Comparison of electrochemically determined metallothionein concentrations in wild freshwater salmon fish and gammarids and their relation to total and cytosolic metal levels

Tatjana Mijošek[1], Vlatka Filipović Marijić[1], Zrinka Dragun[1], Dušica Ivanković[1], Nesrete Krasnići[1], Marijana Erk[1], Sanja Gottstein[2], Jasna Lajtner[2], Mirela Sertić Perić[2], Renata Matoničkin Kepčija[2]

[1]Ruđer Bošković Institute, Division for Marine and Environmental Research, Bijenička cesta 54, 10000 Zagreb, Croatia

[2] University of Zagreb, Faculty of Science, Department of Biology, Division of Zoology, Rooseveltov trg 6, 10000 Zagreb, Croatia

E-mail addresses: tmijosek@irb.hr, vfilip@irb.hr, zdragun@irb.hr, djuric@irb.hr, nkrasnic@irb.hr, erk@irb.hr, sanja.gottstein@biol.pmf.hr, jasna.lajtner@biol.pmf.hr, msertic@biol.pmf.hr, rmatonic@biol.pmf.hr

[*]corresponding author: tmijosek@irb.hr (Ruđer Bošković Institute, Division for Marine and Environmental Research, Bijenička cesta 54, 10000 Zagreb, Croatia)

Abstract

Application of metallothionein (MT) as an early warning sign of metal exposure in aquatic organisms is common in biomonitoring, but there is a huge variability in MT concentrations among different studies. Present research aims to assess MT responses in freshwater fish brown trout (*Salmo trutta* Linnaeus, 1758) and gammarids (*Gammarus balcanicus* Schäferna, 1922 and *Echinogammarus acarinatus* Karaman, 1931) as indicators of metal exposure within the freshwater karst environment (Krka River, Croatia). Sampling was performed upstream (reference site) and downstream (anthropogenically impacted site) of the wastewater discharges in autumn and spring seasons. Brown trout intestine was applied as a bioindicator tissue due to its role in dietborne metal uptake while gammarids were chosen as fish food and potential metal uptake source. Moreover, there is a lack of data on intestinal MT levels, so our results on MT and metal/metalloid concentrations, measured as total and metabolically available cytosolic levels, represent the first data of this kind for the selected indicator species. The results indicated that the ecotoxicological impact of technological and municipal wastewaters on the biota of the karst Krka River was moderate, although higher metal levels at the affected site were evident in both, fish and gammarids. The modified Brdička reaction applied in this study was confirmed as reliable electrochemical technique for MT quantification in both vertebrates and invertebrates, and it indicated higher MT levels in gammarids (1.9-4.1 mg g^{-1} w.w) than in fish intestine (0.5-2.8 mg g^{-1} w.w.). Due to the lack of the data on MT concentrations in *S. trutta* and gammarid species *G. balcanicus* and *E. acarinatus*, presented results can serve as a preliminary data to establish MT background levels in intestine of wild freshwater fish and gammarids. Obtained MT levels showed species-, tissue- and method-specific differences, so comparison between MT levels should always involve the same species, tissue and measurement method.

Keywords: biomarkers, metallothioneins, fish intestine, amphipods, karst aquatic environment

1. Introduction

First measurable changes related to the exposure of contaminants and their impacts on the aquatic organisms are biochemical responses used as cellular and histological biomarkers (Hinton and Lauren, 1990). One of the major biomarkers pointing to metal exposure of aquatic organisms is the increase in metallothionein (MT) levels as a consequence of the induction of metallothionein (MT) synthesis associated with increased capacity for metal binding and MT involvement in protection against metal toxicity (Roesijadi, 1992). MTs are low molecular mass cysteine- and metal-rich proteins containing sulphur-based metal clusters that have significant roles in maintaining the homeostasis of essential trace metals (Zn and Cu), sequestration of toxic metals (e.g., Cd, Ag and Hg), and protection against oxidative damage (Vašák, 2005; Amiard et al., 2006). Although often used as the best known biochemical responses to metal exposure in the environment, MTs are also inducible by other biotic and abiotic factors often contributing to variations of the MT-cellular concentrations, e.g., starvation, freezing (Amiard et al., 2006), reproductive state, age and sex, temperature, seasonal environmental changes (Viarengo et al., 1999; Isani et al., 2000).

As organisms at the top of aquatic food chains, fish are commonly used as bioindicator species for the assessment of metal accumulation. Metal uptake in fish occurs through gills and skin (i.e., sites of waterborne uptake), and intestine (i.e., site of dietborne uptake). Most studies dealing with metal contaminant exposure involved gills (Dragun et al., 2009), liver (Podrug and Raspor, 2009) and kidney (Sevcikova et al., 2013) as indicator organs in fish. The investigations on the uptake and effects of dietary metals in fish and the respective MT responses to metal exposure in fish intestine are still rare (Handy, 1996). It was reported that metals are accumulated in the epithelial cell layer of the intestinal tissue and can be eliminated from the organism by desquamation of mucus layer (Sorensen, 1991). MT induction is evident in intestinal absorptive cells, enterocytes, and serves as a biological mechanism which reduces transfer of metals from the luminal to the serosal side. Most of the previous studies considered laboratory experiments in which applied metal concentrations were often higher than their environmental levels, and metals sourcing from the diet were usually ignored, due to complexity of wild fish nutrition (Schlekat et al., 2005; Giguere et al., 2006).

In the present study MT and metal levels were estimated in the gastrointestinal tissue of the brown trout (*Salmo trutta* Linnaeus, 1758), selected as a widely spread freshwater species

in rivers in Europe. The potential of fish intestinal tissue to be applied as bioindicator organ of metal contamination in the aquatic environment was evaluated. Additionally, the present study included the assessment of the MT and metal levels in amphipod crustaceans, *Gammarus balcanicus* Schäferna, 1922 and *Echinogammarus acarinatus* Karaman, 1931. Crustaceans of the genus *Gammarus* are often used as bioindicators of environmental pollution due to their wide distribution, high abundance, clear sexual dimorphism, easy sampling and identification, and due to their sensitivity to different kinds of toxicants (Geffard et al., 2007). Gammarids often play a central role in freshwater ecosystems because they represent an important link between detritus and fish in the aquatic food webs and a reduction in their number can have deleterious effects on the structures of biological communities (MacNeil et al., 1997; Kunz et al., 2010). Moreover, in a pilot-study of bentos-drift relationship in the Krka River it was suggested that gammarids, which were found most numerous in drift, could be considered as the most suitable bioindicators of a contaminant (i.e., metal) accumulation and mobilization within karst aquifers (Sertić Perić et al., 2018).

Metal exposure assessment of the organisms (i.e., fish, gammarids) was conducted in anthropogenically impacted karst Krka River. The study involved evaluation of MT and metal levels, including total metal concentrations in fish intestine and cytosolic metal concentrations in fish intestine and whole gammarids, as a fraction which presents metabolically available and therefore potentially toxic metals (Caron et al., 2019; Mijošek et al., 2019). Aquatic systems, especially the sensitive karst ecosystems, are nowadays threatened by a variety of contaminants, often originating from different anthropogenic sources. Among them, metals/metalloids represent one of the most troublesome pollutants in the aquatic environment due to their high toxicity, long persistence and tendency of bioaccumulation and biomagnification in the food chain (Eisler, 1993). Heavy metals can originate from direct atmospheric deposition, geologic weathering as natural sources or through the discharge of different waste products; agricultural, municipal or industrial, as one of the main anthropogenic sources (Demirak et al., 2006).

Our main goals were to evaluate the impact of known pollution sources (technological and municipal wastewaters) on the biota inhabiting the Krka River using electrochemically measured MTs as biomarkers of metal exposure, and metal/metalloid concentrations in the whole intestinal tissue of brown trout and additionally in cellular cytosol of fish intestine and whole gammarids. Cytosolic metal fraction involves respective metal levels which are available of binding to biomolecules in the cell cytosol of the organisms and therefore may

constitute metal-sensitive (enzymes) or metal detoxified fraction (metallothioneins) (Filipović Marijić et al., 2010; Caron et al., 2018). Moreover, seasonal and spatial differences in metal and MT levels in fish and gammarids from the two sites (reference site - upstream of the wastewater discharge, and pollution impacted site - downstream of the wastewater discharge) were compared, as well as MT levels measured by electrochemical method among different wild freshwater bioindicator organisms.

