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Temporal variations of metallothionein and metal concentrations in the digestive gland of oysters (*Crassostrea gigas*) from a clean and a metal-rich site

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The concentrations of metallothionein (MT) in bivalves, a potential biomarker of metal pollution, are variable according to specific organs, the highest concentrations being encountered in the digestive glands of oysters. Thus, the present study has been focussed on this organ with a view to validate the use of MT as a biomarker in the field, the temporal changes of metal and metallothionein concentrations have been examined from March to October 1997 in the digestive gland of resident oysters from a clean site (Bay of Bourgneuf, France) and a metal-rich site, the Gironde estuary which has been shown as the most Cd-contaminated marine area in France but is also enriched with Cu and Zn. Moreover, oysters from the clean site have been translocated to the Gironde estuary over the same period. Taking into account all the samples collected over the 7 months of the study, MT concentrations in the digestive gland were positively correlated with weight whereas metal levels were negatively correlated with weight. However, considering monthly samples including specimens from both sites (resident or translocated oysters), a positive correlation was shown between MT and metal concentrations in autumn (September and October) but not in spring and summer. These findings limit the interest of using the digestive gland of oysters as the preferred tissue for the determination of MT concentration as a biomarker. The alternative use of gills should be considered.

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

Introduction

Metallothionein (MT) is a metalloprotein, ubiquitous in vertebrates, invertebrates, plants and microorganisms, the primary role of which is the homeostasis of the essential metals Zn and Cu. Since it is cystein-rich, it is also involved in Ag, Cd or Hg binding and thus contributes to metal detoxification. The literature shows that the synthesis of metallothionein is often induced when organisms are exposed to environments contaminated by heavy metals, the exposure route being more often experimental in the laboratory than natural in the field (Amiard and Cosson 1997, Langston *et al.* 1998). Thus, induction of MT synthesis by metals has been envisaged as a useful tool for biological monitoring of metallic pollution (George and Olsson 1994, Cosson and Amiard 1998, Langston *et al.* 1998). Bivalves such as mussels (George and Olsson 1994) and oysters (Imber *et al.* 1987, Mouneyrac *et al.* 1998) appear as the most promising candidates among aquatic invertebrates even if good relationships between metal and MT levels have

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also been shown in intra-sedimentary bivalves such as the carpet shell *Ruditapes decussatus* (Hamza-Chaffai *et al*. 1999) and the Baltic clam *Macoma balthica* (Mouneyrac *et al*., 2000). Moreover, mussels and oysters have been recognized as the best species to be used in monitoring the quality of coastal and estuarine waters in the framework of the 'Mussel Watch' (NAS 1980) and the data thus obtained for metals and other chemicals may be at least partly transposed for the methodology of biomarkers.

Another common feature with the problematics of the Mussel Watch is to determine the relative influence of either contamination factors or natural factors on the measured parameters, metal concentrations in the framework of the Mussel Watch, MT level in the biomarker approach. Since it is hypothezised that MT concentrations are governed at least partly by concentration of bioaccumulated metals, one may expect that environmental (salinity, season, localization in the intertidal zone, etc.) and biological (mainly sexual maturity) factors which influence metal concentrations (NAS 1980) would probably also influence MT levels. MT biosynthesis is induced not only by metals but also hormones, cytokines, and other endogenous and exogenous agents (Cosson *et al*. 1991, Kägi 1993, George and Olsson 1994). When considering MT levels as a tool in environmental monitoring, it is necessary to determine the signal-to-noise ratio (Cairns 1992). The signal, i.e. the change in MT concentration after metal exposure must be significantly higher than the noise, i.e. MT level changes due to non-metallic factors. Weight has been shown to influence MT concentrations in soft tissues of *M. balthica* and in digestive glands of mussels but not in mussel gills (Amiard-Triquet *et al*. 1998, Mouneyrac *et al*. 2000). Body weight fluctuations associated with reproduction were probably responsible for seasonal changes in MT concentrations observed in Baltic clams from the estuary of the western Scheldt (Bordin *et al*. 1997). The period of sampling, the sex and size of animals affect MT levels in a natural population of clams (*R. decussatus*) collected from the Tunisian coast (Hamza-Chaffai *et al*. 1999). In oysters (*Crassostrea gigas*), weight was shown as an important factor to explain variations of MT concentrations in whole soft tissues (Mouneyrac *et al*. 1998) whereas patterns of metal-binding to metallothioneins were significantly affected by collection period in *Crassostrea virginica* (Roesijadi 1994a).

The rationale for active bio-monitoring by translocation of bivalve molluscs between clean and contaminated sites, compared with collection of feral individuals, has been discussed by De Kock and Kramer (1994). These authors underline the optimization of the resolution power 'by employing statistically similar groups of organisms, with regard to population, size, age, pollution and environmental history, for comparing chemical stress at different locations'. Case studies have been reported, either for the study of chemical accumulation (De Kock and Kramer 1994 and literature cited therein) or, more rarely, for both accumulation and biochemical responses (Johansson *et al*. 1986, Couillard *et al*. 1995, Michel *et al*. 1998).

MT synthesis may vary considerably between tissues. The highest MT concentrations are generally determined in the digestive gland of bivalves compared with gills and remaining tissues (Bebianno *et al*. 1993 in *Ruditapes decussatus*; Géret *et al*. 1998 in *Mytilus edulis* ; Mouneyrac *et al*. 1998 in *Crassostrea gigas*). Thus, it could be hypothesized that determining MT concentrations in the whole soft tissues rather than in the digestive gland would lead to a kind of 'biological dilution' of MT, able to concealed at least partly the changes expected as a consequence of metal exposure. In a literature review, the relative interest of gills and digestive gland as the best biological matrix for MT determination has been discussed. No consistent trend was evidenced since interspecific differences were highlighted (Cosson 2000).

