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Evaluation of Metallothioneins in Blue Mussels (Mytilus galloprovincialis) as a Biomarker of Mercury and Cadmium Exposure in the Slovenian waters (Gulf of Trieste): A Long-term Field Study

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In order to assess the spatial distribution and temporal trends of pollution with metals in the coastal sea of Slovenia, the level of metallothioneins (MT) was determined in blue mussels from three sampling locations, twice per year for a period of 10 years. MT concentrations ranged from 45 to 163 μg g^{-1} w.w. in March, and from 46 to 144 μg g^{-1} w.w. in the September sampling. The metals content was determined in whole mussel tissue once per year at two stations. The cadmium (Cd) concentration was in the range from 0.50 to 1.11 mg kg^{-1}, while the concentration of mercury (Hg) was in the range from 0.70 to 0.237 mg kg^{-1}. Results revealed no significant seasonal differences or differences between sampling sites in MT content, as well as variations in the content of Cd and Hg in mussels' tissue during the examined period. The MT and metal content in mussels are not well correlated and other factors may have caused the fluctuations observed in MT content between years. This supports the notion that biomonitoring data have to be interpreted cautiously, taking into account various other factors that may influence MT induction.

Key words: metallothioneins, metals, *Mytilus galloprovincialis*, biomonitoring, field study, north Adriatic

INTRODUCTION

High anthropogenic pressures (pollution, exploration of natural resources, building, etc.) in the marine environment demand human monitoring. In recent decades European environmental monitoring programs have become more and more sophisticated and comprehensive, culminating with the Water Framework Directive (WFD, 2000/60/EC9) which integrated chemical, biological, physico-chemical, hydro-morphological parameters and ecological status in defining water quality (VINCENT *et al*., 2002). However, the effect of pollutants on biological systems at all levels is still neglected in the WFD. Biomarkers, in their strictest sense are defined as "a xenobiotically induced variation in cellular or biochemical components, and in processes, structures, or functions that are measurable in a biological

system or sample" (NATIONAL RESEARCH COUNCIL, 1987). These biomarkers have been incorporated in several regional programs of biomonitoring carried out under international bodies (UNEP and OSPAR).

Pollution problems and anthropogenic pressures are particularly pronounced in enclosed seas such as the Adriatic Sea. Its northernmost part encompasses the Gulf of Trieste, a shallow marine basin with heavily populated coasts and an industrialized area with many different anthropogenic pressures (construction, maritime, transport, fishing, aquaculture, etc.), and whose southern part is widely open to the rest of the Adriatic Sea. The circulation of water masses depends on weather conditions (strong winds and precipitation), river inflow and the circulation of water masses of the northern Adriatic Sea. The spreading of effluents from a municipal wastewater treatment plant under the influence of two different, but common winds (bora and sirocco) has been studied (MALAČIČ & MOZETIČ, 2005).

The coastal area of Slovenia has been exposed to strong development pressure as shown in the rapid growth of population in recent decades (105,632 permanent inhabitants in 2005, SURS http://www.stat.si/eng/index.asp), town planning and the development of business sectors (traffic, trade, tourism, processing activities and agriculture in the hinterland). The principal industries in the coastal region include metal manufacturing, the production of chemicals and the food industry. However, most of these industries have declined in the last decade.

The main pollution inputs along the Slovenian coast during the '90s were evaluated (TURK & POTOČNIK, 2001) and their effects resulted mainly in eutrophication (reviewed in TURK *et al*., 2007). Discharges from industry, agriculture and municipal sewage plants (partially treated industrial and urban wastewater) are brought by rivers (Rižana, Badaševica and Dragonja) and a few smaller springs. Discharge by rivers along the eastern and southern coastlines is minor in comparison with the western coastline (MALAČIČ *et al*., 2006).

Several studies were devoted to investigating the extent of pollution by metals and organic compounds in the Gulf of Trieste, as well as in the entire Adriatic Sea. Pollution with organochlorine pesticides, PCBs and anionic detergents were localised to the inner part of the Bay of Koper (PLANINC *et al*., 1993). The composition, distribution and sources of polycyclic aromatic hydrocarbons (PAHs) were investigated along the Slovenian coast (BAJT, 2007) and throughout the Gulf of Trieste (NOTAR *et al*., 2001). From the results of these investigations, it was concluded that sediment in the Gulf of Trieste is low to moderately contaminated with polycyclic aromatic hydrocarbons.

Contamination of selected organisms, bottom sediments and water with metals was studied from the '70s, and revealed rather low metal pollution, except for mercury (Hg), lead (Pb) and organotin compounds (ŠČANČAR *et al*., 2007). Mercury distribution and it biogeochemistry have been studied in detail (HORVAT *et al*., 1999; COVELLI *et al*., 2006; FOUCHER *et al*., 2009). The major source of inorganic Hg in the Gulf of Trieste is still the river Soča due to the mining of cinnabar ore in the past (the mining was abandoned in 1977). Moreover, the river Soča drains Hg into the western part of the Gulf of Trieste - approx. 1.5 tonnes of particulate Hg and 0.14 tonnes of dissolved Hg per year (RAJAR *et al*., 2000). These studies have shown that the concentrations of the total Hg were up to 10-fold higher than those found in the central and southern Adriatic Sea (HORVAT *et al*., 2001). Higher concentrations of Hg in the bottom water layers were the consequence of sediment resuspension (FAGANELI *et al*., 2003). The gross flux of liquid emissions of metals from rivers were for nickel (Ni, 1.5t/year), while the gross flux of other metals was much smaller. The Bay of Koper is more burdened than others bays, mostly by Ni, zinc (Zn), copper (Cu), Pb, chromium (Cr) and Hg (TURK & POTOČNIK, 2001; GOSAR & MURI, 2005).

