Biomarkers, July–August 2007; 12(4): 369–383

informa
healthcare

Assessment of heavy metal contamination using real-time PCR analysis of mussel metallothionein *mt10* and *mt20* expression: a validation along the Tunisian coast

M. BANNI[1,2], F. DONDERO[1], J. JEBALI[2], H. GUERBEJ[3], H. BOUSSETTA[2], & A. VIARENGO[1]

[1]*Department of Environmental and Life Science, University of Piemonte Orientale Amedeo Avogadro, Alessandria, Italy,* [2]*Laboratory of Biochemistry and Environmental Toxicology, ESH, Chott-Mariem, Sousse, Tunisia and* [3]*Laboratory of Marine Biotechnology, INSTM, Monastir, Tunisia*

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

Abstract

In mussel *Mytilus galloprovincialis* tissues, metallothionein belongs to two different gene classes, *mt10* and *mt20*, showing differential expression at both basal conditions and under heavy metal challenge. In this study, a new more highly sensitive technique, expression analysis of *mt10* and *mt20* mRNA levels by quantitative reverse transcription polymerase chain reaction, was used to assess the effects of heavy metal contamination in the digestive glands of mussels caged along the Tunisian coast. To validate the new assay, total metallothionein protein, amount of heavy metals (zinc, copper, cadmium), and a biomarker of oxidative stress such as malondialdehyde content, were assessed in the same tissues. At the investigated sites, the molecular assay showed variations of *mt20* relative gene expression levels within one or two orders of magnitude, with maximum values at two sites severely polluted with cadmium, Mahres (100-fold) and Menzel Jemile (165-fold). Changes in *mt10* expression were recorded at all sites where copper had significantly accumulated, although fold induction levels were less pronounced than those of *mt20*. In this paper, gene expression data are discussed in relation to the studied biomarkers, demonstrating that the molecular technique based on the differential expression of *mt10* and *mt20* genes represents (i) a useful and robust tool for studying and monitoring heavy metal pollution under field conditions, and (ii) an improvement in the application of metallothionein as a biomarker of response to exposure to heavy metals in marine mussels.

Keywords: *Biomarker, gene expression, heavy metals, metallothionein, real-time quantitative PCR, mussels*

(Received 30 March 2006; accepted 12 January 2007)

Introduction

Metallothioneins (MTs) are a class of low-molecular-weight, cysteine-rich, inducible, cytosolic proteins well known for their high affinity to heavy metals. MTs are

Correspondence: F. Dondero, Department of Environmental and Life Science, University of Piemonte Orientale Amedeo Avogadro, Via Bellini 25 G, 15100 Alessandria, Italy. Tel: +39 0131360238. Fax: +39 0131360243. E-mail: fdondero@unipmn.it

ISSN 1354-750X print/ISSN 1366-5804 online © 2007 Informa UK Ltd.
DOI: 10.1080/13547500701217061

RIGHTSLINK

implicated in homeostasis of essential heavy metals such as copper (Cu) and zinc (Zn), detoxification of toxic metal cations such as cadmium (Cd) and mercury (Hg) (Amiard et al. 2006), oxidative stress protection (Bebianno et al. 2005, Viarengo et al. 1999a) and possibly in gene transcription regulation (Roesijadi et al. 1998). Two major MT families, mt10 and mt20, have been described in mussels Frazier 1986, Mackay et al. 1993, Barsyte et al. 1999).

The exposure of marine organisms to heavy metals can be assayed indirectly by chemical analysis of sea water and sediment, but these determinations do not take into account the bioavailability of metals that depend on biological and abiotic factors (Hamza-Chaffai et al. 1995). In addition, heavy metal concentrations found in tissues of marine organisms do not always reflect the quantity of metal present in a toxic form within the cell, as demonstrated for Cu, which can be complexed in non-active inorganic complexes (Viarengo & Nott 1993). For some time mussel MT concentration has been used as a biomarker of exposure to heavy metals (Bebiano & Machado 1997, Viarengo et al. 1997, Romeo et al. 1998). The quantification of MTs in the tissues of exposed animals has been challenging and it has led to the development of a large number of methods, such as polarographic (Thompson & Cosson 1984), silver saturation (Scheuhammer & Cherian 1985), immunological (Roesijadi & Unger 1988), chromatographic (Mazzucotelli et al. 1991) and spectrophotometric procedures (Viarengo et al. 1997). Subsequently, a molecular approach, based on amplification of MT transcripts by means of reverse transcription polymerase chain reaction (RT-PCR), has been introduced to evaluate relative expression levels (fold induction) determined by the heavy metal exposure (Lemoine et al. 2000, Lemoine & Laulier 2003, Rebelo et al. 2003, Tom et al. 2004). An improvement of this technique is real-time quantitative PCR, which is currently among the most sensitive and reliable methods for the detection of levels of gene expression, and in particular for low-abundance mRNAs (Orlando et al. 1998). Recently, using such a technique, our research group reported the differential expression and heavy metal regulation of two genes belonging to the *mt10* and *mt20 Mytilus galloprovincialis* MT families (Dondero et al. 2005). The aim of this study was to validate this sensitive and highly accurate technique to detect molecular responses in the mussel *M. galloprovincialis* subjected to a caged experiment for 45 days in seven sites along the Tunisian coastal areas, where heavy metal pollution has been reported previously (Dellali et al. 2001, Smaoui-Damak et al. 2004).

Materials and methods

Animals and treatments

Specimens of *M. galloprovincialis*, 4–5 cm shell length (second age), were purchased from an aquaculture farm at Bizerta (Tunisia). Mussels, in groups of 80–90 individuals caged into a single bow-net, were transplanted to seven different sites along the Tunisian coast during October–November 2003 (Figure 1). Each bow-net was immersed at 2 m depth and fixed using an anchor. After 45 days, mussels were collected and transported to the laboratory in humid/thermostatic chambers at 4°C. Mussel digestive glands were immediately dissected out and washed in ice-cold filtered sea water. One sample set (ten individual entire digestive glands) were briefly rinsed in Dulbecco's phosphate buffer saline and then kept at −20°C in a RNA-preserving solution (RNA Later, Sigma-Aldrich; Milan, Italy) until gene expression