Thus, the present study has been designed to examine under natural conditions the induction of metallothionein in the digestive gland of *Crassostrea gigas* from a metal-rich site (Gironde estuary) compared with specimens from a clean site (Bay of Bourgneuf). The influence of natural factors which can interact with metal exposure in the field will be investigated, particularly weight and season, since the influence of salinity – a major factor in estuarine environments – has been clarified for both MT and metal concentrations (Mouneyrac *et al.* 1998). The Gironde estuary has been shown as the most Cd-contaminated marine area in France but is also enriched with Cu and Zn (RNO 1995, Mouneyrac *et al.* 1998). Moreover, oysters from the clean site have been translocated to the Gironde estuary in consideration of the advantages mentioned for the so-called active bio-monitoring studies (De Kock and Kramer 1994).

Material and methods

Oysters (*Crassostrea gigas*) from a relatively clean coastal area (Bay of Bourgneuf, France) were translocated to a site on the left bank of the Gironde estuary, 25 km upstream from the mouth of the river on March 1997, then monthly sampling was carried out up to October 1997. These will be referred to in the subsequent text as BG (originating from B for Bourgneuf, transferred to G for Gironde). Additional populations were sampled according to the same time-schedule: oysters living in the Bay of Bourgneuf during the same period (group BB, originating from and left in B for Bourgneuf), used as controls, and oysters growing naturally in the Gironde estuary (group GG, originating from and remaining in G for Gironde).

In order to avoid differences in MT and metal concentrations due to the influence of weight (see above), the specimens collected on different sites at different periods of the year were carefully selected: controls and transplants, obtained from an oyster-farm, were 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.

Eight specimens were examined for each sampling date and each group (controls BB, transplants BG and residents GG). They were weighed (total weight), then the soft tissues were separated from the shell, excess fluid removed with absorbent paper, weighed (wet weight) and then dissected in order to recover the whole gills of each individual which were weighed (as well as digestive glands and all remainders). The condition index (CI) was determined according to the recommendation of the French Association for Standardization (AFNOR, NF V45056, Sept. 1985):

$$\text{CI} = \text{Drained weight of soft tissues} \times 100 \times \text{Total weight}^{-1} \qquad \text{(eqn 1)}$$

With a view to metal and MT analyses, digestive glands were homogenized in a buffer solution (20 mM TRIS, 10^{-5} mM β mercaptoethanol, 150 mM NaCl solution adjusted to pH 8.6). The soluble (S1) and insoluble (P1) fractions were separated by centrifugation (25 000 *g* for 55 min). The cytosolic heat-stable compounds including metallothioneins (S2) were isolated by centrifugation of the soluble fraction (12 000 *g* for 10 min) after heat treatment (75 °C for 10 min). The possible contamination of S2 by —SH soluble compounds other than MT has been tested (Berthet, comm. pers.). Gel permation has been performed on heat-denaturated cytosol S2 in order to determine the presence of thiols in fractions corresponding to the whole range of soluble compounds. Thiols have been shown only in those fractions, the molecular mass of which was consistent with the presence of MT. The amount of MT was determined in the heat-denaturated cytosol 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). The PAR Model 174 analyser, the PAR/EG&G Model 303 static mercury drop electrode (SMDE) and an X–Y recorder (RE 0089) were used. The temperature of the cell was maintained at 5 °C. The standard addition method was used for calibration with rabbit liver MT (Sigma Chemical Co., St Louis, MO) in the absence of purified bivalve MT.

The pre-treatment of samples devoted to metal analysis was carried out according to the procedure which best avoided secondary contamination. The soluble and insoluble fractions were digested with suprapure nitric acid at 80 °C for 1 h. Metals were determined in these acid solutions by flameless (Cd in digestive gland of controls) or flame (all other determinations) atomic absorption spectrophotometry using the Zeeman effect (Hitachi Z 8200) according to the analytical method described by Amiard *et al.* (1987), validated by external inter-calibrations (Coquery and Horvat 1996, Coquery *et al.* 1997).

Statistical treatment

Differences between groups of bivalves according to conditions of exposure or natural factors were evaluated by analysis of variance (ANOVA) and post hoc comparisons assessed by the multiple Range Test of Scheffé. These tests were carried out using a standard statistical package (StatView SE = Graphics^{tm}).

Results

Biometric data

Digestive gland weights are shown in figure 1 for the different groups of oysters collected monthly. Analyses of variance showed significant temporal fluctuations in all the three groups ($p = 0.0001$). When differences among months were examined using Scheffé contrasts, the average weight appeared as significantly higher in July compared with most of the other months whereas it was generally significantly lower in September and October.

Coefficients of variation were calculated for each month and each site. They varied from 13.5 to 39.5% in controls (BB), from 13.5 to 35.4% in translocated specimens (BG) and from 13.6 to 42.4% in feral oysters from the Gironde estuary (GG). Analysis of variance did not show any significant difference between these coefficients according to the experimental groups ($p = 0.4682$). It means that the weight of the digestive gland did not fluctuate more in feral specimens than in cultivated oysters having the same age and the same size at the beginning of the study.

