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EXAMINING THE SUITABILITY OF MUSSEL DIGESTIVE GLAND TO SERVE AS A BIOMONITORING TARGET ORGAN*

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In coastal marine areas, the level of metallothioneins (MTs) is monitored as the specific response to trace heavy metals and is considered as a biomarker of metal exposure. Laboratory experiments with bivalves indicate that MT synthesis is induced by Cd, Cu, Hg, Ag and Zn ions. The results of our study, conducted with the mussel *Mytilus galloprovincialis*, deployed 12 months in the "hot spot" area of the Kaštela Bay, Dalmatia, Croatia, are evaluated considering the recommendation of MED POL Programme for estimating exposure of mussels to trace heavy metals by means of MTs. Our data clearly indicate that the MT level in the digestive gland of the Mediterranean mussel depends on the biotic and abiotic parameters. Beside MT level, a number of parameters, like digestive gland mass, shell mass, cytosolic metal concentration is needed in order to define if MT level in the digestive gland of mussels is indicative as a biomarker of cadmium exposure.

KEY WORDS: copper, cadmium, field study, long-term metal exposure, metallothioneins, zinc

Characteristics of metallothionein

The term *metallothionein* was initially used to designate the Cd-, Zn- and Cu-containing cysteine-rich protein isolated from the equine renal cortex (1). Characteristic of this protein are low molecular mass, high metal content, specific amino acid composition (high cysteine content, no aromatic amino acids or histidine), unique amino acid sequence (characteristic distribution of cysteinyl residues such as Cys-X-Cys or Cys-Cys motif), spectroscopic features characteristic of metal thiolates (mercaptides), and metal-thiolate clusters.

Therefore, polypeptides resembling equine renal metallothionein in several of their features can be designated as *metallothioneins* (2).

The discovery of MTs was entirely due to its component metals, Cd in particular, but also Zn. Induction of MTs by metals (Zn, Cd, Cu, Ag and Hg)

was an important milestone in the history of MT research (3). Inducibility of MTs identified unknown potential of this molecule and its genetics. New experimental approaches evolved for identification, isolation and characterisation of MTs in different organs of different species. Widespread distribution and specific response to stress stimuli suggest they are the oldest phylogenetic stress-response systems in animals (4).

Considerable progress has been made in the determination of mammalian MT structure. Both NMR and X-ray crystallography have revealed MT to be a two-domain molecule in which metal-thiolate clusters form inorganic cores of essentially globular domains. One cluster contains three divalent metal ions coordinated to nine cysteines, while the other contains four divalent metal ions coordinated to 11 cysteines (5). Coordination geometry for Cd²⁺,

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Zn²⁺ ions is tetrahedral, while for Cu⁺, Ag⁺ ions is trigonal. Invertebrate MTs are much more variable in their cysteine residues alignment, domain and central domain structure. Particularly high resistance of *Mytilus* sp. to heavy metal toxicity may in part be due to the high copy number of transcripts from several MT isoforms (4). Extensive studies using many different organisms (6) indicate that MTs have three major physiological roles in:

1. Detoxification of heavy metals that penetrate into the cells at toxic concentrations;

2. Internal homeostasis of Cu and Zn by (a) keeping intracellular ion concentrations of Cu and Zn at low level, by binding excess in a non-toxic form, as well as by (b) acting as a storage of Cu and Zn for later use and reactivation of apoproteins that require these metals for their activity; and

3. Participation in metabolic functions, including scavenging of free radicals and, as recently proposed, regulation of gene expression (7).

Biomonitoring programme

Among the biomarkers that are currently used to monitor the quality of the coastal marine areas, the induction of MTs in response to metal exposure is well documented. In the context of MED POL Phase III-Programme for the Assessment and Control of Pollution in the Mediterranean Region (8) biomarkers are proposed as early-warning pollution monitoring tools to signal the onset of harmful effects at the cellular and subcellular levels.

Biological response is evident as the induced rate of synthesis of a specific class of metal-binding proteins, known as MTs, following exposure to certain metals (Cd, Cu, Zn). This is considered to be a sublethal detoxification response, but the induced synthesis of these proteins could result in biological costs which reduce the fitness of the individual. In the framework of MED POL Phase III, MT analysis in the digestive gland of *M. galloprovincialis* has been recommended for monitoring the exposure of indicator organisms to metals. The bivalve species, *M. galloprovincialis*, fulfil most of the required criteria for indicator organisms, that is, that they are: abundant as species; ubiquitous (for comparison between areas, regions); an important link in the food chain; accumulating contaminants ("Mussel Watch"); a sessile type of organism; sufficiently long-lived, of reasonable size; easy to sample all over the year; robust enough to survive the transport; exhibiting

high concentration factors of pollutants; tolerant to brackish water, and easy to identify with certainty.

