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THE INFLUENCE OF THE BIOMETRIC PARAMETERS ON METALLOTHIONEIN AND METAL LEVEL IN THE HEAT-TREATED CYTOSOL OF THE WHOLE SOFT TISSUE OF TRANSPLANTED MUSSELS

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Abstract. The influence of the biometric parameters (shell mass, whole soft tissue mass, condition index) on MT and metal levels in the heat-treated cytosol of the whole soft tissue of transplanted mussels was studied over the period of one year. The positive correlation of three metals (Cd, Fe, Zn) with the shell mass indicated to time-dependent increase of their contents. Strong correlation of Mn content with the whole soft tissue mass ($r = 0.74$, $p < 0.0001$), and almost identical changes of these two parameters over the year make Mn a good indicator of mussel's condition. As opposed to the other metals, Cu content does not exhibit connection with biometry. On the other hand, MTs are highly influenced by biometry. As much as 65% of their variability could be explained by the changes of the shell mass and the whole soft tissue mass. Consequently, it is difficult to distinguish if the obtained positive correlation between Cd and MTs ($r = 0.48$, $p < 0.05$) reflects MT induction by Cd, or Cd accumulation as a result of age-dependent increase of MTs. Due to the strong influence of the biometry on MT level, the whole soft tissue is not considered as the best choice for measuring MTs as a biomarker. Better option would be to isolate a specific tissue that shows indisputable connection between MT induction and metal accumulation.

Keywords: biometric parameters, heat-treated cytosol, metallothionein, *Mytilus galloprovincialis*, trace metals, whole soft tissue

1. Introduction

Metallothioneins (MTs), as the biomarkers of exposure to metals, often are measured in the digestive gland of mussels, the tissue with the highest MT and Cd level (Raspor *et al*., 1999). However, from an operational point of view, dissection of digestive gland, or some other particular mussel's tissue, is time-consuming (Mourgaud *et al*., 2002). Thus, the possibility is considered to use the whole soft mussel tissue, which is easily separated from the shell, to study the changes of MT and metal levels in the cytosol as the response to environmental conditions.

The whole soft mussel tissue reflects the contribution of frequently analysed tissues, like digestive gland and gills, but also of those that play the important role

in mussel physiology, and are still rarely the object of environmental analyses, such as, for example, the mantle (Pavičić *et al*., 1991). The uptake of metals may take place via the gills or digestive gland, but also via the surface of the mantle, depending on the speciation of the metal (Cossa, 1989). The mantle is also important during gametogenesis, since gonadic tissues develop within the mantle and the digestive gland, thus influencing the total mass of mussels (Regoli, 1998). Since the tissue metal concentrations are a function of net metal content, any change in growth or general condition might influence measured body concentrations (Langston *et al*., 1998).

Correction of growth-related variability in metal and MT concentrations is of major consideration in improving the interpretation of field data by reducing the contribution of biometric parameters in order to better resolve the biological effects of accumulated metals (Langston *et al*., 1998).

Martinčić *et al*. (1992) suggested that, because of biological similarities and better reproducibility of the results, the transplanted mussels are more suitable for monitoring of trace metals than indigenous mussels, as originally proposed by the "Mussel Watch" programme (Goldberg *et al*., 1978).

Thus, with the aim to observe the general condition of mussels *Mytilus galloprovincialis*, we have conducted metal and MT analyses in the heat-treated cytosol of the whole soft tissue of mussels that were transplanted and caged over one year at four sites (A, B, C and D) in the semi-enclosed Kaštela Bay in the Adriatic Sea, Croatia (Figure 1). The specific objectives were:

a) to obtain reliable data set on the levels of MTs and metals (Cd, Zn, Cu, Mn, Fe) in the heat-treated cytosol of the whole soft mussel tissue;
b) to define the influence of mussel's age and general condition on the content of MTs and metals in the heat-treated cytosol of the whole soft mussel tissue; and

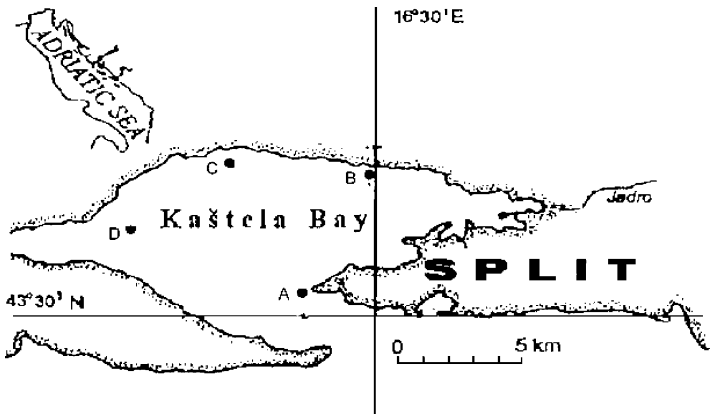

Figure 1. Study area of the semi-enclosed Kaštela Bay in the Adriatic Sea, Croatia, and the mussel deployment sites A to D.

c) to define dependence between MT and metal contents in the heat-treated cytosol of the whole soft mussel tissue.

