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Metallothionein as a tool in biomonitoring programmes

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The biochemical features of metallothioneins and their functional role in the cell are described. On this basis, the potential role of MTs as a biomarker of exposure in aquatic organisms, such as fishes and molluscs, is evaluated in the light of recent knowledge about MT gene regulation and inducibility. It appears that in fish MTs should be considered as a kind of stress protein which is particularly responsive to heavy metals. In molluscs, in particular in mussels, MTs seem more specifically involved in responses to heavy metals and they should therefore be considered a biomarker of exposure to heavy metal pollution. Common techniques for MT evaluation are listed and a simple spectrophotometric method recently developed is also reported. Finally, the correct approach to the use of MTs as a biomarker of exposure in biomonitoring programmes for an assessment of the physiological status of aquatic organisms is discussed.

Keywords: molluscs, fish, metallothionein, biomarker of exposure, heavy metal.

Abbreviations: ARE, antioxidant response element; bp, base pair; BaP, benzo(*a*)pyrene; DTNB, 5,5-dithiobis-2-nitrobenzoic acid; GRE, glucocorticoid response element; MT, metallothionein; MRE, metal response element; MLTF, major late transcription factor; PAH, polycyclic aromatic hydrocarbons; PCB -156, 2, 3, 3´, 4,4´,5-hexachlorobyphenyl; ROS, reactive oxygen species; SDS–PAGE, sodium dodecyl sulphate poly–acrylamide gel electrophoresis.

Introduction

Metallothioneins are a class of metalloproteins first described by Margoshes and Vallee (1957) in the horse kidney. MTs show particular biochemical features: they are of low molecular weight (about 6000–8000 Da, 61 amino acid residues in mammal MTs), soluble, sulphydryl-rich proteins (≈ 30% cysteine content in mammal MTs), with a peculiar amino acid sequence (characteristic distribution of cysteinyl residues such as Cys-X-Cys, Cys-Cys, Cys-XY-Cys, where XY are amino acids different from cysteine) (Kägi and Kojima 1987, Kägi and Shaffer 1988). MTs have a high heavy metal affinity and binding capacity (7–9 g atom mol^{-1} thionein) and are able to chelate both essential (Zn, Cu, etc.) and non-essential metals (Cd, Hg, Ag, etc.) by cysteine tetrathiolate clusters. However, MTs show different affinities for heavy metal cations ($Hg^{2+} > Cu^{+} > Cd^{2+} > Zn^{2+}$) (Viarengo 1989). MTs show virtual lack of light absorbance at 280 nm due to the absence of aromatic amino acids but show typical absorbance spectra due to particular interactions of different cations in metal thiolate clusters (Cd = 254 nm, Cu = 272 nm, Zn = 212 nm). These metallotetrathiolate clusters provide the protein with a highly stable tertiary structure that renders it heat stable (Kägi and Kojima 1987).

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MTs are inducible proteins. Heavy metal cations accumulated within the cells stimulate metalloprotein neosynthesis by enhancing MT gene transcription (Squibb and Cousin 1977, Andersen and Weser 1979). The MT mRNA is translated by cytosolic free ribosomes, leading to an increase of apometallothionein that will rapidly react with free metal cations present in the cytosol. Due to their biochemical and functional characteristics, MTs are able to protect cell structures from non-specific interactions with heavy metal cations and to detoxify metal excess penetrating into the cell (Hamer 1986, Viarengo and Canesi 1991, Roesijadi 1992, Viarengo and Nott 1993). Due to their inducibility to heavy metals, MTs are usually considered an important specific biomarker to detect organism response to inorganic pollutants such as Cd, Hg, Cu, Zn, etc., present in the aquatic environment. Bioindicator organisms that have been commonly employed in the application of MTs as biomarkers are fish (Addison and Clarke 1990, Hylland *et al.* 1992, 1996), molluscs (Engel and Roesijadi 1987, Viarengo 1989) and crustaceans (Pedersen *et al.* 1997). The importance of MTs as a tool for biomonitoring activities is increased by the fact that they are ubiquitary proteins and therefore can be studied in most living organisms (Hamer 1986).