In our study, MT as a molecular biomarker of metal exposure has been validated and combined with the additional data on physical and chemical properties of the ambient water and the cytosolic concentration of metals known as inducers of MTs (Cd, Zn, Cu). For that purpose, a population of mussels was transferred from an aquaculture area to an area under diverse anthropogenic pressures (domestic and industrial waste, transport activities). During the 12 months of mussel deployment, we followed the cytosolic levels of MTs and metals (9). Due to a complex nature of biological responses, monitoring results from the field study were interpreted with caution. The biological responses were assessed in the light of the physiological status of the indicator organisms during the deployment period. Food availability and the gametogenesis cause changes in total body mass, lipid concentration, lipid composition and therefore contaminant levels.

The objective of our study was to follow the recommendations of MED POL Phase III Programme for biological effect methods and estimate the suitability of the digestive gland of *Mytilus galloprovincialis* as target organ for monitoring metal exposure by means of MT. Metallothionein and metal levels, which are known as MT-inducers (Cd, Cu, Zn) were determined in the same heat-treated cytosolic fraction prepared from the composite sample of the digestive glands of Mediterranean mussels, *Mytilus galloprovincialis* and related to biometric data.

MATERIALS AND METHODS

Mussel deployment and sampling

Filter-feeding *M. galloprovincialis* of defined length (5.1 ± 0.2 cm) and age (12 ± 1 months) were transplanted from an aquaculture area and deployed at 4 different sites in the Kaštela Bay (Figure 1, sites A to D), receiving urban and industrial wastewaters. The deployment sites were selected taking into account the hydrographic, chemical and biological characteristics of the Bay (10-12). Within the Bay, the prevailing circulation of water masses is anticlockwise and their average retention time 30 days. According to numerous indicators, including primary production, the Kaštela Bay was categorised as highly a eutrophic area (11).

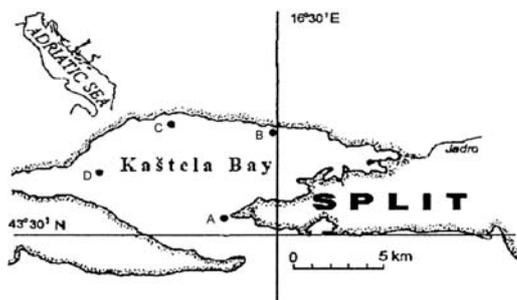


Figure 1 Kaštela Bay, East Adriatic, with the deployment sites (A to D) for mussels *Mytilus galloprovincialis*.

At the deployment sites the water depth was 10 m, while the distance from the shore varied from 50 to 400 m. At each caging site, eight net-like baskets (each one containing 50 specimens) were deployed 1.5 m above the sea bed on 15 September 1997; one basket was sampled at a time. In 1997, samples were taken every month, that is, in October, November and December, to detect any changes which might reflect the acclimation of the Mediterranean mussels to new ambient conditions. In 1998, samples were taken every two months (February, April, June, August). The last sampling took place in September 1998 to end up the round. Within current month, sampling took place on the same date as the deployment date. At stations A and B, sampling was completed (8 samplings), but one sampling at site C and two at site D were not accomplished due to rough weather. Therefore, data are missing for these two locations for particular periods.

After sampling, mussels were kept for 24 hours in filtered (0.45 μm) seawater to depurate the gut content, and then transported to the laboratory in Zagreb, where biometry and tissue dissection took place. Entire glands of 28 specimens per site were pooled to a composite sample, ensuring that the average response counteracted individual variations. Composite samples of mussel digestive glands were deep frozen at $-80\text{ }^{\circ}\text{C}$ until further processing and analysis.

Homogenate and cytosolic fraction

The volume of homogenised buffer 0.02 mol L^{-1} TRIS-HCl buffer, $\text{pH}=8.6$ (at $4\text{ }^{\circ}\text{C}$), containing leupeptine (0.006 mmol L^{-1}), phenylmethylsulphonylfluoride (PMSF, 0.5 mmol L^{-1}) and 2-mercaptoethanol (0.01%), was adjusted to that of the tissue mass at 3:1 ratio. Homogenisation proceeded on an ice-bath with a Potter-Elvehjem type of

homogenizer. The homogenate was centrifuged in the Sorval RC28S centrifuge by Du Pont at 30000 $\times g$ for 40 minutes at $4\text{ }^{\circ}\text{C}$. The isolated supernatant (S30) contained total cytosolic proteins. Further on, S30 was heat-treated at $70\text{ }^{\circ}\text{C}$ for 10 minutes using The Dri Block (Techne), and subsequently centrifuged at 30000 $\times g$ for 20 minutes at $4\text{ }^{\circ}\text{C}$. This supernatant contained MTs as heat-stable cytosolic proteins.

