Accepted manuscript – postprint version. Final form is published in:
Dragun, Z., Filipović Marijić, V., Krasnići, N., Ivanković, D., Valić, D., Žunić, J., … Erk, M. (2018). Total and cytosolic concentrations of twenty metals/metalloids in the liver of brown trout Salmo trutta (Linnaeus, 1758) from the karstic Croatian river Krka. *Ecotoxicology and Environmental Safety*, *147*, 537–549. https://doi.org/10.1016/j.ecoenv.2017.09.005

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# Total and cytosolic concentrations of twenty metals/metalloids in the liver of brown trout *Salmo trutta* (Linnaeus, 1758) from the karstic Croatian river Krka

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**Abstract**

Total and cytosolic concentrations of twenty metals/metalloids in the liver of brown trout *Salmo trutta* (Linnaeus, 1758) were studied in the period from April 2015 to May 2016 at two sampling sites on Croatian river Krka, to establish if river water contamination with metals/metalloids downstream of Knin town has influenced metal bioaccumulation in *S. trutta* liver. Differences were observed between two sites, with higher concentrations of several elements (Ag, As, Ca, Co, Na, Se, Sr, V) found downstream of Knin town, whereas few others (Cd, Cs, Mo, Tl) were, unexpectedly, increased at the Krka River spring. However, total metal/metalloid concentrations in the liver of *S. trutta* from both sites of the Krka River were still mainly below previously reported levels for pristine freshwaters worldwide. The analysis of seasonal changes of metal/metalloid concentrations in *S. trutta* liver and their association with fish sex and size mostly indicated their independence of fish physiology, making them good indicators of water contamination and exposure level. Metal/metalloid concentrations in the metabolically available hepatic cytosolic fractions reported in this study are the first data of that kind for *S. trutta* liver, and the majority of analyzed elements were present in the cytosol in the quantity higher than 50% of their total concentrations, thus indicating their possible availability for toxic effects. However, the special attention should be directed to As, Cd, Cs, and Tl, which under the conditions of increased exposure tended to accumulate more within the cytosol. Although metal/metalloid concentrations in *S. trutta* liver were still rather low, monitoring of the Krka River water quality and of the health status of its biota is essential due to a trend of higher metal/metalloid bioaccumulation downstream of Knin town, especially taking into consideration the proximity of National Park Krka and the need for its conservation.

**Key words**: bioaccumulation, fish, freshwater, inorganic contamination, liver, subcellular distribution

## 1. Introduction

One of the major problems of aquatic systems in the world is their ever-growing contamination originating from different types of anthropogenic activities. Among many types of contaminants, metals/metalloids occupy an important place in the environmental studies. Once introduced in an aquatic system, metals/metalloids are redistributed in the water column between the particulate and dissolved phase, deposited in sediment and accumulated in the organs of various aquatic organisms, including fish, through water filtration, diet or skin absorption (Fichet et al., 1998; Kraemer et al., 2006). Such metal accumulation may leave fish populations at an increased risk of experiencing toxicity (Kraemer et al., 2006), because it has been shown to cause metabolic alterations and disturbances of biological systems (van der Oost et al., 2003).

Since total quantity of metals present in the aquatic environment is not completely bioavailable, one of the most effective ways to evaluate their potential impacts on aquatic biota is to monitor metal concentrations accumulated in an adequate and representative bioindicator organism (Kraemer et al., 2006). Brown trout *Salmo trutta* (Linnaeus, 1758) is widely present in freshwater systems in Europe and around the world. It can be found both in clean and in polluted areas and thus it represents a good species for biomonitoring (Culioli et al., 2009). For example, this species has already been proven as useful bioindicator organism for arsenic accumulation (Culioli et al., 2009). Moreover, *S. trutta* is a part of the human diet, and therefore, their contamination is also a matter of concern for human health (Culioli et al., 2009).

Monitoring of metal/metalloid accumulation in bioindicators is usually carried out by measuring their total concentrations in relevant target organs. Liver and kidney are considered to be the best indicator organs for evaluating long term, chronic exposure to metals (Miller et al., 1992). This is especially true for liver, because it is the main site for metal metabolism and detoxification (Linde et al., 1998), and also has the most effective accumulation ability (Sindayigaya et al., 1994; Papagiannis et al., 2004; Vukosav et al., 2014). As a defence mechanism, hepatocytes, the main cell type in the liver, are equipped with high levels of intracellular binding proteins and peptides, which aid in the metal/metalloid sequestration, thus preventing their interaction with potentially sensitive sites (Di Giulio and Hinton, 2008; Sigel et al., 2009).

Therefore, in addition to measuring total accumulated metal/metalloid concentrations in fish liver, useful information about how aquatic organisms deal with both essential and non-essential metals can be obtained by determining metal/metalloid concentrations at the subcellular level (Barst et al., 2016). After entering the organism, trace metals usually undergo a series of metabolic processes and are subsequently incorporated into various cellular components (Mason and Jenkins, 1995; Wang and Rainbow, 2005; Goto and Wallace, 2010). They might be bound by a variety of biomolecules for metabolic function, storage, detoxification, toxicity, or excretion (Klaassen et al., 1999; Rainbow, 2002). Some metals are sequestered by metal-binding proteins (e.g., metallothioneins) or granular concretions in detoxified forms (Langston et al., 1998; Goto and Wallace, 2007). The others may be incorporated into non-detoxifying cellular components (e.g., enzymes and organelles), which could ultimately result in toxicological effects at various levels of biological organization (Wallace et al., 2003; Sigel et al., 2009; Goto and Wallace, 2010).

This study was performed on *S. trutta* from the Croatian river Krka. The Krka River is a natural karst phenomenon, and a large part of its watercourse was proclaimed a national park in 1985 (web 1). An increase in trace metal concentrations in the upper flow region, as the result of the untreated municipal and industrial waste-water discharge downstream of Knin town (Cukrov et al., 2008), presents a potential threat for its conservation, especially considering that the northern border of National Park Krka is situated only 2 km downstream of Knin town. Although *S. trutta*, which is a representative species in the Krka River, is fish widely used as a bioindicator organism for monitoring metals in freshwater ecosystems, there is only limited number of elements that have been monitored in its organs. For example, so far there is only information on Al, As, Cd, Co, Cu, Se and Zn concentrations in trout liver from different parts of the world (Karlsson-Norrgren et al., 1986; Brotheridge et al., 1998; Linde et al., 1998; Olsvik et al., 2000; Dussault et al., 2004; Vítek et al., 2007; Arribére et al., 2008; Has-Schön et al., 2008; Foata et al., 2009; Can et al., 2012; Herrmann et al., 2016). With the general aim to broaden the existing data pool on metal/metalloid levels in *S. trutta* organs which could be used in the future monitoring as the basis for comparison, we have measured total and cytosolic concentrations of twenty elements (Ag, Al, As, Ca, Cd, Co, Cs, Cu, Fe, K, Mg, Mn, Mo, Na, Rb, Se, Sr, Tl, V, Zn) in the liver of *S. trutta*. Our specific goal was to compare those concentrations at two sampling sites of the Krka River, the Krka River spring as a reference site and the location downstream of Knin town as a contaminated site. We wanted to determine if contamination of the river water have influenced metal/metalloid accumulation in *S. trutta* liver. Since the relationship between metal/metalloid concentrations and several intrinsic factors of the fish can present a confounding factor when using aquatic animals as biomonitors of metal pollution (Linde et al., 1998), we have also tested the seasonal changes of metal/metalloid concentrations in *S. trutta* liver, as well as their association with *S. trutta* sex and size. Additionally, with the aim to assess metabolically available and potentially toxic fractions of metals/metalloids in *S. trutta* liver, we have calculated the proportions of each metal/metalloid present in the cytosolic hepatic fractions, which contain heat-stable and heat-sensitive biomolecules, lysosomes and microsomes (Bonneris et al., 2005; Dragun et al., 2013a). Finally, our overall aim was to evaluate, based on the all gathered information within this study, the current quality status of the Krka River, and the potential threat for the aquatic organisms inhabiting its water.

## 2. Materials and methods

### *2.1. Study area and fish sampling*

The samplings of *S. trutta* were performed at two sampling sites in the Krka River in Croatia (Fig. 1) in four campaigns (April, September, and October 2015, and May 2016). Based on the previously published information (Cukrov et al., 2008; Filipović Marijić et al., 2016; 2017) and this study (Table 1), the Krka River spring was chosen as a reference site, whereas a location downstream of Knin town, situated only 2 km upstream of the northern border of the Krka National Park, was chosen as a contaminated site. In the Knin area, there are two known sources of contamination, industrial wastewater of screw factory and untreated municipal wastewater discharge. The analyses of dissolved metals/metalloids in the river water have indicated a slightly higher concentrations of several trace elements (e.g. Al, As, Ca, Co, Fe, K, Mn, Mo, Na, Rb, Se, Sr, V, and Zn) downstream of Knin town (Table 1; Cukrov et al., 2008; Filipović Marijić et al., 2016; 2017; Sertić Perić et al., 2017). Information about the river water samplings and subsequent measurements of dissolved metals/metalloids were described in details by Filipović Marijić et al. (2017) and Sertić Perić et al. (2017).

For this study we have sampled 135 *S. trutta* specimens by electro fishing, according to the Croatian standard HRN EN 14011 (2005), 14 to 22 from each site in each sampling campaign, as indicated in Table 2. All 135 fish, sampled in all four campaigns, were used for analyses of cytosolic metal concentrations in the liver, whereas total metal concentrations in the hepatic tissue were only determined in 65 fish sampled in the last two sampling campaigns. The captured fish were kept alive in aerated water tank till further processing in the laboratory.

