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The assessment of natural causes of metallothionein variability in the gills of European chub (*Squalius cephalus* L.)

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Abstract

The possible causes of the variability of gill metallothionein (MT) levels were studied on 182 specimens of 2- and 3-year old European chub (*Squalius cephalus* L.) from the Sava River in Croatia. The most pronounced differences in MT levels were obtained between three sampling campaigns, and especially between periods with presumably different metabolic activities (April/May 2006 vs. September 2005 and 2006). Next to the probable influence of metabolic activity on MT level, the correlation analysis indicated to significant association between MTs and the fish size. Differences between males and females, as well as between mature and non-mature fish, were not observed in the young group of studied chub even in the spring reproductive season. Based on the analysis of the site-specific MT variability, it could be concluded that, under the conditions of low dissolved metal concentrations in the river water (as reported for the Sava River), MTs seem to be more affected by different biotic factors than by metal exposure. Therefore, MTs measured in this study were considered as the constitutive gill MT levels of young European chub. The constitutive MT ranges were defined separately for the season of lower metabolic rate (autumn: 1.31-2.00 mg g^{-1}) and the season of higher metabolic acitivity (spring: 2.15-2.95 mg g^{-1}).

34 **Key words**: Cd, chub, Cu, cytosol, gills, metallothionein, river, Zn

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Introduction

Pollution assessment cannot be solely based on chemical analysis of environmental samples since it does not inform us about deleterious effects of contaminants to biota (Livingstone, 1993; Sarkar et al., 2006). Molecular biomarkers are considered as the most sensitive and earliest responses to pollutants (Rodríguez-Ortega et al., 2009), whereas metallothioneins (MTs) are recognized among a suite of ″core biomarkers″, because their induction presents biochemical response to increased bioavailability of metals in the environment (Amiard et al., 2006). MTs are a class of ubiquitously occurring low molecular mass cysteine- and metal-rich proteins containing sulphur-based metal clusters (Vašák, 2005). Important roles of these proteins include their involvement in the homeostasis of essential trace metals, Zn and Cu, or sequestration of environmental toxic metals, Cd and Hg, as well as protection against oxidative damage (Vašák, 2005).

However, the lack of detailed knowledge about the variability of biomarker responses and their possible seasonal variations limits their use (Ringwood et al., 1999). For example, factors unrelated to metal contamination can also induce MT synthesis, such as handling, starvation, anoxia, freezing, and the presence of antibiotics, vitamins or herbicides (Baer and Thomas, 1990; Templeton and Cherian, 1991; Amiard et al., 2006); the level of induction, however, is usually lower than that caused by metals (Kägi, 1993). The best studied MT isoforms, MT-1 and MT-2, are regulated at transcriptional level, and these genes can be induced not only by metals, but also by glucocorticoids, cytokines, and a variety of chemical and physical stress conditions (Vašák, 2005). As reviewed by Amiard et al. (2006), any factor which is able to influence protein metabolism will be able to influence MT directly, whereas factors known to influence metal uptake and accumulation, such as size, sex or sexual maturity, will be able to influence MT indirectly; confounding factor may also result from a combination of physical and biological factors, as exemplified by a seasonal effect. The same authors further emphasized that relative influence of natural and contamination factors limits the possibility of using MT level as a biomarker of metal exposure; consequently, despite many so far published studies on MTs in different tissues of aquatic organisms, the choice of the best species and the most relevant organ for MT determination is still subject for discussion. Studies on MTs under realistic environmental concentrations are needed, especially for the gills, considered the most vulnerable organ for metal toxicity during exposure to waterborne metals (McDonald and Wood 1993; Olsvik et al. 2001).

The aim of this study was to examine different causes of MT variability in the gills of European chub (*Squalius cephalus* L.), as a widespread fish species in European freshwater, and to establish if the chub gill MT is useful as a biomarker of exposure to low metal concentrations in river water, such as reported for Sava River in Croatia (Dragun et al., 2008 and 2009b). Currently, there are no well-established factors that can be used to correct biomarker responses against body size, age, nutritional status, etc.; it is therefore necessary to collect this information with the biomarker samples (Handy et al., 2003). With that in mind, we have examined interannual and interseasonal MT variability, as well as the possible association of fish sex, age, sexual maturity, size and total protein (TP) concentrations with the changes of MT levels. In addition, the spatial variability of chub gill MTs in the Sava River in Croatia was assessed, as well as its association with the gill cytosolic levels of three metals known as inducers of MT synthesis (Zn, Cu, and Cd). Since the most abundant chub specimens in the Sava River are 2- and 3-year old (Habeković et al., 1993), the analysis presented in this paper was directed toward these two age groups. To our knowledge, so far only one study reported the MT levels in the chub gills (Hayes et al. 2004); additional knowledge on MT fluctuations in tissues of this fish species is therefore required.

Materials and methods

Fish sampling

The selected bioindicator organism for this study was European chub, a fish species from the family of carps (*Ciprinidae*), widespread in European freshwater; according to IUCN Red List of Threatened Species, since 2007 a valid name for European chub is *Squalius cephalus* L. instead of a former name *Leuciscus cephalus* L. The fish were caught in Sava River in Croatia, which is the biggest tributary (945 km) to the Danube River. The section of Sava River (Fig. 1), 150 km long, starting at the Croatian-Slovenian state border and ending at the town of Jasenovac (the state border between Croatia and Bosnia and Herzegovina) was chosen for the present study due to the well-defined gradient of pollution ranging from low polluted sites upstream of the city of Zagreb up to the sites affected by the pollution load from the Zagreb and Sisak city areas (Zagreb: 1 million inhabitants, heavily industrialized; Sisak: 50,000 inhabitants, pesticides production facility, ironworks and oil refinery; Krča et al. 2007).

