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Mining waste as a cause of increased bioaccumulation of highly toxic metals in liver and gills of Vardar chub (*Squalius vardarensis* Karaman, 1928)☆

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Freshwater contamination with mining waste can result with high concentrations of toxic metals in the water and in fish organs. In North-Eastern Macedonia, several rivers (e.g., Zletovska, Kriva) are exposed to acid mine drainage from active Pb/Zn mines. Previous studies confirmed high concentrations of dissolved metals in their water. This study was performed in liver and gills of Vardar chub (*Squalius vardarensis* Karaman, 1928) from three Macedonian rivers (Bregalnica, Kriva and Zletovska) in spring and autumn 2012. The aim was to establish if increased exposure to certain metals have resulted with their increased bioaccumulation. The concentrations of 19 elements were measured in cytosolic tissue fractions, to obtain information on metabolically available metal species. The following ranges of cytosolic concentrations of highly toxic elements were measured in the Vardar chub liver (in μg/L): Cd, 1.18–184; Cs, 0.25–25.4; Tl, 0.02–5.80; Pb, 0.70–61.1. Their ranges measured in the gills (in μg/L) were the following: Cd, 0.24–59.2; Cs, 0.39–24.4; Tl, 0.01–1.00; Pb, 0.65–87.2. Although the water of the mining impacted Zletovska River was highly contaminated with several essential metals, especially with Mn and Zn, the majority of essential elements (Na, K, Ca, Mg, Co, Cu, Fe, Mn, Mo, and Zn) did not reflect the exposure level. In contrast, seven nonessential elements reflected the level of exposure in the water. Significantly increased hepatic and gill concentrations of Cs, Rb, Sr, and Tl were detected in Vardar chub from the Zletovska River compared to the other two rivers, of Cd and Pb in the Zletovska and Kriva River compared to Bregalnica, and of V in the Bregalnica River compared to Zletovska and Kriva rivers. Observed significant metal bioaccumulation, in particular of highly toxic elements, as a consequence of exposure to water contaminated with mining waste points to necessity of intensified supervision of mining impacted rivers.

1. Introduction

Contamination of freshwater ecosystems often originates from domestic wastewaters and agricultural runoff, as well as from anthropogenic activities such as industrial production and mining and smelting operations (Sunjog et al., 2016). In active mining areas, the environmental concern is primarily related to spilled mine tailings, emitted dust, and acid mine drainage transported

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into aquatic ecosystems (Riba et al., 2005; Mayes et al., 2010). However, not only currently active mining, but also the mining heritage, should be considered when establishing strategies for long-term environmental management (Monna et al., 2011). A special threat of mining effluents for surrounding aquatic ecosystems comes from the fact that they present a source of toxic metals, which, due to their toxicity, long persistence and tendency to bioaccumulate, pose a potential health risk for aquatic organisms and for humans from fish consumption (Liu et al., 2012; Zhuang et al., 2013). For example, Pb is a very toxic, non-essential element that occurs in a wide variety of minerals, and substantial amounts of Pb have been distributed into the environment from mine and metal smelters (Biuki et al., 2011). In North-Eastern Macedonia, there are several active Pb/Zn mines, which present a risk of metal contamination for local rivers. The study of water metal contamination in the rivers of North-Eastern Macedonia revealed high dissolved concentrations of numerous metals (Cd, Co, Cs, Cu, Mn, Rb, Sr, Tl, and Zn) in the water of the Zletovska River, which is impacted by Pb/Zn mine Zletovo, and somewhat milder Cd and Pb contamination of the Kriva River, which is impacted by the Pb/Zn mine Toranica (Ramani et al., 2014). The adjacent river Bregalnica at the same time presented the case of moderate metal contamination with Ba, Fe, Mo, and V as a consequence of runoff from rice fields (Ramani et al., 2014; Stipaničev et al., 2017).

For the assessment of the quality of aquatic environment scientists largely use fish, which can serve as bioindicators of environmental pollution (Lopes et al., 2000; Williams and Holdway, 2000). The study on native fish populations revealed that the representative fish species in Macedonian rivers, Vardar chub (*Squalius vardarensis* Karaman, 1928), was affected by water contamination in all three above mentioned rivers (Zletovska, Kriva and Bregalnica), just to a different degree, as evidenced by histopathological changes in the gills, liver and kidney, as well as signs of oxidative stress (Barišić et al., 2015; Dragun et al., 2017; Jordanova et al., 2016, 2017). It was, therefore, necessary to provide a link between fish exposure to metals in the water and observed changes in fish health and condition, and to evaluate the actual concentrations of metals accumulated in fish organs. Several studies of metal bioaccumulation have been previously performed on related fish species European chub (*Squalius cephalus*) (Podrug et al., 2009; Dragun et al., 2007, 2009, 2012, 2013a, 2013b, 2016), but so far there are no such information available for Vardar chub.

In this study, metal bioaccumulation in Vardar chub was assessed by analyses of soluble, cytosolic concentrations of 19 elements, 11 essential and 8 nonessential, in two organs, namely liver and gills. Metal bioaccumulation in animals can differ, depending on the metal and organ, due to their different functional statuses (Khadiga et al., 2002). Thus, the liver was chosen due to its importance in the uptake, storage, metabolism, redistribution, and excretion of environmental toxicants (Di Giulio and Hinton, 2008). The gills, on the other hand, are in direct contact with the ambient water and are expected to respond quickly to changes in metal exposure (Kraemer et al., 2005; Dragun et al., 2007). We have applied evaluation of cytosolic metal concentrations instead of evaluation of total metal loads in organs, to evaluate metabolically and trophically available metal levels in liver and gills of Vardar chub (Wallace and Luoma, 2003; Giguère et al., 2006). Soluble cytosolic metal forms are presumably also more available for toxic effects compared to detoxified forms, such as granules, or compared to metals incorporated in the cellular structures (McGeer et al., 2012). Accordingly, the specific aims of this study were: (1) to evaluate the pollution statuses of three rivers in North-Eastern Macedonia (Bregalnica, Kriva and Zletovska) through assessment of metal bioaccumulation in two organs of bioindicator fish species, Vardar chub, with special emphasis on mining impacted rivers; and (2) to broaden the knowledge on bioaccumulation of specific metals in fish as a consequence of exposure to mining waste, with special emphasis on differences between essential and nonessential elements.

2. Materials and methods

2.1. Study period and area

The study of metal bioaccumulation in two organs (liver and gills) of freshwater fish Vardar chub was carried out in May/June and October 2012 in three rivers in North-Eastern Macedonia. One of the studied rivers was characterized by agricultural impact (the Bregalnica River), whereas the other two were rivers influenced by active mining (the Zletovska River and the Kriva River). The metal concentrations in the water of three studied rivers in the exact time of fish sampling (Table 1), the hydrological and geological information, and the information about contamination sources were published and discussed in details by Ramani et al. (2014).

The sampling points selected at three studied rivers were previously described in several of our papers resulting from the same project (Ramani et al., 2014; Barišić et al., 2015; Jordanova et al., 2016; Dragun et al., 2017; Jordanova et al., 2017). The sampling point at the Bregalnica River (N 41°43.57′ E 22°10.27′) was selected at the site primarily influenced by rice fields runoff (Andreevska et al., 2013), but also by municipal sewage and industrial facilities of the Štip town (Rebok, 2013). The sampling point at the Zletovska River (N 40°58.54′ E 21°39.45′) was selected at the site situated approximately 5 km downstream of the input of mining waste from the active Pb/Zn mine Zletovo (Alderton et al., 2005; Dolenec et al., 2005). That site was additionally impacted by municipal and industrial wastewaters of Probištip town (e.g. the waste of battery factory; Spasovski and Dambov, 2009). The sampling point at the Kriva River (N 42°11.39′ E 22°18.34’) was selected at the site situated 15 km downstream of Toranica, still active Pb/Zn mine. The Kriva River flows through land used for gardens and orchards, so it was additionally exposed to the influence of agricultural runoff (Ramani et al., 2014; Dragun et al., 2017). The map of the study area was already published by Ramani et al. (2014).

Table 1
Mean macro and trace element concentrations (n = 3) in water of three Macedonian rivers in spring (May/June) and autumn (October) of 2012 (Ramani et al., 2014).

	Spring 2012			Autumn 2012		
	Bregalnica	Zletovska	Kriva	Bregalnica	Zletovska	Kriva
Essential macro elements (mg/L)						
Na	18.37	79.11	4.76	39.30	117.4	10.67
K	5.60	17.08	1.88	5.88	18.46	3.57
Ca	47.33	197.4	27.99	73.09	332.2	41.82
Mg	11.49	43.49	5.91	21.89	70.36	9.59
Essential trace elements (μg/L)						
Co	0.095	1.51	0.053	0.078	0.841	0.074
Cu	2.16	3.38	1.21	1.07	3.16	1.37
Fe	61.28	12.35	10.01	37.59	5.90	25.67
Mn	13.27	351.9	9.90	4.40	2527	9.65
Mo	0.950	0.278	0.442	1.068	0.136	0.476
Zn	4.97	197.0	22.07	6.14	1507	3.81
Nonessential trace elements (μg/L)						
Ba	55.97	4.63	28.89	79.05	8.07	37.29
Cd	0.032	0.272	0.270	0.021	2.002	0.029
Cs	0.067	1.299	0.026	0.091	1.034	0.015
Pb	0.692	0.313	1.845	0.307	0.748	0.420
Rb	2.03	22.75	0.84	3.20	25.87	1.62
Sr	348.7	2796	121.3	514.8	4307	182.2
Tl	0.014	0.043	0.006	0.008	0.146	0.003
V	1.132	0.040	0.220	0.838	0.029	0.386

2.2. Fish sampling and organ dissection

For the purposes of this study we have caught 158 individuals of Vardar chub (*Squalius vardarensis* Karaman, 1928; family Cyprinidae; class Actinopterygii). In the spring campaign 90 individuals were caught, 30 from each river, whereas in the autumn campaign 68 individuals were caught, 30 from the Bregalnica River, 26 from the Kriva River, and 12 from the Zletovska River (Barišić et al., 2015; Jordanova et al., 2016; Dragun et al., 2017; Jordanova et al., 2017). Fish were sampled by electro fishing, applying the electrofisher Samus 725G and following the guidelines stated in the standard CEN EN 14011:2003. Opaque plastic reservoir was used for transport of sampled fish to the laboratory and for keeping them alive in aerated river water, which was taken from each respective sampling point. Fish were anesthetized with Clove Oil (Sigma Aldrich, USA) prior to killing and dissection. Then followed the measurement of fish total lengths (cm) and total masses (g), based on which Fulton condition indices (FCI) were calculated using the formula from Rätz and Lloret (2003). Fish were then killed, liver, gills and gonads were dissected, and the organ masses were measured. The liver and gills were stored at −80 °C. Fish sex was determined histologically using samples of gonad tissues which were put in Bouin's fixative (Merck, Germany) (Jordanova et al., 2016). Gonadosomatic indices (GSI, %) were calculated according to Şaşi (2004), as ratios of gonad masses to total body masses, multiplied with 100. Fish biometric parameters are presented in Table 2.

