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Comparison of intracellular trace element distributions in the liver and gills of the invasive freshwater fish species, Prussian carp (*Carassius gibelio* Bloch, 1782)

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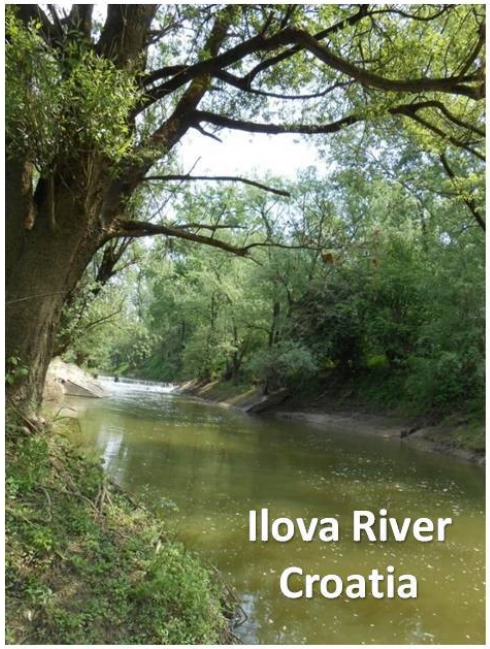


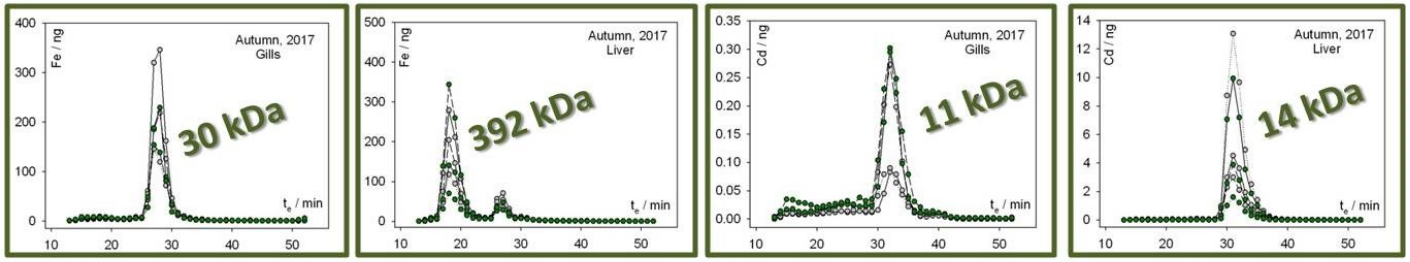
Prussian carp (Carassius gibelio)

Invasive fish species
- high tolerance to contamination

Intracellular metal/nonmetal fate

Cd Cu Zn Fe Mo Se

SEC-HPLC separation → **HR ICP-MS detection**



Fe in gills and liver **Cd in gills and liver**

15

Abstract

Prussian carp (*Carassius gibelio*) is an invasive freshwater fish known for its high tolerance to aquatic pollution. Our aim was to try to clarify its tolerance to increased exposure to metals/nonmetals, by determining their cytosolic distributions among peptides/proteins of different molecular masses (MM), which form a part of the fish protective mechanisms. The applied approach consisted of fractionation of gill and hepatic cytosols of Prussian carp from the Croatian river Ilova by size-exclusion high performance liquid chromatography, whereas Cd, Cu, Zn, Fe, Mo, and Se analyses were done by high resolution inductively coupled plasma mass spectrometry. The results indicated high detoxification of Cd by its binding to metallothioneins (MTs) in both fish organs. In addition, binding to MTs was observed for Cu in both organs and for Zn in the liver, whereas clear Zn binding to MTs in the gills was not recorded. Zinc in the gills was predominantly bound to proteins of higher MM (50-250 kDa) and to biomolecules of MM below 2 kDa. Predominant Fe binding to proteins of MM of ~400 kDa (presumably storage protein ferritin) was observed in the liver, whereas in the gills Fe was mainly associated to proteins of MM of ~15-65 kDa (presumably hemoglobin oligomers). Maximum Mo and Se elutions in the liver were noted at 235 kDa and 141 kDa, respectively, and in the gills below 10 kDa. The striking difference was observed between two organs of Prussian carp, with predominant metal/nonmetal binding to high MM proteins (e.g., enzymes, storage proteins) in the liver, and to very low MM biomolecules (<10 kDa) in the gills (e.g., antioxidants, metallochaperones, nonprotein cofactors). Such metal/nonmetal distributions within the gills, as the first site of defense, as well as association of several metals to MTs, indicated highly developed defense mechanisms in some organs of Prussian carp.