Şaşi (2004) reported that the spawning of European chub takes place in March and April, while Ünver (1998) reported it to start in May and lasts until the end of June, probably

depending on the geographical region. In our study, therefore, three sampling campaigns were conducted: two campaigns in the period which can be regarded as post-spawning (September 2005 and 2006), and one in the period which can be regarded as reproductive (either pre-spawning or spawning period in April/May 2006). The September samplings were completed within 10 days (September 19th to 28th, 2005; September 13th to 22nd, 2006). Due to the unfavourable weather conditions, the sampling conducted in April-May 2006 lasted over one month (April 18th to May 25th). The samplings were performed by electro fishing, according to the Croatian standard HRN EN 14011:2005. Total number of 182 specimens of 2- and 3-year old European chub was caught (73 in September 2005, 70 in April-May 2006 and 39 in September 2006). During the few hours of sampling and transportation, the captured fish were kept alive in tank filled with river water taken from their respective sampling sites, and the water was aerated. In the laboratory, the fish were first anesthetized with MS 222 (tricaine methane sulphonate, Sigma Aldrich) and then sacrificed; the gills were isolated, weighed and stored at -80°C until further analyses.

Determination of chub age, sex, Fulton condition indices and gonadosomatic indices

Age was determined by counting summer and winter growth zones which appear on calcified structures of fish body (scales), using optical microscope BH-2 (Olympus); scales were taken dorsolaterally below the dorsal fin. Sex was determined by histological examination of chub gonads. Tissues were preserved in 10% neutral buffered formaline for at least 48 h, washed in 50% ethanol, and stored in 70% ethanol until histological processing. Samples were embedded with paraffin and serially sectioned at 5 μm thickness. Sections were stained with hematoxylin and counterstained with eosin. Structural and cellular sex-distinguishing markers were identified: stages of oogenesis in females and spermatogenesis in males (Luckenbach et al., 2003). The calculation of Fulton condition indices (FCI, g cm^{-3} x 100) were made according to Rätz and Lloret (2003), based on chub's total mass and length. Gonadosomatic indices (GSI, %) were calculated as ratios between gonad mass (g) and total chub mass (g), multiplied with 100.

Isolation of cytosolic fraction

Isolation of the S50 cytosolic fraction from the chub gill tissue was performed according to SOP (1999) developed at Norwegian Institute for Water Research (NIVA) in the frame of the BEQUALM programme (Biological Effects Quality Assurance in Monitoring Programmes). The gill tissue samples were first diluted 6 times with cooled homogenization buffer,

consisting of 100 mM Tris-HCl/Base (pH 8.1 at 4°C) and the reducing agent 1 mM dithiotreitol (both by Sigma). The diluted tissue samples were then homogenized by Potter-Elvehjem homogenizer (Glas-Col, USA) in ice cooled tube at 6,000 rpm. The homogenates were centrifuged in the Sorval RC28S centrifuge (Kendro, USA) at 50 000×g for 2 h at 4°C. Next, the supernatant (S50), which represents water soluble cytosolic tissue fraction, was separated from the pellet and stored at -80°C for subsequent metal and protein analyses.

Determination of MT levels

For MT determination, the cytosolic fraction (S50) was purified by heat-treatment. Heat-treatment efficiently denatures high molecular mass proteins in S50 supernatant which would otherwise interfere with the electrochemical MT determination (Erk et al., 2002). The cytosolic fraction was first 10 times diluted with 0.9% NaCl (*Suprapur*, Merck), then heat-treated for 10 min at 85°C in The Dri Block (Techne), and subsequently placed on ice for 30 min. The heat-treated cytosol was then centrifuged at 10 000×g for 15 min at 4°C in Biofuge Fresco centrifuge (Kendro, USA). The supernatant obtained after the centrifugation (HT S50) was separated from the pellet and stored at -80°C until further analysis.

MT concentrations were measured in the HT S50 by differential pulse voltammetry (DPV) following the modified Brdička procedure (Raspor et al., 2001) and using 797 VA Computrace (Metrohm, Switzerland) with a three-electrode system (hanging mercury drop electrode, HMDE, with the surface area of 0.40 mm^2 as a working electrode, an Ag/AgCl/saturated KCl reference electrode and a platinum counter electrode). The voltammetric measurements were performed in 10 mL of supporting electrolyte solution (2 M NH_4Cl/ NH_4OH, 5 mL and $1.2x10^{-3}$ M $Co(NH_3)_6Cl_3$, 5 mL; pH=9.5) thermostated at 20°C and deaerated with extra pure nitrogen, to which 20-40 μL of chub gill HT S50 was added. Instrumental parameters for DPV were the following: potential scan from -0.9 V to -1.65 V; scan rate 0.005 Vs^{-1}; voltage pulse amplitude 0.025 V; duration of the pulse application 0.057 s; and a clock time 0.5 s. MT concentrations expressed as μg mL^{-1} were derived from the calibration straight line, which was constructed by using the commercially available, >95% pure, rabbit liver zinc-MT (MT-95-P, Lot 041102; Ikzus Proteomics, Italy) dissolved in 0.25 M NaCl. The final results expressed as mg of MTs per g of gill tissue (wet mass) were obtained by multiplying measured MT concentrations with the tissue dilution factor (6).

Determination of TP concentrations in gill cytosol

TP concentrations were measured in the gill cytosol (S50) according to Lowry et al. (1951). The Bio-Rad DC Protein Assay was applied according to manufacturer's instructions. The measurements were performed on the photometer Microplate Reader HT3 (Anthos, Austria) at 750 nm wavelength. Calibration curve was constructed with five different concentrations (0.2-2.0 mg mL^{-1}) of bovine serum albumin (Serva, Germany) dissolved in the homogenization buffer.