2. Experimental section

2.1. Study area

The samplings of both *S. trutta* and gammarids were performed at the two sampling sites of the Krka River and involved two sampling campaigns, autumn (October 2015) and spring (May 2016). Coordinates for the reference site were 44°04.11' N 16°23.24′ E and for the contaminated site 44°03.37′N 16°19.04′ E.

Krka River watercourse is situated in the Dinaric area of the Republic of Croatia. Due to its unique tufa-barriers, a large part of the watercourse was proclaimed national park in 1985. Based on the previously published data on the physico-chemical water parameters and total dissolved metal levels in the river water (Cukrov et al., 2008; Filipović Marijić et al., 2018), river source was chosen as the reference site, whereas a location downstream of the town of Knin, located 2 km upstream of the northern border of the Krka National Park, was selected as the contaminated site. Krka River is threatened by two known sources of contamination: technological wastewaters of the screw factory and municipal wastewaters of the town of Knin. The wastewaters are released without a proper treatment into the river watercourse just 2 km upstream of the border of the Krka National Park. Krka River water analyses conducted upstream and downstream of the wastewater discharge have shown that metal/metalloid concentrations were increased at the affected site (Dragun et al., 2018; Filipović Marijić et al., 2018; Sertić Perić et al., 2018). Dissolved metals in water with the highest concentrations at the anthropogenically impacted site were Fe, Li, Mn, Mo, Sr, Rb and Ca and among them Fe and Mn are metals specific for technological wastewaters and often used in the manufacture of iron and steel alloys, and manganese products (Dragun et al., 2018; Filipović Marijić et al., 2018; Sertić Perić et al., 2018). All measured physico-chemical water parameters indicated slightly deteriorated environmental conditions at the site downstream of wastewater discharge, of which temperature, conductivity, total dissolved solids and total water hardness

showed significant between-site differences (Sertić Perić et al., 2018). The map and detailed description of the area have already been published (Filipović Marijić et al., 2018).

2.2. Sampling procedure

Sampling was conducted in two campaigns; in the autumn 2015, a total of 36 individuals of brown trout were sampled (16 from the reference site and 20 from the contaminated site), while in the spring 2016, a total of 32 fish were sampled (16 per each site).

Electrofishing was applied as a fish sampling tool, according to the Croatian standard HRN EN 14011. After capture, fish were placed in an opaque plastic tank with aerated river water in order to be kept alive until further processing in the laboratory. Fish were euthanized using freshly prepared anaesthetic tricaine methane sulphonate (MS 222, Sigma Aldrich, USA) and sacrificed. Body mass and the total length of the fish were recorded. The posterior part of the intestinal tissue was dissected, weighed and stored at −80 °C until further analyses.

Gammarids were collected by benthos hand net (625 cm^2 and mesh size: 250 μm) in aquatic macrophytes and on the stony substrate at the same sampling sites as fish. At least 200 individuals of *G. balcanicus* were collected at each location in both seasons, while *E. acarinatus* was recorded only at one location, the Krka River source, and at least 180 individuals were sampled. Gammarids were stored at −80 °C until further analyses in the laboratory. Prior to homogenization process individuals were dried on the filter paper, subjected to manual exclusion of detritus that might contaminate the samples, and pooled together due to their small masses. At the reference site, we obtained 14 pooled samples of *G. balcanicus* in autumn and 10 samples in spring, whereas at the contaminated site, 16 samples were obtained in autumn, and 17 in spring. From the reference site, 5 pooled samples of the *E. acarinatus* were obtained in autumn and 6 in spring. A pooled sample of the *G. balcanicus* from the reference site consisted of 11-15 individuals, whereas 6-12 individuals were pooled at the contaminated site. For the appropriate sample of *E. acarinatus* collected at the reference site, 20-30 individuals were pooled.

2.3. Homogenization of posterior intestinal tissue and whole gammarids

Samples of the fish intestine were cut in small pieces and diluted 6 times with cooled homogenization buffer. The homogenizing buffer contained 100 mM Tris-HCl/base (Merck,

Germany, pH 8.1 at 4 °C) in which 1 mM DTT (dithiothreitol, Sigma, USA) was added as a reducing agent and 0.5 mM PMSF (phenylmethylsulfonyl fluoride, Sigma, USA) and 0.006 mM leupeptin (Sigma, USA) as protease inhibitors (Filipović Marijić and Raspor, 2007). Intestinal tissue was homogenized by 10 strokes of Potter-Elvehjem homogenizer (Glas-Col, USA) in an ice cooled tube at 6000 rpm. Whole gammarids were homogenized in a same way; only pooled samples were 10 times diluted with the homogenization buffer to get enough material for the measurements (Filipović Marijić et al., 2016). Considering fish intestinal tissue, one part of the obtained homogenates was separated and subjected to the digestion procedure, in order to determine the total metal content (insoluble and soluble tissue fraction) in this tissue. The other part of the homogenates was centrifuged to obtain cytosolic cellular fraction, which was also digested for subsequent metal measurements. Cytosolic metals represent only soluble tissue fraction (Wallace and Luoma, 2003). In whole gammarids, due to the existence of chitin exoskeleton metals were measured only in cytosolic fractions.

2.4. Preparation of cytosolic and heat-treated cytosolic fractions

Fish and gammarid homogenates were centrifuged in the Avanti J-E centrifuge (Beckman Coulter, USA) at 50,000×g for 2 h at 4 °C. Resulting supernatants (S50), representing the water soluble tissue fractions (cytosol), were used for metal analyses, while MT measurements were performed in the heat treated S50 fraction (HT S50). Heat-treatment was applied because this procedure denatures high molecular mass cytosolic proteins, which would otherwise interfere with the electrochemical MT determination, while MT as a thermostable protein remains in the solution after heat-treatment (Erk et al., 2002). The cytosolic S50 fraction was firstly 10 times diluted with 0.9 % NaCl (Suprapur, Merck) to prevent co-precipitation of MTs with denatured proteins and then heat-treated at 85 °C for 10 min in the Dri Block (Techne, GB). Afterwards, heat-treated samples were placed on ice at 4 °C for 30 min and centrifuged at 10,000 g in Biofuge Fresco centrifuge (Kendro, USA). The resulting supernatant (HT S50), containing heat-stable proteins was stored at –80 °C until further analyses, while the pellet was discarded.

2.5. Electrochemical determination of MT concentrations

MT concentrations were measured by differential pulse voltammetry (DPV) following the modified Brdička procedure (Raspor et al., 2001). Voltammetric measurements were

performed on 797 VA Computrace (Metrohm, Switzerland) with a three-electrode system (hanging mercury drop electrode, HMDE, as a working electrode, an Ag/AgCl/saturated KCl reference electrode and a platinum counter electrode). Measurements were done in duplicate (A and B subsample) in 10 mL of an electrolyte solution consisting of 5 mL of 2 M NH_4Cl/NH_4OH and 5 mL of 1.2×10^{-3} M Co $(NH_3)6Cl_3$, pH=9.5 which was thermostated to 20 °C and purged with the pure nitrogen. The applied measurement parameters for DPV were the following: potential scan from −0.9 V to −1.65 V; scan rate 0.013 Vs^{-1}; voltage pulse amplitude 0.02502 V; duration of the pulse application 0.057 s and a step time 0.2 s (Mijošek et al., 2018). MT concentrations were derived from the straight calibration line, constructed with the commercially available standard rabbit liver MT-2 (Enzo, USA) dissolved in 0.25 M NaCl. Final results were expressed as mg MT g^{-1} of wet tissue. In order to enable comparison with other available studies reporting MT levels on protein content, our data on MT levels were also standardized by the protein content. Protein concentrations were measured according to Lowry et al. (1951). Calibration was accomplished using a bovine serum albumin (BSA) (Serva, Germany) as a reference standard (0.25–2 mg ml^{-1} BSA).

