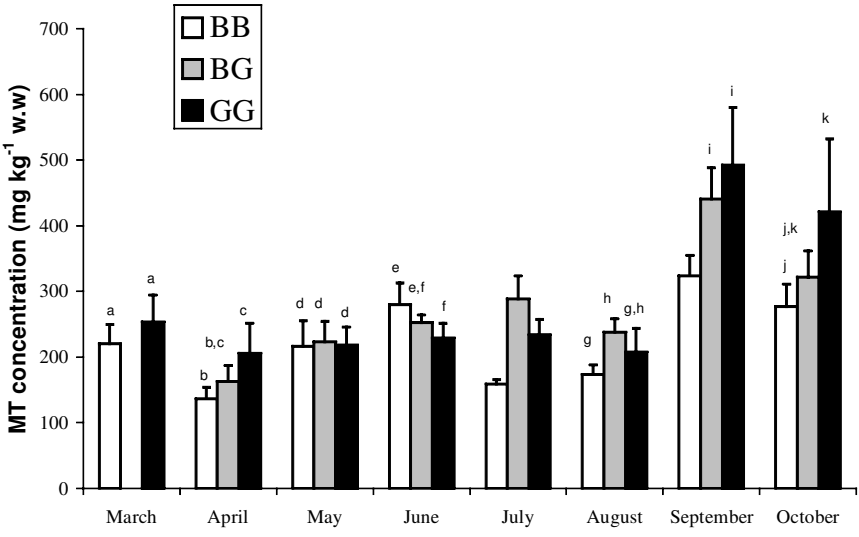

Figure 1. Spatial and temporal variations in MT concentrations in oyster gills (mean +95% confidence interval expressed in mg kg^{-1} w/w). Within each month, groups of data with the same superscripts did not differ significantly at the 95% level. Bars without superscripts are significantly different from the other two.