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

RIGHTSLINK

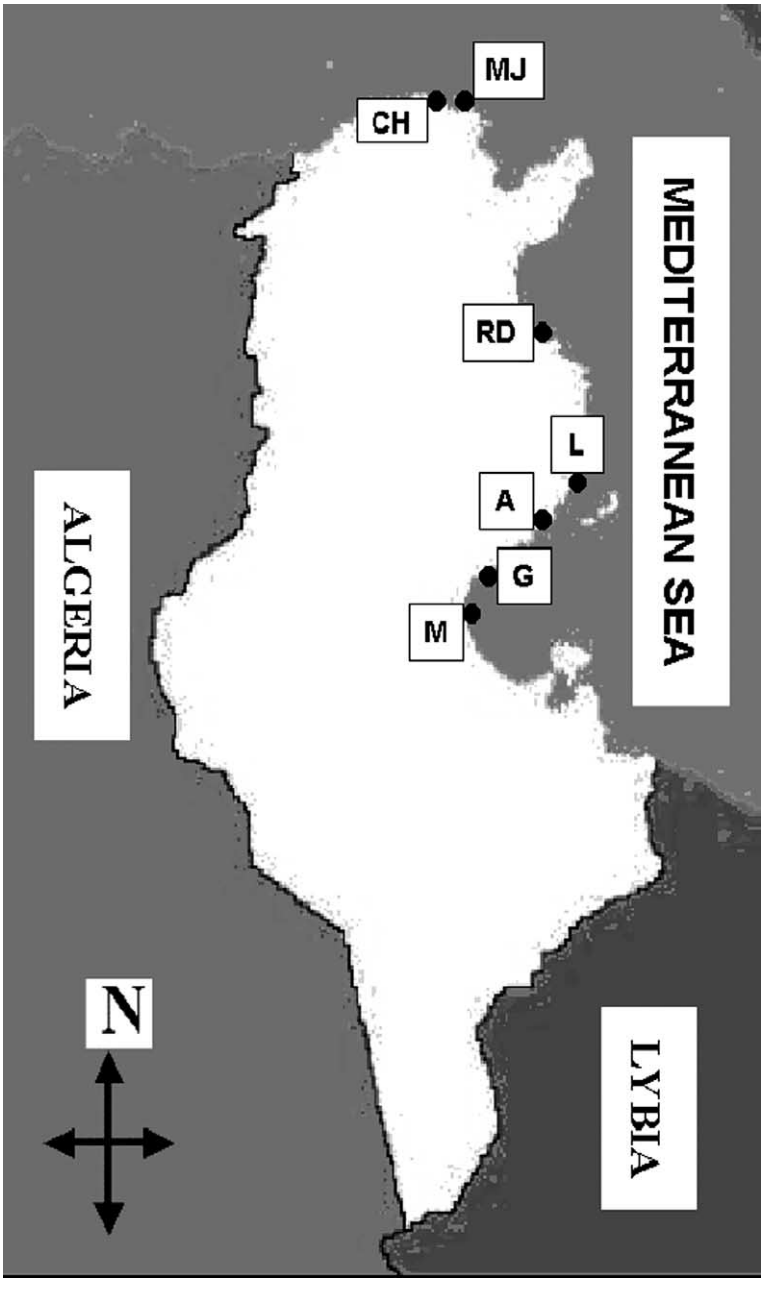

Figure 1. Location of sampling sites along Tunisian coastal areas. CH, Chaara; MJ, Menzel Jemile; RD, Rass Dimes; L, Luza; A, Aouebed; G, Gargour; M, Mahres.

analysis was carried out. The rest of the tissue was sampled as follows: digestive glands were cut into two to three pieces and immediately snap-frozen in liquid nitrogen. Then, tissue pools of about ten digestive gland portions were prepared and stored at $-80^{\circ}C$ until analysis (heavy metal, MT protein and malondialdehyde (MDA) determination). No mortality was observed at any of the sites after the caging period.

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

RIGHTSLINK

Determination of heavy metal content

The digestive gland tissue (five distinct pools prepared as described above) was thawed and dried at 50°C to a constant weight. Digestion of the samples was performed in a microwave oven (CEM-MDS 81D) in high-pressure vessels with concentrated nitric acid (Amiard et al. 1987). Cd, Cu and Zn concentrations were determined by atomic absorption spectrophotography with an acetylene flame for Cu and Zn, and with a graphite furnace for Cd (Amiard et al. 1987). Internal controls based on standard reference materials with certificated values of metal levels and international intercalibration exercises were carried out to validate this procedure. The limit of detection (LOD) of Cd, Cu and Zn was 0.05 μg g^{-1} wet weight.

Malondialdehyde determinations

All procedures were carried out at 0–4°C. Digestive glands (five distinct pools prepared as previously described) were homogenized in a Tris–HCl buffer (Tris–HCl 50 mM, pH 7.4, NaCl 150 mM), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) in a 1/4 ratio (w/v) using a Polytron Ultra-Turrax homogenizer. The homogenates were then centrifuged at 9000*g* for 30 min. Aliquots of the supernatant (S9 fraction) were frozen at −80 °C until analysis. Total proteins were determined according to Bradford (1976). MDA determination was carried out in the digestive gland extracts, using the method developed by Sunderman et al. (1985) known as the measure of thiobarbituric acid-reactive substances (TBARS), as modified by Janero (1998).

Metallothionein determination

MT content was evaluated in the digestive gland tissue (five distinct pools) according to the spectrophotometric method described by Viarengo et al. (1997) based on cysteine residue titration of a partially purified MT extract.

Reverse transcription quantitative PCR analysis

The method of real-time quantitative PCR analysis of the *mt10* and *mt20* isogenes has been previously described (Dondero et al. 2005). Briefly, total RNA was isolated from five distinct mussel digestive gland biopsies (50 mg each) by means of acid phenol extraction using the Trizol reagent (Invitrogen, Milan, Italy). One microgram of total RNA was reverse transcribed using 200 U of Revert Aid RNAse H^- M-MuLV reverse transcriptase (Fermentas, Vilnius, Lithuania), in the presence of 250 ng of random hexamers (Invitrogen, Milan, Italy), following the manufacturer's instructions. Synthesized cDNA was used for real-time PCR. For specific amplification of the *mt10* and *mt20* isogenes, 50 ng RNA reverse-transcribed cDNA was amplified in 96-well optical plates by mean of an Icycler real-time PCR apparatus (Bio-Rad Laboratories, Milan, Italy), using the following reaction mixture: 1X QuantiTect Sybr Green PCR Master Mix (Qiagen, Milan, Italy), 10 nM fluorescein, 0.2 μM each MT Q-PCR specific primer pair (*mt10* sense Q-PCR *mut1*: 5′-GGGCGCCGACTG-TAAATGTTC-3′; *mt10* antisense Q-PCR *mut2*: 5′-CACGTTGAAGGYCCTGTA-CACC-3; *mt20* sense Q-PCR *a2*:5′-GTGAAAGTGGCTGCGGA-3′; *mt20* antisense Q-PCR *a2*: 5′-GTACAGCCACATCCACACGC-3. For gene expression data

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

RIGHTSLINK

normalization between control (Luza) and experimental samples, a ribosomal *18S* gene fragment was amplified starting from 50 pg RNA reverse-transcribed cDNA using the following primer pair (*18S* Q-PCR sense: 5′-TCGATGGTACGT-GATATGCC-3′; *18S* Q-PCR antisense: 5′-CGTTTCTCATGCTCCCTCTC-3′). The thermal protocol for all the amplified targets was as follows: 15 min at 95°C, followed by 40 cycles (15 s at 95°C, 60 s at 59°C, 20 s at 77°C where the signal was acquired). A melting curve of PCR products (59–90°C) was also performed to ensure the absence of artifacts.