No inter-group differences within each month were observed in March, June, July, September and October. In April, resident GG oysters from the Gironde estuary showed average weights significantly lower than in both other groups. In May, resident GG oysters from the Gironde estuary had an average weight significantly lower than in control oysters BB, whereas translocated specimens did not differ significantly from both other groups. In August, the digestive gland of both residents GG and oysters translocated towards the Gironde estuary BG showed significantly lower weights than in control oysters BB.

Temporal variations of MT and metal concentrations

Temporal variations of MT concentrations are shown in figure 2. The most striking feature, common to all the groups of oysters (BB, BG, GG) was the increase of MT levels during late spring and summer and the subsequent decrease observed during autumn. MT concentrations were not systematically significantly higher in oysters from the Gironde estuary (GG) or those translocated to the Gironde estuary (BG) than in controls (BB) (figure 2).

On the other hand, inter-group differences were more important than temporal changes when metal concentrations were considered (figure 3). The level of Cd was about 14 times lower in controls (BB) than in oysters from the Gironde estuary (GG) on an annual average. After 3 months of exposure in the Cd-rich Gironde estuary, the concentrations of this metal were no longer significantly different

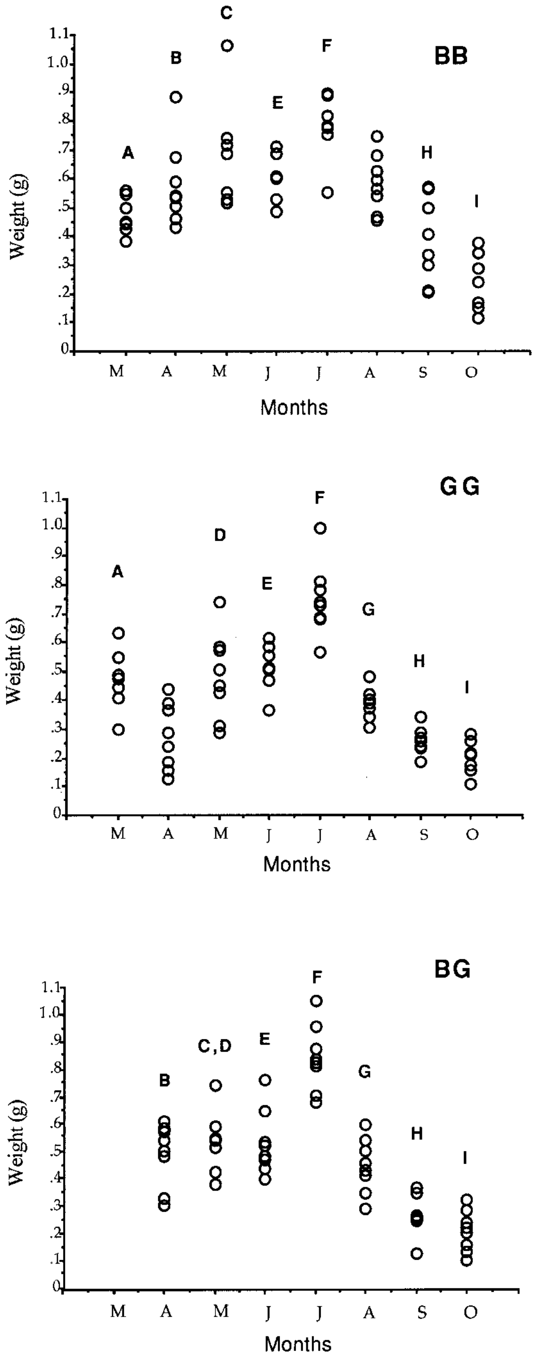

Figure 1. Variations of wet weight in individual oyster digestive glands (expressed in g) in different months March (M) to October (O). BB: controls originating from the Bay of Bourgneuf; GG: residents from the Gironde estuary; BG: transplanted from the Bay of Bourgneuf to the Gironde estuary. Groups of data with same superscripts did not differ significantly for each month the three sites are compared.

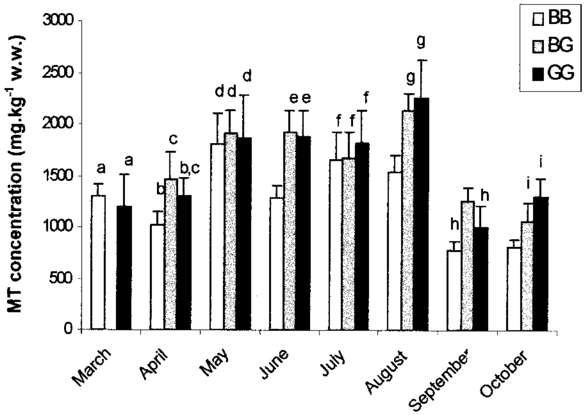

Figure 2. Temporal variations of MT concentrations in digestive gland of oysters (means and confidence intervals at the 95% level expressed in mg kg^{-1} ww). BB: controls originating from the Bay of Bourgneuf; GG: residents from the Gironde estuary; BG: transplanted from the Bay of Bourgneuf to the Gironde estuary. Within each month, groups of data with same superscripts did not differ significantly. Bars without superscripts are significantly different from the other two.

Table 1. Relationship between MT or metal concentrations and the weight of the digestive gland. Values of the correlation coefficient *r* and levels of significance (*$p < 0.05$; **$p < 0.01$) for oysters originating from a clean site (BB, $n = 64$), from the metal-rich Gironde estuary (GG, $n = 62$) or translocated from the clean to the metal-rich site (BG, $n = 66$) or for all the samples (BB + BG + GG, $n = 182$).