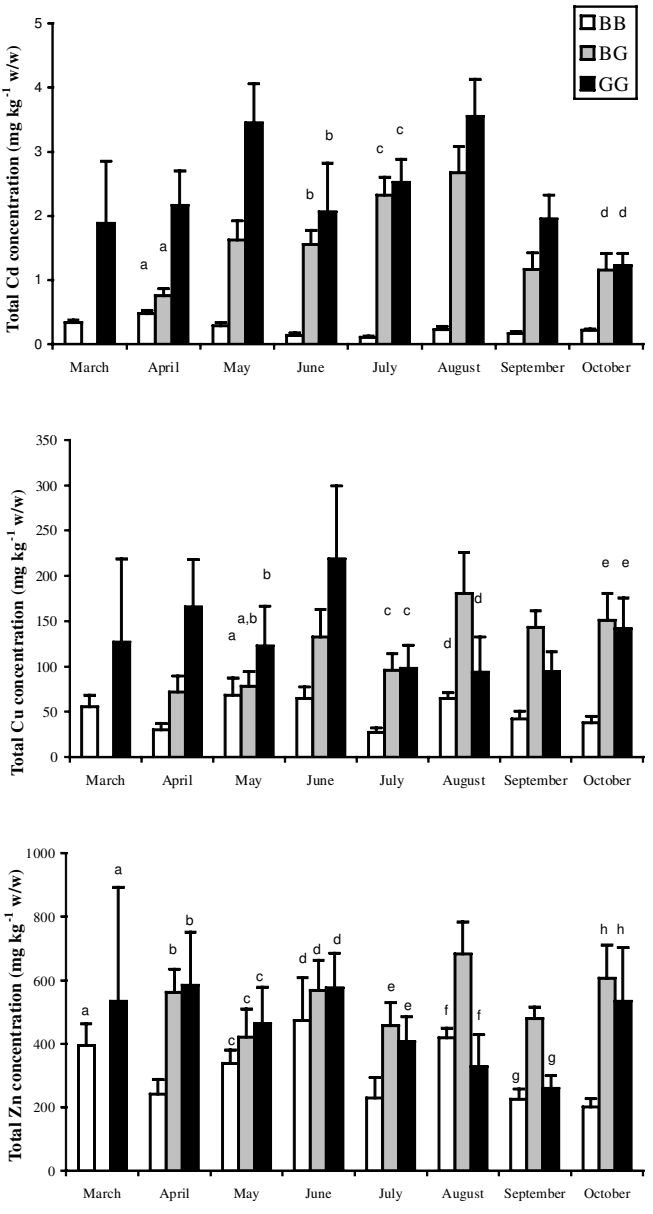

Figure 2. Spatial and temporal variations of total metal (S1 + P1) concentrations in oyster gills (mean and 95% confidence interval expressed in mg kg^{-1} w/w). Within each month, groups of data with the same superscripts did not differ significantly at the 95% level. Bars without superscripts are significantly different from the other two.

The ratio between the annual average metal concentrations in specimens from the clean and the metal-rich sites was as high as 9.8 for Cd, compared with 2.7 for Cu and 1.5 for Zn.

To take into account the potential influence of temporal weight changes on MT and metal concentrations, a correlation matrix was established using linear relationships between these factors in oysters originating from the clean (BB) or

Table 2. Relationship between MT or metal concentrations and the weight of gills: values of correlation coefficient r for oysters from a clean (BB, $n = 64$) and a metal-rich site (GG, $n = 62$)

	Geographical origin	
	BB	GG
MT	-2.66×10^{-3}	-0.237
Total Cd	-0.373^{a}	$+0.072$
Total Cu	-0.020	-0.189
Total Zn	-0.022	-0.164

[a] Significant at the level of 99%.

the metal-rich (GG) site. In these resident oysters it may be considered that there was an equilibrium between incorporated metals and metals in the environment. In contrast, in translocated oysters this equilibrium did not exist, at least during the first months of the experiment, and temporal changes in MT or metal concentrations were likely to depend on supplementary accumulation in the metal-rich site rather than on weight changes. Metal (S1 + P1) and MT concentrations were generally negatively correlated with weight, but the correlations were generally not significant, except for Cd in oysters from the clean site (table 2).

Tissue distribution of metals

The distribution of metals between soluble (S1) and insoluble (P1) fractions was examined as a function of increasing concentrations of the total metal (S1 + P1) in all the individuals collected at different sites and dates (figure 3). The parameters of the equations of the linear regressions between soluble or insoluble metals versus total metals are shown in table 3. The representativeness of the linear model is satisfactory since correlations were always significant at the level of 99%.

With increasing concentrations of total Cd up to 1 mg kg^{-1} or of total Cu up to 100 mg kg^{-1} in the gills, both of these elements were stored increasingly in the cytosolic fraction (figure 3A and B), with slopes consistently higher for the soluble metals (table 3). At higher concentrations (figure 3C and D), the distribution remained steady, with slopes nearly equal for both the soluble and insoluble fractions (table 3). In the specimens that had incorporated globally more Cd and Cu, the predominance of cytosolic Cd and Cu was still observed. On the other hand, Zn was equally distributed between the soluble and insoluble fractions over the whole range of total Zn concentrations (figure 3E).

In figure 4 the temporal changes in the tissue distribution of metals are shown using the percentages of metals present in the soluble fraction. At both sites, temporal changes were moderate, with the exception of maxima observed for all the three metals in June in the Bay of Bourgneuf (BB) and 1 month later in the Gironde estuary (GG), mainly for Cu. No temporal changes were observed in the transplants (BG).