2.6. Digestion of homogenates and cytosolic cellular fractions

Prior to the metal measurement, homogenates of fish intestine and cytosolic fractions of fish intestine and whole gammarids were digested in duplicates by adding the oxidation mixture of concentrated HNO_3 (Rotipuran® Supra 69%, Carl Roth, Germany) and 30% H_2O_2 (Suprapur®, Merck, Germany). In all cases, concentrated acid and hydrogen peroxide were added in the volume ratio of 3:1. Digestion was performed in the laboratory dry oven at 85 °C for 3.5 h. Cooled samples were afterwards diluted with Milli-Q water, 1:20 for Na, K, and Mg analyses, and 1:5 for the remaining elements (Dragun et al., 2018). The validation of acid digestion efficiency was performed by the digestion of dogfish muscle certified reference material for trace metals (DORM-2, National Research Council of Canada, NRC, Canada). Recoveries means of the trace elements studied from the certified reference material ranged from 95 to 105% as follows: As (103%), Cd (105%), Co (99%), Cu (100%), Fe (101%), Mn (101%); Se (102%), Tl (100%) and Zn (95%).

2.7. Determination of total and cytosolic metal concentrations

Elements were analyzed using high resolution inductively coupled plasma mass spectrometer (HR ICP-MS, Element 2; Thermo Finnigan, Germany), equipped with an autosampler SC-2 DX FAST (Elemental Scientific, USA). During the metal measurements, three resolution modes were used. Measurements of ^{82}Se, ^{98}Mo, ^{111}Cd, ^{133}Cs, and ^{205}Tl were all operated in low resolution mode; of ^{23}Na, ^{24}Mg, ^{42}Ca, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{63}Cu and ^{66}Zn in medium resolution mode; and high resolution mode was used for ^{39}K and ^{75}As determination. External calibration for macro elements was made using multielement stock standard solution containing Ca 2.0 g L^{-1}, Mg 0.4 g L^{-1}, Na 1.0 g L^{-1}, and K 2.0 g L^{-1} (Fluka, Germany). Calibration solution for the trace elements was prepared by dilution of multielement stock standard solution (Analitika, Czech Republic) supplemented with Cs (Fluka, Germany). Indium (1 μg L^{-1}, Indium Atomic Spectroscopy Standard Solution, Fluka, Germany) was added to all solutions as an internal standard (Fiket et al., 2007). Quality control samples were used to test the accuracy and the precision of measurements; QC Minerals, Catalog number 8052, UNEP GEMS, Burlington, Canada for the macro elements and QC trace metals, catalog no. 8072, UNEP GEMS, Burlington, Canada for the trace elements. A generally good agreement was observed between our data and certified values, with the following recoveries based on five measurements in the control sample (%): As (101.4 ± 10.3), Ca (95.7 ± 1.3), Cd (95.6 ± 0.6), Co (97.0 ± 1.6), Cu (95.7 ±2.2), Fe (95.4 ± 5.1), K (90.7 ± 5.1), Mg (93.3 ± 2.5), Mn (96.5 ± 1.8), Na (97.3 ± 3.9), Se (99.1 ± 3.6), Tl (96.3 ± 0.8) and Zn (96.9 ± 2.3). Limits of detection (LOD) were calculated based on three standard deviations of ten consecutively determined trace element concentrations in blank sample (100 mM Tris-HCl/Base, 1 mM DTT) which was digested the same way as samples. LODs for trace elements measured within this study were the following (ng g^{-1}): As, 6.72; Cd, 0.430; Co, 0.266; Cs, 0.102; Cu, 13.5; Fe, 141; Mn, 0.810; Mo, 0.680; Se, 2.93; Tl, 0.001 and Zn, 635, while for macro elements (μg g^{-1}): Ca, 1.07; K, 0.112; Mg, 0.024; and Na, 0.320.

2.8. Statistical methods

Basic calculations were performed in Microsoft Office Excel 2007, while SigmaPlot 11.0 (Systat Software, USA) was used for all statistical analyses. Since assumptions of normality and homogeneity of variance were not always met, the significance of differences between seasons or locations was tested by application of Mann-Whitney U-test. Differences were regarded as significant when $p < 0.05$. Correlation between different parameters was calculated using Spearman correlation analysis. Levels of significance of applied statistical

tests were indicated in the text. Fulton condition indices (FCI) were expressed according to Rätz and Lloret (2003), i.e. $K=W/L^3$, where W is the body mass (g) and L is the total length of fish (cm).

3. Results and discussion

3.1. Biological responses in brown trout

Comparison of biometric parameters of sampled fish from two sampling sites indicated comparable total length but higher body mass of fish from the wastewater impacted site in both seasons, although not significantly. Only FCI values were significantly higher at the contaminated site in both investigated seasons (U=6.00; p=0.001 in autumn and U=9.00; p=0.002 in spring). This could be due to the higher fish masses, which are likely a consequence of better nutrient availability (Lambert and Dutil, 1997) originating from municipal and industrial wastewaters discharged into the Krka River water downstream of the town of Knin. Average total length and body mass both pointed to significantly higher fish biometric parameters in the autumn than spring season at both locations (U=14.5; p=0.008 and U=20; p=0.026 for fish length in reference and contaminated site, respectively; U=16; p=0.011 and U=21; p=0.031 for fish mass in reference and contaminated site, respectively). Other than that, FCI values were elevated in spring, although not significantly which could be a result of the seasonal mobilization of energy reserves needed for reproductive development (Maddock and Burton, 1999). However, in different studies the opposite trend of lower FCI values in polluted sites is also often observed (Couture and Rajotte, 2003; Jenkins, 2004; Shobikhuliatul, 2013; Zhelev et al., 2016; 2018). Our results might suggest that the wastewater impact at the contaminated site was not high enough to induce defense mechanism of fish in a way which would require a lot of energy and consequently result in decreased FCI values.

3.1.1. MT concentrations in the heat-treated cytosol of intestine of brown trout

Average fish intestinal MT levels were higher at the contaminated site (downstream of the town of Knin) compared to the river source in both seasons, but the site- or season-specific differences were not proven significant (Fig. 1). Average MT concentrations in the intestine of fish from the reference site in autumn and spring campaign were 0.85 and 0.96 mg

g^{-1} w.w., and from polluted site 1.5 and 1.45 mg g^{-1} w.w., respectively (Fig. 1). To our knowledge, MT levels reported in this study represent the first data set for the intestinal tissue of brown trout measured by electrochemical method DPV. Different research groups in the world use variety of spectrometric, immunochemical and electrochemical methods for MT determination but obtained MTs levels are highly variable depending on the measurement method (Isani et al, 2000; Dabrio et al., 2002; Zorita et al., 2005). Therefore, it would not be relevant or correct to compare our records with the MT levels obtained by different methods. Data on MT levels determined by electrochemical method were reported for different tissues of wild freshwater fish species, i.e., European chub (Dragun et al., 2009; Filipović Marijić and Raspor, 2010; Dragun et al., 2013), rainbow trout (Roch et al., 1982), common carp, perch, pike, bream, roach and rudd (Sevcikova et al., 2013). Filipović Marijić and Raspor (2010) reported average MT concentrations in the intestine of European chub from the Sava River to be around 3 mg g^{-1} w.w., which is therefore 2-3 times higher than values observed for the brown trout from the Krka River in our study, but also higher compared to MT levels in gills (around 2 mg g^{-1} w.w.) and liver (around 1.5 mg g^{-1} w.w.) of the same species (Dragun et al., 2009; Podrug and Raspor, 2009; Dragun et al., 2015). As gastrointestinal tissue and gills are organs which are known to be involved in the uptake, detoxification and excretion processes (Van Cleef et al., 2000), higher MT concentrations observed in these tissues could probably be linked to the important function of MTs in metal homeostasis and detoxification. In humans, higher MT concentrations can even indicate more serious disorders in the body, such as carcinoma (Krizkova et al., 2009b). However, there is no real connection of higher MT concentrations with carcinoma in fish. Barišić et al. (2018) made the investigation on architectural and histopathological biomarkers in the intestine of the same brown trout specimens as used in this research and concluded that serious histopathological lesions, such as neoplasia, were not evident in fish from the Krka River.