Cytosolic MT concentration

MT concentration in the heat-treated supernatant (mg mL^{-1}) was determined by electrochemical method in a differential pulse mode (13), on a Metrohm 290E hanging mercury drop electrode (HMDE). MT calibration straight line was obtained at $7\text{ }^{\circ}\text{C}$ with the commercial rabbit liver MT(I+II) from Sigma.

Heat-treated cytosolic trace metal concentrations

Metal concentrations in the heat-treated S30 fraction ($\mu\text{g mL}^{-1}$) were determined using a Varian double beam flame atomic absorption spectrometer (SpectrAA 220) with multielement lamps and a deuterium lamp for baseline correction. Atomisation of metals was achieved in the air-acetylene flame. Calibration was performed using Merck's standard solutions of Cd, Zn and Cu. Selected concentration ranges of Cd 0.01-0.05 $\mu\text{g mL}^{-1}$, Zn 0.5-2.0 $\mu\text{g mL}^{-1}$ and Cu 0.1-0.5 $\mu\text{g mL}^{-1}$ for the construction of the calibration straight lines were prepared in Tris-HCl buffer (0.004 mol L^{-1}) of the same concentration as the samples, which were prior to metal analysis fivefold diluted with redistilled water. Detection limits of the selected metals were as follows: Cd 0.003 $\mu\text{g mL}^{-1}$, Zn 0.012 $\mu\text{g mL}^{-1}$ and Cu 0.002 $\mu\text{g mL}^{-1}$.

Statistical analysis

All statistical analyses (Kruskal-Wallis test, multiple regression analysis, Pearson correlation coefficients) were performed in SigmaStat for Windows Version 1.0.

RESULTS

Since MTs are heat-stable proteins which are comprised in cytosolic fraction of the tissues, their concentrations were determined in the heat-treated supernatants (S30 fraction) of mussels' digestive glands. The concentrations of metals Cd, Cu, Zn,

which are inducers of MTs, were determined in the same heat-treated tissue fraction. At all four stations, the highest metal concentrations present in the heat-treated cytosol of the digestive glands were those of Zn with median values $\approx 1\text{-}2 \mu\text{g mL}^{-1}$, followed by $\text{Cu} \approx 0.5 \mu\text{g mL}^{-1}$ and $\text{Cd} \approx 0.1 \mu\text{g mL}^{-1}$. The median MT concentration was 0.6 mg mL^{-1} (Table 1). Regarding the metals, Kruskal-Wallis analysis showed no statistically significant differences between the results obtained from the four deployment sites. It might indicate that the input of heavy metals in the water column of the Kaštela Bay is uniform. Thus, for the purpose of the statistical analyses the results obtained at the four deployment sites were treated as the same population of data.

Table 1 The median and the concentration ranges of MTs, Cd, Zn and Cu in the heat-treated cytosol (S30) of the composite sample of the digestive glands of *Mytilus galloprovincialis* Lmk., caged at sites A to D (Figure 1) in the Kaštela Bay. Concentration ranges include the total deployment period of 12 months.

Deployment site	MTs	Cd	Zn	Cu
	mg mL ⁻¹ Median Min-Max	$\mu\text{g mL}^{-1}$ Median Min-Max	$\mu\text{g mL}^{-1}$ Median Min-Max	$\mu\text{g mL}^{-1}$ Median Min-Max
A	0.677 0.525-0.865	0.138 0.084-0.205	1.761 1.355-2.366	0.508 0.374-0.621
B	0.656 0.511-0.817	0.100 0.079-0.120	1.879 1.273-2.245	0.516 0.464-0.653
C	0.637 0.547-0.812	0.123 0.093-0.165	2.070 1.245-2.232	0.532 0.457-0.766
D	0.645 0.524-0.754	0.122 0.094-0.134	2.154 1.313-2.583	0.587 0.363-0.786

Temporal variation of digestive gland mass of deployed mussels

Figure 2a shows the variation in average mussel digestive gland mass over one year at each deployment site. At the four caging sites, the average digestive gland mass of mussels reached maximum in April 1998, when the concentration of MTs was minimal, while the peaks of MT concentration were observed in December 1997 and June 1998 (Figure 2b). Seasonal changes in digestive gland mass are known to be greatly affected by the development of gonadic tissues which penetrate into the digestive gland and make difficult the physical tissue separation by dissection (14). Lower concentrations of trace metals in the gonadic tissues (15-17) cause "biological dilution" of trace metal concentrations in the digestive gland. Thus, the highest metal concentrations can be observed at the end of the reproductive cycle (18).