2.3. Homogenization of Vardar chub livers and gills and isolation of soluble cytosolic fractions

Isolation of soluble cytosolic fractions from liver and gills of Vardar chub was performed according to Standard Operational Procedure (1999), which was developed in the framework of the Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM) (Dragun et al., 2009). Frozen samples of Vardar chub liver and gills were chopped up inside cooled glass dishes, and then cooled homogenization buffer [100 mM Tris-HCl/Base (Sigma, pH 8.1 at 4 °C) containing the reducing agent (1 mM dithiothreitol, Sigma)] was added into the dishes (w/v 1:5). Obtained suspensions were homogenized in glass tube, which was cooled on ice, applying 10 strokes of PTFE-coated pestle at 6000 rpm (Potter-Elvehjem homogenizer, Glas-Col, USA). The homogenates were afterwards centrifuged for 2 h in the Avanti J-E centrifuge (Beckman Coulter) at 50,000×g and 4 °C. Supernatants obtained after centrifugation represented soluble cytosolic fractions of Vardar chub liver and gills, which contained cytosolic biomolecules, as well as microsomes and lysosomes, whereas cell membranes, nuclei, mitochondria and granules were excluded from the samples (Bonneris et al., 2005; Podrug et al., 2009; Dragun et al., 2013a). Cytosolic fractions of Vardar chub liver and gills were then stored at −80 °C for latter macro and trace element analyses.

2.4. Macro and trace element analyses in the cytosolic fractions of Vardar chub liver and gills

Simple sample preparation for macro and trace element analyses was applied, consisting of dilution of cytosolic tissue fractions with Milli-Q water and acidification with HNO_3 (*Suprapur*®, Merck, Germany) (Dragun et al., 2013a). Dilution factors for Na, K, Mg, and Ca were 100, whereas for all the remaining elements they were 10. Final concentration of HNO_3 in the samples was 0.65%. The samples were prepared for measurement in duplicate.

In diluted and acidified samples we have measured 19 macro and trace elements using high resolution inductively coupled plasma mass spectrometer (HR ICP-MS, Element 2, Thermo Finnigan, Germany). The instrument was equipped with an autosampler ASX 510 (Cetac Technologies, USA), whereas the sample introduction kit consisted of SeaSpray nebulizer and cyclonic spray chamber Twister. In all samples we have added In (1 μg/L; Fluka, Germany) as an internal standard (Fiket et al., 2007). Several elements were measured in low-resolution mode (^{82}Se, ^{85}Rb, ^{98}Mo, ^{111}Cd, ^{133}Cs, ^{205}Tl, and ^{208}Pb), several others in medium-resolution mode (^{23}Na, ^{24}Mg, ^{42}Ca, ^{51}V, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{63}Cu, ^{66}Zn, ^{86}Sr, and ^{138}Ba); whereas only ^{39}K was measured in high-resolution mode. For calculation of macro and trace element concentrations we have applied external calibrations using multielement standard solution for macro elements containing Na, K, Mg, and Ca (Fluka, Germany) and multielement standard solution for trace elements (Analytika, Czech Republic) supplemented with Rb (Sigma-Aldrich, Germany) and Cs (Fluka, Germany). All standards were acidified with HNO_3 (*Suprapur*®, Merck, Germany; final acid concentration 1.3%) and supplemented with In (1 μg/L; Fluka, Germany).

We have performed accuracy control of macro and trace element measurements, using quality control samples acquired from UNEP/GEMS (QC trace metals, catalogue no. 8072, lot no. 146142–146143; QC minerals, catalogue no. 8052, lot no. 146138–146139; Burlington, Canada). Ten measurements in control samples were carried out and the following recoveries (%) were obtained: Ba (97.2 ± 3.0), Ca (100.2 ± 2.5), Cd (97.5 ± 1.8), Co (98.4 ± 2.2), Cu (98.2 ± 1.8), Fe (104.1 ± 6.3), K (96.7 ± 11.5), Mg (97.1 ± 2.3), Mn (97.6 ± 1.4), Mo (96.4 ± 2.9), Na (100.1 ± 2.5), Pb (97.5 ± 3.3); Se (100.9 ± 6.7), Sr (99.9 ± 2.9), Tl (100.4 ± 2.3), and V (99.3 ± 1.8). Limits of detection (LOD) were calculated as three standard deviations of ten successive measurements of macro and trace element concentrations in the blank samples (adequately diluted 100 mM Tris-HCl/Base supplemented with 1 mM dithiothreitol). The following LODs (mg/L) were determined for macro elements in the hepatic and gill cytosolic fractions: Na, 1.23; K, 0.216; Mg, 0.047; and Ca, 1.05. The following LODs (μg/L) were determined for trace elements in the hepatic and gill cytosolic fractions: Ba, 0.471; Cd, 0.013; Co, 0.001; Cs, 0.009; Cu, 0.438; Fe, 1.46; Mn, 0.088; Mo, 0.044; Pb, 0.120; Rb, 0.179; Se, 0.378; Sr, 0.451; Tl, 0.020; V, 0.015; and Zn, 95.0. All concentrations measured in

Table 2
Biometric parameters of sampled Vardar chub in three Macedonian rivers in spring (May/June) and autumn (October) of 2012.

	Spring 2012			Autumn 2012		
	Bregalnica	Zletovska	Kriva	Bregalnica	Zletovska	Kriva
n	30	30	30	30	12	26
Total length (cm)	18.59 ± 4.16^{a}	13.91 ± 2.35^{b}	18.84 ± 4.10^{a}	19.79 ± 3.46^{A}	13.36 ± 4.25^{B}	14.33 ± 3.32^{B}
Total mass (g)	86.82 ± 62.51^{a}	29.16 ± 18.99^{b}	90.43 ± 71.87^{a}	89.33 ± 52.67^{A}	27.15 ± 29.64^{B}	33.45 ± 22.42^{B}
GSI (%)	6.16 ± 2.66^{a}	6.58 ± 3.47^{a}	9.82 ± 3.68^{b}	1.87 ± 0.76^{A,B}	3.48 ± 1.98^{A}	1.49 ± 0.86^{B}
FCI	1.16 ± 0.11^{a}	0.99 ± 0.07^{b}	1.15 ± 0.08^{a}	1.05 ± 0.06^{A}	0.85 ± 0.07^{B}	1.00 ± 0.07^{A}
Sex (F/M)	14/16	18/12	10/20	17/13	7/5	9/17

[a,b] statistically significant differences in biometric parameters among three rivers in spring.
[A,B] statistically significant differences in biometric parameters among three rivers in autumn.

Vardar chub liver and gills in this study were presented as µg per L or mg per L of cytosolic tissue fractions.

2.5. Data processing and statistical analyses

For creation of graphs we have used SigmaPlot 11.0 for Windows, whereas Microsoft Office Excel 2007 was used for basic calculations. The data analyses presented in Figs. 1–3 were performed using SAS Studio from SAS University Edition® software. In order to explore the time-spatial distribution patterns and compare response group means among representative rivers and different sampling periods, analysis of covariance (ANCOVA) was performed after adjusting for the covariate - fish mass. As a data pretreatment, natural log transformation (ln(x)) was used to meet the fundamental assumptions required by the model, namely normality and variance homogeneity, but also this transformation improves data linearity. The continuous covariate variable was grand-mean centred (Hofmann and Gavin, 1998; Enders and Tofighi, 2007) in order to ensure numerical stability of the model. The ANCOVA model was utilized using the PROC GLM procedure with REML estimation method and unstructured covariance (option type = UN in random statement) at the 0.05 level of significance. Regression models applied to establish the associations between bioaccumulated metal levels and levels of water contamination (Fig. 4) were obtained using SigmaPlot 11.0 for Windows. The same program was also used for between-site comparisons of biometric parameters (Table 2), which were done by Kruskal-Wallis one-way analysis of variance separately for each sampling season, and for between-organ comparisons of bioaccumulated metal concentrations (Table 3), which were done by Mann-Whitney rank sum test, also separately for each sampling season.

3. Results and discussion

3.1. Fish biometry

Vardar chub sampled in this study varied significantly in size among three rivers (Table 2). In the spring season, fish from the Zletovska River were the smallest, whereas in the autumn season fish from both the Zletovska and the Kriva River were significantly smaller compared to the fish from the Bregalnica River. Fulton condition indices, indicating fish nutritional status, were also the lowest at the Zletovska River in both sampling seasons. The cause of small size and low condition factor of fish from the Zletovska River was possibly high metal exposure reported for the Zletovska River at the time of Vardar chub sampling (Ramani et al., 2014; Jordanova et al., 2016). Additionally, lower condition factor of the fish from the Zletovska River could be also the result of insufficient nutrition (Munkittrick and Dixon, 1988; Jordanova et al., 2016). Having that in mind, we have performed statistical comparisons among sites of metal concentrations in liver and gills using fish masses as a covariance, to annul the impact of fish size on the obtained metal bioaccumulation data for two selected Vardar chub organs. In addition, GSIs were higher in the spring in fish from all three rivers compared to autumn season, confirming spring period as active