2. Materials and Methods

2.1. Mussel caging 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 (the shellfish breeding farm in Mali Ston Bay) and caged at 4 different sites within Kaštela Bay (Figure 1, sites A to D), a recipient of untreated urban and industrial wastewater (Barić *et al.*, 1992). The caging locations were 50 to 400 m distant from the shore. At each caging site 8 net-like baskets (each one containing 50 specimens) were deployed 1.5 m above the sea bottom on September 15th, 1997; one basket was sampled at a time. Zero-point samples, i.e. mussels before transplantation to the Kaštela Bay were not taken and analyzed. Monthly sampling was performed in October, November and December 1997, to detect any changes which might reflect the acclimation of Mediterranean mussels to new ambient conditions. In 1998, bimonthly sampling was performed (February, April, June, August). The last one 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 complete (8 times), while due to rough weather, at site C one sampling and at site D two samplings were not accomplished. Therefore, data at these two locations in particular periods are missing.

After sampling, mussels were kept for 24 hours in the filtered (0.45 μm) seawater to depurate the gut content (Bordin *et al.*, 1992; Odžak *et al.*, 2001), and then transported to the Laboratory in Zagreb, where biometric parameters measurement and tissue dissection took place. Composite samples of mussel tissues were deep frozen at $-80\,^{\circ}$C until further processing and analysis.

2.2. Mussel biometry

At each caging site and sampling period the composite sample of the whole soft mussel tissue was made up on average from 10 specimens. Another 10 specimens were used for determination of condition index, and the rest for target tissue dissection (Dragun *et al.*, 2004; Raspor *et al.*, 2004). Therefore, no replicates per sampling period and site exist, but results represent the average biochemical and chemical responses. Average mass of the whole soft tissue within composite tissue sample was measured in order to observe temporal fluctuations of that tissue, mainly related to reproductive cycle and food availability (Mourgaud *et al.*, 2002). At the beginning of our field study the mussels' age was defined as 12 ± 1 months. Shell mass was measured as an additional indicator of age, due to the fact that the calcareous shell

continues to be formed even when the length increments are not observed (Fischer, 1983). Our observations confirm that during 12 months of caging, mussels' length increased on average from 5.2 to 5.9 cm, while the shell mass increased on average from 4.5 to 7.0 g. Fresh to dry tissue ratio was determined using 10 individuals, and drying for 24 hours at 105 °C. Condition index (%) was determined according to Davenport and Chen (1987), using the following equation:

$$\frac{\text{Dry meat mass}}{\text{Total volume} - \text{shell volume}} \times 100 \quad (1)$$

2.3. HOMOGENATE AND CYTOSOLIC FRACTION

Composite sample of the whole soft tissue was homogenised in three volumes of 0.02 M TRIS-HCl buffer, pH = 8.6, containing leupeptine (0.006 mM), phenylmethyl-sulphonylfluoride (PMSF, 0.5 mM) and 2-mercaptoethanol (0.01%), on an ice-bath with a Potter-Elverhjem type of homogenizer. The homogenate was centrifuged in the Sorval RC28S centrifuge by Du Pont at $30000 \times g$ for 40 minutes at 4 °C. The isolated supernatant (S30) contained total cytosolic proteins (Yang *et al.*, 1995). Further on, S30 was heat-treated at 70 °C for 10 minutes using The Dri Block (Techne), and subsequently centrifuged at $30000 \times g$ for 20 minutes at 4 °C. This supernatant contained MTs as heat-stable cytosolic proteins (Yang *et al.*, 1995).

2.4. MT AND TRACE METAL CONCENTRATIONS IN THE HEAT-TREATED CYTOSOL OF THE WHOLE SOFT TISSUE

The MT concentration (mg ml^{-1}) in the heat-treated supernatant was determined by an electrochemical method in a differential pulse mode (Raspor *et al.*, 2001), on a Metrohm 290E hanging mercury drop electrode (HMDE), on μ Autolab (Eco Chemie, The Netherlands). A MT calibration straight line was obtained at 7 °C with the commercial rabbit liver MT(I + II) from Sigma.