3.1.2. Total and cytosolic metal concentrations in intestine of brown trout

Metal levels measured in digested homogenate, presenting total metal concentrations in insoluble and soluble tissue fractions, were, as expected, higher compared to their levels measured in cytosolic intestinal fraction, i.e. soluble fraction (Table 1). For Ca, Cu, Fe, Mn and Zn less than 50% of the total metal levels were present in the insoluble cellular fractions (Fig. 2a), pointing that these metals are partially present in tissue fraction which is not considered as metabolically available (such as metal rich granules) and partially in cytosolic fraction, which represents potentially toxic part of metals which can bind to physiologically

important molecules (Wallace and Luoma, 2003; Vijver et al., 2004; Caron et al., 2018). The proportion of other measured metals, As, Cd, Cs, Mo, Se, K, Mg, and Na, was over 67% in cytosolic fraction (Fig. 2b), indicating that these metals are mostly found in soluble fraction where they can be bound to cytosolic biomolecules, for example metallothioneins (detoxified metal fraction) or enzymes (metal-sensitive fraction) (Wallace and Luoma, 2003; Caron et al., 2018). Presented relation of total and cytosolic metal/metalloid concentrations in *S. trutta* intestinal tissue is in accordance to the proportions of total metals in hepatic cytosol of the same fish (Dragun et al., 2018). Exceptions were only Co, Cu, Mn and Zn, with around 20% higher proportion of total levels in liver cytosol than intestinal cytosol. Total concentrations of trace elements in *S. trutta* intestinal tissue followed the descending order Zn>Fe>Se>Mn≥Cu>Cd>Co≥Mo>Tl≥As>Cs, which is quite similar to total metal trends observed in hepatic tissues of the same fish (Dragun et al., 2018). Due to the lack of data on cytosolic metal levels in fish intestine, comparison with other literature was only possible for total metal levels and also confirmed the common trend of the highest Fe, Zn, Mn and Cu levels in the intestine of rainbow trout from rivers Augraben and the Leiferer Graben in Italy (Dallinger and Kautzky, 1985), perch from the lake Mondsee in Austria (Sures et al., 1999), different freshwaters fish species in waters of Lithuania (Staniskiene et al., 2006), starlet from the Danube River in Serbia (Jarić et al., 2011), barbel from the Danube River in Bulgaria (Nachev and Sures, 2016) and in *Salmo trutta macrostigma* and rainbow trout from Çatak River in Turkey (Yeltekin and Sağlamer, 2019). In fish from Croatian rivers, average total Cu, Fe and Mn levels in the intestinal tissue of European chub from the lowland Sava River were either comparable or lower than their values in intestine of brown trout from the karst Krka River, depending on the season and location, while total Cd and Zn levels were mostly higher in the intestine of European chub from the Sava River (Filipović Marijić and Raspor, 2012; Dragun et al., 2015).

Despite differences in total and cytosolic metal concentrations, their relation between two locations indicated similar pattern, with higher total and cytosolic concentrations of As, Ca, Co, Se and Zn in brown trout from the contaminated compared to the reference site (Table 1). Such pattern of elevated intestinal metal/metalloid levels at the contaminated site might reflect higher metal/metalloid exposure level in the river water at the location near the town of Knin, influenced by technological and municipal wastewaters, as already reported by Filipović Marijić et al. (2018) and Sertić Perić et al. (2018). Other measured metals, Fe, K, Mg, Mn and Na did not show a clear trend between two locations (Table 1), while Cd, Cs, Mo

and Tl concentrations were higher in the intestinal homogenate and cytosolic fraction of fish from the Krka River source in at least one season (Table 1). Presented results are in accordance with the trend reported for hepatic metal levels of *S. trutta* from the same locations (Dragun et al., 2018) but the exact cause of significantly higher Cd, Cs, Mo and Tl concentrations in fish from the reference site requires further investigation, with special emphasis on river sediment and food as metal sources, considering dietary intake as the important uptake route in fish (Clearwater et al., 2000). Cd, Cs and Tl were also significantly higher in gammarids from the same site which might serve as possible fish prey and consequently as a possible metal source for fish.

Regarding seasonal differences, majority of studied metal/metalloids in intestinal homogenate and cytosolic fraction had higher levels in autumn than spring season. Significant differences were observed in both fractions only for As and Na at the reference site, and for Mo and Cd at the contaminated site, with the elevated metal levels in autumn, except for As (Table 1). Unique seasonal differences at both locations, but without significant differences, were evident as higher Co, Cs, Fe, Mo and Na levels in autumn in intestinal homogenate and as higher Mo, Se and Na levels in autumn in intestinal cytosol (Table 1). Mostly lower intestinal metal levels in spring could be due to the lower dissolved metal levels in the river water accompanied by the more effective self-purification process of the Krka River in that period (Cukrov et al., 2008; Filipović Marijić et al., 2018).

Since one of the main MT roles in the organisms is the regulation of essential metals (Cu and Zn), and detoxification of heavy metals (Cd, Hg, Ag) (Amiard et al., 2006), which increased levels may induce MTs synthesis, we evaluated possible contribution of intestinal metals to the observed MT levels. Spearman correlation analysis confirmed a significantly positive relation of MT with cytosolic Cd ($r=0.762$; $p=0.02$) and Cu levels ($r=0.786$; $p=0.0149$) in fish from the reference site in spring, while in autumn with cytosolic Cu in fish from contaminated site ($r=0.782$; $p=0.005$). Total metal levels did not show significant correlation with MT, probably because cytosolic metals are those which might be directly bound to biomolecules and have impact on their concentrations, activities or structures (Caron et al., 2018). However, metal content obviously cannot completely explain variability and complexity in MT levels, which may be affected by other parameters such as season, temperature, pH values, size, fish gender or nutritional status (Hylland et al., 1998; Dragun et al., 2009; Filipović Marijić and Raspor, 2010). Intestinal MT and metal levels did not show significant correlation with brown trout biometry, what is in agreement with the existing

literature data where intestine has already been reported as an organ with no additional metal accumulation with fish age and growth (Giguére et al., 2004; Filipović Marijić and Raspor, 2007). Of physico-chemical factors, temperature, conductivity, total dissolved solids and total water hardness showed significant differences between the sites and pointed to deteriorated ecological status near the town of Knin (Sertić Perić et al., 2018), possibly influencing MT levels as well. Since in polluted environment organisms are exposed to a mixture of different metals and contaminants, it is generally impossible to connect the elevated MT synthesis only to specific elements, especially knowing that a combination of various biotic and abiotic factors greatly affects MT induction.

3.2. Biological responses in gammarids

In both seasons, individuals of *G. balcanicus* were bigger at the contaminated site with the average weight of 27 and 23 mg in autumn and spring, respectively. At the Krka source, average weights were about 15 mg in both seasons. *E. acarinatus* individuals were sampled only at Krka River source and they were much smaller than *G. balcanicus*, which is the inherent property of this species. The average weights of *E.acarinatus* were of 6 mg in autumn, and 8 mg in spring. Gammarid mass differences were most likely caused by habitat or microhabitat conditions. In the source part of the rivers, higher water velocity takes away nutrients and consequently can affect size of the organisms. Žganec et al. (2016) also observed dominance of smaller species of gammarids in both microhabitat types, stones and mosses, at the upper course of the Krka River, which represents food limited location due to the lack of packs of detritus/leaves – likely as a result of a very strong water current and absence of detritus in upstream sections of the river. Usually, higher abundance and bigger gammarids are found in the downstream parts where more fine particulate organic matter (FPOM) can be found.

3.2.1. MT concentrations in the heat-treated cytosol of *Gammarus balcanicus*

Opposite to the intestinal MT levels in brown trout, MT concentrations in *G. balcanicus* differed significantly between locations and seasons (Fig. 3a). Spatial differences were observed in spring with significantly higher MT levels in gammarids from the wastewater impacted location ($U=23.00$; $p=0.002$), while in autumn MT concentrations were comparable between the reference and contaminated site. Significant seasonal differences were present at

both locations, pointing to increased MT levels in autumn ($U=6.00$; $p<0.001$ in the reference site and $U=32.00$; $p<0.001$ at the contaminated site) (Fig. 3a). Average MT concentration in gammarids in autumn was around 3.30 mg g^{-1} w.w. in both locations while average MT levels in spring were lower, 2.43 mg g^{-1} w.w. in individuals from the reference site and 2.87 mg g^{-1} w.w. in individuals from the Krka near Knin (Fig. 3a). These values were comparable or a bit higher than the MT levels obtained in the research on *G. fossarum* from the Sutla River, where reported average MT values were around 2.50 mg g^{-1} w.w (Filipović Marijić et al., 2016).

