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Size-exclusion HPLC analysis of trace element distributions in hepatic and gill cytosol of Vardar chub (*Squalius vardarensis* Karaman) from mining impacted rivers in north-eastern Macedonia

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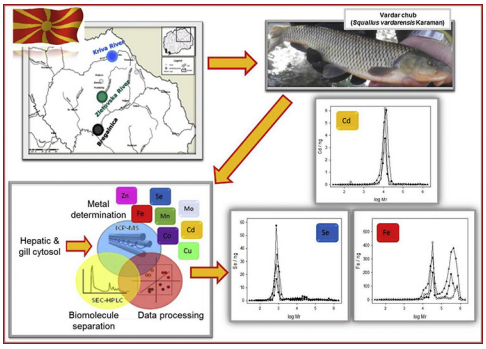
HIGHLIGHTS

- Metallomics approach in monitoring effects of metal pollution on Vardar chub
- Use of SEC-HPLC/HR ICP-MS for determination of metal distributions within cytosol
- Distribution profiles of Cd, Co, Cu, Fe, Mn, Mo, Se and Zn in chub liver and gills
- Changes in the distribution profiles due to increased metal exposure level in water
- Comparison of metal profiles in Vardar and European chub, two related fish species

GRAPHICAL ABSTRACT



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ABSTRACT

Many bioindicators have not yet been well characterized regarding their tendency to bind trace elements by different cytosolic biomolecules in response to trace element exposure. Accordingly, our principal aim was to define the cytosolic distributions of Cd, Co, Cu, Fe, Mn, Mo, Se, and Zn among the biomolecules of different molecular masses in liver and gills of Vardar chub (*Squalius vardarensis* Karaman), a representative fish species of Macedonian rivers, and to determine distribution changes which occur as a consequence of increased exposure to specific trace elements. Additionally, we aimed to confirm the presence of heat-stable biomolecules in chub hepatic and gill cytosols. Distribution profiles were obtained by separation of cytosols and heat-treated cytosols using size-exclusion high performance-liquid chromatography, and by offline determination of trace element concentrations using high resolution inductively coupled plasma-mass spectrometry. Distribution profiles of trace elements were mainly characterized by several peaks encompassing different ranges of molecular masses, as a sign of incorporation of trace elements in various biomolecules within hepatic and gill cytosols. Especially interesting finding was probable binding of Fe to ferritin, which was especially pronounced in the liver, as a sign of important liver function in Fe storage. Furthermore, association with heat-stable proteins, metallothioneins (MT), was indicated for Cd, Cu, and Zn in the hepatic cytosol, as well as for Cd in the gill cytosol, whereas a sign of Zn-MT association was not observed in the gills. The presence of Mo- and Se-binding heat-stable compounds of very low molecular masses (<10 kDa) in the cytosol was determined for both liver and the gills. Trace elements under

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all studied conditions were found associated to the same biomolecules, and only their proportions associated to specific cytosolic compounds have changed as a consequence of their increased bioaccumulation in the liver and gills of Vardar chub.

1. Introduction

Trace element contamination which originates from anthropogenic sources, such as the mining activities (Ramani et al., 2014), presents one of the major concerns for the preservation of high ecological status of aquatic ecosystems. Some trace elements are essential in the development and functions of living organisms (e.g. Zn, Cu, Fe and Se), and they play an important role as cofactors of a number of metalloproteins and enzymes (García-Sevillano et al., 2012). However, in high concentrations they can also compete with the other elements for protein binding sites, and thus may cause toxic effects. A number of the other trace elements (e.g. Ag, Cd, Hg, Pb) are considered non-essential, and are associated with harmful effects in the organisms even in very low concentrations. Therefore, a long term exposure to high levels of trace elements in the water, which can result in the increased bioaccumulation of trace elements in organs of aquatic organisms, can consequently cause various toxic effects, starting with reactions in the cytosol, such as nonspecific binding of trace elements to physiologically important molecules and their consequent inactivation (Mason and Jenkins, 1995).

In the pollution assessment, it is important to analyze not only the concentrations of accumulated trace elements in the organs of aquatic organisms, but also their fate within the cells by studying the biomolecules which bind trace elements, as potential biomarkers of trace element exposure and their possible toxicity. Particularly useful organisms for the assessment of the effect of waterborne and sediment-deposited trace elements are fish, due to their high sensitivity and readily measured responses to trace elements (Goenaga Infante et al., 2003). Analysis of different biomolecules that bind trace elements in fish, known as metalloproteins, can provide insight in the impact of contaminants on fish cellular metabolism and global homeostasis (García-Sevillano et al., 2014). So far only a few fish metalloproteins have been discovered and applied as successful biomarkers of trace element exposure, such as metallothioneins (MTs), but their exact functional roles in fish physiology are not yet well understood (Hu et al., 2013; Lavradas et al., 2016). Therefore, it is important to study metalloproteins in fish in more detail, and the development of metallomic techniques in the recent years offers the possibility to perform such comprehensive studies (Hauser-Davis et al., 2012).

We have applied metallomic techniques in the study of biomolecules that bind trace elements in Vardar chub (*Squalius vardarensis* Karaman) from three mining and agriculturally impacted Macedonian rivers. In Macedonia, mining is still one of the most important industries, with Pb/Zn ores in the north-eastern part of the country being the most significant mineral deposits for exploitation (Midžić and Silajdžić, 2005; Barišić et al., 2015). As a result, many natural freshwater ecosystems, especially in the north-eastern Macedonia, are contaminated with trace elements. Selected fish species, Vardar chub (*S. vardarensis*), is a representative fish in rivers of Vardar basin, as well as a member of genus *Squalius*, wide spread in European rivers, thus providing the possibility for comparison among distant geographical regions (Barišić et al., 2015). The exposure of fish to trace elements can result in their accumulation in different organs, and in this study we have chosen two of them: liver as a metabolic and detoxification centre of an organism (Krasnići et al., 2013; Van Campenhout et al., 2004; Dragun et al., 2012, 2015), as well as a major producer of trace element-binding proteins (Roesijadi and Robinson, 1994); and gills, as a good indicator of current environmental conditions, due to their direct and permanent contact with contaminants in the water (Bernet et al., 1999; Amiri et al., 2011), and their fast response and high sensitivity even to low concentrations of contaminants (Monteiro et al., 2008). To our knowledge, so far there is no information available concerning the accumulation of trace elements and their cytosolic distribution in organs of Vardar chub.

The relationship between environmental exposure, bioaccumulation and distribution among cytosolic biomolecules of seven essential elements (Co, Cu, Fe, Mo, Mn, Se, Zn) and one non-essential element (Cd) in Vardar chub liver and gills was studied applying the separation of cytosolic biomolecules by size-exclusion high performance liquid chromatography (SEC-HPLC) combined with offline determination of trace elements by high resolution inductively coupled plasma mass spectrometry (HR ICP-MS). Our main aim was to define, for the first time, the distribution profiles of studied elements among cytosolic biomolecules of different molecular masses, and to determine changes which occur as a consequence of increased exposure to specific trace elements. Having in mind that some heat-stable biomolecules, such as MT, have important role in trace element detoxification, our additional objective was to analyze cytosolic distribution of trace elements after cytosol heat-treatment, according to the procedure applied for MT analysis (Erk et al., 2002). Similar study was previously performed on mussels by Lavradas et al. (2016), but, to the best of our knowledge, this is the first attempt to determine distribution of selected elements (Cd, Cu, Mo, Se, Zn) among various cytosolic heat-stable biomolecules in organs of fish.

2. Materials and methods

2.1. Fish sampling and organ dissection

Selected bioindicator species, Vardar chub (*S. vardarensis*), was collected in spring (May/June) of 2012 from three rivers in north-eastern Macedonia: the moderately agriculturally contaminated Bregalnica River and two rivers, Zletovska and Kriva, impacted by active Pb/Zn mines Zletovo and Toranica, respectively. In total, 90 fish was sampled, 30 from each river. The map and detailed description of the sampling area were already published (Ramani et al., 2014). The fish were caught by electro fishing, using electrofisher Samus 725G, according to relevant standard (CEN EN 14011:2003). Captured fish were kept alive in a tank with aerated river water until further processing in the laboratory. Fish capture and their handling complied with the current laws of the Republic of Macedonia. Individual fish were anesthetized with Clove Oil (Sigma Aldrich, USA). First, the biometric data were recorded (total length, whole mass), whereas Fulton condition indices (FCI) were calculated according to Rätz and Lloret (2003). After the fish were anesthetized and sacrificed, the liver, gills and gonads were removed. Liver and gills were weighed and stored in liquid nitrogen immediately after sampling and then held at −80 °C until further analyses. Gonads were used for histological examination of sex.

Since previous studies have demonstrated high reliability and repeatability of trace element distribution profiles obtained after the repeated measurements in the same cytosolic samples, or in the samples of the same fish species and target organ, by the same approach as applied in this study (same chromatographic column, HPLC and HR ICP-MS system) (Krasnići et al., 2013, 2014), we have decided to design this study based on the analyses of only three fish per each river, due to the time restrictions and limited resources. The selection of fish for analyses was based on two criteria: 1) the availability of the sample for analysis, which depended on the fish size and 2) cytosolic trace element concentrations in liver and gills. We wanted to study samples with a

wide range of different cytosolic trace element concentrations, from low to high, to be able to define what happens with the additional quantity of trace elements accumulated in Vardar chub liver and gills as a consequence of higher environmental exposure. Since some of the fish selected for hepatic analysis had too low gill cytosolic concentrations, or too small volume of gill cytosol available for analyses, we were forced to select additional fish for gill analyses. Also, due to smaller fish size in the Zletovska River, and thus also smaller volume of gill cytosol, we have analyzed only two gill samples from the Zletovska River, and instead we have analyzed four gill samples from the Kriva River. Therefore, we have analyzed in total 13 specimens of Vardar chub, and their biometric characteristics and total cytosolic protein concentrations are given in Table 1. Hepatic analyses were performed on the nine fish specimens marked with the following ordinal numbers: 1, 2, 4, 5, 6, 7, 8, 10, and 12 (Table 1, Figs. 1–2). Gill analyses were performed on the nine fish specimens marked with the following ordinal numbers: 2, 3, 4, 5, 7, 9, 11, 12, and 13 (Table 1, Fig. 3). Analyses of heat-stable biomolecules were performed on one hepatic sample (No. 8, Table 1, Fig. 4), and on two gill samples (No. 11 and 13, Table 1, Fig. 5). Few profiles were excluded from presentation, due to analytical problems, such as contamination with certain elements during the chromatographic separations or measurement, namely Mo in hepatic samples 4, 7, and 12 (Fig. 2a); Se in hepatic sample 4 (Fig. 2b); Fe in gill sample 11 (Fig. 3a); Zn in gill sample 13 (Fig. 3c); and Cd in gill sample 12 (Fig. 3d).