Determination of cytosolic metal concentrations

The concentrations of three metals known as the inducers of MT synthesis (Zn, Cu, and Cd) were measured in a duplicate in the five times diluted S50 fraction of the chub gills by Varian SpectrAA 220 atomic absorption spectrometer. Flame technique (air/acetylene) was applied for the measurement of Zn (at 213.9 nm), whereas graphite furnace with universal platforms (Varian GTA-100) was used for the measurement of Cu (at 324.8 nm) and Cd (at 228.8 nm). 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 five times diluted homogenization buffer, which was also used as the blank sample. The validation of Cu determination by ETAAS has proven that reliable Cu measurements can be made even in complex organic matrix of undigested chub tissue cytosol (Dragun and Raspor 2008). The detection limits for measured metals were the following: Zn 0.007 mg L^{-1}; Cu 0.270 μg L^{-1}; and Cd 0.023 μg L^{-1}. Since the reference material with a matrix corresponding to the cytosolic fraction 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 and SW-HM-47 by Vituki (Hungary). Furthermore, the accuracy of metal measurements was assured by periodical participation in the international intercalibration studies organized by Vituki (Hungary) and UNEP/GEMS (Burlington, Canada).

Statistical analyses

Statistical analyses were performed using SAS for Windows, SAS 9.1.3 Service Pack 4. The analyses of differences in MT levels between sexes, between two age groups, three sampling campaigns, as well as between sites were performed by one-way and two-way ANOVA on adequately transformed data to obtain normal distribution (confirmed by Shapiro-Wilk test). Post-hoc pairwise comparisons were made by Bonferroni t-test. For correlation analysis we

have used the original data; since they were not normally distributed, the Spearman coefficients were applied.

Results and discussion

The measurement of MTs in different tissues of aquatic organisms as a biomarker of metal exposure is widespread in the environmental studies. Handy et al. (2003) have proposed that standard operating procedures (SOPs) should be developed for the determination of each biomarker, but it has not been done yet for MTs. Different research groups use different purification and analytical procedures for MT determination, which makes impossible the comparison of the obtained results. Therefore, it is important to point out that MT levels reported in this study present the preliminary data set for the gills of European chub measured by electrochemical method. To our knowledge, only so far published gill MT levels for European chub were measured by capillary electrophoresis (Hayes et al., 2004). MT levels reported for the gills of various other cyprinid fish were also obtained by methods other than polarography (cadmium saturation assay: *Cyprinus carpio* - De Smet et al., 2001; *Carassius auratus gibelio* - De Boeck et al., 2003; mercury saturation assay: *Danio rerio* - Gonzalez et al., 2006; ELISA: *Acrossocheilus paradoxus* - Wu et al., 2006).

The levels of MTs obtained in our study in September 2005 were in the range from 1.02-2.85 mg g^{-1}, in April/May 2006 from 1.51-4.13 mg g^{-1}, and in September 2006 from 1.02-2.10 mg g^{-1}. In addition to pronounced differences in MT ranges obtained in three sampling campaigns, the calculated relative standard deviations (RSD) indicated that MT variability within sites (up to 24%) was higher than MT variability between sampling sites (up to 14%). It was an indication of more pronounced interindividual variability of this biomarker compared to the variability possibly caused by different metal exposure. It is, therefore, necessary to characterize basic physiological variability of MTs in the chub gills and to define its causes, before the changes of MT levels could be applied as the indicator of metal exposure (Van Cleef et al., 2000). The possible dependence of gill MT level on biotic factors, such as sex and age, the gill mass, metabolic activity, sexual maturity and reproductive cycle, as well as the cytosolic concentrations of TPs and metals in the gills, will be discussed in detail below.

MT variability associated to sex and age

The analysis of differences in gill MTs between females and males, as well as between two age groups, is presented in Table 1. The number of 2- and 3-year old chub included in this analysis was 41 and 29 for September 2005, 28 and 42 for April/May 2006, and 12 and 23 for September 2006, respectively. The percentage of females was always higher than males and amounted from 59-66%, depending on the sampling campaign, which is consistent with the previously published reports by Ünver (1998) and Şaşi (2004) (68% and 73% of females in the chub populations, respectively). Although it is often described in the literature that biochemical parameters, such as MT, differ between sexes (e.g. MTs in the liver of squirrelfish, Hogstrand et al., 1996), it is dependent on the fish maturity and the selected tissue. Different reports are available in the literature regarding the age when European chub attains sexual maturity. According to Şaşi (2004) both males and females become sexually mature at age of 2; according to Ünver (1998) males at age of 2-3 and females at age of 3-4; and according to Habeković et al. (1993) both sexes at age of 4-5. Fish in this study were therefore on the verge of sexual maturity and the lack of sex differences, as indicated in Table 1, is not surprising. For example, Hansen et al. (2006) also have not found any difference between sexes in gill MT levels in the study on immature brown trout (*Salmo trutta*) from three Norwegian rivers, whereas Van Cleef et al. (2000) have not found differences in gill MT mRNA between sexes even in the mature specimens of common killifish (*Fundulus heteroclitus*) during the reproductive period. In the liver of the same chub specimens as used in this study females had somewhat higher average hepatic MT level in the reproductive period (2.01 mg g^{-1}) than males (1.82 mg g^{-1}; Podrug and Raspor, 2009). The sex differences were probably more evident in the liver than gill tissue, because liver has more important role in the reproduction compared to gills, for example in the process of vitellogenesis (Werner et al. 2003).

Statistically significant difference (p<0.05) in gill MT level between 2- and 3-year old chub was obtained only in the spring period; approximately 10% higher MT levels were obtained in the gills of younger fish (Table 1). Younger and smaller fish are known to have faster filtration and metabolic rates, and consequently higher concentrations of proteins (e.g. metallothioneins) in fish tissues can be expected as the result of homeostatic regulation (Wiener and Giesy, 1979), especially in the period of more intense metabolic activity, such is presumably spring reproductive period.

