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Application of two SH-based methods for metallothionein determination in mussels and intercalibration of the spectrophotometric method: laboratory and field studies in the Mediterranean Sea

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Abstract

Metallothionein (MT) induction is widely used as a biomarker of exposure to metals in mussels. The aims of the present work were first to compare the suitability of spectrophotometry and differential pulse polarography (DPP) for MT detection in mussels exposed to 200 ppb cadmium for 9 days in a laboratory experiment and in mussels sampled in different seasons from expected pollution gradients along the Mediterranean Sea; second, to intercalibrate the widely used spectrophotometric method using mussels from Saronikos Gulf. In the intercalibration of the spectrophotometric method, similar results ($p > 0.05$) were obtained by two different research teams indicating a good reproducibility of the technique. However, polarographic and spectrophotometric methods gave significantly ($p < 0.05$) different results in laboratory and field studies. In the laboratory experiment, MT values detected with DPP were nine times higher than with spectrophotometry. The results obtained by the two methods were significantly correlated. Both methods could discriminate between control and exposed mussels. In field studies, MT values obtained by DPP were 34–38-fold higher than with spectrophotometry, and MT concentrations measured by both methods were not correlated. This discrepancy could be due to several factors, including the low levels of bioavailable metals in the studied areas and the possibility that the different methods can measure MT isoforms differentially. Further work is needed to decipher the functions of MT isoforms in mussels. This information is relevant for the application of MT as a biomarker in biomonitoring programmes.

Keywords: *Metallothionein quantification, differential pulse polarography, spectrophotometry, mussel, digestive gland, cadmium exposure, Mediterranean Sea*

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Introduction

Metals are among the major contaminants reaching the marine environment. Their levels have been increasing over the last decades as a result of technological development. Some molluscs, notably mussels and oysters, have been selected for monitoring purposes in order to assess the bioavailable fraction of metals in the marine environment (Rainbow & Phillips 1993). The use of bivalve molluscs as bioindicators looks attractive as these organisms take up metals from all environmental compartments (i.e. from the aqueous medium and — through ingestion — from food and inorganic particulate material) and concentrate them to a great extent (Phillips 1977, Goldberg et al. 1978). The exposure of marine organisms to certain metals leads to alterations in several biochemical processes that have the potential to be used as biomarkers of exposure and therefore as 'early warning' signals of the presence of these particular contaminants (Bayne et al. 1988, Roesijadi 1992, Cajaraville et al. 2000).

Metallothioneins (MTs) are one of the most widely used exposure biomarkers for metals since the exposure to metals induces MT expression in different species and tissues. MTs are low molecular weight, cysteine-rich cytosolic proteins that bind up class 1B (i.e. copper (Cu) and silver (Ag)) and 2B metals (i.e. zinc (Zn), cadmium (Cd) and mercury (Hg)) (Kägi & Kojima 1987, Olsson et al. 1998). MTs play a role in multiple biological processes such as homeostasis of essential metals (Zn and Cu), detoxification of toxic metals (Cd, Ag and Hg) and cell protection against oxidative stress caused by free radicals (Roesijadi 1994, Langston et al. 1998, Klaasen et al. 1999, Viarengo et al. 2000a, Dabrio et al. 2002). MT induction has been included in international marine monitoring programmes such as OSPAR JAMP (Joint Assessment and Monitoring Programme) (Stagg 1998) and MED POL (Mediterranean Pollution Biomonitoring Programme) (UNEP/RAMOGE 1999) as a biomarker of exposure to assess metal pollution in aquatic ecosystems. Both metal and MT levels have been shown to vary depending on body weight, gender, reproductive stage, size, season and water temperature (Hylland et al. 1992, Roesijadi 1994, Olsson et al. 1995, 1996, Bordin et al. 1997, Serra et al. 1999, Serafim et al. 2002). Consequently, these natural fluctuations have strong implications for the use of MTs as a biomarker for the determination of metal pollution (Olsson et al. 1998).

In addition, the interpretation of MT data is complicated by the variability in the results obtained with the same method and by the diverse methodologies used to isolate and quantify MTs. UV-Vis spectrophotometry, electroanalytical techniques, metal saturation assays and immunological techniques such as enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) have been successfully applied for the quantification of total MTs (see reviews by Cosson 2000, Isani et al. 2000, and Dabrio et al. 2002). Quality assurance and MT standard reference materials are required to validate some of these methods (Cajaraville et al. 2000, Viarengo et al. 2000b). Moreover, different methods use different units to express MT concentrations, making it difficult to compare the results obtained by different research teams. Consequently, there is a strong need to make comparable the results obtained using different methods and to validate the methods more widely used for MT determination.

Methods available for MT detection in molluscs are those based on the determination of sulphydryl levels such as differential pulse polarography (DPP) and spectrophotometric method using Ellman's reagent (Bebianno & Langston 1989,

Viarengo et al. 1997). These seem to be the most promising techniques for routine evaluation of MTs in mussel samples (Ivankovic et al. 2002). The present work aims to achieve two main objectives: (1) to compare the suitability of the spectrophotometric and polarographic methods to measure MT levels in mussels from a laboratory experiment and from selected sites of the Mediterranean Sea; and (2) to intercalibrate the spectrophotometric method using mussels collected in the Saronikos Gulf.

Material and methods

Laboratory experiment

Mussels, *Mytilus galloprovincialis* (4–5 cm shell length), were collected from Plentzia, Biscay Gulf (43°24′90N; 2°56′90W). The animals were kept unfed in an aquarium at a constant 15°C for 1 week for acclimatization before the start of the experiment. Natural seawater for all the experiments was previously sterilized with ultraviolet light, filtered through active charcoal and changed daily. During the experiments the water was constantly aerated (dissolved oxygen 7.6–8.3 mg l^{-1}; pH 7.9–8.1; salinity 35‰; temperature 15°C and with a 12-h light/dark cycle). After acclimatization, mussels were fed daily with a commercial food mixture (Marine Invertebrate Diet, Burlington, NC, USA).

Eighty mussels were exposed to 200 ppb (1.785 μM) Cd for 9 days and another 80 mussels were kept as control mussels. The metal was added daily to the seawater in the form of Cd chloride ($CdCl_2$).