2.2. Isolation of cytosolic fractions from Vardar chub liver and gills

Whole frozen livers and gills of Vardar chub (0.84–5.46 g and 0.29–2.31 g, respectively) 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 dithiothreitol, Sigma)] was added (*w*/*v* 1:5), which was followed by homogenization in an ice cooled Potter-Elvehjem homogenizer (Glas-Col, USA) applying 10 strokes of motor-driven PTFE-pestle at 6000 rpm. The homogenates were centrifuged (Avanti J-E centrifuge, Beckman Coulter) at 50,000 × *g* for 2 h at 4 °C. The obtained supernatants, which correspond to water-soluble cytosolic tissue fractions (Van Campenhout et al., 2010) containing lysosomes and microsomes (Bonneris et al., 2005), were aliquoted and stored at −80 °C for separation by size exclusion high performance liquid chromatography (SEC-HPLC) and for analyses of trace elements (Krasnići et al., 2013, 2014).

2.3. Heat-treatment of hepatic and gill cytosols

The heat-treatment of hepatic and gill cytosols was performed according to Erk et al. (2002), with slight modifications. The cytosols were heat-treated at 70 °C for 10 min using The Dri Block (Techne) (Bibby Scientific Limited, Staffordshire, UK). After the heat-treatment, cytosols were placed on ice and kept for 30 min at 4 °C, and subsequently centrifuged at 10,000 × *g* for 15 min at 4 °C (Heraeus Biofuge Fresco, Kendro, USA). Supernatants obtained after this step were stored at −80 °C until further analysis. This heat-stable fraction is expected to show lower protein content than cytosolic fraction, due to denaturation of thermo-labile and high molecular mass proteins from cytosol samples at temperatures above 60–70 °C (Krizkova et al., 2011). Heat-treated cytosols, for example, contain heat-stable cytosolic proteins MTs (Yang et al., 1995).

2.4. SEC-HPLC analysis of cytosols and heat-treated cytosols from chub liver and gills

Distribution of trace elements among biomolecules of different molecular masses in the cytosols and heat-treated cytosols from Vardar chub livers and gills was studied by SEC-HPLC (Perkin Elmer HPLC system, series 200, USA), as previously described in Krasnići et al. (2013, 2014). Hepatic and gill cytosols and heat-treated cytosols were separated into fractions containing biomolecules of different molecular masses (MM) using two types of size exclusion columns (SEC): prepacked Tricorn™ Superdex 200 10/300 GL (GE Healthcare Biosciences, USA) with a separation range of 10 kDa–600 kDa and Tricorn™ Superdex 75 10/300 GL column (GE Healthcare Biosciences, USA) with a separation range of 3 kDa–70 kDa, for globular proteins. Immediately before application on the column, cytosol samples were centrifuged at 10,000 × *g* for 10 min at 4 °C (Heraeus Biofuge Fresco, Kendro, USA). The sample volumes of 100 μL were applied on the column. For each sample, two consecutive chromatographic runs were performed, i.e. 200 μL in total of each cytosol sample was run through the column. The separation was achieved using 20 mM Tris-HCl/Base (Sigma, pH 8.1 at 22 °C, flow 0.5 mL min^{-1}) as a mobile phase (isocratic mode). The fractions were collected at 1 min intervals in the plastic tubes using a fraction collector (FC 203B, Gilson, USA). In total 40 fractions were collected for each sample, starting with 13th minute to 52nd minute. For column calibration, several protein standards (thyroglobulin, apoferritin, β-amylase, alcohol dehydrogenase, conalbumin, bovine albumin, ovalbumin, carbonic anhydrase, cytochrome C, vitamin B12, Sigma, USA) were run through the column under the same conditions as the samples (Table 2). The

Table 1
Basic biometric characteristics and total cytosolic proteins in livers and gills of 13 specimens of Vardar chub (*Squalius vardarensis*) used in this study, which were caught in moderately contaminated Bregalnica River and two mining impacted rivers (Zletovska and Kriva) in north-eastern Macedonia in spring (May/June) of 2012.

Fish no.	River	Length/cm	Mass/g	Sex	FCI/%	Total proteins/mg mL^{-1}	
						Liver	Gills
1	Bregalnica	21.1	108.1	F	1.15	23.6	17.6
2	Bregalnica	22.0	137.3	F	1.29	22.4	20.1
3	Bregalnica	20.4	105.8	F	1.25	24.7	21.2
4	Bregalnica	17.5	58.6	F	1.09	21.4	16.3
5	Zletovska	21.3	106.7	F	1.10	16.1	15.1
6	Zletovska	15.2	37.4	F	1.06	16.1	–
7	Zletovska	19.4	69.3	F	0.95	19.7	16.0
8	Kriva	30.2	350.0	F	1.27	17.3	17.3
9	Kriva	20.1	99.9	F	1.23	19.3	17.5
10	Kriva	27.5	231.5	F	1.11	21.1	15.5
11	Kriva	22.5	138.3	F	1.21	19.3	22.1
12	Kriva	26.0	210.6	M	1.20	24.1	24.2
13	Kriva	21.4	125.1	F	1.28	20.2	22.8

Table 2
Elution times (t_e) and molecular masses (MM) of seven proteins used as standards for calibration of Superdex™ 200 10/300 GL and Superdex™ 75 10/300 GL size exclusion columns, as well as of rabbit metallothionein standard (Enzo Metallothionein-1, Enzo Metallothionein-2).

	Protein	t_e/min	MM/kDa	Concentration/ mg mL^{-1}
Superdex™ 200 10/300 GL	Thyroglobulin	16.08	669	8
	Apo-ferritin	17.80	443	10
	β-amylase	20.36	200	4
	Alcohol dehydrogenase	21.72	150	5
	Bovine albumin	23.13	66	10
	Superoxide dismutase	27.87	32	1.25
	Carbonic anhydrase	29.66	29	3
	Metallothionein - 2	31.22	6.1	1
	Metallothionein - 1	32.32	6.1	1
Superdex™ 75 10/300 GL	Conalbumin	18.57	75	3
	Ovalbumin	17.74	43	4
	Superoxide dismutase	19.97	32	1.25
	Carbonic anhydrase	21.26	29	5
	Cytochrome C	24.80	12	3
	Metallothionein-2	23.02	6.1	1
	Metallothionein-1	24.06	6.1	1
	Vitamin B12	36.14	1.35	3

equation of the calibration straight line for Superdex™ 200 was: y = −0.2808× + 1.6469; and for Superdex™ 75: y = − 0.3343× + 1.6664; y = Kav; x = log MM. In addition, elution times were also determined for superoxide dismutase (Sigma, USA), MT-1 and MT-2 (Enzo Life Sciences, USA). The void volume was determined by use of blue dextran (defined MM: 2000 kDa), which was eluted in columns Superdex™ 200 and Superdex™ 75 at 16.3 min and 15.6 min, respectively.

2.5. Determination of trace element concentrations in chub hepatic and gill cytosols and in the fractions obtained by SEC-HPLC separation

The concentrations of eight trace elements (Co, Cu, Fe, Mo, Mn, Se, Zn and Cd) were determined within this study. For determination of cytosolic trace element concentrations in the liver and gills of Vardar chub (Table 3), cytosols were ten times diluted with Milli-Q water and acidified with HNO_3 (*Suprapur*, Merck, Germany; final acid concentration in the samples: 0.65%). Fractions of liver and gill cytosols obtained after SEC-HPLC separation were only acidified with HNO_3 (*Suprapur*, Merck, Germany, final acid concentration in the samples 0.16%) prior to offline measurement of trace elements. Indium (Fluka, Germany) was added to all samples as an internal standard (1 μg L^{-1}). The measurements were performed using high resolution inductively coupled plasma mass spectrometer (HR ICP-MS, Element 2, Thermo Finnigan, Germany), equipped with an autosampler SC-2 DX FAST (Elemental Scientific, USA) 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). Measurements of ^{82}Se, ^{98}Mo, ^{111}Cd were operated in low-resolution mode, whereas ^{55}Mn, ^{56}Fe, ^{59}Co, ^{63}Cu, and ^{66}Zn were measured in medium resolution mode. External calibrations were performed using multielement standard solution for trace elements (Analitika, Czech Republic). All standards were prepared in 1.3% HNO_3 (*Suprapur*, Merck, Germany) and supplemented with In (1 μg L^{-1}; Fluka, Germany). The accuracy of trace element measurements by HR ICP-MS was checked by analysis of quality control sample (QC for trace elements, catalog no. 8072, lot no. 146142-146143, Burlington, Canada). A generally good agreement was observed between our data and the certified values, with the following recoveries (%) (based on 23 measurements in control sample for trace elements): Cd (99.2 ± 3.7), Co (100.0 ± 4.9), Cu (100.7 ± 11.2), Fe (92.3 ± 7.3), Mn (102.3 ± 13.2), Mo (95.9 ± 2.4), Se (95.4 ± 3.9), and Zn (97.7 ± 27.7). Somewhat higher deviation of Zn recoveries in comparison to other analyzed elements was due to much lower Zn concentrations in the control sample than in the gill and hepatic cytosols. Limits of detection (LODs) were determined based on three standard deviations of ten consecutively determined trace element concentrations in the blank sample (Tris-HCl/Base, dithiothreitol, HNO_3). LODs for trace elements measured within this study were as follows (in μg L^{-1}): Cd, 0.005; Co, 0.002, Cu, 0.037; Fe, 0.084; Mn, 0.002; Mo, 0.004; Se, 0.138; and Zn, 2.40 (Krasnići et al., 2013, 2014).

Table 3
Total trace element concentrations (ng mL^{-1} or μg mL^{-1}) in analyzed hepatic and gill cytosols of Vardar chub (*Squalius vardarensis*) which were caught in moderately contaminated Bregalnica River and two mining impacted rivers (Zletovska and Kriva) in north-eastern Macedonia in spring (May/June) of 2012.

