Combined use of bioindicators and passive samplers for the assessment of the river water contamination with metals

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Abstract

In the autumn 2005, the site-specific variability of cytosolic metal and protein concentrations in the gills of the European chub from the Sava River could be mostly associated to the gill mass variability. In the spring 2006, the correlations of metals and proteins with the gill mass were mainly non significant, and their site-specific variability could be presumably associated to the river water pollution. The spring cytosolic concentrations of Zn, Cu and Mn have not differed significantly between the sites (median: 8.37-11.34 μg mL^{-1}, 68.2-86.2 ng mL^{-1}, 55.9-68.6 ng mL^{-1}, respectively), while increased cytosolic Cd concentrations were obtained at Oborovo and Lukavec Posavski (median: 4.01 ng mL^{-1}), the sites influenced by the pollution sources from two major urban areas, compared to the remaining sampling sites (median: 1.93 ng mL^{-1}). The cytosolic Fe concentrations were almost twice higher at Jasenovac (median: 11.98 μg mL^{-1}) compared to the concentrations at Sava-Zagreb (median: 6.72 μg mL^{-1}). The labile Fe concentrations measured in the river water with passive samplers indicated that cytosolic Fe concentrations in the spring possibly reflected the water-borne Fe-uptake. The spring cytosolic protein concentrations decreased from upstream (Otok Samoborski: 27.2±5.6 mg mL^{-1}) towards the downstream sites (Lukavec Posavski: 14.8±2.8 mg mL^{-1}), possibly due to the influence of organic pollution and water toxicity. The spring period seemed to be more appropriate season for the assessment of the river water pollution, if the chub gills are used as the target organ.

Key words: metals, proteins, gill cytosol, European chub, River Sava

Introduction

The simple approaches of chemical water analyses often fail to detect environmental changes that are harmful for aquatic organisms; the complex mixture of pollutants in natural waters, with their synergistic and antagonistic effects, combined with variable physico-chemical conditions make it difficult to predict the impact of toxicants on the ecosystem. Bioindicators and, in particular, long-living organisms such as fish are sensitive to the impact of a complex mixture of chemicals on a specific aquatic system, while tissue concentrations of chemicals present excellent indicators of their bioavailability in the environment (Chovanec et al. 2003).

In the freshwater fish, two different routes of uptake are important for metals: directly from the water via the gills and from the food via the digestive tract. The gills make a good indicator tissue based on the several reasons: for example, large respiratory surface and a high pumping rate of water enhances the direct uptake of water-borne toxicants (Chovanec et al. 2003); furthermore, the gills of various fish species were previously reported to reflect the metal contamination gradient in the ambient water (Andres et al. 2000), as well as to respond quickly to changes in metal exposure (Kraemer et al. 2005). Laboratory exposures have shown that metal concentrations will likely be higher at the site of uptake at the beginning of exposure (Giguère et al. 2004), and therefore, after short-term exposure, gills, digestive tract, and liver usually show a high load of toxicants, whereas concentrations in kidney, bones and muscles increase more slowly after a time-lag (Olson et al. 1978; Köck et al. 1996). Consequently, the metal concentrations in the gills are expected to reflect the short-term metal exposure in the water, contrary to the metal concentrations in several other organs which represent the long-term storage of metals (Roméo et al. 1999).

Accordingly, the main objective of this study was to assess the metal pollution status of Sava River water by supplementing the available information on the water contamination with metals (Dragun et al., 2008a; Dragun et al., 2008b) with the information on metal levels in the gills of European chub (*Squalius cephalus*). Typically, 50-80% of the metals stored in cells are found in the cytosol; during chronic exposure to metals the portion of cytosolic metals increases in relation to the total metal load of the cell (Olsson et al. 1998). The importance of the cytosol is associated to the fact that it comprises two important cellular fractions: heat-sensitive proteins, such as enzymes, and heat-stable proteins, such as metallothioneins (Wallace and Luoma 2003). Therefore, it comprises the metal fraction which is expected to be associated with some biological impairment (bound to heat-sensitive proteins) (Wallace et al. 2000), but also the metal fraction which is considered as detoxified and not available to more sensitive cellular fractions (bound to heat-stable proteins) (Kreamer et al. 2005). In this study, the concentrations of nonessential metal Cd and four essential metals were, thus, determined in the cytosolic fraction of the chub gills. Although Zn, Cu, Fe and Mn are essential for the health of most organisms, forming integral components of proteins involved in all aspects of biological function, in excess they can also be toxic, binding to biologically sensitive molecules or forming dangerous free radicals (Bury et al. 2003).

Due to the direct or indirect influence of very large set of abiotic and biotic factors on metal concentrations in fish tissues (Andres et al. 2000), we have first analyzed the variability of cytosolic metal and protein concentrations that could be associated to the season and the biotic factors, such as age and size (Dragun et al. 2007). The data analysis in this paper was, therefore, focused on clarifying the site-specific variability of the cytosolic metal and protein concentrations in the chub gills, with the general aim to assess the Sava River water contamination with metals. Additionally, the cytosolic metal concentrations in the chub gills

were compared with labile metal concentrations in the Sava River water, which were measured simultaneously with fish sampling using the passive samplers for metals (diffusive gradients in thin films, i.e. DGTs) (Dragun et al. 2008a).