After completion of the experiment, digestive glands from 80 Cd-treated mussels were dissected out and divided in half. One half was used to measure MT levels by DPP. The other half was reserved for spectrophotometry. In order to avoid variability between individuals, the same digestive glands were used to measure MT levels by both techniques. Control mussels were processed in the same way. In total, four replicates of approximately 1 g were analysed per experimental group.

Field studies

In order to quantify MT levels by spectrophotometry and DPP, mussels, *M. galloprovincialis* (4–5 cm shell length), were collected in September 2001 in four stations of the Ligurian Sea (Italy) and in four stations on the Saronikos Gulf (Greece). In September 2002 and May 2003, mussels were also collected in 12 stations along the north-western Mediterranean Sea in France, Italy and Spain. At each station, for the spectrophotometric method, 24 mussel digestive glands were dissected out and frozen in liquid nitrogen. Four replicates of six digestive glands each were prepared for this method. For MT determination by DPP, 12 frozen mussel digestive glands were divided in three replicates.

For the intercalibration of the spectrophotometric method, mussels, *M. galloprovincialis* (4–5 cm shell length), were sampled in four stations of the Saronikos Gulf in May 2003. In order to obtain four similar replicates per station for MT determination, 48 mussel digestive glands were dissected out, halved and measured by two different research groups: the University of the Basque Country and the Hellenic Centre for Marine Research. In addition, the whole soft tissue of 15–20 mussels was collected

Table I. Description of the sampling sites of the four studied areas of the Mediterranean Sea.

Site	Station	Coordinates	Description
1. Catalonian coast	Cala Montjoy	42°15′00N, 3°14′50E	tourist area in the summer
	Fangar	40°46′40N, 0°45′60E	rice agriculture, marine farms, fishing
	Alfacs	40°36′70N, 0°36′30E	rice agriculture, marine farms, fishing
	Barcelona	41°22′55N, 2°11′80E	intense maritime traffic, industrial effluents
2. Gulf of Fos-sur-mer/Marseilles	Aragnon	43°18′70N, 5°04′26E	reference station
	Lavera	43°25′00N, 4°53′25E	near oil refineries
	Fos harbour	43°25′00N, 4°53′25E	refineries and oil tankers
	Cortiou	43°11′50N, 5°23′00E	sewage effluents from Marseille
3. Western Ligurian coast	Portofino	44°18′62N, 9°12′80E	marine reserve park
	Voltri	44°25′12N, 8°46′65E	moderate industrial input, oil tankers
	Genoa inside	44°24′73N, 8°54′99E	intense maritime traffic, industrial effluents
	Genoa outside	44°23′44N, 8°55′83E	shipwreck of the oil tanker *Haven*
4. Saronikos Gulf	Megara	37°59′01N, 23°24′49E	mussel farm, reference station
	Skaramagas	37°59′95N, 23°35′03E	shipyard
	Piraeus	37°56′47N, 23°38′19E	harbour, intense maritime traffic
	Epidavros	37°45′69N, 23°07′55E	reference station
	Agios Kosmas	37°52′68N, 23°43′61E	reference station
	Anavissos	37°43′45N, 23°54′18E	reference station

per station in order to determine the metal content by atomic absorption spectrophotometry (AAS).

Sampling sites with the selected stations are shown in Table I and in Figure 1. In France, the Gulf of Fos-sur-mer/Marseille is a site characterized by one of the most important petrochemical activities in France, intensive maritime traffic and the presence of wastewater collectors from Marseille. In Italy, the western part of the Ligurian coast is a highly impacted area in which there is a petrochemical terminal and a number of industrial effluents. In addition, previous industrial settlement involving discharge of heavy metals, mainly chromium, produced a high storage of these compounds in the sediment. On the Catalonian coast (Spain), Barcelona harbour has intense maritime traffic and is impacted anthropogenically (Baumard et al. 1999, EEA 1999). In Greece, the Saronikos Gulf is mainly influenced by the sewage outfall of the Athens–Piraeus metropolitan area where there are no river discharges and agricultural activities are limited. Elefsis Bay is in the north part of Saronikos Gulf and is located along the North Coast of the bay.

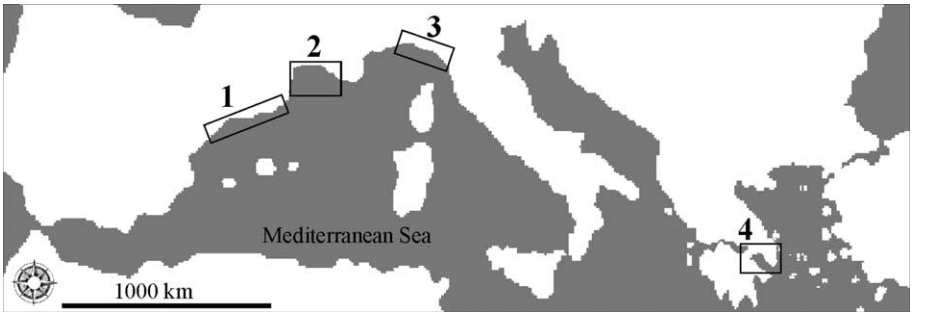

Figure 1. Map of the four studied areas of the Mediterranean Sea: 1, Catalonian coast; 2, Gulf of Fos-sur-mer/Marseille; 3, western Ligurian coast; and 4, Saronikos Gulf.

