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Assessment of low-level metal contamination using the Mediterranean mussel gills as the indicator tissue[#]

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Abstract

Purpose: The aim of this study was to compare the level of metal contamination in two bays in the middle part of the Eastern Adriatic coastal zone in Croatia using the gills of mussels *Mytilus galloprovincialis* as indicator tissue. Despite the existing sources of contamination, previous studies with caged mussels only indicated moderate metal contamination of the Kaštela Bay, contrary to the Trogir Bay in which marina and shipyard present a probable source of Cu- and Zn-contamination.

Methods: The measurements of metallothioneins (MTs) and metals that induce MT synthesis (Cu, Zn and Cd) were performed in the heat-treated gill cytosol, and total proteins (TPs) in the untreated gill cytosol. MTs were determined by differential pulse voltammetry, Cu and Zn by flame atomic absorption spectrometry (AAS), Cd by graphite furnace AAS and TPs by Bradford spectrophotometric procedure.

Results: The results collected in four sampling campaigns (autumn periods from 2001-2004) indicated that MT levels in mussel gills (expressed on dry mass basis 2.3±0.3 mg g^{-1}) were comparable with basal levels reported in the literature (2.5±0.8 mg g^{-1}). Observed interindividual, temporal and spatial MT variability could be associated with different confounding factors, such as the time of sampling, total protein concentration and mussel size rather than cytosolic levels of Cu and Zn. Metal levels, expressed on wet mass basis, in the heat-treated gill cytosol ranged from 1.33-11.31 μg g^{-1} for Zn, from 0.72-2.96 μg g^{-1} for Cu and from 0.036-0.100 μg g^{-1} for Cd. The highest Zn level was measured at Vranjic (Kaštela Bay) - the site influenced by untreated domestic wastewater, while somewhat increased Zn and the highest Cu levels were found at marina and shipyard locations (Trogir Bay). The highest Cd level was measured at Inavinil (Kaštela Bay).

Conclusions: The observed association of gill MT levels with several biotic and abiotic factors, limits its use as the biomarker of low-level metal exposure. Therefore, the use of the metal concentrations in the heat-treated gill cytosol of Mediterranean mussels should be considered for the assessment of the low-level metal contamination of coastal marine areas.

Key-words: *Mytilus galloprovincialis*, gill cytosol, metallothioneins, copper, zinc, cadmium

1. Introduction

Next to the natural sources, a large number of metals is present in the environment as a result of human activities (e.g. industries, agriculture, mining, harbour activities, dumping, oil spills). Because of the metal toxicity, persistence and bioaccumulation, metal contamination is of major concern in many industrialized countries. Molluscs, especially bivalves living in the littoral zone are known to accumulate high levels of metals in their tissues and yet survive in these contaminated environments (Goldberg et al., 1978; Cantillo, 1998). As such, these animals have been used widely for monitoring of contamination in marine ecosystems (Zorita et al. 2007). Their tolerance to metal contaminants depends on the ability to regulate metals in their tissues and cells and to accumulate excess metals in non-toxic forms (Viarengo and Nott 1993). In our study, gills were selected as the indicator tissue for metal contamination assessment because they are an important route for metal uptake since they are directly exposed to the external medium as a respiratory organ (Widdows et al. 1995). In addition, the gill mass generally shows the smallest seasonal variability compared to other mussel tissues (Martinčić et al. 1987; Regoli 1998; Dragun et al. 2004). Mussels, as filter-feeders, have high filtration rates (*Mytilus edulis*: 52-196 L g^{-1} d^{-1}, Widdows et al. 1995) and daily process large volumes of water. Hence, the uptake of metals from the dissolved phase significantly contributes to their accumulation in the gill tissue.

Furthermore, as a tissue directly involved in the metal uptake, storage and excretion, gills have a high capacity to synthesize metallothioneins (MTs) (Sarkar et al. 2006), specific soluble ligands which have high affinity for binding metals and therefore present an important metal homeostasis and detoxification mechanism in marine invertebrates (Viarengo and Nott 1993, Amiard et al. 2006). Many biological functions of MTs have been reported, including their primary role in maintaining cellular homeostasis of essential metals, Zn and Cu; detoxification of non-essential metals (e.g., Cd, Hg and Ag); as well as a role in the inflammation processes and free radical scavenging (Vallee and Maret 1993). Within the suite of biomarkers, MTs are recommended by ICES WGBEC (2007) for measuring exposure and disturbance of Cu and Zn metabolism in *Mytilus* sp. However, as a biomarker MT has to be used with a caution. Other factors can contribute to MT variability, such as organism handling, starvation, anoxia, and the presence of antibiotics, vitamins or herbicides (Amiard et al. 2006). The influence of food abundance, reproductive cycle, and seasonal and spatial factors on the MT levels have been reported for marine bivalves (Serafim et al., 2002; Raspor et al. 2004; Ivanković et al., 2005). Due to a number of so far identified confounding factors (Amiard et al. 2006), different MT levels in organisms from distinct locations may erroneously be interpreted as a consequence of anthropogenic activities.