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, 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 (1 million inhabitants, heavily industrialized) up to the sites affected by the pollution load from the Zagreb and Sisak city areas (Krča et al. 2007). Five sampling sites were selected (Figure 1, Table 1): Otok Samoborski - low polluted, reference location 10 km upstream of the city of Zagreb; Sava in Zagreb - located within the Zagreb city area, but 20 km upstream of the main household and industrial wastewater outlets; Oborovo - 15 km downstream of the main effluent outlet of the city Zagreb (mixed industrial and municipal wastewater) and 5 km downstream of the wastewater outlet of the city Velika Gorica (60,000 inhabitants); Lukavec Posavski - 15 km downstream of the city of Sisak (50,000 inhabitants, pesticides production facility, ironworks and oil refinery); Jasenovac - 50

km downstream of the city of Sisak, close to the confluence of the Una River, Sava River right tributary (Krča et al. 2007). The basic physico-chemical parameters of Sava River water were measured at the selected sampling sites (Table 1). The measurements of the dissolved oxygen level in the river water and the water temperature were carried out *in situ* using the portable device MO 128 (Mettler Toledo), while for the determination of pH (pH-meter MP120, Mettler Toledo) and conductivity (conductivity meter S30 Seven Easy, Mettler Toledo) river water was sampled in the plastic bottles.

The samplings were conducted in two seasons, autumn 2005 and spring 2006. The autumn sampling was accomplished within 10 days, at the end of September 2005 (September 19th to 28th), after the spawning period. Due to the unfavourable weather conditions, the spring sampling, conducted during April and May 2006, lasted over one month (April 18th to May 25th). The sampling was performed by electro fishing, according to the Croatian standard HRN EN 14011:2005. In autumn 2005, the following number of European chub specimens was caught: 22 at Otok Samoborski, 15 at Sava in Zagreb, 15 at Oborovo, 16 at Lukavec Posavski, and 10 at Jasenovac. In spring 2006, 14 specimens were caught at Otok Samoborski, 18 at Sava in Zagreb, 12 at Oborovo, 15 at Lukavec Posavski, and 14 at Jasenovac. The captured fish were kept alive in aerated water tank till further processing in the laboratory. After the fish were anesthetized with MS 222 (tricaine methane sulphonate, Sigma Aldrich) and sacrificed, the gills were isolated, weighted and stored at -80°C until further analyses.

Isolation of cytosolic fraction

Isolation of the S50 cytosolic fraction from the chub gill tissue was performed according to SOP (1999) developed at NIVA in the frame of the BEQUALM programme. 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-Elverhjem 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 total cytosolic protein concentration

Total cytosolic protein 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 measurement was 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 five metals (Zn, Fe, Cu, Mn and Cd) were measured in a duplicate in the five times diluted S50 fraction of chub gills by Varian SpectrAA 220 atomic absorption spectrometer. Flame technique (air/acetylene) was applied for the measurement of Zn (at 213.9 nm) and Fe (at 248.3 nm), while graphite furnace with universal platforms (Varian

GTA-100) was used for the measurement of Cu (at 324.8 nm) (Dragun and Raspor 2008), Mn (at 279.5 nm) and Cd (at 228.8 nm). Deuterium lamp was used for background correction. External calibration was performed for each metal. The calibration standards were prepared using the metal stock solutions (Zn, Fe, Cu, Mn or Cd, 1000 mg L^{-1} by Merck) and five times diluted homogenization buffer, which was also used as the blank sample. The detection limits for five measured metals were the following: Zn 0.007 mg L^{-1}; Fe 0.015 mg L^{-1}; Cu 0.270 μg L^{-1}; Mn 0.224 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. 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 standard statistical package SigmaStat for Windows, Version 1.0. The gill masses and cytosolic metal and protein concentrations measured in the chub gills at different sites were compared using Kruskal-Wallis test followed by Dunn's test. The levels of significance for Kruskal-Wallis test are given throughout the text, while the level of significance for Dunn's test was always $p<0.05$. The association of the chub size with the metal and protein concentrations was previously tested using the Pearson correlation and linear regression analyses, while the differences between two seasons were tested using two-way ANOVA and Bonferroni t-test; the results of those analyses were presented in detail in Dragun et al. (2007).

Results and Discussion

According to our previous reports on heavy metal concentrations in the Sava River water, this river is exposed to anthropogenic influence, but is still comparable with relatively unpolluted world rivers (Dragun et al. 2008a; Dragun et al. 2008b). Therefore, we had the opportunity to study the changes of the metal concentrations in the chub gill cytosol in only moderately polluted river system, and to establish if the cytosolic metal levels reflect subtle differences in metal concentrations in the river water observed between different sites.