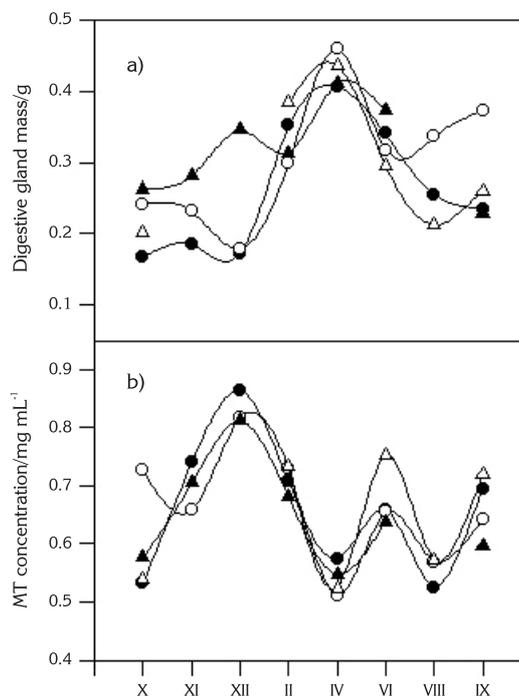


Figure 2 Temporal variation of the digestive gland mass (g) and MT concentration (mg mL^{-1}) in the heat-treated cytosol (S30) of the digestive gland of mussels *Mytilus galloprovincialis* deployed at four sites (A=●; B=○; C=▲ and D=△) in the Kaštela Bay for 12 months.

Literature reports (19-22) that variation in soft tissue mass influences MT concentration in the bivalves. The "biological dilution" is thus a valid explanation for the MT concentration in the digestive gland as well. Kruskal-Wallis test of MT concentrations at the four deployment sites in the Kaštela Bay did not show statistically significant differences. Cytosolic concentrations of MT were comparable, which suggests that the living conditions for the mussels were also comparable.

Figure 3 (a-c) shows temporal variation in metal concentrations (Cd, Cu and Zn) analysed in the heat-treated cytosol (S30) of the digestive gland of deployed mussels. A comparison between the temporal variation of cytosolic trace metal concentrations and MT concentrations, which were analysed in the same biochemical fraction, shows an obvious and well-resolved peak in June, co-occurring for both the metals, especially Cd (Figure 3a), and MTs (Figure 2b). A shift in the maximum at the site C for Cu (Figure 3b) and sites C and B for Zn (Figure 3c) led in June to a less pronounced maximum for Cu and Zn than for Cd.

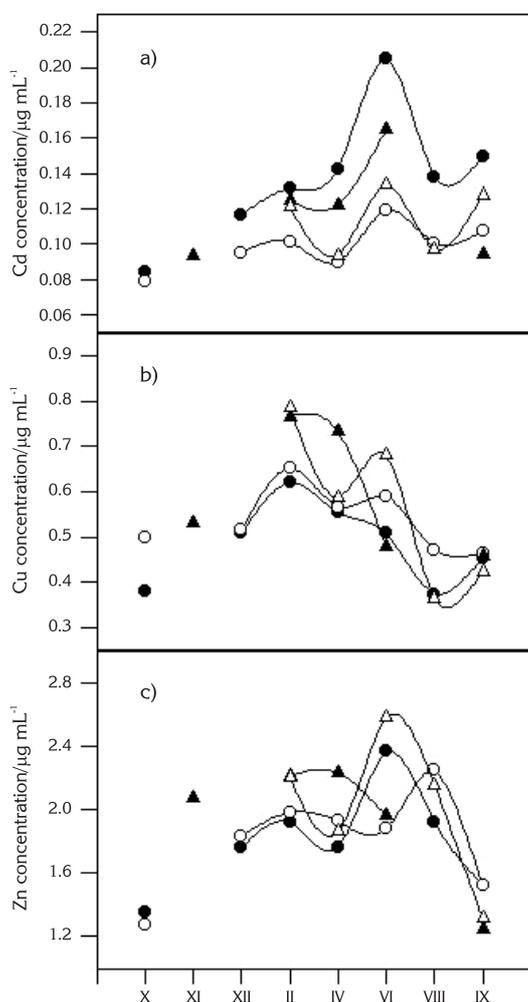


Figure 3 Temporal variation of a) Cd, b) Cu, and c) Zn concentrations ($\mu\text{g mL}^{-1}$) in heat-treated cytosol (S30) of the digestive gland of mussels *Mytilus galloprovincialis* deployed at four sites (A=●; B=○; C=▲ and D=△) in the Kaštela Bay for 12 months.

DISCUSSION

In situ exposure of mussels deployed in the Kaštela Bay provides useful information which cannot be obtained from laboratory bioassays and characterizations of indigenous communities. Laboratory bioassays are mostly performed under controlled abiotic conditions (temperature, salinity, contaminant level), while organisms are starving during the exposure period. In contrast, an *in situ* study makes it possible to evaluate data for the change across defined space and time. It is a great challenge to replace laboratory research with the one in the field. The results indicate complex biological

responses and the need to understand the physiology and metabolism of indicator organisms.