MT variability associated to gill mass

In spite of the attempts made in the monitoring programmes to catch the fish of comparable size at different sites, it is usually hard to achieve. In this study 2- and 3-year old fish also varied in size at different sites in all three sampling campaigns (total length: 18.5±2.8 cm, 18.7±2.4 cm, and 20.0±3.5 cm, respectively; total mass: 65.6±32.5 g, 73.6±30.8 g, and 83.8±45.6 g, respectively). It could affect the assessment of metal exposure based on MT as a biomarker, providing there was an association between size and MT level. We have, therefore, examined this association by use of Spearman correlation coefficients (Table 2). The gill mass was used as the indicator of the fish size, due to the high positive and statistically significant correlations (r= 0.919-0.995; p<0.0001; n=39-73) obtained between three biometric parameters associated to the chub size (length, total mass and gill mass), among which the gill mass showed the highest variability (Dragun et al. 2007). In both September sampling campaigns (Table 2), we have obtained positive, statistically significant correlations between MTs and the gill masses on the level of all data gathered within each season. The correlations calculated separately for each sampling site were not always significant, probably because at some sites the range of gill masses was rather constrained, while in all three sampling campaigns the gill masses were evenly distributed from approximately 0.2-1.5 g when the whole data sets were considered (Table 2). Contrary to September campaigns, April/May sampling resulted with the statistically significant negative correlation between MT level and the gill mass, which is consistent with the observation previously made for TPs: positive association with the gill mass obtained in both September campaigns and negative in April/May 2006 (Dragun et al., 2007). It was hypothesized that, because of the increased metabolic activity, the important factor governing the protein synthesis in the spring time is the metabolic rate. The metabolic rate is negatively correlated with the fish size (Neely, 1979), which could cause the negative association of TP concentrations with the gill mass in the spring, in contrast to positive association observed in the autumn period (Dragun et al., 2007). The same explanation is plausible for MTs, as a protein fraction. However, although the statistical analysis indicated significant correlation between MTs and the gill mass (Table 2), it should be highlighted that only small portion of MT variability could be associated with the chub size, smaller in the spring (10%) than autumn periods (22% and 36%, respectively).

Interseasonal MT variability

The comparison of gill MT levels measured in three sampling campaigns (one reproductive and two non-reproductive periods) is presented in Table 1. The differences between all three campaigns were statistically significant, indicating to the presence of both interseasonal (average MT level 45% and 76% higher in April/May 2006 compared to September 2005 and 2006, respectively) and interannual variability (average MT level 22% higher in September 2005 compared to September 2006), with interseasonal variability being more pronounced. Although significant association was established between MTs and the gills mass (Table 2), analysis of differences in gill masses between seasons indicated the comparable chub size in all three sampling campaigns (Table 3), thereby excluding the possibility that MT levels varied between seasons as a consequence of size differences. Interseasonal variability was previously established for several other parameters measured in the gills of these same chub, such as cytosolic concentrations of TPs, Zn and Fe (Dragun et al., 2007). Based on the literature data (McCoy et al., 1995; Andres et al., 2000), the spring increase of metal and protein concentrations in gills was explained as a consequence of increased metabolic and feeding activity (Dragun et al., 2007). In the liver of the same chub specimens as used in this study significantly higher average MT levels (1.93 mg g^{-1}) were also obtained in the spring 2006 compared to average MT level for both autumn seasons (1.63 mg g^{-1}), but the difference was not as high as in the gills, and amounted to approximately 20% (Podrug and Raspor, 2009). The seasonal variability in liver MT was attributed to different phases of reproductive cycle, with higher levels obtained in the pre-spawning/spawning period due to the process of vitellogenesis (Podrug and Raspor, 2009). The reproductive cycle should therefore be considered as a possible additional cause of MT increase even in the chub gills. However, MT level is known to increase 2-3 times in fish liver during the reproductive phase (in red mullet *Mullus barbatus*: Benedicto et al., 2001; Filipović Marijić and Raspor, 2008; in striped mullet *Mugil cephalus*: Gorbi et al., 2005), and therefore small MT increase reported by Podrug and Raspor (2009), as well as comparable GSI in reproductive and non-reproductive sampling campaigns (Table 3) further confirmed that 2-3 year old chub were mainly not mature. Literature based GSI for mature chub during the reproductive phase is above 3% for females and above 1.5% for males (Şaşi, 2004). Only 8 specimens in our study complied with that requirement. Comparison of spring gill MTs of mature and non-mature chub (GSI<1.5% (n=62): MT = 2.57±0.42 mg g^{-1}; GSI>1.5% (n=8): MT = 2.44±0.22 mg g^{-1}) indicated that in the young chub gill MT levels were higher in the spring than autumn period regardless of fish sexual maturity. Therefore, the pronounced spring MT increase in the gills of 2- and 3-year old chub could not be associated to the reproductive cycle.

Altered feeding was previously suggested as a possible influential factor on MT level (George and Olsson, 1994; McCoy et al., 1995). Enhanced feeding in the spring period, which was confirmed by significantly higher Fulton condition indices compared to both autumn seasons (Table 3 and Dragun et al., 2007), could cause increase of metabolic activity. Metabolic stimulation subsequently causes accelerated gill ventilation, and thereby also the enhanced uptake of essential, as well as toxic metals (Chovanec et al., 2003). It is, therefore, possible that more pronounced spring increase of MT level in gills than in liver is an outcome of its important role in uptake of metals, such as Zn (Bury et al., 2003). Zinc concentrations in chub gills were also significantly higher in the spring than autumn periods (50-70%, Table 4 and Dragun et al., 2007), while differences in cytosolic Zn concentrations in chub liver between spring and autumn were much lower (<10%) and not significant (Podrug and Raspor, 2009). Based on the fact that gills appear to be a major site of uptake of dissolved metals in aquatic environments (Olsson and Hogstrand, 1987), Van Cleef et al. (2000) have already proposed that observed increase of MT mRNA in spawning compared to non-spawning killifish in gills and not in the liver tissue was a consequence of physiological changes involving intracellular metal regulation.