Metallothionein determination

Differential pulse polarography. MT content was evaluated according to the Brdicka reaction (Brdicka 1933) modified by Olafson and Olsson (1991). Digestive gland tissues were homogenized in 4 vols 20 mM Tris-HCl buffer, pH 8.6, then centrifuged at 30 000*g* for 1 h at 4°C in a Kontron ultracentrifuge (Kontron Instruments S.p.A., Milan, Italy). Dithiothreitol was not added to the homogenizing buffer since it provoked interference in the MT measurement (see also Erk & Raspor 2000). The supernatant (cytosol) was separated from the pellet. An aliquot was reserved for the determination of protein concentration using the DC protein assay (BioRad, Richmond, CA, USA) based on the method of Lowry et al. (1951) with γ-globulin as standard. Another aliquot was diluted ten times with saline solution (NaCl 0.9%). The diluted sample was heated at 80°C for 10 min to precipitate high molecular weight (HMW) proteins and centrifuged at 30 000*g* for 1 h at 4°C (Bebianno & Langston 1989). Aliquots of 50 µl of the heat-treated cytosol were taken for MT quantification by DPP by the standard addition method, using rabbit liver MT (MT-I+MT-II; Sigma Chemical Co., St Louis, MO, USA) as reference standard material (Olafson & Olsson 1991). The analysis was performed in 10 ml of the supporting electrolyte: 1 M NH_4, 1 M NH_4OH and 1.2 mM $[Co(NH_3)_6Cl_3]$; to which 100 µl aliquot of triton X-100 (working solution of 1.25×10^{-2}%, v/v) was added. All voltammetric measurements were performed using a 757 VA Computrace (Metrhom, Switzerland) at 7°C on a differential pulse mode with a static Hg drop electrode (SMDE). The software used was 6.6032.000 VA Computrace Software 1.0. The instrumental measuring conditions were the following: range of scan −1.3 to −1.6 V versus Ag/AgCl; pulse amplitude, 50 mV; voltage step, 5 mV; voltage step time of 1 s; a pulse time of 0.05 s and a resulting scan rate of 5 mV s^{-1}. Results are expressed as MT concentrations: MT µg protein mg^{-1}. For comparison purposes, MT values were converted to MT µg wet weight tissue g^{-1}.

Spectrophotometric method. MT content was evaluated according to the method of United Nations Environment Programme (UNEP)/RAMOGE (1999) modified from Viarengo et al. (1997). Pools of six mussels were used in order to obtain enough sample for the measurement. Pooled digestive gland tissues (1 g) were homogenized in 3 vols 0.5 M sucrose, 20 mM Tris-HCl buffer, pH 8.6, containing 0.006 mM leupeptine, 0.5 mM phenylmethylsulphonylfluoride (PMSF) and 0.01% β-mercaptoethanol. The homogenate was centrifuged at 30 000*g* for 20 min. To the 1 ml 30 000*g* supernatant, 1.05 ml cold −20°C ethanol and 80 µl chloroform were added; the samples were then centrifuged at 6000*g* for 10 min at 4°C. The collected supernatant was combined with 1 mg RNA and 40 µl 37% HCl and subsequently with 3 vols cold ethanol (to a final concentration of 87%). The sample was maintained at −20°C for 1 h , then centrifuged at 6000*g* for 10 min. The MT-containing pellet was washed with 87% ethanol/1% chloroform, centrifuged at 6000*g* for 10 min, then dried under nitrogen gas stream. The pellet was resuspended in 150 µl 0.25 M NaCl and 150 µl 1 N HCl containing 4 mM ethylenediaminetetra acetic acid. A volume of 4.2 ml 2 M NaCl containing 0.43 mM DTNB (5.5-dithiobis-2-nitrobenzoic acid) buffered with 0.2 M Na-phosphate, pH 8 (Ellmann 1958), was added to the sample at room temperature. The sample was centrifuged at 3000*g* for 5 min, and the supernatant absorbance evaluated at 412 nm.

MT concentration was estimated using reduced glutathione (GSH) as a reference standard. The amount of MT was calculated assuming an arbitrary sulphydrylic (SH) content of 21 SH/mole with a molecular weight of 8600 Da for mussel MT (Mackay et al. 1993). Results are expressed as MT concentrations: MT μg wet weight tissue g^{-1}. For comparison purposes, the values were converted to MT μg protein mg^{-1}.

Metal content determination

Mussels from the Saronikos Gulf were dissected out and pooled samples from the whole body tissue of 15 −20 individuals were freeze dried. Approximately 1.2 g dried tissue were digested with 12.5 ml nitric acid into Teflon vessels in a MDS 2100 microwave digestion system from CEM (Matthews, NC, USA). Cu and Zn concentrations were determined by a Varian Spectr AA 20 Plus flame atomic absorption spectrophotometer (AAS, Victoria, Australia) while the determination of Cd was done by graphite furnace AAS, using a Perkin-Elmer 4100 equipped with a HGA 700 (Boston, MA, USA). The accuracy and precision of the method was verified with the reference material Dorm-2 (dogfish muscle tissue) provided by the National Research Council of Canada, Institute for National Measurement Standards (Ottawa, Canada) with the following results in μg dry weight g^{-1} ($n = 6$ for measured values): Cu 2.29 ± 0.08 measured value versus 2.34 ± 0.16 assigned value, Zn 22.9 ± 0.7 measured value versus 25.6 ± 2.3 assigned value and Cd 0.037 ± 0.002 measured value versus 0.043 ± 0.008 assigned value.

Statistical analysis

Statistical analyses were carried out with the aid of the statistical package SPSS® 10.0 for Windows (SPSS, Inc., Chicago, IL, USA). In the intercalibration of the spectrophotometric method and in the laboratory experiment, the statistical analysis was performed in four replicates from pooled tissue of 1 g digestive gland.

Results were reported as means ± standard deviations (SD). Data were analysed by the *t*-test at a significance level of $p < 0.05$. In field studies, MT levels were presented as means ± SD of three to four replicates. Data were analysed for homogenity of variances (Levene's test) and normality (Kolmogorov–Smirnov test). Data were logarithmically transformed when required in order to obtain a normal distribution of the data and one-way analysis of variance (ANOVA) was performed. Significant differences between pairs of means were established at $p < 0.05$ level using Duncan's test. Pearson's correlation was applied in order to investigate the possible correlation between MT values obtained by both methods and between MT levels and metal content at $p < 0.05$ level.