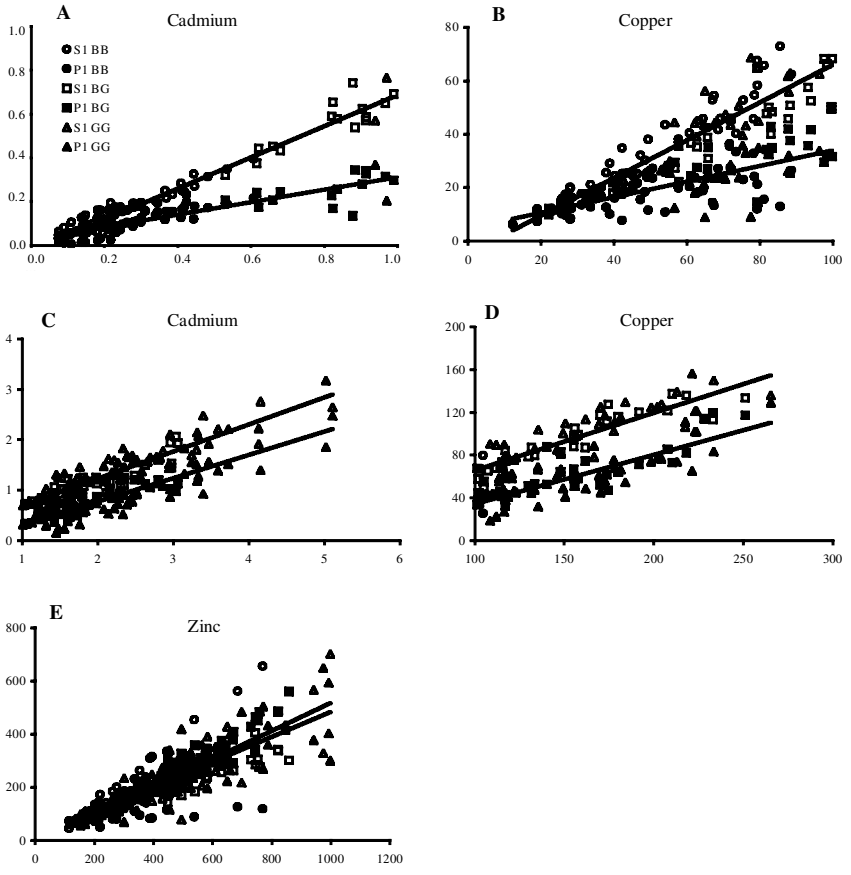

Figure 3. Relative importance of soluble and insoluble tissue fractions in bioaccumulation of metals in oyster gills. *x* axis, total metal (S1 + P1) concentration expressed in $mg\,kg^{-1}$ w/w. *y* axis, concentration of metals in soluble (S1, open symbols) and insoluble (P1, filled symbols) forms expressed in $mg\,kg^{-1}$ w/w. The parameters of the straight line equations are shown in table 3.

Table 3. Parameters of the equations ($y = ax + b$) of the linear regressions between concentration of soluble or insoluble metals *y* versus concentration of total metal *x* shown in figure 3

x axis	*y* axis	*n*	*a*	*b*	r^{2a}
Cd < $1\,mg\,kg^{-1}$	S1	80	0.714	−0.026	0.959
	P1	80	0.286	+0.026	0.790
Cd > $1\,mg\,kg^{-1}$	S1	100	0.537	+0.151	0.830
	P1	100	0.463	−0.151	0.784
Cu < $100\,mg\,kg^{-1}$	S1	107	0.708	−4.73	0.851
	P1	107	0.292	+4.73	0.494
Cu > $100\,mg\,kg^{-1}$	S1	72	0.538	+12.1	0.775
	P1	72	0.462	−12.1	0.718
Zn	S1	180	0.471	+11.7	0.707
	P1	180	0.529	−11.7	0.752

[a]Significant at the level of 99% for all the equations.