Metal concentrations (μg ml^{-1}) were also determined in the heat-treated S30 fraction of the whole soft tissue, to obtain data only on the fraction of metals that could be associated to MTs. Methodological study on the distribution of metals (Zn, Cu and Cd) and proteins in differently treated S30 fractions from digestive gland of *M. galloprovincialis* indicated that Cd is predominantly associated with MT fraction in both untreated and differently treated (temperature or solvent precipitation) S30 cytosol (Ivanković *et al.*, 2003).

Varian double beam flame atomic absorption spectrometer (SpectrAA 220) with multielement lamps and a deuterium lamp for baseline correction was used for metal analyses in the heat-treated S30 fractions. Atomisation of metals was achieved in the air-acetylene flame. Calibration was performed using Merck's standard solutions of Cd, Zn, Cu, Mn and Fe prepared in Tris-HCl buffer of the same concentration

(0.004 M) as the diluted samples. Detection limits of the selected metals were as follows: Cd 0.003 μg ml^{-1}; Zn 0.012 μg ml^{-1}; Cu 0.002 μg ml^{-1}; Mn 0.002 μg ml^{-1}; and Fe 0.009 μg ml^{-1}.

Mass partition of MTs and metals in the whole soft tissue was expressed on a dry tissue mass basis (mg g^{-1} and μg g^{-1}, respectively), multiplying concentrations determined in the heat-treated cytosol of the whole soft tissue by a factor of four which corresponds to homogenate dilution, and by fresh to dry tissue ratio (median value equals 8.1). Contents of MTs and metals (mg and μg, respectively) were obtained by multiplying their mass partition by the dry mass of the whole soft tissue.

2.5. Statistical treatment of results

All statistical analyses (descriptive statistics, Kruskal-Wallis test, correlation and linear regression analysis, multiple linear regression) were performed in SigmaStat for Windows Version 1.0, except the principal component analysis (PCA), which was performed in SPSS®10.0 for Windows.

3. Results and Discussion

3.1. The mass partition of MTs and metals in the heat-treated cytosol of the whole soft mussel tissue

The mass partition in the heat-treated cytosol was determined for five metals. Three of them are known inducers of MT synthesis (Cd, Zn, Cu), while Fe and Mn were chosen for analysis as metals essential for mussel's metabolic processes. Among these five metals, the highest mass partition (based on the dry tissue mass) was observed for Zn, with the median value of 30–33 μg g^{-1}, depending on the deployment site. Compared to Zn, the levels of other metals were considerably lower, with the highest level of Fe (median $\approx$ 15–20 μg g^{-1}), followed by Cu (median $\approx$ 4–6 μg g^{-1}), Mn (median $\approx$ 4–5 μg g^{-1}) and Cd (median $\approx$ 1.5–2.0 μg g^{-1}). The median value of MT mass partition was approximately 5.5–7.5 mg g^{-1} (Table I). Kruskal-Wallis test showed that the levels of metals and MTs were not significantly different at different deployment sites, same as the shell mass and the dry soft tissue mass, indicating that living conditions were uniform throughout the Bay which allowed us to treat all data as the single population. Nevertheless, some differences were observed, although the test showed that they are not statistically significant. For example, at the site D the lowest mass partition of Cu was determined. At the same station, low Cu level was also determined in the marine sediment (Odžak *et al.*, 2001).

TABLE I

Median, minimum and maximum values of the shell mass (g), dry whole soft tissue mass (g), and mass partition of MTs and metals (mg g^{-1} and $\mu g\ g^{-1}$, respectively, based on dry tissue mass, dm) in the heat-treated cytosol of the whole soft tissue of mussels *Mytilus galloprovincialis* Lmk. transplanted to four stations in Kaštela Bay (viz. Figure 1), comprising the period of 12 months