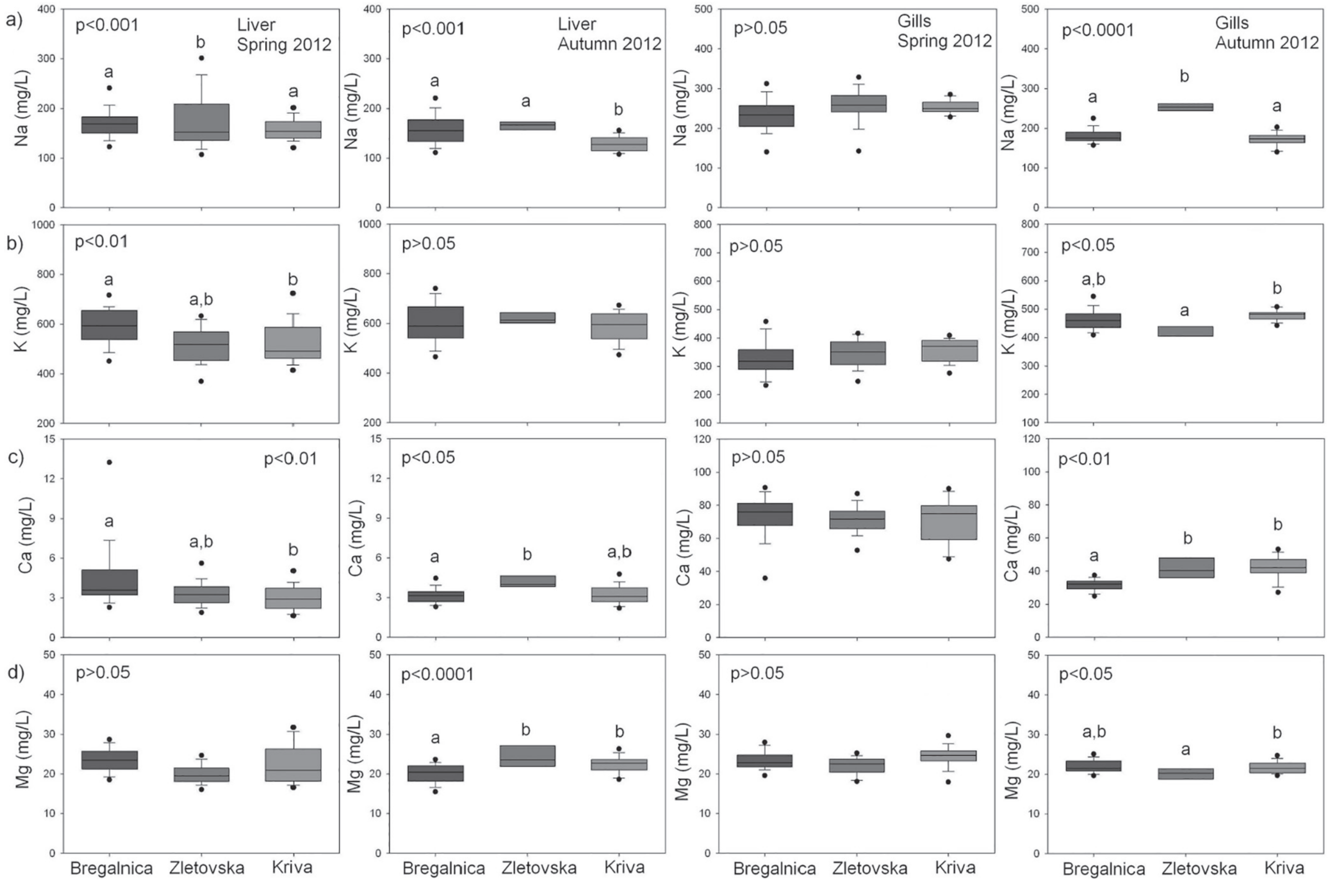

Fig. 1. The cytosolic concentrations (mg/L) of four essential macro elements. The results refer to liver and gills of Vardar chub caught in three Macedonian rivers in two sampling campaigns (spring and autumn 2012): a) Na, b) K, c) Ca, d) Mg. The results are presented as box-plots. The boundaries of box-plot indicate 25th and 75th percentiles; a line within the box marks the median value; whiskers above and below the box indicate 10th and 90th percentiles, whereas the black dots indicate 5th and 95th percentiles. Differences between sites for each organ within each season are indicated with different letters (a, b, c).

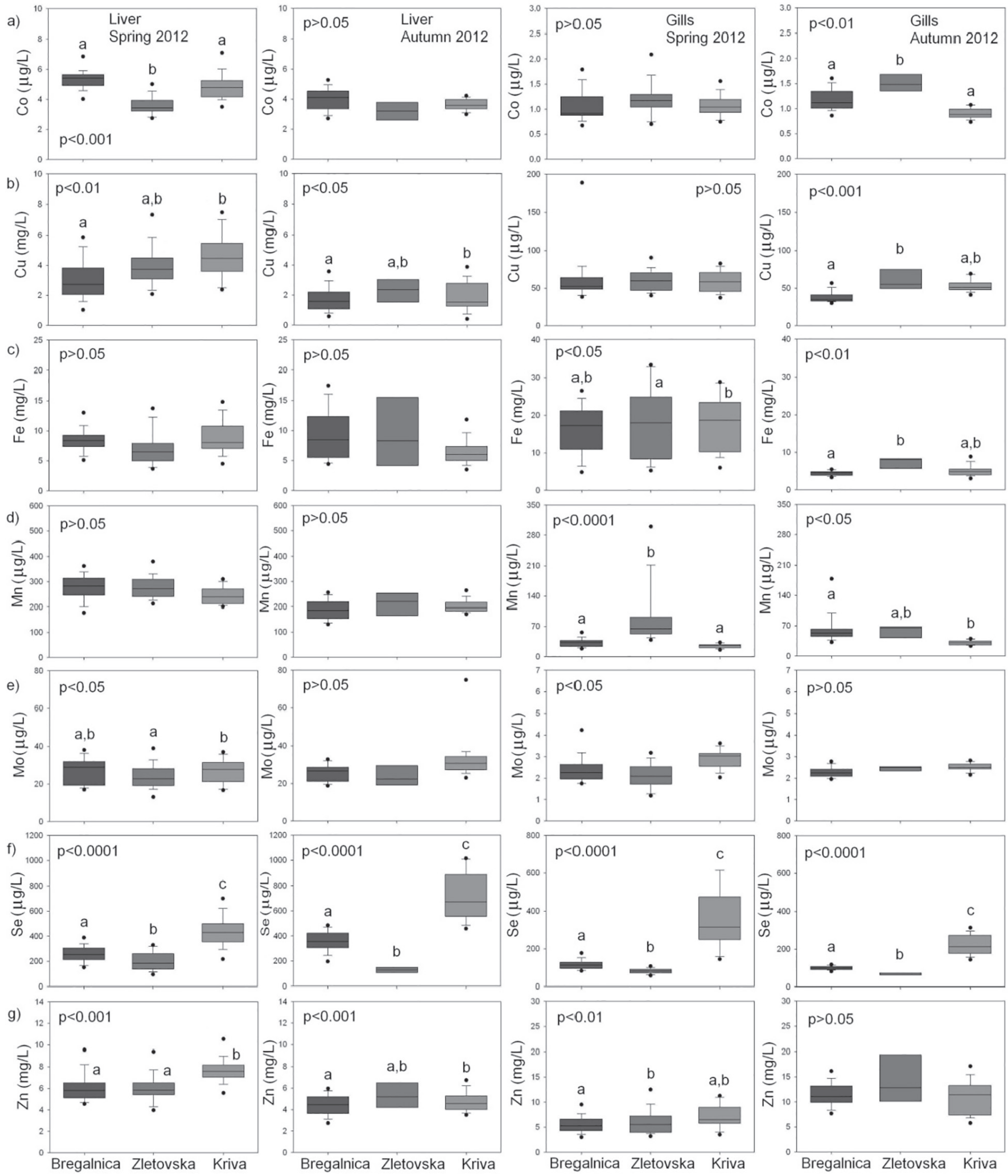

Fig. 2. The cytosolic concentrations (μg/L or mg/L) of seven essential trace elements. The results refer to liver and gills of Vardar chub caught in three Macedonian rivers in two sampling campaigns (spring and autumn 2012): a) Co, b) Cu, c) Fe, d) Mn, e) Mo, f) Se, g) Zn. The results are presented as described in the caption of Fig. 2.

reproductive period for Vardar chub (Table 2). The sampled populations at all three rivers consisted of both females and males, although in different proportions (Table 2).

3.2. Association of metal bioaccumulation in liver and gills of Vardar chub with increased metal exposure in the river water

Cytosolic concentrations of 19 elements in liver and gills of Vardar chub in spring and autumn 2012 are given in Table 3, and present the first data of that kind for the selected fish species. Cytosolic concentrations of several of these elements were previously published for liver and gills of related fish species, European chub (*S. cephalus*), from two moderately contaminated rivers in Croatia – the Sava River and the Sutla River, and can serve as a basis for comparison with the results of this study. Average cytosolic metal concentrations in gills of *S. cephalus* from the Sava River were as follows: Cd 2.9–3.6 μg/L, Cu 68.4–79.0 μg/L, Fe 3.9–9.6 mg/L, Mn 55.0–63.5 μg/L, and Zn 6.3–10.3 mg/L (Dragun et al., 2007),

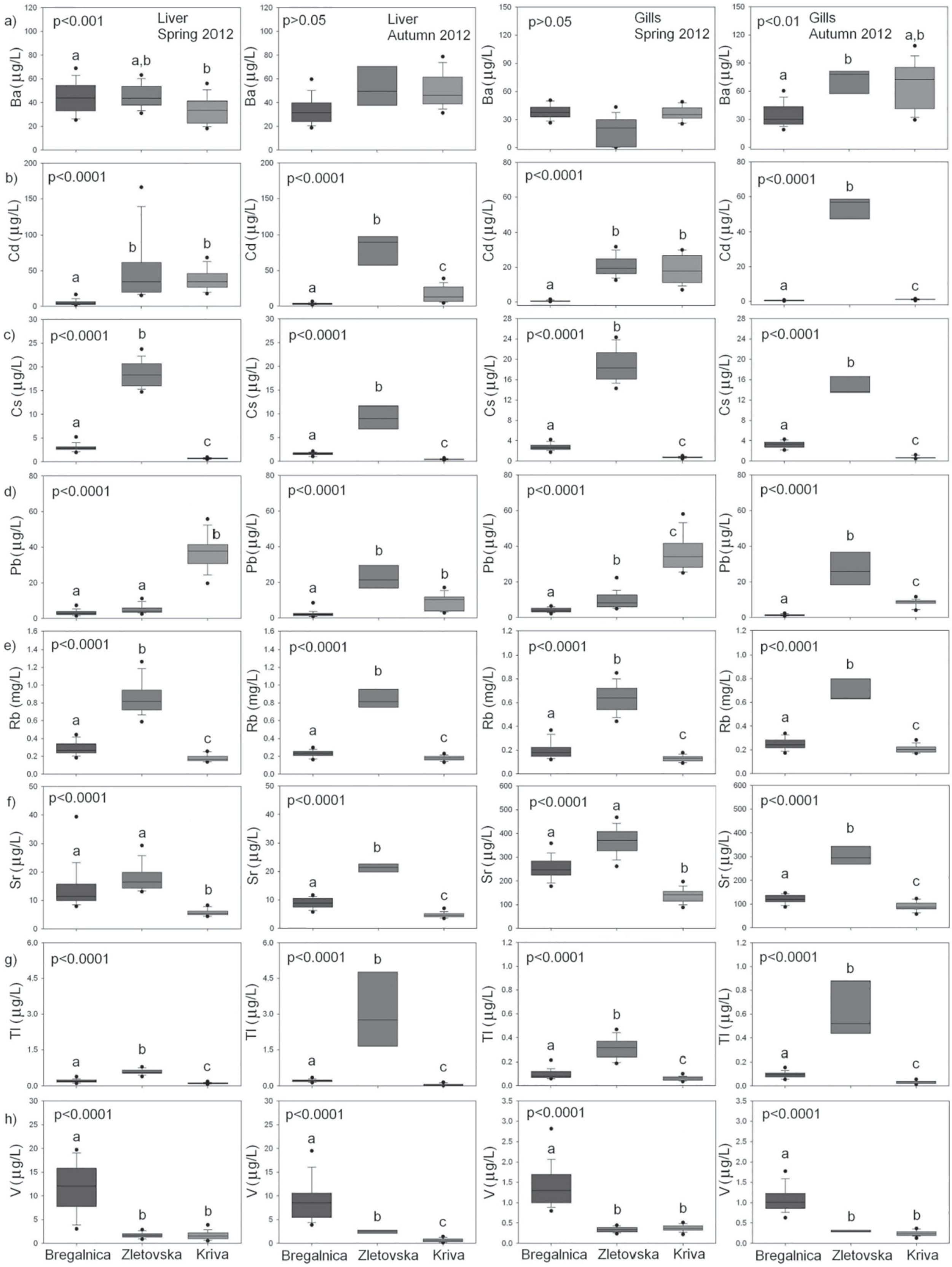

Fig. 3. The cytosolic concentrations (µg/L or mg/L) of eight nonessential trace elements. The results refer to liver and gills of Vardar chub caught in three Macedonian rivers in two sampling campaigns (spring and autumn 2012): a) Ba, b) Cd, c) Cs, d) Pb, e) Rb, f) Sr, g) Tl, h) V. The results are presented as described in the caption of Fig. 2.