If a biomarker of metal exposure, such as MT, which is a specific metal-binding and metal-induced protein, is selected to define natural variability (spatial and temporal) and baseline level, the method applied has to be sensitive enough to enable undisputable determination of that biomarker, satisfactory repeatability and reproducibility, to resolve natural variability of MT level and not just the changes due to method performance. The results of this study confirm with certainty that the electrochemical determination of MT levels fulfils the criteria of sensitivity, repeatability and reproducibility, needed for a reliable field study.

The mussel digestive gland plays a role in the intracellular and extracellular digestion and storage of nutritive substances (23). It accumulates substances needed for gonad development (glycogen, lipids and proteins), thereby stimulating the process of gametogenesis. The digestive gland mass reached a pronounced maximum in April 1998, which reflects the process of gametogenesis, followed by spawning. The abundance of food from February to June enabled the accumulation of substances needed for gonad development (glycogen, lipids and proteins), thereby stimulating the process of gametogenesis. Glycogen constitutes the dominant energy reserve fueling gametogenesis. When stored glycogen, lipids and proteins are exhausted, gametogenesis may be fuelled directly by circulating metabolites originating from the transformation of ingested food (24). Intensified food supply increases the uptake of metals, some of which are known as MT inducers. Compared to the whole soft tissue and the gills, mass partition of MT in the digestive gland of mussels is the highest (25). Langston *et al.* (26) also reported that, compared with other tissues, basal MT level in the digestive gland of *Mytilus spp.* is relatively high i.e. $\sim 8 \text{ mg g}^{-1}$ dry mass. Applying the conversion factor of 4 for dry-to-wet digestive gland mass, basal mass partition of MT in the digestive gland amounts to 2 mg g^{-1} wet mass. In this study, one month after mussel deployment (October 1997), the average mass partition of MT was 2.2 mg g^{-1} wet mass (Figure 2b). Concentrations of MT shown in Figure 2b were converted to mass partition by multiplication with 4, which corresponds to tissue dilution with the homogenising buffer. Comparing the results obtained by Langston *et al.* (26) with ours, there is a high coincidence between them, which supports the view that reported MT levels may be considered as baseline. Further comparison with 2.1 mg g^{-1} wet

mass reported by Pavičić *et al.* (27) for mussels from an aquaculture site (Lim Bay) on the East Adriatic coast additionally supports the view that the MT level of $\sim 2 \text{ mg g}^{-1}$ wet mass may be considered baseline. As a result of high basal physiological pool of MTs, a presence of free binding sites on MT molecules can be assumed. After saturating free SH-sites, metal ions such as Cd and Cu(I), which have higher stability constants than thiolate clusters, are able to displace Zn ions from a basal MT pool (28).

Pearson's coefficients (Table 2) show a statistically significant positive correlation for the following pairs of parameters: Cu/Zn (0.53); Cu/digestive gland mass (0.51) and shell mass/digestive gland mass (0.48).

Table 2 Correlation between metal, MT concentrations and biometric data analysed using Pearson's coefficients (level of significance * $p < 0.05$; ** $p < 0.01$).

	MT	Cd	Zn	Cu	Digestive gland mass
MT	1				
Cd	0.09	1			
Zn	0.01	0.36	1		
Cu	0.24	0.07	0.53**	1	
Digestive gland mass	-0.30	0.22	0.38	0.51*	1
Shell mass	-0.27	0.35	0.16	-0.19	0.48**

It is interesting to note that Cd concentration increased with time at all four deployment sites, reaching peak values in June 1998 (Figure 3a). A positive correlation between Cd concentration and shell mass (0.35, $p < 0.10$), which is an indicator of the mussel's age, further confirms these findings (Table 2). The increase in Cd concentration with the mussel's age in the cytosol of digestive gland is consistent with the known affinity of this metal for MTs. Since the mussels were transplanted from the same aquaculture site to four different sites in the Kaštela Bay, Cd concentration determined in October 1997 was practically identical at all four sites (Figure 3a). If cytosolic Cd concentration is expressed as mass partition (conversion factor of 4), it would yield the average value of $0.32 \mu\text{g g}^{-1}$ wet mass. The reference basal value of Cd in the digestive gland tissue for mussels from the aquaculture site in Lim Bay, East Adriatic coast has been reported by Martinčić *et al.* (29) and is on average $0.37 \mu\text{g g}^{-1}$ wet mass. The fact that our results refer to cytosolic Cd suggest that Cd content in the digestive gland is predominantly related

to the cytosolic fraction. The differences in the Cd concentration in the digestive gland of mussels caged at the four sites became evident with time, probably due to different bioavailability of Cd at different sites. The differences were particularly clear in June 1998, when the Cd content was at its maximum, dropping in the following order: $A > C > D \approx B$. The highest Cd concentration at site A can be explained by the fact that site A receives water masses from the Split channel, where the urban runoff is discharged through mixed sewer systems into the Split harbour (30).