	Shell mass/ g	Whole soft tissue mass/ g dm	MT/ mg g^{-1} dm	Cd/ $\mu g\ g^{-1}$ dm	Zn/ $\mu g\ g^{-1}$ dm	Cu/ $\mu g\ g^{-1}$ dm	Mn/ $\mu g\ g^{-1}$ dm	Fe/ $\mu g\ g^{-1}$ dm
	Median Min-Max	Median Min-Max	Median Min-Max	Median Min-Max	Median Min-Max	Median Min-Max	Median Min-Max	Median Min-Max
Station A	5.94	0.41	5.69	1.98	32.21	5.23	4.39	15.84
	4.59–6.81	0.27–0.52	3.95–22.01	1.15–3.51	22.38–49.90	4.49–13.10	3.13–5.85	12.36–24.06
Station B	5.95	0.48	6.49	1.52	29.84	5.50	4.04	14.70
	4.20–8.04	0.28–0.82	5.30–13.40	0.73–2.33	13.90–44.95	2.73–9.01	3.29–6.80	10.04–21.01
Station C	5.74	0.40	7.57	1.85	32.87	5.81	4.73	20.34
	4.31–6.80	0.29–0.84	4.30–16.78	0.98–2.54	19.08–46.74	4.02–10.27	3.77–6.38	11.44–25.20
Station D	6.17	0.44	7.40	2.12	32.10	3.94	3.99	17.26
	4.29–6.76	0.27–0.48	5.67–18.36	1.20–2.91	23.80–57.00	3.14–7.33	3.86–5.30	9.89–19.54

3.2. BIOMETRIC PARAMETERS

Figure 2 is showing the temporal pattern of the shell mass and the dry mass of the whole soft mussel tissue at each deployment site (A, B, C and D). Evidently, the shell mass has increased during one year of observation, thus reflecting the ageing process of mussels, as was previously suggested by Fischer (1983). Dry whole soft tissue mass varies within the deployment period, but it has not increased after one year at any of the deployment sites. The whole soft tissue mass was the same, or sometimes even lower, at the end of the study period.

Linear regression analysis (Figure 3) showed that relationship between dry whole soft tissue mass and the shell mass ($R^2 = 0.12$, $p = 0.07$) is rather weak, as also implied by the graphs in Figure 2. The variations in the soft tissue mass are, thus, not strongly related to age. Some other factors, like difference in food availability and different phases of the reproductive cycle, probably play more important role in the whole soft tissue mass variability (Mourgaud *et al*., 2002).

According to Fischer (1983), in very large mussels *Mytilus edulis* (shell mass above 20 g) there is no more net growth of the soft body, while shell mass continues to increase. But, in the small (shell mass up to 5 g) and medium individuals (shell mass in the range from 5–20 g), soft body mass still increases, in the first case faster, and in the second slower than the shell mass (Fischer, 1983). Thus, when the mussels' shell masses are in the range from 4.5 to 8.0 g, as in our study, it is still

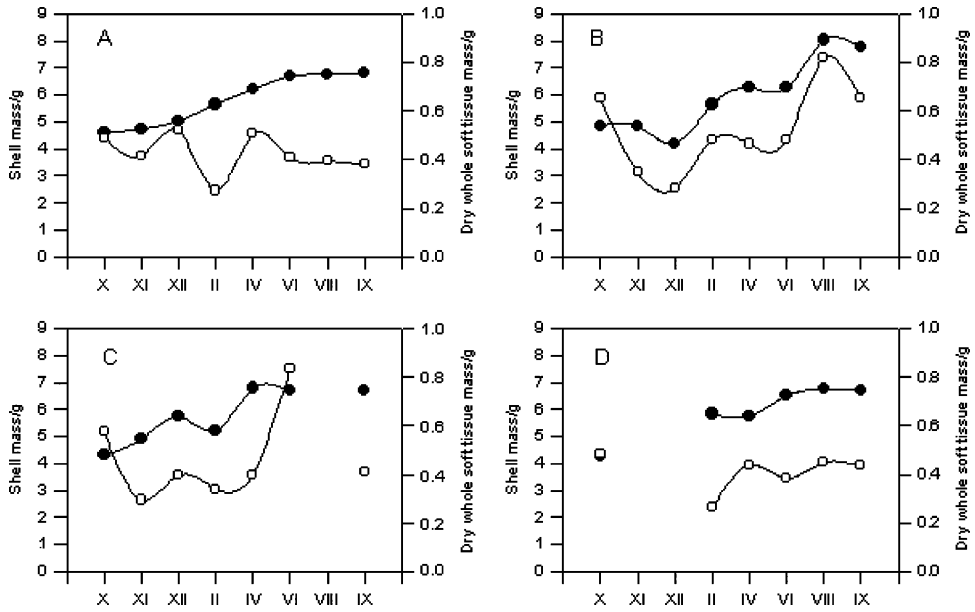

Figure 2. Temporal distribution of the shell mass and dry mass of the whole soft tissue of mussels *Mytilus galloprovincialis* Lmk. deployed at four sites (A, B, C, D) in the semi-enclosed Kaštela Bay over the period of one year. ● Shell mass; ○ Soft tissue mass.