The variability of the cytosolic metal and protein concentrations in the autumn period

In the autumn period, strong positive correlations with the gill mass were obtained for the cytosolic concentrations of total proteins (r=0.327 to 0.885), Mn (r=0.295 to 0.794) and Zn (r=0.216 to 0.657) (Dragun et al. 2007). The gill mass was used as the indicator of the fish size, due to the high positive and statistically significant correlations (r= 0.720 to 0.994; $p<0.01$) obtained between three biometric parameters associated to the chub size (length, total mass and gill mass) (Dragun et al. 2007). Accordingly, the site-specific distribution of total proteins, Mn and Zn (Figure 2c, Figure 3 a and c) was comparable to the site-specific distribution of the chub gill masses, which were the highest at Oborovo and Sava-Zagreb, and the lowest at Lukavec Posavski (Figure 2a, statistically significant differences, $p<0.001$). Although the statistically significant differences in total protein and Mn ($p<0.0001$), as well as Zn concentrations ($p<0.01$) were also obtained between different sites, the chub size have to be considered as a possible cause of these differences, and not necessarily different fish exposure to metals in water. The differences between sites in the cytosolic concentrations of remaining two essential metals, Fe and Cu, were also statistically significant ($p<0.05$ and $p<0.01$, respectively). Their correlations with the gill mass were, however, weaker, positive in

the case of Fe (r=0.258 to 0.505) and negative in the case of Cu (r=-0.026 to -0.594) (Dragun et al. 2007). Accordingly, somewhat higher cytosolic Fe concentrations (Figure 4a) and lower Cu concentrations (Figure 3e) were found at Oborovo, the site where the biggest fish were caught. For both Fe and Cu, cytosolic concentrations measured at four remaining sites were comparable. Only for nonessential metal Cd, due to the lack of clear association with the gill mass (Dragun et al. 2007), it could be assumed that statistically significant differences between sites (p<0.0001) possibly originated from different exposure in water (Figure 4c). The sampling site Oborovo, which is located downstream of the municipal sewage outlets of cities Zagreb and Velika Gorica, was the site with the highest cytosolic Cd concentration in the chub gills, about 100% higher compared to the lowest measured cytosolic Cd concentration at the sampling site in the city of Zagreb.

The variability of the cytosolic metal and protein concentrations in the spring period

The seasonal changes of metal concentrations in the fish tissues can arise due to the changes of the feeding and growth rates, as well as the result of the changes in the fish condition (McCoy et al. 1995; Farkas et al. 2002). Fulton condition indices of European chubs caught in the spring period (1.07±0.10%) were higher compared to the autumn period (0.96±0.07%; Dragun et al. 2007), indicating to more intense recent feeding activity (Lambert and Dutil 1997). Furthermore, the liver mass, as well as the cytosolic Mn and metallothionein concentrations in the liver of the same chub specimens as used in this study were also higher in the spring than autumn period (Podrug and Raspor 2008). Since the metal concentrations, especially for essential metals like Zn, increase following the increase of the fish metabolic activity (Andres et al. 2000), it can be hypothesized that higher Zn and Fe concentrations in the chub gill cytosol in the spring period compared to the autumn (Figures 3c-d and 4a-b) were an indication of the increased metabolic and feeding rates of European chub, as

previously elaborated (Dragun et al. 2007). Consequently, due to the well known negative association of metabolic and filtration rates with the fish size (Neely 1979), the positive correlations between the gill mass and the cytosolic metal and protein concentrations obtained in the autumn have attenuated in the spring; they were mainly non significant (Dragun et al. 2007). Although Kruskal-Wallis test has indicated to the significant differences between the gill masses obtained at different sites ($p<0.05$), the gill size was more uniform in the spring compared to the autumn period (Figure 2a-b). It can be, thus, assumed that possible differences of the cytosolic metal levels between sites in the spring period could be attributed to different metal bioavailability from river water. However, the concentrations of three essential metals (Mn, Zn and Cu) varied very little between sites (Figures 3 b, d, f), and only statistically significant difference was associated to lower Zn concentration at Lukavec Posavski compared to three other sites (Figure 3d). It can be explained by the fact that the concentrations of essential elements, such as Zn and Cu, are generally efficiently regulated in the fish tissues by homeostatic processes, except at highly polluted sites (Andres et al. 2000; Sorensen 1991). Andres et al. (2000), for example, reported that although Zn concentrations in the river water varied greatly between sites in river Lot and its affluent, Zn bioaccumulation in chub gills varied only slightly. Similar observation was made by Giguère et al. (2004) for Cu concentrations in the gills of yellow perch (*Perca flavescens*) in Canadian lakes, while Amundsen et al. (1997) reported that both Zn and Cu in the gills of whitefish (*Coregonus lavaretus*) in river Pasvik did not reflect increase of Zn and Cu concentrations in the water in the vicinity of smelters. Among the essential metals measured in this study, the cytosolic Fe concentrations exhibited the most pronounced site-specific variability in the spring period (Figure 4b; $p<0.0001$). Although the significant differences between seasons indicated that Fe concentrations are strongly influenced by the fish physiological status

(Dragun et al. 2007), the site differences within spring season possibly reflected different Fe-exposure in water at different sites.