The kinetics of MT metabolism and associated metal flux are important in determining Cd tissue burden and toxicity. Turnover rates for MT and Cd in *M. edulis*, expressed as half-lives, are 25 and 300 days, respectively. The slow rate of Cd elimination is explained by the fact that, as MT degrades, the released Cd induces synthesis of a new protein, to which the metal becomes re-sequestered (31). Localisation of Cu-MT within lysosomes has been reported for the digestive glands of mussels (32), suggesting that this is the principal site of degradation of the metal-binding protein. Differences in the distribution and flux of Cd, Cu and Zn may, therefore, be due to different susceptibility to lysosomal degradation and varying turnover of their respective MTs (33). This may explain why Cu is eliminated more rapidly from mussels than Zn or Cd (half-lives 10, 60 and 300 days, respectively) (26).

Table 3 Multiple linear regression analysis; dependence of MT concentration on the following independent variables: the heat-treated cytosolic (S30) trace metal concentrations, digestive gland mass and shell mass.

Variable	Coefficient	p
Constant	0.64	< 0.001
Cd	0.98	0.122
Zn	-0.04	0.483
Cu	0.53	0.016*
Digestive gland mass	-0.83	0.005*
Shell mass	-0.01	0.655

($R^2 = 0.54$)

* statistically significant dependence.

Since MT concentration did not show statistically significant correlation with any analysed parameter (Table 2), we performed multiple linear regression analysis. The obtained model (coefficients are shown in Table 3) explains 54 % of MT concentration variability, where statistically significant influence was

determined for Cu (0.53, $p=0.016$) and digestive gland mass (-0.83 , $p=0.005$). Relatively high basal MT level in the digestive gland of *Mytilus spp.* reflects its role in Cu metabolism, in addition to its protective role against cytotoxicity (26). The negative coefficient for MT concentration and digestive gland mass confirms the already observed effect of inverse dependence of both parameters (Figure 2a and 2b).

Although time-related accumulation of Cd, as toxic metal, occurs differently at four caging sites, these differences are not reflected in the level of MTs (Figure 2b). The conclusion is that Cd in the digestive gland of mussels did not reach the level at which it could induce additional MT synthesis, because the basal physiological pool of MTs in the digestive gland was already high and was related to high tissue concentration of essential metals, Zn and Cu (34). The existing level of MTs was therefore sufficient to detoxify the levels of cytosolic metals (Cd, Cu, Zn) reported in this study, without the need for additional induction. A similar observation was reported for the digestive gland of *Littorina littorea* and *Ruditapes decussata*, where only small overall increase in MT levels over the year could be detected, in spite of relatively high Cd influx, because Cd was sequestered by high levels of MTs inherent to that tissue, probably in relation to the storage and turnover of essential metals (35, 36). Geffard et al. (34) came to a similar conclusion for *Crassostrea gigas*, emphasizing that the digestive gland of this oyster has a limited value as a tissue for biomonitoring MTs as biomarkers. As suggested by Bebianno et al. (36) for *R. decussata*, the low level of significant *de novo* MT synthesis renders the digestive gland of *Mytilus galloprovincialis* unsuitable for detecting responses to sublethal levels of Cd. The observed increase in the digestive gland mass during gametogenesis leads to a "biological dilution" of MT which is analysed as the biomarker of metal exposure.

CONCLUSION

The digestive gland of mussel *Mytilus galloprovincialis* is often selected as a target tissue for the analysis of MTs as biomarkers of metal exposure. It is of special interest for environmental research to be able to assess toxic effects of cadmium by means of MT levels. The digestive gland as a storage organ for nutrients has the highest total protein content of all other mussel tissues and, consequently, the

highest content of specific metal-binding proteins (MTs). Although time-related accumulation of cadmium was different at the four caging sites in our study, these differences did not reflected on the MT levels. Since the basic MT pool in the digestive gland of *M. galloprovincialis* is high due to high cytosolic content of Cu and Zn that induce MT synthesis, cadmium content did not reach the level that would induce additional MT synthesis. In spite of high metal accumulation in the digestive gland, this organ does not meet the criteria as a tissue of choice for estimating exposure of *M. galloprovincialis* to sublethal levels of Cd. The physiological changes caused by gonadal development and food abundance contribute to the changes in MT levels more than bioavailable Cd concentrations.