The first attempts to establish a National Biomonitoring Programme in Slovenian coastal waters started in the framework of the UNEP/MAP MEDPOL programme in 1999, with the aim of fulfilling the obligations set out in

the Barcelona Convention. According to the concern over Hg pollution in the discussed area, metallothioneins were included in biomonitoring from the very beginning.

Metallothioneins (MT) are low molecular weight proteins, with most of their amino acids being cysteines residues and of extraordinarily high metal content. Another characteristic is their ability to be induced by metals and many other metabolites e.g. glucocorticoids, catecholamines, progesterone, estrogene, etc. Metallothionein induction is an indicator of exposure to metal pollution, especially to cadmium (Cd), mercury (Hg), silver (Ag), gold (Au), cobalt (Co), nickel (Ni), bismuth (Bi), and perhaps copper (Cu) and different metal mixtures (KÄGI, 1991, BEBIANNO & SERAFIM, 1998; MOURGAUD *et al*., 2002). MT induction has been observed in populations of living organisms from polluted areas or in those that have been exposed to different metals in laboratory trials. More recently, their induction is widely used in biomonitoring programmes such as OSPAR, UNEP/MAP and Mussel Watch Programme (UNEP/RAMOGE, 1999; CARAJAVILLE *et al*., 2000; AMIARD *et al*., 2006). In a polluted environment, metals or their mixtures such as Cd and Hg bind to MT by displacing Zn from existing Zn-thioneins in the cells, and new synthesis occurs when there is a saturation of existing MT. Significant correlations between metals (Cd, Cu and Zn) and MT concentrations in bivalve molluscs were found in laboratory studies and field experiments (AMIARD *et al*., 2006 and references therein). Conversely, there are no biochemical interactions between some metals (arsenic (As), chromium (Cr), and Pb) and MT (MORGAUD *et al*., 2002).

MTs have been detected in different tissues in blue mussels but the use of the digestive gland alone in blue mussels reduced the effects of seasonal changes in weight associated with the ripening of gametes (MOURGAUD *et al*., 2002; RASPOR *et al*., 2004), and eliminated interferences between physiological factors and metal contamination, according to the concept of the signal-to-noise ratio proposed by CAIRNS (1992). The changes in MT concentration after metal exposure were significantly higher than MT level changes due to non-metallic factors (GEFFARD *et al*., 2001; COSSON, 2000; AMIARD *et al*., 2006). The importance of these proteins as a protecting agent against metal cytotoxicity was confirmed by research conducted on lysosomal membrane stability (VIARENGO *et al*., 1985a).

The aim of the present study is to determine spatial and temporal differences in the MT level of blue mussels from different sampling sites along the Slovenian coast over a period of 10 years. Moreover, the results of this MT biomonitoring are discussed with other available results, using the Mediterranean scale for comparison.

MATERIALS AND METHODS

Sampling sites

Three sites along the Slovenian coastline were included in the biomonitoring programme (Fig. 1). One station in the Bay of Koper (Station 1) is located in the vicinity of the marina and the Port of Koper. The marina has 75 moorings and 30 places for vessels up to 18 m on land. The municipal port of Koper has 194 anchoring sites. In 2008 the Port of Koper surpassed more than 16 million tonnes of cargo. Most of the cargo is declared as dry bulk cargo (7 million tonnes in 2008), while 2 million tonnes of liquid cargo mostly comprises of chemicals, minerals and vegetable oils. The total cargo transfer increased from year 2000 from 9 million tonnes to 16 million tonnes in 2008 (http://www.luka-kp.si/eng/). The city of Koper has 24,658 inhabitants. The main discharges from industry, municipal sewage plants and agriculture were cleaned mechanically and then released to the Rižana River.

In the Bay of Strunjan (Station 2) there is a blue mussel farm, and part of the bay has been declared as a natural reserve (the central area is forbidden for anchoring). Besides a blue mussel farm, there is tourist resort with a beach for swimming and permanent anchoring for dozens of boats. The local point source of pollution is a creek, Strunjanski potok, which flows alongside the main road and drains waters

Fig. 1. A map of the Gulf of Trieste with stations included in the biomonitoring programme with geographical coordinates: Station 1: 13° 43,36' N, 45° 33,00 E; Station 2: 13° 35,40' N, 45° 31,93' E; Station 3: 13° 35,00' N, 45° 29,40' E

from local agriculture. The impact of this inflow is significant after a rainy period; otherwise the stream flow is quite small. Station 2 is 11.4 km away from Station 1, and 9.2 km away from Station 3. The content of Cd in the sediment was in the range from 0.155 mg/kg to 0.112 mg/kg, and in the sea water was from 2.3 μg/L to <0.1 μg/L. Moreover, the level of Hg in the sediment was in the range from 0.533 mg/kg to 0.15 mg/kg, and in the sea water from 0.4 μg/L to 0,00109 μg/L (ARSO, 2010).