Data analysis and statistics

Expression levels of *mt10* and *mt20* mRNAs were analysed using the Relative Expression Software Tool (REST) (Pfaffl 2001, Pfaffl et al. 2002), in which the mathematical model used is based on mean threshold cycle differences between the sample and the control group. Five different cDNA preparations obtained from individual digestive gland biopsies (biological replicates) were analysed in triplicate Q-PCR reactions. The PCR efficiency (E) for each target was calculated as described by Rasmussen (2001), using the median value among different analyses. REST was also used to perform a randomization test with a pair-wise reallocation in order to assess the statistical significance of differences in expression between control and experimental samples.

Heavy metals, MT and MDA contents were analysed from five different pools obtained as described in the section Animals and treatments. Data were expressed as the mean values, plus standard deviations. Differences in such parameters were analysed using the Holm-Sidak ANOVA multiple comparison statistics by means of the SigmaStat 3.0 software (SYSTAT Software Inc., USA). Statistical significance was accepted at $p < 0.05$ ($n = 5$).

Factor analysis of the variables analysed was carried out by means of the principal component analysis (PCA) method with orthogonal rotation (Varimax) using the Systat 11 software (SYSTAT Software Inc.). (Varimax minimizes the number of variables that have high loadings on each factor.) Because the units of these measurements differ, we analysed a correlation matrix.

Results

After 45 days of the caged experiment, mussels sampled at Menzel Jemilee and Mahres (see Figure 1 for spot locations) showed the highest heavy metal accumulation rate (Figure 2). Mussels caged in these two sites showed the highest content of Cd, Cu and Zn: 0.19, 2.13 and 23.58 μg g^{-1} wet weight (w.w.), respectively, in the mussel digestive glands from Menzel Jemile, and 0.24, 2.42 and 32.47 μg g^{-1} w.w., respectively, in the mussels caged at Mahres. Cd concentration was about twofold lower at sites Chaara and Gargour with values of 0.11 and 0.09 μg g^{-1} w.w., respectively. Conversely, no Cd accumulation was registered at Rass Dimes, Luza and Aouebed. The last two sites also displayed the lowest Cu content (0.29 and 0.49 μg g^{-1} w.w., respectively), while Ras Dimes and Chaara showed Cu loads comparable with Menzel Jemile and Mahres (respectively, around 1.5 and over 2 μg g^{-1} w.w.). In general, Zn amounts were comparable in all sites, except Mahres were a significantly

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

RIGHTSLINK

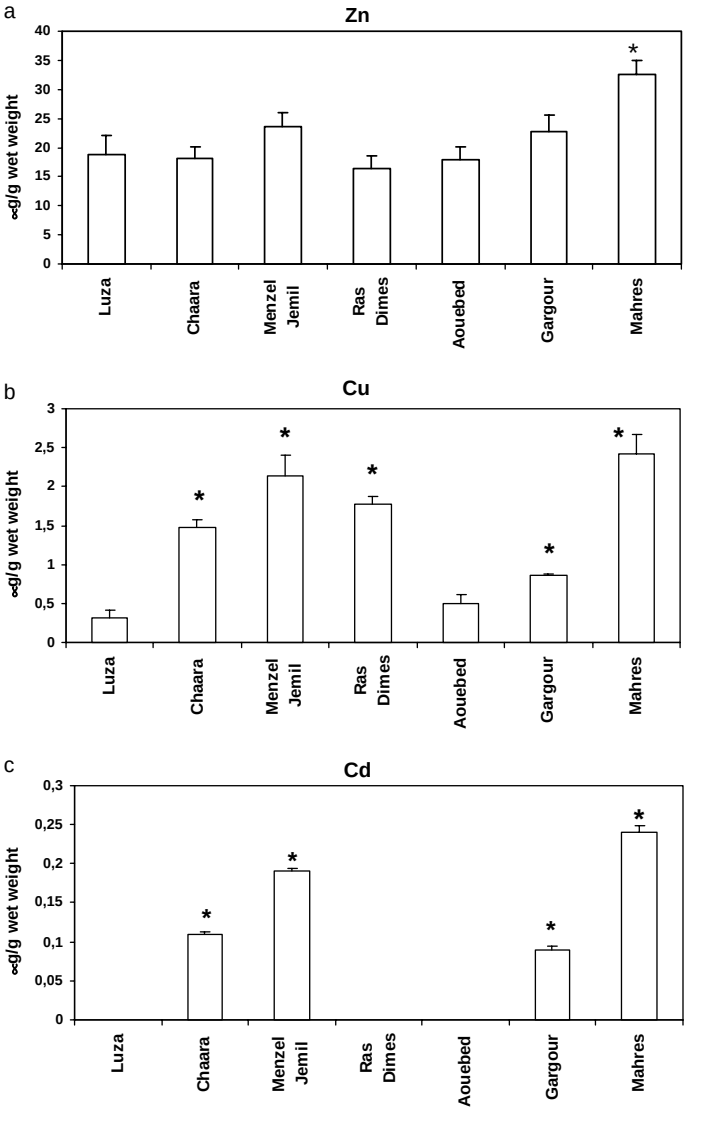

Figure 2. Heavy metal content (µg g^{-1} wet weight) in the digestive gland of *Mytilus galloprovincialis* from the seven different sites after 45 days of the caged experiment. (A) Zinc (Zn) concentration, (B) copper (Cu) concentration and (C) cadmium (Cd) concentration. Analyses were performed by means of atomic absorption spectrophotometry. The limit of detection for the three metals was 0.05 µg g^{-1} wet weight. *Significant differences, Holm-Sidak ANOVA multiple comparison test versus Luza ($n = 5$, $p < 0.05$). Luza was used as the control site because it had the lowest values for heavy metals.

higher content was found. As Luza showed the least amounts of heavy metals, it was chosen as the reference site for further biomarker and gene expression analysis.