Intraseasonal MT variability associated to TP and metal concentrations

Since MTs showed comparable association with the gill mass and variability between seasons as TP and Zn concentrations, further step was to examine their mutual relationship within each season, as well as MT association with two other metals, also known as inducers of teleost MT synthesis (Cu and Cd; George and Olsson, 1994). Cytosolic concentrations of total protein and three metals in chub gills are presented in Table 4. Depending on the sampling period, MTs in the chub gills constitute on average 1.7-2.7% of total cytosolic proteins. The percentage partition of MTs in the chub gill tissue was, therefore, somewhat higher compared with the liver tissue of chub (1.3%; Podrug and Raspor, 2009) and of red mullet (*Mullus barbatus*; 0.9%; Filipović Marijić and Raspor, 2006). Even higher MT partition was obtained in the intestine of these same chub (3-4%, Filipović Marijić, 2009). Since gill and intestinal epithelial tissues are involved in the uptake, detoxification and excretion processes (Van Cleef et al., 2000), higher MT presence in those tissues is probably associated with the important function of MTs in metal uptake, as well as their protective role against excessive uptake.

The association between MTs and TPs was analyzed for each sampling campaign by Spearman correlation coefficients, using the complete data sets. As expected, in all three campaigns the correlation between TPs and MTs was positive and statistically significant (Table 5). However, it is important to recall that both parameters share a common influencing factor, the gill mass; the correlations of MTs and TPs with the gill mass were positive in September campaigns and negative in April/May campaign (Tables 2 and 5; Dragun et al., 2007), which possibly account for at least part of the positive correlation between MTs and TPs. Furthermore, MT correlation with the gill mass was (Table 2) always stronger than MT correlation with TPs (Table 5).

After exposure to low dissolved metal concentrations in river water, as reported for the river Sava (Dragun et al., 2008 and 2009b), the induction of MT synthesis by toxic metals is not necessarily anticipated. In such cases the positive association with essential elements Zn and Cu is more often reported in different fish tissues, because of MT involvement in their regulation (Nordberg, 1998). Accordingly, based on the complete data sets, the correlations between MTs and nonessential metal Cd were very low, explaining only up to 6% of MT variance (Table 5). The correlations between MTs and essential metals, positive with Zn and negative with Cu, were somewhat higher, but statistically significant only in September campaigns (Table 5). However, same as in the case of TPs, it could be presumed that these correlations were partially influenced by a common factor, i.e. the gill mass; positive correlation of gill mass with MTs and Zn, and negative with Cu (Tables 2 and 5; Dragun et al., 2007), could strenghten the association between MTs and these essential metals.

Site-specific MT variability

The differences in MT levels between sampling sites were less pronounced than, for example, the differences within sites or between sampling campaigns (Table 1). According to one-way ANOVA, differences between sites were statistically significant in September, 2005 (Fig. 2a) and April/May, 2006 (Fig. 2b). In September 2005, maximal median MT level measured at Oborovo was approximately 40% higher than minimal level measured at Jasenovac; in April/May 2006, the difference between maximal and minimal median MT level was only 26%, and it dropped to 18% in September 2006, when even ANOVA did not indicate significant differences between sites (Fig. 2c). Using the Spearman correlation coefficients between the median values obtained at each site for MTs and for several analyzed parameters (gill mass, FCI, GSI, TPs, Zn, Cu, and Cd) we intended to isolate those which showed the

best agreement with MT spatial variability (Table 6). In both September campaigns, total protein concentrations, Fulton condition indices and the gill masses could be extracted as parameters having similar site-specific distribution as MTs, indicating to strong MT association with fish size and condition. The highest MT levels in September 2005 were obtained at Oborovo, the site where the biggest chub were caught (Dragun et al. 2009a), while in September 2006 MTs were the highest at Otok Samoborski and Jasenovac, again following the spatial variability of the gill mass. This influence has attenuated in the spring period, leading to overall lesser association between spatial distribution of MTs and other measured parameters (Table 6). However, MTs in the spring period seem to be more closely related to the changes of the cytosolic concentrations of essential metals Zn (positive association) and Cu (negative association) than of the biometric parameters. It is important to point out that the concentrations of essential metals (like Zn and Cu) in gill cytosol seem to be regulated at low level of metal exposure as reported for river Sava (Dragun et al., 2008 and 2009b), and do not reflect fine differences in ambient metal concentrations (Dragun et al., 2009a). Therefore, MT association with cytosolic Zn and Cu concentrations is more probably related to its function in their regulation, than to the level of exposure in water. The spatial distribution of cytosolic Cd concentrations, on the other hand, indicated significant increase of Cd uptake at Oborovo and Lukavec Posavski in the spring period (Table 4 and Dragun et al., 2009a), which was not always accompanied by increase in MT levels (Figure 2b, Table 6).

In addition to our results which emphasized vague site-specific association between chub gill MTs and cytosolic metal levels, especially for non-essential metal Cd, Hayes et al. (2004) have also questioned the use of MTs from chub tissues as a biomarker of metal exposure. Their study was conducted in three rivers in England, with the lowest dissolved Zn and Cd concentrations in the Blythe River (4.66 $\mu g\ L^{-1}$ and 0.083 $\mu g\ L^{-1}$, respectively), intermediate in the Cole River (12.97 $\mu g\ L^{-1}$ and 0.100 $\mu g\ L^{-1}$, respectively) and the highest in the Tame River (36.12 $\mu g\ L^{-1}$ and 0.138 $\mu g\ L^{-1}$, respectively) (Garofalo et al. 2004; Matthew Winter and Elisabetta Garofalo, *personal communications*). MT induction was observed only in the gills of feral European chub from the most contaminated Tame River, but even that increase was not statistically significant because of high interindividual variability.