The concentrations of metals accumulated in different tissues of bivalves are also frequently used as the indicators of metal bioavailability from the seawater (e.g. Sunlu 2006). Contrary to usually reported whole body burdens, the cytosolic metal concentrations are often more responsive to contamination, and therefore more representative of the bioavailable concentrations in the water (Langston et al. 1998). For example, Cd concentrations in the gills of *Ruditapes decussatus* from contaminated area increased only in the heat-treated fraction, and not in the insoluble or high molecular weight fractions (Bebianno and Serafim 2003). The same authors have also reported that gill fraction of the clam *R. decussatus* containing thermo-stable compounds have a particularly important role in the Cu accumulation kinetics; compared to insoluble and thermo-labile fractions, this fraction had higher percentage of accumulated Cu, which increased linearly with the time of exposure showing the existence of available ligand for Cu binding; in addition, regardless of analyzed tissue, Cu was eliminated more quickly from thermo-stable fraction ($t_{1/2}$=4-7 days) than from thermo-labile fraction ($t_{1/2}$=7-18 days) (Serafim and Bebianno 2009). Therefore, in our study the concentrations of metals known as the inducers of MT synthesis (Cd, Cu and Zn) were determined in the heat-treated cytosolic fraction (HT-S50) of the gills of native mussels *Mytilus galloprovincialis* from the coastal area of the Eastern Adriatic Sea.

The general aim of our study was to apply the mussel gills as indicator tissue for the assessment of the low-level metal contamination. The specific objectives were:

a) to determine the concentrations of total proteins (TPs) in the gill cytosol, as well as the concentrations of MTs and metals (Cu, Zn and Cd) in the heat-treated gill cytosol; and

b) to define the interdependence between total protein, MT and metal concentrations, as well as their association with abiotic and biotic factors, such as the sampling period, gill mass, salinity and water temperature.

2. Materials and methods

2.1. Study area and periods

Native Mediterranean mussels *M. galloprovincialis* were collected at four sampling sites in Kaštela Bay and Trogir Bay (Figure 1). The sampling area is located in the middle part of the Eastern Adriatic coast (Dalmatia, Croatia) in the vicinity of urban centres Split (188 694 inhabitants; Central Bureau of Statistics 2001) and Trogir (12 995 inhabitants; Central Bureau of Statistics 2001).

A semi-enclosed Kaštela Bay is a recipient of municipal and industrial wastewater (6.4×10^6 m^3 per year) (Barić et al. 1992) and characterized by continuously increasing eutrophication level (Vukadin et al. 2003). The sampling sites within Kaštela Bay, Vranjic (VR) and Inavinil (IV), were classified as the sites occasionally influenced by freshwater (Ivanković et al. 2005): Vranjic from small river Jadro and Inavinil from freshwater source situated next to the ex-chlor-alkali plant (active in the period from 1950 to 1990). However, despite the existing sources of contamination in the Kaštela Bay, the study conducted in 1997/1998 with transplanted mussels indicated that metal concentrations in mussel tissues, and especially in the gills, still did not reflect the anthropogenic influence; total and cytosolic metal levels in mussel tissues were uniform throughout the Bay, in contrast to pronounced variations of metal levels in the sediment (Odžak et al. 2001; Dragun et al. 2004). Based on these studies, the Kaštela Bay was selected as the area less contaminated by metals.

Marinas and shipyards are generally recognized as the sites of intense human activities and consequently as the point sources of marine contamination. High Cu concentrations are reported in the sediments and seawater of ports and marinas, as well as in mooring places used by tourist vessels (Warnken et al. 2004), probably associated to the use of Cu-based antifouling paints. As the use of TBT antifouling paints was ceasing, the use of copolymer paints that consist of up to 30% of Cu became common, as well as the use of Zn-containing antifoulants, such as zinc pyrithione and zinc ethylenebis(dithiocarbamate) (zineb) (Ambrose 1994; Stephenson and Leonard 1994; Comber et al. 2001; Warnken et al. 2004). Particles of antifouling paints that have been discarded after boat hull cleaning or that have flaked off submerged structures represent an important, but little studied source of contamination in many coastal waters; because of their nature and function to leach biocides at a controlled rate into ambient water it is reasonable to expect that they pose a threat to aquatic organisms (Turner et al. 2009). Therefore, in our study the sampling sites at Trogir Marina (TM) and Trogir Shipyard (TSH) were chosen as potentially Cu- and Zn-contaminated areas.

This investigation, conducted as the part of the CroWat project, comprised the studies on both mussels and fish (red mullet, *Mullus barbatus*). Therefore, four sampling campaigns were conducted in the autumn periods (November 2001 and October 2002-2004), after the fish were already spawned, to avoid the influence of the enzymatic changes specific for the spawning period (Filipović Marijić and Raspor 2007). Salinity and temperature of the ambient seawater were measured *in situ* on each sampling campaign (Table 1) using a

portable conductivity meter MC126 (Mettler Toledo). Salinity was additionally checked with the salinity refractometer.