Expression of *mt10* and *mt20* transcripts in digestive gland RNA extracts of mussels was performed by real-time quantitative PCR, using *18S* rRNA as a reference gene for data normalization. It should be noted that, in all the investigated sites, threshold cycles of the amplified *mt10* and *mt20* targets were viewed in relation to Luza's values (the reference site), indicating higher expression levels for both genes (Table I). The

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

RIGHTSLINK

Table I. Output of the real-time polymerase chain reaction (PCR) expression analysis of mussel *mt10* and *mt20* genes. Shown are the mean threshold cycle (Ct), the normalized relative expression level with respect to the reference site (Luza), *p* values,% Ct coefficient of variation (CV) obtained from the amplification of the two metallothionein (MT) genes and a *18S* ribosomal target used for normalization of expression data. The Relative Expression Software Tool (REST) described by Pfaffl et al. (2002) was used to achieve normalized relative expression ratio (fold induction) as group-wise comparison and to perform a non-parametric statistic test based on the pair-wise fixed reallocation randomization test ($n = 15$). The PCR efficiencies used to calculate relative fold induction levels in relation to the control represent the median value obtained from at least four independent experiments, and they were 2.0, 1.96 and 1.95, respectively, for 18S, *mt10* and *mt20*. The threshold line for the calculation of Ct was set arbitrarily at 113.3 AU.

Site	Target	Sample Ct	Normalized relative expression	*p* Values	%CV
Luza	18*S*	12.70			0.44
	*mt*20	28.56			0.36
	*mt*10	21.03			2.77
Chaara	18*S*	12.54			2.82
	*mt*20	22.08	68.91	0.001	7.89
	*mt*10	18.05	6.58	0.001	0.11
Menzel Jemile	18*S*	13.78			0.55
	*mt*20	22.07	165.45	0.001	1.04
	*mt*10	19.56	5.68	0.001	0.40
Ras Dimes	18*S*	13.20			0.89
	*mt*20	25.95	8.08	0.001	3.23
	*mt*10	21.03	5.37	0.001	0.79
Aouebed	18*S*	12.26			0.54
	*mt*20	23.70	19.06	0.003	2.11
	*mt*10	20.80	0.87	0.652	1.06
Gargour	18*S*	12.32			0.37
	*mt*20	23.42	23.79	0.001	1.03
	*mt*10	17.68	7.03	0.001	0.42
Mahres	18*S*	14.08			0.89
	*mt*20	23.10	100.02	0.001	0.15
	*mt*10	18.73	12.36	0.001	0.66

highest values for the *mt20* target were recorded at sites Menzel Jemile, Mahres and Chaara, with 165.45, 100.02 and 68.91 fold induction, respectively. In contrast, mussels collected from Rass Dimes, Aouebed and Gargour displayed lower levels by about one order of magnitude, 8.08, 19.06 and 23.78 fold, respectively (Table I, Figure 3). Patterns of the *mt10* gene were quite different. In fact, *mt10* was upregulated at most sites: Mahres, Gargour, Chaara, Menzel Jemile and Rass Dimes, but to a much lower extent with respect to *mt20* values (12.36, 7.03, 6.58, 5.68 and 5.37 fold, respectively). Conversely, at Aouebed no significant effect was noticed (Table I, Figure 3).

In addition, total MT protein content was evaluated in the digestive gland of caged mussels from the different investigated sites (Figure 4). A significant increase in MT levels with respect to the reference site (Luza) was registered at Mahres (6-fold) Menzel Jemile (4.7-fold), Chaara (2.9-fold) and Gargour (1.7-fold). Rass Dimes and Aouebed exhibited only moderate increases, below twofold (1.5 and 1.6, respectively).

Analysis of lipid peroxidation levels was performed by evaluating the MDA content in the digestive gland tissue (Figure 5). The highest MDA accumulation was recorded

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

RIGHTSLINK

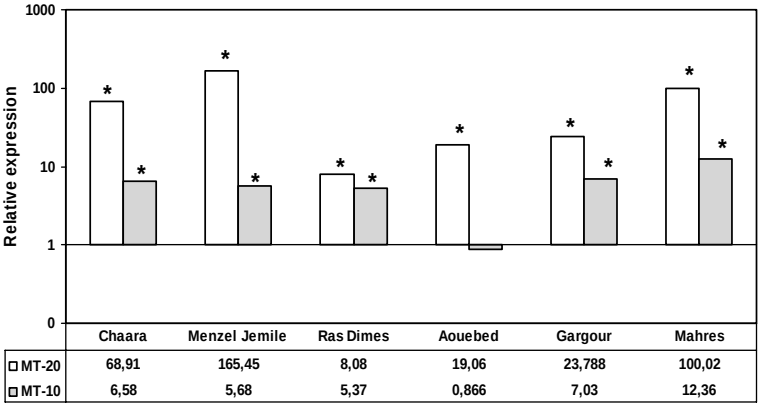

Figure 3. Quantitative real-time PCR expression analysis of the mussel *mt10* and *mt20* metallothionein genes. Data represent logarithmic relative expression levels with respect to Luza samples. cDNA aliquots obtained from mussel digestive gland total RNA, were amplified in a real-time PCR apparatus, in the presence of the intercalating dye Sybr Green-I^{TM}. See Table I for more details.

in Mahres with 39.6 nmole mg^{-1} protein and to a lesser extent in Gargour (33.22 nmole mg^{-1} protein) and Aouebed (28.48 nmole mg^{-1} protein). Rass Dimes (20.06 nmole mg^{-1} protein), Mezel Jemile (19.93 nmole mg^{-1} protein) and Chaara (15.06 nmole mg^{-1} protein) exhibited lower MDA contents, similar to the value recorded in animals from the reference site Luza (12.01 nmole mg^{-1} protein).

A PCA of the different variables analysed was also carried out (Figure 6), showing three principal components which each account for 39.0%, 27.1% and 30.3% of the total variance.