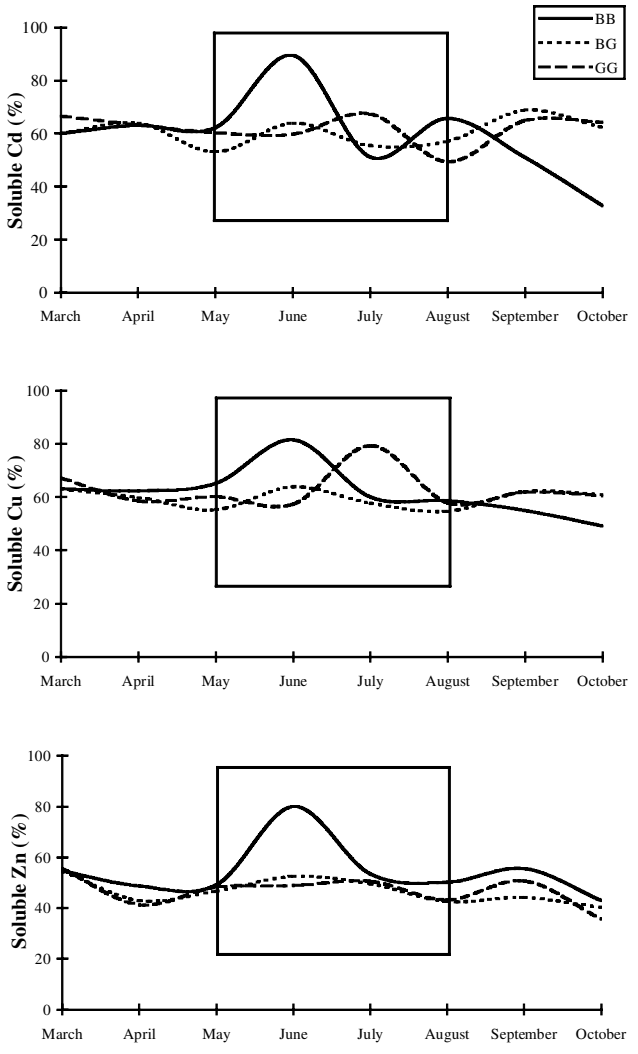

Figure 4. Temporal and spatial variations in the percentages of metals in soluble forms in oyster gills.

Relationship between metal and MT concentrations

Because MT is a cytosolic heat-stable protein, it should be preferable to examine the relationship between metal and MT levels, taking into account metal concentrations in the supernatant S2 obtained after heat denaturation of the cytosol. However, the fate of metals during heating is a matter of question, since the analysis of metals in chromatographic fractions separated from total cytosol S1 and heat-denaturated cytosol S2 has revealed differences in metal binding to cytosolic ligands (Berthet, personal communication). Metal levels in cytosol S1 versus MT are depicted in figure 5. Linear regressions were calculated for each set of monthly data, including controls, transplants and residents from the Gironde estuary. Each curve is shown only between limits corresponding to the minimum and maximum values for x determined for each given month among the three groups of oysters. MT concentrations were often correlated significantly and positively to soluble metal concentrations, with the exception of May and June for