2.2. Isolation and heat-treatment of cytosolic fraction

Four composite samples, containing gill tissue of five individuals, were prepared for each sampling site in each sampling campaign, except for Inavinil in 2002. The samples were weighed and stored at -80°C, and for each composite sample the average gill mass (g) was calculated (Table 1). The determination of the condition indices (CI) was introduced in the study in two latter sampling campaigns (2003-2004); CI were calculated as the ratios of the dry whole soft tissue masses and the shell masses (Davenport and Chen 1987) of 10 mussels and expressed as a single value (Table 1). Tissue homogenization was performed using a PTFE pestle and motorized homogenizer in 20 mM Tris-HCl buffer (pH 8.6 at 4°C; tissue wet mass: buffer volume = 1:3) containing 0.006 mM leupeptine and 0.5 mM phenylmethyl-sulphonylfluoride (protease inhibitors), as well as 0.01% β-mercaptoethanol (reducing agent). The homogenate was centrifuged at 50,000×g for 120 minutes at 4°C. The resulting supernatant, i.e. the cytosolic fraction, was then 10 times diluted with 0.9% NaCl (Suprapur, Merck) and heat treated using the Dri Block (Techne) at 85°C for 10 minutes to remove high molecular mass proteins from the samples which would otherwise interfere with the electrochemical MT determination (Erk et al. 2002). Subsequently, the samples were placed in the refrigerator at 4°C for 30 minutes and then centrifuged at 10,000×g for 15 minutes at 4°C. The resulting supernatant, comprising heat-stable MTs, was stored at -80°C.

2.3. Determination of TPs and MTs

The concentrations of TPs were determined in the cytosolic fractions according to Bradford procedure (1976), while MTs were quantified in the heat-treated cytosol, based on the modified Brdička procedure (Raspor 2001; Raspor et al. 2001) using a differential pulse voltammetry (DPV). This is an electrochemical method for quantification of MTs, in which an electrochemical signal evolves from the reduction of thiol-groups (SH-groups) on cystein residues.

MT measurements were performed using μAutolab instrument (Eco Chemie, the Netherlands) with hanging mercury drop electrode (HMDE, Metrohm 290E, drop surface area 2.20±0.05 mm^2) as a working electrode, an Ag/AgCl/saturated KCl reference electrode and a platinum auxiliary electrode. MT concentrations expressed as $\mu g\ mL^{-1}$ were derived from the calibration straight line, which was constructed by using purified rabbit liver MT

I+II standard material (MT 7641, Sigma). The final results expressed as mg of MTs per g of gill tissue (on wet mass basis) were obtained by multiplying measured MT concentrations with cytosol dilution factor (10) and the tissue dilution factor (4).

2.4. Metal determination

The concentrations (μg mL^{-1}) of three metals known as the inducers of MT synthesis (Zn, Cu, and Cd) were measured in the same fraction as MTs, namely in the NaCl containing heat-treated cytosol of mussel gills by Varian SpectrAA 220 atomic absorption spectrometer. The final results expressed as μg of cytosolic metal per g of gill tissue (on wet mass basis) were obtained by multiplying measured metal concentrations in the heat-treated cytosol with the cytosol dilution factor (10) and tissue dilution factor (4). Flame technique (air/acetylene) was applied for the measurement of Zn (at 213.9 nm) and Cu (at 324.8 nm). The concentrations of Cd were determined by graphite furnace AAS (Varian GTA-100; at 228.8 nm; argon as a purge gas) with the addition of Na_2H_2EDTA as a modifier to efficiently separate Cd signal from the NaCl background signal (Dragun and Raspor 2005); Cd concentrations were measured only in two latter sampling campaigns (2003-2004). A deuterium lamp was used for the background correction. External calibration was performed for each metal. The calibration standards were prepared using the metal stock solutions (Zn, Cu, Cd, 1000 mg L^{-1} by Merck) and 0.9% NaCl, which was also used as the blank sample. The detection limits for measured metals were the following: Zn 0.012 mg L^{-1}; Cu 0.002 mg L^{-1}; and Cd 0.010 μg L^{-1}. Since the reference material with a matrix corresponding to the heat-treated cytosol was not commercially available, the quality control of the metal determination was regularly performed using the water control samples, such as WP-036 by US EPA. Furthermore, the accuracy of metal measurements was assured by periodical participation in the international intercalibration studies organized by Vituki (Hungary).

2.5. Statistical analyses

Statistical analyses were performed using SAS for Windows, SAS 9.1.3 Service Pack 4. One-way ANOVAs were applied to compare the levels of MTs, TPs, gill mass, Zn, Cu and Cd measured at different sites and in different sampling campaigns; if necessary, data were adequately transformed to obtain normal distribution (confirmed by Shapiro-Wilk test); *post-hoc* pairwise comparisons were made by Tukey's method. For correlation analysis we used the original data; since mainly they were not normally distributed, the Spearman coefficients were applied. Multiple regression analysis was performed on the standardized values (z) to enable

the assessment of the relative contributions of independent variables to the variance of MTs, as dependent variable; the residuals were normally distributed according to Shapiro-Wilk test.