Discussion

MTs have been used largely as biomarkers of response to exposure to heavy metal contamination under field conditions, either in fresh water or in sea water ecosystems (Bebianno et al. 1997, Viarengo 1999b, Linde et al. 1999). Although MT genes are

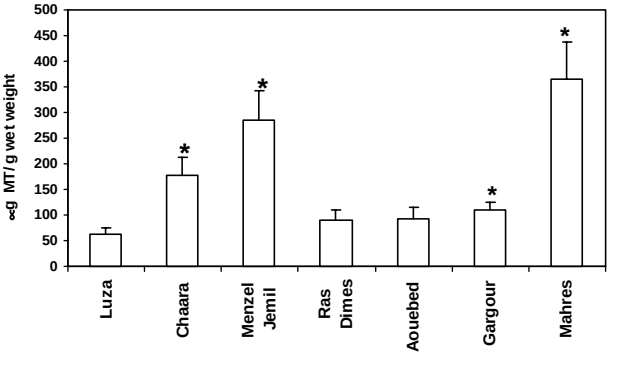

Figure 4. Total metallothionein (MT) content analysis. MT content (µg g^{-1} wet weight) was evaluated in the digestive gland tissue of mussels subjected to the caged experiment (45 days) along the Tunisian coastal areas. Data represent means ± SD. *Significant differences, Holm-Sidak ANOVA multiple comparison test versus Luza ($n = 5$, $p < 0.05$).

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

RIGHTSLINK

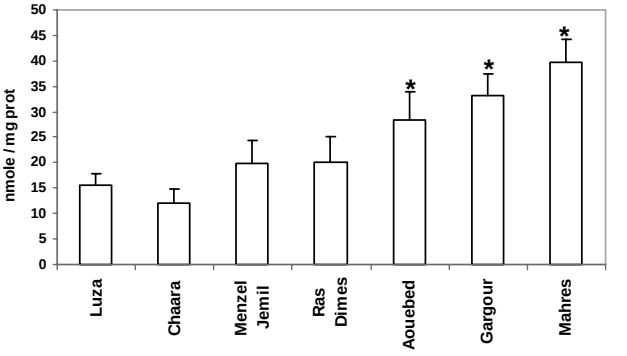

Figure 5. Accumulation of malondialdehyde (MDA) in the digestive gland tissue of mussels subjected to the caged experiment along the Tunisian coastal area. Shown are means ± SD of MDA values expressed in nmole mg^{-1} protein. MDA determination was carried out using the method developed by Sunderman et al. (1985) and now known as the measure of thiobarbituric acid-reactive substances (TBARS). *Significant differences, Holm-Sidak ANOVA multiple comparison test versus Luza ($n = 5$, $p < 0.05$).

primarily controlled at the level of transcription (Durnam & Palmiter 1981, Thiele 1992), so that their mRNA levels display a remarkable increase following heavy metal exposure, the most common practice to utilize this parameter in biomonitoring programmes has been the evaluation of the total MT protein content from a partially purified cytosolic extract (Roesijadi & Unger 1988, Bebianno & Machado 1997, Viarengo et al. 1999b). In the past, these biochemical methodological approaches satisfied the demands of low cost, low sophistication, reproducibility, and wide

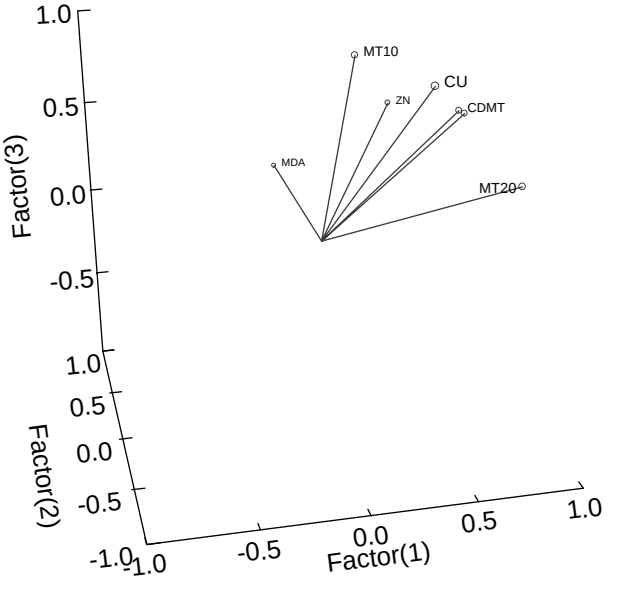

Figure 6. Principal component analysis (PCA) of the variables obtained from heavy metals, biomarkers and gene expression analysis. Multivariate analysis (PCA) provided three principal factors which essentially describe: the correlation between cadmium (Cd), *mt20* and metallothionein (MT) (Factor 1); the low correlation between MDA and the other variables, except zinc (Zn) (Factor 2); and the correlation between copper (Cu) and *mt10* (Factor 3).

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

RIGHTSLINK

applicability of the same technique to different organisms. In fact, a biochemical technique usually did not require the knowledge of species-specific amino acid protein and/or cDNA sequence. However, the biochemical determination of MT can present disadvantages over a molecular approach based on the mRNA level determination. In mussels, detoxification routes of Cd and Cu are quite divergent. In fact, Cu is rapidly extruded through the vacuolar–lysosomal system bound to a Cu thionein with a half-life of about 6 days, while Cd is accumulated long-term in the cytosol where it is found bound to a Cd thioneins with a half-life of more than 7 months (Viarengo et al. 1985a, Viarengo 1989). It appears clear that the determination of MT as a cytosolic soluble protein, as described in most routine procedures, may be biased by the metal bound to the protein, and therefore the application of MTs as biomarker of response to exposure to heavy metals can be impaired by the type of metals present in the environment, i.e. this parameter is mostly sensitive to Cd and to a much lower extent to other metals. Our data are in accordance with this hypothesis, in fact the comparison of Figure 2c and Figure 5 indicates that only at sites where Cd accumulation in mussel tissues was reported, was a significant overexpression of MT proteins also present. The main goal of this study was to validate this technique, whereas the use of gene expression analysis of two differentially expressed and regulated mussel MT genes belonging to the *mt10* and *mt20* families could represent a notable improvement of the application of MT as a biomarker of response to exposure to heavy metals. First of all, we selected seven sites along the Tunisian coastal areas where mussels were transplanted for 45 days, a period long enough to permit the tissue accumulation of even low Cd amounts present in the water, and detect chronic pollution due to Cu and Zn. Sampling sites were chosen because of their geographical distribution near urban, industrial, agricultural and remote areas, thus providing different levels of contamination (Figure 1). Chaara and Menzel Jemile are located in the north of Tunisia in the Bizerta Mediterranean lagoon. The latter ecosystem is subjected to urban and agricultural pollution, and can experience eutrophication phenomena during the summer (Dellali et al. 2001). Rass Dimes, which is located near Sousse city, is subjected to urban pollution due to the presence of waste water discharges. The other four sites belong to South Tunisia and are represented by Luza, Aouebed, Mahres and Gargour. The latter two are located south of Sfax, which is the most important industrial site of the country, and close to a phosphogypsum plant, which can be a source of heavy metal release (Smaoui-Damak et al. 2003) (Figure 1).