For nonessential metals, such as Cd, the concentration gradient in water can be also expected in fish organs (Andres et al. 2000), because Cd tissue concentrations are independent of strict physiological control which is characteristic for the majority of essential metals (Sorensen 1991). Therefore, it can be assumed that higher Cd concentrations measured in the fish gills reflect higher bioavailability in the ambient water, as previously confirmed by Kraemer et al. (2005) in the study on yellow perch (*P. flavescens*) in Canadian lakes, by Andres et al. (2000) in the study on chub (*S. cephalus*), roach (*Rutilus rutilus*), perch (*Perca fluviatilis*) and bream (*Abramis brama*) in river Lot, and by Amundsen et al. (1997) in the study on whitefish (*C. lavaretus*) in river Pasvik. In our study, statistically significant differences of the cytosolic Cd concentrations in the chub gills between sites were obtained again in the spring period (p<0.0001), and, same as in the autumn, indicated to increased Cd bioavailability at Oborovo (Figure 4c-d). However, in the spring period, increased cytosolic Cd was also found at Lukavec Posavski, and the differences between sites were more pronounced than in the autumn (Figure 4d). Therefore, the hypothesis can be made that in the spring period increased water filtration and feeding rates lead to increased uptake of metals, which resulted in easier identification of metal contaminated sites, at least for nonessential metals, such as Cd, and only for a particular essential metal, i.e. Fe.

In the spring period, distinctive site-specific distribution was furthermore observed for total cytosolic proteins. The highest protein level was obtained at the most upstream sampling site Otok Samoborski (27.2±5.6 mg mL^{-1}), which is assumed to have the least polluted river water, and then gradually decreased towards the lowest level of 14.8±2.8 mg mL^{-1} at Lukavec

Posavski (Figure 2d), and the differences were statistically significant (p<0.0001). The protein concentrations were significantly higher in the spring period than in the autumn at all sampling sites (Figure 2c-d; Dragun et al. 2007), but the relative increase was more pronounced at the reference site Otok Samoborski (2.7 times) compared to downstream sampling sites (about 1.5 times). As seen from Figures 2d and 4d, the sites with the lowest protein concentrations were at the same time the sites with the highest cytosolic Cd concentrations. Metal exposure has been shown in the laboratory to affect protein metabolism; study on yellow perch (*Perca flavescens*) in metal contaminated Whitson lake, for example, suggested that metal exposure could be related to lower tissue protein concentrations or lower biosynthetic capacities (Audet and Couture 2003). However, due to only moderate metal pollution reported for Sava River water (Dragun et al. 2008a; Dragun et al. 2008b), it is more probable that decreased total protein concentrations in the chub gills at the sites downstream of the city of Zagreb, and especially at Oborovo and Lukavec Posavski, were not solely associated to metal pollution, but rather reflected the combination of different types of pollution. This conclusion can be supported by the results of several chemical and toxicological assays performed during the SARIB project (European FP6 project - Sava River Basin: Sustainable Use, Management and Protection of Resources), which have indicated to moderate organic pollution (PCB, PAH) and water toxicity at the sites downstream of the city of Zagreb, most probably as a reflection of enhanced hydrocarbon input by main municipal sewage vent at Oborovo, and the oil refinery and ironworks situated in the city of Sisak (Čalić et al. 2006; Čalić et al. 2007; Krča et al. 2007; Källqvist et al. 2008; Grung et al. 2007). More prominent influence of the polluted water on the protein concentrations observed in the chub gill cytosol in the spring than autumn period is probably the result of stronger impact of the environment on the fish organism in the period of more intense metabolism, due to the accelerated gill ventilation and uptake of different toxicants (Chovanec et al. 2003).

The comparison of the cytosolic metal concentrations in the chub gills with labile metal concentrations in the river water

It is assumed that the labile metal concentrations are proportional to biological response of aquatic organisms (Campbell 1995). Therefore, to establish if indeed some cytosolic metal concentrations measured in the spring period in the chub gills present potential indicators of metal bioavailability from the river water, they were compared with the labile metal concentrations simultaneously measured in the river water using DGTs at three out of five fish sampling sites (Sava in Zagreb, Oborovo and Lukavec Posavski; Table 2; Dragun et al. 2008a). Labile Mn concentrations were 30-50% higher at Lukavec Posavski, while labile Cu concentrations were 40-60% higher at Oborovo compared to the remaining two sampling sites (Table 2). In the same period Mn and Cu cytosolic concentrations were comparable at all three sites (Figure 3 b and f), further supporting the assumption that the concentrations of these essential metals are regulated in the chub gills up to a certain level of exposure. The concentration increase for majority of essential metals in fish tissues obviously can be expected only after fish are exposed to rather high concentrations of those metals in the ambient water. Accordingly, increased Cu concentrations in the gills of North African catfish (*Clarias gariepinus*) and Mozambique Tilapia (*Oreochromis mossambicus*) were reported for river Olifants at dissolved Cu concentrations in water higher than 10 μg L^{-1} (Kotze et al. 1999), which is approximately 20 times higher than dissolved Cu concentrations measured in the river Sava (0.54±0.14 μg L^{-1}; Dragun et al. 2008b). In the same study, Kotze et al. (1999) reported increased gill concentrations of Zn at dissolved Zn concentrations in water higher than 100 μg L^{-1}. Andres et al. (2000) observed increased concentrations of Zn in the chub gills after exposure to extremely high concentration of dissolved Zn in river Lot water (890 μg L^{-1}), while increase was still not observed at water Zn concentration of 45 μg L^{-1}. Dissolved Zn