3. Results and discussion

The complete data set of all parameters is presented in Table 1. Metallothionein levels in Kaštela and Trogir Bay, expressed on wet mass basis (MT_{wm}), varied from 0.334 to 0.589 mg g^{-1}, which is consistent with our previously reported results for MT levels in the gills of mussels caged over 12 months in the Kaštela Bay (0.31-0.68 mg g^{-1}; Dragun et al. 2004). Mussel gill MTs measured in this study were even lower than MT levels reported for the gills of clam *R. decussatus* collected at the reference site (annual average: 1.0±0.5 mg g^{-1}) (Smaoui-Damak et al. 2009). In order to compare our results with MT levels in the gills of mussels *M. galloprovincialis* unexposed to metals (Bebianno and Serafim 1998), we have expressed the average MT level on a dry mass basis (MT_{dm}), by multiplying it with average fresh to dry tissue mass ratio obtained for the whole soft tissue (5.2). The average MT_{dm} value obtained in this study (2.26±0.32 mg g^{-1}) was comparable to the basal MT value of 2.5±0.8 mg g^{-1} proposed by Bebianno and Serafim (1998). Accordingly, it was assumed that considerable MT variations between sites and sampling campaigns observed in the course of the study (Table 1) could be attributed to different biotic and abiotic factors, rather than to the metal exposure. In the field studies where many parameters can randomly affect MT levels, the endogenous regulation of MTs must be considered before using MTs as an indicator of heavy metal exposure (Smaoui-Damak et al. 2009).

To define the factors showing the strongest association with MT_{wm}, multiple linear regression analysis was performed on the whole data set (n=60). Mussel gill MT_{wm} (dependent variable, y) was defined as a function of the several measured biotic and abiotic parameters (independent variables, x_1-x_5). The following model was statistically significant (p<0.0001; Table 2) and explained 70% of MT_{wm} variance:

$$MT_{wm\text{-}st} = -1.076 + 0.217\times GM_{st} + 0.872\times TP_{st} - 0.092\times Zn_{st} + 0.028\times Cu_{st} + 1.468\times SP$$

where $MT_{wm\text{-}st}$ represents standardized value of gill MT concentration, GM_{st} standardized value of gill mass, TP_{st} standardized value of gill total protein concentration, Zn_{st} and Cu_{st} standardized values of Zn and Cu concentrations in gill cytosol, and SP the sampling period. The significant association with MT_{wm} was obtained for:

1) the sampling period,

2) total cytosolic protein concentration, and

3) the gill mass,

whereas the associations between MT_{wm} and cytosolic Cu and Zn concentrations were not statistically significant. Based on the ratios of the squared standardized coefficients (Table 2), the sampling period showed the strongest association with MT_{wm} level, TP concentration three times weaker, and the gill mass the weakest - 16 times weaker than TPs.

3.1. The sampling period

The sampling period was included in the multiple regression analysis as a binary variable (Table 2), to examine the possible differences between November and October sampling campaigns. The analysis indicated significant association between MT_{wm} level and the time of sampling. Metallothioneins present a small fraction of cytosolic proteins; in this study, mussel gill MTs amounted from 2.3 to 3.9% of TPs (Table 1). In three October sampling campaigns both MT_{wm} and TPs gradually increased from 2002 to 2004, leading to stable average MT fraction (average MT_f: 3.2-3.3%, Table 3). However, in November sampling campaign (2001), in contrast to the lowest MT_{wm} level, the highest gill TP concentration was measured, which resulted in significantly lower average MT_f (2.6%) compared to three successive October samplings. Serafim et al. (2002) reported higher background level of MTs in mussel gills at higher temperatures. However, small differences in the water temperature between November and October sampling campaigns (November 2001: 17.4-19.0°C, October 2002-2004: 17.3-21.6°C; Table 1) excluded this parameter as a direct cause of MT variability associated to the time of sampling. Therefore, the sources of temporal variability of MTs in mussel gills should be further investigated.

3.2. Total cytosolic proteins

According to multiple regression analysis, the second strongest association with MT variability was obtained for TP concentrations (Table 2). It was further confirmed by positive statistically significant correlations obtained between MT_{wm} and TPs in three sampling campaigns (2001: r=0.685, p<0.01; 2003: r=0.530, p<0.05; 2004: r=0.867, p<0.0001). Analysis of site-specific differences performed on whole data set indicated overall highest MT_{wm} (0.470 mg g^{-1}), TP (16.33 mg g^{-1}) and Zn level (5.42 μg g^{-1}) at Vranjic (Table 4). According to Ivanković et al. (2005), as well as the preliminary report of the Croatian national monitoring programme Adriatic for the period from 1999 to 2001, Vranjic can be characterized as moderately eutrophycated coastal location with