### *2.2. Fish dissection*

Fish were euthanized with freshly prepared anaesthetic tricaine methane sulphonate (MS 222, Sigma Aldrich) which was added directly to the water in which fish were held, in accordance with the Ordinance on the protection of animals used for scientific purposes (NN 55/2013). Fish total mass and length were recorded, then the liver and the gonads were dissected and weighed, and the liver were stored at -80°C for further analyses. Hepatosomatic (HSI) and gonadosomatic indices (GSI) were calculated based on the ratio of liver and gonad mass to total *S. trutta* mass, respectively. Fulton condition indices (FCI) were calculated according to Rätz and Lloret (2003), using the following equation: [(mass in grams × 100) / (length in centimetres)$^3$]. Sex was determined by both macroscopic and microscopic examination of gonads. For microscopic identification of sex, a section of gonad tissue from each fish was placed on a microscope slide, and the slides were observed under a 40× and 100× magnifications using optical microscope BH-2 (Olympus).

*2.3. Tissue homogenization and isolation of soluble cytosolic tissue fractions*

Isolation of soluble cytosolic fraction from *S. trutta* liver was performed according to the Standard Operational Procedure (1999), which was developed at Norwegian Institute for Water Research in the framework of the Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM) (Dragun et al., 2009). The samples of hepatic tissue were cut into small pieces. Then cooled homogenization buffer [100 mM Tris-HCl/Base (Sigma, pH 8.1 at 4°C) supplemented with reducing agent (1 mM dithiotreitol, Sigma)] was added (w/v 1:5), followed by homogenization with 10 strokes of Potter-Elvehjem homogenizer (Glas-Col, USA) in an ice cooled tube at 6,000 rpm. The homogenates were subsequently centrifuged (Avanti J-E centrifuge, Beckman Coulter) at 50,000×g for 2 h at 4°C. Soluble cytosolic hepatic fractions, i.e. supernatants obtained after centrifugation of tissue homogenates at 50,000×*g*, contained cytosolic biomolecules, lysosomes and microsomes, and excluded cell membranes, nuclei, mitochondria and granules (Bonneris et al., 2005; Dragun et al., 2013a; Podrug et al., 2009).

*2.4. Preparation of hepatic homogenates and cytosolic fractions for metal/metalloid measurement*

During liver homogenization, an aliquot of each homogenate was set aside for subsequent digestion. Digestion procedures used in this study were modified from previously described procedures (Dragun et al., 2013a; Filipović Marijić et al., 2013). Hepatic homogenates were digested by addition of oxidation mixture (v/v 1:3), which contained concentrated $HNO_3$ (*Rotipuran® Supra* 69%, Carl Roth GmbH + Co. KG, Germany) and 30% $H_2O_2$ (*Suprapur®*, Merck, Germany) (v/v 3:1). Digestions were performed in a laboratory dry oven at 85°C for 3.5 h.

Cytosolic fractions from the first two sampling campaigns (April and September 2015) were only diluted with Milli-Q water and acidified with $HNO_3$ (*suprapur*, Merck, Germany; final acid concentration in the samples 0.65%) prior to measurement. Dilution factor was 100 for Na, K, and Mg, and ten for the remaining elements (Dragun et al., 2013a). Cytosolic fractions from third and fourth sampling campaign (October 2015 and May 2016) were digested in duplicate by addition of oxidation mixture (v/v 1:1), which contained concentrated $HNO_3$ (*Rotipuran®*

*Supra* 69%, Carl Roth GmbH + Co. KG, Germany) and 30% $H_2O_2$ (*Suprapur®*, Merck, Germany) (v/v 3:1). Digestion was performed in laboratory dry oven at 85°C for 3.5 h.

Following digestions of homogenates and cytosolic fractions, samples were diluted with Milli-Q water, 1:5 prior to Ca and trace element analyses, and 1:20 for Na, K and Mg analyses. Although two approaches were used for preparation of hepatic cytosols for analyses (dilution in the first two samplings and digestion in two latter), the results could be comparatively analyzed. Previously performed methodological study demonstrated comparability of the results obtained after application of sample dilution and of sample digestion prior to metal measurement by high-resolution inductively coupled plasma mass spectrometer (HR ICP-MS) (Dragun et al., 2013a).

### *2.5. Metal and metalloid analyses*

Twenty trace and macro elements were analyzed using HR ICP-MS (Element 2, Thermo Finnigan, Germany) equipped with an autosampler SC-2 DX FAST (Elemental Scientific, USA) and sample introduction kit consisting of a SeaSpray nebulizer and cyclonic spray chamber Twister. Typical instrumental conditions and measurement parameters were reported previously (Fiket et al., 2007). Indium (1 µg/L; indium atomic spectroscopy standard solution, Fluka, Germany) was added in all samples as an internal standard (Fiket et al., 2007). Measurements of $^{82}$Se, $^{85}$Rb, $^{98}$Mo, $^{109}$Ag, $^{111}$Cd, $^{133}$Cs, and $^{205}$Tl were operated in low-resolution mode; of $^{23}$Na, $^{24}$Mg, $^{27}$Al, $^{42}$Ca, $^{51}$V, $^{55}$Mn, $^{56}$Fe, $^{59}$Co, $^{63}$Cu, $^{66}$Zn, and $^{86}$Sr in medium resolution mode; and of $^{39}$K and $^{75}$As in high resolution mode. External calibrations were performed using a multielement standard containing Na, K, Mg, and Ca (Fluka, Germany), a standard containing Ag (Fluka, Germany), and a multielement standard solution for trace elements (Analytika, Czech Republic) supplemented with Rb (Sigma-Aldrich, Germany) and Cs (Fluka, Germany). All standards were prepared in 1.3% $HNO_3$ (*Suprapur®*, Merck, Germany) and supplemented with In (1 µg/L; Fluka, Germany).

All measurements were performed in duplicate. For checking the accuracy of HR ICP-MS measurements, quality control samples obtained from UNEP/GEMS (QC trace metals, catalogue no. 8072, lot no. 146142-146143; QC minerals, catalogue no. 8052, lot no. 146138-146139; Burlington, Canada) were used. A generally good agreement was observed between our data and certified values, with the following recoveries (%) (based on seven measurements in control sample for trace elements and five measurements for macro elements): Ag (87.3 ± 8.1), Al (101.4 ± 9.1), As (97.5 ± 9.1), Ca (98.8 ± 6.5), Cd (97.4 ± 2.6), Co (97.4 ± 3.8), Cu (95.0 ± 3.4), Fe (85.9 ± 16.6), K (93.4 ± 8.7), Mg (94.2 ± 5.9), Mn (96.6 ± 5.1), Na (99.1 ± 4.9), Se (94.3 ± 3.8), Sr (98.4 ± 1.6), Tl (100.5 ± 5.4), V (97.2 ± 3.5), and Zn (111.6 ± 19.8).

Limits of detection (LOD) were calculated as three standard deviations of ten consecutive trace element determinations in the blank sample (100 mM Tris-HCl/Base, 1 mM dithiotreitol) digested according to the procedure for cytosols. Limits of detection for macro elements, in µg/g, were as follows: Ca, 1.07; K, 0.112; Mg, 0.024; and Na, 0.320. Limits of detection for trace elements, in ng/g, were as follows: Ag, 0.255; Al, 44,0; As, 6.72; Cd, 0.430; Co, 0.266; Cs, 0.102; Cu, 13.5; Fe, 141; Mn, 0.810; Mo, 0.680; Rb, 0.339; Se, 2.93; Sr, 1.09; Tl, 0.001; V, 2.86; and Zn, 635. Limits of detection for metal/metalloid concentrations in homogenates were twofold higher, in accordance with applied digestion procedure.

The results obtained for digested homogenates are referred to as total metal/metalloid concentrations, whereas the results obtained for cytosolic fractions are referred to as soluble, cytosolic metal/metalloid concentrations. All concentrations obtained in this study are presented as ng/g or µg/g of wet tissue, in the same way as all the cited metal concentrations. The proportions of metals/metalloids present in the soluble tissue fractions of *S. trutta* liver were calculated as the ratios of cytosolic to total metal/metalloid concentrations in *S. trutta* liver, multiplied by 100, and expressed in percentages.

### *2.6. Data processing and statistical analyses*

SigmaPlot 11.0 for Windows was used for statistical analysis and creation of graphs, whereas basic calculations were performed in Microsoft Office Excel 2007. We have used nonparametric statistical tests, because assumptions of normality and homogeneity of variance were not always met. The level of significance was set at 95% ($p<0.05$).

Comparisons of values obtained at two different sites for fish biometric characteristics, total and cytosolic metal/metalloid concentrations were performed by Mann-Whitney rank sum test, separately for each season. Total metal/metalloid concentrations measured in two sampling campaigns (seasons) were compared by Mann-Whitney rank sum test, separately for each sampling site. Fish biometric characteristics in four sampling campaigns (seasons) were compared by Kruskal-Wallis one-way analysis of variance, separately for each sampling site. Correlation between fish length and total metal/metalloid concentrations was calculated by Spearman correlation coefficient. Comparison of total metal/metalloid concentrations between females and males was performed by Mann-Whitney rank sum test on the whole data set. In several samples, the concentrations of As and V were below their LODs and for purposes of statistical analyses these values were substituted with LODs of As and V, respectively.

## 3. Results and discussion

### *3.1. Biometric characteristics of S. trutta sampled in the Krka River*

Biometric characteristics of fish used in this study are presented in Table 2. In general, fish size and HSI were comparable at two sampling sites, except in the second sampling campaign (September 2015), when fish were significantly bigger at the sampling site downstream of Knin town compared to the reference site. Fulton condition indices tended to be higher downstream of Knin town compared to the Krka River spring, and the differences between locations were significant in the last two samplings (Table 2). Bigger fish and especially their consistently higher FCI at the contaminated site could be associated to higher availability of nutrients at that site (Lambert and Dutil, 1997), which could refer to organic matter originating from municipal and industrial wastewaters regularly discharged into the Krka River water downstream of Knin town. Furthermore, the higher GSI, which indicates the period of gonad development in fish, was significantly different at the reference site in comparison to the contaminated site only in September 2015 (Table 2). It may indicate that the onset of the active reproductive period was somewhat delayed in the fish caught in the Krka River downstream of Knin town, possibly induced by water contamination. In the next sampling campaign, in October of the same year, however, comparable increase of GSI in all fish has indicated that *S. trutta* entered the reproductive phase at both sampling sites.

