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Metallothionein concentration in the mussel *Mytilus galloprovincialis* as a biomarker of response to metal contamination: validation in the field

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Mussels were translocated from a shell-fish breeding area (Sète, on the French Mediterranean coast) to sites exposed to trace element inputs in April 2000. They were recovered 3 months later. Whole soft tissues from all of the sites ($n = 97$) were analysed for arsenic, cadmium, chromium, copper, mercury, nickel, lead and zinc. Metallothioneins (MTs) were also measured in the digestive gland and in the remaining tissues (allowing calculation of whole soft tissue concentrations) at 22 of the 97 sites. MT concentrations in the digestive gland and the whole soft tissues were strongly correlated. The condition index varied with food availability at different sites. This did not influenced MT concentrations in the whole soft tissues, whereas the condition index was negatively correlated to trace element concentrations. A model is proposed to minimize this influence of condition. Metal concentrations adjusted using this model showed significant correlations with MT levels for those metals (cadmium, copper, nickel and zinc) that are known to bind to this protein, with the exception of mercury. Even in moderately contaminated sites, measurement of the MT level in the soft tissues of mussels was generally able to discriminate between different levels of contamination, allowing the use of a simplified procedure compared with dissection of the digestive gland. It is recommended to avoid translocation and sampling during the reproductive period, which is well documented for commercial species such as *Mytilus* sp.

Keywords: mussel, metallothionein, biomarker, metals

Introduction

Among the biomarkers that are currently used to monitor the quality of the marine environment, the induction of metallothioneins (MTs) in response to metal exposure is well documented (see reviews by Roesijadi 1992, George and Olsson 1994, Cosson and Amiard 2000). However, the choice of the best biological matrix for the determination of MTs as a biomarker of response to metallic contamination is still a topic of discussion. In invertebrates, metal detoxification is realized through several different processes, involving a large range of cellular or extracellular ligands (Mason and Jenkins 1995), in contrast to vertebrates, in which metal-binding to MTs is thought to be the major route for detoxification. George and Olsson (1994) have suggested that fish species would be better candidates for

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monitoring use than invertebrates, although they recognized that *Mytilus* sp. were the only promising candidate. However, due to the ability of sedentary bivalves to represent the local situation and the experience gained from chemical biomonitoring (NAS 1980, RNO 2000), the possible use of bivalve MTs as a biomarker has given rise to a large number of studies, which have been recently reviewed (Langston *et al*. 1998, Cosson 2000, Isani *et al*. 2000).

MT level varies considerably between tissues within the same bivalve species, as has been shown for the clam *Ruditapes decussatus* (Bebianno *et al*. 1993), the Asiatic clam *Corbicula fluminea* (Baudrimont *et al*. 1997), the Japanese oyster *Crassostrea gigas* (Mouneyrac *et al*. 1998, Geffard *et al*. 2001, 2002), and the mussels *M. galloprovincialis* (Raspor *et al*. 1999a,b) and *M. edulis* (Geffard 2001). In addition, Pavicic *et al*. (1993) have shown in mussels that the most marked induction by metals was in the digestive gland, and that even when significant seasonal fluctuations were observed they did not concealed the response due to exposure in a metal-rich site (Geffard 2001). MT analysis in the digestive gland has been recommended in the framework of the Mediterranean Action Plan (UNEP/RAMOGE 1999). The existing data suggest that MT analyses in the digestive gland are most probably reliable, but may still be improved.

The weight of the soft tissues is a major factor governing metal levels (Amiard *et al*. 1986, Amiard and Berthet 1996). The weight varies during the reproductive cycle and/or with the food abundance at different sites. Thus, Andral and Stanisière (1999) have proposed a correction factor to minimize the influence of weight changes on the level of metals in mussels translocated from a reference site to a range of sites contaminated by metals to a greater or lesser degree (Andral and Stanisière 1999) and of differing trophic status. Considering that MT is induced by exposure to metals (George and Olsson 1994; Cosson and Amiard 2000), it seems probable that factors influencing metal levels would also influence MT concentrations. In the present study, the first aim was to verify whether the correction factor proposed for metals is relevant for MT in order to distinguish between variations due to weight changes and variations due to metal contamination of studied sites. From an operational point of view, it would be advantageous to avoid the time-consuming dissection of mussels to recover the digestive gland, and so the present study also investigated the relevance of using whole soft tissues as the biological matrix for MT determination. This study was therefore designed to examine both of these aspects and to validate the use of mussel MTs to monitor metal exposure on a large scale, involving 22 sites along about 430 km of the French Mediterranean coast.