occasional freshwater influence, causing variable salinity (Table 1). Brackish water is favourable habitat for mussels principally due to the increased food levels in such environment. Discharge of untreated domestic wastewater presents an additional food source at Vranjic. In consistence with well documented relationship between nutrient load and feeding physiology in bivalves (Scholten and Smaal 1999), mussels from Vranjic were reported to have approximately 35% higher maximal condition indices in autumn seasons in the period from 1999 to 2001 compared to mussels from Inavinil (Ivanković et al. 2005). Similar results were also obtained in our study in the last two sampling campaigns, with condition indices on average 30% higher at Vranjic (average CI: 19.1%) than Inavinil (average CI: 14.9%). Higher food availability in this environment, which enhances somatic growth and protein synthesis in general, could also affect the quantity of MTs (Mourgaud et al. 2002), which explains the highest MT_{wm} levels measured at Vranjic.

3.3. Gill mass

The significant association was also obtained between MT level and the gill mass, although considerably weaker compared with the sampling period and total protein concentration (Table 2). Higher MT_{wm} level was measured at Trogir Shipyard (0.429 mg g^{-1}) compared to Trogir Marina (0.409 mg g^{-1}) and Inavinil (0.402 mg g^{-1}) (Table 4), but it could not be associated with TP level, which was the lowest at that site (12.93 mg g^{-1}). Consequently, the mussels from Trogir Shipyard had significantly higher average MT fraction (3.4%) compared to mussels from the remaining sites (2.9-3.0%; Table 4), as well as significantly higher gill masses (TSH: 0.77 g; the remaining sites: 0.44-0.62 g). Contrary, although Vranjic was singled out as the site with the highest MT_{wm}, it also had the highest total protein concentration resulting with the lowest MT fraction (2.9%) in the combination with the lowest gill mass (0.44 g) (Tables 1 and 4). Therefore, it can be hypothesized that MT fraction in TPs is associated with the gill mass, i.e. with the size of mussels, with higher percentage partition in bigger specimens. The size of mussels, on the other hand, can be influenced by different factors, such as age and seawater salinity. Our previous study with the transplanted mussels deployed over 12 months in the Kaštela Bay, showed the positive association of the shell mass with the gill mass ($r=0.61$; $p<0.001$) and MT level ($r=0.38$; $p<0.05$), indicating that both gills and MTs are increasing concurrently with the increase of the shell mass, i.e. with mussels' age (Dragun et al. 2004). Therefore, the positive association between mussels' age and MT fraction can be presumed. On the other hand, decrease in salinity has a detrimental effect on mussels' growth (Almada-Villela 1984). Accordingly, bigger mussels were collected at Trogir Shipyard as the site with constantly high

salinity (28-38 psu), whereas small specimens were collected at Vranjic, the site with fluctuating and sometimes very low seawater salinity (8-36 psu) (Table 1).

The positive association between the mussel size and MT level was previously suggested in the literature (Serafim et al. 2002), and therefore, it was recommended to collect the mussels of similar age and size, to minimize the natural variability in the level of measured biomarker. However, Lobel et al. (1991) pointed out that it is not sufficient to collect mussels of similar length since mussels of similar length from different sites do not necessarily have similar ages, soft tissue weights or conditions. Therefore, they have recommended that mussels should only be collected from the sites with similar maximum shell lenghts, to ensure similar growth rates. Unfortunately, this requirement is hard to achieve because it is not always possible to find evenly matched sites (Lobel et al. 1991).

3.4. Metal levels in the heat-treated cytosol

On average, Cu level was higher at Trogir Shipyard (1.79 μg g^{-1}) and Trogir Marina (1.69 μg g^{-1}) compared to Vranjic (1.41 μg g^{-1}) and Inavinil (1.01 μg g^{-1}) (Table 4). The lowest Cu level at Inavinil was consistent with low Cu level obtained in our previous study (0.54 μg g^{-1}) in the heat-treated gill cytosol of mussels deployed at Inavinil over 12 months (Dragun et al. 2004). As already mentioned, Zn level was the highest at Vranjic (5.42 μg g^{-1}), followed by Trogir Marina (4.22 μg g^{-1}) and Trogir Shipyard (3.58 μg g^{-1}), whereas the lowest concentrations were also measured at Inavinil (2.94 μg g^{-1}) (Table 4). Comparable Zn level at Inavinil (2.78 μg g^{-1}) was previously reported for the heat-treated gill cytosol of caged mussels (Dragun et al. 2004). If each campaign was assessed separately, Cu level in the heat-treated gill cytosol was the highest at Trogir Shipyard in the first two campaigns (2001-2002; Table 1) when sampling was performed next to the constructing ship, whereas it was the highest at Trogir Marina in the last two campaigns (2003-2004; Table 1). It could be explained by the fact that in 2003 campaign, the sampling location within Trogir Shipyard had to be moved. At new shipyard location, Cu level was lower compared to the original sampling site (Table 1). At Trogir Marina the sampling location also had to be changed in the 2002 to the mooring place, with resulting increase of Cu level (Table 1). Our results are consistent with previous reports on leaching from antifouling paints as a source of increased Cu and Zn level in mussel tissues at marinas and shipyards (Stephenson and Leonard 1994; Comber et al. 2001).