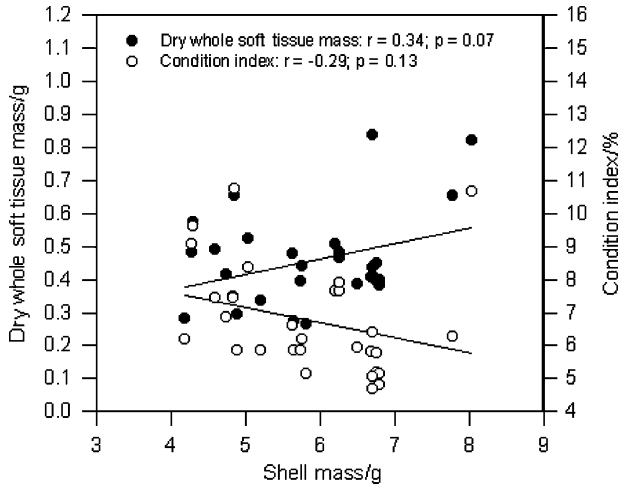

Figure 3. Linear regression graphs showing the influence of the shell mass on the dry whole soft tissue mass and condition index. Data from all four deployment sites were treated as one population of data. ● Dry whole soft tissue mass; ○ Condition index.

expected to observe the increase of soft tissue mass in relation to mussel's ageing. The absence of the increase after one year is probably caused by the reduced food availability as the result of cage fouling. Consequently, mussel condition deteriorated over the year, based on the negative correlation ($r = -0.29$; $p = 0.13$) of condition index with the shell mass (Figure 3). According to observations made by scuba-diver, who has been retrieving the samples, the cages were increasingly overgrown with algae over the time. This is not surprising since the Kaštela Bay is categorized as highly eutrophic area (Barić *et al.*, 1992). Thus, the cages with mussels that were sampled at the end of the study period, which have been deployed continuously in the seawater for a year, were heavily overgrown with algae at the collection time. The circulation of water through the cages was probably decreased due to the obstruction of slits with algal overgrowth, which would also cause lower food availability for mussels. This occurrence would eventually end up in the absence of expected somatic growth. Therefore, the conclusion could be made that the long-term caging (e.g. over one year) in eutrophic area is unfavourable for mussels. Otherwise, cleaning of the cages without the interruption of exposure should be organized. Also, the mass of the whole soft tissue should not be used as an indicator of mussel's age, especially for normalisation of contents of those metals that accumulate proportionally with mussel's age.

3.3. Influence of age and the changes of the whole soft tissue mass on metal and MT content

Figure 4 shows log-log regression graphs for cytosol based metal contents as a function of shell mass, as the indicator of mussel's age. Three of five analysed

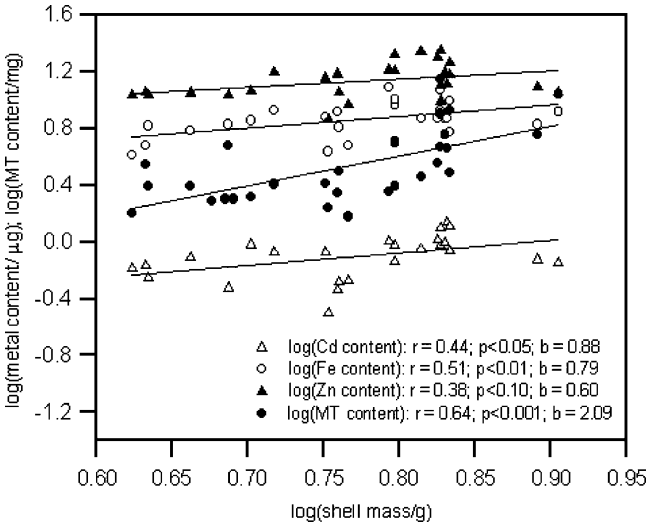

Figure 4. Log-log linear regression graphs showing the influence of the shell mass on metal (Cd, Fe, Zn) and MT contents in the heat-treated cytosol of the whole soft mussel tissue. Data from all four deployment sites were treated as one population of data. ● MT; ○ Fe, ▲ Zn, △ Cd.

metals correlate positively with the shell mass, Cd, Fe and Zn. The correlation of Cd and Fe with the shell mass is statistically significant ($p < 0.05$), and the slopes ($b = 0.88$ and 0.79, respectively) indicate to accumulation of these metals almost directly proportional to the age of the mussels. Zinc content correlates positively with the shell mass, but with the lower level of significance ($p < 0.10$). Lower slope ($b = 0.60$) indicates that this metal does not follow the intensity of Cd and Fe accumulation. Mn and Cu do not show a significant dependence on mussel's age. This time-dependence of metal accumulation is not the only explanation of metal variability, since data are widely dispersed around the regression graphs (see Figure 4). Abiotic factors, like metal concentration in ambient water, food or sediment, obviously, play an important role in metal uptake and accumulation, too. For example, it is well established that the absorption of Cd in the genus *Mytilus* is proportional to the exposure time and to its concentration in the environment (Cossa, 1988, 1989).