Fish no.	Organ	Co	Cu	Fe	Mn	Mo	Se	Zn	Cd
		ng mL^{-1}	μg mL^{-1}	μg mL^{-1}	ng mL^{-1}	ng mL^{-1}	ng mL^{-1}	μg mL^{-1}	ng mL^{-1}
1	Liver	4.77	1.67	13.5	322.4	26.5	228.0	5.17	2.69
2	Liver	5.54	2.87	8.47	288.2	19.5	254.0	6.31	2.38
	Gills	–	–	18.3	–	–	88.5	4.28	0.34
3	Gills	–	–	17.3	–	–	93.4	5.28	0.41
4	Liver	5.21	3.66	6.61	408.6	–	–	6.93	6.40
	Gills	–	–	11.1	–	–	89.1	5.61	0.57
5	Liver	3.34	3.10	7.06	231.7	13.2	85.8	3.51	36.9
	Gills	–	–	5.48	–	–	57.9	14.4	33.8
6	Liver	2.56	2.35	6.40	214.5	12.6	115.1	5.21	18.0
7	Liver	3.39	4.19	12.7	233.2	–	124.6	5.51	33.8
	Gills	–	–	8.57	–	–	70.3	11.7	22.6
8	Liver	4.22	6.54	7.44	237.1	26.6	402.7	7.61	40.5
9	Gills	–	–	9.70	–	–	178.9	12.0	10.5
10	Liver	5.27	3.70	7.45	250.4	21.2	289.8	8.37	28.7
11	Gills	–	–	–	–	3.05	300.8	5.76	22.9
12	Liver	4.43	4.86	14.7	257.6	–	631.1	7.87	68.2
	Gills	–	–	28.5	–	–	469.0	6.23	–
13	Gills	–	–	22.6	–	2.55	449.7	6.23	30.0

2.6. Data processing

Chromatographic results were processed using TotalChrom Version 6.3.1 software (Perkin Elmer, USA). All basic calculations were done in Microsoft Excel 2007, whereas graphs were created using the statistical program SigmaPlot 11.0.

3. Results and discussion

This research presents the first attempt to gain information on the influence of water contamination and of the subsequent trace element bioaccumulation on the distribution of trace elements among unknown cytosolic biomolecules in two organs, gills and liver, of Vardar chub (*S. vardarensis*). Information on cytosolic trace element distribution of this type was previously reported for gills and liver of European chub (*Squalius cephalus*) from moderately contaminated Sutla River in Croatia (Krasnići et al., 2013, 2014), but still does not exist for Vardar chub. Thirteen selected specimens of Vardar chub, analyzed in this study, whose biometric characteristics and total cytosolic protein concentrations are presented in Table 1, were caught in three rivers in north-eastern Macedonia, displaying different degrees and types of environmental contamination: three specimens were caught in the Zletovska River and six specimens in the Kriva River, both being contaminated by waste of active Pb/Zn mines, and four specimens in the Bregalnica River, as river less contaminated with trace elements and impacted by agricultural runoff. Severe trace element contamination of the Zletovska River and slighter contamination of the Kriva River were confirmed by water analysis performed in parallel with fish sampling (Ramani et al., 2014). Consequent harmful impact on the fish was also reported, which was reflected in observable histopathological damages on gills, liver and spleen of Vardar chub (Barišić et al., 2015; Jordanova et al., 2016, 2017).

We have defined the basic cytosolic distributions of trace elements among biomolecules of different molecular masses in the liver and gills of Vardar chub, which are characteristic for low exposure to trace elements. According to Langston et al. (2002), increased environmental concentrations of trace elements may cause shifts in their distribution profiles among cytosolic ligands, and thus the distribution changes as a consequence of increased accumulation of trace elements in the selected chub organs were also determined. The technology applied for that purpose consisted of SEC-HPLC, using Superdex™ 200 column with a linear separation range between 10 and 600 kDa, and HR ICP-MS, coupled offline. Several studies have demonstrated the potential of SEC-HPLC/ICP-MS coupling as a sensitive multi-elemental approach for the quantitative analysis of metalloproteins (Mason and Storms, 1993; Ferrarello et al., 2000; de la Calle Guntiñas et al., 2002; Montes-Bayón et al., 2003). The elution times of analyzed trace elements were associated to specific protein molecular masses by use of calibration straight line, obtained by chromatographic analysis of standard proteins. For the easier handling of the obtained results, cytosolic biomolecules were categorized in four groups, according to their molecular masses (MM), as previously defined by Krasnići et al. (2013, 2014): high MM (HMM), >100 kDa; medium MM (MMM), 30–100 kDa; low MM (LMM), 10–30 kDa; and very low MM (VLMM), <10 kDa.

3.1. Distributions of trace elements in hepatic and gill cytosols of Vardar chub

As defined by Bonneris et al. (2005), tissue cytosols obtained after centrifugation of tissue homogenates at 50,000 ×*g* (a protocol applied in our study) contain both heat-sensitive and heat-stable biomolecules that bind trace elements, with latter being considered as a detoxified trace element forms (Giguère et al., 2006; Rosabal et al., 2015). Analysis of trace element distributions among cytosolic biomolecules therefore gave insight into both potentially toxic and detoxified cellular pools of trace elements.

It is well known that bioaccumulation of trace elements depends on different factors, such as fish species, nature of trace elements, as well as the organ in which accumulation occurs. The cytosolic concentrations of many trace elements in Vardar chub gills were much lower compared to the liver, as can be seen from cytosolic concentrations listed in Table 3. This could be explained by the ability of gills to transfer absorbed trace elements by blood to the liver as a main detoxification and storage organ in fish (Souza et al., 2013; Amiri et al., 2011). Much higher hepatic than gill cytosolic concentrations of several elements were the reason why we were able to describe cytosolic distributions for as much as eight elements in the liver (Co, Cu, Fe, Mn, Mo, Se, Zn, and Cd), whereas in the gills the same thing was done for only four elements (Fe, Se, Zn, and Cd). Cytosolic distributions of trace elements in the Vardar chub liver are presented in the Figs. 1 and 2, and in the gills in the Fig. 3, whereas their elution times and molecular masses of corresponding biomolecules are given in Table 4. Each studied element will be separately discussed further in the text.

3.1.1. Cobalt

Cobalt distribution profiles were determined only for the hepatic cytosol (Fig. 1a). In general, the toxicity of Co to fish seems to be quite low compared with the effects of the other metal ions, especially during in situ environmental exposures (Marr et al., 1998; Kubrak et al., 2011). Nevertheless, Co has also been recognized as a stress inducing factor, which can participate in free radical processes, resulting in reactive oxygen species (ROS) production (Wang et al., 1993; Olivieri et al., 2001; Battaglia et al., 2009). Cobalt was eluted in four peaks, and the major proportion of the cytosolic Co in liver was eluted within the HMM peak (~110–380 kDa), with a maximum corresponding to biomolecules of MM ~230 kDa (Table 4). It is consistent with previous reports that ions of Co, as an essential element, have a high affinity for binding to high MM enzymatic proteins (Paustenbach et al., 2013; Wojcieszek and Ruzik, 2016). Much smaller amounts of Co were present in three remaining peaks, associated with MMM biomolecules (~30–80 kDa, with a maximum at 66 kDa), and with VLMM biomolecules distributed in two peaks (2–5 kDa, with a maximum at 4 kDa; and 0.7–2 kDa, with a maximum at 1.2 kDa) (Table 4). This last peak, with a maximum at 1.2 kDa, could possibly correspond to Co-containing compound cobalamin, which has a MM of 1.3 kDa (Kirschbaum, 1981). The studies on mussels also showed that a considerable proportion of Co was associated to biomolecules of MM below 4 kDa (Ferrarello et al., 2000). Although the main described role of Co in the fish organism is associated with its constitutive role in cobalamin, i.e. the vitamin B12 (Blust, 2012; Krasnići et al., 2013), and although the liver plays a major role in the vitamin B12 metabolism (Wang et al., 2001), only minor part of Co present in the Vardar chub hepatic cytosol was eluted within the fraction presumably containing cobalamin. Distribution profile of Co in Vardar chub liver was further compared to previously published Co hepatic profile of European chub (*S. cephalus*), and they were almost identical, with the only exception that Co HMM and MMM peaks in the hepatic cytosol of European chub were merged together and were not clearly resolved (Krasnići et al., 2013).

Nine studied specimens of Vardar chub from three rivers had quite narrow range of cytosolic Co concentrations (2.6–5.5 ng mL^{-1}, Table 3), and therefore the differences between the profiles were hardly even notable. Cobalt elution associated to HMM biomolecules was found to increase only slightly with increasing cytosolic Co