Heavy metal analysis clearly showed different degrees of heavy metal loads in the digestive gland tissue of mussels subjected to the 45-day caged experiment at the Tunisian sites (Figure 2). Zn content was only slightly higher at Marhes, but within physiological values (Viarengo et al. 1997). Cd was under the limit of detection in individuals from three sites, Rass Dimes, Luza and Aouebed, the latter two showing also the lowest amounts of Cu, below 0.5 $\mu g\ g^{-1}$ w.w. In contrast, Chaara, Menzel Jemile, Rass Dimes and Mahres were characterized by relatively high heavy metal loads (Cd and Cu), as previously reported by several authors. In fact, Dellali et al. (2001) reported values up to 1 $\mu g\ g^{-1}$ Cd dry weight and 67 $\mu g\ g^{-1}$ dry weight Cu, in sediments from Chaara and Menzel Jemile stations. Others studies performed at the Gargour and Mahres sites demonstrated the presence of high concentrations of such heavy metals with a corresponding increase of MT protein levels in tissues of *Ruditapes decussatus* (Banni et al. 2003, Hamza-Chaffai et al. 2003, Banni et al. 2005) and the fish *Scorpaena porcus* (Hamza-Chaffai et al. 1995).

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

RIGHTSLINK

The originality and the real improvement of the new molecular technique herein proposed is that it is based on the determination of the expression level of two mussel genes, showing a differential expression towards specific metal ions. This feature seems to be typical of *mt* genes, and it was first described for human *MT-I* and *MT-II* genes (Sadhu & Gedamu 1988). The mussel genes can be grouped in two multicomponent clusters, *mt10* and *mt20*. In general, in the digestive gland *mt10* genes seem to be highly expressed at basal level, and they can respond to both essential (Cu, Zn) and non-essential (Cd, Hg) heavy metals (Barsyte et al. 1999, Dondero et al. 2005). From laboratory exposures a scale of *mt10* transcriptional activation was proposed, suggesting that Cd is more effective than Cu, Zn and Hg, in order. Conversely, *mt20* appeared as a rare transcript with a very low basal expression level (few copies per cell). Its expression could be dramatically activated, up to thousands fold, in the presence of Cd, and to a lesser extent also by Hg and Zn. Although this gene should be considered merely not sensitive to Cu, as after a prolonged exposure to this metal its mRNA was less than twice that of the control, the concomitant exposure to Cu with hydrogen peroxide could give rise to a considerable transcriptional activation, suggesting a role of the hydroxyl radical in *mt20* activation (Dondero et al. 2005).

In our approach, the real-time PCR quantification analysis was performed in the relative expression mode, where the gene of interest (GOI) threshold cycle of an 'experimental' sample is compared with that of a reference one (Bustin 2002). In our study, GOIs were the *mt10* and *mt20* genes, while the reference was represented by gene expression levels in individuals collected at Luza, a site that according to our chemical and biological analyses should be considered free of heavy metal contamination (under the LOD). It is interesting to note that mussels caged at sites showing a notable Cd contamination (Figure 2c), also displayed the highest *mt20* relative expression levels, in particular at Chaara, Mahres and Menzel Jemile, where such values are in the order of a hundred fold over the control (Table I, Figure 3). At Gargour, where Cd uptake was lower than the previously discussed sites, the *mt20* gene displayed, accordingly, a lower regulation level. However, at Aouebed and Rass Dimes, where Cd was also not detected, *mt20* exhibited a mild activation, 19- and 8-fold induction, respectively. This increase could be due to (i) the accumulation of Zn (33% and 25% more than control), (ii) the presence of mixed heavy metals (mainly Cu)/organic pollution that might give rise to reactive oxygen species, as clearly suggested by high MDA accumulation in Aouebed (Figure 4), or (iii) the presence of even moderate Hg or other heavy metal concentrations (a hypothesis that we did not test). The evaluation of lipid peroxidation products, in terms of MDA accumulation in the mussel digestive gland, indicated that probably at some investigated sites pollution was not only due to heavy metals but also to some organic aromatic compounds able to evoke lipid peroxidation of biological membranes (Romeo et al. 1998, Geret et al. 2003). Indeed, transition metals such as Cu may stimulate lipid peroxidation in mussel tissues (Viarengo et al. 1990) by acting as a redox-cycling catalyst in the formation of oxygen radicals, e.g. by the Fenton reaction (Halliwell & Gutteridge 1984). The presence of high MDA levels in individuals at Aouebed, where a physiological amount of Cu was reported (Figure 2B), could be due to the presence of other sources of lipid peroxidation-generating compounds, but in particular their interaction with even minimal Cu concentrations, giving rise to additive/synergic effects on *mt20* expression, as reported for H_2O_2 by Dondero et al. (2005). In fact, it

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

RIGHTSLINK

should mentioned that certain types of organic pollutants, such as the crude oil preparation North Sea Oil, were not able to induce *mt20* expression (nor *mt10*), but conversely such genes were slightly downregulated (Dondero et al. 2006). In addition, paraquat a well known pro-oxidant in vertebrate cells was not able to sustain the accumulation of MT proteins in mussel digestive gland (Cavaletto et al. 2002).

Due to its different expressional behaviour, the *mt10* gene showed more limited responses to heavy metal contamination, in general contained within one order of magnitude (Table I, Figure 3). A significant upregulation was observed at all sites except Aouebed, where, interestingly, Cu accumulation was negligible in respect to Luza. Individuals from Mahres displayed the maximum relative expression level, in accordance with the highest Cu load (Figure 2B, Table I). At other sites, mussels showed similar *mt10* expression levels (between 5- and 7-fold), in accordance with a significant increase of Cu content in the digestive gland. In another study carried out in the same period (November 2003), mussels (*M. edulis)* were transplanted along a Cu-pollution gradient at Visnes, Norway (North Sea), but no modulation of *mt10* was observed using the same technique, although Cu was highly accumulated in soft tissues (Dondero et al. 2006). The occurrence of such a difference is likely to depend on seasonal effects linked to the different habitats tested (the Mediterranean Sea or the North Sea) and due to the different species used (*M. edulis* or *M. galloprovincialis*) In particular, in *M. galloprovincialis*, the transcriptional susceptibility of *mt10* to heavy metals shows divergent trends during the progress of the annual cycle. In fact, in contrast to what was found during autumn (October) by Dondero et al. (2005), in spring (April) neither the exposure of mussels to high Cd (200 $\mu g\ l^{-1}$) nor Cu (45 $\mu g\ l^{-1}$) could give rise to even a moderate increase of the *mt10* gene expression level (Dondero et al. unpublished data). In April, *mt10* increases its basal level over tenfold, reaching a level typical of mussels exposed to Cd during October. Such levels are similar to the ones displayed by *M. edulis* during the Visnes campaign. Taking these data together, it can be argued that in *M. galloprovincialis mt10* can be used as an estimator of heavy metal pollution, but its responsiveness to heavy metals being dependent on the basal expression level, can be biased by seasonal factors, and possibly by other ecological and physiological aspects. As a corollary, in *M. edulis* and in general in mussels from higher latitudes (North Sea, Atlantic Ocean), *mt10* does not seem to represent a good biomarker of response to exposure to Cu pollution, as already demonstrated by other authors (Lemoine et al. 2000, 2003).