REFERENCES

1. Kägi JHR, Vallee BL. Metallothionein: a cadmium and zinc-containing protein from equine renal cortex. *J Biol Chem* 1960;235:3460-3465.
2. Kojima Y, Binz P-A, Kägi JHR. Nomenclature of metallothionein: Proposal for a revision. In: Klaassen CD, editor. *Metallothionein IV*. Basel: Birkhäuser Verlag; 1999, p. 3-6.
3. Vallee BL, Maret W. The functional potential and potential functions of metallothioneins: A personal perspective. In: Suzuki KT, Imura N, Kimura M, editors. *Metallothionein III, Biological Roles and Medical Implications*. Basel: Birkhäuser Verlag; 1993, p. 1-27.
4. Baršyte D, White KN, Lovejoy DA, Cloning and characterization of metallothionein cDNAs in the mussel *Mytilus edulis* L. digestive gland. *Comp Biochem Physiol Part C* 1999;122:287-296.
5. Mackay EA, Overnell J, Dunbar B, Davidson I, Hunziker PE, Kägi JHR, Fothergill JE. Complete amino acid sequences of five dimeric and four monomeric forms of metallothionein from the edible mussel *Mytilus edulis*. *Eur J Biochem* 1993;218:183-194.
6. Hamer DH. Metallothionein. *Ann Rev Biochem* 1986;55:913-951.
7. Roesijadi G, Bogumil R, Vasak M, Kägi JHR. Modulation of DNA binding of a tramtrack zinc finger peptide by the metallothionein-thionein conjugate pair. *J Biol Chem* 1998;273:17425-17432.
8. UNEP/MAP. MED POL Phase III, Programme for the assessment and control of pollution in the Mediterranean region. MAP Tech. Rep. Ser. No. 120. Athens: UNEP; 1999.
9. Raspor B, Dragun Z, Erk M, Ivanković D, Pavičić J. Is the digestive gland of *Mytilus galloprovincialis* a tissue of