Materials and methods

Within the framework of an active biomonitoring programme along the French Mediterranean coast (RINBIO for the Biological Integrator Network, financed by the Agence de l'Eau Rhône Méditerranée Corse and driven by IFREMER, the French Research Institute for Sea Tapping), mussels (*M. galloprovincialis*) were translocated from a shell-fish breeding area (Sète) to 97 sites (approximately 150 individuals at each site) (figure 1) located 3 km from the coast and potentially impacted by urban and/or industrial effluents or inputs from river basins. Specimens of the same age were selected according to their size using a 20 mm meshed grid, and submerged 6 m under the surface from April to July 2000. Mussels were collected for metal analysis at all 97 experimental sites, and at 22 sites for MT analysis (figure 1).

The pretreatment of samples for metal analysis was carried out using procedures that best avoided secondary contamination. All laboratory ware was soaked in 10% HCl, rinsed three times with dejonized

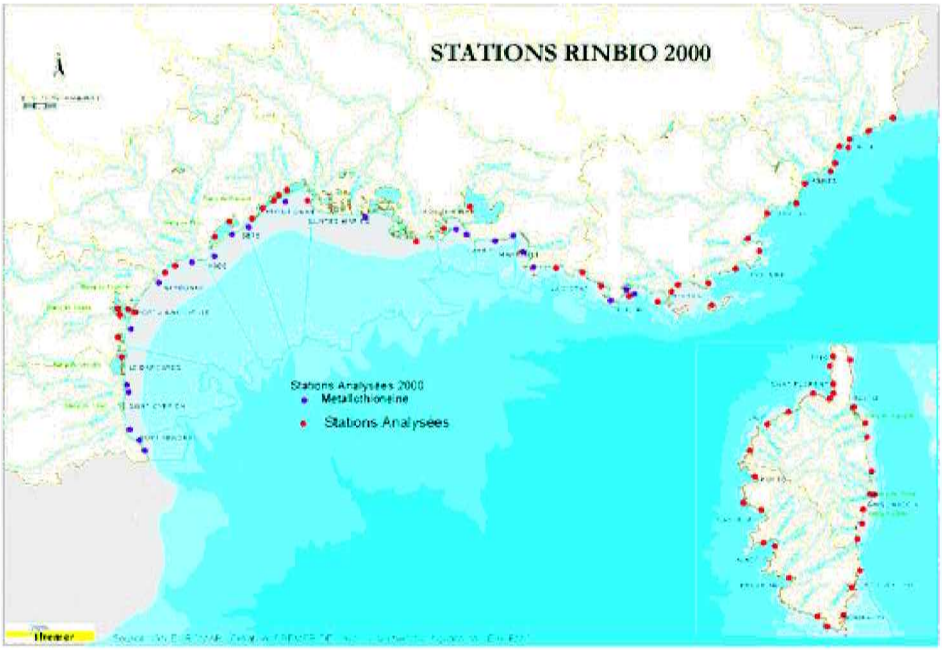

Figure 1. Map of the translocation study. Mussels originating from the shell-fish breeding area of Sète were translocated and sampled after 3 months for metal analyses (red points) and both metal and MT analyses (blue points).

water and dried in a dessicator. The byssus was removed and the whole soft tissues were carefully separated from the shell and then drained. A sufficient number of specimens were dissected in order to reach a total volume of approximately 135 ml (about 30 individuals). The flesh was weighed before and after freeze-drying (thus metal concentrations may be expressed either in mg kg^{-1} dry weight or wet weight), and the corresponding shells were weighed after drying at 60°C. The condition index CI_{metal} was calculated as follows:

$$CI_{metal} = \text{dry weight of the flesh/dry weight of the shell} \quad (1)$$

The analytical method for metal determination has been described previously by Aminot and Chaussepied (1983). The soft tissues were heated with acid to solubilize them. Cadmium, lead, copper, zinc, nickel and chromium were quantified in these acid solutions using flame or flameless atomic absorption spectroscopy (AAS) or inductively coupled plasma-atomic emission spectrometry (ICP-AES) according to their level in the samples, whereas arsenic and mercury were analysed using atomic fluorescence after successive phases of oxidizing and reducing. The laboratory in charge of this work (Municipal and Regional Laboratory of Rouen, France) is accredited by COFRAC and is involved in international intercalibration programmes (QUASIMENE).