Finally, we analysed total MT protein content from the same tissues to make a direct comparison with the novel molecular approach. We have already emphasized that MT analysis has a bias in favour of Cd accumulation and against other heavy metals, as also shown by the PCA plot where Cd and MT almost overlap (Figure 6). In the proposed technique, this aspect is compensated for by the concomitant determination of two genes, one being also fairly sensitive to Cu exposure, *mt10*. A further confirmation is given by the PCA analysis in which Factor 3 describes the correlation of Cu with only *mt10*.

Another advantage of the molecular approach concerns fold induction levels observed at the investigated sites. In fact, MT protein levels were much lower than *mt20* ones, even at Cd-polluted sites. The highest values were obtained at Mahres, Menzel Jemile and Chaara (6-, 4.7- and 2.9-fold, respectively), where Cd was found at higher levels. These data, furthermore, strengthen the hypothesis that, in mussels, Cd is the preferential inducer of both MT neosynthesis and further accumulation in the

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

RIGHTSLINK

cytosol. By contrast, gene expression measurements were also clearly responsive at sites where heavy metals, both Cd or Cu, were accumulated to low extents, such as Gargour (23.8- and 7.03- vs. 1.7-fold induction, for *mt20*, *mt10* and MT proteins, respectively).

Conclusion

In conclusion, the new molecular technique developed in mussels and based on the evaluation of dual MT relative expression levels represents an accurate, sensitive and robust approach to assess the biological responses to heavy metal contamination, and it can validate an advance of the use of MT as a biomarker of response to exposure to heavy metals in marine environments.

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

Acknowledgements

This study was supported by a fund from the Ministry of Scientific Research and Technology, Tunisia (Unité de Recherche en Biochimie et Toxicologie Environnementale), the 5th UE Framework Program project Biological Effects of Environmental Pollution in marine coastal ecosystems (BEEP) (Contract N°EVK3-2000-00543) and the UNEP/MAP/MEDPOL-II Project.

References

Amiard JC, Amiard-Triquet C, Barka S, Pellerin J, Rainbowd P-S. 2006. Metallothioneins in aquatic invertebrates: Their role in metal detoxification and their use as biomarkers. Aquatic Toxicology 76:160–202.

Amiard JC, Pineau A, Boiteau H-L, Metayer C, Amiard-Triquet C. 1987. Application de la spectrométrie d'absorption atomique Zeeman aux dosages de huit éléments traces (Ag, Cd, Cr, Cu, Mn, Ni, Pb et Se) dans les matrices biologiques solides. Water Research 21:693–697.

Banni M, Jebali J, Daubez M, Clerandeaux C, Guerbej H, Narbonne JF, Boussetta H. 2005. Monitoring pollution in Tunisian coasts: application of a classification scale based on biochemical markers. Biomarkers 10:105–116.

Banni M, Ben Dhiab R, El Abed A, Boussetta H. 2003. Genotoxicity, catalase and acetylcholinesterase in the assessment of the Tunisian coastal areas. Archive of Environmental Contamination and Toxicology 5:157–165.

Barsyte D, White KN, Lovejoy DA. 1999. Cloning and characterization of metallothionein cDNAs in the mussel *Mytilus edulis* L. digestive gland. Comparative Biochemistry and Physiology C 122:287–296.

Bebianno MJ, Company R, Serafima L, Camus L, Cosson RP, Fiala-Medoni A. 2005. Antioxidant systems and lipid peroxidation in *Bathymodiolus azoricus* from Mid-Atlantic Ridge hydrothermal vent fields. Aquatic Toxicology 75:354–373.

Bebianno MJ, Machado LJ. 1997. Concentrations of metals and metallothioneins in *Mytilus galloprovincialis* along the south coast of Portugal. Marine Pollution Bulletin 34:666–671.

Bradford MM. 1976. A rapid and sensitive method for the quantification of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72:248–254.

Bustin SA. 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. Journal of Molecular Endocrinology 29:23–39.

Cavaletto M, Ghezzi A, Burlando B, Evangelisti V, Ceratto N, Viarengo A. 2002. Effect of hydrogen peroxide on antioxidant enzymes and metallothionein level in the digestive gland of *Mytilus galloprovincialis*. Comparative Biochemistry and Physiology Part C 131:447–455.

Dellali M, Gnassia Barelli M-B, Romeo M, Aissa P. 2001. The use of acetylcholinesterase activity in *Ruditapes decussatus* and *Mytilus galloprovincialis* in the biomonitoring of Bizerta lagoon. Comparative Biochemistry and Physiology Part C 130:227–235.

RIGHTSLINK

Dondero F, Dagnino A, Jonsson H, Capri F, Gastaldi L, Viarengo A. 2006. Assessing the occurrence of a stress syndrome in mussels (*Mytilus edulis*) using a combined biomarker/gene expression approach. Aquatic Toxicology 78S:S13–S24.

Dondero F, Piacentini L, Banni M, Rebelo M, Burlando B, Viarengo A. 2005. Quantitative PCR analysis of two molluscan metallothionein genes unveils differential expression and regulation. Gene 345:259–270.

Durnam DM, Palmiter R-D. 1981. Transcriptional regulation of the mouse metallothionein-I gene by heavy metals. Journal of Biological Chemistry 256:5712–5716.

Frazier JM. 1986. Cadmium–binding proteins in the mussel, *Mytilus edulis*. Environmental Health Perspective 65:39–43.

Geret F, Serafim A, Bebianno MJ. 2003. Antioxidant enzyme activities, metallothioneins and lipid peroxidation as biomarkers in *Ruditapes decussates*. Ecotoxicology 12:417–426.

Halliwell B, Gutteridge J-M. 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochemical Journal 219:1–14.