Results

Laboratory experiment

Total MT levels quantified by DPP (expressed as MT μg protein mg^{-1}) and spectrophotometry (expressed as MT μg wet weight g^{-1}) are shown in Figure 2a, b. MT values obtained by the two methods were significantly higher in Cd-exposed animals in comparison with controls. However, the ratio between MT in exposed mussels and MT in controls was higher using the spectrophotometric method than with DPP. Both methods could distinguish significant differences between Cd-treated

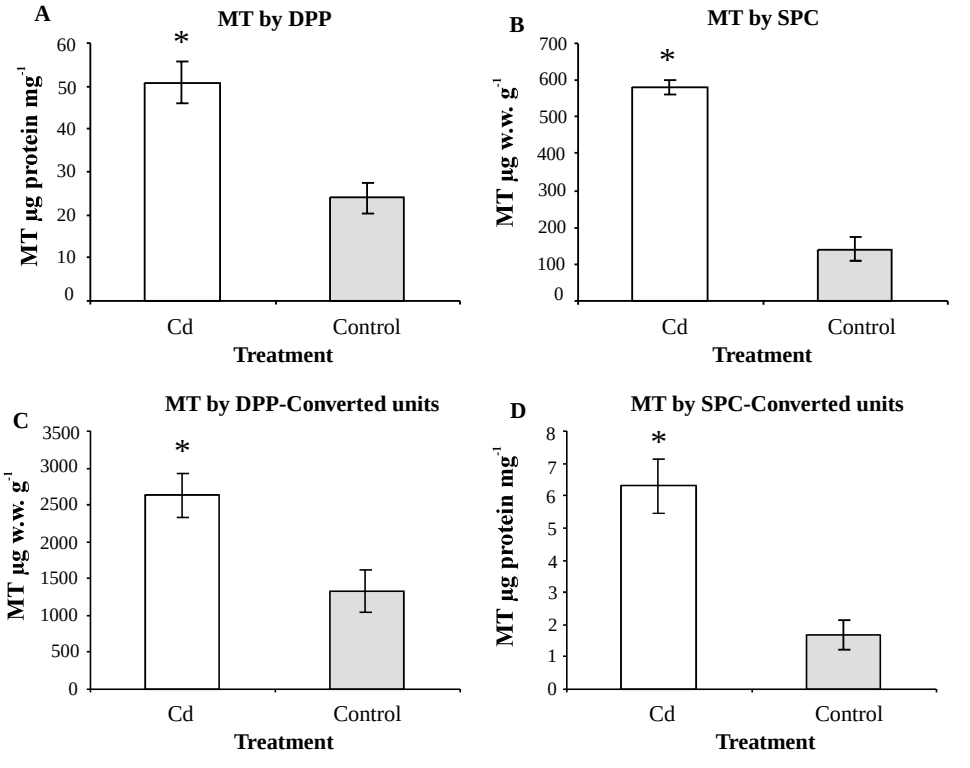

Figure 2. Quantification of MT concentrations (mean ± SD) in mussels exposed to 200 ppb (1.785 µM) Cd for 9 days and in control mussels: (a) MT determination by DPP, MT µg protein mg^{-1}; (b) MT quantification by spectrophotometry, MT µg w.w. g^{-1}; (c) MT determination by DPP, converted units, MT µg w.w. g^{-1}; and (d) MT quantification by spectrophotometry, converted units, MT µg protein mg^{-1}. *Significant differences between control and exposed mussels based on a *t*-test ($p < 0.05$). DPP, differential pulse polarography; SPC, spectrophotometry.

and control mussels, but differences were more prominent using spectrophotometry than with DPP. In the former case, Cd-treated mussels presented MT values five times higher than those recorded in control mussels. In order to compare MT values obtained by the two methods units were converted (Figure 2a to c, and b to d). Values from Figure 2a were compared with those from Figure 2d, and similarly, values from Figure 2b were compared with values from Figure 2c. After unit conversion, DPP MT values were nine times higher than with spectrophotometry. MT values obtained by the two methods were significantly correlated ($R^2 = 0.973$, $p < 0.01$).

Field studies

Field study in September 2001. Results obtained by DPP did not reveal significant differences among Italian stations, although values of MT showed a trend to increase in mussels from Genova compared with those from Portofino and Voltri (Figure 3a). In Saronikos Gulf, MT levels were higher in mussels from Megara when compared with the MT values found in mussels from Agios Kosmas (Figure 3a). Spectrophotometry indicated significant higher MT levels in mussels from Genova harbour ($p < 0.0001$) when compared with mussels from Portofino and Voltri and in mussels