However, the results of some other studies showed that the level of metal exposure which can induce additional MT synthesis, as well as a time lag between the exposure and biological

effect, can be regarded as important factors, and not only the selected fish species. For example, in spite of the steady increase of gill Cd concentrations and induced transcription of MT-A gene during the exposure period, Hansen et al. (2007) also did not obtain the increase of MT levels in gills of another fish species, brown trout (*Salmo trutta*), after transplantation from reference Stribekken River to Cd/Zn contaminated Naustebekken River, at total Cd concentration in river water equal to 0.147 μg L^{-1}. Lange et al. (2002), on the other hand, after exposure to much higher concentrations of Cd (10 μg L^{-1}), obtained the significant increase of MT gene expression in the gills of rainbow trout (*Oncorhynchus mykiss*) only after 28 days of exposure, whereas the significant effect was not observed after 14 days of exposure, in spite of the obvious accumulation of Cd in gills. Contrary, clear increase in MT levels was reported by Gonzalez et al. (2006) for gills of zebrafish (*Danio rerio*) already after seven days of fish exposure to approximately 2 μg Cd L^{-1}, which resulted in two fold, and 10 μg Cd L^{-1}, which resulted in five fold MT increase. Cadmium concentrations in the study by Gonzalez et al. (2006) were approximately 14 and 70 times higher than Cd concentrations to which brown trout was exposed in the study reported by Hansen et al. (2007), but comparable to the level of Cd exposure applied in the study by Lange et al. (2002), indicating the dependence of MT induction in gills on both the level of metal exposure and the investigated fish species. Another important factor is the ability of particular metal to induce MT synthesis. For example, native brown trout (*Salmo trutta*) from Cu contaminated Rugla River (total Cu: 17.4 μg L^{-1}; labile Cu: 4.6 μg L^{-1}) did not have increased gill MT compared to the reference river, which can be explained by prevention of Cu uptake in brown trout chronically exposed to Cu (Hansen et al., 2006), but also by known fact that Cu is inferior inducer of MT synthesis than Cd and Zn (Klaassen et al., 1999). In a conclusion, although so far conducted studies on chub gill MT indicated that this biomarker is not applicable for monitoring in the freshwater with low concentrations of dissolved metals, the possibility to use the chub gill MT as the indicator of higher level of metal exposure should be further investigated.

Constitutive MT levels in the gills of 2- and 3-year old European chub

In the Sava River, even maximal dissolved concentrations of Zn and Cd reported for the spring period of 2006 (8.74 μg L^{-1} and 0.020 μg L^{-1}, respectively; Dragun et al. 2009b) were lower than the concentrations reported by Garofalo et al. (2004) for the Cole River, in which the increase of chub gill MT level was still not observed (Hayes et al. 2004). Therefore, MT levels measured in the gills of chub caught in moderately contaminated Sava River can be

regarded as the constitutive MT levels. In the literature, the basal levels of MTs have been shown to vary with time of year, reproductive state, water temperature and developmental state (George and Olsson, 1994). Therefore, we have defined the constitutive gill MT ranges for young specimens of European chub (2-3 years old) of both sexes separately for periods of lower (autumn) and higher metabolic activity (spring). Constitutive MT range representative for the slowed down metabolism of chub in the post-spawning period was defined as 1.31-2.00 mg g^{-1}, and is based on the MT levels measured in the gills of 112 chub specimens caught in both September sampling campaigns (68% of measured MT values: average ± 1 standard deviation). It is comparable with constitutive MT range in the chub liver characteristic for the non-reproductive period (1.24-2.02 mg g^{-1}; Podrug and Raspor, 2009). Constitutive MT range representative for the period of higher metabolic activity was defined as 2.15-2.95 mg g^{-1}, based on the measurements in the gills of 70 chub specimens caught in the Sava River in spring 2006; only 3% of studied specimens in the spring period had gill MT levels within the constitutive range defined for autumn period.

Conclusions

The study on MT levels in the gills of European chub (*Squalius cephalus*) revealed high interindividual, interannual, and especially interseasonal variability of this biomarker. The most prominent difference was obtained between spring and autumn periods, probably because of the differences in chub metabolic activity and gill ventilation rate. Spring increase of MT level was accompanied by increase in TP and Zn concentrations in the gill cytosol. In the gills of 2- and 3-year old chub specimens, MT levels did not differ between males and females, as well as between mature and non-mature fish. Among analyzed biotic factors, the gill mass showed the strongest association with MT levels, positive in autumn periods, and negative in the spring period, consistant with the previous observations made for total cytosolic proteins. In September sampling campaigns, which were considered as representative for non-reproductive period of lower metabolic activity, site-specific MT variability was largely comparable to spatial variations of total cytosolic proteins, the gill mass and Fulton condition indices. Contrary, in the spring, as period of higher metabolic acitivity, spatial distribution of MTs was more related to variations of essential metals Zn and Cu, probably reflecting MT role in their regulation. At low level of dissolved Zn, Cu and Cd in river water chub gill MT is not useful as a biomarker of metal exposure. Gill MT levels measured in this study were, therefore, considered as constitutive levels of young chub

504 specimens, and the basal ranges were defined separately for periods of lower and higher
505 metabolic activity (autumn and spring, respectively).
506

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678

Figure Captions

Figure 1. The map of the studied section of the Sava River in Croatia, with marked sampling sites: Otok Samoborski (OS, N 45° 50,543' E 15° 43,497') - low polluted, reference location 10 km upstream of the city of Zagreb; Sava in Zagreb (SZ, N 45° 46,572' E 15° 56,524') - 20 km upstream of the main household and industrial wastewater outlets; Oborovo (OB, N 45° 41,286' E 16° 14,875') - downstream of the main effluent outlets of the cities of Zagreb and Velika Gorica (15 and 5 km, respectively); Lukavec Posavski (LP, N 45° 24,081' E 16° 32,337') and Jasenovac (JAS, N 45° 15,825' E 16° 53,658') - 15 and 50 km downstream of the city of Sisak, respectively.

Figure 2. Metallothionein levels in the gills of the European chub caught in the Sava River at five sites in three sampling campaigns: a) September, 2005; b) April/May, 2006, c) September, 2006; data are presented as the boxes whose boundaries indicate 25^{th} and 75^{th} percentiles; a line within the box marks the median value; whiskers below and above the box indicate 10^{th} and 90^{th} percentiles; the outliers are presented with black dots; the differences between sites were evaluated using one-way ANOVA on transformed data ($\log_{10}$), and the level of significance (p) is given in the figure; *post hoc* comparisons were made by Bonferroni t-test, and the sites that differ significantly in MT level are specified in the figure.