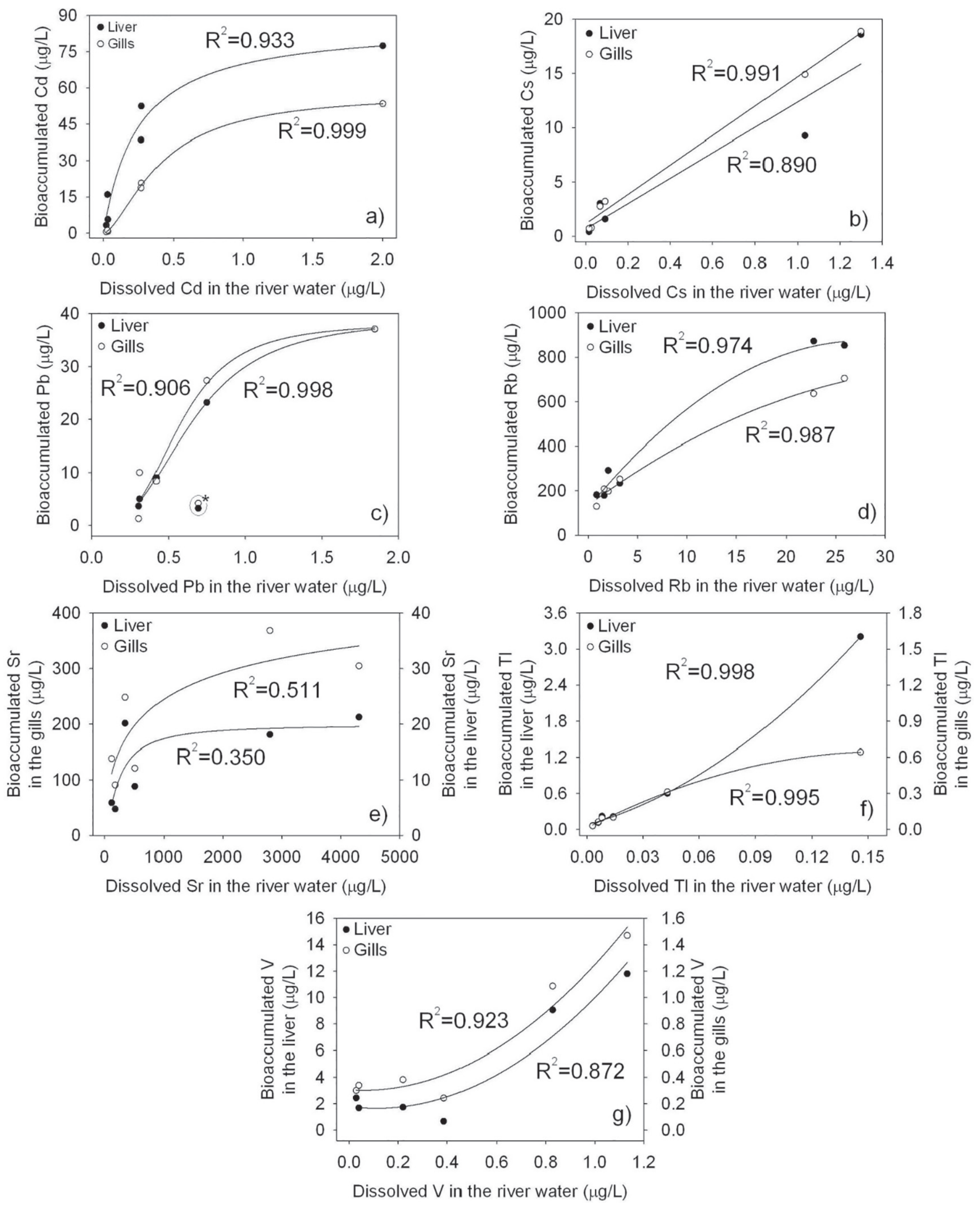

Fig. 4. Graphical presentation of associations between bioaccumulated metal concentrations in liver and gills of Vardar chub (µg/L) and dissolved metal concentrations in the river water (µg/L). The results refer to the following seven metals: a) Cd; b) Cs; c) Pb; d) Rb; e) Sr; f) Tl; g) V. The models were obtained using mean concentrations calculated for three rivers in two time points (n = 6). For Cd, Pb, and Sr, sigmoidal logistic nonlinear regression was used, for Cs, polynomial linear regression, whereas for Rb, Tl and V, polynomial quadratic nonlinear regression was applied. Adjusted coefficients of determination (R^2) are given for each curve within the figure. *Only for Pb concentrations, data obtained for the Bregalnica River in the spring period were excluded from the curve fitting, with an assumption that observed increase of dissolved Pb in the river water was probably just momentary, with no effect on Pb bioaccumulation.

whereas average cytosolic concentrations in the liver of the same fish specimens were: Cd 8.0 µg/L, Cu 1.5 mg/L, Fe 5.0 mg/L, Mn 150.0 µg/L, and Zn 5.0 mg/L (Podrug et al., 2009). The ranges of cytosolic concentrations of several other hepatic metals in *S. cephalus* from the Sava River were: Co 4.1–5.1 µg/L, Mo 22.8–30.6 µg/L, Pb 1.0–5.9 µg/L, Sr 5.5–10.5 µg/L, and V 2.9–11.5 µg/L (Dragun et al., 2013a). Average cytosolic metal concentrations in gills of *S. cephalus* from the Sutla River were as follows: Pb 0.85–17.3 µg/L (Dragun et al., 2012), and Cd 0.68 µg/L, Cu 42.6 µg/L, and Zn 14.3 mg/L (Dragun et al., 2013b); whereas the ranges of cytosolic concentrations of several other elements in gills were the following: Ba 13–67 µg/L, Ca 19–62 mg/L, Co 0.7–2.7 µg/L, Cs 0.2–1.9 µg/L, Fe 1.6–6.4 mg/L, K 225–895 mg/L, Mg 13–47 mg/L, Mn 16–69 µg/L, Mo 1.3–16 µg/L, Na 78–366 mg/L, Rb 164–1762 µg/L, Sr 24–81 µg/L, and V 0.1–1.8 µg/L (Dragun et al., 2016). Average cytosolic concentrations in the liver of the same fish specimens

Table 3
Macro and trace element concentrations (mean ± standard deviation) in liver and gills of Vardar chub from all three rivers together in spring (May/June) and autumn (October) of 2012. Comparison was made between two organs within the same fish specimens, i.e. only for those fish for which data were available for all elements in both organs ($n_{spring} = 79$; $n_{autumn} = 46$).

	Spring 2012		Autumn 2012	
	Liver	Gills	Liver	Gills
Essential macro elements				
Na (mg/L)	169.1 ± 40.0^a	246.9 ± 43.0^b	148.3 ± 30.6^A	183.7 ± 26.3^B
K (mg/L)	547.9 ± 89.7^a	343.2 ± 58.5^b	606.0 ± 79.0^A	466.7 ± 35.1^B
Ca (mg/L)	4.09 ± 6.26^a	71.50 ± 13.99^b	3.31 ± 0.71^A	35.54 ± 7.43^B
Mg (mg/L)	22.11 ± 4.17^a	23.39 ± 2.98^b	21.42 ± 2.97	21.85 ± 1.68
Essential trace elements				
Co (µg/L)	4.60 ± 1.05^a	1.13 ± 0.44^b	3.85 ± 0.72^A	1.12 ± 0.26^B
Cu (µg/L)	3826 ± 1566^a	77.51 ± 164.0^b	1929 ± 957^A	44.43 ± 12.34^B
Fe (mg/L)	8.23 ± 2.72^a	17.26 ± 7.67^b	8.77 ± 4.04^A	4.86 ± 1.36^B
Mn (µg/L)	264.4 ± 59.0^a	52.36 ± 62.82^b	194.9 ± 39.8^A	54.16 ± 36.37^B
Mo (µg/L)	25.60 ± 7.32^a	2.75 ± 2.39^b	27.18 ± 5.58^A	2.38 ± 0.26^B
Se (µg/L)	306.1 ± 144.8^a	208.4 ± 190.6^b	464.2 ± 239.2^A	132.7 ± 65.7^B
Zn (mg/L)	6.68 ± 1.68	6.41 ± 2.42	4.68 ± 1.14^A	11.72 ± 3.02^B
Nonessential trace elements				
Ba (µg/L)	40.58 ± 13.60^a	32.29 ± 14.27^b	38.62 ± 14.13	46.80 ± 24.57
Cd (µg/L)	31.37 ± 33.15^a	13.47 ± 10.78^b	14.31 ± 23.66^A	4.17 ± 13.27^B
Cs (µg/L)	6.66 ± 7.82	6.72 ± 8.17	1.69 ± 2.05^A	3.20 ± 3.40^B
Pb (µg/L)	16.59 ± 17.35	18.26 ± 17.41	7.04 ± 9.43^A	5.16 ± 7.34^B
Rb (µg/L)	420.1 ± 322.3^a	300.3 ± 236.1^b	258.8 ± 167.0	268.1 ± 129.1
Sr (µg/L)	14.12 ± 23.84^a	240.9 ± 108.4^b	8.48 ± 4.11^A	124.2 ± 54.1^B
Tl (µg/L)	0.285 ± 0.219^a	0.151 ± 0.124^b	0.312 ± 0.607^A	0.112 ± 0.161^B
V (µg/L)	5.14 ± 5.87^a	0.739 ± 0.694^b	6.13 ± 5.42^A	0.784 ± 0.488^B

^{a,b} statistically significant differences between concentrations in the liver and gills in spring.
^{A,B} statistically significant differences between concentrations in the liver and gills in autumn.

from the Sutla River were: Pb 1.2–18.1 µg/L (Dragun et al., 2012), and Cd 19.4 µg/L, Cu 1.5 mg/L, and Zn 6.6 mg/L (Dragun et al., 2013b). If we compare those numbers with the results of the present study (Table 3), we can see that cytosolic concentrations of highly toxic elements (Cd, Cs, Pb and Sr) in the gills and liver of Vardar chub reached higher levels compared to European chub from moderately contaminated Sava and Sutla rivers in Croatia, indicating that increased metal exposure caused by mining contamination of the Macedonian rivers resulted with an observable impact on metal bioaccumulation in fish organs.