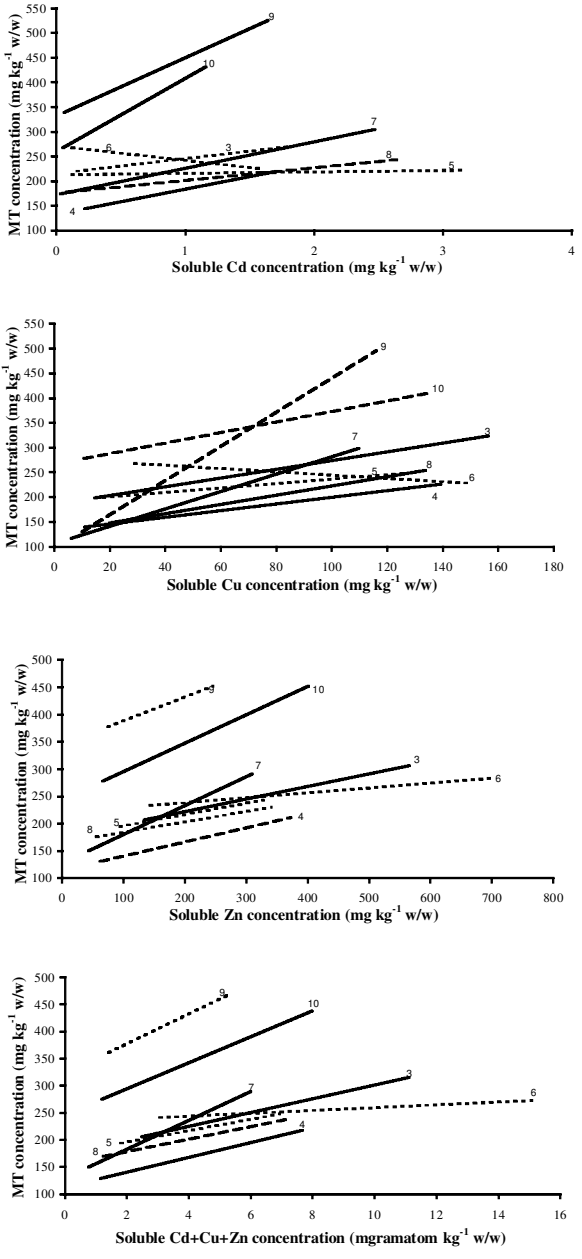

Figure 5. Relationships between metal (individual or added) and MT concentrations in oyster gills. Best-fit straight lines were calculated monthly (each month indicated with its rank in the year) for controls, residents and transplants from the Gironde estuary together. Continuous line, correlation significant at the level of 99%; broken line, correlation significant at the level of 95%; dotted line, non-significant correlation.

all of the three studied metals; this absence of significant correlation was also observed in March for Cd and in August and September for Zn.

All three of the studied metals (Cd, Cu and Zn) bind to MT and might therefore contribute concomitantly to MT induction, so soluble metal concentra-

tions were calculated by adding together soluble Cd, Cu and Zn concentrations expressed in mgramatoms. MT concentrations were positively correlated to metal concentrations but the correlation was not significant in May, June, and September. However, this global relationship was strongly influenced by the relationship between MT and Zn, since this metal made the highest contribution to the total metal content.

Discussion

The high concentrations of Cd, Cu and Zn in oysters from the Gironde estuary are consistent with previous reports of elevated concentrations of these metals in this area (RNO 1995, Amiard-Triquet *et al.* 1998b, Mouneyrac *et al.* 1998), whereas the lower concentrations determined in specimens from the Bay of Bourgneuf are in agreement with the findings of Amiard and Berthet (1996), who considered that this area, devoted to oyster culture, is nearly free from anthropogenic metallic pollution.

The so-called 'Mussel Watch' (NAS 1980) and its French version 'RNO' (national observation web of the marine environmental quality) is based on metal analysis in mussels as a bioindicator species, but in some areas, where mussels are missing, oysters have been used in their place. The same problem arises with the methodology of biomarkers and thus it is important to determine the best procedure in order to use oyster MT in biomonitoring. The first question is probably the choice of tissue on which the analysis will be performed. Due to high concentrations of both metals and MT in the digestive gland, and considering the precedent of mussels (Amiard-Triquet *et al.* 1998a, Raspor *et al.* 1999, UNEP/RAMOGE 1999), the feasibility of MT analysis was first examined in this tissue, but the study concluded that it had limited usefulness (Geffard *et al.* 2001). Important seasonal variations in weight were recognized in the digestive gland that were positively correlated with MT concentrations but negatively correlated with metal concentrations. Thus fluctuations in MT concentrations did not consistently reflect changes in metal exposure and incorporation, even if significant relationships were demonstrated in September and October.

A major argument for the potential use of gills for monitoring MT induction was their lesser contribution to the reproductive process and associated weight fluctuations. The present study is in agreement with this presupposition, since both spatial and temporal fluctuations of gill weight were limited. As a consequence of these moderate changes, the concentrations of MT and metals were not strongly affected by weight and, moreover, the trend was the same for the protein and the contaminants, a slight decrease in concentrations being observed in specimens with the highest weights. In the case of seasonal and inter-annual variations, de-seasonalizing methods and analysis of time-series data may be used (Cossa 1989).