concentrations in the river Sava were much lower, and amounted to 2.27±1.53 μg L^{-1} (Dragun et al. 2008b). That is why it is surprising that the lowest concentration of cytosolic Zn was measured at Lukavec Posavski (Figure 3d), the same site where the lowest concentration of labile Zn was found (approximately twice lower compared to Sava in Zagreb and Oborovo; Table 2). However, the most of the cellular Zn, due to its presence in over 300 Zn dependent enzymes (Coyle et al. 2002), is bound to high molecular mass protein fraction, as shown by Roch et al. (1982) in the study on rainbow trout. Therefore, it is possible that low cytosolic Zn at Lukavec Posavski can actually be associated to lower protein concentration at that site (Figure 2d). The labile Fe concentrations were 2.5-3 times higher at Oborovo than at the other two sites (Table 2), and corresponded well with higher cytosolic Fe at Oborovo compared to Sava in Zagreb and Lukavec Posavski (Figure 4b). Unfortunately, the labile Fe concentration was not determined in the water at Jasenovac, where the highest cytosolic Fe was found, so further investigation is required to confirm with certainty the association of cytosolic Fe concentrations in the chub gills with the Fe-exposure from the river water. Labile Cd concentrations were very low in the Sava River water (<2 ng L^{-1}) and comparable at all three sampling sites (Table 2), while cytosolic Cd was two times higher at Oborovo and Lukavec Posavski than at Sava in Zagreb (Figure 4d). Cadmium, as non-essential metal, tends to accumulate in tissues of aquatic organisms even at relatively low water concentrations; however, in water with low metal level, metal uptake from food prevails (Dallinger and Kautzky 1985). Accordingly, Harrison and Klaverkamp (1989) reported significant Cd accumulation in the gills after fish were exposed to Cd from food. In the study on three-spined stickleback (*Gasterosteus aculeatus*), positive correlation of gill Cd was observed with Cd in food (invertebrates), and not with Cd in water; as a result of low concentrations of dissolved Cd in water (0.3-1.3 μg L^{-1}), Cd uptake from food was predominant (Bervoets et al. 2001). This was also a possible explanation for increased cytosolic Cd at Oborovo and

Lukavec Posavski observed in our study, since dissolved Cd concentrations in Sava River water were not higher than 0.025 μg L^{-1} (Dragun et al. 2008b). However, there is another possibility: it is known that metabolic stimulation during stress can accelerate gill ventilation and thus also the uptake of toxicants (Chovanec et al. 2003). Therefore, organic pollution and water toxicity at Oborovo and Lukavec Posavski (Čalić et al. 2006; Čalić et al. 2007; Krča et al. 2007; Källqvist et al. 2008; Grung et al. 2007), could have caused higher water filtration rate at these two sites compared to remaining sampling sites, resulting with higher Cd accumulation in the chub gills. In each case, increased cytosolic Cd in the chub gills can be used as an indicator of unfavourable living conditions in the ambient water.

Conclusions

In the autumn period, the site-specific variability of the cytosolic concentrations of proteins and essential metals Zn, Fe and Mn in the gills of European chubs caught in the river Sava was comparable to the site-specific variability of the chub gill masses, while Cu concentrations showed the opposite trend. In the spring period, the cytosolic concentrations of Zn, Cu and Mn seemed to be regulated in the chub gills at the moderate level of metal pollution observed for the River Sava, while the concentrations of total cytosolic proteins, Cd and Fe could presumably be associated to the level of water pollution. The cytosolic protein concentrations in the chub gills could be associated to the pollution gradient in the Sava River water, with decreased level observed at the sites affected by organic pollution and higher water toxicity. Increased cytosolic Cd concentrations were probably caused by dietary uptake or by stress-induced increase of the gill ventilation at the sampling sites affected by the pollution sources from Zagreb and Sisak areas. And, based on the comparison with the labile metal concentrations in the river water, our data indicated that cytosolic Fe concentrations

possibly reflected the water-borne Fe-uptake. Therefore, the spring period can be recommended as more appropriate season for the assessment of the river water pollution, if the gills are used as the target organ.

Acknowledgements

The financial support by the Ministry of Science, Education and Sport of the Republic of Croatia (project No. 098-0982934-2721) is acknowledged. This study was carried out as a part of the European FP6 project **SA**va **RI**ver **B**asin: Sustainable Use, Management and Protection of Resources (INCO-CT-2004-509160). The authors are thankful to all SARIB project participants for the help in the fish sampling and dissection.

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Table 1. Fish sampling sites, the coordinates recorded with GPSMAP 76CS (Garmin International, USA), the sampling dates, and basic physico-chemical parameters of Sava River water.