In the present study, four analyzed macro elements, Na, K, Ca and Mg, were in both sampling periods present in the highest concentrations in the river water of the mining impacted Zletovska River (Table 1; Ramani et al., 2014). The dissolved concentrations of Na were higher in the Zletovska River compared to the other two rivers up to 10–15 times, depending on the season, whereas for dissolved K, Ca and Mg the difference was somewhat smaller and amounted to 3–9 times (Table 1; Ramani et al., 2014). Nevertheless, bioaccumulated concentrations of these four elements in liver and gills of Vardar chub did not reflect the increased exposure from the river water (Fig. 1). With few exceptions, the highest concentrations of these elements were generally not found at the Zletovska River either in Vardar chub liver or gills, in either one of two studied seasons. Although statistically significant differences among the sites were sometimes observed, the differences were generally rather small and the concentrations of each of these four elements were placed within the narrow ranges indicating strong intracellular regulation.

The second group of the studied elements was composed of seven trace elements that are essential for functioning of fish metabolism, namely Co, Cu, Fe, Mn, Mo, Se and Zn. It has been reported that such metals, that are required as essential nutrients for various biochemical and physiological functions (WHO, 1996), can become toxic under conditions of increased environmental concentrations (Anke, 2004). Four of these elements, Co, Cu, Mn and Zn, were increased in the river water of the mining impacted Zletovska River, whereas two of them, Fe and Mo, were increased in the river water of the agriculturally impacted Bregalnica River (Table 1, Ramani et al., 2014). In the case of Se, we do not have the information on its concentrations in the water of the studied rivers at the time of the fish sampling. Increase of the dissolved Fe and Mo in the Bregalnica River, as well as Cu in the Zletovska River, in comparison to the other rivers was only moderate, and amounted to 2–6 times, 2–8 times, and 2–3 times, respectively (Table 1; Ramani et al., 2014). In contrast, the increase of dissolved Co, Mn and Zn in the Zletovska River compared to the other two rivers was much more pronounced, and was as high as 28–40 times in the spring period, depending on the metal (Table 1; Ramani et al., 2014). In the autumn period, characterized by extremely low water level (Ramani et al., 2014), this increase was even more obvious for dissolved Mn and Zn, and was equal to maximally 570 and 400 times, respectively (Table 1; Ramani et al., 2014). Despite this enormous increase in the exposure level, bioaccumulated concentrations of analyzed essential trace elements in the liver and gills of Vardar chub showed the similar behaviour as the macro elements. Although some statistically significant differences were observed among the sites for both organs and in both seasons, the observed trends mainly could not be associated to the exposure from the water, again indicating probable strong regulation of essential elements in the fish organism, already reported in numerous studies (Olsvik et al., 2000; Moiseenko and Kudryavtseva, 2001; Monna et al., 2011). The only indication of higher bioaccumulation of essential elements in connection to increased exposure in water was recorded in the gills of Vardar chub, in the autumn for Co (Fig. 2a) and Cu (Fig. 2b), and in the spring for Mn (Fig. 2d) and Zn (Fig. 2g), with significantly higher gill concentrations at the Zletovska River. However, it could not be deduced without a doubt, even for those mild differences observed for essential metals, that they were the results of higher exposure in the water. Dissolved Mn and Zn concentrations were, as already mentioned, much higher in the water in the autumn sampling, and thus manifold increase of gill accumulation of Mn and Zn at the Zletovska River would be expected in the autumn sampling compared to spring, which was not the case (Fig. 2d and g). Such differences, as observed in the gills, were not at all observed in the liver; in contrast, hepatic Co (Fig. 2a) was even the lowest in the Vardar chub caught at the site with the highest exposure, i.e. at the Zletovska River. Brumbaugh et al. (2005) also reported that Zn in the liver of common carp (*Cyprinus carpio*) did not reflect water contamination near the former site of Pb/Zn mining, Casiot et al. (2009) reported absence of Cu, Mn and Zn bioaccumulation in the liver of chub (*Leuciscus cephalus*) downstream of the point of contamination by acid mine drainage originating from a former Pb/Zn mine, whereas Zhuang et al. (2013) reported that Zn bioaccumulation factors in the various fish tissues from mining influenced ponds showed a small range (8–29%) suggesting that Zn was regulated to maintain a homeostatic status. Furthermore, bioaccumulated Fe (Fig. 2c) and Mo (Fig. 2e) concentrations did not reflect the exposure in either of the target organs of Vardar chub. Their highest bioaccumulation would be expected in Vardar chub from the Bregalnica River which had the highest exposure level, but that was not confirmed by the obtained results. The differences among sites were actually very small for Fe and Mo in both organs and in both seasons (Fig. 2c and e). This was opposite to previous study on related fish species, European chub (*S. cephalus*), when evident Fe bioaccumulation in the gills was reported as a consequence of exposure in the river water (Dragun et al., 2016). The only element that stands out from this group of essential elements is Se (Fig. 2f), which was markedly increased in both seasons and in both organs in fish from the mining and agriculturally impacted Kriva River; however, due to the lack of information about the Se level in the river water we can only hypothesize that the cause was higher exposure level at the Kriva River. This contrast between Se and most of the other essential elements was already described in the literature. Although Se is an essential micronutrient that is vital to biological systems in small amounts, it has a narrow range between essentiality and toxicity in fish (Wilber, 1980). Even at low aqueous concentrations ($\leq$1 μg/L) in freshwater ecosystems, Se can bioaccumulate in aquatic organisms and cause harmful effects at higher trophic levels in organisms such as fish (Lemly, 1985, 1993; Bowie et al., 1996; Hamilton, 2004). Thus, whole-body Se concentrations of lake chub (*Couesius plumbeus*) significantly increased after exposure to uranium mill effluent (Phibbs et al., 2011). In the study by Bubach et al. (2015), Se concentrations in the liver of rainbow trout (*Oncorhynchus mykiss*) were doubled at certain areas, whereas at the same time K, Zn, and Fe did not show significant differences among differently contaminated areas. Increased Se accumulation was also previously reported in several studies in the liver of metal exposed fathead minnows (*Pimephales promelas*) (Rozon-Ramilo et al., 2011; Ouellet et al., 2013). Various authors have reported different bioaccumulation trends for essential metals at comparable exposure levels (Rozon-Ramilo et al., 2011; Liu et al., 2012; Ouellet et al., 2013), and thus some other factors, and not only the exposure level, probably can influence bioavailability and accumulation of essential metals in fish organs, such as fish species (i.e. physiological specificity; Anke, 2004) and metal speciation in the water (i.e. physico-chemical properties of metals; Garofalo et al., 2004). Accordingly, an explanation for differences in bioaccumulation between Se and other essential elements may be found in water chemistry. Water chemistry parameters (such as pH and Ca content) can make metals more or less bioavailable and toxic for animals (Monna et al., 2011). Selenium availability is the highest at basic pH, whereas the availabilities of the majority of other elements are higher in acidic waters (Eisler, 1993; Ezoe et al., 2001). In our study, all rivers had pH that was either close to neutral (6.52–6.88 in the Zletovska River) or slightly basic (7.90–8.12 in the Bregalnica and the Kriva River) (Ramani et al., 2014), which was evidently favourable for Se bioaccumulation.

The third group of the studied elements was composed of eight trace elements that have no known functions in fish metabolism and that are mostly highly toxic, namely Ba, Cd, Cs, Pb, Rb, Sr, Tl and V. Such elements, like Cd and Pb, are considered systemic toxicants that are known to induce multiple organ damage even at very low exposure levels (Tchounwou et al., 2012). Two of these elements were increased in the river water of the agriculturally impacted Bregalnica River, namely Ba and V, and their increase amounted to 2–12 times for Ba and 2–29 times for V, compared to the two other rivers (Table 1; Ramani et al., 2014). The other elements were mainly increased in the river water of the mining impacted Zletovska River (Table 1; Ramani et al., 2014). In the spring period, dissolved Cd and Tl were increased in the Zletovska River compared to one or the other remaining river up to 7–8 times, dissolved Rb and Sr up to 23–27 times and dissolved Cs as much as 50 times (Table 1; Ramani et al., 2014). In the autumn period, increases of several of those elements were even more pronounced, especially of dissolved Cd, Cs and Tl, amounting to as much as 95, 70 and 50 times, respectively, in comparison to the other rivers. Even dissolved Pb, which was rather low in the Zletovska River in the spring, was somewhat increased in the autumn season, probably due to extremely low water level of that river (Ramani et al., 2014). In the Kriva River, which was also impacted by mining waste, increased concentrations of dissolved Cd and Pb were found only in the spring period. At that time, concentration of dissolved Cd in the Kriva River was comparable to the Zletovska River and 8 times higher than in the Bregalnica River, whereas dissolved Pb concentration was 2.5–6 times higher compared to both other rivers (Table 1; Ramani et al., 2014). The only nonessential element that did not reflect the exposure in the water was Ba. The highest hepatic and gill concentrations of that metal were not found as expected in the fish from the Bregalnica River (Fig. 3a). In contrast to our findings, Rašković et al. (2018) found increased Ba bioaccumulation in the liver and gills of chub (*S. cephalus*) after an accident at an abandoned mining site. However, gill Ba concentrations were previously shown to exhibit much higher concentrations in smaller than in bigger fish in the study on European chub (*S. cephalus*) from the Sutla River, and also did not reflect the level of exposure in the water (Dragun et al., 2016). Therefore, despite of the fact that Ba is a nonessential element, the absence of association between water exposure and consequent bioaccumulation which was observed for Ba in this study possibly occurred due to combined effects of low exposure level in Macedonian rivers and certain level of physiological regulation, possibly because of chemical similarity between Ba and Ca, both being alkaline earth metals (Dragun et al., 2016). All the other nonessential elements clearly reflected the exposure from the water, in both seasons and in both organs of Vardar chub (Fig. 3b–h). Monroy et al. (2014) also reported that the levels of non-essential elements, such as Cd and Pb, in various fish species generally followed the peaks of environmental metal contamination more closely than the levels of essential elements. The highest hepatic and gill V concentrations were always found in the Vardar chub from the Bregalnica River (Fig. 3h), whereas the highest hepatic and gill Cs (Fig. 3c), Rb (Fig. 3e), Sr (Fig. 3f), and Tl (Fig. 3g) concentrations were always found in the Vardar chub from the Zletovska River. Association between Cs bioaccumulation and level of water exposure was already reported for gills of European chub (*S. cephalus*) (Dragun et al., 2016). Similar finding was reported for Rb and Cs concentrations in the liver of rainbow trout (*O. mykiss*) as a consequence of fish exposure to water contaminated by a volcanic source, with strong association between hepatic Rb and Cs concentrations and the distance to the volcano (Bubach et al., 2015). Ouellet et al. (2013) reported increased accumulation of Rb in the liver and Tl in the gills of fathead minnows (*P. promelas*) during chronic exposure to a metal mine effluent. Campbell et al. (2005) even reported tendency of Rb and Cs to consistently biomagnify in diverse food webs. Furthermore, hepatic and gill Cd (Fig. 3b) and Pb (Fig. 3d) concentrations in Vardar chub followed the pattern of changeable water contamination. Higher hepatic and gill Cd concentrations were found in Vardar chub from both Zletovska and Kriva River compared to the Bregalnica River in the spring period, whereas in the autumn period the highest Cd concentrations were found only in liver and gills of Vardar chub from the Zletovska River, which was in accordance with the levels of exposure recorded in the river water. Hepatic and gill Pb concentrations were the highest in Vardar chub from the Kriva River in the spring period, and from the Zletovska River in the autumn, again in agreement with the current river water contamination with Pb. Similar bioaccumulation patterns were reported by several authors and for various fish species. For example, Biuki et al. (2011) observed increases of Cd and Pb in the liver of juvenile milkfish (*Chanos chanos*) following the concentration increase of these pollutants; Casiot et al. (2009) reported a twofold increase of Cd, a threefold increase of Tl and a sixfold increase of Pb in the liver of chub (*L. cephalus*) exposed to acid mine drainage of former Pb/Zn mine, whereas Monna et al. (2011) reported three orders of magnitude greater hepatic Cd and Pb concentrations in brown trout (*Salmo trutta fario*) from mining influenced sites compared to those of commercial trout produced by fish farming. Increased Cd was also reported in the liver of *S. cephalus* from the contaminated site of the

Sava River (Podrug et al., 2009), whereas increased Pb concentrations were found in both liver and gills of grass carp (*C. idellus*) from a freshwater ecosystem influenced by a copper mine (Liu et al., 2012) and of European chub (*S. cephalus*) from a Pb contaminated site of the Sutla River (Dragun et al., 2012). Moreover, Ebrahimi and Taherianfard (2010) reported increased hepatic Pb concentrations in two cyprinid fish species (*Cyprinus carpio* and *Capoeta* sp.) from a Pb contaminated section of the Kor River in Iran.