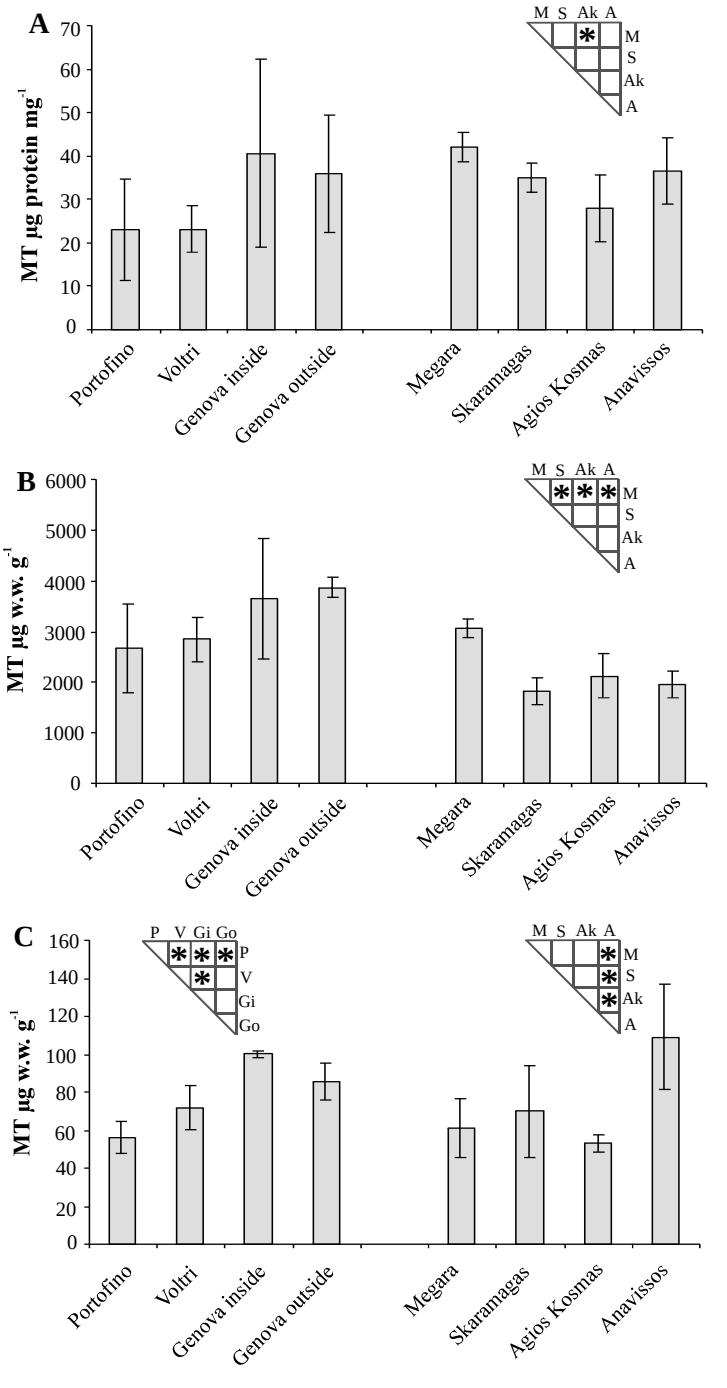

Figure 3. MT quantification (mean ± SD) in mussels sampled in the western Ligurian coast and in the Saronikos Gulf in September 2001: (a) MT determination by DPP, MT µg protein mg^{-1}; (b) MT quantification by DPP, converted units, MT µg w.w. g^{-1}; and (c) MT levels measured spectrophotometrically, MT µg w.w. g^{-1}. *Significant differences between pairs of means for each area based on Duncan's tests ($p < 0.05$). DPP, differential pulse polarography; P, Portofino; V, Voltri; Gi, Genova inside; Go, Genova outside; M, Megara; S, Skaramagas; Ak, Agios Kosmas; A, Anavissos.

from Genova outside when compared with mussels from Portofino (Figure 3c and Table I). In the Saronikos Gulf, mussels from Anavissos exhibited the highest MT levels when compared with the other Greek stations (Table I). In order to compare MT values measured by the two methods, results obtained by DPP were converted into units used in spectrophotometry, μg w.w. g^{-1} (Figure 3b). MT concentrations quantified by DPP were approximately 38 times higher than with spectrophotometry (Figure 3b, c). MT levels measured in the Ligurian coast and the Saronikos Gulf by both methods were not significantly correlated ($p > 0.05$).

Field study in September 2002. The DPP method produced significantly higher MT values in Cortiou ($p = 0.008$) compared with the French stations and in Barcelona ($p = 0.019$) with respect to the Spanish stations (Figure 4a). No significant differences were recorded among the Italian stations ($p = 0.212$). The spectrophotometric method produced the highest MT values in mussels from the reference stations, Portofino ($p = 0.004$) and Cala Montjoy ($p = 0.014$). The lowest MT values were found in mussels collected in Fos harbour ($p = 0.032$) and Voltri ($p = 0.004$) (Figure 4c). After unit conversion, the DPP method gave MT values approximately 35 times higher than spectrophotometry (Figure 4b, c). There was no significant correlation in MT levels measured in the three selected areas by the two methods ($p > 0.05$).

Field study in May 2003. The DPP method produced no significant differences between stations of each area, in France $p = 0.131$, Italy $p = 0.616$ and Spain $p = 0.101$. Conversely, spectrophotometry produced significantly higher MT levels in mussels from Barcelona ($p = 0.006$) compared with mussels collected in Cala Montjoy and Fangar (Figure 5c). After unit conversion (Figure 5b), DPP produced MT values approximately 34 times higher than by spectrophotometry. In general, MT values measured by both methods were higher and more variable in May 2003 than in September 2002 (compare the scales in Figures 5 and 4, respectively). In May 2003, there was no correlation in MT levels measured in the three sampling areas (Table I) by the two methods.

Intercalibration study. In May 2003, additional samples were obtained in the Saronikos Gulf in Piraeus, Skaramagas, Megara and Epidavros for an intercalibration study of the spectrophotometric method. MT values obtained by two research groups: the University of the Basque Country and the Hellenic Centre for Marine Research, did not significantly differ ($p > 0.05$) (Figure 6). Mussels from the harbour of Piraeus presented significantly higher MT levels than those of Skaramagas, Megara and Epidavros. These results agreed well with the higher levels of Cu and Zn (but not Cd) in mussels from Piraeus (Table II). There was a significant correlation between MT values and Cu levels measured in mussels from the Saronikos Gulf ($R^2 = 0.707$, $p < 0.05$). MT levels were not significantly correlated with Zn ($R^2 = 0.328$, $p = 0.215$) and Cd ($R^2 = 0.202$, $p = 0.454$) concentrations found in the whole soft tissue of mussels from the Saronikos Gulf.

Discussion

Induction of MT synthesis is a biomarker widely used in environmental monitoring programmes (UNEP/RAMOGE 1999, Cajaraville et al. 2000). One of the aims of