The coastline of the Bay of Piran (Station 3) is surrounded by many tourist resorts on both sides of the bay. The river Dragonja flows into the Bay of Piran and is loaded with agricultural and domestic wastes from the hinterland. A blue mussel and fish farm is situated in the inner part of the bay. This station has similar content of Cd and Hg in the sediment and in the sea water. The Cd content in the sediment was in the range from 0.157 mg/kg to 0.11 mg/kg, and in the sea water from 3.2 μg/L to <0.1 μg/L. Hg in the sediment was in the range from 0.136 mg/kg to 0.095 mg/kg, while the sea water had a content of Hg from 0.1 μg/L to 0.00045 μg/L. The concentration of metals was measured in non-filtered sea water and in sediments using atomic absorption spectrometry (ARSO, 2010).

Blue mussels, *Mytillus galloprovincialis* Lam. were collected from the previously described sites (Fig. 1.) along the Slovenian coast twice a year (in March and September), from September 1999 to September 2008.

Blue mussels from Station 1 were collected from their natural beds between the Koper marina and that of the Port of Koper. Blue mussels from Station 2 and Station 3 were collected from a blue mussel farm. The size of collected blue mussels was from 4 to 6 cm, where anteroposterior (length of shell) and dorsoventral length (width of shell) were measured. Concentrations of Cd and Hg were measured once a year in blue mussels collected in September at Station 1 and Station 2. The temperature of the sea water, salinity, and oxygen saturation were measured at a depth of 2 m at all stations (Table 1).

A condition index was calculated as the ratio between wet mass of whole mussel and shell length of the individual mussels used in the MT analyses. The water content was calculated as the difference between wet tissue weight and dried tissue weight, after drying in a lyophilizer.

Metallothionein determination

Digestive glands from 10 blue mussels were pooled in a single sample that was homogenised and five samples from each station were used to measure the MT concentrations and reduced glutathione was used as a standard. Concentrations were expressed as micrograms of metallothionein per gram of tissue (wet weight). The metallothionein content was analysed according to the method of VIARENGO *et al.*, (1997) and the laboratory procedure was strictly followed as described previously.

Determination of the concentrations of Cd and Hg in blue mussel samples

All analyses of the concentrations of Cd and Hg in blue mussel samples and reference

		March						September					
Name of station	Year of sampling	MT (µg/gw.w) mean± SD	Mean length (mm)	CI (wet mass/shell length)	O_2 mg/L	T of sea water (°C)	Salinity PSU	MT (µg/gw.w) mean+- SD	Mean length (mm+-SD)	CI (wet mass/shell length)	O_2 mg/L	T of sea water (°C)	Salinity PSU
Station 1	1999*	ND	ND	ND	ND	ND	ND	92±23	54±2	0.293±0.039	6.35	23	37.6
	2000*	102±7	60±4	ND	9.9	10	37.4	114±12	48±4	ND	6.2	23	37.3
	2001	102±10	64±3	0.329±0.051	7.7	11	37.0	81±11	60±4	0.329±0.053	7.0	22	36.3
	2002	145±10	54±4	0.234±0.049	7.6	10	37.8	111±5	49±4	0.212±0.033	7.3	23	38.2
	2003	93±4	54±4	0.232±0.031	8.5	9	38.0	61±13	43±4	0.132±0.036	7.3	21	38.2
	2004	53±7	49±2	0.134±0.021	9.2	10	37.8	46±7	56±2	0.217±0.031	4.8	23	37.3
	2005	149±5	60±2	0.223±0.027	6.4	9	38.1	100±7	56±1	0.284±0.035	5.3	24	36.8
	2006	101±10	55±2	0.210±0.032	7.5	8	38.1	54±8	56±1	0.241±0.033	6.9	23	37.0
	2007	68±11	53±2	0.182±0.026	8.9	12	37.4	100±4	55±3	0.243±0.032	6.8	21	36.3
	2008	125±14	57±2	0.197±0.039	8.5	9	37.8	135±16	57±2	0.273±0.040	6.4	25	37.4
Station 2 (reference site)	1999*	ND	ND	ND	ND	ND	ND	70±10	56±2	0.301±0.060	6.3	23	36.5
	2000	121±13	65±3	ND	9.8	9	37.8	115±12	52±5	ND	6.9	22	37.0
	2001	66±17	56±3	0.207±0.025	7.3	10	37.0	64±22	55±4	0.192±0.028	6.9	21	36.7
	2002	120±7	59±5	0.207±0.153	9.1	9	37.6	107±5	53±5	0.181±0.031	7.5	22	37.4
	2003	81±7	59±5	0.228±0.064	8.6	9	38.0	83±10	51±5	0.180±0.058	7.3	21	38.2
	2004	66±14	56±3	0.139±0.016	9.5	10	37.6	53±5	58±2	0.191±0.022	4.7	23	37.5
	2005	163±11	58±2	0.149±0.016	6.4	9	38.1	113±13	58±2	0.274±0.064	5.3	24	36.9
	2006	78±5	57±2	0.148±0.021	7.2	7	37.9	87±25	55±2	0.174±0.017	7.3	24	37.3
	2007	64±6	60±3	0.170±0.031	8.7	12	37.4	103±13	65±3	0.224±0.030	6.9	21	38.0
	2008	123±24	57±2	0.160±0.020	8.3	8	37.7	144±13	58±2	0.167±0.024	6.4	25	37.2
Station 3	1999*	ND	ND	ND	ND	ND	ND	78±11	55±2	0.286±0.044	6.3	23	36.6
	2000	104±6	45±3	ND	9.8	10	37.2	138±57	48±3	ND	6.9	22	37.5
	2001	97±6	59±4	0.192±0.040	7.1	12	36.6	83±4	55±3	0.186±0.035	7.0	22	36.2
	2002	115±5	52±4	0.165±0.026	6.9	10	37.9	113±7	58±4	0.178±0.040	7.5	23	38.2
	2003	85±8	64±4	0.232±0.033	8.8	10	37.9	88±11	56±4	0.155±0.015	7.1	21	38.2
	2004	45±9	54±2	0.133±0.018	9.9	11	37.5	47±4	64±2	0.249±0.028	4.7	24	37.5
	2005	150±13	61±2	0.186±0.027	6.6	9	38.1	118±21	64±2	0.250±0.050	4.8	24	36.9
	2006	105±28	65±2	0.221±0.031	6.9	8	38.0	69±24	60±2	0.153±0.015	6.9	23	37.3
	2007	77±13	51±2	0.123±0.018	9.2	12	37.6	108±7	56±2	0.173±0.018	6.6	21	37.9
	2008	126±25	59±2	0.205±0.038	8.3	8	37.5	117±7	63±3	0.198±0.027	6.1	25	37.2