If we take in consideration such observable bioaccumulation of highly toxic elements (Cd, Cs, Pb, Rb, Sr, and Tl) in organs of Vardar chub from the Zletovska River, it is not surprising that fish from the Zletovska River, the same individuals as analyzed in this study, were more intensely affected with macroscopically visible disorders, which reflect an advanced stage of toxicant impact, compared to the fish from the other two rivers (Jordanova et al., 2016). Furthermore, the highest relative volumes of immune cells, pigmented macrophages, in the kidney (Jordanova et al., 2017), as well as higher frequency and intensity of histopathological alterations in the gills (Barišić et al., 2015), were also noticed in the fish from the Zletovska River, which had higher levels of bioaccumulated toxic metals, and that was especially evident in autumn season during the period of increased metal exposure (Ramani et al., 2014).

3.3. Differences in metal bioaccumulation between two target organs

We have further compared, separately for each sampling season, the bioaccumulated concentrations of 19 elements in liver and gills of Vardar chub, as organs of specialized and different functions in the aquatic organisms. Gills are principal respiratory organ of fishes, the primary site of osmoregulation and nitrogen excretion, and they are involved in acid-base balance and hormone metabolism (Graham, 2006). Gills directly accumulate toxicants dissolved in the water thanks to their large respiratory surface and high water-pumping rate (Chovanec et al., 2003). Therefore, gills respond quickly to changes in the level of metal exposure in the water (Kraemer et al., 2005) and will likely reflect acute, short-term metal exposure (Roméo et al., 1999). In contrast, liver is not an organ initially exposed to contaminants, but it is an organ which may accumulate, metabolize and detoxify various contaminants from different sources or their metabolites (Hinton et al., 2001; Hedayati, 2016), and it is considered as one of the best indicator organs for evaluation of chronic exposure to metals (Miller et al., 1992). The majority of studied elements have differed statistically significantly between two target organs (Table 3), some with higher concentrations in the liver and some in the gills, probably in some way connected to specific functions of each of these two organs. For example, in both seasons we have found significantly higher concentrations of K, Co, Cu, Mn, Mo, Se, Cd, Tl and V in the liver, and significantly higher concentrations of Na, Ca and Sr in the gills. The rest of the elements were in one season higher in the liver, and in the other season in the gills, or even comparable in both organs. Although there were significant differences in concentrations of all studied elements between organs of Vardar chub either in just one or in both seasons (Table 3), only few elements showed really high tendency for bioaccumulation in the liver and those were Cu with 43–50 times higher cytosolic concentrations in the liver, then Mo and V, with 7–11 times higher cytosolic concentrations in the liver, and finally Co and Mn, with 3.5–5 times higher cytosolic concentrations in the liver than in the gills, depending on the metal and the season. Much higher total concentrations in chub (*S. cephalus*) liver than in gills, approximately 10–40 times, were already reported for Cu (Sunjog et al., 2016; Rašković et al., 2018), and approximately 2–20 times for Mo (Sunjog et al., 2016). Similar differences, with almost ten times higher Cu concentrations in the liver than in the gills, were also observed in the grass carp (*C. idellus*) (Liu et al., 2012). In contrast, there were two elements with much higher bioaccumulation in the gills than in the liver of Vardar chub, and those were Ca and Sr, with 11–17 times higher cytosolic concentrations in the gills, depending on the metal and the season (Table 3). Higher Ca concentrations in the gills compared to the liver are consistent with involvement of gills, as ion-transporting tissue, in processes of Ca homeostasis (Takei and Loretz, 2006), whereas Sr, due to similar chemical properties to Ca, often follows Ca bioaccumulation trends, as previously described in the case of European chub gills (Dragun et al., 2016). Total Sr concentrations in the gills of chub (*S. cephalus*) were previously reported to be around 100–450 times higher than in the liver (Sunjog et al., 2016; Rašković et al., 2018). Higher bioaccumulation of Pb in the gills than in the liver of freshwater fish *Colisa fasciatus* (Kumar and Mathur, 1991) and of grass carps (*C. idellus*) (Liu et al., 2012) was previously reported, whereas in our study the differences in Pb concentrations between two organs were rather small (Table 3). All the other differences, although statistically significant, were much less pronounced. Such different accumulation levels of certain elements in different fish organs may be primarily caused by different metabolic activities of those organs (Biuki et al., 2011).

Furthermore, we have also compared the bioaccumulation rates observed in two organs for seven highly toxic metals that reflected the exposure from the river water (Fig. 3b–h), since it is known that metal accumulation in tissues of fish is dependent upon the rate of uptake, storage, and elimination (Heath, 1995; Biuki et al., 2011). Although it is not possible to create completely reliable models of metal bioaccumulation kinetics if the study is not performed under controlled laboratory conditions, we have analyzed the association between metal exposure levels and the consequent levels of bioaccumulated metals in the organs of Vardar chub, to draw the preliminary conclusions about bioaccumulation rates of seven above mentioned metals. We have used mean bioaccumulated metal concentrations calculated for each studied river at each time of sampling, and based on altogether six data points for each organ, we have fitted the curves that best described the metal bioaccumulation rates under the studied exposure conditions (Fig. 4). It could be seen that only bioaccumulated Cs levels in both liver and gills have increased proportionally to the increase of water contamination, with a constant bioaccumulation rate (Fig. 4b). Hansman et al. (2018) reported that dissolved Cs uptake was linear and did not reach a steady state in the olive flounder (*Paralichthys olivaceus*) over the course of the 8-day exposure period to radioactive Cs. For the majority of the other elements, namely Cd (Fig. 4a), Pb (Fig. 4c), Rb (Fig. 4d) and Sr (Fig. 4e), bioaccumulated levels in both liver and gills have increased linearly up to a certain threshold point of water contamination, and thereafter the rate of bioaccumulation declined, which was especially evident for Sr and Cd. Similarly to our study, Cd and Pb accumulation in liver of juvenile milkfish (*Chanos chanos*) showed lower accumulation factors for both metals at higher exposure concentrations (Biuki et al., 2011). In laboratory experiments (Kumar and Mathur, 1991; Biuki et al., 2011), fish are usually exposed to much higher metal concentrations than usually found in the realistic environmental situations, and thus bioaccumulation plateau, which they described to occur at exposure level of several milligrams per litre, in our study was observed already at the exposure level of few micrograms per litre. This can probably be explained by the fact that fish in mining contaminated freshwater environments are chronically exposed to moderately increased metal concentrations. Such chronic exposure could lead to a certain degree of fish acclimation, for which various explanations were so far suggested in the literature. Hollis et al. (1999), for example, have suggested that chronic sublethal waterborne exposure can induce the gills to function as a barrier in Cd-acclimated rainbow trout (*O. mykiss*), minimizing internal Cd uptake as a result of decrease in affinity and an increase in capacity of the gill surface for Cd. The cause of such gill functioning, according to Hollis et al. (1999), would be a large burden of Cd already contained within the gills of chronically exposed fish. On the other hand, it is possible that lower metal bioaccumulation in fish chronically exposed to metals occurs due to retardation of metabolic and feeding rates, which in our study could be corroborated by the observed inferior Vardar chub sizes and conditions at the mining impacted Zletovska River compared to the other two rivers (Table 2). Similar hypothesis was proposed by Szebedinszky et al. (2001), who observed that high levels of dietary exposure to Cd resulted in mortalities and lower fish growth rates of rainbow trout (*O. mykiss*). They proposed that reduced growth in fish exposed to elevated levels of dietary Cd may occur as a consequence of physiological stress that results in decreased nutrient absorption and/or increased consumption of energy which would normally be used for growth (Szebedinszky et al., 2001). Thus, restriction of excessive metal accumulation in chronically metal-exposed fish could be expected in the events of periodical short-term exposures to extremely high metal concentrations in the river water, such as observed for Cd in our study in the autumn period of very low water discharge (Ramani et al., 2014). This is consistent with previous reports of increased fish resistance to acute Cd incidents after chronic sublethal exposure to waterborne and dietborne Cd (McDonald and Wood, 1993; Szebedinszky et al., 2001).

In addition, Cd accumulation in the liver of milkfish was higher than Pb accumulation under similar exposure conditions (Biuki et al., 2011), same as observed in our study for Vardar chub liver, where bioaccumulated Cd concentrations were approximately twice higher compared to Pb at the same exposure level (Fig. 4a and c). The differences between Pb and Cd accumulation levels in the liver possibly originate from different mechanisms involved in their sequestration in fish (Biuki et al., 2011). Absorbed Cd accumulates highly in the liver due to its association to metallothioneins, whereas absorbed Pb is quickly distributed to other tissues rather than accumulated mainly in the liver (Roesijadi, 1992; Odžak and Zvonarić, 1995; Kljaković Gašpić et al., 2002).

Thallium bioaccumulation presented an interesting case, because it differed between two organs (Fig. 4f). For Tl in the gills, the trend of decreasing bioaccumulation rate following the increase of water contamination was recorded, similarly to Cd, Pb, Rb and Sr. In the liver the situation was opposite, i.e. bioaccumulation rate increased with increasing level of exposure in the river water, which can be especially perilous considering the high toxicity of Tl (Bertram and Bertram, 2004). Finally, bioaccumulation of V in both organs was not evident at all up to a certain threshold point of water contamination (Fig. 4g), indicating a partial regulation of V uptake and elimination. Only after that threshold was overpassed, increases of bioaccumulated V were observed in both organs.