Fish were generally of greater size in two autumn samplings (September and October 2015) compared to two spring samplings (April 2015; May 2016) (Table 2), and the differences were significant, for both fish length and mass, between specimens caught in May 2016 and those caught in both autumn samplings ($p<0.05$). The same was observed for GSI, which was higher in autumn samplings compared to spring (Table 2), but the differences were statistically significant between specimens caught in April 2015 and those caught in both autumn samplings ($p<0.05$). These results are in accordance with typical period of gonadal development of *S. trutta*, which occurs in autumn (Hajirezaee et al., 2012), and which possibly affects not only GSI, but also the size of the fish. Consequently, due to lower fish mass in the spring samplings, the opposite was found for HSI, which was higher in spring samplings compared to autumn, and the differences were statistically significant if the comparison was made with October 2015. FCI was significantly higher in September 2015 compared to all the other samplings, even to October 2015, indicating that the reason for FCI increase could not be found in the season of the year, but possibly in the food availability, and therefore also in the transient increase of contamination intensity.

### *3.2. Differences in total and cytosolic metal/metalloid concentrations between the reference and the contaminated sampling site*

Comparison of total metal/metalloid concentrations in the liver of 65 *S. trutta* specimens from two sampling sites indicated three spatial patterns: 1) some elements had comparable concentrations at both sites; 2) some elements had higher concentrations at the contaminated site; and, 3) some elements had higher concentrations at the reference site (Table 3). That finding was further confirmed by spatial patterns of cytosolic metal/metalloid concentrations, which represent metabolically available metal/metalloid fraction in the cells and which were analyzed in the liver of 135 *S. trutta* specimens from the reference and the contaminated site of the Krka River. In addition, although cytosolic concentrations of several metals/metalloids in the fish liver were previously reported for *S. cephalus* (Podrug and Raspor, 2009; Podrug et al., 2009; Dragun et al., 2012; 2013a, b), cytosolic concentrations presented in this paper represent the first data of such kind for *S. trutta*, and for trout in general.

Significant differences of total concentrations in *S. trutta* liver between two sites were not observed for the following eight metals: Al, Cu, Fe, K, Mg, Mn, Rb, and Zn (Table 3). Similarly, generally comparable cytosolic concentrations in *S. trutta* liver or absence of clear trend were observed for the following five metals: Al, Ca, Fe, Mg, and Mn (Fig. 2). This may have occurred because metal exposure levels in the river water did not differ enough between two sampling sites (e.g. for Al, Cu, K, Mg, Rb) (Table 1; Filipović Marijić et al., 2016; 2017). Another possibility is that physiological regulation of metal concentrations in *S. trutta* was efficient enough (e.g. for Fe, Mn, Zn). So even though concentrations of these metals in the river water were higher at the contaminated sampling site (Table 1; Filipović Marijić et al., 2016; 2017), they were not excessively accumulated in the *S. trutta* liver. Dussault et al. (2004) have found that, although there was a significant relationship between hepatic Al concentrations in rainbow trout *Oncorhynchus mykiss* and waterborne Al, Al accumulation occurred only after prolonged exposure to Al concentrations in the water higher than 20 μg/L. Low dissolved Al concentrations (below 6 μg/L) generally reported for the Krka River water downstream of Knin town (Table 1; Filipović Marijić et al., 2017) could thus explain the absence of association between waterborne and hepatic Al concentrations in our study.

Higher total hepatic concentrations of the other set of eight elements (Ag, As, Ca, Co, Na, Se, Sr, and V) were found in *S. trutta* from the site downstream of Knin town in comparison to the reference site (Table 3), which was mainly in accordance with the previously published information on the Krka River water contamination (Filipović Marijić et al., 2016; 2017) and with the dissolved metal/metalloid concentrations found in the river water in the time of *S. trutta* sampling (Table 1). Out of these eight elements, dissolved concentrations of four (As, Co, Sr, and V) were even significantly higher in the Krka River water downstream of Knin town compared to the reference site in the first two sampling campaigns in 2015 (Filipović Marijić et al., 2016; 2017). Therefore, it can be reasonably concluded that the observed higher hepatic concentrations of several above mentioned elements were a consequence of the higher metal/metalloid exposure level in the river water downstream of Knin town. It is consistent with the report of Deniseger et al. (1990) that the increase in metal concentrations in the water environment is accompanied by increased metal concentrations in salmonid tissues, as well as with reported strong positive correlation observed between As concentrations in water and in *S. trutta* liver in As contaminated rivers of Corsica (Culioli et al., 2009). However, in our study, differences between sites in total hepatic concentrations were significant in both sampling campaigns only for Co and Sr, and their values at the contaminated site were higher compared to reference site 50-70% in autumn, and three to four times in spring (Table 3). Among remaining six elements, five were significantly higher at the contaminated site only in spring, with V being twice higher, and As, Ca, Na and Se 25-70% higher (Table 3). The only exception was Ag, which was significantly higher at the contaminated site only in autumn campaign, with values twice higher compared to the reference site (Table 3). Similarly, generally higher cytosolic concentrations in *S. trutta* liver at the site downstream of Knin town in comparison to the reference site, as a sign of higher water contamination, were observed for nine elements (Ag, As, Co, Cu, Na, Se, Sr, V, and Zn; Fig. 3). Differences were statistically significant in all sampling campaigns only for As, Co and Sr, and, depending on the campaign, amounted to two to six times for As, two to three times for Co, and two times for Sr. The remaining elements were significantly higher up to two times in only one (Ag and Cu), two (Na, V, and Zn) or three campaigns (Se).

Interesting and quite unexpected finding were, however, higher total concentrations of four elements (Cd, Cs, Mo, and Tl, Table 3) and cytosolic concentrations of six elements (Cd, Cs, K, Mo, Rb and Tl, Fig. 4) in the liver of *S. trutta* from the Krka River spring. The differences in total Cd and Tl concentrations between two sites were statistically significant in both studied campaigns, with seven to tenfold higher values of Cd at the reference site and two times in the case of Tl (Table 3). Cytosolic Cd and Tl concentrations were significantly higher at the reference site in all four or three studied campaigns, respectively (Fig. 4). Cytosolic concentrations of Cd were 8-12 times higher at the reference site compared to the contaminated one, and two to six times for Tl (Fig. 4). On the other hand, total Cs and Mo significantly differed between two sites in only one studied campaign, spring and autumn, respectively, and the differences amounted to 20-50% (Table 3). Cytosolic concentrations of Cs, on the other hand, were significantly higher at the Krka River spring in three studied campaigns, and the differences amounted to 50-100% (Fig. 4). Cytosolic concentrations of K, Mo and Rb significantly differed between sites in only one campaign, and their values were higher only 20-60% at the reference site (Fig. 4).

The dose-dependent increases of Cd concentration in liver, with linear accumulation pattern were previously reported for juvenile *O. mykiss* (Kamunde, 2009), indicating that higher Cd concentrations were probably caused by higher exposure level in the river water. Vukosav et al.

(2014) have also found a significant correlation between Cd in water and Cd in liver of *S. trutta* from the Plitvice Lake National Park, in Croatia. However, in our study, none of these six elements had higher dissolved concentrations in the river water at the reference site (Table1; Filipović Marijić et al., 2016; 2017; Sertić Perić et al., 2017), which could possibly serve as an explanation of their observed higher accumulation in the *S. trutta* liver. Quite contrary, K, Mo, and Rb concentrations in the Krka River water were even somewhat higher downstream of Knin town (Table 1). Therefore, the cause of these high concentrations should be further investigated, considering the food and the sediment as their possible sources, and dietary intake as the possible uptake route, since fish can accumulate metals not only from the dissolved water phase, but also from the sediment and food (Van Campenhout et al., 2009). Dietary intake can even be a major route of exposure to some metals (Lapointe et al., 2009a). For example, aqueous Tl appears to be better regulated than the dietary form in juvenile fathead minnows *Pimephales promelas*, which is the reason why diet-borne Tl may represent a greater threat for those fish than aqueous Tl (Lapointe et al., 2009a). Based on the analysis of metals in gut content, Filipović Marijić and Raspor (2014) have demonstrated the importance of diet-borne metal intake in European chub *Squalius cephalus*, especially in those fish specimens that reside in only moderately contaminated natural waters. Several scientists have even proposed that it should be evaluated whether water quality guidelines established only for dissolved metals are sufficiently protective (Fisher and Hook, 2002; Hare et al., 2003; Lapointe et al., 2009a).