Table 1. Comparison of mean MT concentrations, mean length of blue mussel, temperature, salinity, and dissolved oxygen in seawater from three stations along the Slovenian coast in the period from 1999 to 2008. ND = not done

materials were performed in triplicate, with each sample consisting of 25 pooled mussels. The concentration of Cd and Hg was measured in the whole blue mussel tissue. Total Hg was analysed by cold vapour atomic absorption spectrometry (CV AAS). Aliquots (100-200 mg) of the representative samples were weighed directly in a Pyrex digestion vessel and, after addition of 2 mL of concentrated HNO_3, the vessel was sealed and the mixture was left to react at room temperature overnight. Digestion was finished by heating in an aluminium block at 70°C for 12 hours. The tubes were then cooled in liquid nitrogen and opened. The digest was transferred to a 50 mL volumetric flask and diluted with doubly distilled water to the mark. An aliquot of the digest was added to the reduction vessel and after reduction with $SnCl_2$, Hg was swept from the solution by aeration and concentrated on a gold trap. Hg was then released from the gold trap by heating and measured on an LDC Milton Roy instrument by CV AAS. A detailed description of the method can be found elsewhere (HORVAT *et al*., 1986; HORVAT *et al*., 1991). The detection limit of the procedure is 0.2 ng Hg mL^{-1}. The precision varies from 2 to 5%.

Other aliquots of the representative samples were used for the determination of Cd in blue mussel tissue. Approximately 1 g of moist blue mussel tissue was weighed into a Teflon beaker. Afterwards 4 mL of HNO_3 (s.p.) was added and the sample was subjected to closed vessel microwave digestion at maximal power of 1200 W: 20 min ramp to temperature of 180°C, pressure 10 bar, hold 20 min, cooling 20 min. The clear solution was quantitatively transferred into 25 mL polyethylene graduated tubes and filled to the mark with water doubly distilled in quartz. The same procedure (acid without sample) was applied for the blank sample. The Cd was determined by electrothermal atomic absorption spectrometry (ETAAS) under optimal measurement conditions using Zeeman background correction.

The accuracy of determination of total Cd and Hg concentrations was controlled by the analysis of NIST standard reference materials NIST 2977 (Mussel Tissue) and SRM 1566 b (Oyster tissue), respectively. The results of analyses indicated a stable measurement process and accurate data for both metals analysed in SRMs.

Data analysis

The results of MT concentration in digestive glands in blue mussels are presented as the means of three replicates from the five pooled samples (+/-standard deviation of concentration). The statistical differences in MT concentrations among samples from three sites and during years were tested by the analysis of variance (three factorial ANOVA, Tukey *post-hoc* HSD ($p<0.05$)) or less considered statistically significant). Similarly, concentrations of Cd and Hg were tested in different years and at both stations by the analyses of variance.

RESULTS

Spatial and temporal differences in MT concentrations in blue mussels

Concentrations of MT were represented by station and according to sampling season in either March or September. Table 1 shows mean MT concentrations in samples from each station together with abiotic parameters (sea temperature, salinity and oxygen content). MT concentrations in blue mussels collected from Station 1 were lowest in March 2004 (53 μg/g w.w.) and highest (149 μg/g w.w.) in 2005 in the same season. The measured concentrations of MTs in blue mussels from Station 1 each September spanned from 46 μg/g w.w. to 135 μg/g w.w., with the lowest value recorded in 2004 and the highest one in 2008. Further, MT concentrations from blue mussels originating from Station 2 in the Bay of Strunjan ranged from 64 μg/g w.w. to 163 μg/g w.w. In September values ranged from 53 μg/g w.w. to 144 μg/g w.w in 2004 and 2008, respectively.