Table 1. The analysis of the differences in MT levels (mg g^{-1}) between three sampling campaigns (two-way ANOVA with period and site as independent factors) and analysis of sex-age differences (two-way ANOVA with sex and age as independent factors).

	All specimens	2-year old			3-year old			[b]p			
	Least square mean	Response means		95% CI	Response means		95% CI	Model	Age	Sex	Age×Sex
	95% CI[a]	Males	Females	All	Males	Females	All				
September, 2005	[c]1.71 1.63-1.79	1.73	1.70	1.59-1.84	1.65	1.86	1.61-1.90	0.402	0.171	0.314	0.646
April/May, 2006	[c]2.53 2.42-2.64	2.54	2.74	2.46-2.82	2.41	2.47	2.30-2.55	<0.05	<0.05	0.147	0.589
September, 2006	[c]1.43 1.34-1.53	1.44	1.35	1.28-1.53	1.42	1.53	1.38-1.58	0.160	0.250	0.953	0.149

[a] 95% confidence interval

[b] the level of significance for sex-age analysis

[c] statistically significant difference was obtained between three sampling campaigns ($p<0.0001$); the pairwise comparisons made with Bonferroni *t*-test indicated to significantly higher MT level in April/May sampling compared with two September samplings ($p<0.05$) at all five sites; the differences between September samplings were significant at three sites (Sava-Zagreb, Oborovo, Lukavec Posavski)

Table 2. The gill masses of European chub and Spearman correlation coefficients between MTs and the gill mass (GM) for each of five sites in three sampling campaigns; the gill masses are expressed as average ± standard deviation, whereas for all sites together minimum and maximum values are also provided; the levels of significance for correlation coefficients are given below the table.

	September, 2005			April/May, 2006			September, 2006		
	n	**GM / g**	**r**	**n**	**GM / g**	**r**	**n**	**GM / g**	**r**
OS	21	0.59±0.39	0.376	15	0.52±0.28	[b]-0.804	11	0.92±0.32	0.364
SZ	15	0.70±0.20	0.009	16	0.55±0.25	-0.187	10	0.44±0.23	0.430
OB	15	0.94±0.32	[a]0.522	11	0.75±0.33	-0.109	9	0.35±0.21	[b]0.817
LP	16	0.37±0.13	0.338	15	0.58±0.15	-0.014	6	0.31±0.12	0.257
JAS	6	0.46±0.21	0.371	13	0.80±0.20	-0.382	3	0.97±0.11	0.500
All sites	**73**	**0.62±0.34 0.17-1.55**	**[c]0.474**	**70**	**0.63±0.26 0.17-1.37**	**[b]-0.319**	**39**	**0.58±0.36 0.08-1.43**	**[c]0.603**

[a] p<0.05 [b] p<0.01 [c] p<0.0001

Table 3. The analysis of differences in chub gill masses (GM), gonadosomatic indices (GSI) and Fulton condition indices (FCI) between three sampling campaigns (one-way ANOVA on transformed data: square root for GM, inverse of square root for GSI, and $\log_{10}$ for FCI; Welch's variance-weighted ANOVA was used for FCI comparison, due to unequal variances). The pairwise *post-hoc* comparisons were made by Bonferroni t-test.

	Gill mass / g p>0.05		**Gonadosomatic index / % p>0.05**		**Fulton condition index / g×100/cm^3, p<0.0001**	
	Least square mean	**95% CI**	**Least square mean**	**95% CI**	**Least square mean**	**95% CI**
September, 2005	0.58	0.51-0.65	0.59	0.52-0.66	0.95	0.93-0.97
April-May, 2006	0.60	0.53-0.67	0.53	0.47-0.60	[a]1.06	1.04-1.08
September, 2006	0.52	0.43-0.62	0.51	0.43-0.59	0.94	0.92-0.97

[a] significantly higher FCI ($p<0.05$) in April/May 2006 compared to both September samplings

Table 4. Total protein, Zn, Cu and Cd concentrations in the gill cytosol of 2- and 3-year old European chub caught in Sava River at five sampling sites in three sampling campaigns: median, minimum and maximum values.

Site	Total proteins / mg mL^{-1})			Zn / μg mL^{-1})			Cu / ng mL^{-1})			Cd / ng mL^{-1})		
	Sep/'05	**Apr/'06**	**Sep/'06**	**Sep/'05**	**Apr/'06**	**Sep/'06**	**Sep/'05**	**Apr/'06**	**Sep/'06**	**Sep/'05**	**Apr/'06**	**Sep/'06**
OS	9.95 7.80-12.4	25.5 21.8-38.8	14.8 12.6-16.8	4.71 2.91-8.25	11.3 7.83-15.6	6.94 5.00-10.9	64.6 44.6-126.8	77.4 47.6-94.9	29.6 25.9-40.9	2.79 1.73-6.39	1.77 1.30-2.33	1.09 0.83-1.33
SZ	13.7 9.35-18.0	20.9 16.5-28.6	13.6 9.14-15.1	6.96 3.70-12.6	10.6 8.81-16.2	7.10 5.94-7.96	67.9 47.6-107.3	67.6 49.7-92.2	33.3 23.6-54.8	1.97 1.69-3.21	1.93 1.51-2.29	0.96 0.88-1.08
OB	12.4 8.67-14.4	16.8 10.4-25.5	13.2 10.8-15.5	7.06 5.54-10.3	10.9 7.38-12.4	7.29 5.54-8.52	41.1 33.8-75.3	69.4 40.4-148.2	41.6 34.6-48.8	3.87 3.18-7.78	4.31 2.84-8.42	1.09 1.05-1.39
LP	9.86 7.73-11.5	14.7 9.90-19.1	14.6 12.0-18.3	4.95 3.08-8.40	8.37 5.30-12.8	4.11 3.60-6.59	70.9 29.0-178.8	72.1 46.6-117.2	42.3 38.6-53.3	2.56 1.85-3.10	3.93 2.86-26.6	1.24 1.07-1.26
JAS	9.59 7.82-13.4	18.2 12.5-22.8	16.5 14.2-16.6	5.98 3.75-13.5	9.70 7.97-15.4	8.13 6.73-8.59	71.6 35.1-87.6	88.5 57.5-182.0	33.7 33.2-35.1	2.17 1.40-3.19	2.08 1.37-9.96	1.22 1.03-1.26

Table 5. The Spearman correlation coefficients of total cytosolic proteins and gill cytosolic concentrations of three metals with MTs and the gill mass for each sampling campaign (based on the whole data sets); the levels of significance for correlation coefficients are given below the table.