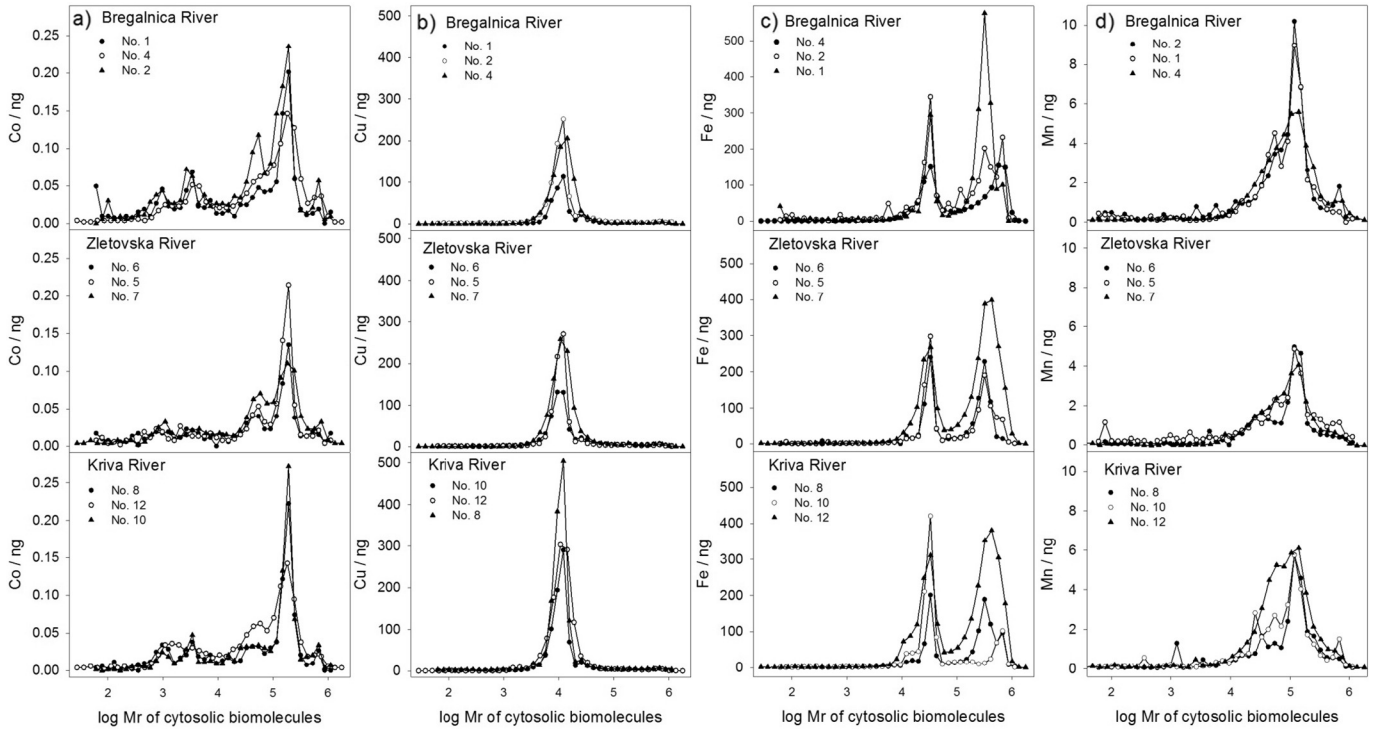

Fig. 1. Hepatic distribution profiles of four selected trace elements (a - Co, b - Cu, c - Fe, and d - Mn) among cytosolic proteins of different molecular masses in Vardar chub (*Squalius vardarensis*) caught in a moderately contaminated Bregalnica River and two mining impacted rivers, Zletovska and Kriva. The profiles were obtained by separation of hepatic cytosols on SEC-HPLC with Superdex™ 200 10/300 GL column and measurement of trace elements on HR ICP-MS. The results are presented as nanograms of trace elements eluted in the fractions containing proteins of specific molecular masses. Nine fish were used for these analyses (No.1, 2, 4, 5, 6, 7, 8, 10, and 12, Table 1), and their total trace element concentrations in hepatic cytosols are presented in Table 3.

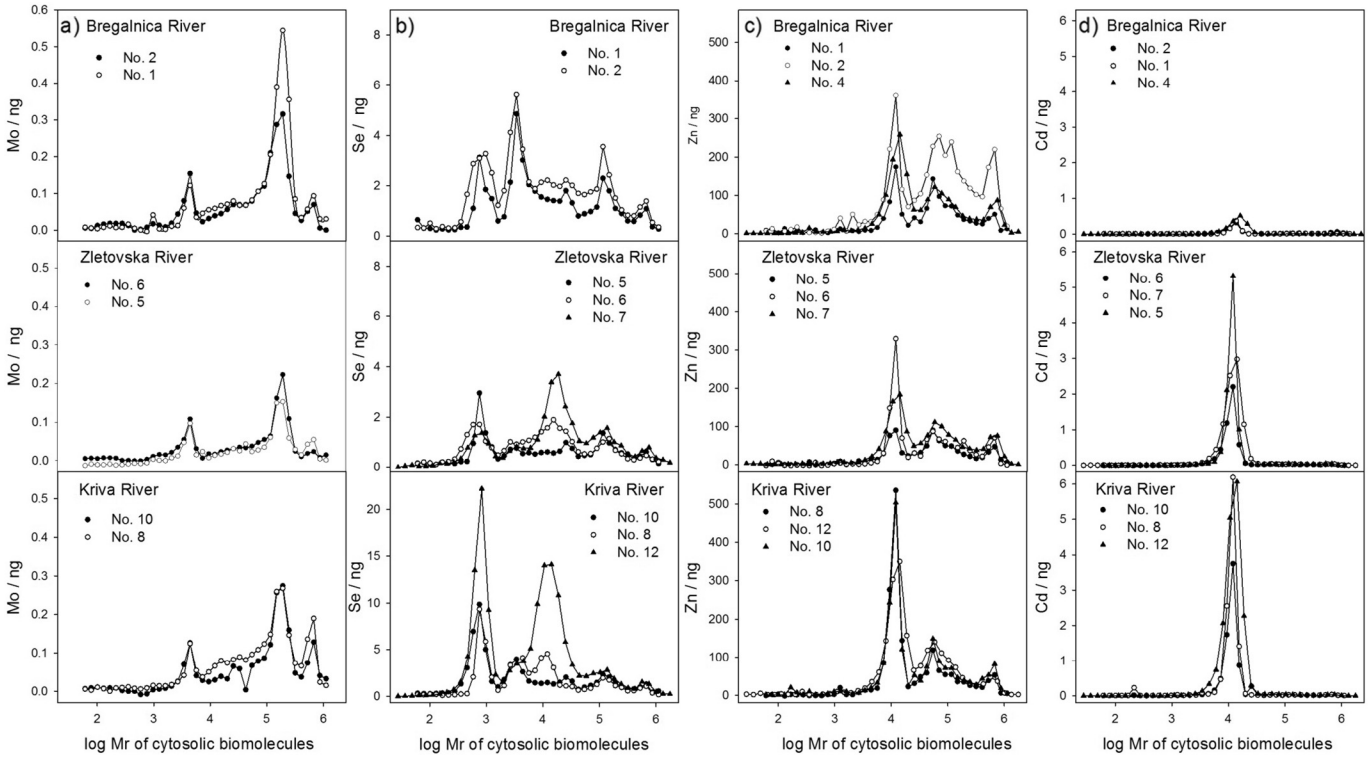

Fig. 2. Hepatic distribution profiles of four selected trace elements (a - Mo, b - Se, c - Zn, and d - Cd) among cytosolic proteins of different molecular masses in Vardar chub (*Squalius vardarensis*) caught in a moderately contaminated Bregalnica River and two mining impacted rivers, Zletovska and Kriva. The profiles were obtained by separation of hepatic cytosols on SEC-HPLC with Superdex™ 200 10/300 GL column and measurement of trace elements on HR ICP-MS. The results are presented as nanograms of trace elements eluted in the fractions containing proteins of specific molecular masses. Nine fish were used for these analyses (No.1, 2, 4, 5, 6, 7, 8, 10, and 12, Table 1), and their total trace element concentrations in hepatic cytosols are presented in Table 3.

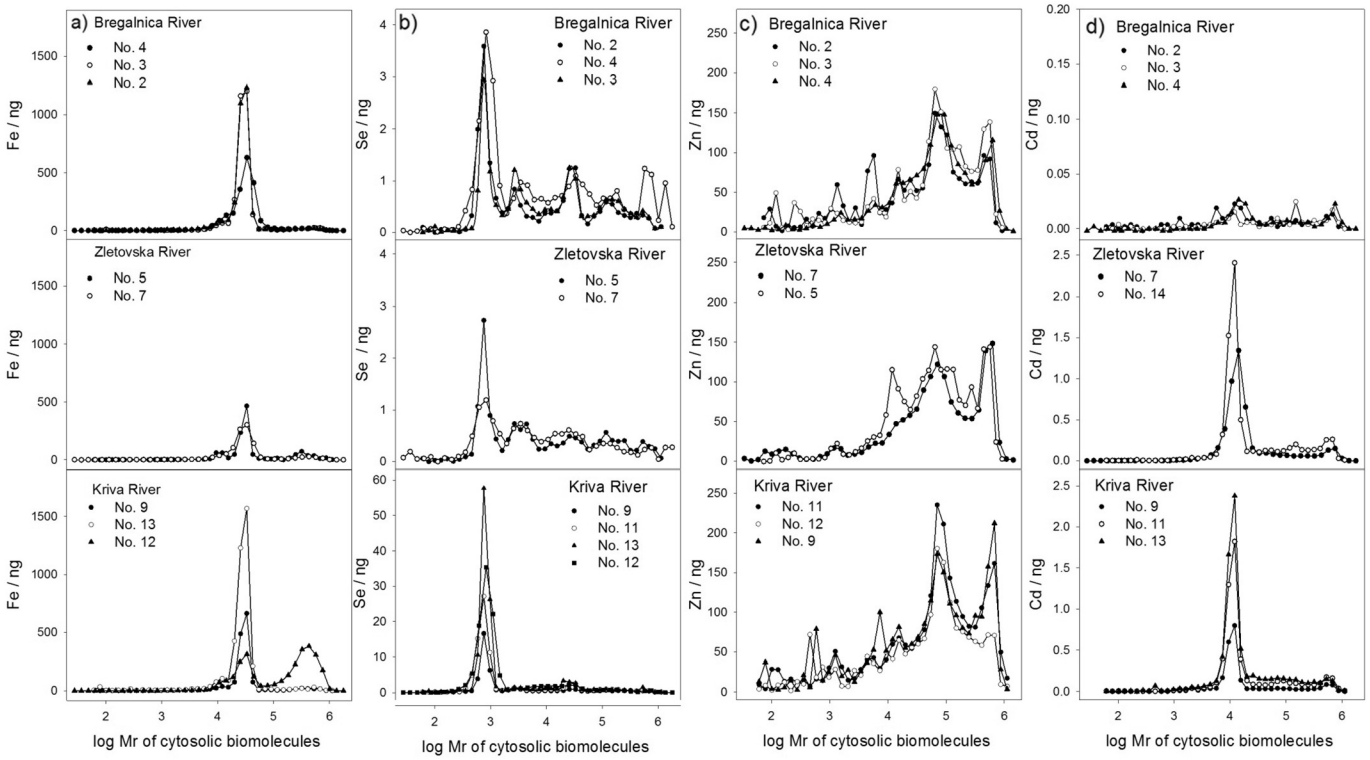

Fig. 3. Gill distribution profiles of four selected trace elements (a - Fe, b - Se, c - Zn, and d - Cd) among cytosolic proteins of different molecular masses in Vardar chub (*Squalius vardarensis*) caught in a moderately contaminated Bregalnica River and two mining impacted rivers, Zletovska and Kriva. The profiles were obtained by separation of gill cytosols on SEC-HPLC with Superdex™ 200 10/300 GL column and measurement of trace elements on HR ICP-MS. The results are presented as nanograms of trace elements eluted in the fractions containing proteins of specific molecular masses. Nine fish were used for these analyses (No. 2, 3, 4, 5, 7, 9, 11, 12 and 13, Table 1), and their total trace element concentrations in gill cytosols are presented in Table 3.