MT regulation

Although MTs have been widely utilized to identify specific responses to heavy metal pollution, there is now a body of evidence demonstrating that in vertebrates (mammals and fish) MT synthesis is stimulated by different endogenous and exogenous agents (Kägi 1993), e.g. glucocorticoid hormones, various kinds of stress (cold, heat, extreme exercise), cytokines and in particular compounds leading to production of reactive oxygen species (ROS) (Dalton *et al.* 1994). Therefore, in mammals and fish, not only inorganic pollutants such as heavy metals but also organic contaminants such as paraquat may activate MT neosynthesis (Wormser and Calp 1988, Sato *et al.* 1989, Bauman *et al.* 1991, Pedrajas *et al.* 1995).

The molecular mechanisms by which different agents can induce MT synthesis in vertebrate cells have been recently investigated by studies concerning the structure of the MT gene promoter. Mapping of mammalian MT gene 5´ flanking regions has greatly contributed to the understanding of the molecular basis of their inductive properties. Cis-acting sequences, termed metal response elements (MREs), located in multiple copies along the promoter allow heavy metal ion induction of MT transcripts (Stuart *et al.* 1985, Hamer 1986). In the mouse, a metal response transcription factor (MTF-1) binds to MREs, activating the MT gene by a mechanism that is not fully understood (Palmiter 1994). Glucocorticoid response elements (GREs) have been well documented in the human and mouse MT gene (Karin *et al.* 1984, Kelly *et al.* 1997). In addition, genetic elements responsive to ROS-producing agents have been described (Dalton *et al.* 1994). Deleted mutagenesis of the mouse MT-I gene promoter and transient transfection assays in hepatoma cells have demonstrated that the stimulation of transcripts by ROS production needs the involvement of a composite major late transcription factor/antioxidant response element (MLTF/ARE) (Carthew *et al.* 1987), and of metal response promoter elements, suggesting a synergetic action between the regulatory elements of the MT promoter (Dalton *et al.* 1994, 1996). The ARE is an element which was first characterized in the promoter of the human phase II drug-metabolizing enzyme gene which is responsible for responses to xenobiotics and

hydrogen peroxide (Li and Jaiswal 1992). The transcription factor(s) responsible for transactivation through the ARE are not well defined. Some authors report a role for AP-1 complexes, while others claim the involvement of a novel factor (Jaiswal 1994). It has been found that in the mouse MT-I promoter, AP-I can bind to ARE but further investigations are required for a full understanding of the molecular mechanisms by which the MT gene is induced under pro-oxidant conditions (Dalton *et al*. 1996).

More recent studies have demonstrated that the MT gene promoter of fish show elements which are similar to those of mammals (figure 1) (Kille *et al*. 1993, Olsson *et al*. 1995, Samson and Gedamu 1995). Heavy metal gene induction in fish has been widely investigated (Roche and McCarter 1984, George 1989, Olsson *et al*. 1989a, Hogstrand *et al*. 1996, Marr *et al*. 1996, Schlenk *et al*. 1997, Lam *et al*. 1998), including the molecular mechanisms of gene activation (Samson and Gedamu 1995). However, authors have also focused their interest on the response of MT to inductors other than heavy metals. Olsson *et al*. (1995) demonstrated that in RTH-149 cells hydrogen peroxide could activate MT gene transcription probably through an ARE consensus sequence. Similar *in vitro* experiments concerning organic xenobiotics are lacking, but it can be inferred from *in vivo* experiments on fish that polar xenobiotics such as paraquat (Pedrajas *et al*. 1995), but not apolar xenobiotics such as BaP or PCB-156 (George 1989, Hylland *et al*. 1996), can stimulate MT neosynthesis. Moreover, it has been shown that the pre-treatment of fish with BaP or PCB-156 prior to heavy metals can inhibit MT