To put our results in wider perspective, we have compared total metal/metalloid concentrations in *S. trutta* liver (Table 3) with the available information on hepatic metal/metalloid concentrations for trout from differently contaminated rivers worldwide, and came to the conclusion that concentrations of metals bioaccumulated in the liver of *S. trutta* from the Krka River were still rather low. In general, total metal/metalloid concentrations were present in *S. trutta* liver in the following decreasing order: K > Na > Mg > Fe > Ca > Cu > Zn > Rb > Se > Mn > Al > Ag > Tl > Mo > Sr > Cd > Co > As > V > Cs (Table 3). The concentrations of Cd measured in our study in *S. trutta* liver at both pristine and contaminated site were lower than Cd hepatic concentrations of 710 ng/g in *S. trutta* from the Rugla River in Norway, characterized by low waterborne Cd and Zn (Olsvik et al., 2000), and Buško Blato reservoir in Bosnia and Herzegovina (144 ± 191 ng/g) (Has-Schön et al., 2008), whereas hepatic Cd concentrations in *S. trutta* from the Krka River spring were somewhat higher compared to *S. trutta* from uncontaminated Esva River in Spain (75 ± 60 ng/g$^{-1}$) (Linde et al., 1998), Munzur stream, Turkey (109 ± 36 ng/g) (Can et al., 2012), and the control site at Loučka River in Czech Republic (107 ± 66 ng/g) (Vítek et al., 2007). Hepatic concentrations of Zn in *S. trutta* from both pristine and contaminated site of the Krka River were lower than Zn hepatic concentrations in *S. trutta* from control site at the Otra River in Norway (42.8 ± 5.2 µg/g)

(Brotheridge et al., 1998), the Rugla River (33.3 µg/g) (Olsvik et al., 2000), the control site at Loučka River (30.7 ± 3.6 µg/g) (Vítek et al., 2007), and Buško Blato reservoir (59.9 ± 16.8 µg/g) (Has-Schön et al., 2008). Hepatic Cu, on the other hand, was somewhat higher in our study at both sites compared to *S. trutta* from the Esva River, control site at Otra River and Munzur stream (23.2 ± 5.1 µg/g, 29.6 ± 12.3 µg/g, and 18.2 ± 8.1 µg/g, respectively) (Brotheridge et al., 1998; Linde et al., 1998; Can et al., 2012), but still lower compared to trout from mining contaminated site at Otra River, Naustebekken River in Norway – characterized by low waterborne Cu, and control site at Loučka River (117.2 ± 33.5 µg/g, 87.0 µg/g, and 60.0 ± 39.0 µg/g, respectively) (Brotheridge et al., 1998; Olsvik et al., 2000; Vítek et al., 2007). The concentrations of Co in *S. trutta* from the Krka River at both sites were lower than hepatic Co in *S. trutta* from the control site of the Otra River (60 ± 30 ng/g) (Brotheridge et al., 1998). Hepatic Al concentrations in *S. trutta* from both sites of the Krka River were far below hepatic Al concentrations (12-60 µg/g) reported for *O. mykiss* exposed to waterborne Al of 20-80 µg/L (Dussault et al., 2004), but also below Al concentrations reported for the liver of farmed *S. trutta* (1.9-4.6 µg/g) (Karlsson-Norrgren et al., 1986). Similarly, hepatic Se in *S. trutta* in our study at both sites was much lower than hepatic Se concentrations reported for *S. trutta* and *O. mykiss* from pristine Patagonian lakes in Argentina (~80 µg/g) (Arribére et al., 2008), and for *S. trutta* from urbanized stream in Colorado, USA (3.1 ± 2.3 µg/g) (Herrmann et al., 2016), and higher only compared to *S. trutta* from Munzur stream (25 ± 39 ng/g) (Can et al., 2012). Moreover, As concentrations in *S. trutta* liver in our study at both sites were lower than hepatic As concentrations reported for liver of *S. trutta* from Buško Blato reservoir (76 ± 14 ng/g) (Has-Schön et al., 2008), reference Bravona River in Corsica (44 ± 14 ng/g) (Foata et al., 2009) and Munzur stream (46 ± 31 ng/g) (Can et al., 2012), whereas As concentrations in *S. trutta* liver from mining contaminated Corsican Presa River were much higher (1.17 ± 0.57 µg/g) (Foata et al., 2009). Therefore, although there were differences between two studied sites, based on the obtained data and above presented comparisons with other published reports on metals/metalloids in *S. trutta* liver, it can be concluded that in the most of the cases, the level of metal/metalloid accumulation detected in this study still does not present a serious concern, but only an indication that monitoring of the water quality and of the aquatic biota in the Krka River is vitally needed downstream of Knin town.

### *3.3. Differences in total metal/metalloid concentrations between sampling campaigns*

Seasonal changes in fish physiology, such as gonad development, spawning or metabolic rate, can also influence metal accumulation in fish organs. Therefore, comparison of total metal/metalloid concentrations measured in October 2015 and May 2016 was made, indicating comparable levels of several analyzed elements (K, Mg, Mo, Cs, Tl, Al, V, Fe, and Zn) in both sampling campaigns (Table 3). Significantly higher total concentrations in *S. trutta* liver were found in autumn campaign in comparison to spring for Se, Rb, Cd and Sr, but only at the reference site, and for Cu at the contaminated site, whereas higher concentrations of Na, Ca, Mn and Co were found in spring campaign in comparison to autumn, but only at the contaminated site. Only for Ag and As pronounced differences between campaigns were observed at both sampling sites, specifically higher levels in autumn for Ag, and in spring for As. Based on the established differences between two sampling campaigns, which did not show clear seasonal trend for majority of studied metal/metalloid concentrations, it can be presumed that the cause of these differences cannot be found in the physiological variability of metal accumulation in different seasons of the year, but rather in the sporadic increase of metal exposure in the water, sediment or food in certain moments at specific sites. The exception was Ag, for which the explanation for seasonal increase in the autumn period could be found in *S. trutta* physiology. Nichols and Playle (2004) reported the positive influence of increased ambient temperature on Ag accumulation in the liver of *O. mykiss*, probably due to increased fish metabolic rates at higher water temperatures, whereas the same effect was not observed on Ag elimination; therefore, they found higher Ag concentrations in the liver of *O. mykiss* kept at higher compared to lower water temperatures. Although water temperatures in autumn and spring samplings are usually comparable (Filipović Marijić et al., 2017), higher hepatic Ag levels in *S. trutta* in the autumn compared to spring in our study could be explained by the fact that autumn sampling occurred after long period of high summer temperatures.

### *3.4. Association of total metal/metalloid accumulation in liver with fish physiological characteristics*

To evaluate the other possible influences on metal/metalloid accumulation in trout liver beside the exposure level, we have determined sex related differences in metal/metalloid hepatic accumulation, as well as the correlation of total hepatic metal/metalloid concentrations with the fish size. Although it was previously reported that As and Cu concentrations in the liver of *S. trutta* were sex dependent (Foata et al., 2009; Monna et al., 2011), in our study significant association with fish sex was not established for any of analyzed metals/metalloids in *S. trutta* liver, meaning that there were no differences between males and females. The absence of sex dependence was also reported by Monna et al. (2011) for Cd and Zn in *S. trutta fario* liver. Since fish length and fish mass have shown high and strong mutual correlation ($r=0.962-0.989$, $p<0.0001$, depending on the sampling site and period), association with fish size was determined only by use of fish length. The majority of analyzed metals/metalloids have shown absence of correlation with fish size. Similar observation was reported for Se in the liver of *S. trutta* and *O. mykiss* from Patagonian lakes and for As in the liver of *S. trutta* from rivers of Corsica, where no correlation was observed between metalloid concentration and fish length

(Arribére et al., 2008, Culioli et al., 2009; Foata et al., 2009). The absence of metal/metalloid association with both fish sex and size makes increase of their total concentrations in *S. trutta* liver a good and reliable indicator of exposure, considering that the most important characteristic for a metal biomonitoring species is that metal concentrations in their tissues are directly correlated to those in the environment (Kraemer et al., 2006), without additional influential factors.

Significant association with fish size was established for only three metals, namely Co, Cu and Zn. The association of Co with *S. trutta* length was negative and rather strong, meaning that higher concentrations were found in smaller specimens. However, this correlation was determined in both sampling campaigns only at the contaminated site, downstream of Knin town (r = -0.659 and -0.720; $p<0.01$), where Co accumulation in *S. trutta* liver was significantly higher compared to the reference site (Table 3), same as Co exposure level in the river water (Table 1; Filipović Marijić et al., 2017; Sertić Perić et al., 2017). Therefore, it can be presumed that in the conditions of increased exposure to Co, smaller *S. trutta* specimens accumulate higher amounts of this metal than the bigger ones. This is in accordance with known fact that if the growth rate of the biomonitoring species is faster than the rate of accumulation, the metal concentration will decrease due to growth dilution (Kraemer et al., 2006). In the conditions of low exposure, therefore, there were no differences between smaller and bigger specimens.

Conversely, hepatic concentrations of Cu and Zn were positively correlated to fish length, and significantly if the complete data set was considered (r = 0.529, $p<0.0001$ and r = 0.293, $p<0.05$, respectively). If each period and site were considered separately, these positive associations were rather weak and statistically significant only in autumn, at the Krka River spring for Cu (r = 0.509, $p<0.05$), and at the site downstream of Knin town for Zn (r = 0.525, $p<0.05$). Considering that body size is directly correlated with fish age (Reyes-Gavilan et al., 1995), the relationship between body size and metal concentrations could be a simple indication of the exposure time, i.e. the age, as the real factor influencing fish metal content (Linde et al., 1998). In other words, bigger and older *S. trutta* have accumulated higher concentrations of Cu and Zn in the liver, suggesting time-dependent accumulation. Linde et al. (1998) have also previously reported that Cu content in the liver of *S. trutta* from northern Spain increased with fish age.

*3.5. Proportions of metals/metalloids present in the soluble tissue fractions of S. trutta liver*

As a next step, the proportions of each element in the soluble tissue fraction were evaluated (Table 4), based on the ratios between cytosolic and total metal/metalloid concentrations in *S. trutta* liver. The percentages of analyzed elements present in the soluble, cytosolic hepatic fraction of *S. trutta* from the Krka River spring decreased in the following order: Na, K

(≥100%) > Cd, Rb, V (90-99%) > Co, Cs, Se (80-89%) > Cu, Fe, Mn, Mo, Tl, Zn (60-69%) > Ag, As, Mg, Sr (50-59%) > Al, Ca (≤40%). Similarly, with only few changes in their order, the percentages of analyzed elements present in the soluble, cytosolic hepatic fraction of *S. trutta* from the Krka River downstream of Knin town decreased in the following order: Na, K (≥100%) > Rb (90-99%) > As, Cd, Co, Cs, Se, V (80-89%) > Cu, Mn, Mo, Tl, Zn (60-69%) > Ag, Fe, Mg (50-59%) > Al, Ca, Sr (<50%).