Table 4
Elution times (t_e) and molecular masses (MM) of cytosolic proteins from liver and gills of Vardar chub (*Squalius vardarensis*) contained within the fractions in which respective elements were eluted, after separation of cytosols by size exclusion HPLC with Superdex™ 200 10/300 GL column. Presented data refer to maximums of trace element peaks (i.e. to fractions with the highest trace element concentrations), whereas the numbers within the brackets refer to the beginnings and the ends of trace element peaks.

Element			VLMM peak 1[a]		VLMM peak 2[a]		LMM peak[b]		MMM peak[c]		HMM peak 1[d]		HMM peak 2[d]	
		Organ	t_e/min	MM/kDa	t_e/min	MM/kDa	t_e/min	MM/kDa	t_e/min	MM/kDa	t_e/min	MM/kDa	t_e/min	MM/kDa
Essential elements	Co	Liver	41 (43–40)	1.2 (0.7–2)	36 (38–35)	4 (2–5)			25 (28–24)	66 (31–85)	20 (23–18)	231 (109–383)		
	Cu	Liver					31 (34–27)	15 (7–40)						
	Fe	Liver							27 (29–26)	40 (24–51)			18 (20–16)	383 (231–633)
		Gills							27 (29–26)	40 (24–51)			18 (20–16)	383 (231–633)
	Mn	Liver							25 (29–24)	66 (24–85)	22 (24–20)	140 (85–382)		
	Mo	Liver			35 (37–34)	5 (3–7)					20 (24–18)	231 (85–382)	15 (17–14)	814 (492–1047)
	Se	Liver	42 (44–39)	1 (0.6–2)	36 (38–35)	4 (2–5)	28 (29–26)	31 (24–51)			22 (23–19)	140 (109–298)		
		Gills	42 (44–40)	1 (0.6–2)	37 (38–35)	3 (2–5)	28 (30–26)	31 (19–51)						
	Zn	Liver					31 (34–29)	15 (7–24)	25 (28–18)	66 (31–383)			15 (17–14)	814 (492–1047)
		Gills							24 (32–19)	85 (11–298)			15 (18–14)	814 (383–1047)
Non-essential element	Cd	Liver					31 (34–29)	15 (7–24)						
		Gills					31 (34–29)	15 (7–24)						

[a] VLMM peak – a peak of trace element concentration in the cytosolic fractions with a maximum in very low molecular mass protein region (<10 kDa).
[b] LMM peak – a peak of trace element concentration in the cytosolic fractions with a maximum in low molecular mass protein region (10–30 kDa).
[c] MMM peak – a peak of trace element concentration in the cytosolic fractions with a maximum in medium molecular mass protein region (30–100 kDa).
[d] HMM peak – a peak of trace element concentration in the cytosolic fractions with a maximum in high molecular mass protein region (>100 kDa).

concentrations, and in general this increase showed a good relationship with cytosolic Co levels (Fig. 1a), indicating that additional attention should be dedicated to study of HMM Co-binding proteins.

3.1.2. Copper

Same as for Co, Cu distribution profiles were also determined only for the hepatic cytosol (Fig. 1b). Copper is an essential trace element for the most living organisms, necessary for certain metabolic processes (e.g., formation of connective tissue, formation and maintenance of myelin, cellular respiration, scavenging of the free radical superoxide; Gaetke et al., 2014), because it plays an important role as a cofactor in a number of enzymes and metalloproteins (Hauser-Davis et al., 2012). Accordingly, Cu was eluted mainly within one single LMM peak (7–40 kDa), with a maximum corresponding to biomolecules of MM of 15 kDa (Table 4), coinciding with the elution time of MT (Table 2), but also encompassing the MM of the other known Cu-containing biomolecules, such as superoxide dismutase (SOD; MM 32 kDa) (Table 2), which indicates Cu essential role in the protection against oxidative stress (Sanchez et al., 2005; Krasnići et al., 2013). Our results, however, indicate that the major part of Cu was probably associated with MTs. MTs constitute a family of low MM, cysteine-rich proteins involved in the binding, regulation and storage of essential metals such as Cu and Zn, and the detoxification of non-essential metals such as Cd (Coyle et al., 2002; Mason et al., 2004). The capacity for MT induction is the greatest in tissues that are active in uptake, storage and excretion of trace elements, such as gills, intestine, liver and kidney (Roesijadi and Robinson, 1994). Cu distribution profile in liver of Vardar chub was in agreement with the previous results of the investigation on Cu-binding biomolecules in hepatic cytosol of European chub (Krasnići et al., 2013), where the most of Cu was detected in LMM protein region (~7–27 kDa), and had the same elution time as MT standard (Krasnići et al., 2013). Copper association with MTs was also confirmed in the liver of eel (*Anguilla anguilla*; Rodríguez-Cea et al., 2003).

In the liver of nine studied specimens of Vardar chub from three rivers, Cu cytosolic concentrations were in rather narrow range, from 1.7 to 6.5 ng mL^{-1} (Table 3). Positions of Cu peaks within the distribution profiles were comparable in Vardar chub from all three rivers (Fig. 1b), whereas increasing cytosolic concentrations resulted with increased elution of Cu in MT region, which was especially observable at the Kriva River. Peak widening towards the region of higher MM was also observed, which could possibly indicate that Cu was also associated to the other cytosolic biomolecules, when present in the cell in higher concentrations.

3.1.3. Iron

Many proteins contain essential metal Fe in ionic form, either within their own structures or bound to their active sites (del Castillo Busto et al., 2010). Thus, under physiological conditions, the majority of Fe is bound to proteins and, to a lower extent, to the other small biomolecules (e.g., citrates), to decrease the free metal ion toxicity (del Castillo Busto et al., 2010). Iron distribution profiles were recorded for both hepatic and gill cytosols of Vardar chub (Figs. 1c and 3a), and were characterized with two Fe containing peaks. Positions of the peaks were comparable in both organs. The first peak was positioned in the MMM region and covered the range of MM from ~25–50 kDa, with a maximum at the MM of 40 kDa (Table 4), encompassing with its borders MM of known Fe-containing biomolecules, such as enzyme catalase (60 kDa) and transport protein myoglobin (17 kDa) (Wolf et al., 2007). The second peak was located in the HMM region and covered the range of MM from ~230–630 kDa, with a maximum corresponding to biomolecules of MM amounting to ~380 kDa (Table 4), which approximately corresponded to ferritin, the major Fe storage protein in almost all living organisms (450 kDa; Szpunar and Lobinski, 1999).

The difference between two organs was in the fact that the majority of Fe in the gill cytosol was eluted within the first MMM peak (Fig. 3a), whereas in the liver the quantity of Fe eluted within the second HMM

peak was either comparable or even higher than in the first MMM peak (Fig. 1c). In the gills, clear presence of Fe in the HMM region was recorded in only one sample with the highest gill cytosolic Fe concentration (28.5 μg mL^{-1}; Fig. 3a). The lack of HMM-Fe peak in the gill samples with lower Fe cytosolic concentrations lead to a conclusion that Fe association with storage protein ferritin was more strongly expressed in the liver than in the gills, even though cytosolic Fe concentrations were lower in the liver (6.4–14.7 μg mL^{-1}, Table 3) compared to gills (5.5–28.5 μg mL^{-1}, Table 3). This was a confirmation of more pronounced role of liver than gills in Fe storage. Distribution profiles of Fe in Vardar chub liver and gills were comparable to previously reported Fe profiles in the liver and gills of European chub, where stronger Fe association with HMM proteins, probably ferritin, was also observed in the liver (Krasnići et al., 2013, 2014).

Positions of Fe peaks within the hepatic and gill distribution profiles were comparable in Vardar chub from all three rivers (Figs. 1c and 3a). The increase of hepatic Fe concentrations resulted with the increased Fe elution in HMM peak. This was an indication that in the case of increasing Fe concentration in the liver, the part of Fe probably bounds to ferritin. Synthesis of ferritin is known to be induced by increased Fe availability, whereas it is repressed under iron deprivation conditions (Torti and Torti, 2002). Contrary, in the gills, increase of cytosolic Fe mainly resulted with the increased Fe elution in MMM peak.

3.1.4. Manganese

Distribution profiles for essential element Mn were determined only for the hepatic cytosol (Fig. 1d). Manganese was eluted within two poorly resolved peaks covering MMM and HMM regions. Manganese in MMM peak was associated to biomolecules of MM in the range from 24 to 85 kDa, with a maximum at 66 kDa, whereas HMM peak encompassed biomolecules of MM from ~85–230 kDa, with a maximum at 140 kDa (Table 4). These peaks comprised MM of albumin (66 kDa, Table 2) and transferrin (80 kDa; Martin-Antonio et al., 2009). Albumin is a protein involved in the Mn transport from intestine to liver, whereas in the liver, Mn binds to transferrin, and in that form presents a source of Mn for delivery to the other tissues (Krasnići et al., 2013; Schäfer, 2004). Obtained MMM and HMM peaks in the distribution profiles of Mn in the liver of Vardar chub were consistent with Mn association with MMM biomolecules of ~35–60 kDa and HMM biomolecules of ~60–400 kDa in hepatic cytosol of European chub (Krasnići et al., 2013). The only difference between two species was lack of clear Mn binding to LMM biomolecules (<20 kDa) in Vardar chub, which was observed in European chub (Krasnići et al., 2013).

Positions of Mn peaks within the hepatic distribution profiles were comparable in Vardar chub from all three rivers (Fig. 1d). The cytosolic Mn concentrations in liver of all nine analyzed Vardar chub were within the following range: 215–409 ng mL^{-1} (Table 3). The differences associated to concentration changes could not be clearly determined, since in some cases there was an increase in MMM peak, in the others in HMM peak, whereas mostly the peaks were nearly identical (Fig. 1d).