3.2.2. Cytosolic metal concentrations in *Gammarus balcanicus*

Reported differences in MT levels might be, to some extent, linked to cytosolic metal/metalloid concentrations, which were higher in *G. balcanicus* from the contaminated site, and this trend was proven significant for Co, Fe, Mn, Mo, K and Na in both seasons and for As, Cu and Zn in one season (Table 2). Therefore, a significant difference in MT levels between the two sites in spring could be linked to the much higher concentrations of Cu and Zn as important MT inducers at the location downstream of the town of Knin (Table 2). Zn was also significantly correlated to MT levels in *G. balcanicus* in autumn at the contaminated site ($r=0.621$; $p=0.0101$). On the other hand, Cd, Cs and Tl levels were significantly elevated in *G. balcanicus* from the reference site in both seasons, the same as recorded in fish intestine (Table 1), and Ca and Se only in autumn (Table 2). Again, as dissolved metal concentrations in water do not follow such pattern, the exact cause of these higher concentrations at the reference site needs to be further investigated. Ternjej et al. (2014) reported total metal levels in *G. balcanicus* from the Kosovčica River, which is Krka tributary, and also pointed to higher Cd levels in gammarids from the river spring compared to the pollution impacted river watercourse.

Significant seasonal differences in metal accumulation in *G. balcanicus* were observed for As, Cd, Cu, Fe, Mn, Mo, Ca, Mg and Na at the reference site with mostly higher values in autumn. Only As and Na levels were higher in spring, similar to As concentrations in fish intestine (Table 1). At the contaminated site, statistically significantly higher Cd, Cs and Mo levels were evident in autumn and Co, Se, Tl, Zn and Na levels in spring (Table 2). In addition, cytosolic As and Ca levels were higher in spring than autumn, but without

484 significant differences, what is in accordance to the seasonal trend of As found in intestine of
485 brown trout (Table 1).
486 Correlation of Cd, Cu or Zn with MT was mostly not significant, except Zn (r=0.621;
487 p=0.0101) in autumn at Knin location, but as in other organisms, MT induction in gammarids
488 might be impacted by other factors such as season, temperature, size, gender or reproductive
489 status (Rainbow and Moore, 1986; Correia et al., 2004; Geffard et al., 2007). However, data
490 on the impact of these parameters are not always consistent. For example, Geffard et al.
491 (2007) concluded that MTs levels in *G. pulex* were significantly negatively related to the
492 organism weight, while on the other hand, Filipović Marijić et al. (2016) have not observed
493 any significant differences in MT concentration in relation to the *G. fossarum* size. No
494 significant differences in MT levels were observed between the different age-groups of *G.*
495 *locusta* either (Correia et al., 2004).
496
497 There is not much detailed data on the life cycle of *G. balcanicus* in the world, but in
498 the area of Bieszczady Mountains in Poland, the breeding period of *G. balcanicus* lasts from
499 the beginning of April to the end of October (Zieliński, 1995). However, depending on the
500 water temperature and geographical region, in localities with constant water temperatures, this
501 species may have acyclic breeding without a winter pause (Dedju, 1980). In the case of the
502 Krka River, the exact life cycle of the species is not known yet, but observed seasonal
503 differences in MT levels might be associated with the different reproductive stages. Levels of
504 MT were significantly higher in autumn at both locations, likely during the reproduction
505 period for this species. Generally, as most of the gammarids have similar life-cycles, MT
506 synthesis is directly related to seasons, with higher values in autumn and winter and lower in
507 spring, as for example observed in *G. pulex* (Geffard et al., 2007). Many studies on different
508 invertebrate species like *Corbicula fluminea* (Baudrimont et al., 1997), *Mytilus*
509 *galloprovincialis* (Raspor et al., 2004; Ivanković et al., 2005) or *Mytilus edulis* (Geffard et al.,
510 2005) have also already shown that variations in MT levels are often related to the
511 physiological conditions of organisms, among which especially to their reproductive stage.
512
513 3.2.3. MT concentrations in the heat-treated cytosol of *Echinogammarus acarinatus*
514
515 Krka River source has already been reported as a habitat of another two gammarid
516 species - *Echinogammarus acarinatus* Karaman, 1931 and *Fontogammarus dalmatinus*

krkensis S. Karaman, 1931, both being endemic species in Dinaric karst rivers (Gottstein et al., 2007; Žganec et al., 2016). These species do not inhabit the area of the chosen contaminated site, so in our research results on *E. acarinatus* are presented only for the reference location in October 2015 and May 2016, whereas *F. dalmatinus krkensis* was not recorded in macrophytes of the Krka spring, but reaches the highest densities in the moss microhabitats of the spring head (Žganec et al., 2016). Absence of *E. acarinatus* and *F. dalmatinus krkensis* in the anthropogenically impacted area of the Krka River was already reported and explained as a consequence of their sensitivity on pollution impact, so their habitat in the Krka River comprises only clean parts of the watercourse (Gottstein et al., 2007).

MT levels in *E. acarinatus* were higher in autumn, the same as in *G. balcanicus*, but the seasonal differences were not significant. Average MT values in *E. acarinatus* were 2.94 and 2.53 mg g^{-1} w.w. in autumn and spring, respectively (Fig. 3b). These values were also similar and comparable to the MT concentrations observed in *G. balcanicus*, so the average MT levels were not significantly different between the two gammarid species at the Krka source in any season (Fig. 3).

3.2.4. Cytosolic metal concentrations in *Echinogammarus acarinatus*

Since *E. acarinatus* species only inhabit unpolluted area of the Krka River, cytosolic metals were presented regarding their seasonal differences in gammarids from the river source. Among investigated metals only As and Na were significantly increased in the spring campaign, while Cd, Cs, Mn, Mo, Tl, Ca, K and Mg were significantly increased in autumn (Table 2). Such pattern was found in *G. balcanicus*, in which As and Na were the only elements elevated in spring (Table 2), as also showed for As in fish intestine (Table 1). Significant differences between the two gammarid species were observed for As, Co, Cs, Mn and Tl in both seasons, while for Cd, Ca and Na only in the spring season. All of these elements had higher concentrations in *E. acarinatus* (Table 2). As *E. acarinatus* individuals were much smaller, the differences in metal accumulation might be caused by the gammarid size differences. For example, Rainbow and Moore (1986) showed that the smallest amphipods accumulated the highest concentrations of Cu, Zn, Fe and Pb. Moreover, even closely related species like these two gammarid species may be feeding on different food sources which results in different dietary inputs of metals (Rainbow and Moore, 1986). If we

consider Cd, Cu and Zn, as the main MT inducers, their levels were not significantly correlated with MT levels in *E. acarinatus*, as already stated for other gammarid species, *G. balcanicus* (Table 2).

3.3. Comparison of cytosolic metal concentrations in intestine of freshwater fish and whole gammarids

Most of the cytosolic metal levels were higher in gammarids than in brown trout intestine, therefore confirming that most of the metals are not expected to biomagnify in aquatic food webs (Mathews and Fisher, 2008). The highest difference existed for Ca, Cu and As, which average levels were around 50, 15 and 8 times higher in gammarids than in fish cytosolic fraction, respectively. Twice as higher cytosolic Cd and Mg levels were recorded in gammarids than in fish intestine, while few metals showed the opposite pattern, i.e. K, Se and Cs levels were 2-3 times lower and Fe and Zn about 5 times lower in gammarids (Tables 1, 2). Such results are in accordance to trophic transfer factors obtained for metals in marine food chain, which indicated that possible biomagnification is specific for Cs, Se and Zn (Mathews and Fisher, 2008). Descending order of cytosolic metal levels in intestine of brown trout from the Krka River was the following: K>Na>Mg>Ca>Zn>Fe>Se (average metal levels higher than 1000 $\mu g\ kg^{-1}$) and Cu≥Mn>Cd≥Co>Mo>As>Tl>Cs (average metal levels lower than 1000 $\mu g\ kg^{-1}$) (Table 1). Comparison of cytosolic metal levels between two gammarid species, *E. acarinatus* and *G. balcanicus* indicated higher metal levels in *E. acarinatus*, but the concentration range in both species was comparable. Thus, descending order of cytosolic metal levels in both gammarid species from the Krka River was the following: Ca>K>Na>Mg>Zn>Cu>Fe (average metal levels higher than 1000 $\mu g\ kg^{-1}$) and Mn>Se>As≥Cd>Mo>Co≥Tl>Cs (average metal levels lower than 1000 $\mu g\ kg^{-1}$) (Table 2).