Since increased environmental metal concentrations may cause shifts in metal distribution profiles among cytosolic ligands (Langston et al., 2002), we have analyzed the association between total accumulated metal concentrations in the *S. trutta* liver and their percentage presence in the hepatic cytosol. For the majority of analyzed elements, this association was negative (Table 4). Specifically, correlation coefficients (r) between these two parameters were negative, higher than 0.5, and statistically significant ($p<0.05$) for the following elements: Ag, Al, Co, Fe, Mg, Mn, and Sr (Table 4). Accordingly, higher total hepatic concentrations of these elements were generally associated with their lower presence in the cytosol, meaning that higher *S. trutta* exposure to these metals and their consequent higher accumulation in the *S. trutta* liver have resulted with metal storage within the cell in the insoluble form, possibly by being detoxified in a form of the granules.

Opposite trend, i.e. positive associations between metal percentage presence in soluble fraction and their total hepatic concentrations were determined only for As, Cd, Cs, and Tl (Table 4). In their case, increased exposure and accumulation have resulted in metal storage within the cytosol, thus either making them available for toxic effects or detoxified by binding to some cytosolic components, for example metallothioneins (e.g. Cd). Increased binding of Cd to metallothioneins after increased Cd accumulation was confirmed in the liver of *S. cephalus*, applying separation of cytosolic biomolecules by size-exclusion high performance liquid chromatography (SEC-HPLC) and subsequent measurement by HR ICP-MS (Krasnići et al., 2013).

However, high metal/metalloid percentage in the cytosolic fractions does not necessarily imply a certainty of metal/metalloid toxicity in the cells, because cytosolic fractions contain organelles microsomes and lysosomes, and both heat-denaturable proteins sensitive to metals, and heat-stable proteins, such as metallothioneins, which represent a route of metal detoxification (Bonneris et al., 2005; Dragun et al., 2013a). Therefore, a part of metals/metalloids present in the cytosol is probably detoxified. Accordingly, taking in consideration existing literature and reports of previous studies, we could hypothesize about the risks of high presence of specific metals/metalloids in the hepatic cytosol of *S. trutta*.

The reports on subcellular Cd distribution are always consistent with its well-known detoxification by MTs (Langston et al., 2002). High sequestration of Cd in the heat-stable MT pool was reported for liver of yellow perch *Perca flavescens*, juvenile *O. mykiss*, eels *Anguilla anguilla* and *Anguilla rostrata*, and *S. cephalus* (Kraemer et al., 2006; Campbell et al., 2008; Van Campenhout et al., 2008; Kamunde, 2009; Krasnići et al., 2013; Rosabal et al., 2015). Van Campenhout et al. (2008) and Kraemer et al. (2006) have also found that proportion of Cd bound to heat-stable proteins increased following increasing Cd exposure. This is similar to our results, which showed high percentage of Cd in the cytosolic fractions of *S. trutta* liver (more than 90%) and significant increase of Cd presence in the cytosol caused by increase of the exposure level. However, the fact that high proportion of cytosolic Cd is generally bound to MTs point to its high detoxification level, thus decreasing the risk of toxic effects in the conditions of moderate exposure. For example, Cd concentration in the metal sensitive, heat-denaturable, protein fraction did not increase with increasing total hepatic concentrations below a threshold of 10 µg/g (on dry mass) of total hepatic Cd in *P. flavescens* liver (Kraemer et al., 2006). However, Kamunde (2009) suggested caution when talking about threshold level and spillover theory, because in the juvenile *O. mykiss*, there was no exposure concentration or internal accumulation at which Cd was not found in potentially metal-sensitive compartments.

Contrary to Cd, in the case of As, the organelles fractions (mitochondria, microsomes and lysosomes), as well as metal-sensitive fraction containing heat-denaturated proteins, were reported as the major As binding compartment in the liver of *A. anguilla* and *A. rostrata* (Rosabal et al., 2015). Such As distribution within the cytosol can be an indication that high presence of As in *S. trutta* hepatic cytosol (around 80% at the site downstream of Knin town, Table 4) could point to high probability of occurrence of toxic effects, especially considering that higher exposure to this element lead to its higher presence in the soluble cell fractions.

Approximately the same amount of Se in Arctic char *Salvelinus alpinus* liver was associated to heat-stable proteins as to heat-denaturable proteins, microsomes and lysosomes together (Barst et al., 2016), indicating that about half of cytosolic Se is probably present in the detoxified form. Selenium was present in the granule-like fraction in *S. alpinus* liver in a very low amount (Barst et al., 2016), which can also explain high presence of Se in the cytosolic fraction of *S. trutta* liver (80-89%) found in our study (Table 4).

In the case of Cu, its substantial amounts found in hepatic subcellular compartments of juvenile *O. mykiss* comprising metabolically active components (organelles and heat-denaturated proteins) highlighted Cu essentiality for normal metabolism (Kamunde and MacPhail, 2008). However, specificity of Cu in the liver of *O. mykiss* was that most of this metal in metal unexposed specimens was present in the organelles and in the heat-stable fraction, whereas additional exposure to Cu resulted with Cu accumulation mainly in the heat-stable fraction, probably due to its predominant binding to MTs or glutathione (GSH) (Kamunde and MacPhail, 2008; Eyckmans et al., 2012). Kraemer et al. (2006) also found high proportion of hepatic Cu in the heat-stable protein fraction in *P. flavescens*. High presence of Cu in the cytosolic fractions of *S. trutta* liver (more than 60%, Table 4) could probably also point to significant Cu binding to MTs, and thus also to its partial detoxification, as was previously confirmed for liver of *S. cephalus* by combined application of SEC-HPLC and HR ICP-MS (Krasnići et al., 2013).

In *S. alpinus* liver, high Fe presence was found in microsomes and lysosomes, as well as bound to heat-stable proteins (Barst et al., 2016). Therefore, it can be presumed that high Fe presence in the cytosol found in *S. trutta* liver (50-60%) partially points to detoxified Fe forms. Iron was probably mainly accumulated in lysosomes, because lysosomes within macrophages are known to play important role in the metabolism and storage of Fe (Kurz et al., 2011; Barst et al., 2016), for example through autophagocytosis of macromolecules containing Fe, such as ferritin (Kurz et al., 2008). Study on Fe distribution among cytosolic molecules of different molecular masses in the liver of *S. cephalus* also confirmed increase of Fe binding to ferritin following increased Fe accumulation in that organ (Krasnići et al., 2013).

Lapointe et al. (2009a,b) reported predominant binding of Tl in liver of *P. promelas* to heat-stable proteins and granules, whereas Barst et al. (2016) and Rosabal et al. (2015) reported the most important role of heat-stable proteins in Tl binding in the liver of *S. alpinus*, *A. anguilla* and *A. rostrata*. Major association of Tl with heat-stable proteins is consistent with rather high presence of this metal in the hepatic cytosol of *S. trutta* (60-69%, Table 4) in our study. Considering the possibility of predominant detoxification of Tl by binding to the heat-stable proteins, Tl high presence in the cytosol does not have to represent high risk of possible toxic effects after only moderate exposure to this metal. However, the report that increased exposure to Tl resulted in lower proportion of detoxified metal in the liver of *P. promelas* (Lapointe et al., 2009b) and that significant amount of Tl was found associated to metal sensitive organelles mitochondria in the liver of *S. alpinus* (Barst et al., 2016), suggests that this metal can spill over into metal-sensitive fractions if fish detoxification capacity had been exceeded (Lapointe et al., 2009b).

Subcellular Zn distribution has been described as a dynamic process with high Zn levels occurring in the cytosol, bound to cytosolic biomolecules, but also in the nucleus and in the lysosome fractions (Jeng et al., 1999). Van Campenhout et al. (2010) reported 60-70% of Zn in the hepatic cytosol of Prussian carp *Carassius auratus gibelio*, and a large part of it bound to MTs. Study of Zn distribution among cytosolic biomolecules in the liver of *S. cephalus* indicated Zn binding to MTs, but also to a large number of proteins in the wide range of molecular masses (10-600 kDa), which is consistent with Zn constitutive and catalytic roles in many proteins and enzymes (Krasnići et al., 2013). That information corresponds well with our results for *S. trutta* liver, in which more than 60% of Zn was found in the cytosolic fraction (Table 4). Taking into consideration that Zn is needed for metabolic processes in rather high quantity, and that there is a homeostatic control of Zn internal concentrations due to its essentiality (Monna et al., 2011), high presence of Zn in the cytosol and probable significant association with MTs render Zn an element for which there is no concern needed under the conditions of moderate exposure in the environment.

In the livers of *A. anguilla* and *A. rostrata*, heat-stable proteins had a predominant role in sequestering Ag (Rosabal et al., 2015). According to Mason and Jenkins (1995), Ag shows preference for binding with the sulfhydryl groups, which explains its association with thermo-stable, cysteine-rich, low molecular mass proteins, such as MTs. Moreover, Langston et al. (2002) reported that 92% of cytosolic Ag was found in MT pool in livers of *A. anguilla*. More than 50% of Ag found in the cytosolic fractions of *S. trutta* liver (Table 4), therefore, probably does not pose a high risk for development of toxic effects under the studied exposure conditions.

Although it has been previously shown that many metals present in the cytosol, especially after only a moderate exposure to metals, are detoxified through binding to heat-stable proteins, such as MTs, MT-like proteins, GSH and free amino acids, still at least a small part of their total amount in the cell was always found bound to metal sensitive, heat-denaturable proteins, indicating that their detoxification was incomplete (Kraemer et al., 2006; Kamunde and MacPhail, 2008; Lapointe et al., 2009a,b; Rosabal et al., 2015; Barst et al., 2016). Campbell et al. (2008) reported that there was no accumulation threshold below which Cd binding to the metal-sensitive fractions (heat-denaturable proteins and organelles) did not occur, even for low exposure concentrations and low hepatic accumulation in *P. flavescens*, and the same was reported by Kamunde (2009) for juvenile *O. mykiss*. Accumulation of metals and metalloids, especially nonessential ones, in the sensitive subcellular compartments, where these elements can block functional groups, displace essential metals or modify the active conformation of biomolecules, could be indication of possible metal/metalloid toxicity (Mason and Jenkins, 1995). Therefore, high presence of metals/metalloids in the cytosolic fractions certainly can be considered as a sign of higher potential for toxicity, especially for elements such as As, Cd, Cs and Tl, for which higher exposure level and consequent higher accumulation in the liver of *S. trutta* resulted with higher presence in the hepatic cytosol.