Eight mussels were used for MT determination at each studied site. They were weighed (total weight), then the soft tissues were separated from the shell, the excess fluid was removed with absorbent paper, and then dissected in order to recover the whole digestive gland of each individual. This was then weighed, as was the remaining tissue. The condition index CI_{MT} was determined according to the following equation:

$$CI_{MT} = \text{wet weight of the soft tissues/total weight} \quad (2)$$

where total weight = wet weight of the soft tissues + shell + palleal liquid.

Individual digestive glands and remaining tissues were homogenized in a buffer solution (20 mM TRIS, 10^{-5} mM β-mercaptoethanol, 150 mM NaCl solution adjusted to pH 8.6). The cytosolic fraction was recovered by centrifugation (25 000 *g* for 55 min). The heat-stable MTs were isolated by centrifugation of the cytosolic fraction (15 000 *g* for 10 min) after heat-treatment (75°C for 10 min). The amount of MT was determined in the heat-denaturated cytosol using differential pulse polarography, a technique based on −SH compound determination according to the Brdicka reaction (Brdicka 1933) as described by Thompson and Cosson (1984). Although MT is probably not the only heat-stable

sulphydryl-containing compound remaining in the solution to be analysed, other species that are potentially present (glutathione, free cysteine, β-mercaptoethanol) had no effect on the polarographic response compared with MT (Olafson and Olsson 1991). 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, Missouri, USA) in the absence of purified bivalve MT. The ISOMer institute in which MT analysis was carried out is involved in BEQUALM (Biological Effects Quality Assurance in Monitoring) programmes (Mathiessen 2000).

Since partial purification of MT was carried out using wet tissues, MT concentrations were expressed in mg kg^{-1} wet weight. The MT concentration in the whole soft tissues was calculated by summing the amounts measured in the digestive gland and in the remaining tissues for each individual.

Differences between MT concentrations in different groups of mussels were studied using analysis of variance (ANOVA) and *post hoc* comparisons were assessed by the multiple range test of Scheffé, using a standard statistical package (StatView SE + Graphics). Linear regressions and correlation coefficients were determined using Excel97.

Results

MT concentrations

MT concentrations varied moderately in the whole soft tissues of mussels (figure 2); concentrations were significantly higher than at the control site (Sète) at only six sites. Similar results were obtained for the digestive gland (data not shown). The ratios between the maximum and minimum values were 1.9 and 1.7, respectively, for the whole soft tissues and the digestive gland. The mean concentration (x) was significantly higher ($p = 0.001$) in the digestive gland ($x = 1366$ mg kg^{-1} wet weight, $\delta_{n-1} = 243$) than in the whole soft tissues ($x = 573$, $\delta_{n-1} = 99$). However, the intersite variations showed a high degree of similarity for both the digestive gland and the soft tissues, and the correlation between these two sets of values was highly significant ($r = 0.63$, $n = 22$).