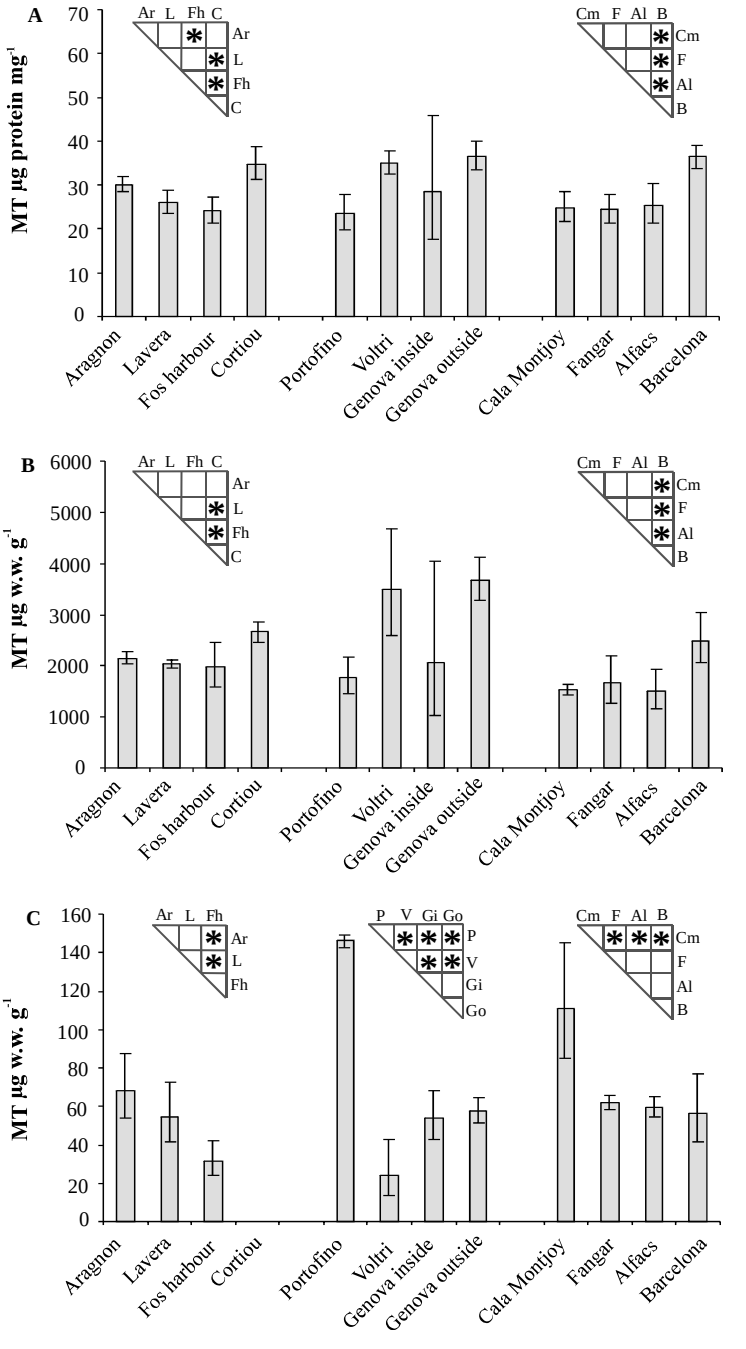

Figure 4. MT quantification (mean ± SD) in mussels sampled in the Gulf of Fos-sur-mer/Marseille, western Ligurian coast and Catalonian coast in September 2002: (a) MT levels quantified by DPP, MT µg protein mg^{-1}; (b) MT determination by DPP, converted units, MT µg w.w. g^{-1}; and (c) MT content measured by spectrophotometry, MT µg w.w. g^{-1}. There were no mussels available in Cortiou for MT quantification by spectrophotometry. *Significant differences between pairs of means for each area based on Duncan's tests ($p < 0.05$). DPP, differential pulse polarography; Ar, Aragnon; L, Lavera; Fh, Fos harbour; C, Cortiou; P, Portofino; V, Voltri; Gi, Genova inside; Go, Genova outside; Cm, Cala Montjoy; F, Fangar; Al, Alfaques; B, Barcelona.

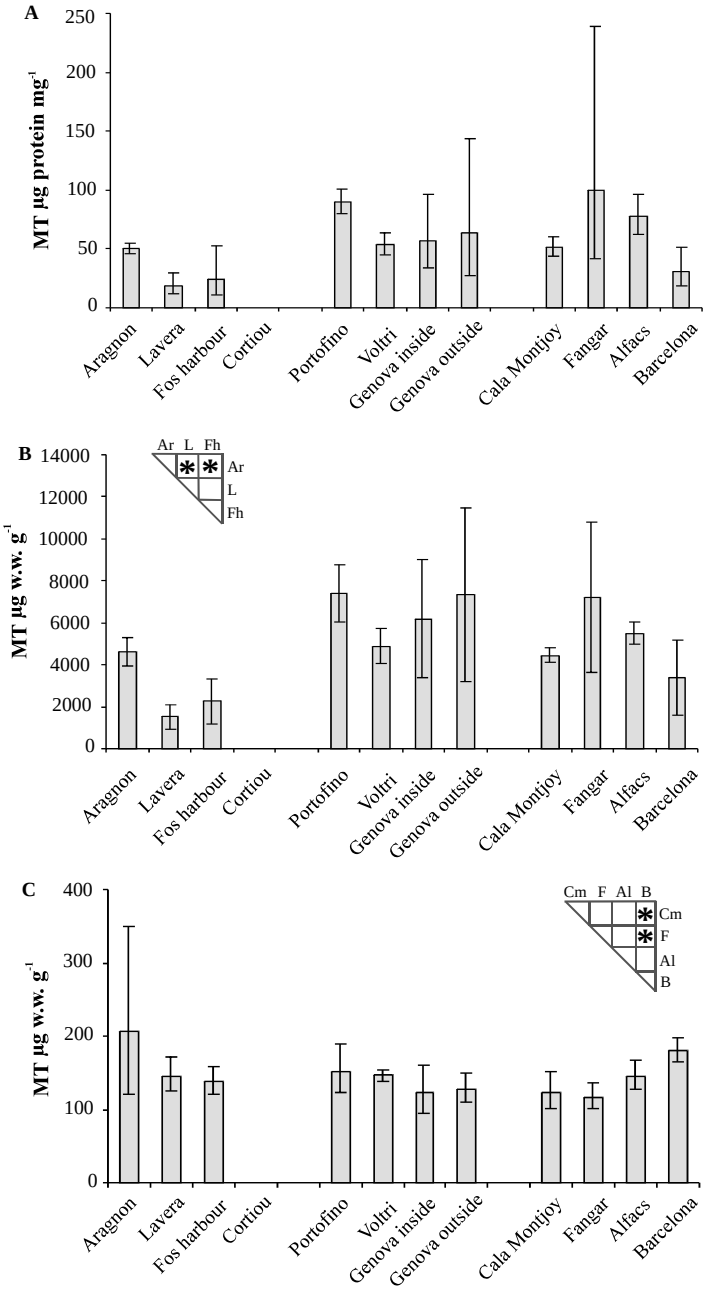

Figure 5. MT quantification (mean ± SD) in mussels sampled in the Gulf of Fos-sur-mer/Marseilles, western Ligurian coast and Catalonian coast in May 2003: (a) MT levels quantified by DPP, MT µg protein mg^{-1}; (b) MT determination by DPP, converted units, MT µg w.w. g^{-1}; and (c) MT content measured by spectrophotometry MT µg w.w. g^{-1}. In this sampling, no mussels were found in Cortiou. *Significant differences between pairs of means for each area based on Duncan's tests ($p < 0.05$). DPP, differential pulse polarography; Ar, Aragnon; L, Lavera; Fh, Fos harbour; Cm, Cala Montjoy; F, Fangar; Al, Alfaques; B, Barcelona.