To our knowledge, comparison of intestinal cytosolic metal/metalloid concentrations in brown trout with other fish species was possible only for cytosolic metal levels in the intestinal tissue of European chub from the Sava River, which showed the same descending order of investigated metal levels and mostly comparable concentrations (Zn>Fe>Cu>Mn>Cd) (Filipović Marijić and Raspor, 2012). Cytosolic metal levels in gammarids can be compared with levels in *G. fossarum* from the Sutla River where Cs, Cu, Mn and Zn levels were approximately 2 times higher than in our research, while the levels of Ca and Tl were about 2 and 6 times higher in gammarids from the Krka River, respectively

583 (Filipović Marijić et al., 2016). Such differences in cytosolic metal levels between different
584 gammarid species are probably influenced by variability in metal exposure and environmental
585 conditions of their habitat.
586
587 **3.4. Comparison of MT concentrations measured by electrochemical methods in**
588 **freshwater fish and gammarids from different studies**

589 Modified Brdička reaction is recognized as a commonly and widely used
590 electrochemical method for MT determination in biological samples (Fabrik et al., 2008;
591 Dragun et al., 2009; Krizkova et al., 2009a; Filipović Marijić et al., 2016). In our research,
592 newly modified Brdička method (Mijošek et al., 2018) was confirmed as a fast and reliable
593 technique for quantification of MTs in both intestinal fish tissue and the whole individuals of
594 gammarids species. One of the main advantages of the applied method is that it requires a
595 small amount of the sample to conduct the assay. Our results on MT concentrations were
596 compared to other so far published data on MT levels in natural populations of organisms
597 measured by electrochemical method in order to get an overview on MT levels in different
598 freshwater fish and gammarid species (Table 3). For the purposes of correct comparison, MT
599 levels (mg g^{-1} w.w.) from our study were additionally divided with the total cytosolic protein
600 concentrations, resulting in the average concentrations in brown trout intestine of around 20.5
601 µg mg^{-1} proteins and in gammarids of around 60 µg mg^{-1} proteins. Also, MT levels (mg g^{-1}
602 w.w.) were divided with MT molecular weight of 6600 Da, resulting in MT average
603 concentrations in brown trout intestine of around 20 nmol g^{-1} and in gammarids of around 40
604 nmol g^{-1}. So far, intestinal MT levels were only reported for the European chub from the Sava
605 River (Croatia), which MT levels (2.9-3.1 mg g^{-1} w.w.) were twice as high as in brown trout
606 intestine (0.8-1.5 mg g^{-1} w.w.) (Table 3). In other fish tissues electrochemically determined
607 MTs ranged 0.3-2 mg g^{-1} w.w. and 2-7 µg mg^{-1} prot. in gills; 2-12 mg g^{-1} w.w. and 5-18 µg
608 mg^{-1} prot. in liver; 9-16 mg g^{-1} w.w. and 1-10 µg mg^{-1} prot. in kidney (Table 3). In *G. pulex*
609 from La Bourbre River (France) and *G. fossarum* from the Sutla River (Croatia) MTs ranged
610 1-4 mg g^{-1} w.w. (Table 3), what is comparable to our data reported for MT levels in *G.*
611 *balcanicus* and *E. acarinatus* from the Krka River (3 mg g^{-1} w.w.).
612

613 **4. Conclusions**

Obtained MT concentrations in the intestinal tissue of *S. trutta* and two gammarid species from the karst Krka River in Croatia revealed that anthropogenic impact near the wastewater outlets was evident, although not significantly in all cases. Wastewaters impact was also confirmed regarding metal concentrations in all organisms, and comparison of total and cytosolic metal levels in fish intestine showed that As, Cd, Cs, Mo, Se, K, Mg, and Na were present mostly in the cytosolic fraction (over 67%), pointing that these metals are present in metabolically available intestinal fraction, where they can be bound to important biomolecules (enzymes) or might be detoxified by MT.

Electrochemically obtained MT levels in vertebrate and invertebrate organisms were species specific, showing higher MT concentrations in the gammarids than in the fish intestine. Despite variable MT levels, both bioindicator organisms pointed to the same trend, with higher MT values in the organisms from the contaminated compared to the reference site. Therefore, in freshwater salmon fish and gammarids MTs reflected metal contamination in the aquatic environment, so electrochemical method was confirmed as a sensitive tool in biomonitoring studies of metal exposure. Comparison of MT levels from our study with the literature data pointed to variability in MT concentrations among native fish and gammarid species, as well as among different fish tissues. Thus, proposed electrochemical method can be applied in biomonitoring studies as a tool for detecting MT changes in relation to anthropogenic impact on aquatic ecosystems and biota, but the interpretation should be done with caution knowing all the factors affecting MT levels. Advantage of the used electrochemical method is that requires a small amount of the sample, but it also needs specialized and sensitive laboratory equipment. Presented results indicated that MT levels are species- and tissue-specific, so the comparison between MT levels should always be performed for the same species, tissue and measurement method.

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Figure captions:

Figure 1. MT levels (mg g^{-1} w.w., mean values and standard deviations) in intestinal tissue of *S. trutta* from the Krka River at two sampling sites (reference site: Krka River source; contaminated site: town of Knin) in two samplings (autumn 2015 and spring 2016).

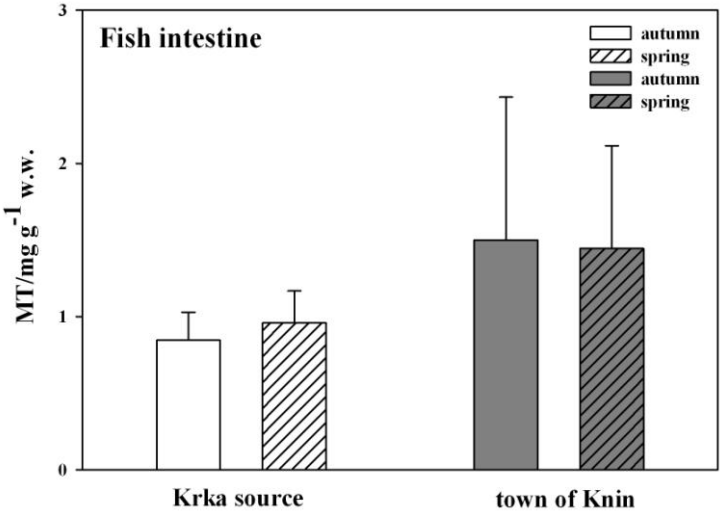

Figure 2. Proportions (%) of metal/metalloid levels present in cellular cytosolic fraction (soluble) and insoluble fraction of the intestine of *S. trutta* caught in the Krka River: a) metals present in cytosolic fraction up to 60% and b) metals present in cytosolic fraction above 60%.