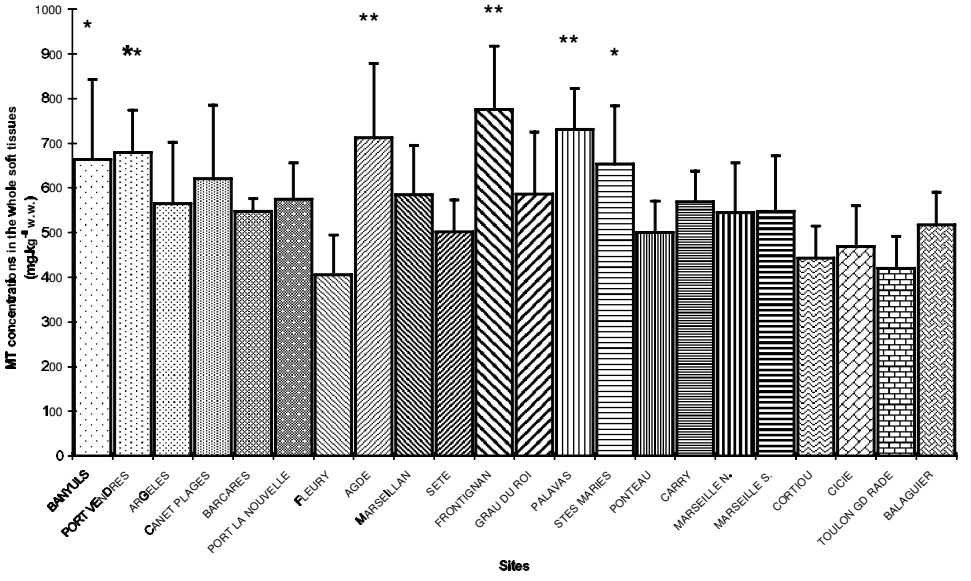

Figure 2. MT concentrations (mean + SD, $n = 8$) in the whole soft tissues of mussels originating from a shell-fish breeding area (Sète) and translocated to differently impacted sites along the French Mediterranean coast. MT concentrations significantly higher than at the control site (Sète) are indicated (*95% level; **99% level). w.w., wet weight.

Table 1. Measured metal concentrations (mean, mg kg^{-1} wet weight) determined in the soft tissues of mussels originating from a shell-fish breeding area (Sète) and translocated to differently impacted sites along the French Mediterranean coast.

Site	Arsenic	Cadmium	Chromium	Copper	Mercury	Nickel	Lead	Zinc
Banyuls	9.01	0.23	0.20	1.26	0.01	0.33	0.33	55.21
Port Vendres	10.21	0.23	0.31	1.64	0.02	0.42	0.46	48.93
Argelès	6.31	0.16	0.16	1.15	0.01	0.22	0.22	31.17
Canet Plages	6.00	0.14	0.14	0.93	0.01	0.19	0.19	28.39
Barcares	6.23	0.16	0.16	1.02	0.01	0.26	0.23	35.25
Port La Nouvelle	5.43	0.19	0.25	0.94	0.01	0.31	0.25	32.34
Fleury	4.57	0.13	0.21	0.95	0.01	0.26	0.21	30.31
Agde	3.47	0.12	0.19	0.81	0.01	0.19	0.19	25.39
Marseillan	4.88	0.17	0.20	1.17	0.01	0.30	0.20	33.77
Sète	4.53	0.19	0.19	0.96	0.01	0.29	0.19	35.38
Frontignan	6.05	0.21	0.17	1.31	0.02	0.30	0.21	48.26
Grau du Roi	4.44	0.17	0.17	1.24	0.01	0.33	0.17	30.29
Palavas	6.30	0.20	0.20	1.53	0.02	0.35	0.23	37.35
Stes Maries	5.15	0.16	0.28	1.14	0.01	0.37	0.20	34.60
Ponteau	6.25	0.19	0.19	1.02	0.03	0.25	0.28	39.29
Carry	9.37	0.22	0.22	1.33	0.02	0.30	0.37	39.27
Marseille Nord	10.69	0.22	0.18	1.40	0.03	0.26	0.66	40.41
Marseille Sud	9.31	0.17	0.19	1.11	0.02	0.19	0.33	28.74
Cortiou	12.19	0.20	0.17	1.05	0.02	0.22	0.35	41.46
Cicie	13.43	0.19	0.15	0.88	0.02	0.19	0.24	34.33
Toulon Gd Rade	12.33	0.23	0.29	1.27	0.03	0.29	0.43	44.87
Balaguier	9.15	0.19	0.24	1.74	0.07	0.27	1.45	47.78

Metal concentrations

Measured metal concentrations in the soft tissues did not vary much from site to site (table 1). For most of the studied metals, the ratio between the maximum and minimum values was approximately 2 (cadmium, chromium, copper, nickel and zinc), whereas for arsenic it reached 3.9. For mercury and lead, if the high concentrations determined in mussels from Balaguier were not included, the ratios were 3.0 and 3.9, respectively.