	Groups of oysters			
Concentrations	BB	BG	GG	BB + BG + GG
MT	+0.499**	+0.3676**	+0.2725*	+0.2674**
Total Cd	–0.4149**	–0.1412	–0.2081	–0.2967**
Total Cu	–0.2575*	–0.6208**	–0.2578*	–0.3896**
Total Zn	–0.1919	–0.5909**	–0.1832	–0.4524**

between oysters translocated from the clean site (BG) and residents (GG). The pattern was globally similar for Cu and Zn but the differences between sites were not so large, with ratios between mean annual concentrations in groups BB and GG reaching about 3 and 2 respectively.

Relationships between MT and metal concentrations vs weight

A correlation matrix has been obtained, based upon linear relationships between these factors. The correlation coefficients are shown in table 1. The results of the statistical analysis did not show major differences according the data sets considered, either those of individual groups (BB, BG or GG) or the whole data set (BB+BG+GG). The temporal changes of MT concentrations paralleled those of the weight of the digestive gland as shown above in figures 1 and 2 and confirmed since these factors were positively correlated (table 1). Conversely, for all three studied metals, concentrations were negatively correlated with the weight of the digestive gland (table 1).

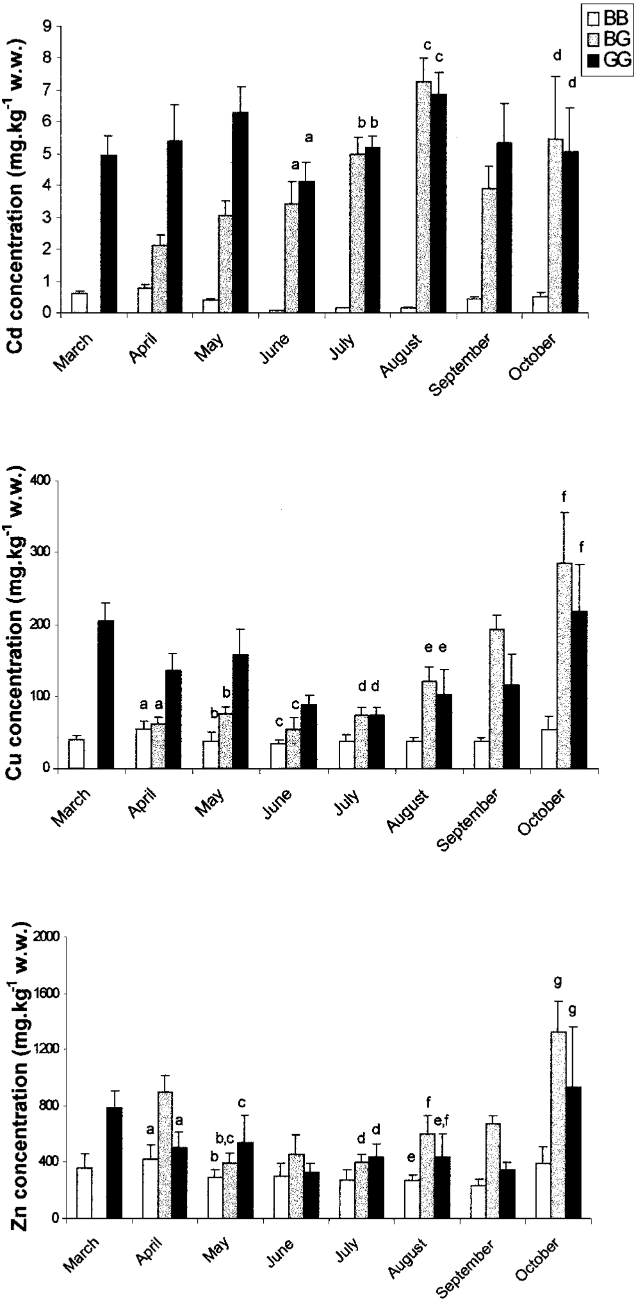

Figure 3. Temporal variations of metal concentrations in digestive glands of oysters (means and confidence intervals at the 95% level expressed in mg kg^{-1} ww). BB: controls originating from the Bay of Bourgneuf; GG: residents from the Gironde estuary; BG: transplanted from the Bay of Bourgneuf to the Gironde estuary. Within each month, groups of data with same superscripts did not differ significantly. Bars without superscripts are significantly different from the other two.