In agreement with our findings, Roesijadi (1994a) has suggested that using oyster gills for MT determination allows the high degree of natural variability in oysters to be minimized. In *C. gigas* exposed to waterborne Ag, Cd, Cu, Hg or Zn, a significant increase in MT concentration was observed for all of the five studied metals in the gills, whereas no additional MT synthesis was observed in the digestive gland except in specimens exposed to Cd (Cosson 2000), suggesting again

that gills must be preferred to the digestive gland as the biological matrix for MT determinations in biomonitoring programmes.

Considering either individual soluble metals or the sum of Cd, Cu and Zn (all of them able to contribute to MT induction), a significant positive correlation was observed in most of the monthly samples collected from the spring to the autumn. The major exception was the absence of significant correlations in May and June for all three metals studied, when considered individually or together. This period corresponds to the ripening of sexual products, leading to an important increase in the condition index and a change in tissue distribution of metals both in the digestive gland (Geffard *et al.* 2001) and in the gills (present study). Since MTs are involved in the homeostasis of essential elements such as Cu and Zn, it is not surprising that they may intervene in processes needing the contribution of essential elements at key periods of the biological cycle such as sexual maturity.

The strong relationship between metal and MT concentrations in oyster gills is a good argument for the possible use of gill MT concentrations as a biomarker of metallic pollution. Moreover, the relative importance of the cytosolic fractions of Cd and Cu increased with the degree of contamination, at least until a threshold (about $1\,mg\,kg^{-1}$ for Cd and $100\,mg\,kg^{-1}$ for Cu), then remained predominant compared with the insoluble fractions. However, it must be kept in mind that MT is only one class of metal-binding compounds among several different potential ligands. In the nearly allied species *C. virginica*, the fraction of total Cd bound to MT in the gill reached 20%, but it was much greater than the fractions of Cu and Zn, being 1% and 0.9%, respectively (Roesijadi 1994b).

Even when increases in MT concentrations paralleled metal changes, the range of fluctuations were not the same. The ratio between the annual average MT concentration in specimens from the clean and the metal-rich sites was 1.3, whereas it reached 1.5, 2.7 and 9.8, respectively, for Zn, Cu and Cd. The ratios were in the same order of magnitude for MT and essential metals, whereas the differences registered for Cd varied by one order of magnitude. This is not surprising, even if Cd has been claimed as the best inducer of MT synthesis in mussels and oysters (Géret 2000). This assumption was based on laboratory exposures to high Cd doses, whereas in the field the concentration of this metal in marine organisms is always low compared with those of essential metals (Eisler 1981).

The limits of the methodology of oyster translocation may be revisited in light of the findings of the present study. De Kock and Kramer (1994) have underlined the advantage of employing 'statistically similar groups of organisms with regard to population, size, age, pollution and environmental history'. This is particularly relevant in the case of *C. gigas*, since in specimens experimentally exposed to Ag, metal bioaccumulation can vary by one order of magnitude according to the origin of the population (Berthet *et al.* 1992). In this species, it is also important to have samples as homogeneous as possible since the influences of age and/or size and/or weight on both MT and metal concentrations are well documented (Amiard and Berthet 1996 and literature cited therein, Mouneyrac *et al.* 1998).

Months are necessary to reach equilibrium in terms of either the total concentration of metals (Geffard *et al.* 2001 and present study) or their tissue distribution (Mouneyrac *et al.* 1999), and thus seasonal effects cannot be avoided. Moreover, the physiological status of the caged bivalves may be influenced by site-specific environmental conditions. This may be particularly important when

caging is carried out in areas where biomonitoring would be impossible due to the absence of feral populations of bivalves, generally linked to adverse natural conditions. A caging experiment carried out in 1996 with mussels at 84 sampling sites over the 1800 km long Mediterranean French coast has confirmed the predominant influence of the bioenergetic status of the bioaccumulator species on micropollutant incorporation, and mathematical modelling has been proposed to break free from this phenomenon (Andral and Stanisière 1999). The adaptation of this model to MT concentrations should be considered.

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