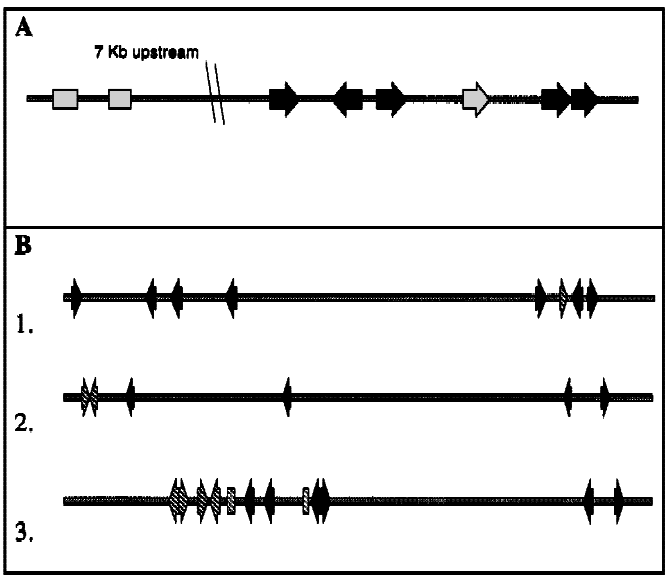

Figure 1. Diagrams of different vertebrate MT gene promoters. Panel A. Mouse MT-I gene promoter: scheme of the regulative elements involved in transcriptional induction. The proximal promoter (≅ 200 bp) contains cis-acting sequences responsive to heavy metal ions (MRE) or to oxidative stress (MLTF/ARE). A pair of adjacent glucocorticoid response elements (GRE) located 7 kb upstream the MT-I gene allow response to steroid hormone stimulation (references in the text). Panel B. Three recently sequenced fish MT gene promoters: (1) stone loach (≅ 700 bp) (2) pike (≅ 1000 bp), (3) rainbow trout (MT-A, ≅ 1000 bp). Putative cis-acting regulatory elements resembling consensus sequences of the mammalian MRE, GRE and ARE are show (modified by Olsson *et al*. 1995). (⬟) MREs; (⇨) AREs; (▭) GREs.

induction (Sandvik *et al*. 1997), showing that the use of MT as a biomarker of exposure to heavy metal can be complicated by interactions with other chemicals.

Endocrine functions can greatly influence MT response in fish, even though the molecular mechanisms involved and their physiological meaning often remain unclear. For instance, during sexual maturation female individuals can show high hepatic zinc-MT levels (Olsson *et al*. 1986, Overnell *et al*. 1987). The same effect was obtained by Olsson *et al*. (1989b) who demonstrated that in juvenile rainbow trout, MT transcripts and Zn-thionein increased after a single intraperitoneal injection of 17-β-oestradiol. Moreover, it must also be mentioned that the injection of 17-β-oestradiol in the rainbow trout completely altered the tissue distribution of cadmium, redirecting the metal from liver to gills, gut, and muscle (Valencia *et al*. 1998). Such findings could be relevant in biomonitoring, as xenoestrogen pollutants, a new class of contaminants, could influence MT response to heavy metals in fish liver, the tissue commonly used for biomarker analyses. Also the responsiveness to heavy metal induction can vary between males and females belonging to the same population (Hamza-Chaffai *et al*. 1997).

As reported above, GRE consensus sequences were found in the rainbow trout MT-A gene promoter (figure 1), suggesting that glucocorticoid hormones could have a role in the regulation of the MT gene in fish. However, treatments with glucocorticoids made on cultured cells or *in vivo* yielded conflicting results. Primary trout hepatocytes exposed to the stress hormone cortisol gave rise to elevated MT accumulation, whereas RTH-149 cells did not (Olsson *et al*. 1990, 1995). Moreover, *in vivo* glucocorticoid treatment did not produce any increase in MT transcripts in the rainbow trout (Olsson *et al*. 1990).

MTs in the mussel

As for marine molluscs, and mussels in particular, it is known that not only heavy metals but also strong changes in environmental parameters such as temperature, oxygen and salinity are able to increase the cellular concentration of MTs (Viarengo *et al*. 1988a) (table 1). Moreover, mussel MT levels also show seasonal changes, following total zinc concentration in tissues (Viarengo *et al*. 1997). However, little is known about the structure of the mussel MT gene promoter, whose inductive properties can only be inferred from indirect data. Recent experiments have demonstrated that exposure in aquarium to 4 mg l^{-1} paraquat for 1–7 days does not stimulate MT neosynthesis, although it strongly affects mussel physiology, as indicated by lysosome membrane destabilization in

Table 1. Effects of environmental parameter changes on the level of MTs in the digestive gland of *Mytilus galloprovincialis* Lam.

Environmental parameters	Percent MT variation
T (13–23 °C)	100%
Salinity (22–36‰)	50%
O_2 (±)	300%
Heavy metals	up to 2,000%

Modified from Viarengo *et al*. (1988a).

digestive gland cells (Viarengo, unpublished data). By contrast, in mammals and fish exposure to paraquat stimulates MT neosynthesis. (Bauman *et al*. 1991, 1992a, b, Pedrajas *et al*. 1995).