3.1.5. Molybdenum

Distribution profiles for essential element Mo were also determined only for the hepatic cytosol (Fig. 2a). Molybdenum was eluted within three peaks, one VLMM peak and two HMM peaks. Minor part of Mo, shown as a first peak, was eluted within VLMM biomolecule region (Fig. 2a; 3–7 kDa), with a maximum at 5 kDa (Table 4). The major part of Mo, shown as a second peak, was eluted within HMM biomolecule region (Fig. 2a; ~85–380 kDa), with a maximum at ~230 kDa (Table 4). According to Beers and Berkow (1998), Mo serves as a cofactor of different enzymes, such as aldehyde oxidase (~130 kDa; Uchida et al., 2003), sulphide oxidase (~120 kDa; Johnson and Rajagopalan, 1976), and Fe-Mo flavoprotein xanthine oxidase (275 kDa, Truglio et al., 2002) (Krasnići et al., 2013), which were all encompassed by the second Mo peak. A small amount of Mo was eluted within the void volume of the column (Fig. 2a), as a sign of Mo association to HMM biomolecules above ~500 kDa (Table 4), possibly containing protein complexes and aggregates. Molybdenum distribution profiles in the liver of Vardar chub were similar to those previously reported for the liver of European chub, where Mo was also mostly bound to biomolecules of HMM (~60–400 kDa), whereas only minor Mo part was associated to VLMM biomolecules (4–12 kDa) (Krasnići et al., 2013).

Positions of Mo peaks within the hepatic distribution profiles were comparable in Vardar chub from all three rivers (Fig. 2a). The range of cytosolic Mo concentrations in liver of six analyzed Vardar chub was the following: 12.6–26.6 ng mL^{-1} (Table 3) and the increase of cytosolic Mo concentrations was reflected in the increased Mo elution within HMM peaks, confirming the predominant Mo association to HMM biomolecules.

3.1.6. Selenium

Selenium is a nonmetal, an essential micronutrient for the synthesis of selenoproteins, which plays an important role in the overall metabolism (Rayman, 2012; Braga et al., 2017). However, very little is known about how Se is metabolized in fish, and for the most fish selenoproteins functions are yet not known (Hauser-Davis et al., 2012). Selenium distribution profiles were determined for both hepatic and gill cytosols of Vardar chub (Figs. 2b and 3b). Hepatic profiles of Se were characterized with four Se containing peaks. The first two peaks were located within the VLMM region, and the first one corresponded to biomolecules of MM in the range from 0.6–2 kDa, with a maximum at 1 kDa, whereas the second VLMM peak corresponded to biomolecules of MM from 2 to 5 kDa, with a maximum at 4 kDa (Table 4, Fig. 2b). The other two Se hepatic peaks were eluted in LMM and HMM protein regions (~25–50 kDa, with a maximum at ~30 kDa, and ~110–300 kDa, with a maximum at 140 kDa, respectively; Table 4, Fig. 2b). Gill Se distribution profiles were almost identical to hepatic profiles, with the only exception that they were lacking clear HMM peak (Fig. 3b). The main characteristic of gill profiles was that majority of Se was eluted in detached and sharp peak within the first VLMM region (0.6–2 kDa). The association of Se with VLMM biomolecules was already reported by many other authors. For example, Se presence in the cell in the form of selenomethionine (~0.2 kDa) was reported by Klotz et al. (2003), whereas very low MM organic selenocompound selenoneine (~0.5 kDa), effective in the defense against oxidative stress by acting as a strong free radical scavenger, was identified in bluefin tuna (*Thunnus orientalis*) by Yamashita and Yamashita (2010) and Yamashita et al. (2012) (Krasnići et al., 2014). On the other hand, the higher proportion of Se eluted in LMM region in the liver compared to the gills, could be associated to several selenoproteins catalytically active in redox processes, such as glutathione peroxidase, iodothyronine deiodinase, and thioredoxin reductase (Hauser-Davis et al., 2012). Hepatic and gill Se profiles obtained for Vardar chub were mainly comparable with those previously reported for European chub (Krasnići et al., 2013, 2014).

Positions of Se peaks within the hepatic and gill distribution profiles were comparable in Vardar chub from all three rivers (Figs. 2b and 3b). However, notable quantitative difference was observed in the hepatic profiles of Vardar chub from the Bregalnica River in comparison to the Zletovska and the Kriva rivers. In the liver of Vardar chub from the Bregalnica River pronounced Se elution was observed within the second VLMM peak (2–5 kDa) whereas much lower Se proportion was eluted within the LMM region. In the chub from the other two rivers situation was opposite: the second VLMM peak was negligible, and Se was eluted in high quantity in LMM region (~25–50 kDa). This finding could be possibly associated to the type of pollution, since Bregalnica is contaminated with agricultural runoff, whereas the Zletovska and the Kriva rivers are contaminated with mining waste. The influence of Se environmental speciation on its fate in the fish organism should be further explored. The range of cytosolic Se concentrations in the liver of eight analyzed Vardar chub (Fig. 2b) was the following: 85.8–631.1 ng mL^{-1} (Table 3), whereas Se concentrations in the gills of

nine Vardar chub (Fig. 3b) ranged from 57.9–469.0 ng mL^{-1} (Table 3). Increasing cytosolic concentrations of Se in the liver of chub from mining impacted rivers have accordingly resulted with increased Se elution in LMM region and within the first VLMM peak (<2 kDa), whereas in the case of the gills increased Se bioaccumulation resulted exclusively with obvious sharp increase of the first VLMM peak (<2 kDa). The same was found for the liver and gills of European chub, with reported increases in LMM and VLMM regions, respectively (Krasnići et al., 2013, 2014).

3.1.7. Zinc

Zinc is an essential metal which has constitutive and catalytic roles in many proteins and enzymes (de la Calle Guntiñas et al., 2002). Zinc distribution profiles were determined for both hepatic and gill cytosols of Vardar chub (Fig. 2c and 3c). Zinc distribution profiles in liver were characterized by three peaks. The majority of Zn was eluted in narrow, sharp first peak associated to LMM biomolecules (7–24 kDa, with a maximum at 15 kDa) (Table 4, Fig. 2c), which coincided with the elution time of known Zn-binding protein, MT (Table 2). Furthermore, a considerable amount of hepatic Zn was found in MMM biomolecules region (~30–400 kDa, with a maximum at 66 kDa; Table 4, Fig. 2c), coinciding, for example, with elution time of standard protein alcohol dehydrogenase (Table 2), which is well known Zn-containing protein (Krasnići et al., 2013; Szpunar and Lobinski, 1999). The third Zn peak appeared within the void volume, and could be associated with HMM biomolecules (above ~500 kDa; Table 4, Fig. 2c). The comparison of hepatic and gill Zn profiles of Vardar chub (Figs. 2c and 3c) showed that Zn was much better resolved in the liver than in the gills. In the gills, it was eluted within two major peaks, mostly within one broad peak covering both LMM and MMM region (~10–300 kDa, with a maximum at 85 kDa, Table 4, Fig. 3c). Unlike in the liver, the clear peak which would indicate Zn binding to MTs was not observed in the gills, but MT elution time was still encompassed by the edge of this wide LMM-MMM peak. A portion of Zn was eluted in HMM region (above 400 kDa), same as observed in the hepatic Zn profile (Figs. 2c and 3c). Similar results as for Vardar chub were previously obtained for the liver and gills of European chub, where clear MT peak was observed only in Zn hepatic profiles (9–27 kDa), whereas in the gills MT peak was missing (Krasnići et al., 2013, 2014). Only difference between Zn profiles in Vardar and European chub was the presence of two high VLMM peaks in the gills (<5 kDa) only in European chub, which increased following the increase of Zn cytosolic concentrations (Krasnići et al., 2014).

Positions of Zn peaks within the hepatic and gill distribution profiles were comparable in Vardar chub from all three rivers (Figs. 2c and 3c). In the liver of nine analyzed Vardar chub (Fig. 2c) cytosolic Zn concentrations were within rather narrow range (3.5–8.4 μg mL^{-1}, Table 3), similar as in the case of gills of eight analyzed Vardar chub (Fig. 3c) (4.3–14.4 μg mL^{-1}, Table 3), and therefore it was not possible to comment on the changes which would occur in the case of increased Zn bioaccumulation.

3.1.8. Cadmium

Cadmium is an element without known functions in the organisms, and therefore possibly toxic already in very low concentrations. Cadmium distribution profiles were determined for both hepatic and gill cytosols of Vardar chub (Figs. 2d and 3d). Similarly to Cu, Cd was eluted within single clear and sharp peak in the LMM region (7–24 kDa, with a maximum at 15 kDa) in both organs of Vardar chub (Figs. 2d and 3d; Table 4). This peak had maximum at elution time of 31 min, which corresponded to elution time of standard MT-2 (Table 2). Obtained results confirmed the known high affinity of MTs for Cd binding, as a mechanism of protection against Cd toxicity (Roesijadi, 1992; Park et al., 2001). The same observations as made for Vardar chub liver and gills were previously made for European chub, with the major part of Cd bound to biomolecules of molecular masses and elution times which corresponded to MTs, both in the liver (9–27 kDa) and in the gills (4–35 kDa) (Krasnići et al., 2013, 2014).

Positions of Cd peaks within the hepatic and gill distribution profiles were comparable in Vardar chub from all three rivers (Figs. 2d and 3d). In the liver of nine analyzed Vardar chub (Fig. 2d), cytosolic Cd concentrations amounted to 2.4–68.2 ng mL^{-1} (Table 3), whereas in the gills of eight Vardar chub (Fig. 3d) they amounted to 0.3–33.8 ng mL^{-1} (Table 3). The range of cytosolic Cd concentrations was rather wide, thus enabling determination of the Cd distribution changes caused by prominent increase of Cd bioaccumulation. Increase of cytosolic Cd in both liver and gills of Vardar chub from mining impacted Zletovska and Kriva rivers resulted only with the increase of Cd elution within the probable Cd-MT peak, which was well coordinated with the cytosolic concentration changes. The presence of Cd-MT peak at all studied concentrations, from low in the Bregalnica River, to rather high in the Zletovska and Kriva rivers, indicated almost complete Cd detoxification in both organs of Vardar chub under the studied conditions, as previously observed by Lavradas et al. (2016) in the study on mussels. According to Goenaga Infante et al. (2003), MTs were found to be the most important Cd, Cu, and, to a lesser extent, Zn-binding compounds, and, therefore, MT induction can serve as a biological marker of metal exposure in fish.