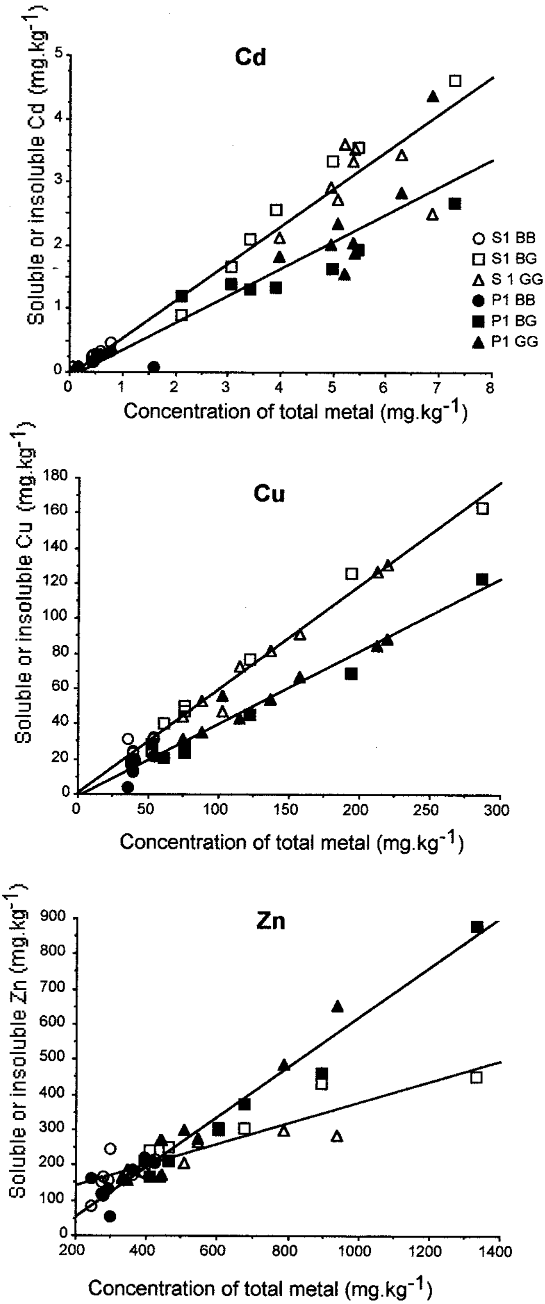

Figure 4. Relative importance of soluble and insoluble tissue fractions in bioaccumulation of metals in oyster digestive glands. x-axis: total metal concentrations in mg kg^{-1} ww. y-axis: concentrations of metals under soluble (open symbols) and insoluble (filled symbols) forms in mg kg^{-1} ww. Circles: BB, controls originating from the Bay of Bourgneuf; squares: GG, residents from the Gironde estuary; triangles: BG, transplanted from the Bay of Bourgneuf to the Gironde estuary.

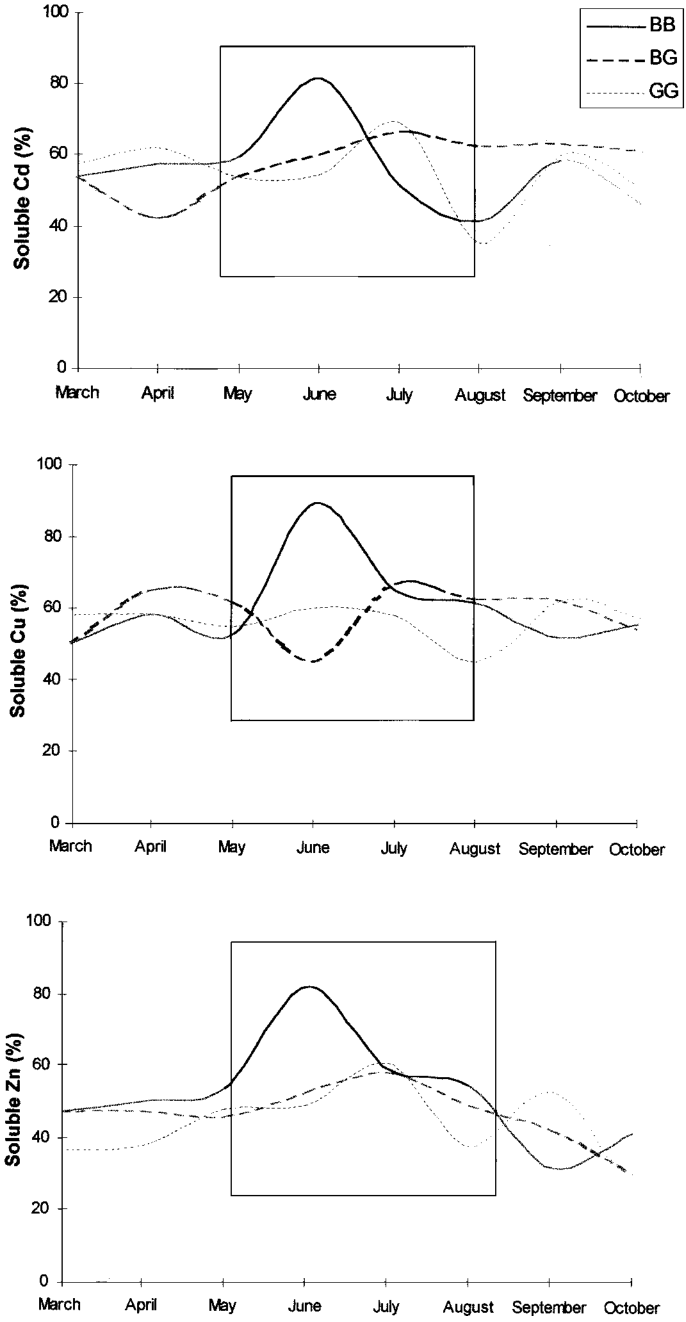

Figure 5. Temporal variations of the percentages of Cd, Cu or Zn determined in the cytosolic fraction of the digestive gland of oysters. BB: controls originating from the Bay of Bourgneuf; GG: residents from the Gironde estuary; BG: transplanted from the Bay of Bourgneuf to the Gironde estuary.