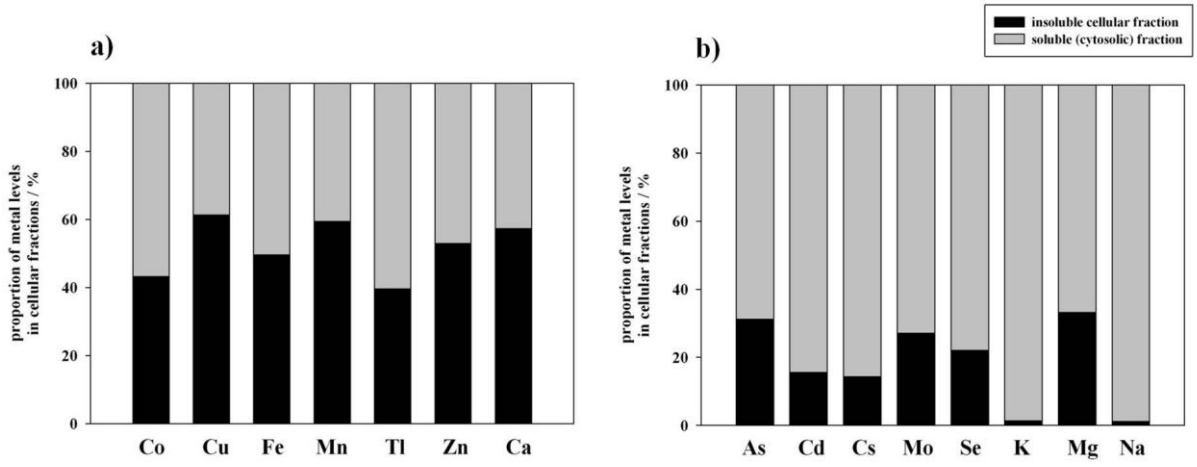

Figure 3. MT levels (mg g^{-1} w.w., mean values and standard deviations) in a) *G. balcanicus* and b) *E. acarinatus* from the Krka River at two sampling sites (reference site: Krka River source; contaminated site: town of Knin) in two samplings (autumn 2015 and spring 2016). Statistically significant differences (Mann-Whitney *U* test) at $p<0.05$ level between two seasons at each sampling site are marked with solid line and asterisk (*) and between two

sampling sites within the same season are assigned with dashed line and different superscript letters (A and B).

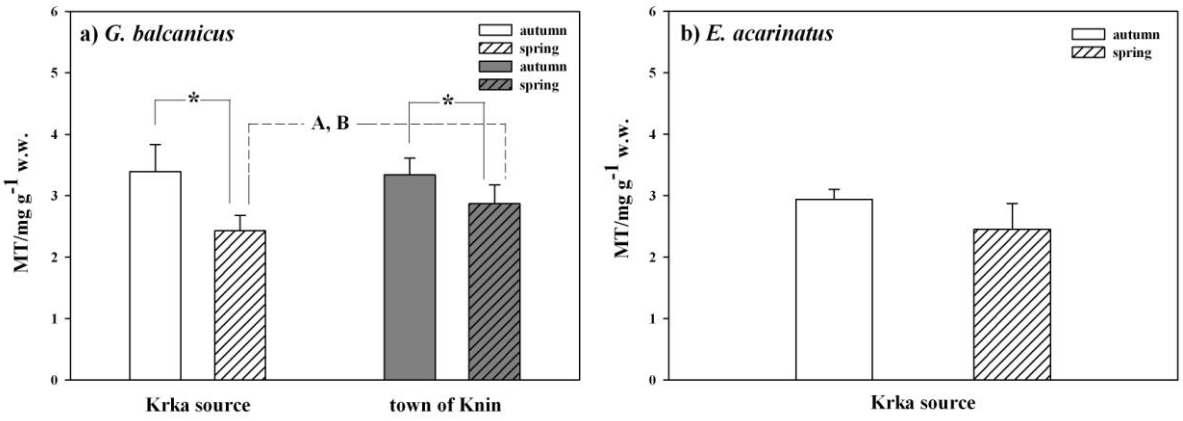

Table 1. Total and cytosolic metal and metalloid concentrations (μg kg^{-1} or mg kg^{-1} (macroelements)) in the intestinal tissue of *S. trutta* from the Krka River at two sampling sites in two sampling campaigns. For each element first row represents the total levels and the second cytosolic levels. Results are showed as mean values ± standard deviations.

		Krka River source		town of Knin	
		October 2015	**May 2016**	**October 2015**	**May 2016**
		For all elements: first row - total levels; second row - cytosolic levels			
As	**μg kg^{-1}**	20.27±12.14*	32.53±9.24*	30.28±14.73	42.83±17.35
		12.45 ± 7.19$^{*, A}$	20.63 ± 7.40*	33.94 ± 34.94^{B}	37.06 ± 15.03
Cd		88.87±123.78	135.47±125.79^{A}	27.68±25.30*	3.81±2.90$^{*, B}$
		64.76 ± 91.80^{A}	85.39 ± 89.94^{A}	30.80 ± 51.54$^{*, B}$	3.12 ± 2.58$^{*, B}$
Co		39.12±17.42	24.33±58.86^{A}	61.34±37.73	58.86±27.45^{B}
		15.38 ± 11.57^{A}	13.73 ± 5.93^{A}	46.33 ± 56.26^{B}	57.62 ± 45.03^{B}
Cs		10.03±2.18^{A}	7.82±1.83	5.79±4.50^{B}	5.97±1.84
		9.28 ± 2.31$^{*, A}$	7.01 ± 1.93$^{*, A}$	4.25 ± 2.86^{B}	4.82 ± 1.70^{B}
Cu		777.88±242.17	942.37±221.62	966.58±413.62	897.67±312.04
		253.10 ± 126.32^{A}	356.22 ± 115.68	597.41 ± 560.92^{B}	345.58 ± 158.98
Fe		19116.76±11560.96	11009.87±2281.04	17529.05±4137.32	14749.16±5247.41
		8185.41 ± 3138.92	5939.63 ± 3153.94	6614.67 ± 3321.66	7037.54 ± 1597.50
Mn		921.23±478.61	783.62±139.55	881.87±209.90	953.15±435.44
		399.26 ± 308.03	282.13 ± 60.16	266.48 ± 112.80	316.30 ± 115.86
Mo		50.90±39.22	48.28±13.06^{A}	42.48±8.90*	31.00±5.31$^{*, B}$
		31.96 ± 10.89	30.67 ± 8.55	41.10 ± 20.60*	23.81 ± 7.72*
Se		807.83±323.81^{A}	845.26±172.90^{A}	1201.36±385.90^{B}	1173.95±292.24^{B}
		721.83 ± 329.89^{A}	677.11 ± 69.57^{A}	1120.86 ± 511.20^{B}	1056.38 ± 296.96^{B}
Tl		45.97±31.73^{A}	44.62±12.96^{A}	19.24±7.90^{B}	19.89±8.15^{B}
		29.62 ± 15.38^{A}	30.78 ± 10.62^{A}	8.68 ± 4.04^{B}	11.76 ± 5.53^{B}
Zn		98677.54±39032.26	107033.66±49100.97	138929.69±86549.18	124701.27±23088.43
		42579.36 ± 12009.36	45995.21 ± 12593.36	46981.93 ± 20645.43	54950.47 ± 6834.28
Ca	**mg kg^{-1}**	221.28±160.72	136.74±49.24^{A}	292.98±298.14	245.27±93.49^{B}
		91.37 ± 93.31	53.95 ± 18.77^{A}	112.42 ± 117.82	94.67 ± 41.56^{B}
K		2935.06±357.63	2887.32±364.96	2938.30±430.18	2911.28±364.25
		2842.10 ± 326.10	2749.35 ± 171.76	2681.73 ± 402.69	2811.05 ± 287.30
Mg		154.53±21.07	163.59±21.51	164.01±23.72	148.39±24.96
		103.16 ± 18.64	103.52 ± 11.10	100.45 ± 15.67	100.55 ± 23.17
Na		1107.76±132.20*	932.87±177.15*	1117.55±115.57*	974.32±162.13*
		1071.33 ± 111.14*	904.32 ± 125.92*	981.17 ± 173.13	976.78 ± 127.09

Significant difference at $p<0.05$ level between two seasons at each sampling site is marked with asterisk (*) and significantly different values at two sampling sites within the same sampling campaign are assigned with different superscript letters (A and B).

Table 2. Cytosolic metal and metalloid concentrations (μg kg^{-1} or mg kg^{-1} (macroelements)) in *G. balcanicus* from the Krka River at two sampling sites in two sampling campaigns and *E. acarinatus* from the Krka River source in two campaigns. Results are showed as mean values ± standard deviations.