At Station 3 in the Bay of Piran concentrations were from 45 μg/g w.w. to 150 μg/g w.w. in March in 2004 and 2005, respectively. In September they ranged from 47 μg/g w.w. to 138 μg/g w.w. in 2004 and 2000, respectively. Noteworthy, the lowest MT value was recorded

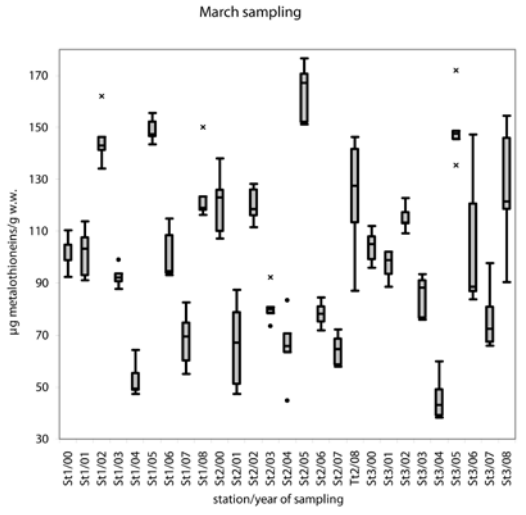
in 2004, regardless of sampling station and season.

In addition, abiotic factors were measured at the sampling stations: the temperature of the surrounding water, salinity and oxygen at the time of sampling. During winter (March) the temperature and salinity were rather uniform, and no substantial deviations were found over the entire period of 10 years. The water temperature spanned from 7 to 12°C from 2000 to 2008, the salinity ranged from 36.6 PSU to 38.1 PSU and the dissolved oxygen was from 6.4 mg/L to 9.9 mg/L. The values of parameters were similar between stations during the sampled season. During September the temperature lay in the interval from 21 to 25°C, the salinity ranged from 36.3 to 38.2 PSU and the dissolved oxygen ranged from 4.7 to 7.5 mg/L. In March 2005 the MT concentrations were the highest in the entire period of biomonitoring at all stations, with seawater O_2 content ranging from 6.4 mg/L to 6.6 mg/L and a high salinity of 38.1 PSU.

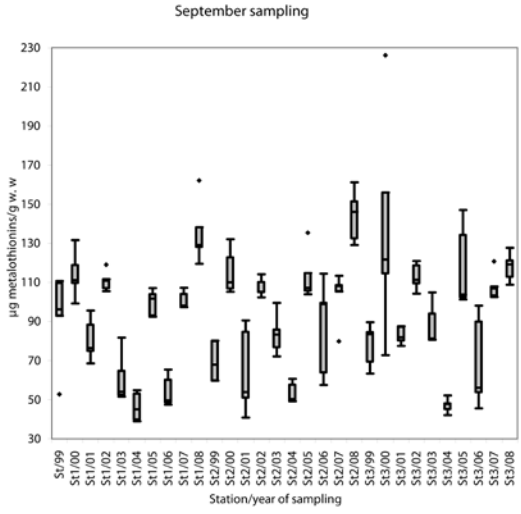
The CI of blue mussels collected in the March sampling were the lowest during the entire 10 year period (value at Station 3 was 0.123 and 0.139 at Station 2). This particular year was characterised by high O_2 content, ranging from 9.2 mg/L to 9.9 mg/L in March, and low O_2 content, ranging from 4.7 to 4.8 mg/L at all stations in September. This was the lowest O_2 content measured in the entire period of biomonitoring.

When we tested differences in MT concentrations between sampling stations we did not find any significant differences between the three stations. Apart from a few cases, no significant seasonal differences were found when MT concentrations were tested in the same year and station. The most significant differences in the MT concentrations were found among consecutive years at the same station, and within the sampling period (see Fig. 2).

Comparison between years and sampling seasons at Station 1 revealed that the most prominent years were: 2002 with high MT concentrations in both seasons; 2004 with very low concentrations of MT (again in both seasons), and 2008 again with high MT concentrations. Comparison at Station 2 highlighted 2001 and 2004 with low MT concentrations in both seasons, and 2008 with higher MT concentrations in both seasons (as well as concentrations of Cd and Hg). At Station 3 2000, 2002 and 2005 were significant years with high MT concentrations in both seasons,



a) sampling in March.



b) sampling in September

Fig. 2. Box plot graphs indicating metallothionein concentrations in blue mussels at three stations during the period from 1999 to 2008. Boxes represent the interquartile range; line inside the boxes: median; small diamonds: extremely high and low values, respectively

and 2004 with low MT concentration in both seasons.