Hamza-Chaffai A, Cosson RP, Amiard Triquet C, El Abed A. 1995. Physicochemical forms of storage of metals (Cd, Cu and Zn) and metallothionein-like proteins in gills and liver of marine fish from Tunisian coast: ecotoxicological consequences. Comparative Biochemistry and Physiology Part C 111:329–341.

Hamza-Chaffai A, Pellerin J, Amiard JC. 2003. Health assessment of marine bivalve *Ruditapes decussatus* from the gulf of Gabès. Environment International 28:609–617.

Janero D. 1998. Malondialdehyde and thiobarbutiric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissues injury. Free Radical Biology and Medicine 9:515–540.

Lemoine S, Bigot Y, Sellos D, Cosson R-P, Laulier M. 2000. Metallothionein isoforms in *Mytilus edulis* (Mollusca, Bivalvia): complementary DNA characterization and quantification of expression in different organs after exposure to cadmium, zinc, and copper. Marine Biotechnology 2:195–203.

Lemoine S, Laulier M. 2003. Potential use of the levels of the mRNA of a specific metallothionein isoform (MT-20) in mussel (*Mytilus edulis*) as a biomarker of cadmium contamination. Marine Pollution Bulletin 46:1450–1455.

Linde AR, Sanchez-Galan S, Vales-Mota P, Garcia Vazquez E. 1999. Metallothionein as bioindicator of fresh water metal pollution: European Eel and Brown trout. Ecotoxicology and Environmental Safety 40:60–63.

Mackey EA, Overnell J, Dunbar B, Davidson I, Hunziker PE, Kägi JH, Fothergill JE. 1993. Complete amino acid sequences of five dimeric and four monomeric forms of metallothionein from the edible mussel *Mytilus edulis*. European Journal of Biochemistry 218:183–194.

Mazzucotelli A, Viarengo A, Canesi L, Ponzano E, Rivaro P. 1991. Determination of trace amounts of metalloprotein species in marine mussel samples by high-performance liquid chromatography with inductively coupled plasma atomic emission spectrometric detection. The Analyst 116:605–608.

Orlando C, Pinzani P, Pazzagli M. 1998. Developments in quantitative PCR. Clinical Chemistry and Laboratory Medicine 36:255–269.

Pfaffl M-W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29:e45.

Pfaffl M-W, Horgan G-W, Dempfle L. 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Research 30:e36.

Rasmussen R. 2001. Quantification on the Light Cycler. In: Meuer S, Wittwer C, Nakagawara K, editors. Rapid Cycle Real-time PCR, Methods and Applications. New York: Springer-Verlag.

Rebelo MF, Pfeiffer W, Da Silva H, O Morales M. 2003. Cloning and detection of metallothionein mRNA by RT-PCR in mangrove oyster (*Crassostrea rhizophorae*). Aquatic Toxicology 64:359–362.

Roesijadi G, Bogumil R, Vasak M, Kagi JH. 1998. Modulation of DNA binding of a tramtrack zinc finger peptide by the metallothionein–thionein conjugate pair. Journal of Biological Chemistry 28:17425–17432.

Roesijadi G, Unger ME. 1988. Immunochemical quantification of metallothioneins of a marine mollusc. Canadian Journal of Fish and Aquatic Science 45:1257–1263.

Romeo M, Hoarau P, Garello G, Gnassia-Barelli M, Girard JP. 1998. Mussel transplantation and biomarkers as useful tools for assessing water quality in the NW Mediterranean. Environmental Pollution 122:369–378.

Sadhu C, Gedamu L. 1988. Regulation of human metallothionein (MT) genes. Differential expression of MTI-F, MTI-G, and MTII-A genes in the hepatoblastoma cell line (HepG2). Journal of Biological Chemistry 263:2679–2684.

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

RIGHTSLINK

Scheuhammer AM, Cherian GM. 1985. Quantification of metallothionein by a silver-saturation method. Toxicology and Applied Pharmacology 82:417–425.

Smaoui-Damak W, Hamza-Chaffai A, Berthet B, Amiard J-C. 2003. Preliminary study of the clam *Ruditapes decussatus* exposed in situ to metal contamination and originating from the Gulf of Gabés, Tunisia. Bulletin of Environmental Contamination and Toxicology 71:961–970.

Smaoui-Damak W, Hamza-Chaffaia A, Bebianno M-J, Amiard J-C. 2004. Variation of metallothioneins in gills of the clam *Ruditapes decussates* from the Gulf of Gabes (Tunisia). Comparative Biochemistry and Physiology Part C 139:181–188.

Sunderman FW, Marzoul A, Hopfer SM, Zaharia O, Reid MC. 1985. Increased lipid peroxidation in tissues of nickel chloride-treated rats. Annals of Clinical and Laboratory Science 15:229–236.

Thiele DJ. 1992. Metal regulated transcription in eukaryotes. Nucleic Acids Research 20:1183–1191.

Thompson JAJ, Cosson RP. 1984. An improved electro-chemical method for the quantification of metallothionein in marine organisms. Marine Environmental Research. 11:147–152.

Tom M, Chen N, Segev M, Herut B, Rinkevich B. 2004. Quantifying fish metallothionein transcript by real time PCR for its utilization as an environmental biomarker. Marine Pollution Bulletin 48:705–10.

Viarengo A, Palermo S, Zanicchi G, Capelli R, Vaissiere R, Orunesu M. 1985a. Role of metallothioneins in Cu and Cd accumulation and elimination in the gill and digestive gland cells of *Mytilus galloprovincialis* Lam. Marine Environmental Research 23–36.

Viarengo A. 1989. Heavy metals in marine invertebrates, mechanisms of regulation and toxicity at cell level. CRC Critical Review in Aquatic Science 1:295–317.

Viarengo A, Canesi L, Pertica M, Poli G, Moore M-N, Orunesu M. 1990. Heavy metal effect on lipid peroxidation in the tissues of *Mytilus galloprovincialis* L. Comparative Biochemistry and Physiology Part C 97:37–42.

Viarengo A, Nott JA. 1993. Mechanisms of heavy metals homeostasis in marine invertebrates. Comparative Biochemistry and Physiology Part C 104:355–372.

Viarengo A, Ponzano E, Dondero F, Fabbri R. 1997. A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic molluscs. Marine Environmental Research 44:69–84.

Viarengo A, Burlando B, Cavaletto M, Marchi B, Ponzano E, Blasco J. 1999a. Role of metallothionein against oxidative stress in the mussel *Mytilus galloprovincialis*. American Journal of Physiology 277:1612–1619.

Viarengo A, Burlando B, Dondero F, Marro A, Fabbri R. 1999b. Metallothionein as a tool in biomonitoring programmes. Biomarkers 4:455–466.

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

RIGHTSLINK