It has been demonstrated that in the mouse ARE-mediated gene induction by planar aromatics requires cytochrome p450 activity (Friling *et al*. 1990). As is known, in mussel cells there is a low level of mixed function oxygenase activity (about 1% compared with mammalian MFO activity) and tissue accumulation of organic xenobiotic compounds does not strongly enhance the cytochrome p450 level (Ade *et al*. 1984, Livingstone 1988). Hence, even though the mussel MT gene promoter might be ROS-activated, organic pollutants seem to be unable to stimulate MT synthesis possibly due to a scarce induction of p450 mediated ROS production.

The fact that MT concentrations in mussel tissues may vary in response to strong fluctuations of environmental parameters could introduce a bias in biomonitoring data. However, this can be minimized by using standard animals caged for a brief period of time (about 1 month) in sites where uniform environmental parameters can guarantee comparable field conditions (temperature, salinity, oxygen concentration, depth, tidal effects, etc.). Experiments carried out at different clean sites along the Ligurian coast (Mediterranean Sea) have shown that caging for 1 month can induce minimal variations in mussel physiology, as the different biomarkers tested have shown a tendency to maintain stable control values (Gabrielides 1997a, b, Viarengo *et al*. 1998, Viarengo unpublished data).

MT quantification

Different methods have been developed for MT evaluation in mussel tissues: chromatographic separation of soluble cytosolic MT-containing fraction associated with the evaluation of the metal concentration, HPLC–AAS (Suzuki 1980, Lehman and Klaassen 1986) and HPLC–ICP (Sunaga *et al*. 1987, Mason *et al*., 1990, Mazzucotelli *et al*. 1991), metal substitution assays (Piotrowski *et al*. 1973, Eaton and Toal 1982, Scheuhammer and Cherian 1986, Lobel and Payne 1987, Martinez *et al*. 1993), radioimmunological techniques (Nolan and Shaikh 1986, Roesijadi *et al*. 1988, Hogstrand and Haux 1990a, b), and electrochemical analyses (Olafson and Sim 1979, Thompson and Cosson 1984). Most of these methods yield excellent results but are expensive and/or too sophisticated to be used as routine analyses in environmental biomonitoring programmes. However, recently a spectrophotometric method has been developed which is simple, highly sensitive, low-cost and allows accurate MT quantification in the tissues of marine organisms (Viarengo *et al*. 1997). This method involves the evaluation of the MT concentration in a partially-purified metalloprotein-containing fraction obtained by acidic ethanol/chloroform fractionation of tissue homogenate. The procedure includes precautions for obtaining a complete MT precipitation, and for avoiding sulphydryl oxidation and contamination by soluble low molecular weight thiols and enzymatic protein degradation, which can occur during sample preparation. In the extracts MTs are denatured by low pH and high ionic strength and quantified spectrophotometrically by using the Ellman's sulphydryl reagent DTNB. The procedure utilized to obtain the MT-containing fraction and to evaluate the MT content in the extracts is summarized in figure 2. The reliability of this method has been confirmed by SDS–PAGE separation and fluorimetric analysis of the proteins