Although significant differences in Cu and Zn concentrations in the heat-treated gill cytosol were obtained between sampling sites, the multiple regression analysis did not indicate the significant association of these metals with MT_{wm} level (Table 2). An increase of MTs following mussel exposure to Cu was previously experimentally obtained (Roesijadi et al. 1988), but the relationship between MTs and Cu does not have to be so evident at low levels of Cu exposure (Langston et al. 1998). For example, the synthesis of MTs in whole soft tissue of *M. edulis* was not induced even after seven days of exposure to 68.1 μg Cu L^{-1} (Brown et al. 2004). In this study, Cu was not determined in the seawater of selected sampling sites, but the reported Cu concentrations in marinas (total Cu: 10.3 μg L^{-1}; Cmuk 2005) and small ports (total Cu: 11.4-15.0 μg L^{-1}; Cmuk 2005; Louis et al. 2008) in the nearby area, indicate to the low exposure of mussels to Cu in the studied area. A fast turnover rate of Cu-thionein in *M. edulis*, with the half-life of 10 days, might also explain the low net increase of MTs associated with essential metals (Langston et al. 1998). Similarly, the laboratory exposure of mussels to a high level of Zn (250 μg L^{-1}) showed only a limited MT induction in gills (Roesijadi et al. 1988). Langston et al. (1998) also pointed out that in some molluscs metabolically functional Cu and Zn constitute the dominant pool which, when combined with an effective regulatory mechanism, masks the detection of contamination at all but the most heavily contaminated sites.

In addition to the concentrations of essential elements Cu and Zn, which are characteristic contaminants for marinas and shipyards, in the last two years the study was expanded on the measurement of non-essential metal Cd. The site with the lowest Cu (1.01 μg g^{-1}) and Zn concentrations (2.94 μg g^{-1}) was at the same time the site with the highest Cd concentration in the heat-treated gill cytosol (Table 4). Although the differences between sites were rather low, Cd level at Inavinil (0.064 μg g^{-1}) was significantly higher compared to Trogir Marina (0.047 μg g^{-1}), opposite to site-specific distribution of MT_{wm} with the lowest level measured at Inavinil (0.40 mg g^{-1}). It can be concluded that Cd levels measured in the heat-treated gill cytosol in our study were not high enough to induce additional MT synthesis. Smaoui-Damak et al. (2009) also have obtained comparable gill MT concentrations in the clams *R. decussatus* from two differently contaminated sites despite the significant differences in cytosolic Cd level (reference site: 0.08 μg g^{-1}; Cd-contaminated site: 0.20 μg g^{-1}). The same authors have previously emphasized the absence of relationship between MTs and Cd in the gills of clams collected from their natural habitat where many biotic and abiotic factors may influence the synthesis of MTs (Smaoui-Damak et al. 2004). In addition, Geffard et al. (2005) have reported the absence of MT induction in the

gills of mussel *M. edulis* after the transplantation from clean to metal-rich site, despite the evident accumulation of three metals, Cd, Cu and Zn.

4. Conclusions

The results of this study indicated that there are numerous confounding factors which can complicate the assessment of the low-level metal contamination by measurement of MTs in the mussel gills, such as the time of sampling, total cytosolic proteins and the size of mussels. Although there are known sources of water contamination in the Kaštela and especially Trogir Bay, MT levels measured in this study were comparable with previously published basal gill MT levels. On the other hand, the prominent changes in the cytosolic metal concentrations following the changes of the sampling sites, as well as the characteristic site-specific differences, pointed to Cu and Cd levels in the heat-treated gill cytosol as possibly more promising indicators of low-level metal exposure from the seawater than MTs. However, an investigation including simultaneous measurement of metal concentrations in both, mussel gill cytosol and the seawater, is necessary to further explore and confirm this assumption.

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Figure caption

Figure 1. A map of the study area, Kaštela Bay and Trogir Bay, located in the middle part of the Eastern Adriatic coastal zone, with indicated sampling sites.

Figure 1.