According to Boyden (1974), the whole soft tissue mass also has a strong influence on metal concentrations in many aquatic molluscs. The metal content is related to whole soft tissue mass by the metabolic power equation $Y = aW^b$, leading to metal mass partitions varying with the body mass according to $[Y] = aW^{b-1}$, where Y is metal content, [Y] is metal mass partition, W is the whole soft tissue mass, while after logarithmic transformation b and (b − 1) are slopes of regression graphs. In our study, statistically significant influence of the whole soft tissue mass was obtained in the case of Mn and Fe content ($r = 0.74$; $p < 0.0001$ and $r = 0.50$; $p < 0.01$, respectively) (Figure 5). The absence of the increase reported

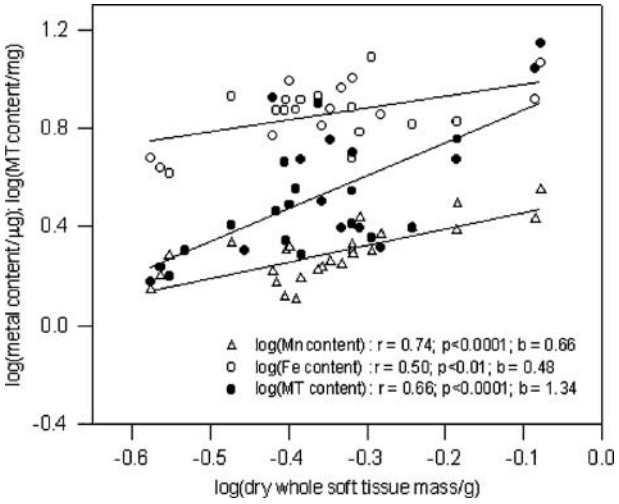

Figure 5. Log-log linear regression graphs showing the influence of the dry whole soft tissue mass on metal (Mn, Fe) and MT contents in the heat-treated cytosol of the whole soft mussel tissue. Data from all four deployment sites were treated as one population of data. ● MT, ○ Fe, △ Mn.

for the mass of the whole soft mussel tissue at the end of the deployment period was also noticed for Mn content. At some deployment sites even lower contents of Mn were obtained at the end of the monitoring period, compared to the values at the beginning. The slope of the log-log regression graph (b) for Mn equals to 0.66, and 0.48 for Fe, meaning that Mn and Fe contents do not increase proportionally with the increase of soft tissue mass. Relationship of metal content to body mass characterized with regression coefficient $b = 0.77$ (range found for different species and different elements was 0.67 to 0.85) implies a connection of metal content with the metabolism (Boyden, 1974), since many metabolic processes, such as oxygen consumption in a variety of bivalves, are characterised by dependance on the body mass by the power of $\sim$0.75 (Bourdelin, 1996). Thus, a conclusion can be made that Mn is probably metabolically regulated in the whole soft tissue of mussels *Mytilus galloprovincialis*. According to the coefficient of determination, as much as 55% of Mn variability can be explained by the changes of the whole soft tissue mass. Other metals known as MT inducers, i.e. Cd, Zn and Cu, do not exhibit dependence on the changes of the whole soft tissue mass.

Following the similar idea for the metallothioneins, the regression of MT content versus whole soft tissue mass could be examined using the same metabolic equation as for metal contents (Bordin *et al*., 1997). Positive correlation of MT content with the whole soft tissue mass ($r = 0.66$; $p < 0.0001$; Figure 5) implies that MT content is strongly influenced by the changes of the whole soft tissue mass. The regression slope of 1.34 indicates that, contrary to metals, not only MT content, but even mass partition of MTs increases with increasing body mass. It is true that MT is a cellular

ligand whose primary function is in homeostasis and detoxification of metals, but it is also a protein, and it may be assumed that any factor which affects general protein metabolism may also affect MT level. For example, the higher food availability in the environment, which enhances somatic growth and, thereby, also the synthesis of total proteins, would be expected to affect the quantity of MTs, too (Mourgaud *et al.*, 2002).