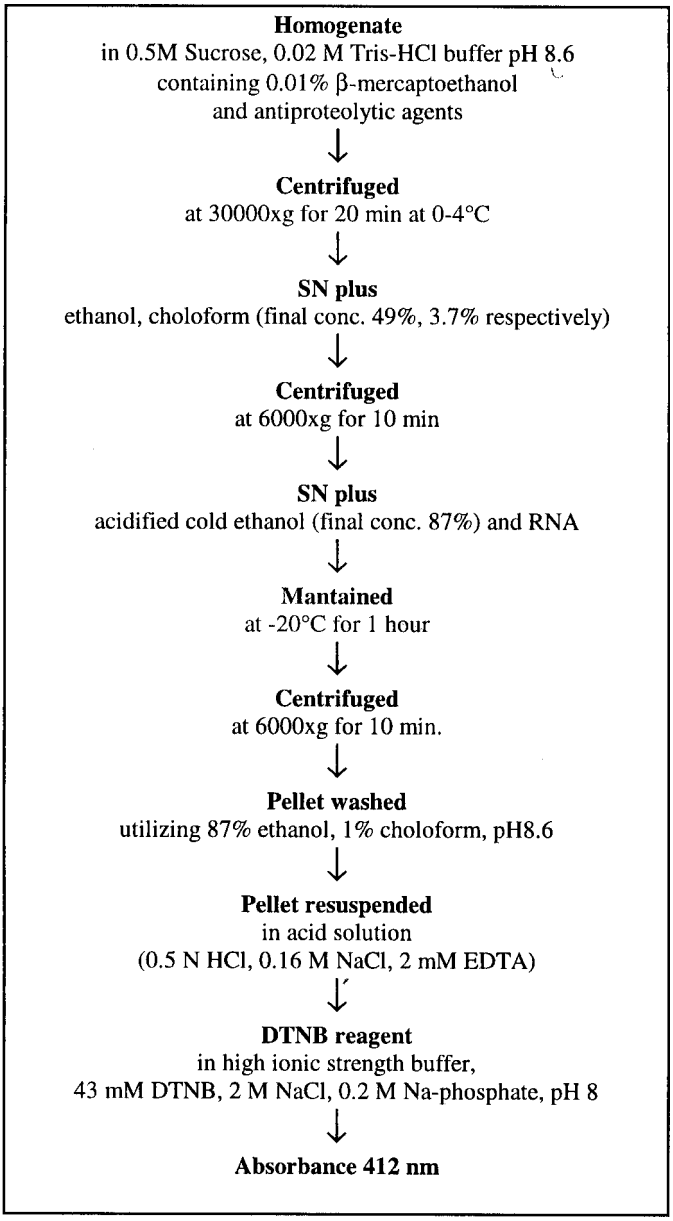

Figure 2. Schematic protocol of the spectrophotometric method for MT evaluation reported by Viarengo *et al*. (1997).

present in the MT-containing fraction, labelled with the fluorescent sulphydryl reagent bromobimane (Kosower and Kosower 1987), showing minimal amounts of SH-rich proteins different from MTs.

Use of mussel MT in biomonitoring

Due to the fact that the mussels are often used as sentinel organisms in

biomonitoring programmes, a number of data are currently available concerning the concentration of MTs in the tissues of mussels living in clean or polluted water (Viarengo *et al*. 1988b, Gabrielides 1997a b, Viarengo *et al*. 1998). The results reported in figure 3 show MT concentrations in the digestive gland of mussels caged for 21 days in different sites along the Ligurian coast. These data belong to a pilot study for the realization of a large biomonitoring programme along the coast of the North Thyrrenian Sea (Mediterranean). In mussels sampled at two polluted sites, the oil terminal Porto Petroli and Lavagna harbour, there is a slightly higher MT concentration (≅ 20%) with respect to values found in animals sampled in clean water at the reference site of Paraggi. However, only at the Lavagna harbour is the level of MT content significantly different from Paraggi. It is important to note that chemical analyses have shown that heavy metal concentration (Zn, Cu, Cd) in mussels from polluted sites is 20% higher than in mussels from Paraggi. On the other hand, PAH concentration in mussels is 10 times higher at Lavagna and 20 times higher at Porto Petroli with respect to Paraggi (data not shown). Hence, the level of MTs is not directly correlated with organic pollutant concentrations, confirming that mussels MTs should be considered stress proteins primarily able to point out the biological effects of inorganic pollutants.

Besides a variable responsiveness to different pollutants, other factors call for a thoughtful interpretation of MT data in biomonitoring programmes. In fact, the various heavy metals able to induce MTs show different biological half lifes. As reported by Viarengo *et al*. (1985), in mussel digestive gland copper shows a biological half life of 9–10 days, while the half life of cadmium is 4 months (figure 4). Considering that in mussel tissues MT levels follow metal concentrations, rises in MT could have a very different ecological meaning. In the case of copper, an increase in MT indicates a pollution event that is existent or occurred at most 1–2 weeks before mussel sampling. In the case of cadmium, high levels of MT could even be related to a seawater contamination which happened months before. Therefore, in the application of MT as a biomarker of heavy metal exposure the use of caged mussels seems to be the best approach in order to obtain replicable and comparable results that are easy to interpret. However, the contemporaneous use of caged and wild animals can also provide important additional information about the dynamics of pollution events.

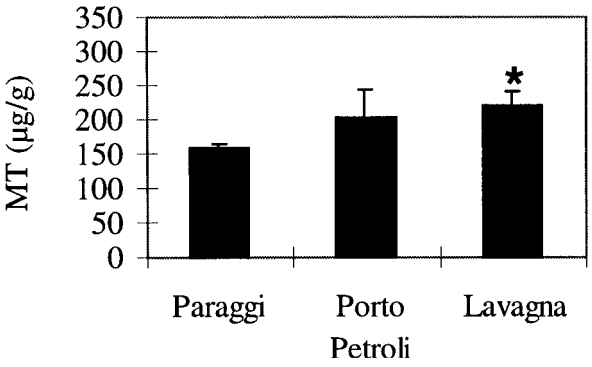

Figure 3. MT concentration in the digestive gland of mussels caged for 21 days in two polluted sites (Porto Petroli and Lavagna) and a reference site (Paraggi) along the Ligurian coast (Mediterranean Sea). MT content was evaluated as described in Viarengo *et al*. (1997). * = significantly different from the reference site of Paraggi ($P < 0.05$, Mann–Whitney test).