Fish sampling sites	Autumn, 2005					Spring, 2006				
	Date	[a]WT	pH	[b]O_2	[c]Cond.	Date	[a]WT	pH	[b]O_2	[c]Cond.
Otok Samoborski N 45° 50.543' E 15° 43.497'	Sep-19 Sep-26	14.4 15.6	- 7.84	83.1 77.6	- 410	Apr-24	12.8	7.87	97.9	465
Sava in Zagreb N 45° 46.572' E 15° 56.524'	Sep-22	13.2	7.92	77.7	377	Apr-21	11.5	7.86	93.7	473
Oborovo N 45° 41.286' E 16° 14.875'	Sep-20	13.7	7.84	79.7	346	Apr-18 Apr-25	12.1 -	7.76 7.82	84.7 -	507 486
Lukavec Posavski N 45° 24.081' E 16° 32.337'	Sep-21 Sep-28	13.9 15.1	7.83 7.82	76.2 69.8	346 408	Apr-26 May-2 May-8	17.1 11.7 14.8	7.85 7.68 7.59	82.9 80.5 80.9	491 415 495
Jasenovac N 45° 15.825' E 16° 53.658'	Sep-23	13.3	7.89	79.5	352	May-25	19.5	7.59	76.0	403

[a]WT: water temperature, °C;
[b]O2: dissolved oxygen, %;
[c]Cond.: water conductivity, μS cm^{-1}.

Table 2. The labile metal concentrations (μg L^{-1}) in Sava River water measured simultaneously with the fish sampling at three selected sites in the spring period of 2006 (March 29th - June 21st), using the diffusive gradients in thin films; DGTs were deployed 2-3 times per site, and deployments lasted from 20 to 36 days; details about the deployment, measurements and calculations are given in Dragun et al. (2008a)

	Number of deployments	Zn	Fe	Cu	Mn	Cd
Sava-Zagreb	2	1.38±1.28	3.41±4.75	0.128±0.014	1.90±0.69	0.0014±0.0003
Oborovo	3	1.15±0.27	8.20±6.73	0.185±0.082	2.19±0.92	0.0015±0.0003
Lukavec Posavski	2	0.63±0.09	2.81±0.96	0.116±0.013	2.88±0.69	0.0017±0.0005

Figure captions

Figure 1. The map of the selected section of the Sava River in Croatia, with marked sampling sites (OS: Otok Samoborski, SZ: Sava in Zagreb; OB: Oborovo; LP: Lukavec Posavski; JAS: Jasenovac).

Figure 2. The gill masses (a, b) and total cytosolic proteins (c, d) measured in the gills of the European chubs caught at five sites in the Sava River, in autumn 2005 and spring 2006. Data are presented as the boxes whose boundaries indicate 25^{th} and 75^{th} percentiles; a square within the box marks the median value; whiskers above and below the box indicate the non-outlier minimum and maximum. The statistically significant differences between sites ($p<0.05$) according to Dunn's test are indicated by different letters.

Figure 3. The cytosolic concentrations of essential metals Mn (a, b), Zn (c, d) and Cu (e, f) measured in the gills of the European chubs caught at five sites in the Sava River, in autumn 2005 and spring 2006. Data are presented as in Figure 2. The statistically significant differences between sites ($p<0.05$) according to Dunn's test are indicated by different letters.

Figure 4. The cytosolic concentrations of essential metal Fe (a, b) and nonessential metal Cd (c, d) measured in the gills of the European chubs caught at five sites in the Sava River, in autumn 2005 and spring 2006. Data are presented as in Figure 2. The statistically significant differences between sites ($p<0.05$) according to Dunn's test are indicated by different letters.

Figure 1.


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

Figure 2.