		Krka River source		Town of Knin		Krka River source	
		October 2015	May 2016	October 2015	May 2016	October 2015	May 2016
		Gammarus balcanicus				*Echinogammarus acarinatus*	
As	**μg kg^{-1}**	79.11 ± 19.89$^{*, A}$	152.70 ± 24.5*	135.17 ± 11.60^{B}	147.18 ± 23.88	157.46 ± 23.51*	220.35 ± 13.36*
Cd		204.30 ± 38.05$^{*, A}$	100.18 ± 12.11$^{*, A}$	15.81 ± 3.71$^{*, B}$	12.42 ± 2.62$^{*, B}$	180.79 ± 23.42*	121.98 ± 14.67*
Co		17.87 ± 2.0^{A}	19.09 ± 1.94^{A}	34.41 ± 3.16$^{*, B}$	70.07 ± 11.11$^{*, B}$	24.57 ± 8.22	21.96 ± 0.59
Cs		4.01 ± 0.4^{A}	3.84 ± 0.25^{A}	3.03 ± 0.46$^{*, B}$	2.62 ± 0.22$^{*, B}$	4.43 ± 0.28*	4.10 ± 0.16*
Cu		5365.41 ± 1207.69*	4184.47 ± 563.15$^{*, A}$	5525.71 ± 529.37	5684.54 ± 651.55^{B}	4464.71 ± 1106.95	4345.17 ± 299.37
Fe		1417.96 ± 552.66$^{*, A}$	977.09 ± 126.11$^{*, A}$	2124.95 ± 353.67^{B}	1972.47 ± 359.78^{B}	1638.18 ± 497.07	1284.98 ± 509.41
Mn		335.80 ± 37.43$^{*, A}$	281.40 ± 22.21$^{*, A}$	648.00 ± 88.88^{B}	622.53 ± 78.17^{B}	433.74 ± 30.44*	325.71 ± 28.83*
Mo		39.25 ± 6.85$^{*, A}$	27.60 ± 3.06$^{*, A}$	68.06 ± 8.70$^{*, B}$	57.02 ± 7.94$^{*, B}$	35.80 ± 5.58*	26.71 1.54*
Se		321.81 ± 45.96^{A}	299.68 ± 44.38	290.65 ± 31.94$^{*, B}$	323.91 ± 47.21*	322.77 ± 18.79	330.11 ± 18.23
Tl		19.41 ± 2.49^{A}	18.55 ± 1.02^{A}	5.57 ± 0.77$^{*, B}$	7.38 ± 1.20$^{*, B}$	27.66 ± 1.51*	22.83 ± 2.18*
Zn		6806.64 ± 719.10	6675.64 ± 595.40^{A}	6794.99 ± 393.87*	8061.22 ± 838.43$^{*, B}$	7252.82 ± 886.40	6883.24 ± 596.36
Ca	**mg kg^{-1}**	4698.53 ± 322.75$^{*, A}$	4041.23 ± 293.38*	4140.15 ± 252.09^{B}	4310.13 ± 415.00	5084.05 ± 454.71*	4414.88 ± 335.72*
K		1627.99 ± 206.60^{A}	1530.02 ± 70.29^{A}	1762.77 ± 145.89^{B}	1746.65 ± 95.18^{B}	1726.35 ± 120.58 *	1579.29 ± 53.14*
Mg		269.72 ± 27.09$^{*, A}$	220.81 ± 19.49$^{*, A}$	228.92 ± 18.45^{B}	239.34 ± 19.50^{B}	280.43 ± 24.66*	224.40 ± 18.67*
Na		1178.64 ± 70.27$^{*, A}$	1352.78 ± 87.68$^{*, A}$	1060.26 ± 73.87$^{*, B}$	1291.18 ± 50.19$^{*, B}$	1244.49 ± 100.92*	1491.57 ± 75.54*

Significant difference at $p<0.05$ level between two seasons at each sampling site is marked with asterisk (*) and significantly different values at two sampling sites within the same sampling campaign are assigned with different superscript letters (A and B).

Table 3. Metallothionein concentrations reported in different tissues of freshwater fish (liver, kidney, gills and the intestine) and crustaceans (whole organisms) species from natural populations obtained by electrochemical methods.

Species	Tissue	MT concentration	Reference
Rainbow Trout *(Salmo gairdneri)*	Liver	58-269 nmol g^{-1}	**Roch et al., 1982**
European eel *(Anguilla anguilla)*	Liver	4.37-12.60 mg g^{-1} w.w.	**Ureña et al., 2007**
	Kidney	9.35-15.86 mg g^{-1} w.w.	
	Gills	0.30-0.50 mg g^{-1} w.w.	
European chub - Sava River *(Squalius cephalus)*	Liver	1.6-1.9 mg g^{-1} w.w.	**Podrug and Raspor, 2009** **Dragun et al., 2009** **Filipović Marijić and Raspor, 2010** **Dragun et al., 2015**
	Gills	1.3-2.0 mg g^{-1} w.w.	
	Intestine	2.9-3.1 mg g^{-1} w.w.	
European chub - Sutla River *(Squalius cephalus)*	Liver	0.80-3.73 mg g^{-1} w.w.	**Dragun et al., 2013**
	Gills	0.66-2.35 mg g^{-1} w.w.	
Asp *(Leuciscus aspius)*	Liver	7.4-7.5 µg mg^{-1} prot.	**Sevcikova et al., 2013**
	Gills	3.6-3.9 µg mg^{-1} prot.	
	Kidney	1.4-2.3 µg mg^{-1} prot.	
Pike-perch *(Sander lucioperca)*	Liver	6.4-7.0 µg mg^{-1} prot.	
	Gills	3.9-5.0 µg mg^{-1} prot.	
	Kidney	3.4-9.4 µg mg^{-1} prot.	
Perch *(Perca fluviatilis)*	Liver	4.8-8.3 µg mg^{-1} prot.	
	Gills	4.0-5.5 µg mg^{-1} prot.	
	Kidney	1.3-2.8 µg mg^{-1} prot.	
Pike *(Esox lucius)*	Liver	11.0-18.1 µg mg^{-1} prot.	
	Gills	2.4-5.4 µg mg^{-1} prot.	
	Kidney	3.3-6.8 µg mg^{-1} prot.	
Bream *(Abramis brama)*	Liver	5.3-10.1 µg mg^{-1} prot.	
	Gills	4.0-4.7 µg mg^{-1} prot.	
	Kidney	2.5 µg mg^{-1} prot.	
Chub *(Squalius cephalus)*	Liver	4.8-7.1 µg mg^{-1} prot.	
	Gills	2.0-2.9 µg mg^{-1} prot.	
	Kidney	2.9-4.4 µg mg^{-1} prot.	
Roach *(Rutilus rutilus)*	Liver	5.7-12.3 µg mg^{-1} prot.	
	Gills	3.4-4.3 µg mg^{-1} prot.	
	Kidney	1.7-2.3 µg mg^{-1} prot.	
Silver bream *(Blicca bjoerkna)*	Liver	7.5 µg mg^{-1} prot.	
	Gills	4.5 µg mg^{-1} prot.	
	Kidney	8.9 µg mg^{-1} prot.	
Common carp *(Cyprinus carpio)*	Liver	8.5 µg mg^{-1} prot.	
	Gills	4.1 µg mg^{-1} prot.	
	Kidney	5.0 µg mg^{-1} prot.	
Rudd *(Scardinius erythrophthalmus)*	Liver	9.6 µg mg^{-1} prot.	
	Gills	6.5 µg mg^{-1} prot.	
	Kidney	10.3 µg mg^{-1} prot.	
Brown trout *(Salmo trutta)*	Intestine	0.85 - 1.5 mg g^{-1} w.w. 18-25 µg mg^{-1} prot. 21-38 nmol g^{-1}	**This study**
Gammarus pulex	Whole organism	1.25-3.25 mg g^{-1} w.w.	**Geffard et al., 2007**
Gammarus fossarum	Whole organism	1.55-3.65 mg g^{1} w.w.	**Filipović Marijić et al., 2016**

Gammarus balcanicus	Whole organism	2.43-3.39 mg g^{-1} w.w 52-70 µg mg^{-1} prot. 37- 51 nmol g^{-1}	**This study**
Echinogammarus acarinatus	Whole organism	2.53-2.94 mg g^{-1} w.w 55-60 µg mg^{-1} prot. 38-45 nmol g^{-1}	