3.4. PRINCIPAL COMPONENT ANALYSIS

Three principal components were extracted by the principal component analysis of following parameters: the shell mass, the condition index, and the contents of five metals and MTs. The first principal component accounts for 35.7%, the second one 21.6%, and the third one for 14.8% of data variability (Table II). Strong correlation of shell mass with the first component (0.74) allowed us to define it as a component associated to mussel's age. The second component highly correlates with the condition index (0.86), and therefore it was designated as a condition component. Third component is associated with the changes of Cu content.

The "age" component encompasses metals Cd, Zn and Fe (coefficients: 0.80, 0.76 and 0.78, respectively), as was previously established by the log-log regression analysis (Figure 4). Same as these three metals, MT content also increases with mussel's age, which is evident from its high correlation with the first principal

TABLE II

Parameters variability (shell mass, condition index, trace metal and MT contents) resolved in three principal components, by Varimax rotation method and Kaiser normalisation. Cumulative % of variance amounts to 72.1%. First component (35.7% of variance) is associated to the mussel's age, the second one (21.6% of variance) to mussels' condition, while third one accounts for 14.8% of variance, and is associated to the changes of Cu content

	Correlation coefficients		
	Component 1	Component 2	Component 3
Shell mass (g)	**0.74**	−0.15	0.11
Condition index (%)	−0.24	**0.86**	−0.24
Cd (μg)	**0.80**	−0.25	0
Zn (μg)	**0.76**	−0.05	−0.03
Cu (μg)	0.06	0.03	**0.95**
Mn (μg)	0.12	**0.85**	**0.30**
Fe (μg)	**0.78**	0.22	0.09
MT (mg)	**0.65**	**0.38**	**0.32**
% of variance	35.7	21.6	14.8

component (coefficient: 0.65), and also with the shell mass ($r = 0.64$, $p < 0.001$; Figure 4). The second component, i.e. the "condition" component, comprises Mn (coefficient: 0.85), as a metal strongly connected to nutritional and reproductive status of mussels, as stated above. The level of Mn is positively influenced by the gonadal maturation (Frias-Espericueta *et al*., 1999). It was suggested by Paez-Osuna *et al*. (1995) that Mn plays an important role during gametogenesis, possibly as an enzymatic catalyst. Thus, almost identical changes of Mn content and the whole soft tissue mass over the time, observed at all deployment sites, make Mn a good indicator of mussel's condition. MTs also exhibited correlation with this component, although somewhat weaker (coefficient: 0.38), showing that MT level in the whole soft tissue is also affected by mussel's condition.

Third component highly correlates with the copper content (coefficient: 0.95), and it can be assumed that this component reflects some unknown factor causing variations of Cu level. According to our results, Cu content is not influenced by mussel biometry. But, some previous investigations indicate that Cu level in the mussel tissues is related to Cu level in the marine sediment (Odžak *et al*., 2001). Furthermore, Langston (1986) showed that concentrations of Cu in the estuarine benthic organisms exhibited a concentration gradient similar to that of Cu concentrations in sediments. Wright and Zamuda (1987) observed a significant association between the accumulation of Cu by oysters and clams and its concentrations in the sediments. Weaker connection with this component is also exhibited by Mn (coefficient: 0.30) and MTs (coefficient: 0.32).

High percentage of MT variability can be explained through the changes of the shell and dry soft tissue masses (according to multiple regression analysis, approximately 65%). Nevertheless, cadmium, as toxic metal and known MT inducer, also exhibits a positive, statistically significant correlation ($r = 0.48$; $p < 0.05$) with MT content (Figure 6). As indicated above (Figure 4), both Cd and MT contents positively correlate with mussel's age, i.e. the shell mass ($r = 0.44$, $p < 0.05$ and $r = 0.64$, $p < 0.001$, respectively), and have increased during the year. The accumulation of Cd takes place simultaneously with the enhanced synthesis of MTs. Thus, it could be either a cause of MT induction, or a consequence of the MT increase due to the ageing of mussels. Since the results obtained in this study indicate that the biometry was a predominant factor influencing the MT content, it may be concluded that Cd accumulation is a consequence of the age-induced MT synthesis, which increases Cd biological half-life time. It is known that metal accumulation is enhanced by the synthesis of metal-binding proteins, like MTs (Dallinger, 1995; Langston *et al*., 1998). On the other hand, MT mass partition obtained in this study (median value approximately 5.5–7.5 mg g^{-1}) was twice as high as the MT mass partition (2–3 mg g^{-1}) reported by Bebianno and Langston (1992) as typical for the whole soft tissue of mussels *Mytilus galloprovincialis* collected at sites free from cadmium pollution. Furthermore, maximal mass partitions of MTs in summer months at different sites in Kaštela Bay were from 13–22 mg g^{-1}, which is even higher than reported by Bebianno and Langston (1992) (12 mg g^{-1}) in the experiment with