	Correlation with MTs				Correlation with the gill mass		
	Total proteins	**Zn**	**Cu**	**Cd**	**Total proteins**	**Zn**	**Cu**
September 2005	[c]0.425	[d]0.500	[a]-0.299	[a]0.249	[d]0.692	[d]0.548	[d]-0.477
April-May 2006	[a]0.268	0.213	-0.165	0.231	[a]-0.297	-0.096	-0.130
September 2006	[c]0.514	0.329	[b]-0.488	-0.011	[a]0.373	[b]0.459	[c]-0.567

[a] $p<0.05$ [b] $p<0.01$ [c] $p<0.001$ [d] $p<0.0001$

Table 6. The variability of MT levels between sampling sites: correlations with the gill masses, Fulton condition indices (FCI), gonadosomatic indices (GSI), the concentrations of total proteins and three metals in the chub gill cytosol in three sampling campaigns; Spearman correlation coefficients (*r)* are based on the median values calculated for each sampling site (within each sampling campaign: n=5).

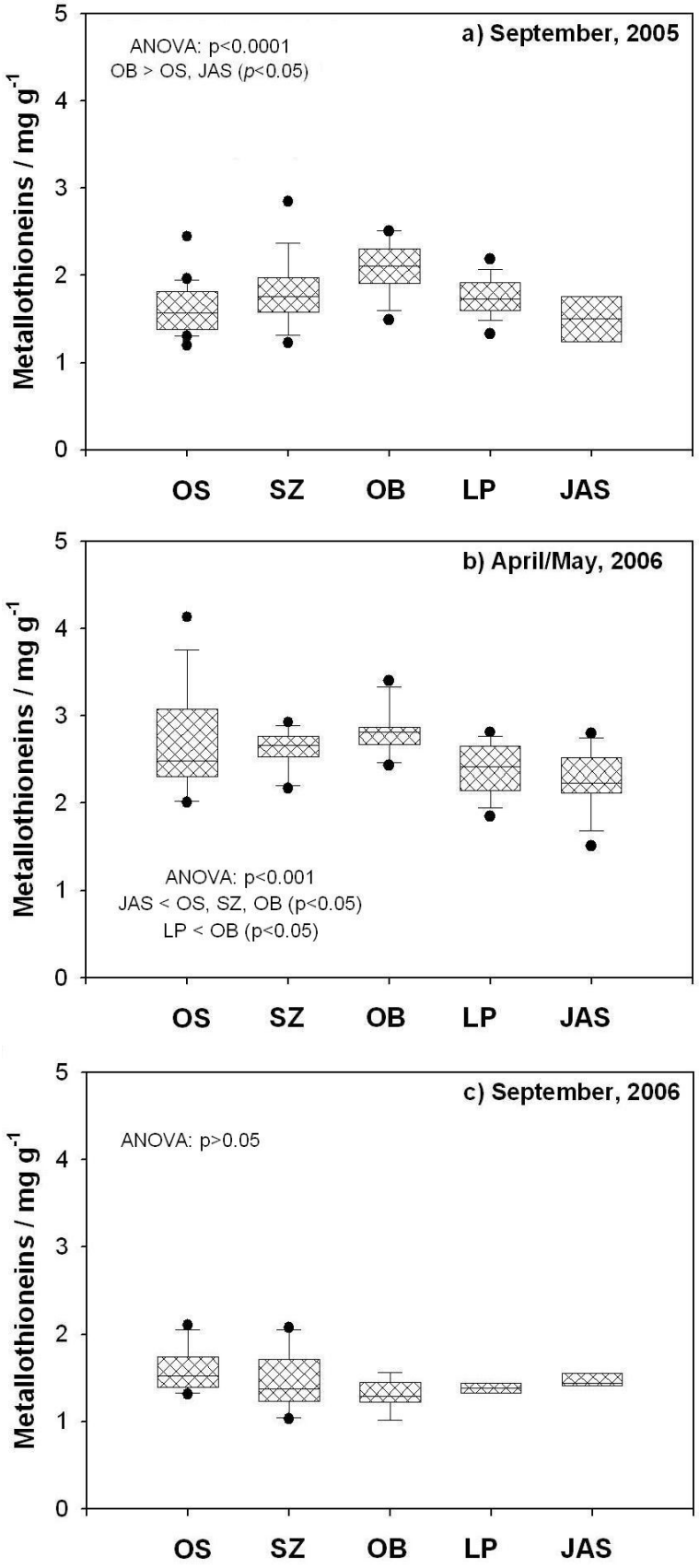
	Gill mass	FCI	GSI	Total proteins	Zn	Cu	Cd
September, 2005	0.700	0.700	0.700	0.800	0.700	-0.700	0.300
April-May, 2006	-0.300	-0.500	0.100	0.100	0.600	-0.800	0.200
September, 2006	0.600	0.700	0.100	[a]0.900	-0.200	-0.500	0.500

[a] $p<0.05$

Figure 1.


30 km
Slovenia
Hungary
river Sava
OS
SZ
Zagreb
OB
Velika Gorica
Croatia
Sisak
LP
JAS
river Sava
Bosnia and Herzegovina

Figure 2.


a) September, 2005
ANOVA: p<0.0001
OB > OS, JAS (p<0.05)
b) April/May, 2006
ANOVA: p<0.001
JAS < OS, SZ, OB (p<0.05)
LP < OB (p<0.05)
c) September, 2006
ANOVA: p>0.05
Metallothioneins / mg g-1
0
1
2
3
4
5
OS
SZ
OB
LP
JAS