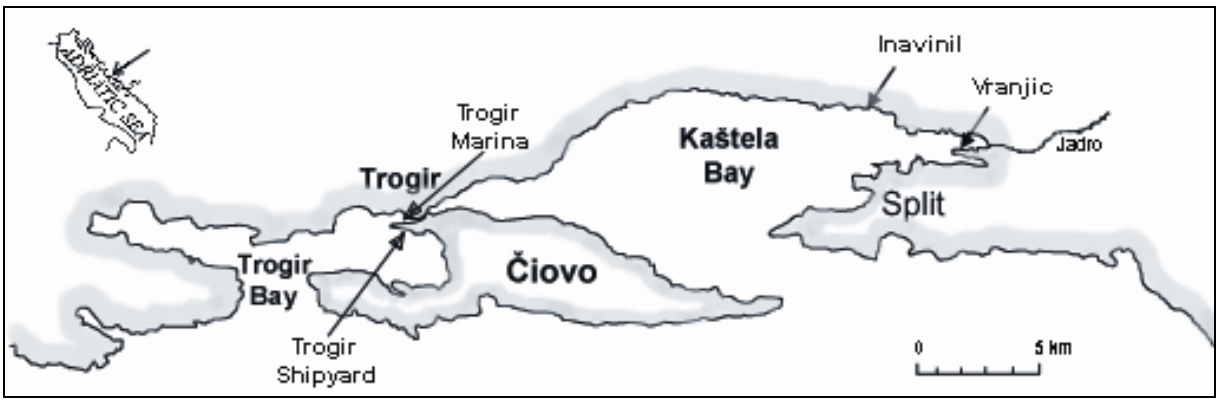

Table 1. Data set collected during the four year of investigation (2001-2004) at four sampling sites: metalothioneins (MT), total cytosolic proteins (TP), as well as Zn, Cu and Cd levels in the heat-treated cytosol expressed on wet mass basis, MTs expressed as percentage partition in total cytosolic proteins, and the gill mass - presented medians (minimum-maximum) are based on the results obtained for four composite gill samples per site in each year; salinity and water temperature are given as the single values for each site in each year.

		MT / mg g^{-1}	MT / %	TP / mg g^{-1}	Zn / μg g^{-1}	Cu / μg g^{-1}	Cd / μg g^{-1}	Gill mass / g	Condition index / %	Salinity / psu	Water temperature / °C
Inavinil	**2001**	0.396 (0.334-0.425)	2.5 (2.3-2.6)	16.3 (12.8-17.1)	2.51 (1.33-4.69)	0.76 (0.72-1.16)	-	0.61 (0.56-0.66)	-	34.0	18.0
	2002	-	-	-	-	-	-	-	-	-	-
	2003	0.402 (0.383-0.429)	3.4 (3.3-3.4)	12.0 (11.3-12.8)	3.16 (2.54-3.62)	1.21 (0.95-1.42)	0.060 (0.056-0.081)	0.60 (0.41-0.76)	17.8	35.8	19.8
	2004	0.395 (0.392-0.490)	3.0 (2.8-3.2)	13.8 (12.2-15.4)	3.46 (2.35-4.44)	1.06 (0.91-1.15)	0.063 (0.047-0.100)	0.49 (0.46-0.51)	11.9	18.4	21.0
Vranjic	**2001**	0.398 (0.379-0.416)	2.5 (2.3-2.6)	16.5 (14.9-17.1)	8.30 (2.39-11.3)	1.26 (0.88-1.39)	-	0.45 (0.43-0.46)	-	31.0	19.0
	2002	0.430 (0.363-0.476)	3.1 (2.8-3.7)	13.1 (12.5-14.7)	3.53 (3.10-6.95)	1.42 (1.31-1.67)	-	0.49 (0.47-0.54)	-	8.2	19.0
	2003	0.512 (0.483-0.569)	3.0 (2.7-3.2)	17.1 (16.2-19.3)	5.61 (5.32-7.28)	1.50 (1.33-1.71)	0.041 (0.037-0.053)	0.37 (0.32-0.38)	17.6	35.8	20.7
	2004	0.561 (0.533-0.589)	3.1 (2.8-3.4)	18.3 (16.6-20.1)	5.57 (4.65-6.46)	1.51 (1.44-1.73)	0.054 (0.047-0.062)	0.49 (0.40-0.56)	20.6	23.7	21.6
Trogir Marina	**2001**	0.366 (0.361-0.404)	2.6 (2.5-2.9)	14.1 (13.9-14.4)	4.25 (2.01-6.88)	1.08 (0.89-1.45)	-	0.65 (0.61-0.69)	-	38.0	17.4
	[a]2002	0.389 (0.366-0.403)	3.2 (3.0-3.3)	12.3 (11.7-13.1)	2.24 (1.76-3.53)	1.91 (1.66-2.01)	-	0.46 (0.44-0.73)	-	21.3	17.3
	2003	0.395 (0.355-0.422)	2.9 (2.7-3.1)	13.4 (13.0-13.7)	7.08 (5.23-8.02)	1.89 (1.57-2.72)	0.049 (0.046-0.065)	0.76 (0.71-0.82)	16.4	31.4	20.0
	2004	0.498 (0.493-0.502)	3.2 (3.1-3.7)	15.6 (13.5-16.4)	5.26 (3.82-5.95)	1.95 (1.48-2.82)	0.042 (0.039-0.048)	0.57 (0.56-0.60)	18.1	31.0	21.0
Trogir Shipyard	**2001**	0.389 (0.358-0.411)	2.8 (2.4-3.0)	13.9 (12.8-16.0)	3.25 (1.37-3.52)	2.08 (1.73-2.96)	-	0.76 (0.60-0.92)	-	38.0	18.4
	2002	0.415 (0.387-0.429)	3.7 (3.3-3.8)	11.5 (10.1-12.3)	2.46 (1.99-2.73)	2.08 (1.85-2.37)	-	0.85 (0.71-0.94)	-	28.1	20.4
	[b]2003	0.479 (0.467-0.515)	3.8 (3.6-3.9)	12.9 (12.3-13.3)	4.80 (3.72-5.26)	1.59 (1.33-1.84)	0.044 (0.036-0.064)	0.93 (0.93-0.95)	13.9	36.9	21.3
	2004	0.436 (0.435-0.457)	3.3 (3.2-3.4)	13.5 (12.9-13.7)	5.45 (4.56-6.38)	1.43 (1.40-1.49)	0.059 (0.043-0.071)	0.60 (0.51-0.68)	19.1	32.0	21.0