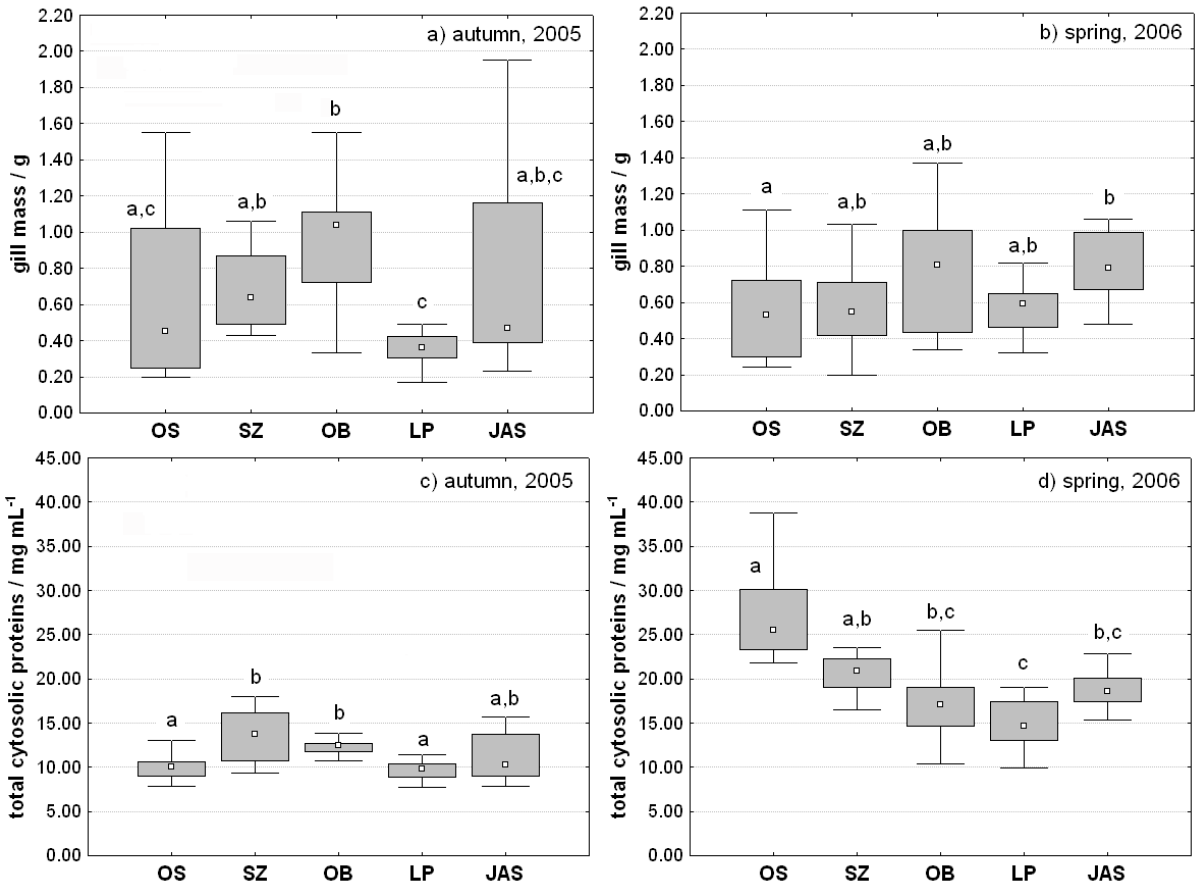

a) autumn, 2005
b) spring, 2006
c) autumn, 2005
d) spring, 2006
gill mass / g
total cytosolic proteins / mg mL-1
OS
SZ
OB
LP
JAS
a,c
a,b
b
c
a,b,c
a
a,b
a,b
b
a
b
b
a
a,b
a
a,b
b,c
c
b,c

Figure 3.