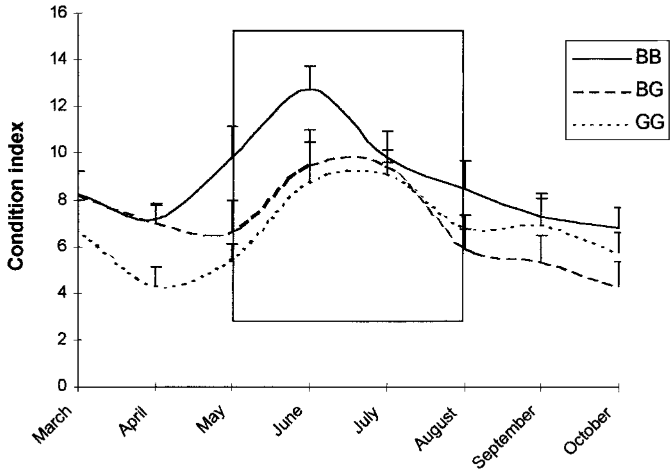

Figure 6. Temporal variations of the condition index of oysters. Means and confidence intervals at the 95% level. BB: controls originating from the Bay of Bourgneuf; GG: residents from the Gironde estuary; BG: transplanted from the Bay of Bourgneuf to the Gironde estuary.

Distribution of metals between the cytosolic and the insoluble fractions of digestive glands

The distribution of metals between soluble (S1) and insoluble (P1) fractions is shown in figure 4 for increasing global concentrations (S1 + P1). Due to the large number of determinations ($n = 182$), the monthly means have been shown, thus allowing differences between sites to be distinguished. Cd and Cu were mainly found in the cytosol, even if at low total Cd concentrations (controls BB), the difference between these two forms of storage were negligible. Zinc was equally distributed between soluble and insoluble fractions in most cases, with the exception of specimens showing the highest levels of total Zn which exhibited higher concentrations of insoluble Zn compared with soluble forms (figure 4).

The percentages of metals associated with the soluble fraction of the digestive gland are shown in figure 5. No strong temporal changes or inter-site differences were in evidence with the exception of a maximum value registered for all of the three metals in June for controls BB and, at a lesser degree, in July for oysters collected in the Gironde estuary (BG and GG) (see boxes in figure 5). These peaks were concomitant with the maximum values of the condition index which were observed in June for controls and in July for specimens originating from the Gironde (figure 6).

Relationship between MT and metal concentrations

Because MT is a cytosolic heat-stable protein, it is logical to consider the relationship between MT concentrations in an organ and metal concentrations in the supernatant obtained after heat-denaturation of the cytosol. However, during heating, the distribution of metals among cytosolic compounds may be modified for the analysis of Cd in fractions separated from S1 or S2 by gel chromatography has shown differences (Berthet, pers. comm.); thus, figure 7 depicts the relationships between MT concentrations and metal contents in the supernatant before heat treatment. All three of the metals studied (Zn, Cu, Cd) bind to MT and might therefore contribute in association to MT induction (Amiard and Cosson 1997). Thus, the total metal content was calculated as the sum of concentrations of soluble Cd, Cu and Zn expressed in mgatom kg^{-1} and the relationship between MT and the sum of metals has also been shown (figure 7). 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 (BB, BG, GG).

Considering either individual metals or the sum of Cd, Cu and Zn, the most striking feature is that MT concentrations were generally not positively correlated with metal concentrations in the digestive glands of oysters except in autumn. In the case of Cd, significantly positive correlations were also shown in June and August but not in September.

From April to August, the range of soluble Cu concentrations was limited, a phenomenon which could contribute to the lack of a significant relationship with MT concentrations and partly to the lack of relationship between the sum of metals and MT since Cu concentrations represented an important contribution of the total metal content. As a matter of fact, in the digestive gland of controls one atom of soluble Cd corresponded to 202 atoms of Cu and 1303 atoms of Zn. In oysters from the Gironde estuary, the ratios were 1, 58, and 216 respectively.

Discussion

Due to the high percentage of metals in the cytosol and to the strong relationship between metal and MT concentrations in the whole soft tissues, the use of MT as a biomarker of metallic pollution seems dependable if oysters are chosen as the preferred species for the determination of this biochemical parameter (Mouneyrac *et al.* 1998 and references cited therein). However, in bivalves, MT concentrations vary according to different tissues, the highest concentrations being generally encountered in digestive glands in different species (Bebianno *et al.* 1993, Géret *et al.* 1998) including oysters (*Crassostrea gigas*) (Mouneyrac *et al.* 1998).

When the whole soft tissues of oysters were examined, weight appeared as an important factor to explain variations of MT levels: when the weight was doubled, MT concentration decreased two-fold (Mouneyrac *et al.* 1998). On the contrary, when digestive gland was examined separately in the present study, MT concentration was positively correlated with weight whereas metal levels were negatively correlated with weight. The latter is classically observed and generally explained by weight increases due to the abundance of food in spring and autumn with phytoplanktonic blooms and/or to the ripening of sexual products (NAS 1980, Amiard and Berthet 1996 and references cited therein). Similarly, Bordin *et al.* (1997) have proposed that seasonal changes in MT concentrations observed in the intra-sedimentary bivalve *Macoma balthica* may be explained by body weight fluctuations associated with reproduction. However, in all these studies, weight explained only a certain percentage of variations in metal or MT concentrations, leaving possibilities that other factors intervene, such as metabolic needs, independent of metals either essential or toxic. Thus, in estuarine crabs, MT levels seemed more linked to changes in general protein metabolism than to changes in metal concentrations since increases of total protein and MT concentrations were observed with increasing salinity whereas metal concentrations decreased (Legras *et al.* 2000).