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[a] - change of sampling location within Trogir Marina; [b] - change of sampling location within Trogir Shipyard

Table 2. Multiple regression model for MTs (mg g^{-1}, wet mass) as dependent variable: adjusted R^2=0.703 (p<0.0001); analysis was performed on standardized values; the residuals were normally distributed, according to Shapiro-Wilk test. Sampling period was included in the multiple linear regression analysis as a binary variable (November sampling (2001) - 0; October samplings (2002-2004) - 1).

	Coefficients (squares)	***p***	**Ratios of squared coefficients**
Intercept	-1.076 (1.16)	<0.0001	-
Sampling period (SP)	1.468 (2.16)	<0.0001	SP:TP = 3
Total cytosolic proteins (TP)	0.872 (0.76)	<0.0001	TP:GM = 16
Gill mass (GM)	0.217 (0.047)	<0.05	GM:Zn = 6
Zn in HT S50	-0.092 (0.008)	0.248	Zn:Cu = 8
Cu in HT S50	0.028 (0.001)	0.747	-

Table 3. The analysis of differences in the measured levels of several parameters between four sampling campaigns (all sites within sampling campaign grouped together): one-way ANOVA was performed on transformed data to obtain normal distribution (inverse for MTs expressed on wet mass basis and TPs; $\log_{10}$ for MTs expressed as percentage partition in TPs, gill mass (GM), Zn, Cu, and Cd). The comparison of least square means is presented in the table.

	Number of samples	**MT / mg g^{-1}**	**MT / %**	**TP / mg g^{-1}**	**GM / g**	**Zn / µg g^{-1}**	**Cu / µg g^{-1}**	**Cd / µg g^{-1}**
		p<0.0001	p<0.0001	p<0.0001	p=0.421	$^{*}p$<0.01	p<0.05	$^{**}p$=0.553
2001	16	^{a}0.385	^{a}2.6	^{a}14.90	0.60	^{a,b}3.65	^{a}1.24	-
2002	12	^{a,b}0.405	^{b}3.3	^{b}12.24	0.59	^{b}2.83	^{b}1.78	-
2003	16	^{b,c}0.442	^{b}3.2	^{a,b}13.62	0.62	^{a}4.88	^{a,b}1.53	0.051
2004	16	^{c}0.472	^{b}3.2	^{a}14.92	0.53	^{a}4.73	^{a,b}1.46	0.054

* variance weighted ANOVA

** *t*-test

^{a,b,c} different letters represent statistically significant differences (p<0.05; Tukey's method)

Table 4. The analysis of differences in the measured levels of several parameters between four sampling sites (all sampling campaigns at each site grouped together): one-way ANOVA was performed on original data for MTs expressed as percentage partition in TPs and for TPs; and on the transformed data (log_{10}) for MTs expressed on wet mass basis, gill mass (GM), Zn, Cu, and Cd. The comparison of least square means is presented in the table.

	Number of samples*	**MT / mg g^{-1}**	**MT / %**	**TP / mg g^{-1}**	**GM / g**	**Zn / μg g^{-1}**	**Cu / μg g^{-1}**	**Cd / μg g^{-1}**
		$p<0.01$	$p<0.01$	$p<0.0001$	$p<0.0001$	$p<0.01$	$p<0.0001$	$p<0.05$
Inavinil	12	^{a}0.402	^{a}3.0	^{a}13.83	^{a}0.56	^{a}2.94	^{a}1.01	^{a}0.064
Vranjic	16	^{b}0.470	^{a}2.9	^{b}16.33	^{b}0.44	^{b}5.42	^{b}1.41	^{a,b}0.049
Trogir Marina	16	^{a}0.409	^{a}3.0	^{a}13.79	^{a}0.62	^{a,b}4.22	^{b,c}1.69	^{b}0.047
Trogir Shipyard	16	^{a,b}0.429	^{b}3.4	^{a}12.93	^{c}0.77	^{a}3.58	^{c}1.79	^{a,b}0.051

^{a,b,c} different letters represent statistically significant differences ($p<0.05$, Tukey's method)

* number of samples for Cd is 8 at each site