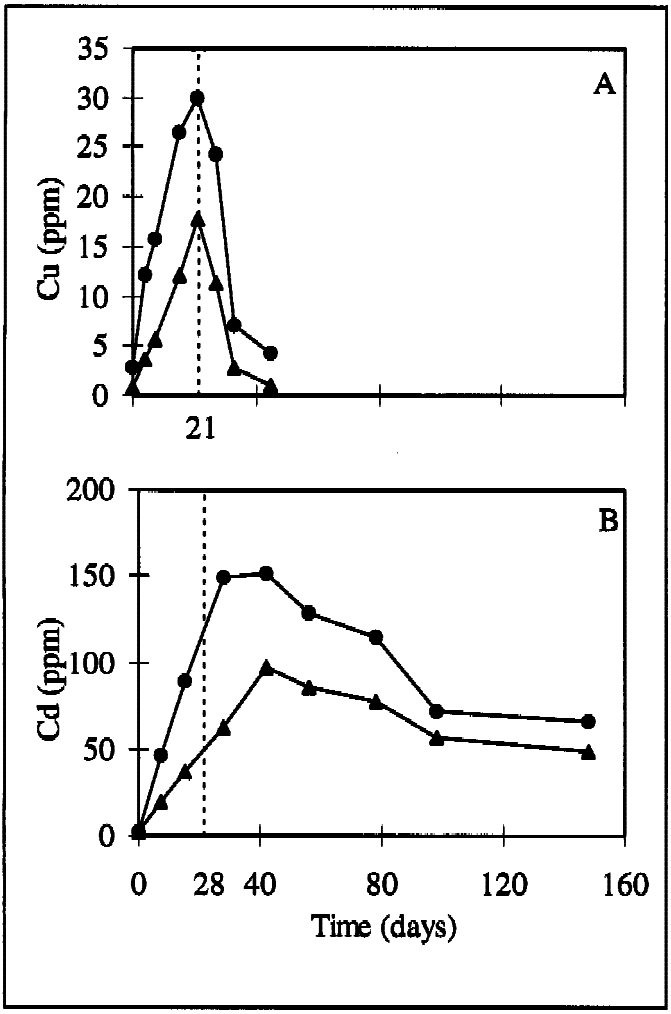

Figure 4. Time variations of heavy metals and metallothioneins in the digestive gland of mussels exposed to Cu or Cd and then detoxified (modified from Viarengo *et al.* 1985). Panel A: concentration of copper (v) and Cu-thionein (¶) in mussels exposed to copper (0.04 ppm) for 21 days and then detoxified for different periods of time. Panel B: concentration of cadmium (v) and Cd-thionein (¶) in mussels exposed to cadmium (0.2 ppm) for 28 days and then detoxified for different periods of time. The MT concentration was evaluated as the metal bound to the MT-containing fraction, obtained by gel filtration analysis of the heat-treated cytosol. The experiment is described in Viarengo *et al.* (1985).

Conclusions

The use of a simple, accurate and sensitive technique for MT detection (such as the simple spectrophotometric method here described) will probably stimulate basic research on MT, which in turn will help in the utilization of MTs as biomarkers of exposure. Concerning this last point, it is important to emphasize that further research is needed to improve the use of MT for an early warning of the biological effects of heavy metals on aquatic organisms, in order to allow the identification of coastal areas where inorganic contamination is relevant. In particular, the use of MT in biomonitoring should be always supported by knowledge about the physiology of

stress responses in bioindicator organisms. For instance, as mentioned above, MT induction in fish should be considered a general stress response, particularly sensitive to heavy metals. Conversely, in mussels and possibly in other molluscs, MTs seem to represent a more selective biological response to heavy metals. It is finally important to emphasize that a correct approach in biomonitoring consists of the use of a battery of biomarkers: therefore MT should be always used in association with other biomarkers of exposure such as MFO activity, acetylcholinesterase activity and biomarker of stress such as destabilization of lysosomal membranes, lipofuscin accumulation and DNA damage.

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