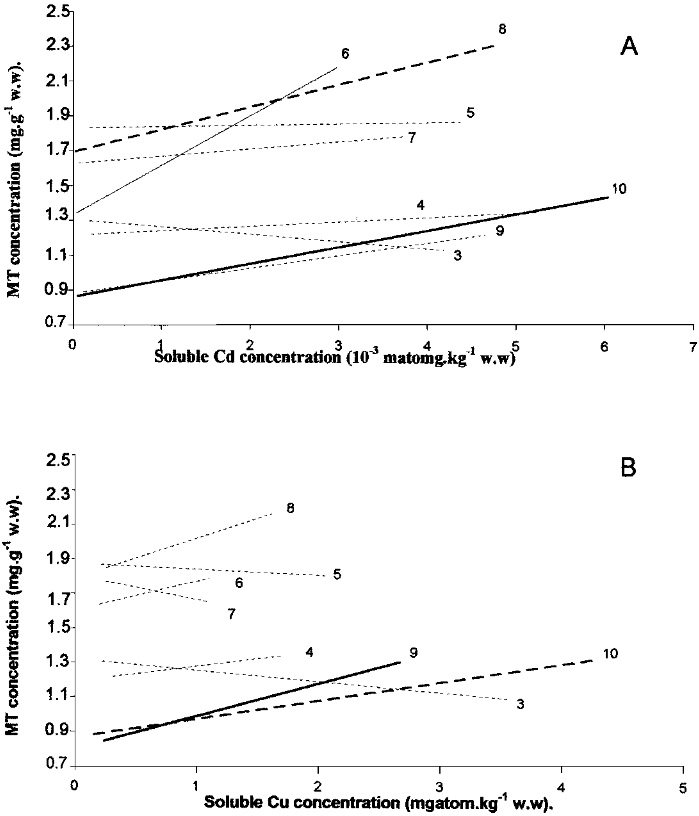

Figure 7. Relationships between metal (individual or added) and MT concentrations in the digestive gland of oysters. Best-fit straight lines calculated monthly for controls (BB) and exposed oysters (residents GG and transplanted specimens BG) together. Continuous line: correlation significant at the 99% level; broken line : 95% level; dotted line : non-significant correlation. (A) Cd; (B) Cu; (C) Zn; (D) Cd + Cu + Zn. Numbers represent the month of analysis (e.g. 9 = September).

The influence of the period of sampling on MT concentrations has been recognized in *M. balthica* (Bordin *et al.* 1997), *Ruditapes decussatus* (Hamza-Chaffai *et al.* 1999) whereas in *Crassostrea virginica* it affects the pattern of metal-binding to MT (Roesijadi 1994a). In the present study, temporal variations of MT concentrations in digestive glands of oysters (*C. gigas*) were also observed but the most striking feature was their importance compared with inter-site differences in spite of marked differences between metal contamination in the 'clean' (Bay of Bourgneuf) and the metal-rich (Gironde estuary) sites. The data here-obtained by determining Cd, Cu and Zn concentrations in the digestive glands of oysters are in agreement with those published previously (RNO 1995). Whereas the comparison of metal concentrations in digestive glands clearly differentiate between sites, MT concentrations were not systematically higher in oyters from the Gironde estuary (or in those translocated to the Gironde estuary) than in controls.

The level of MT is already high in clean animals, a fact which could be related to their high tissue content of Cu and Zn. Similar data were obtained by Imber *et al.* (1987) using the same determination technique excepted that partial purification

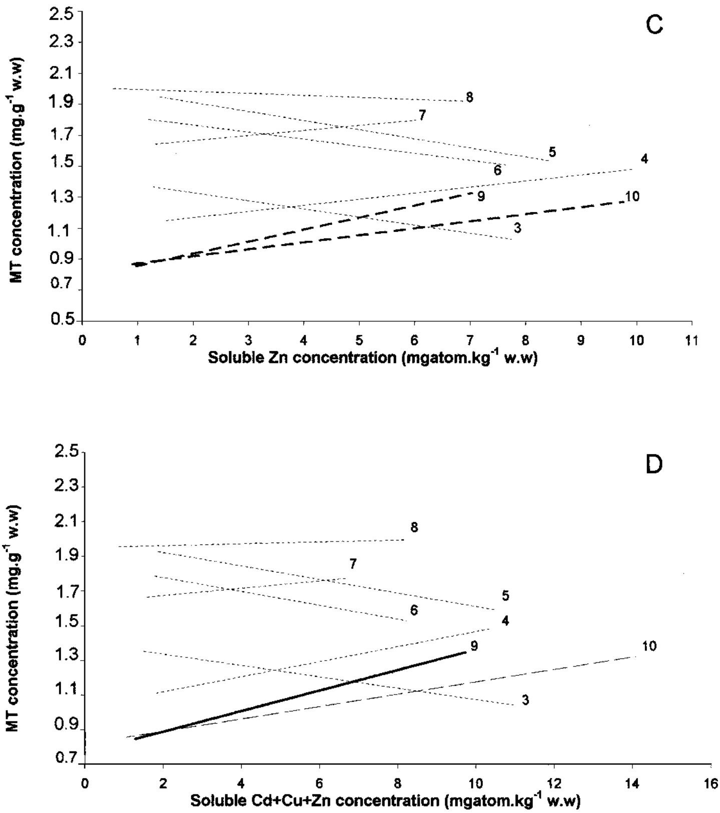

Figure 7. For legend see facing page.

of MT was performed using ethanol-denaturation instead of heat-denaturation. However, it has been shown that in the digestive gland of oysters, the response of the polarographic assay is identical for both of these modes of denaturation (Géret 2000). In spite of levels of metals considerably lower in digestive glands of mussels, MT concentrations are equivalent or even higher than in oysters, the inter-specific comparison being based on specimens samples exposed under identical conditions in the field (Geffard *et al.* submitted). An increase in metal content does not necessarily mean a corresponding increase in MT concentration. It may be hypothezised that organisms can cope, increasing the turn-over of MT rather that its global concentration. In agreement with that, Roesijadi (1994b) has shown that the half-life of MT was either 4 or 20 days depending on whether the turn-over was measured respectively during or immediatly after cessation of exposure to Cd.