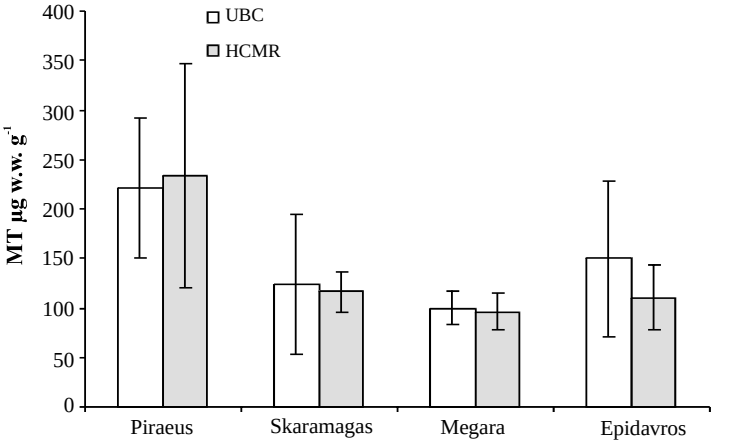

Figure 6. Intercalibration of the spectrophotometric method. Quantification of MT levels expressed as MT µg w.w. g^{-1} (mean ± SD) in digestive glands of mussels collected in the Saronikos Gulf. UBC, University of the Basque Country; HCMR, Hellenic Centre for Marine Research.

such programmes is the comparison of MT levels measured in marine organisms from different geographical sites in order to assess metal pollution (Bebianno & Machado 1997, Mourgaud et al. 2002). However, due to the variability in methods used for MT analysis (George & Olsson 1994, Viarengo et al. 1997, Cosson 2000), intersite comparisons are possible only if methods are consistent. The lack of a generalized approach for the purification and quantification of MTs (Geret et al. 1998, Cosson 2000) and the use of different units makes comparison of MT values recorded in different biomonitoring programmes difficult. Therefore, intercalibration exercises are essential for the quality control of biological data collected in large biomonitoring programmes (Viarengo et al. 2000b) and they have been recognized to be a fundamental step for the correct application of a biomonitoring programme in the framework of the OSPAR/ICES (OSPAR Convention for the Protection of the Marine Environment of the North-East Atlantic/International Council for the Exploration of the Sea).

Under the framework of the MED POL biomonitoring programme, several Mediterranean laboratories intercalibrated the spectrophotometric method for MT determination according to Viarengo et al. (1997). The study concluded that the methodology was easy, repeatable and able to discriminate between Cd-exposed and control mussels (Viarengo et al. 2000b). As our research groups did not participate in

Table II. Metal content (µg d.w. g^{-1}) measured in the whole soft tissue of mussels collected in the Saronikos Gulf.

	Copper		Zinc		Cadmium	
Station	Mean	SD	Mean	SD	Mean	SD
Piraeus	31.71	2.64	116.64	13.34	0.29	0.10
Skaramagas	19.40	3.48	108.37	49.05	0.31	0.08
Megara	5.13	0.34	70.24	13.08	0.17	0.01
Epidavros	4.98	0.27	58.26	5.63	0.26	0.05

Sample size, $n = 4$.

that intercalibration exercise, we repeated the same experimental conditions in the present laboratory study. In agreement, our MT values were in the same range as the values of the MED POL intercalibration exercise, indicating that our results were also comparable and consistent with the ones of the rest of participants. Once the spectrophotometric method was successfully intercalibrated in the laboratory, we decided to intercalibrate it using field samples collected from the Saronikos Gulf during May 2003. MT values obtained spectrophotometrically by our two research groups were very similar and did not significantly differ, suggesting a good reproducibility of the technique. In addition, both research groups detected higher MT levels in mussels from the harbour of Piraeus when compared with the other stations. These higher MT levels were correlated with a higher bioavailability of Cu at this station ($R^2 = 0.707$, $p < 0.05$). In contrast, no significant correlation was found between MT levels and Zn and Cd concentrations measured by atomic absorption spectrophotometry (AAS) in the whole soft tissue of mussels. Similarly, a relationship between MT and Cu concentrations has been demonstrated in other species such as the gibel carp, *Carassius auratus gibelio*, and the common carp, *Cyprinus carpio* (De Boeck et al. 2003), the clam, *Ruditapes decussatus* (Hamza-Chaffai et al. 1999, 2000), the zebra mussel, *Dreissena polymorpha* (Lafontaine et al. 2000), and the oyster, *Crassostrea gigas* (Mouneyrac et al. 1998).

Regarding the differential pulse polarography or DPP method, in 2000 we participated in the intercalibration of MT analysis in fish liver under the framework of the European Union-funded project BEQUALM (Biological Effects Quality Assurance in Monitoring). Results revealed that our MT values were in the expected range, indicating a good discriminatory power and reproducibility of the technique (BEQUALM, 2000). The expression of MT units was also investigated and it was concluded that the best approach to express MT levels measured by DPP was MT μg protein mg^{-1} (even if some variations were recorded in protein determination). Filipovic and Raspor (2003) compared the two approaches for expressing MT levels and also recommended the units MT μg protein mg^{-1} since MTs are part of total cytosolic proteins in the specific tissue and expressing the MT levels on a wet weight tissue basis would include the organelles, which are not related to cytosolic proteins.

In the present work, we have applied the already intercalibrated and standardized protocols of DPP and spectrophotometry in mussels exposed to 200 ppb (1.785 μM) Cd for 9 days in a laboratory experiment and in mussels collected in different field studies carried out along the Mediterranean Sea. Some features of the two SH-based methods are compared in Table III.