Spatial and temporal differences in Cd and Hg concentrations in blue mussels

Concentrations of Cd and Hg were measured in a composite sample of 25 blue mussels collected every September at Station 1 and Station 2 in three replicates. The water content in blue mussels was calculated and the concentration of metals was given per dry weight (Table 2). The data about concentrations of some other metals (Pb, Ni, Cr, Zn and Cu) measured in the whole mussel tissue were available only from spring 2003 at Stations 2 and 3 (Table 3). Concentrations of Cr, Ni, Pb, and Zn were higher in blue mussels collected at Station 2 while only the concentration of Cu was higher at Station 3. The concentrations of Cd at Station 1 ranged from 0.60 ± 0.01 mg kg^{-1} to 1.11 ± 0.04 mg kg^{-1}. Comparison between years at Station 1 revealed significantly higher concentrations of Cd in 2000 and 2008 with respect to other years. The lowest concentrations were from 2003 to 2006.

At Station 2 concentrations of Cd in blue mussels ranged from 0.50 ± 0.02 to 1.09 ± 0.03 mg kg^{-1}. Comparisons of Cd concentrations at both Stations 1 and 2 revealed that the highest concentrations of Cd were in 2000 and 2008, while the lowest concentrations of Cd were in 2005.

Statistical analyses between stations and years revealed that concentrations of Cd at Station 1 were significantly lower than those measured in specimens from Station 2 in years 2002 or 2001, 2003, 2004 and 2006. Meanwhile, significantly lower concentrations were found at Station 2 in comparison to Station 1 in 2002 and 2007. Concentrations of Hg at Station 1 were in the range from 0.070 mg kg^{-1} ± 0.003 to 0.130 mg kg^{-1} ± 0.005. Statistical analysis of data obtained from mussels collected from Station 1 revealed that Hg concentration lay at its lowest value in 2001 (0.070 mg kg^{-1}), and the highest value (0.130 mg kg^{-1}) was noted in 2002 and 2004. The concentrations of Hg in mussels from Station 2 during the same period ranged from 0.084 to 0.237 mg kg^{-1}. The samples collected in 2000 showed the highest concentration of Hg while the lowest value was again recorded in 2001. Comparison between stations revealed that Station 2 was more polluted by Hg than Station 1, although this does not apply for years 2002 and 2004.

The concentrations of Hg at Station 2 during the examined period were in the range from 0.084 mg kg^{-1} ± 0.006 to 0.237 mg kg^{-1} ± 0.007. The samples collected in 2000 revealed significantly higher concentrations of Hg at Station 2 compared to other years of sampling at

	Station 1			Station 2		
Year of sampling	Cd (mg kg^{-1}) mean± SD	Hg (mg kg^{-1}) mean± SD	Mean length (mm±SD)	Cd (mg kg^{-1}) mean± SD	Hg (mg kg^{-1}) mean± SD	Mean length (mm±SD)
2000	1.11±0.04	0.116±0.013	55±3	1.04±0.04	0.237±0.007	50±2
2001	0.78±0.04	0.070±0.003	59±3	0.96±0.04	0.084±0.006	52±3
2002	0.98±0.02	0.130±0.013	55±5	0.66±0.02	0.110±0.004	51±3
2003	0.60±0.01	0.111±0.008	40±2	1.04±0.01	0.142±0.008	55±3
2004	0.63±0.01	0.130±0.005	56±4	0.92±0.02	0.111±0.002	65±4
2005	0.61±0.01	0.097±0.007	52±3	0.50±0.02	0.109±0.006	62±5
2006	0.62±0.02	0.107±0.009	64±3	0.80±0.03	0.101±0.005	65±3
2007	1.05±0.03	0.117±0.005	60±3	0.84±0.03	0.151±0.003	61±4
2008	1.10±0.03	0.126±0.006	68±3	1.09±0.03	0.155±0.005	74±3

Table 2. Concentrations of Cd and Hg in blue mussels collected from two stations in the Slovenia Sea each September from 1999 to 2008,. Results are expressed as the mean of three samples per dry weight

	Pb# (mg kg^{-1}) mean ±SD	Ni# (mg kg^{-1}) mean ±SD	Cr# (mg kg^{-1}) mean ±SD	Zn* (mg kg^{-1}) mean ±SD	Cu* (mg kg^{-1}) mean ±SD
Station 2	0,84±0,02	1,70±0,05	0,95±0,02	108±2	6,4±0,1
Station 3	0,64±0,02	1,06±0,04	0,32±0,009	95±2	7,2±0,1

*Legend: *FAAS and ETAAS# analytical method*

Table 3. Concentrations of metals in the blue mussels collected from two stations in the Slovenia Sea during spri ng sampling in 2003. Results are expressed as the mean of three samples per dry weight

this particular station. Comparison between both stations (1 and 2) together with years revealed that the concentration of Hg in 2000 (0.237 mg kg^{-1} ± 0.007) was significantly highest at both stations and during all years.

DISCUSSION

The small and enclosed Gulf of Trieste is very vulnerable to many different pressures and anthropogenic impacts that could lead to extensive and irreversible changes in biota. The main recognised pollution problems in the Gulf of Trieste were eutrophication, and moderate pollution with hydrocarbons and metals, especially with Hg. From this point of view it is important to set up an efficient, reliable and comprehensive biomonitoring programme which can, at an early stage, detect local pollution effects. Moreover, there is a strong need to evaluate data from biomonitoring on a wider geographical scale in order to recognise the local baseline with respect to pollution effects and shifts.