**4. Conclusions**

The levels of 20 metals/metalloids accumulated in the liver of *S. trutta* from karstic Krka River in Croatia were still not high enough to raise concern, either for the health of *S. trutta* itself, or for the consequent welfare of the humans. However, comparison between two sampling sites, the Krka River spring as the reference site, and the site downstream of Knin town contaminated by municipal and industrial wastewaters, indicated higher hepatic accumulation of several elements (Ag, As, Ca, Co, Na, Se, Sr, and V) in *S. trutta* caught at the contaminated site. That finding represented justifiable ground for implementation of more strict and regular monitoring of water quality and health of aquatic organisms in the Krka River. Such measures would be especially important, considering that the National Park Krka is situated only 2 km downstream of Knin town. Unexpectedly, the hepatic concentrations of Cd, Tl, Cs and Mo were found to be higher at the reference site. Since the dissolved concentrations of these metals in the river water at that site were extremely low, it remains to be revealed in future studies what is the cause for higher metal accumulation in *S. trutta* liver at pristine location, with special reference to metal content in sediments and food as possible sources of this phenomenon. Furthermore, it was established that the majority of analyzed metals/metalloids do not show significant association with *S. trutta* biometric characteristics (sex and size) and season of the year, whereas they reflect the water concentrations, what makes their hepatic concentrations promising and reliable indicators of metal/metalloid exposure in the water. Determined percentage presence of analyzed elements in the soluble cytosolic tissue fractions indicated that large proportions (mainly above 50%) of the majority of studied metals/metalloids were present in this metabolically available and potentially toxic fraction of the liver. Special attention should be put on elements such as As, Cd, Cs and Tl, for which higher exposure level and consequent higher accumulation in the liver resulted with higher presence in the hepatic cytosol.

**Acknowledgements**

This study was carried out in the scope of two projects: 1) “Accumulation, subcellular mapping and effects of trace metals in aquatic organisms” (Project no: IP-2014-09-4255), funded by the Croatian Science Foundation; and 2) “Assessment of water quality in the Krka River and the potential risks for the Krka National Park by using new methods and bioindicators”, funded by the Adris Foundation. The financial support of the Ministry of Science and Education of the Republic of Croatia for institutional funding of the Laboratory for Biological Effects of Metals is also acknowledged.

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**Table 1.** Dissolved metal/metalloid concentrations in the water (µg/L or mg/L) of the Krka River at two sampling sites (reference site: Krka River spring; contaminated site: Krka downstream of Knin town) in two sampling campaigns (October 2015 and May 2016), measured after filtration (pore diameter 0.45 µm) and acidification (2% $HNO_3$, *suprapur*) of river water samples. The results are presented as means ± standard deviations of three replicates.

| | **October 2015** | | **May 2016** | |
|---|---|---|---|---|
| | **Krka River spring** | **Krka downstream of Knin** | **Krka River spring** | **Krka downstream of Knin** |
| **Ag (µg/L)** | <0.100 | <0.100 | <0.100 | <0.100 |
| **Al (µg/L)** | 2.20 ± 0.11 | 5.40 ± 0.48 | 2.72 ± 0.07 | 2.38 ± 0.63 |
| **As (µg/L)** | 0.130 ± 0.029 | 0.200 ± 0.028 | 0.101 ± 0.019 | 0.145 ± 0.014 |
| **Ca (mg/L)** | 69.03 ± 0.56 | 83.09 ± 0.02 | 58.65 ± 1.69 | 69.88 ± 1.06 |
| **Cd (µg/L)** | 0.010 ± 0.003 | 0.010 ± 0.004 | 0.005 ± 0.001 | 0.005 ± 0.002 |
| **Co (µg/L)** | <0.019 | 0.196 ± 0.010 | <0.019 | 0.211 ± 0.033 |
| **Cs (µg/L)** | <0.001 | <0.001 | <0.001 | <0.001 |
| **Cu (µg/L)** | <0.401 | <0.401 | <0.401 | <0.401 |
| **Fe (µg/L)** | 0.910 ± 0.370 | 4.88 ± 0.37 | 4.04 ± 0.31 | 5.16 ± 0.85 |
| **K (mg/L)** | 0.337 ± 0.005 | 0.667 ± 0.017 | 0.285 ± 0.007 | 0.391 ± 0.001 |
| **Mg (mg/L)** | 9.52 ± 0.14 | 9.06 ± 0.08 | 9.90 ± 0.28 | 10.08 ± 0.09 |
| **Mn (µg/L)** | 0.100 ± 0.008 | 3.86 ± 0.15 | 0.031 ± 0.005 | 2.97 ± 0.26 |
| **Mo (µg/L)** | 0.210 ± 0.004 | 0.410 ± 0.005 | 0.378 ± 0.087 | 0.515 ± 0.032 |
| **Na (mg/L)** | 1.36 ± 0.01 | 1.85 ± 0.04 | 1.94 ± 0.05 | 3.57 ± 0.05 |
| **Rb (µg/L)** | 0.250 ± 0.003 | 0.450 ± 0.007 | 0.260 ± 0.001 | 0.316 ± 0.019 |
| **Se (µg/L)** | 0.080 ± 0.022 | 0.100 ± 0.014 | <0.059 | 0.088 ± 0.059 |
| **Sr (µg/L)** | 67.71 ± 0.38 | 112.8 ± 0.6 | 85.49 ± 0.17 | 168.8 ± 13.2 |
| **Tl (µg/L)** | 0.004 ± 0.000 | 0.005 ± 0.000 | 0.005 ± 0.000 | 0.005 ± 0.001 |
| **V (µg/L)** | 0.520 ± 0.011 | 0.680 ± 0.003 | 0.482 ± 0.012 | 0.617 ± 0.044 |
| **Zn (µg/L)** | <7.34 | 20.41 ± 5.15 | 11.07 ± 5.02 | 17.87 ± 1.26 |

**Table 2.** Biometric parameters of *S. trutta* caught in the Krka River at two sampling sites (reference site: Krka River spring; contaminated site: Krka downstream of Knin town) in four sampling campaigns (April, September, and October 2015, and May 2016). The results are presented as medians, with minima and maxima within brackets.

| | **April 2015** | | **September 2015** | | **October 2015** | | **May 2016** | |
|---|---|---|---|---|---|---|---|---|
| | **Krka River spring** | **Krka downstream of Knin** | **Krka River spring** | **Krka downstream of Knin** | **Krka River spring** | **Krka downstream of Knin** | **Krka River spring** | **Krka downstream of Knin** |
| **n** | 18 | 16 | 14 | 22 | 16 | 18 | 16 | 15 |
| **Total length (cm)** | 20.0 (14.0-30.5) | 18.4 (13.0-58.0) | **19.0[a] (15.0-29.5)** | **25.0[b] (15.0-37.0)** | 24.8 (18.0-30.8) | 25.0 (15.0-31.8) | 17.9 (15.2-22.1) | 19.1 (13.8-26.7) |
| **Total mass (g)** | 89.2 (29.5-350) | 60.8 (17.9-1870) | **79.0[a] (38.6-277.1)** | **204[b] (40.4-598)** | 143 (59.5-304) | 174 (35.9-424) | 59.2 (36.6-107) | 83.0 (31.5-201) |
| **HSI (%)** | 1.24 (0.45-2.71) | 1.11 (0.87-3.52) | 0.95 (0.74-1.64) | 1.04 (0.70-3.29) | 0.90 (0.53-1.36) | 0.99 (0.77-1.81) | 1.19 (0.88-1.97) | 1.44 (1.06-1.80) |
| **GSI (%)** | 0.28 (0.16-1.03) | 0.23 (0.03-0.67) | **4.81[a] (0.16-11.9)** | **0.22[b] (0.07-11.6)** | 4.19 (0.11-8.08) | 2.35 (0.02-7.59) | **0.22[a] (0.13-1.39)** | **0.14[b] (0.08-0.25)** |
| **FCI (%)** | 1.10 (0.92-2.65) | 1.12 (0.22-1.48) | 1.15 (1.03-1.32) | 1.22 (0.98-1.46) | **1.02[a] (0.84-1.14)** | **1.10[b] (0.98-1.38)** | **1.04[a] (0.95-1.16)** | **1.19[b] (1.05-1.37)** |
| **Sex (F/M)** | 9/9 | 10/5* | 3/11 | 12/10 | 5/10* | 9/9 | 6/10 | 8/7 |

[a,b]The values which are significantly different at two sampling sites within certain sampling campaign are written in bold, and asigned with different superscript letters (a or b), indicating $p<0.05$ according to Mann-Whitney rank sum test.
*One fish specimen within the group was of undetermined sex.