3.2. Changes in the cytosolic distribution profiles of Cu, Zn, Cd, Mo, and Se after the heat-treatment

Heat-stable proteins and peptides are considered as detoxified fractions of trace elements in the cell, whereas heat-denaturated biomolecules are defined as fraction sensitive to trace elements, and a possible target of their toxicity (Giguère et al., 2006; Goto and Wallace, 2010; Rosabal et al., 2015). Applying the heat-treatment procedure used for analysis of MTs, which is based on cytosol heating at 70 °C for 10 min (Erk et al., 2002), we have removed the heat-sensitive proteins from the cytosol, and further on analyzed only trace element distribution among heat-stable biomolecules of different molecular masses. For that purpose, we have used SEC-HPLC with Superdex™ 75 column (linear separation range between 3 and 75 kDa), coupled offline with HR ICP-MS detection. In the Figs. 4 and 5, and Tables 5 and 6, we have presented only the information on the distribution profiles of those elements for which the clear presence of the heat-stable biomolecules was confirmed in the cytosols of liver (Cu, Zn, Cd, Mo, and Se) and of gills (Zn, Cd, Mo, and Se). The peaks obtained before and after the heat-treatment of the same samples were at first visually compared (Figs. 4 and 5). We have further calculated the percentage decreases or increases of eluted quantities of specific trace elements at specific elution times, which have occurred after the heat-treatment (Table 6), to determine if analyzed trace elements were bound to heat-stable biomolecules (unaltered peaks) or heat-sensitive biomolecules (decreased peaks). With the exception of the detailed studies of the heat-stable protein metallothionein (e.g., Rodríguez-Cea et al., 2003; Mason et al., 2004; Goenaga Infante et al., 2006; Hauser-Davis et al., 2012), to our knowledge the information on heat-stable proteins and peptides that bind specific trace elements in fish organs, and therefore possibly can serve in the process of detoxification, is yet not available in the scientific literature.

3.2.1. Distribution of Cu, Zn, and Cd in the heat-treated hepatic and gill cytosols

In the region of the studied molecular masses (approximately from 0.5 to 140 kDa, Table 5), Cu (Fig. 4a), Zn (Fig. 4d), and Cd (Fig. 4e) in the untreated hepatic cytosol were all eluted within the same LMM peak (~10–30 kDa, with the maximum at 20 kDa, Table 5), having the same elution time as MT-2 standard (Table 2). Only Zn was eluted within the additional MMM peak (~40–110 kDa, with the maximum at ~70 kDa, Table 5). After the heat-treatment, Zn-MMM peak was almost completely removed (Zn quantity decreased for 76%, Table 6), pointing to heat-sensitivity of those biomolecules, whereas metal-binding biomolecules within LMM peak were confirmed as heat-stable, based on

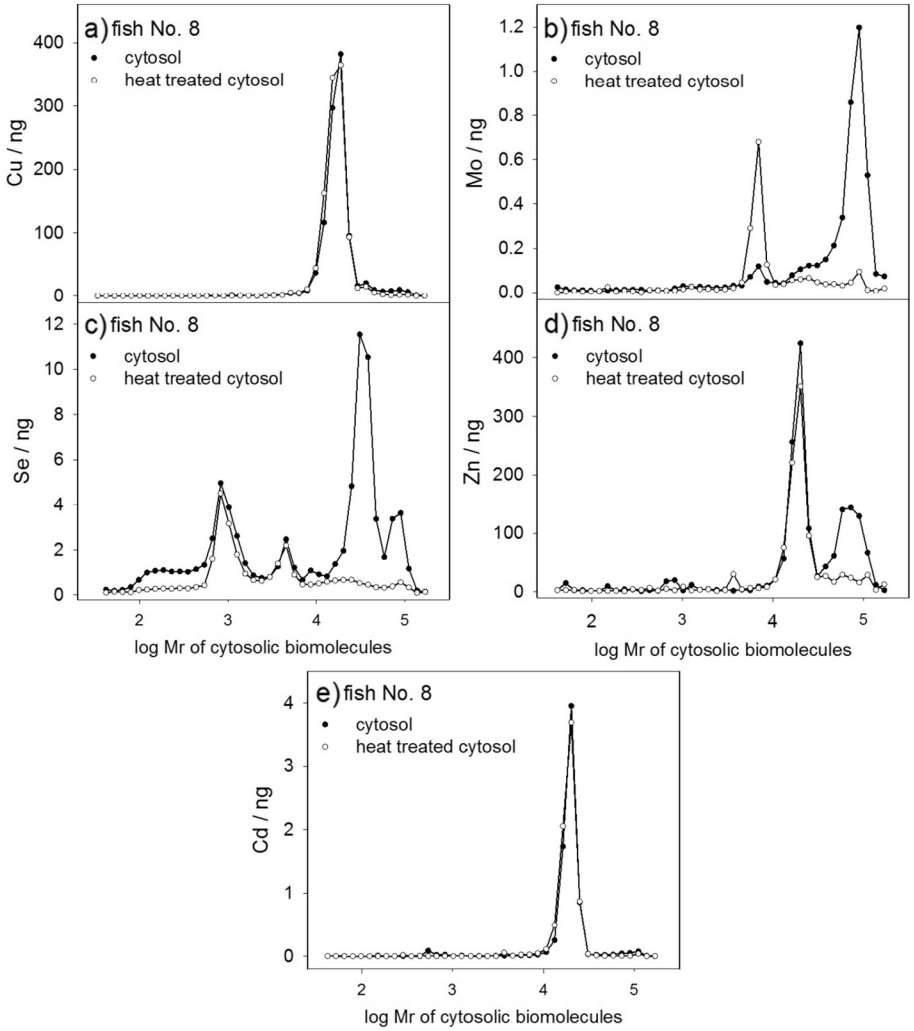

Fig. 4. Influence of heat-treatment on hepatic distribution profiles of five selected trace elements (a - Cu, b - Mo, c - Se, d - Zn, and e - Cd) among cytosolic proteins of different molecular masses in Vardar chub (*Squalius vardarensis*). The profiles were obtained by separation of hepatic cytosol and heat-treated cytosol on SEC-HPLC with Superdex™ 75 10/300 GL column and measurement of trace elements on HR ICP-MS. The results are presented as nanograms of trace elements eluted in the fractions containing proteins of specific molecular masses. One fish was used for these analyses (No. 8, Table 1), and its total trace element concentrations in hepatic cytosol are presented in Table 3.

the changes of metal quantity eluted within that peak which generally have not surpassed 10% (Table 6), and which further corroborated above suggested binding of Cu, Zn and Cd to MTs in the liver of Vardar chub.

In the gills, Cu profiles were not determined, due to rather low cytosolic Cu concentrations in the gills, both before and after the heat-treatment. In both gill samples, all of Zn (Fig. 5e, f) in untreated cytosols was eluted within the MMM peaks (~30–140 kDa, with the maximum at ~90 kDa, Table 5), which were almost completely removed after the heat-treatment (Zn quantity decreased for 76 and 86%, Table 6), and which coeluted with MMM peak of the hepatic cytosol. On the other hand, in each untreated gill sample Cd was eluted within two peaks (Fig. 5g, h). LMM peaks (~10–30 kDa, with the maximum at 20 kDa, Table 5), as already observed in the liver, indicated Cd binding to MTs, whereas the additional MMM peaks (~60–110 kDa, with the maximum at ~90 kDa, Table 5) indicated that part of Cd, which was accumulated in the gills, was not detoxified. Cadmium-LMM peaks were confirmed to contain heat-stable proteins, most probably MTs, since the decrease of Cd quantity eluted within that region after the heat-treatment amounted to ~10% (Table 6), whereas Cd-MMM peaks obviously comprised heat-sensitive proteins, since decrease of Cd quantity eluted within MMM region after the heat-treatment amounted to 65–75%.

Binding to heat-stable proteins, likely MTs, was thus demonstrated for Cu, Zn, and Cd in the liver, as well as for Cd in the gills of Vardar chub, whereas previously observed absence of Zn binding to MTs in the gills of Vardar chub in this study (Fig. 3c), and the European chub from the Sutla River (Krasnići et al., 2014), was further confirmed. Various authors have reported Cu, Zn and Cd association to MT proteins. In the liver of eel (*A. anguilla*) MTs were proven as the most important Cu-binding compounds and, to a lesser extent, Zn-binding compounds (Goenaga Infante et al., 2003). Lavradas et al. (2016), in the investigation of heat-stable metalloproteins in *Perna perna* mussels, also found that Cu was mostly bound to the proteins with lower MM (13 kDa).

3.2.2. Distribution of Mo in the heat-treated hepatic and gill cytosols

After the separation of untreated hepatic cytosol of Vardar chub on Superdex™ 75 column, Mo was eluted within two peaks (Fig. 4b). The first one was barely visible VLMM peak (5–9 kDa, with the maximum at 7 kDa, Table 5), whereas the second one was high and sharp MMM peak (~50–110 kDa, with the maximum at ~90 kDa, Table 5). After the cytosol heat-treatment, however, MMM peak was almost completely removed (decrease of 92%, Table 6), whereas VLMM peak has significantly increased (almost 300%, Table 6), as an indication that possibly due to heat-treatment, heat-sensitive MMM proteins or peptides have decomposed to small biomolecules of very low molecular masses, which are heat-stable. Contrary, in the gills, cytosolic Mo was eluted within single VLMM peaks (5–11 kDa, with the maximum at 7 kDa, Table 5) in both analyzed samples, even before the heat-treatment.