Ten years after setting up a biomonitoring programme, results of the determination of Cd and Hg in whole blue mussel tissue and MT in their digestive glands were summarised and statistically analysed in order to establish the relationship between metals in the marine environment, and MT concentrations as a biomarker of environmental pollution. A statistical evaluation of the results was also used to recognise spatial and temporal trends in marine pollution. For this purpose, three locations in the Slovenian coastal sea were included in the biomonitoring programme and samplings were performed twice a year. The evaluation of the results after a decade of biomonitoring in the Slovenian coastal waters revealed: (1) no significant seasonal differences between sampling seasons (March versus September); (2) no differences between the three sampling stations; (3) significant variations in MT concentrations between consecutive years of sampling; (4) the highest concentrations of Cd in blue mussels were found at both stations in 2000 and 2008 and (5) the highest concentrations of Hg were found at both stations in 2002 and 2004.

Two years were exceptional with regard to the concentration of MT and content of O_2 in the seawater. Besides very low concentrations of MT in 2004 at all stations and in both seasons, there was a high concentration of O_2 in March and low CI in the mussels. In the same year, low MT concentrations in September were accompanied by low concentration of O_2 and at Station 1 with low concentrations of Cd. Moreover, in the same mussels higher concentrations of Hg were found. Conversely, high MT concentrations in March 2005 were related to low O_2 and high salinity (see Table 1), together with low concentrations of Cd in blue mussels.

A comparison of MT values among stations did not reveal any significant differences between them. Several facts support this finding. At present, no one station received pollutants (e. g. metals) from a highly polluted point source, although the entire study area had a higher level of Hg due to natural pollution (more pronounced on the western side of the gulf and which is not included in the present study). Stations were located closely together, at a total distance of approx. 20 km, and with a distance between stations of 10 km. The main input of metals and

other pollutants were the Rižana, Badaševica and Soča rivers, the last of which has an estuary on the opposite side of the Gulf of Trieste (the main source of Hg in the Gulf of Trieste). The measured yearly input of Cd was, for instance, only 0.1 kg at Station 1 and consequently the pollution with metals is declared as of only minor concern (GOSAR & MURI, 2005). Moreover, measured concentrations of metals (Cd and Hg) in blue mussels were not elevated during 10 years of monitoring.

Our results showed a higher level of MT concentrations in the digestive gland, from 45 ±ug/g w.w. (Station 3 in March 2004) to 163±11 ug/g w.w. (Station 2 in March 2005), in comparison to the results obtained in the four year study conducted by PETROVIĆ *et al*., (2001) on the eastern coastal sea of Istria and Kvarner (north Adriatic Sea). They reported MT concentrations in digestive glands of blue mussels ranging from 50 to 100 μg g^{-1} w.w. In this study, concentrations of Cd and Hg in the whole tissue of blue mussels were lower than those in the presented study. Another 3-year study from the central part of the eastern Adriatic coast revealed similar MT concentrations ranging from 100 - 180 μg/g w.w. (IVANKOVIĆ *et al*., 2005). MT concentrations were also evaluated in natural blue mussels from an unpolluted area close to Ancona (on the Adriatic Sea), with concentrations found to be between 5 and 6.5 nmol/mg protein (expressed in equivalents of GSH) (BOCCHETTI & REGOLI, 2006). Several studies from Greece reported on MT concentrations measured at sites with different pollution loads and seasons (spring, summer and autumn). The MT concentrations at polluted sites ranged from 148 ± 12 ug/g w.w. to 248 ± 7 ug/g w.w., while values at the reference site were from 65 ± 8 ug/g w.w. to 107 ± 5 ug/g w.w. (KALPAXIS *et al*., 2004). Another study in Greek waters in the Gulf of Patras revealed MT concentration in the range from 70 to 170 ug/g w. w. (PYTHAROPOULOU et al., 2006), and 200 ug/g w.w. to 400 ug/g w.w. in the Gulf of Thermaikos and Strymonikos (DOMOUHTSIDOU *et al*., 2004). Anthropogenic impacted sites in Greek coastal waters had mussels with high MT concentrations ranging from 152 to 225 ug/g w.w., while mussels from other sites contained MTs ranging from 94 to 138 ug/g w.w. (TSAGARIS *et al*., 2010).

It must be stressed that the induction of MT can also be the result of a mixture of metals in the water which bind to the MT with different affinity, and with some being more potent inducers than others. It is important to note that Hg is not known as a strong inducer of MT, but has strong binding affinity to MT and could displace other metals bound to MT. These properties of Hg could explain the results obtained from blue mussels collected from Gulf of Trieste, the area having the highest content of Hg in the Adriatic (HORVAT *et al*., 2001). No correlation was found between MT and Hg and some other metals (such as Cr, Pb and As), with the remark that an adjusted concentration for metals was used in this study. A condition index (CI) was used to normalise the influence of reproduction cycle and food availability, which resulted in weight changes, influencing metal intake and MT synthesis (PERCEVAL *et al*., 2002). In blue mussels there is a negative correlation between CI and metal concentrations (MORGAUD *et al*., 2002).