Condition indices

At the 22 sites where both CI_{metal} (equation 1) and CI_{MT} (equation 2) were calculated, the relationship between these two indices was examined. This is described by the following equation:

$$CI_{metal} = 0.96\, CI_{MT} \quad (3)$$

The correlation between CI_{metal} and CI_{MT} was highly significant, allowing them to be used indifferently, despite the fact that the former was based on dry weight and the latter on wet weight.

The mean CI_{MT} value obtained at each of the 22 sites of translocation were compared (ANOVA, with p at the 95% level). Compared with the site of origin of the translocated mussels (Sète), the mean CI_{MT} (based on wet weight) was significantly different (higher) at seven sites only, namely Argelès, Canet Plages, Barcares, Fleury, Agde, Palavas and Marseille Sud. These variations in mean CI_{MT} did not influence MT levels (in mg kg^{-1} wet weight) in the soft tissues (figure 3a). In contrast, when elemental concentrations (in mg kg^{-1} dry weight) were considered at the same 22 sites, a negative relationship with the mean CI_{metal}

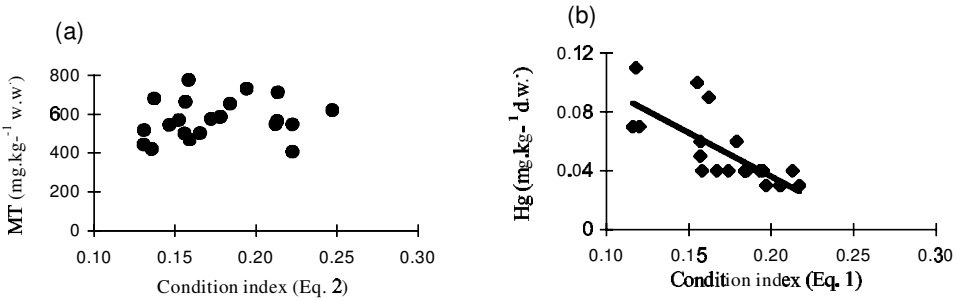

Figure 3. Relationship between CI and (a) MT concentrations and (b) measured metal concentrations (in this case mercury) in the soft tissues of mussels collected from 22 sites along the French Mediterranean coast. Each point represents the mean of eight individuals. w.w., wet weight; d.w., dry weight.

Table 2. Regression models between CI_{metal} (equation 1) and metal concentrations (mg kg^{-1} dry weight) for mussels collected from 97 sites on the French Mediterranean coast.

Metal	Model	r
Arsenic	$[As] = 1.32 \times (1/CI_{metal}) + 11.37$	0.45
Cadmium	$[Cd] = 0.12 \times (1/CI_{metal}) - 0.2$	0.94
Chromium	$[Cr] = 0.03 \times (1/CI_{metal}) + 0.47$	0.23
Copper	$[Cu] = 0.08 \times (1/CI_{metal}) + 3.22$	0.42
Mercury	$[Hg] = 0.005 \times (1/CI_{metal}) - 0.02$	0.75
Nickel	$[Ni] = 0.09 \times (1/CI_{metal}) + 0.32$	0.63
Lead	$[Pb] = 0.08 \times (1/CI_{metal}) + 0.23$	0.69
Zinc	$[Zn] = 14.13 \times (1/CI_{metal}) + 27.32$	0.94

(based on dry weight) was demonstrated (e.g. results for mercury are shown in figure 3) for all of the eight studied elements.

For the metals, analyses were carried out at all of the 97 sites of the RINBIO network, and the relationships between the metal concentrations (mg kg^{-1} dry weight) and the CI_{metal} values were calculated (table 2). This relationship was used to establish a correction factor that limits the effect of trophic conditions at each site of translocation. The new values of metal concentrations thus obtained for each site will be referred below as 'adjusted concentrations', and were calculated as follows:

$$\text{adjusted concentration} = \text{measured concentration} + [a \times (0.2 - CI_{metal})] \quad (4)$$

where a is the slope for each metal described by the equations in table 2; 0.2 is the reference CI derived from the typical values determined in mussels living in mesotrophic areas (the major trophic type along the North Atlantic coasts). Adjusted concentrations obtained in mg kg^{-1} dry weight were transformed to mg kg^{-1} wet weight by taking into account the wet and dry weights measured at each site (see Materials and methods).