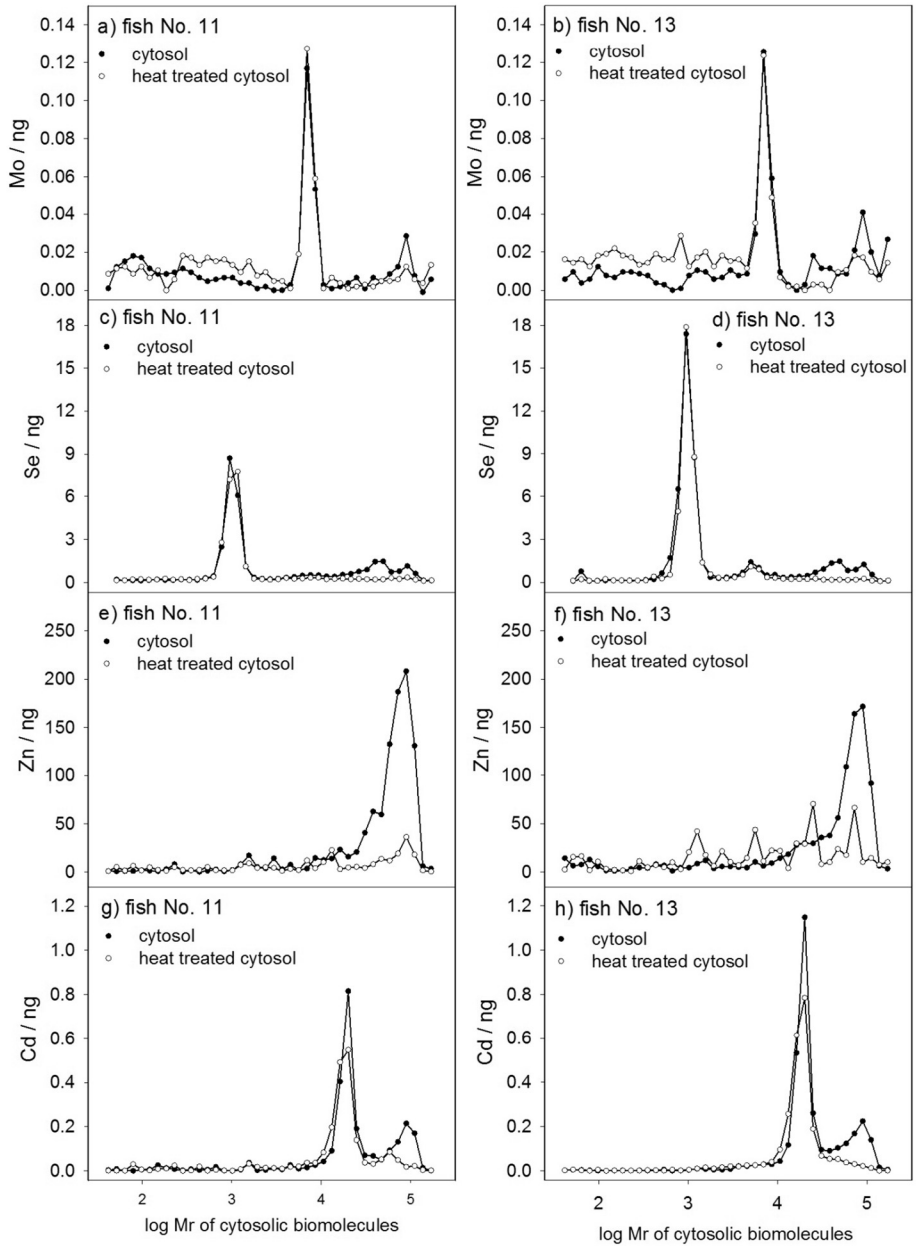

Fig. 5. Influence of heat-treatment on gill distribution profiles of four selected trace elements (a, b - Mo, c, d - Se, e, f - Zn, and g, h - Cd) among cytosolic proteins of different molecular masses in Vardar chub (*Squalius vardarensis*). The profiles were obtained by separation of gill cytosol and heat-treated cytosol on SEC-HPLC with Superdex™ 75 10/300 GL column and measurement of trace elements on HR ICP-MS. The results are presented as nanograms of trace elements eluted in the fractions containing proteins of specific molecular masses. Two fish were used for these analyses (No. 11 and 13, Table 1), and their total trace element concentrations in gill cytosols are presented in Table 3.

After the heat-treatment, these VLMM peaks remained practically unchanged (7–11% peak increase) (Table 6, Figs. 5a, b), thus proving the heat-stability of the compounds comprised within them. Since there is no information available about heat-stable biomolecules that bind Mo, it would be very interesting to further investigate the fate and character of Mo-binding compounds in the hepatic and gill cytosol of the fish.

3.2.3. Distribution of Se in the heat-treated hepatic and gill cytosols

Separation of untreated hepatic cytosol of Vardar chub on Superdex™ 75 column resulted with four Se peaks (Fig. 4c). The first two peaks were eluted within VLMM region (0.5–1.6 kDa, with the maximum at 0.8 kDa; and 3–6 kDa, with the maximum at 5 kDa; Table 5). The most of Se was eluted within the third, high and sharp, MMM peak (~20–50 kDa, with the maximum at ~30 kDa, Table 5). The fourth peak was also eluted within the MMM region (~60–110 kDa, with a maximum at ~90 kDa), but it was much smaller and corresponded to void volume of the column. After the cytosol heat-treatment, both MMM peaks were almost completely removed (decreases of approximately 90%, Table 6), whereas VLMM peaks were only slightly decreased (10–26%, Table 6), indicating that a portion of hepatic Se binds to small, heat-stable compounds within the cytosol. In both untreated gill samples, cytosolic Se was mostly eluted within the single, sharp and narrow VLMM peaks (0.5–1.3 kDa, with a maximum at 0.8 kDa; Fig. 5c, d), which coincided with the first peak in the hepatic cytosol (Fig. 4c; Table 5), and corresponded well with the results obtained for the gills after the cytosol separation on Superdex™ 200 column (Fig. 3b). These VLMM peaks were not affected by the heat-treatment (peak variations within 10%, Table 6). Selenium binding to VLMM compounds in both liver and gills of Vardar chub might correspond to some Se-containing peptides or selenoamino acids, and based on the analyses performed after the cytosol heat-treatment, it can be concluded that those compounds are heat-stable.

Table 5
Elution times (t_e) and molecular masses (MM) of cytosolic proteins from liver and gills of Vardar chub (*Squalius vardarensis*) contained within the fractions in which respective elements were eluted, after separation of cytosols and heat-treated cytosols by size exclusion HPLC with Superdex™ 75 10/300 GL column. Presented data refer to maximums of trace element peaks (i.e. to fractions with the highest trace element concentrations), whereas the numbers within the brackets refer to the beginnings and the ends of trace element peaks.

Element		Organ	VLMM peak 1[a]		VLMM peak 2[a]		LMM peak[b]		MMM peak 1[c]		MMM peak 2[c]	
			t_e/min	MM/kDa	t_e/min	MM/kDa	t_e/min	MM/kDa	t_e/min	MM/kDa	t_e/min	MM/kDa
Essential elements	Cu	Liver					23 (26–21)	20 (11 − 31)				
	Mo	Liver			28 (30–27)	7 (5–9)					16 (19–15)	89 (47–111)
		Gills			28 (30–26)	7 (5–11)						
	Se	Liver	38 (40–35)	0.8 (0.5–1.6)	30 (32–29)	5 (3–6)			21 (23–19)	31 (20–47)	16 (18–15)	89 (58–111)
		Gills	38 (40–36)	0.8 (0.5–1.3)								
	Zn	Liver					23 (26–21)	20 (11–31)			17 (20–15)	72 (38–111)
		Gills									16 (14–21)	89 (31–137)
Non-essential element	Cd	Liver					23 (25–21)	20 (13 − 31)				
		Gills					23 (25–21)	20 (13–31)			16 (18–15)	89 (58–111)

[a] VLMM peak – a peak of trace element concentration in the cytosolic fractions with a maximum in very low molecular mass protein region (<10 kDa).
[b] LMM peak – a peak of trace element concentration in the cytosolic fractions with a maximum in low molecular mass protein region (10–30 kDa).
[c] MMM peak – a peak of trace element concentration in the cytosolic fractions with a maximum in medium molecular mass protein region (30–100 kDa).

4. Conclusions

This comprehensive study of cytosolic distributions of eight trace elements (Co, Cu, Fe, Mo, Mn, Se, Zn, and Cd) in the liver and gills of Vardar chub (*S. vardarensis*) from three differently contaminated Macedonian rivers by use of metallomics approach, based on SEC-HPLC cytosol separation and HR ICP-MS detection, has enabled determination of the ranges of molecular masses of biomolecules that bind trace elements under different conditions of environmental exposure. Especially interesting findings were: (i) probable binding of Fe to storage protein ferritin observed in much higher proportion in the liver than in the gills; (ii) association with metallothioneins observed for Cd, Cu and Zn in the liver, and Cd in the gills; (iii) Se binding to compounds of very low molecular masses, below 5 kDa, observed in both organs, but in higher proportion in the gills. Additional study of distributions of trace elements in the hepatic and gill cytosols after their heat-treatment further confirmed binding of Cd, Cu and Zn to heat-stable proteins, probably MTs, as well as existence of the heat-stable cytosolic compounds of very low molecular masses (<10 kDa) that bind Mo and Se. Analysis of the influence of increased environmental exposure to trace elements on their cytosolic distributions in liver and gills of Vardar chub revealed that the changes were generally of quantitative and not qualitative nature. In other words, trace elements under all studied conditions were found associated to the same biomolecules, and only their proportions associated to specific cytosolic compounds have changed as a consequence of their increased bioaccumulation in the liver and gills of Vardar chub. Comparison of the results obtained for the liver and gills of Vardar chub from the Macedonian rivers with previously published information on the same organs of European chub (*S. cephalus*) from the Sutla River (Croatia) indicated that distributions of trace elements are generally comparable in these two species from the same genus, *Squalius*, which enables their comparative use in the monitoring programmes, even in the distant parts of the world.

Table 6
Heat-treatment induced changes in distribution profiles of Cu, Mo, Se, Zn and Cd in cytosol of chub liver and gills, after separation by SEC-HPLC with Superdex™ 75 10/300 GL column. The results are presented as percentage decrease (−) or increase (+) of metal quantity in certain peaks which occurred after heat treatment of the cytosol.

	Protein MM	Liver	Gills	
		Fish No. 8	Fish No. 11	Fish No. 13
Cu	11–31 kDa	+8%		
Mo	5–9 kDa	+272%	+11%	+7%
	47–111 kDa	−92%		
Se	0.5–1.6 kDa	−26%	+2%	−6%
	3–6 kDa	−10%		
	20–47 kDa	−89%		
	58–111 kDa	−92%		
Zn	11–31 kDa	−11%		
	38–111 kDa	−76%	−86%	−76%
Cd	13–31 kDa	+5%	−10%	−11%
	58–111 kDa		−65%	−75%

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