Data from biomonitoring have to be interpreted cautiously, because various factors have an impact on MT concentration in blue mussels (VIARENGO *et al*., 1999; RASPOR *et al*., 2004). Different environmental parameters such as temperature, salinity and O_2 are able to increase MT concentration, but the impact of those environmental variables on MT induction cannot be easily evaluated. Such variables must be followed during a longer time period than the half life of MT if one wishes to evaluate their influence. In this regard is important to find if there are any seasonal changes in the MT level. If found they should be avoided by careful sampling design. VIARENGO *et al*. (1997) measured the seasonal variation in total Zn content and metallothionein content in the digestive gland of wild blue mussels. They found that metallothionein content increased from January to July (during the period of higher metabolic activity and reproduction). Generally, the results of studies regarding MT content

in blue mussels from field experiments and biomonitoring programmes, especially those examining seasonal variations, are contradictory. A lower level of MT in blue mussels was found during the summer sampling and higher during the winter and early spring samples in the eastern Adriatic. Local site-specific characteristics did not conceal the seasonal influence on MT and metal concentrations (IVANKOVIĆ *et al.*, 2005). On the other hand, evidence from field experiments in Greece, from the Adriatic, and in the present study from Slovenia did not find any difference between seasons in MT concentrations (DOMOUHTSIDOU *et al.*, 2004; PHYTHAROPOULOU *et al.*, 2006; PETROVIĆ *et al.*, 2001; BOCCHETTI and REGOLI, 2006). For instance, the study of BOCCHETTI & REGOLI (2006) revealed seasonal variations in several biomarkers in blue mussels, though never in MTs. They explain that due to the different physiological roles of MT and their protein metabolism, MT itself could exhibit different variations toward the same environmental factors. In more detail, the temperature of sea water had a significant effect on the metabolism of metals and MT induction as proved in laboratory experiments and in the field. A positive relationship was confirmed between water temperature, size of the blue mussel shell, uptake of metals and the induction of MT in gills in the laboratory (SERAFIM *et al.*, 2002). In contrast SERRA *et al.* (1999), observed that the high temperature of sea water in August causes a decrease in metal uptake (e.g. Cd) and MT concentration, accompanied by low levels of adenylate energy charge, probably indicating that animals were already stressed (ISANI *et al.*, 2000).

Metals able to induce metallothioneins show different biological half-lives. In mussel digestive gland, copper shows a biological half-life of 9-10 days, while the half life of cadmium is 4 months (VIARENGO *et al.*, 1985b). Estimated half-life of metallothioneins in mussels exposed to cadmium is 25 days (BEBIANNO & LANGSTON, 1993). During this period it would be possible to detect acute pollution effects. Comparison of results from biomonitoring through the years revealed the baseline level of MT at the studied area, thus allowing the detection of deviations to higher levels due to pollution effects. Metallothionein analysis in marine molluscs has been recommended by UNEP as a part of biomonitoring in the framework of the Mediterranean Action Plan (UNEP/RAMOGE 1999; VIARENGO *et al.*, 2000) and also by other pollution surveys (CARAJAVILLE *et al.*, 2000; MATHIESSEN, 2000). The same methodology used in biomonitoring of the Mediterranean Sea enables the pinpointing of areas which are more impacted by metals. At the level of the Adriatic Sea, the baseline of MT concentrations spans from 50 to 180 ug/g w.w., according to the published data (see above). The measured values of MT in the Slovenian coastal sea were in the range of values measured around the Mediterranean Sea.

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Procjena metalotioneina u dagnjama (*Mytilus galloprovincialis*) kao biomarkera žive i kadmija u slovenskim vodama tršćanskog zaljeva: studija dugoročnog istraživanja

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SAŽETAK

Kako bi se mogao ocijeniti prostorni raspored i vremenski trendovi onečišćenja metalima u priobalnom moru Slovenije, razina metalotioneina (MT) utvrđena je u dagnjama na tri mjesta uzorkovanja, dva puta godišnje za razdoblje od 10 godina. Koncentracije MT kretale su se od 45 do 163 μg g^{-1} mokre težine u ožujku, te od 47 do 144 μg g^{-1} mokre težine u rujanskom uzorkovanju. Sadržaj metala utvrđen je u cijelom tkivu dagnji na dvjema postajama, jednom godišnje. Koncentracija kadmija (Cd) kretala se u rasponu od 0,50 do 1,11 mg kg^{-1}, dok je koncentracija žive (Hg) kolebala u rasponu od 0,70 do 0,237 mg kg^{-1}. Rezultati nisu pokazali značajne sezonske razlike ili razliku između postaja glede sadržaja metalotioneina tijekom uzorkovanja, dok se sadržaj kadmija i žive u tkivu školjki mijenjao tijekom promatranog razdoblja. Prisutnost metalotioneina i metala u dagnjama nije usko povezana što ukazuje na druge moguće čimbenike kao uzroke fluktuacija promatranih tijekom godina. To podržava ideju da se podaci iz biomonitoringa moraju tumačiti oprezno, uzimajući u obzir razne druge čimbenike koji mogu utjecati na indukciju metalotioneina (MT).

Ključne riječi: metalotioneini, metali, *Mytilus galloprovincialis*, biomonitoring, terensko istraživanje, sjeverni Jadran