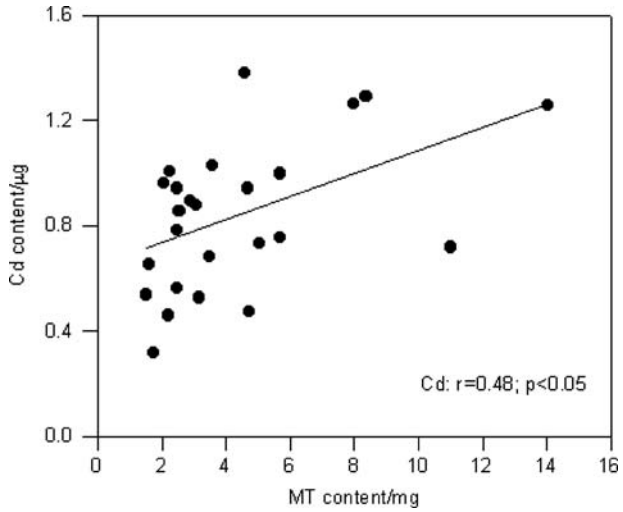

Figure 6. Linear regression graph showing the dependence between Cd and MT contents in the heat-treated cytosol of the whole soft mussel tissue. Data from all four deployment sites were treated as one population of data.

mussels exposed for 40 days to sublethal Cd level of 400 μg L^{-1}. Therefore, it is not likely that this high level of MTs is just a consequence of variations in soft tissue mass, especially taking in concern that overall increase of the whole soft tissue mass after one year was not registered. The whole soft tissue encompasses different organs with various biochemical functions. For example, in gill tissue low levels of accumulated Cd can cause an increase of MTs (Dragun *et al*., 2004), while in the digestive gland of different bivalve species Cd accumulation to a certain extent does not cause corresponding increase of MT level (Bebianno *et al*., 1993; Geffard *et al*., 2001; Raspor *et al*., 2004). Furthermore, the lower concentrations of trace metals were reported for gonadic tissue in comparison with the other mussel tissues (George and Coombs, 1977; La Touche and Mix, 1982; Lobel and Wright, 1982). The combined effect of different biochemical responses in different tissues leads to weaker relationship ($R^2 = 0.23$, $p < 0.05$) between MTs and Cd in the whole soft tissue. Nevertheless, this connection exists and indicates to a possibility of MT increase as a result of Cd accumulation in some part of the whole soft tissue, which contribution is partially masked by the absence of MT induction in some other tissue. Based on these findings, the whole soft tissue is not considered as the best choice for measuring MTs as a biomarker of metal exposure. Better option would be to isolate a specific tissue that shows indisputable connection between MT induction and metal accumulation. Those tissues that, due to high basal MT level, are able to detoxify sublethal levels of accumulated metals without a need for further induction should be eliminated (Raspor *et al*., 2004), as well as those that do not accumulate metals. Gills are already proven to be a good tissue for measurement

of MT level as a biomarker of metal exposure in different bivalve species (Bebianno *et al*., 1993; Geffard *et al*., 2002; Dragun *et al*., 2004). But, some other tissues, like mantle, that showed marked MT induction after laboratory exposure to Cd (Pavičić *et al*., 1991), also deserve further investigation.

4. Conclusions

The mass of the whole soft tissue of medium sized mussels is still expected to increase in the course of one year. Comparison of the soft tissue mass at the beginning and at the end of our caging experiment has not exhibited increment in the soft tissue mass. Consequently, condition index has decreased after one year of caging. Therefore, the long-term caging is not favourable for mussels, due to the reduced food availability as the result of obstruction of slits on cages with algal overgrowth.

The analysis of metal dependence on biometric parameters lead to following conclusions: three metals (Cd, Fe, Zn) correlate positively with mussel's age, meaning that their contents have increased over the period of one year; the content of manganese is strongly influenced by the changes of the whole soft tissue mass; this metal, thus, presents a good indicator of mussel's condition; and, Cu content, as opposed to other analyzed metals, does not exhibit connection with biometry.

MT content in the heat-tretaed cytosol of the whole soft mussel tissue has increased with mussel's age, but it was, at the same time, influenced by mussel's condition. Strong influence of biometry on MT level in the whole organism is a cause of a problem when interpreting the connection between metals and MTs. Consequently, it is not considered as a good choice to measure MTs as a biomarker of metal exposure in the whole soft tissue of mussels.

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