Relationship between MT and metal levels

The relationships between MT concentration and elemental concentrations were examined using either the measured concentrations (shown in table 1) or the

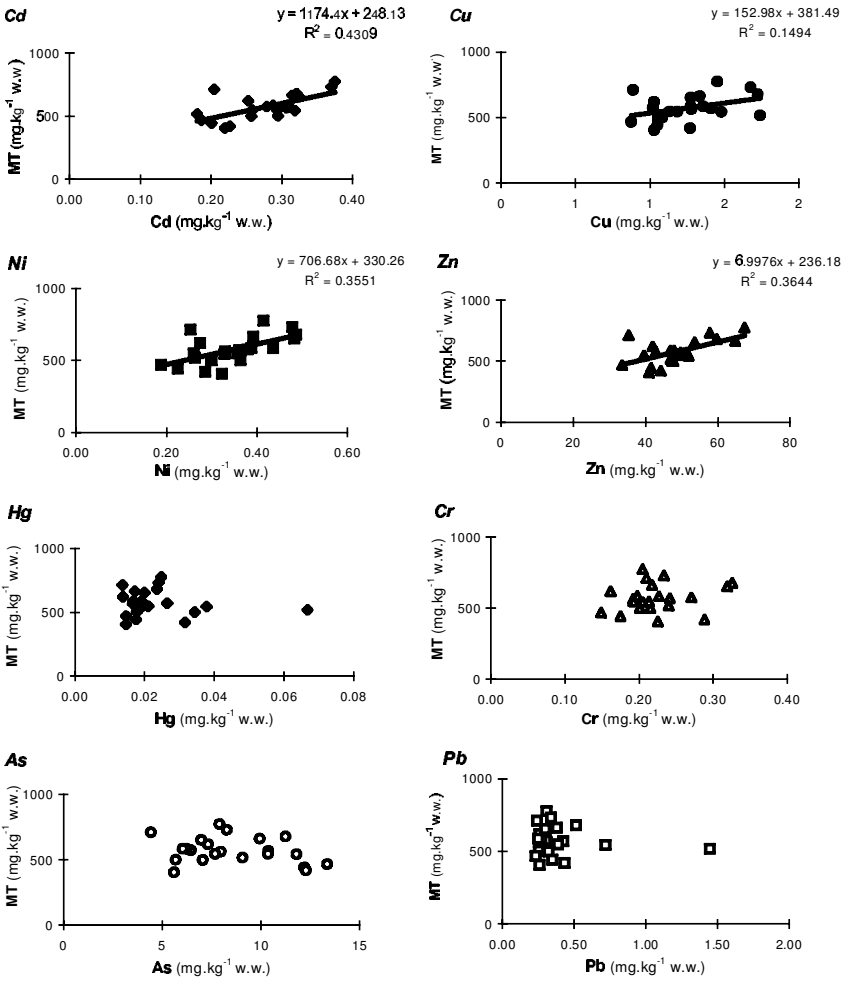

Figure 4. Relationship between measured MT concentrations and adjusted metal concentrations in the soft tissues of mussels collected from 22 sites along the French Mediterranean coast. Each point represents the mean of eight individuals. Closed symbols are used for elements known to bind to MTs, and opened symbols are used for elements not known to interact with MTs. w.w., wet weight.

adjusted concentrations. No significant relationships were obtained using the measured concentrations (data not shown). Using the adjusted concentrations, the results varied for the different elements (figure 4). No significant relationships were shown for those elements not known to have interactions with this cytosolic ligand (i.e. chromium, lead and arsenic). In contrast, a significant relationship was demonstrated for most of the metals that are considered as potential inducers of MT and/or are able to bind to this protein (i.e. cadmium, copper, nickel and zinc), with the exception of mercury (figure 4).