**Table 3.** Total metal and metalloid concentrations (µg/g or ng/g; on wet mass basis) in hepatic tissue of *S. trutta* caught in the Krka River at two sampling sites (reference site: Krka River spring; contaminated site: Krka downstream of Knin town) in two sampling campaigns (October 2015 and May 2016). The results are presented as medians, with minima and maxima within brackets.

| | **October 2015** | | **May 2016** | |
|---|---|---|---|---|
| | **Krka River spring** | **Krka downstream of Knin** | **Krka River spring** | **Krka downstream of Knin** |
| **Ag (ng/g)** | **299[a] (23.9-463)** | **605[b] (30.5-3370)** | 135 (46.7-242) | 145 (74.5-407) |
| **Al (µg/g)** | 0.613 (0.242-3.11) | 0.395 (0.109-7.04) | 0.528 (0.221-1.36) | 0.462 (0.298-3.28) |
| **As (ng/g)** | 17.6 (13.4-34.6) | 20.3 (13.4-112) | **23.2[a] (13.5-39.0)** | **39.3[b] (24.2-60.4)** |
| **Ca (µg/g)** | 57.3 (39.7-114) | 49.1 (33.7-90.4) | **55.1[a] (40.3-67.8)** | **68.3[b] (51.0-158)** |
| **Cd (ng/g)** | **132[a] (77.3-327)** | **12.3[b] (6.30-25.7)** | **92.5[a] (26.4-149)** | **12.6[b] (4.80-46.9)** |
| **Co (ng/g)** | **19.7[a] (14.5-28.3)** | **29.0[b] (21.3-84.1)** | **20.2[a] (15.7-29.6)** | **76.9[b] (22.0-220)** |
| **Cs (ng/g)** | **6.33[a] (3.60-8.88)** | **4.17[b] (0.420-9.36)** | 5.76 (4.44-13.9) | 5.58 (2.58-8.46) |
| **Cu (µg/g)** | 34.8 (3.63-242) | 72.6 (7.56-138) | 27.1 (6.65-59.8) | 31.2 (13.8-71.5) |
| **Fe (µg/g)** | 85.9 (40.9-351) | 94.7 (50.7-183) | 62.7 (25.1-185) | 72.8 (41.3-116) |
| **K (µg/g)** | 3690 (2791-4356) | 3645 (2736-4952) | 3728 (3141-4520) | 3514 (2165-4655) |
| **Mg (µg/g)** | 167 (129-186) | 161 (127-233) | 176 (159-197) | 177 (126-236) |
| **Mn (µg/g)** | 1.12 (0.703-1.92) | 1.18 (0.811-2.10) | 1.35 (0.921-1.64) | 1.45 (1.14-2.35) |
| **Mo (ng/g)** | 158 (83.5-182) | 131 (83.5-267) | **164[a] (93.7-223)** | **139[b] (113-180)** |
| **Na (µg/g)** | 939 (626-1297) | 928 (572-1153) | **760[a] (587-1164)** | **1161[b] (670-1584)** |
| **Rb (µg/g)** | 3.98 (2.44-7.91) | 3.41 (1.22-8.98) | 3.13 (2.06-9.79) | 3.73 (1.89-5.30) |
| **Se (µg/g)** | 2.17 (0.940-5.23) | 2.97 (1.21-5.71) | **1.49[a] (0.674-1.99)** | **2.35[b] (1.42-4.00)** |
| **Sr (ng/g)** | **56.5[a] (36.9-120)** | **95.8[b] (55.7-300)** | **33.9[a] (25.0-66.6)** | **96.9[b] (53.8-182)** |
| **Tl (ng/g)** | **293[a] (121-700)** | **121[b] (9.12-343)** | **400[a] (193-859)** | **204[b] (52.5-359)** |
| **V (ng/g)** | 7.71 (5.70-15.1) | 7.59 (5.70-82.6) | **7.26[a] (5.70-193)** | **16.7[b] (5.76-89.9)** |
| **Zn (µg/g)** | 18.2 (13.8-28.5) | 19.3 (13.6-51.8) | 20.6 (14.1-60.9) | 17.9 (15.4-24.8) |

[a,b]The concentrations which are significantly different at two sampling sites within certain sampling campaign are written in bold, and asigned with different superscript letters (a or b), indicating $p<0.05$ according to Mann-Whitney rank sum test.

**Table 4.** The proportions of total metal/metalloid amount, expressed as percentage (%), present in the soluble, cytosolic fractions of liver of *S. trutta* caught in the Krka River at two sampling sites (reference site: Krka River spring; contaminated site: Krka downstream of Knin town) in two sampling campaigns (October 2015 and May 2016). Data gathered in two sampling campaigns were pooled in one data set, separately for each sampling site. The results are presented as means ± standard deviations. Additionally, in the last column, the Spearman coefficients of correlation (r) between total concentrations and percentage of metals/metalloids present in the soluble fractions are presented, along with accompanying *p* values.

| | **Krka River spring** | **Krka downstream from Knin** | **r; *p*** |
|---|---|---|---|
| **Ag** | 58.6 ± 8.0 | 53.8 ± 11.4 | -0.530; <0.001 |
| **Al** | 35.1 ± 11.2 | 45.2 ± 28.8 | -0.686; <0.001 |
| **As** | 57.5 ± 18.9 | 80.3 ± 13.9 | 0.468; <0.001 |
| **Ca** | 40.2 ± 5.4 | 41.4 ± 6.2 | -0.342; <0.010 |
| **Cd** | 93.0 ± 6.4 | 87.0 ± 11.3 | 0.417; <0.001 |
| **Co** | 86.0 ± 5.0 | 79.7 ± 12.3 | -0.595; <0.001 |
| **Cs** | 87.3 ± 6.0 | 80.9 ± 10.7 | 0.330; <0.010 |
| **Cu** | 63.6 ± 6.2 | 63.9 ± 7.5 | -0.339; <0.010 |
| **Fe** | 59.8 ± 18.1 | 57.1 ± 12.5 | -0.638; <0.001 |
| **K** | 100.1 ± 6.8 | 102.8 ± 12.1 | -0.473; <0.001 |
| **Mg** | 55.2 ± 4.3 | 57.0 ± 6.8 | -0.534; <0.001 |
| **Mn** | 67.5 ± 4.3 | 62.7 ± 7.9 | -0.528; <0.001 |
| **Mo** | 60.0 ± 5.9 | 60.5 ± 8.4 | -0.494; <0.001 |
| **Na** | 120.5 ± 10.5 | 117.7 ± 11.8 | -0.420; <0.001 |
| **Rb** | 94.2 ± 4.5 | 93.6 ± 8.9 | -0.127; 0.313 |
| **Se** | 85.1 ± 9.1 | 89.1 ± 11.0 | -0.317; <0.050 |
| **Sr** | 51.8 ± 8.9 | 46.7 ± 8.3 | -0.559; <0.001 |
| **Tl** | 66.5 ± 7.4 | 63.0 ± 8.3 | 0.244; 0.050 |
| **V** | 95.5 ± 39.9 | 81.2 ± 17.8 | -0.292; <0.050 |
| **Zn** | 64.0 ± 4.3 | 66.7 ± 7.6 | 0.079; 0.592 |

**Figure captions**

**Figure 1.** Study area with marked sampling sites on the Krka River (1 – Krka River spring; 2 – Krka River downstream of Knin town), and marked position of Croatia within Europe.

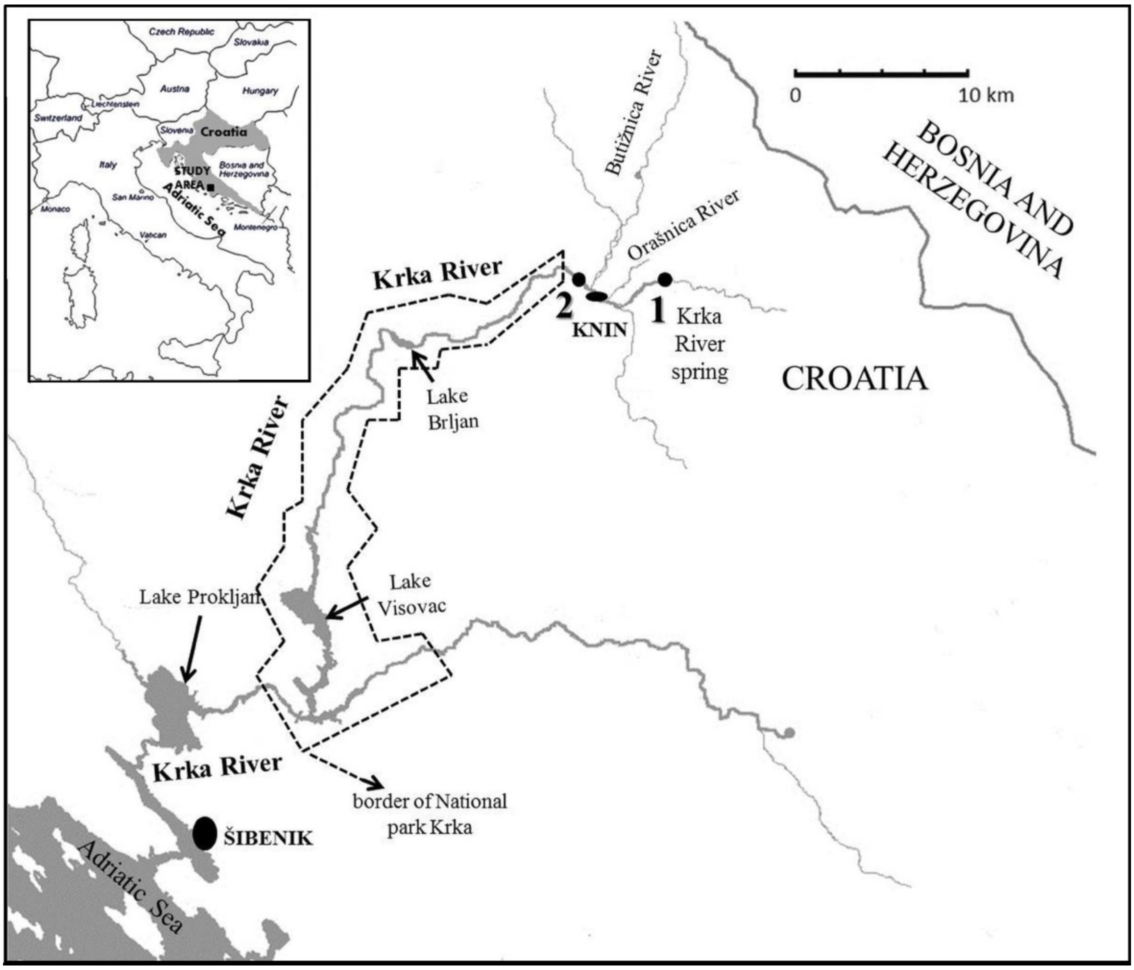

**Figure 2.** Concentrations (ng/g or µg/g on wet mass basis) of five metals in hepatic cytosolic fractions of brown trout *Salmo trutta* caught at two sites in the Krka River (reference site: Krka River spring; contaminated site: Krka downstream of Knin town) in four sampling campaigns (April, September, October 2015, and May 2016), and characterized by comparable values at both sites: a) Al, b) Ca, c) Fe, d) Mg, e) Mn. Differences between sites within each season are indicated with different letters (a, b), based on Mann-Whitney rank sum-test ($p$<0.05). Season legend: white – spring; grey – autumn; site legend: clear boxes – Krka River spring; boxes with pattern – Krka downstream of Knin town.