In oysters from both sites, Cd, Cu and Zn were mainly cytosolic in digestive gland with the exception of Zn in a small number of samples in which the level of this metal was particularly high. This is in agreement with previous studies on bivalves (Langston and Zhou 1987, Pavicic *et al.* 1989, Evtushenko *et al.* 1990, Géret *et al.* 1998, Mouneyrac *et al.* 1998). In the American oyster *C. virginica*, only 23% of Cu and 28% of Zn were present in the soluble fraction whereas cytosolic Cd was highly variable (29–66%) according to site and season (Roesijadi 1994a). In the present study, seasonal variations of the distribution between soluble and insoluble fractions of the soft tissues were also evident but they appeared concomitantly for all three of the metals studied, considering each site (Bay of Bourgneuf and Gironde estuary) separately. At each site, the maximum percentage of cytosolic metals was observed when the condition index was at its highest, in June for the clean site, in July for the metal-rich site, suggesting a link between the general metabolism of oysters and metal metabolism, such as a special need for essential elements during sexual maturity.

From a physiological point of view it would be interesting to consider the relationship between MT and metal concentrations in the heat-stable cytosolic fraction. However, since it has been shown that heating influences the fate of metals, this relationship has been examined for the total cytosol. But, with a view to its use as a biomarker, MT has to reflect the gross metallic concentration in the tissues. According to Johansson *et al.* (1986), the concentration of metals in the cytosol usually reflects the concentration in the whole tissues, although variability has been noted when the concentrations were low. Similarly, in oysters collected at sites in the Gironde estuary differing through salinity, the relationships between MT concentrations and either total metal levels or cytosolic metal levels were identical (Mouneyrac *et al.* 1998). In this latter study carried out in November 1995, a strong relationship had been shown generally between metal and MT concentrations. In the present study, when this relationship was calculated over a larger range of metal concentrations since the data included determinations in a clean site, a positive correlation was shown during autumn (September and October) but not in spring and summer, a fact which limits the interest of using the digestive gland of oysters as the chosen tissue for the determination of MT concentrations as a biomarker.

Thus, despite the high level of MT in the digestive gland of the oyster *C. gigas* and if one considers further; (a) the limited inter-site variations of MT compared with the marked intra-site differences in metal concentrations, (b) the lack of a clear relationship between MT and metal levels over a large period of the year, (c) the importance of temporal changes of its weight and their influence on MT concentrations, all these data indicate clearly that the digestive gland of the oyster *C. gigas* has limited value as a tissue for biomonitoring through the measurement of MT as a biomarker. This conclusion is in agreement with the findings of Bebianno *et al.* (1993) for the clam *R. decussatus* who considered that the low levels of significant *de novo* synthesis of metallothionein in response to experimental Cd exposure in the whole soft tissues and digestive gland reduce the value of these tissues as materials for biomonitoring in this species but gills appear to be a more suitable tissue. On the contrary, from a translocation study from a clean to a metal-rich site, it may be concluded that for mussels (*M. edulis*), analysis of the digestive gland seems more relevant than analysis of the gills whereas seasonal metabolic changes limit the efficiency of the use of MT as a biomarker at certain periods of the year (Amiard *et al.* 1998). In mussels from the Arctic, changes in MT concentrations among sites were more important in digestive glands than in gills (Amiard-Triquet *et al.* 1998).

The relevance of translocation experiments must be examined taking into account the equilibrium concept which considers both chemical accumulation processes (De Kock and Kramer 1994) and changes in biochemical compound levels usable as biomarkers such as metallothionein. The sub-cellular distribution of metals is governed by numerous factors, particularly the length and the level of exposure: the latency for metallothionein induction and the relative importance of this ligand in metal-binding have given rise to highly differing estimations in both field and laboratory studies (Couillard *et al.* 1995, Amiard-Triquet and Amiard 1998, Mouneyrac *et al.* 1999 and literature cited therein). Carrying a field transplantation of the freshwater bivalve *Pyganodon grandis* across a metal contamination gradient, Couillard *et al.* (1995) have estimated the response time of gill MT to the change in ambient Cd water to be 90 days. In *Crassostrea gigas*, four classes of Cd-binding cytosolic compounds were separated and their relative importance in metal-binding was found to differ among resident and transplanted oysters even after 3 months of the experiment (Mouneyrac *et al.* 1999).

Among the advantages of active biomonitoring investigations through translocation experiments in the case of chemical monitoring of biota, De Kock and Kramer (1994) have underlined 'the optimization of the resolution power by employing statistically similar groups of organisms with regard to population, size, age, pollution and environmental history'. However, if the initial inter-site variability is reduced, seasonal variability is obviously not affected by this procedure and seasonal changes (of weight mainly) may be governed by local conditions (temperature, food availability, etc.). In order to avoid this source of fluctuations, the length of translocation must be restricted, but, however, one has to take into account the time necessary to reach equilibrium in translocated specimens. Complementarily, the experiments have to take place during those periods of the year where changes in the general metabolism are limited (i.e. beyond the periods of reproduction and/or of phytoplanktonic blooms leading to a marked increase of available food and weight of bivalves). The alternative use of gills as a biological matrix for the validation of metallothionein as a biomarker of metal contamination has to be investigated since, due to its different biological role, this organ is less affected than the digestive gland by seasonal changes.

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