Since these metals might act together in the induction of MT, the total content of cadmium + copper + mercury + nickel + zinc was calculated in mmol kg^{-1}. The intersite fluctuations in this factor for each site with regard to MT concentrations are shown in figure 5. MT concentrations strikingly paralleled total metal

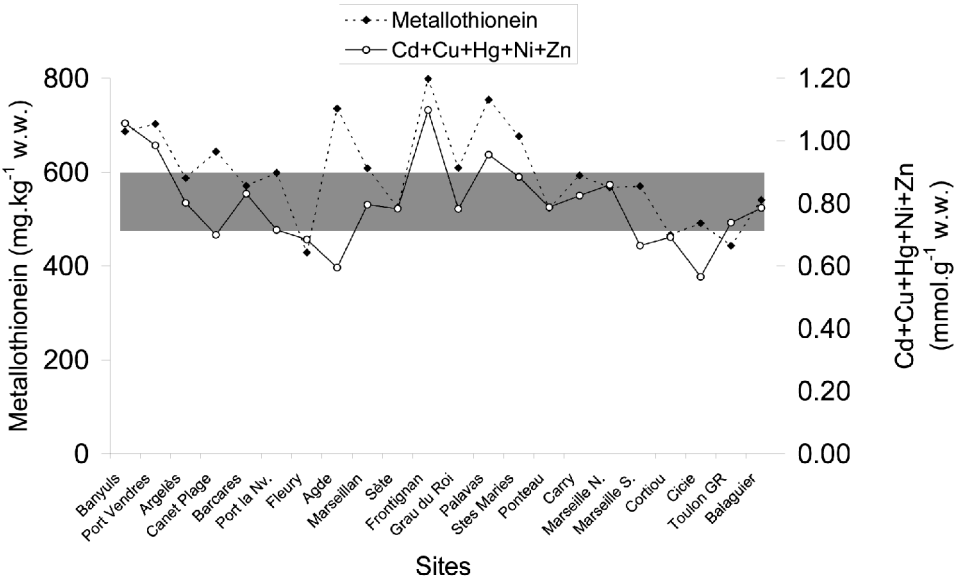

Figure 5. Comparison of geographic variations of MT and combined metal concentrations in the soft tissues of mussels collected from 22 sites along the French Mediterranean coast. Each point represents the mean of eight individuals. The grey strip represents the interval of median quartiles for the MT concentrations. w.w., wet weight.

concentrations except at one site (Agde), and a significantly positive correlation ($r = 0.6$) was found between these two parameters.

Discussion

Mussel species have been described as the best potential candidates among invertebrates for monitoring uses in the marine environment, based on MT as a biomarker of exposure to metals (George and Olsson 1994); later works by several authors have confirmed this point of view. More precisely, the digestive gland of mussels has been proposed as the best biological matrix for MT determination (Pavicic *et al*. 1987, Raspor and Pavicic 1991, Amiard *et al*. 1998, Raspor *et al*. 1999a,b, UNEP/RAMOGE 1999, Geffard 2001). This choice was based partly on the fact that MT induction was most marked in this organ. The results of the present study were in agreement with these data, as the concentration of MT in the digestive gland was more than two times higher than that in the whole soft tissues.

Since MT concentrations often parallel metal concentrations (Bebianno and Machado 1997, Amiard *et al*. 1998, Geffard 2001), it may be hypothesized that any factor that influences the bioconcentration of metals would also affect MT induction. Unexpectedly, the CI of Mediterranean mussels in the present study was strongly and inversely correlated with metal concentrations but not with MT levels. The increase in metal concentrations with decreasing weight and/or CI is well documented (Amiard *et al*. 1986, Hummel *et al*. 1997 and literature cited therein). This means that physiological changes have only a small effect on the metal body burden (the total metal quantity per individual). Although MT is a cellular ligand involved in homeostasis and detoxification of metals, it is also a protein, and it may be hypothesized that any factor that affects protein metabolism

may affect MT concentration. In some cases changes in MT concentrations are linked more to changes in general protein metabolism than to changes in metal accumulation (e.g. in crabs living in a metal-rich estuary; Legras *et al.* 2000). When the CI decreases, the fact that MT concentration remains stable indicates a decrease in the quantity of MT, which could be the consequence of environmental pressures such as less available food. Moreover, it must be kept in mind that MT-bound metals represent only a fraction of the total pool of metals. In the adult mussel *M. edulis* exposed experimentally to a high cadmium dose ($400\,\mu g\,l^{-1}$ for 65 days), 40% of the total cadmium in the whole soft tissues were bound to MT (Bebianno and Langston 1991). In the American oyster *Crassostrea virginica*, MT binds up to 20% of the cadmium in the gills, a much higher proportion than that found in *Ostrea edulis,* whereas the proportions of copper (1%) and zinc (0.3%) bound to MT were very low (Roesijadi 1994). In pre-veligers of the mussel *M. galloprovincialis*, the MT-detoxified pool reached 12% for zinc and 50% for cadmium (Pavicic *et al.* 1994). This suggests that the effect of the condition may differ between MT on the one hand and total metals on the other.