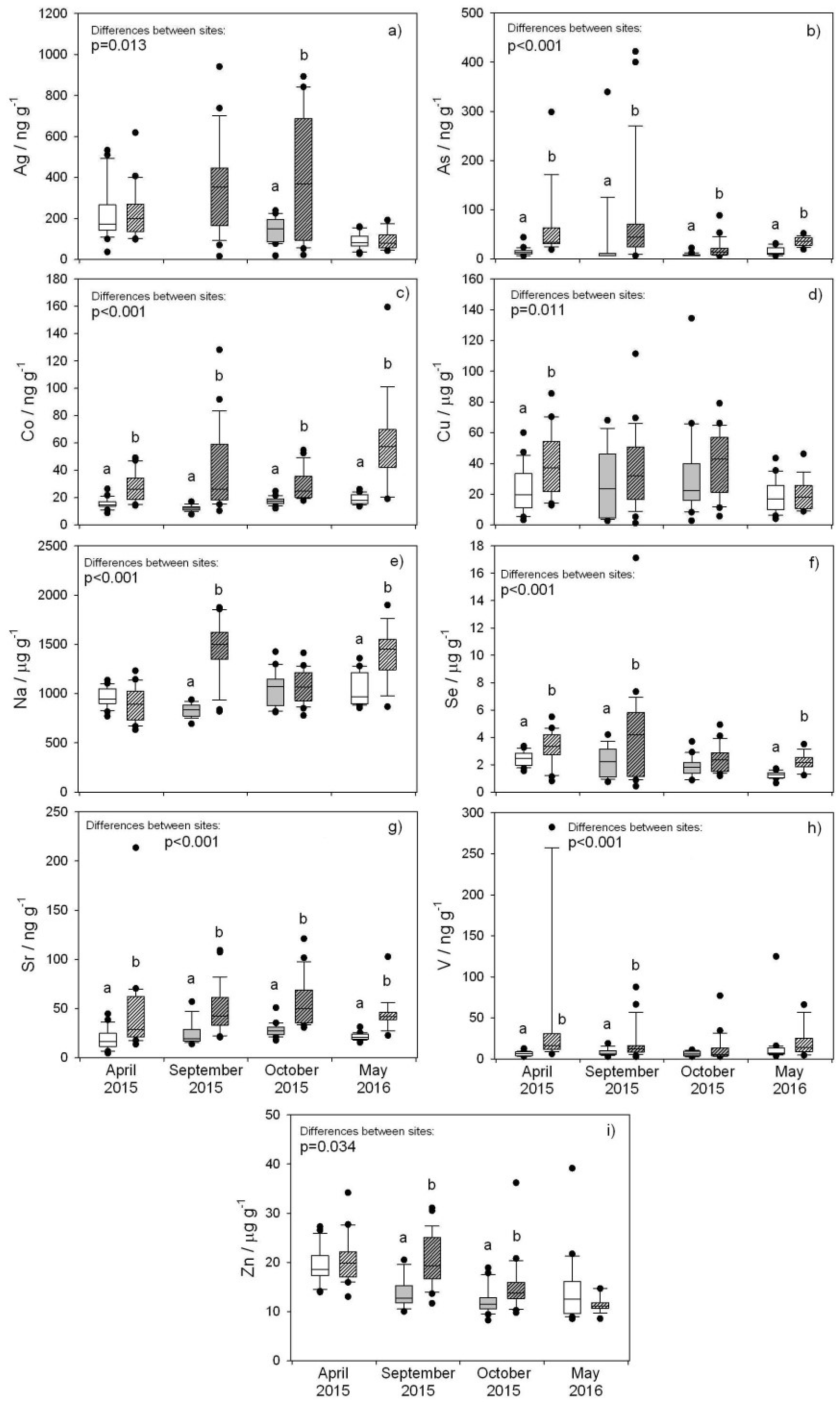

**Figure 3.** Concentrations (ng/g or µg/g on wet mass basis) of nine metals/metalloids in hepatic cytosolic fractions of brown trout *Salmo trutta* caught at two sites in the Krka River (reference site: Krka River spring; contaminated site: Krka downstream of Knin town) in four sampling campaigns (April, September, October 2015, and May 2016), and characterized by higher values at contaminated site: a) Ag, b) As, c) Co, d) Cu, e) Na, f) Se, g) Sr, h) V, and i) Zn. Differences between sites within each season are indicated with different letters (a, b), based on Mann-Whitney rank sum-test ($p$<0.05). Season legend: white – spring; grey – autumn; site legend: clear boxes – Krka River spring; boxes with pattern – Krka downstream of Knin town.

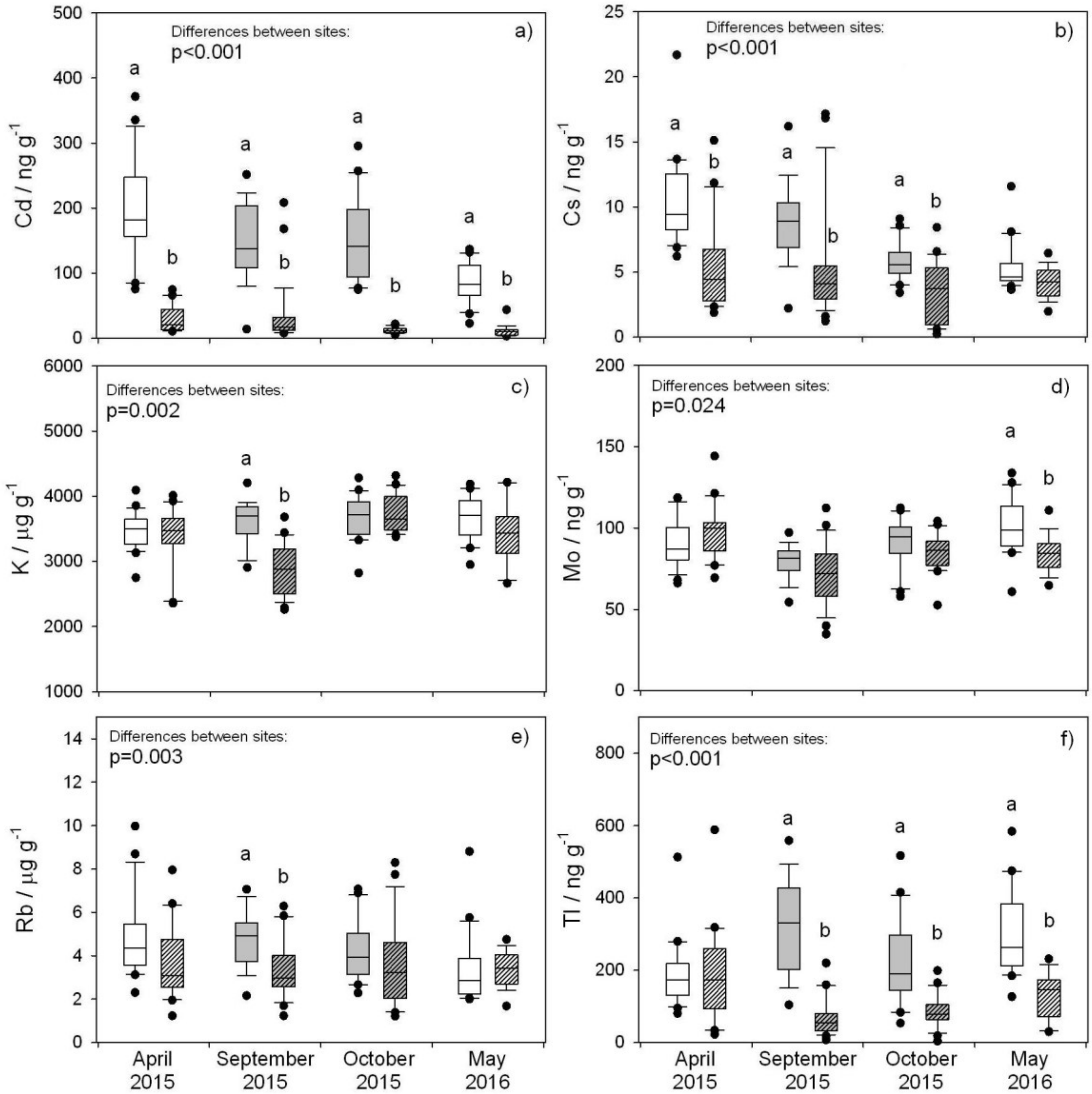

**Figure 4.** Concentrations (ng/g or µg/g on wet mass basis) of six metals in hepatic cytosolic fractions of brown trout *Salmo trutta* caught at two sites in the Krka River (reference site: Krka River spring; contaminated site: Krka downstream of Knin town) in four sampling campaigns (April, September, October 2015, and May 2016), and characterized by higher values at reference site: a) Cd, b) Cs, c) K, d) Mo, e) Rb, f) Tl. Differences between sites within each season are indicated with different letters (a, b), based on Mann-Whitney rank sum-test ($p$<0.05). Season legend: white – spring; grey – autumn; site legend: clear boxes – Krka River spring; boxes with pattern – Krka downstream of Knin town.