4. Conclusions

The study of bioaccumulation of 19 elements in the liver and gills of Vardar chub from three differently contaminated Macedonian rivers revealed the following: (1) in general, essential elements (Na, K, Ca, Mg, Co, Cu, Fe, Mn, Mo, and Zn) were regulated and kept in narrow ranges in both organs of selected bioindicator species, even in the conditions of extremely high water exposure, as was especially evident for Mn and Zn; (2) bioaccumulated concentrations of nonessential and highly toxic elements (Cd, Cs, Pb, Rb, Sr, Tl, and V) in general reflected the level of exposure in the water. The exception to the rule was essential element Se, which was markedly increased in the organs of fish from one river (namely, the Kriva River), probably as a consequence of increased water contamination with that element. The other exception was nonessential element Ba, which hepatic and gill concentrations did not follow the pattern of water contamination, possibly due to previously described pronounced dependence of Ba bioaccumulation on fish physiology. The comparison of bioaccumulation in two selected target organs showed much higher tendency of Cu, Mo, V, Co, and Mn to accumulate in the liver than in the gills, whereas Ca and Sr exhibited the opposite tendency, with much higher concentrations in the gills. It is interesting to notice that, although bioaccumulation of nonessential elements increased following the increase of exposure level, that increase was not proportional to increase of water contamination, indicating a certain degree of fish acclimation to adverse living conditions, as well as restriction of metal accumulation in fish organism during acute contamination incidents. Still, significantly higher concentrations of toxic metals in liver and gills of Vardar chub from mining impacted Zletovska River, in combination with previously reported histopathological alterations in the same fish, present an indication of threat for aquatic organisms in that river and eventually for health of humans that consume them. Thus, strict supervision of the Zletovska River and other mining impacted freshwater ecosystems should be considered, along with adequate remediation measures.

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References

Alderton, D.H.M., Serafimovski, T., Mullen, B., Fairall, K., James, S., 2005. The chemistry of waters associated with metal mining in Macedonia. Mine Water Environ. 24, 139–149.
Andreevska, D., Menkovska, M., Andov, D., 2013. Overview of the current condition, in production consumption and the research potential of the rice crop in the Republic of Macedonia. Maced. J. Anim. Sci. 3, 219–228.
Anke, M.K., 2004. Essential and toxic effects of macro, trace, and ultratrace elements in the nutrition of animals. In: Merian, E., Anke, M., Ihnat, M., Stoeppler, M. (Eds.), Elements and Their Compounds in the Environment. Volume 1: General Aspects. Wiley-VCH Verlag, Weinheim, pp. 305–341.
Biuki, N.A., Savari, A., Mortazavi, M., Zolgharnein, H., Salamat, N., 2011. Accumulation and elimination of cadmium and lead in juvenile milkfish during sublethal exposure. Toxicol. Environ. Chem. 93, 2022–2033.
Barišić, J., Dragun, Z., Ramani, S., Filipović Marijić, V., Krasnići, N., Čož-Rakovac, R., Kostov, V., Rebok, K., Jordanova, M., 2015. Evaluation of histopathological alterations in the gills of Vardar chub (*Squalius vardarensis* Karaman) as an indicator of river pollution. Ecotoxicol. Environ. Saf. 118, 158–166.
Bertram, H.P., Bertram, C., 2004. Thallium. In: Merian, E., Anke, M., Ihnat, M., Stoeppler, M. (Eds.), Elements and Their Compounds in the Environment. Volume 1: Metals and Their Compounds. Wiley-VCH Verlag, Weinheim, pp. 1099–1112.
Bonneris, E., Giguère, A., Perceval, O., Buronfosse, T., Masson, S., Hare, L., Campbell, P.G.C., 2005. Sub-cellular partitioning of metals (Cd, Cu, Zn) in the gills of a freshwater bivalve, *Pyganodon grandis*: role of calcium concretions in metal sequestration. Aquat. Toxicol. 71, 319–334.
Bowie, G.L., Sanders, J.G., Riedel, G.F., Gilmour, C.C., Breitburg, D.L., Cutter, G.A., Porcella, D.B., 1996. Assessing selenium cycling and accumulation in aquatic ecosystems. Water Air Soil Pollut. 90, 93–104.
Brumbaugh, W.G., Schmitt, C.J., May, T.W., 2005. Concentrations of cadmium, lead, and zinc in fish from mining-influenced waters of Northeastern Oklahoma: sampling of blood, carcass, and liver for aquatic biomonitoring. Arch. Environ. Contam. Toxicol. 49, 76–88.
Bubach, D.F., Macchi, P.J., Pérez Catán, S., 2015. Influence of volcanic activity and antrophic impact in the trace element contents of fishes from the North