In this study, a mathematical model was used to calculate a correction factor that takes into account the effect of trophic conditions on metal concentrations at each site of translocation. In contrast, however, these conditions were shown not to affect MT concentrations, and thus it was not useful to apply such a model in this case. Thus adjusted metal concentrations and measured MT concentrations were able to be used to examine the relationship between these contaminants and the protein they induce.

With regard to the metals that are known to interact with MT, the fact that their concentrations correlated with MT concentrations in the whole soft tissues is a good argument for the use of MT levels in this matrix as a biomonitoring tool, even if the absence of correlation with mercury levels remains unexplained. The absence of correlation between MT concentrations and concentrations of arsenic, chromium and lead are in agreement with the present state of knowledge, since no biochemical interactions have been shown between MT and these elements. MT has been described as a biomarker of exposure to 'metals', but it is worth noting, particularly by end-users, that some elements cannot be revealed using this methodology. Therefore MTs should be regarded as biomarkers of response to certain metals and metal mixtures.

From an operational point of view, it would be faster to avoid the dissection of the digestive gland and to carry out MT determination after having just taken the soft tissues off the shell. In the present study, MT levels in the digestive gland and the total soft tissues were positively and significantly correlated, with intersite differences in exposure resulting in similar MT responses in both matrices. However, it must be remembered that this study took place from April to July, i.e. outside the period of sexual product ripening in the Northern Mediterranean Sea. In mussels, the sexual products are localized in the mantle. Thus, using the digestive gland alone allows the avoidance of seasonal changes in weight associated with the ripening of gametes, and thus interferences between this natural factor and metal contamination, according to the concept of the signal-to-noise ratio proposed by Cairns (1992). In oysters, which are also currently used for biomonitoring purposes, the gills have been preferred to the digestive gland because they are less influenced by temporal changes linked to sexual maturity (Geffard *et al.* 2001, 2002). However, no significant correlations were observed, even in the gills

between MT and metal levels in May and June, i.e. during the period that corresponds to sexual product ripening. Thus it may be concluded that, in general, it may be preferable to avoid translocation and sampling during the reproductive period, which is associated with both weight fluctuations and profound metabolic changes. In the case of commercial bivalves such as mussels, many studies of the natural reproductive cycle are available since they are important in indicating when the population can be harvested most effectively (Seed and Suchanek 1992).

When the relative efficiency of biomonitoring based on the metal or the MT determinations was examined by comparing the intersite changes revealed by one set of values or the other, the information derived from either metal or MT levels was generally similar, with the highest concentrations registered at the same places. Among the six sites showing the highest concentrations of MT (Banyuls, Port-Vendres, Agde, Frontignan, Palavas and Les Saintes Maries), five also showed the highest metal concentrations. The exception was at Agde, where one of the highest values of MT concentrations in mussels from the French Mediterranean coast was determined but the combined concentration of cadmium, copper, mercury, nickel and zinc was one of the lowest. This high MT level could be due to the presence of a metal that was not analysed in the present study but is able to induce MT, such as silver. Silver has a higher affinity for MT than copper, zinc, cadmium or mercury (Mayer *et al*. 1996) and has been shown to induce MT in a copepod crustacean (Barka *et al*. 2001). Non-metallic factors have also been recognized as being able to enhance MT induction (Kägi 1993), even if they are generally considered to be less potent than metals. The possibility of interactions between environmental microcontaminants with regard to biochemical responses used as biomarkers suggests that the best procedure would be the concomitant use of several biomarkers (Narbonne *et al*. 1999). The present study reinforces previous findings showing that MT may be considered as a core biomarker, along with ethoxyresorufin-*O*-deethlyase (EROD), acetylcholinesterase (AChE) and malondialdehyde (MDA).

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