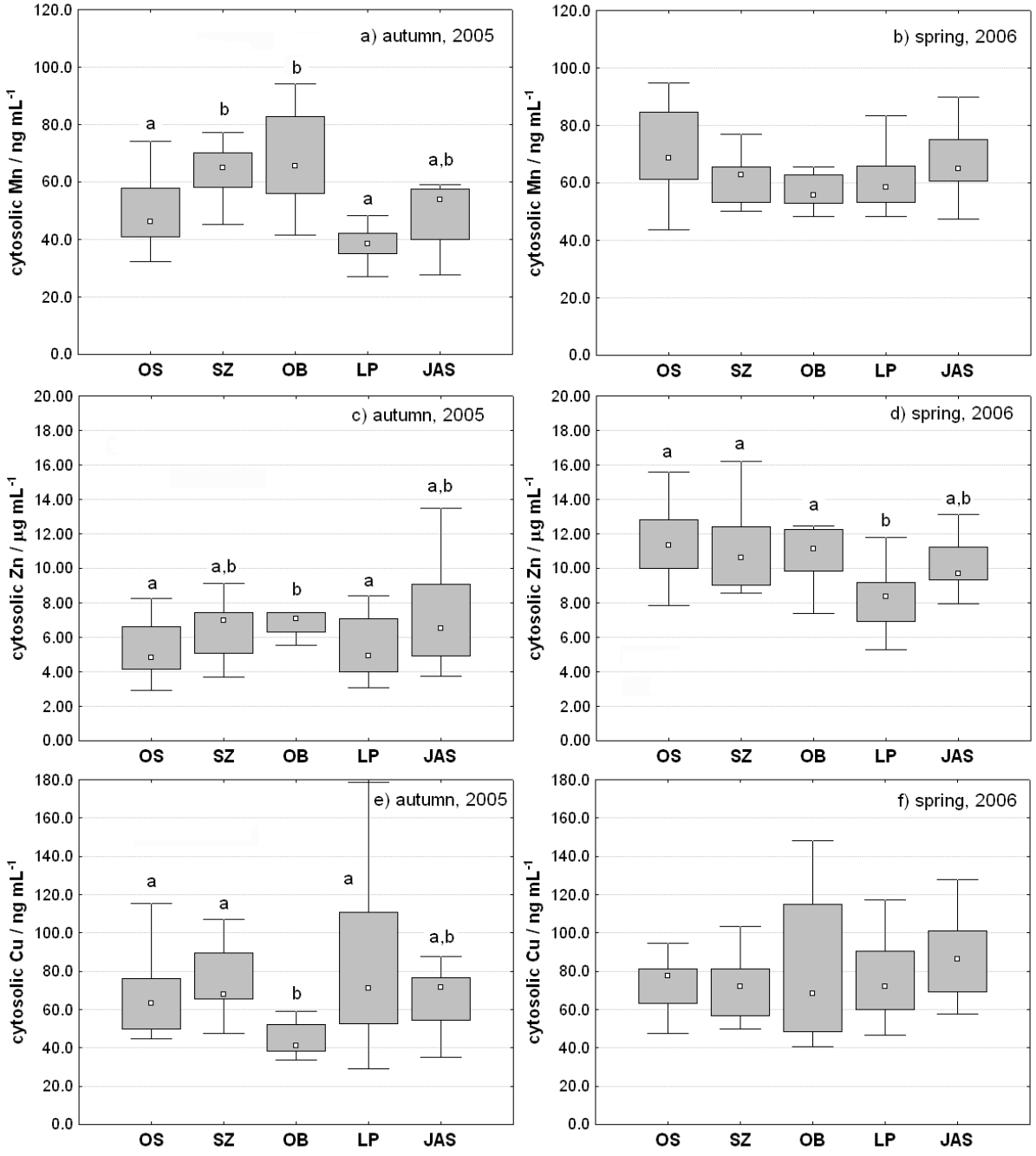
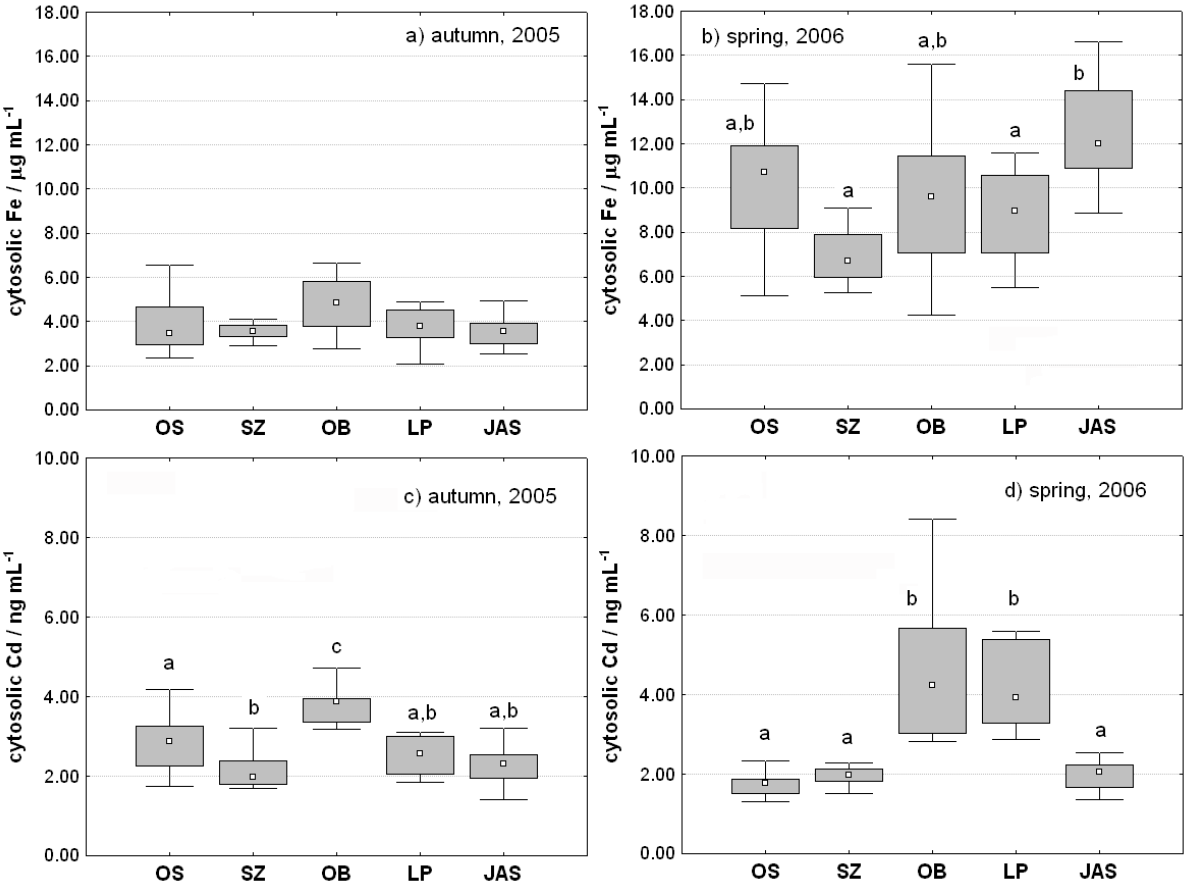

a) autumn, 2005
b) spring, 2006
c) autumn, 2005
d) spring, 2006
e) autumn, 2005
f) spring, 2006
cytosolic Mn / ng mL-1
cytosolic Zn / µg mL-1
cytosolic Cu / ng mL-1
OS
SZ
OB
LP
JAS

Figure 4.


a) autumn, 2005
b) spring, 2006
c) autumn, 2005
d) spring, 2006
cytosolic Fe / µg mL-1
cytosolic Cd / ng mL-1
OS
SZ
OB
LP
JAS
a
b
c
a,b