Patagonia in a global context. Environ. Monit. Assess. 187, 710.
Campbell, L.M., Fisk, A.T., Wang, X., Köck, G., Muir, D.C.G., 2005. Evidence for biomagnification of rubidium in freshwater and marine food webs. Can. J. Fish. Aquat. Sci. 62, 1161–1167.
Casiot, C., Egal, M., Elbaz-Poulichet, F., Bruneel, O., Bancon-Montigny, C., Cordier, M.-A., Gomez, E., Aliaume, C., 2009. Hydrological and geochemical control of metals and arsenic in a Mediterranean river contaminated by acid mine drainage (the Amous River, France); preliminary assessment of impacts on fish (*Leuciscus cephalus*). Appl. Geochem. 24, 787–799.
Chovanec, A., Hofer, R., Schiemer, F., 2003. Fish as bioindicators. In: Markert, B.A., Breure, A.M., Zechmeister, H.G. (Eds.), Bioindicators and Biomonitors: Principles, Concepts and Applications. Elsevier, pp. 639–676.
Di Giulio, R.T., Hinton, D.E., 2008. The Toxicology of Fishes. Taylor & Francis Group, Boca Raton, FL, p. 1101.
Dolenec, T., Serafimovski, T., Dobnikar, M., Tasev, G., Dolenec, M., 2005. Mineralogical and heavy metal signature of acid mine drainage impacted paddy soil from the western part of the Kočani field (Macedonia). RMZ Mater. Geoenviron. 52, 397–402.
Dragun, Z., Fiket, Ž., Vuković, M., Raspor, B., 2013a. Multielement analysis in the fish hepatic cytosol as a screening tool in the monitoring of natural waters. Environ. Monit. Assess. 185, 2603–2614.
Dragun, Z., Filipović Marijić, V., Kapetanović, D., Valić, D., Vardić Smrzlić, I., Krasnići, N., Strižak, Ž., Kurtović, B., Teskeredžić, E., Raspor, B., 2013b. Assessment of general condition of fish inhabiting a moderately contaminated aquatic environment. Environ. Sci. Pollut. Res. 20, 4954–4968.
Dragun, Z., Filipović Marijić, V., Krasnići, N., Ramani, S., Valić, D., Rebok, K., Kostov, V., Jordanova, M., Erk, M., 2017. Malondidaldehyde concentrations in the intestine and gills of Vardar chub (*Squalius vardarensis* Karaman) as indicator of lipid peroxidation. Environ. Sci. Pollut. Res. 24, 16917–16926.
Dragun, Z., Krasnići, N., Strižak, Ž., Raspor, B., 2012. Lead concentration increase in the hepatic and gill soluble fractions of European chub (*Squalius cephalus*) – an indicator of increased Pb exposure from the river water. Environ. Sci. Pollut. Res. 19, 2088–2095.
Dragun, Z., Podrug, M., Raspor, B., 2009. Combined use of bioindicators and passive samplers for the assessment of river water contamination with metals. Arch. Environ. Contam. Toxicol. 57, 211–220.
Dragun, Z., Raspor, B., Podrug, M., 2007. The influence of the season and the biotic factors on the cytosolic metal concentrations in the gills of the European chub (*Leuciscus cephalus* L.). Chemosphere 69, 911–919.
Dragun, Z., Tepić, N., Krasnići, N., Teskeredžić, E., 2016. Accumulation of metals relevant for agricultural contamination in gills of European chub (*Squalius cephalus*). Environ. Sci. Pollut. Res. 23, 16802–16815.
Ebrahimi, M., Taherianfard, M., 2010. Concentration of four heavy metals (cadmium, lead, mercury, and arsenic) in organs of two cyprinid fish (*Cyprinus carpio* and *Capoeta* sp.) from the Kor River (Iran). Environ. Monit. Assess. 168, 575–585.
Eisler, R., 1993. Zinc hazards to fish, wildlife, and invertebrates: a synoptic review. In: Contaminant Hazard Reviews (1-35), Biological Report 10. U.S. Fish and Wildlife Research Center. http://www.pwrc.usgs.gov/eisler/CHR_26_Zinc.pdf.
Enders, C.K., Tofighi, D., 2007. Centering predictor variables in cross-sectional multilevel models: a new look at an old issue. Psychol. Methods 12, 121–138.
Ezoe, Y., Lin, C.H., Mochioka, N., Yoshimura, K., 2001. The distribution of trace elements in tissues of fish living in acid environments of Yangmingshan National Park, Taiwan. Anal. Sci. 17, 813–816.
Fiket, Ž., Roje, V., Mikac, N., Kniewald, G., 2007. Determination of arsenic and other trace elements in bottled waters by high resolution inductively coupled plasma mass spectrometry. Croat. Chem. Acta 80, 91–100.
Garofalo, E., Ceradini, S., Winter, M., 2004. The use of diffusive gradients in thin-film (DGT) passive samplers for the measurement of bioavailable metals in river water. Ann. Chim. Rome 94, 515–520.
Giguère, A., Campbell, P.G.C., Hare, L., Couture, P., 2006. Sub-cellular partitioning of cadmium, copper, nickel and zinc in indigenous yellow perch (*Perca flavescens*) sampled along a polymetallic gradient. Aquat. Toxicol. 77, 178–189.
Graham, J.B., 2006. Aquatic and aerial respiration. In: Evans, D.H., Claiborne, J.B. (Eds.), The Physiology of Fishes, third ed. Taylor & Francis, Boca Raton, pp. 85–118.
Hamilton, S.J., 2004. Review of selenium toxicity in the aquatic food chain. Sci. Total Environ. 326, 1–31.
Hansman, R.L., Metian, M., Pouil, S., Oberhänsli, F., Teyssié, J.-L., Swarzenski, P.W., 2018. A double-tracer radioisotope approach to assess simultaneous bioaccumulation of caesium in the olive flounder *Paralichthys olivaceus*. J. Environ. Radioact. 190–191, 141–148.
Heath, A.G., 1995. Water Pollution and Fish Physiology. CRC Press, Boca Raton.
Hedayati, A., 2016. Liver as a target organ for eco-toxicological studies. J. Coast. Zone Manag. 19, e118.
Hinton, D.E., Segner, H., Braunbeck, T., 2001. Toxic responses of the liver. In: Schlenk, D., Bensen, W.H. (Eds.), Toxicity in Marine and Freshwater Teleosts, vol. 1. Taylor & Francis, London, pp. 224–268.
Hofmann, D.A., Gavin, M.B., 1998. Centering decisions in hierarchical linear models: implications for research in organizations. J. Manag. 24, 623–641.
Hollis, L., McGeer, J.C., McDonald, D.G., Wood, C.M., 1999. Cadmium accumulation, gill Cd binding, acclimation, and physiological effects during long-term sublethal Cd exposure in rainbow trout. Aquat. Toxicol. 46, 101–119.
Jordanova, M., Rebok, K., Dragun, Z., Ramani, S., Ivanova, L., Kostov, V., Valić, D., Krasnići, N., Filipović Marijić, V., Kapetanović, D., 2016. Histopathology investigation on the Vardar chub (*Squalius vardarensis*) populations captured from the rivers impacted by mining activities. Ecotoxicol. Environ. Saf. 129, 35–42.
Jordanova, M., Rebok, K., Dragun, Z., Ramani, S., Ivanova, L., Kostov, V., Valić, D., Krasnići, N., Filipović Marijić, V., Kapetanović, D., 2017. Effects of heavy metal pollution on pigmented macrophages in kidney of Vardar chub (*Squalius vardarensis* Karaman). Microsc. Res. Tech. 1–6, 2017.
Khadiga, G.A., Sherifa, S.H., Hama, M.I., Ramadan, A.S., 2002. Impaired function in Nile tilapia, *Oreochromis niloticus* from polluted waters. Acta Hydrochim. Hydrobiol. 29, 278–288.
Kljaković Gašpić, Z., Zvonarić, T., Vrgoč, N., Odžak, N., Barić, A., 2002. Cadmium and lead in selected tissues of two commercially important fish species from Adriatic Sea. Water Res. 36, 5023–5028.
Kraemer, L.D., Campbell, P.G.C., Hare, L., 2005. Dynamics of Cd, Cu and Zn accumulation in organs and sub-cellular fractions in field transplanted juvenile yellow perch (*Perca flavescens*). Environ. Pollut. 138, 324–337.
Kumar, A., Mathur, R.P., 1991. Bioaccumulation kinetics and organ distribution of lead in a freshwater teleost, *Colisa fasciatus*. Environ. Technol. 12, 731–735.
Lemly, A.D., 1985. Toxicology of selenium in a freshwater reservoir: implications for environmental hazard evaluation and safety. Ecotoxicol. Environ. Saf. 10, 314–338.
Lemly, A.D., 1993. Teratogenic effects of selenium in natural populations of freshwater fish. Ecotoxicol. Environ. Saf. 26, 181–204.
Liu, F., Ni, H.-G., Chen, F., Luo, Z.-X., Shen, H., Liu, L., Wu, P., 2012. Metal accumulation in the tissues of grass carps (*Ctenopharyngodon idellus*) from fresh water around a copper mine in Southeast China. Environ. Monit. Assess. 184, 4289–4299.
Lopes, P.A., Pinheiro, T., Santos, M.C., da Luz Mathias, M., Collares-Pereira, M.J., Viegas-Crespo, A.M., 2000. Response of antioxidant enzymes in freshwater fish populations (*Leuciscus alburnoides* complex) to inorganic pollutants exposure. Sci. Total Environ. 280, 153–163.
Mayes, W.M., Potter, H.A.B., Jarvis, A.P., 2010. Inventory of aquatic contaminant flux arising from historical metal mining in England and Wales. Sci. Total Environ. 408, 3576–3583.
McDonald, D.G., Wood, C.M., 1993. Branchial mechanisms of acclimation to metals in freshwater fish. In: Rankin, J.C., Jensen, F.B. (Eds.), Fish Ecophysiology. Chapman & Hall, London, UK, pp. 297–321.
McGeer, J.C., Niyogi, S., Smith, D.S., 2012. Cadmium. In: Wood, M., Farrell, A.P., Brauner, C.J. (Eds.), Homeostasis and Toxicology of Non-essential Metals, Fish Physiology Series, 31B. Elsevier, Amsterdam, pp. 125–184.
Miller, P.A., Munkittrick, K.R., Dixon, D.G., 1992. Relationship between concentrations of copper and zinc in water, sediment, benthic invertebrates, and tissues of white sucker (*Catastomus commersoni*) at metal contamination sites. Can. J. Fish. Aquat. Sci. 49, 978–984.
Moiseenko, T.I., Kudryavtseva, L.P., 2001. Trace metal accumulation and fish pathologies in areas affected by mining and metallurgical enterprises in the Kola Region, Russia. Environ. Pollut. 114, 285–297.
Monna, F., Camizuli, E., Revelli, P., Biville, C., Thomas, C., Losno, R., Scheifler, R., Bruguier, O., Baron, S., Chateau, C., Ploquin, A., Alibert, P., 2011. Wild brown trout affected by historical mining in the Cévennes National Park, France. Environ. Sci. Technol. 45, 6823–6830.
Monroy, M., Maceda-Veiga, A., de Sostoa, A., 2014. Metal concentration in water, sediment and four fish species from Lake Titicaca reveals a large-scale environmental concern. Sci. Total Environ. 487, 233–244.
Munkittrick, K.R., Dixon, D.G., 1988. Growth, fecundity, and energy stores of white sucker (*Catosomus commersoni*) from lakes containing elevated levels of copper and zinc. Can. J. Fish. Sci. 45, 1355–1365.
Odžak, N., Zvonarić, T., 1995. Cadmium and lead uptake from food by the fish, *Dicentrarchus labrax*. Water Sci. Technol. 32, 49–55.
Olsvik, P.A., Gundersen, P., Andersen, R.A., Zachariassen, K.E., 2000. Metal accumulation and metallothionein in two populations of brown trout, *Salmo trutta*, exposed to different natural water environments during a run-off episode. Aquat. Toxicol. 50, 301–316.
Ouellet, J.D., Dubé, M.G., Niyogi, S., 2013. The influence of food quantity on metal bioaccumulation and reproduction in fathead minnows (*Pimephales promelas*) during chronic exposures to a metal mine effluent. Ecotoxicol. Environ. Saf. 91, 188–197.
Phibbs, J., Wiramanaden, C.I.E., Hauck, D., Pickering, I.J., Liber, K., Janz, D.M., 2011. Selenium uptake and speciation in wild and caged fish downstream of a metal mining and milling discharge. Ecotoxicol. Environ. Saf. 74, 1139–1150.
Podrug, M., Raspor, B., Erk, M., Dragun, Z., 2009. Protein and metal concentrations in two fractions of hepatic cytosol of the European chub (*Squalius cephalus* L.). Chemosphere 75, 843–849.
Ramani, S., Dragun, Z., Kapetanović, D., Kostov, V., Jordanova, M., Erk, M., Hajrulai-Musliu, Z., 2014. Surface water characterization of three rivers in the lead/zinc mining region of northeastern Macedonia. Arch. Environ. Contam. Toxicol. 66, 514–528.
Rašković, B., Poleksić, V., Skorić, S., Jovičić, K., Spasić, S., Hegediš, A., Vasić, N., Lenhardt, M., 2018. Effects of mine tailing and mixed contamination on metals, trace elements accumulation and histopathology of the chub (*Squalius cephalus*) tissues: evidence from three differently contaminated sites in Serbia. Ecotoxicol. Environ. Saf. 153, 238–247.
Rätz, H.-J., Lloret, J., 2003. Variation in fish condition between Atlantic cod (*Gadus morhua*) stocks, the effect on their productivity and management implications. Fish. Res. 60, 369–380.
Rebok, K., 2013. A Toxicopathology Survey of the River Bregalnica Using the Barbel (*Barbus Peloponnesius*, Valenciennes 1844) as Bioindicator. Doctoral Thesis.

University Ss. Cyril and Methodius, Skopje, Macedonia.
Riba, I., Blasco, J., Jimenez-Tenorio, N., DelValls, T.A., 2005. Heavy metal bioavailability and effects: I. Bioaccumulation caused by mining activities in the Gulf of Cadiz (SW, Spain). Chemosphere 58, 659–669.
Roesijadi, G., 1992. Metallothioneins in metal regulation and toxicity in aquatic animals. Aquat. Toxicol. 22, 81–114.
Roméo, M., Siaub, Y., Sidoumou, Z., Gnassia-Barelli, M., 1999. Heavy metal distribution in different fish species from the Mauritania coast. Sci. Total Environ. 232, 169–175.
Rozon-Ramilo, L.D., Dubé, M.G., Rickwood, C.J., Niyogi, S., 2011. Examining the effects of metal mining mixtures on fathead minnow (*Pimephales promelas*) using field-based multi-trophic artificial streams. Ecotoxicol. Environ. Saf. 74, 1536–1547.
Şaşi, H., 2004. The reproduction biology of chub (*Leuciscus cephalus* L., 1758.) in topçam dam lake (Aydin, Turkey). Turk. J. Vet. Anim. Sci. 28, 693–699.
Spasovski, O., Dambov, R., 2009. Heavy metals in the water of the river Kalnistanska and the vicinity. In: Balkanmine 2009 - 3rd Balkan Mining Congress. Izmir, Turkey, pp. 667–670.
Standard Operational Procedure, 1999. Preparation of S50-fraction from fish tissue (unapproved rev. 01). In: 1st Workshop in the Frame of BEQUALM Programme. NIVA, Oslo (September 13-14 1999).
Stipaničev, D., Dragun, Z., Repec, S., Rebok, K., Jordanova, M., 2017. Broad spectrum screening of 463 organic contaminants in rivers in Macedonia. Ecotoxicol. Environ. Saf. 135, 48–59.
Sunjog, K., Kolarević, S., Kračun-Kolarević, M., Višnjić-Jeftić, Ž., Skorić, S., Gačić, Z., Lenhardt, M., Vasić, N., Vuković-Gačić, B., 2016. Assessment of status of three water bodies in Serbia based on tissue metal and metalloid concentration (ICP-OES) and genotoxicity (comet assay). Environ. Pollut. 213, 600–607.
Szebedinszky, C., McGeer, J.C., McDonald, D.G., Wood, C.M., 2001. Effects of chronic Cd exposure via the diet or water on internal organ-specific distribution and subsequent gill Cd uptake kinetics in juvenile rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem. 20, 597–607.
Takei, Y., Loretz, C.A., 2006. Endocrinology. In: Evans, D.H., Claiborne, J.B. (Eds.), The Physiology of Fishes, third ed. Taylor & Francis, Boca Raton, pp. 271–318.
Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K., Sutton, D.J., 2012. Heavy metal toxicity and the environment. In: Luch, A. (Ed.), Molecular, Clinical and Environmental Toxicology. Springer, Basel, pp. 133–164.
Wallace, W.G., Luoma, S.N., 2003. Subcellular compartmentalization of Cd and Zn in two bivalves. II. Significance of trophically available metal (TAM). Mar. Ecol. Prog. Ser. 257, 125–137.
WHO, 1996. Trace Elements in Human Nutrition and Health. World Health Organization, Geneva.
Wilber, C.G., 1980. Toxicology of selenium: a review. Clin. Toxicol. 17, 171–230.
Williams, N.D., Holdway, D.A., 2000. The Effects of pulse-exposed cadmium and zinc on embryo hatchability, larval development, and survival of Australian crimson spotted rainbow fish (*Melanotaenia fluviatilis*). Environ. Toxicol. 15, 165–173.
Zhuang, P., Li, Z., McBride, M.B., Zou, B., Wang, G., 2013. Health risk assessment for consumption of fish originating from ponds near Dabaoshan mine, South China. Environ. Sci. Pollut. Res. 20, 5844–5854.