- choice for estimating cadmium exposure by means of metallothioneins? *Sci Tot Environ*, 2004;333:99-108.
10. Kušpilić G, Marasović I, Vukadin I, Odžak N, Stojanoski L. Hydrographic conditions and nutrient requirements during red tide in the Kaštela Bay (middle Adriatic). *Acta Adriatica* 1991; 32:813-826.
 11. Barić A, Marasović I, Gačić M. Eutrophication phenomenon with special reference to the Kaštela Bay. *Chem Ecol* 1992; 6: 51-68.
 12. Beg-Paklar G, Gačić M. The wind effect on the Kaštela Bay current field. *Acta Adriat* 1997; 38: 31-43.
 13. Raspor B, Paić M, Erk M. Analysis of metallothioneins by the modified Brdička procedure. *Talanta* 2001; 55: 109-115.
 14. Regoli F, Orlando E. Seasonal variation of trace metal concentrations in the digestive gland of the Mediterranean mussel *Mytilus galloprovincialis* – comparison between a polluted and a non-polluted site. *Arch Environ Contam Toxicol* 1994; 27: 36-43.
 15. George SG, Coombs TL. The effects of chelating agents on the uptake and accumulation of cadmium by *Mytilus edulis*. *Mar Biol* 1977; 39: 261-268.
 16. La Touche YD, Mix MC. Seasonal variation of arsenic and other trace elements in bay mussels (*Mytilus edulis*). *Bull Environ Contam Toxicol* 1982; 29: 665-670.
 17. Lobel PB, Wright DA. Gonadal and nongonadal zinc concentrations in mussels. *Mar Pollut Bull* 1982; 13: 320-323.
 18. Cardellicchio N, Brandini E, Dileo A, Giandomenico S, Annicchiarico C. The influence of environmental and physiological factors on the accumulation of heavy metals in mussels (*Mytilus galloprovincialis*). *Ann Chim* 1998; 88: 253-260.
 19. Bordin G, McCourt J, Cordeiro Raposo F, Rodriguez AR. Metallothionein-like metalloproteins in the Baltic clam *Macoma balthica*: seasonal variations and induction upon metal exposure. *Mar Biol* 1997; 129: 453-463.
 20. Amiard-Triquet C, Rainglet F, Larroux C, Regoli F, Hummel H. Metallothioneins in Arctic bivalves. *Ecotoxicol Environ Saf* 1998; 41: 96-102.
 21. Mouneyrac C, Amiard JC, Amiard-Triquet C. Effects of natural factors (salinity and body weight) on cadmium, copper, zinc and metallothionein-like protein levels in resident populations of oysters *Crassostrea gigas* from a polluted estuary. *Mar Ecol Prog Ser* 1998; 162: 125-135.
 22. Mouneyrac C, Geffard A, Amiard JC, Amiard-Triquet C. Metallothionein-like proteins in *Macoma balthica*: effects of metal exposure and natural factors. *Can J Fish Aquat Sci* 2000; 57: 34-42.
 23. Bayne BL, Thompson RJ, Widdows J. Physiology: I. In: Bayne BL, editor, *Marine mussels: their ecology and physiology*. Cambridge: Cambridge University Press; 1976, p. 121-206.
 24. De Zwaan A, Mathieu M. Cellular biochemistry and endocrinology. In: Gosling E, editor, *The mussel Mytilus: Ecology, physiology, genetics and culture*. Amsterdam: Elsevier Science Publishers B.V.; 1992, p. 223-307.
 25. Raspor B, Erk M, Pavičić J, Jurić D, Kwokal Ž, Odžak N. Metallothionein as biomarker of mussel exposure to heavy metals. *Marine pollution, IAEA-TECDOC-1094*, July 1999, p. 151-156.
 26. Langston WJ, Bebianno MJ, Burt GR. Metal handling strategies in molluscs. In: Langston WJ, Bebianno MJ, editors, *Metal Metabolism in Aquatic Environments*. London: Chapman & Hall; 1998, p. 219-283.
 27. Pavičić J, Raspor B, Martinčić D. Quantitative determination of metallothionein-like proteins in mussels. Methodological approach and field evaluation. *Mar Biol* 1993; 115: 435-444.
 28. Viarengo A, Palmero S, Zanicchi G, Capelli R, Vaissiere R, Orunesu M. Role of metallothioneins in Cu and Cd accumulation and elimination in the gill and digestive gland cells of *Mytilus galloprovincialis* Lam. *Mar Environ Res* 1985; 16: 23-36.
 29. Martinčić D, Nürnberg HW, Stoeppeler M, Branica M. Bioaccumulation of heavy metals by bivalves from Lim Fjord (North adriatic Sea). *Mar Biol* 1984; 81:177-188.
 30. Bogner D, Juračić M, Odžak N, Barić A. Trace metals in fine-grained sediments of the Kaštela Bay, Adriatic Sea. *Wat Sci Technol* 1998; 38:169-175.
 31. Bebianno MJ, Langston WJ. Turnover rate of metallothionein and cadmium in *Mytilus edulis*. *BioMetals* 1993; 6: 239-244.
 32. Viarengo A, Pertica M, Canesi L. et al. Purification and biochemical characterization of a lysosomal copper-rich thionein-like protein involved in metal detoxification in the digestive gland of mussels. *Comp Biochem Physiol* 1989; 93C: 389-395.
 33. George SG. Biochemical and cytological assessments of metal toxicity in marine animals. In: Furness RW, Rainbow PS, editors, *Heavy Metals in the Marine Environment*. Boca Raton, Florida: CRC Press Inc; 1990, p. 123-142.
 34. Geffard A, Amiard-Triquet C, Amiard JC, Mouneyrac C. Temporal variations of metallothionein and metal concentrations in the digestive gland of oysters (*Crassostrea gigas*) from a clean and a metal-rich site. *Biomarkers* 2001; 6: 91-107.
 35. Bebianno MJ, Langston WJ, Simkiss K. Metallothionein induction in *Littorina littorea* (Mollusca: Prosobranchia) on exposure to cadmium. *J Mar Biol Assoc UK* 1992; 72: 329-342.
 36. Bebianno MJ, Nott JA, Langston WJ. Cadmium metabolism in the clam *Ruditapes decussata*: the role of metallothioneins. *Aquat Toxicol* 1993; 27: 315-334.

Sažetak

PRIHVATLJIVOST CILJANOG ORGANA (PROBAVNE ŽLIJEZDE) DAGNJE ZA PROVOĐENJE BIOMONITORINGA

U priobalnim morskim područjima razina metalotioneina (MT) prati se kao specifični odgovor na teške metale u tragovima te se smatra biomarkerom izloženosti metalima. Laboratorijski eksperimenti sa školjkama pokazuju da sintezu MT induciraju ioni Cd, Cu, Hg, Ag i Zn. Rezultati našeg istraživanja, koje je provedeno s dagnjama, *Mytilus galloprovincialis*, izloženim tijekom 12 mjeseci u području "vrućih točaka" u Kaštelanskom zaljevu, Dalmacija, Hrvatska, razmatrani su u skladu s preporukama MED POL programa za procjenu izloženosti dagnja teškim metalima u tragovima putem MT. Naši podaci jasno ukazuju da razina MT u probavnoj žlijezdi mediteranske dagnje ovisi o biotičkim i abiotičkim parametrima. Osim razine MT, potrebni su brojni parametri, poput mase probavne žlijezde, mase ljuštore te koncentracije metala u citosolu kako bi se moglo definirati da li razina MT u probavnoj žlijezdi dagnja upućuje na njihovu izloženost kadmiju.

KLJUČNE RIJEČI: bakar, cink, dugotrajna izloženost metalima, kadmij, metalotioneini, terensko istraživanje

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