In the laboratory experiment the same mussel digestive glands were used for both methods in order to avoid variability between individuals. Results obtained by both methods were significantly correlated ($R^2 = 0.973$, $p < 0.01$). Differences between control and Cd-exposed mussels were more prominent with spectrophotometry than with DPP. However, DPP detected more sulphydrylic groups giving MT values nine times higher than spectrophotometry. This discrepancy could be attributed to the fact that the polarographic method assesses the overall MT content irrespective of the degree of sulphydryl-metal saturation (Olafson & Sim 1979, Raspor 2001) compared with the spectrophotometric method which is based on the metal-free thiolic content (Viarengo et al. 1997). Consequently, the degree of metal dissociation may have a greater implication on spectrophotometric than on polarographic determination. In addition, Ivankovic et al. (2002) found that the higher levels of MT measured by DPP,

Table III. Comparison of the polarographic and spectrophotometric methods to quantify MT levels.

Features	DPP	Spectrophotometry
1	Relies on the detection of SH-groups (a)	Relies on the detection of SH-groups (b)
2	Sensitive, repeatable and less time consuming than spectrophotometry	Sensitive, repeatable and lower cost than DPP
3	Minimum 100 mg	Minimum 400 mg
4	Tris buffer	Tris buffer + β-mercaptoethanol + antiproteolytic agents
5	Ultracentrifuge/polarograph	Ultracentrifuge/spectrophotometer
6	Heat treatment (c) (a)	Solvent precipitation (b)
7	Rabbit liver MT (a)	Glutathion (b)
8	No. After heat treatment MT20 isoform is significantly reduced (d)	No. After solvent precipitation MT20 isoform is drastically reduced (d)
9	Measures overall MT content. Possibility to measure metals in the same homogenate (e) (f)	Measures metal-free MT (b)
10	Available, BEQUALM	Available, UNEP/RAMOGE
11	Possible incomplete removal of interfering SH-containing substances (g) MT oxidation (i)	Possible incomplete SH-metal dissociation (g) Possible removal of dimeric or higher polymeric MT forms with organic solvents (b) (h) Possible co-precipitation of MTs with organic solvents MT oxidation (i)

Features compared are: 1, basis of the method; 2, sensitivity, repeatability and cost; 3, sample required; 4, homogenization buffer; 5, infrastructure needed; 6, isolation procedure; 7, reference standard; 8, distinction of MT isoforms; 9, type of measurement; 10, standard protocol; and 11, problems related to the technique. (a) Bebianno and Langston (1989); (b) Viarengo et al. (1997); (c) Thompson and Cosson (1984); (d) Erk et al. (2002); (e) Olafson and Sim (1979); (f) Raspor (2001); (g) Ivankovic et al. (2002); (h) Hamza-Chaffai et al. (1999); (i) Cosson (2000).

independently of the sample treatment applied (heat treatment or ethanol precipitation), could be due to an incomplete removal of possible interfering SH-containing substances. However, the relatively high level of residual thiolic, non-MT polarographic activity, could not entirely explain the large discrepancy. Ivankovic et al. (2002) partly attributed this discrepancy to calibration with different reference standards. In contrast, the spectrophotometric method can be affected by the removal of dimeric or higher polymeric MT forms during MT isolation by organic solvents, which could lead to a lower MT content (Viarengo et al. 1997, Hamza-Chaffai et al. 1999). Our results confirm that MT concentrations in molluscan tissues are higher when determined by polarograpic methods than by other quantification procedures (Viarengo et al. 1997, Geret et al. 1998, Hamza-Chaffai et al. 1999, Cosson 2000, Isani et al. 2000, Ivankovic et al. 2002).

In field studies, results obtained by the two methods were not statistically correlated. MT values obtained by DPP were 34–38 times higher than those obtained by spectrophotometry. In addition to reasons discussed above, the lack of correlation between MT levels measured by the two methods in field versus laboratory studies could be due to the use of different set of mussels for each method in field studies. Another possible explanation could be the induction of specific MT isoforms by Cd in the laboratory experiment whereas in the field the pattern of expression of different isoforms could be affected by a variety of interacting factors including synergistic or antagonistic effects between contaminants (Hamza-Chaffai et al. 1998, Legras et al.

2000, Serafim et al. 2002). In the present study, some significant differences were detected in MT levels between stations, although the biological relevance of those differences remains unclear because MT levels at all stations were low when compared with mussels exposed to Cd in the laboratory. The metal content measured by AAS in the whole soft tissue of mussels sampled in the same stations was also relatively low (personal communication with Dr G. Boquené, Ifremer, Nantes, France). The higher MT levels found in May compared with September could be related more to the reproductive cycle than to changes in metal bioavailability in the studied areas. Accordingly, MT levels in mussels from the Mediterranean Sea reached their maximum peak in late spring/early summer (Viarengo et al. 1997).

The exact biological role of MT isoforms is not known as different isoforms of MT may accomplish different functions with different degrees of induction (Mackay et al. 1993, Dallinger et al. 1997, Erk & Raspor 1999, Lacorn et al. 2001, Chabicovsky et al. 2003). The existence of at least two interactive pools of MT in the cell's cytosol has been postulated; one, possibly MT-20, is affected by transient changes in metal concentrations in the cytosol due to environmental fluctuations; the other, MT-10, is involved in the physiological metal regulatory processes (Vasak & Hasler 2000). In Cd-exposed mussels, a higher proportion of Cd was bound to the MT-20 component than to the MT-10 component, suggesting that the dimeric isoform MT-20 may be considered as Cd-specific MT (Ivankovic et al. 2002, Lemoine & Laulier 2003). The higher proportion of dimeric MT forms, which appear to be transcriptionally induced by metal ions (Barsyte et al. 1999), may indicate metal exposure of mussel field populations. However, the most routinely applied techniques for MT detection in biomonitoring programmes such as differential pulse polarography and spectrophotometry are not able to distinguish different MT isoforms. In this respect, Erk et al. (2002) found that the MT-10 fraction is not affected by any purification procedure and remains unchanged while the MT-20 form is drastically reduced when using solvent precipitation (Table III). This finding may have practical implications for the selection of the most accurate method to measure MT levels as a biomarker of metal exposure in biomonitoring programmes. Further work is needed to decipher the function(s) of mussel MT isoforms. The application of molecular biology tools to measure differential expression of mussel MT isoforms could be relevant in future biomonitoring programmes.

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