Key words: antioxidants, cytosol, enzymes, metallothionein, metals, size-exclusion

1. Introduction

Prussian carp (*Carassius gibelio* Bloch, 1782) is an invasive fish species which is a member of the Cyprinidae family; it is dominating in stagnant and slow-flowing waters and has very wide ecologic tolerance (Ergüden, 2015), including extreme tolerance to hypoxia-anoxia, ammonia, various water pollutants and temperature variability (Perdikaris et al., 2012; Topić Popović et al., 2016). It has widely spread in freshwaters of southern Europe following the accidental introduction (Leonardos et al., 2008). Since it hinders the growth of native fish populations, through competition for food and space and inhibition of their reproductive activity, it can even cause disappearance of native fish species (Ergüden, 2015; Topić Popović et al., 2016). Accordingly, it can rapidly increase in number in the inland waters (Ergüden, 2015), and become the dominant species in new habitat in a short time (Yerli et al., 2014).

In the study of the influence of treated wastewaters of municipal, hospital and industrial origin on Prussian carp, that fish species showed the ability to compensate for adverse environmental changes and demonstrated a high capacity to live in the waters of diminished quality, which were contaminated, among others, with several metals (Topić Popović et al., 2016). Ever increasing presence of metals in freshwater ecosystems is a consequence of various human-related activities, such as mining, metal smelting and other industries (Nriagu et al., 1998; Rosabal, 2015) and can result with metal bioaccumulation in the organs of freshwater biota (e.g. fish) through water and/or food, finally leading to toxic effects (Luoma et al., 2005, 2008; Rosabal, 2015). Tolerance to metal toxicity observed in some organisms, like Prussian carp, can be related to a variety of protective mechanisms that control the uptake, distribution and excretion of metals, and include metal binding to a variety of cellular ligands (e.g., metal-binding proteins) (Rainbow, 2002; Rosabal, 2015).

With the aim to identify metal species present in a cell or tissue, and determine their localization and quantity, a field of investigation was developed rather recently, called metallomics, which also includes the study of metal-binding proteins or metalloproteins (Lavradas et al., 2016). One of the preferred approaches in the study of metalloproteins is the combination of their chromatographic separation by size exclusion high performance liquid

chromatography (SEC-HPLC) and detection by inductively coupled plasma mass spectrometry (ICP-MS) (Lavradas et al., 2016). Such methodological approach have been applied for determination of trace metals bound to cytosolic biomolecules of different sizes in many aquatic organisms (Goenaga Infante et al., 2003), for example in bivalve molluscs such as mussels (*Mytilus edulis*; Ferrarelo et al., 2000; *Perna perna*; Lavradas et al., 2016; *M. galloprovincialis*; Strižak et al., 2014), and in fish such as common carp (*Cyprinus carpio*; Goenaga Infante et al., 2002; Van Campenhout et al., 2004), European and Vardar chub (*Squalius cephalus* and *Squalius vardarensis*; Krasnići et al., 2013, 2014, 2018, 2019), brown trout (*Salmo trutta*; Dragun et al., 2018), yellow perch (*Perca flavescens*; Caron et al., 2018), white sucker (*Catostomus commersonii*; Urien et al., 2018), and European eel (*Anguilla anguilla*; Goenaga Infante et al., 2003).

The general aim of this study was to try to partially elucidate the metal/nonmetal handling strategies of Prussian carp, which present the fundamental part of its high tolerance to detrimental environmental conditions. Specifically, we wanted to describe the cytosolic distributions of several trace elements, namely of nonessential metal Cd, four essential metals (Cu, Zn, Fe, and Mo) and essential nonmetal Se among biomolecules of different molecular masses in two organs of this invasive fish species inhabiting the Ilova River in Croatia. Our hypothesis is that the changes in the metal/nonmetal exposure and bioaccumulation will cause the observable changes in their intracellular distributions, either of qualitative or quantitative nature. The selected organs for the analyses were the gills, as the organ of direct contact with the aquatic environment and the site of metal/nonmetal waterborne uptake (Bury et al., 2003; Sauliutė and Svecevičius, 2015), and the liver as the central metabolic, detoxification and storage organ of fishes that has numerous anabolic and catabolic functions (Jordanova et al., 2016; Peters et al., 1987). Our additonal aim was to establish the differences in metal/nonmetal behaviour between the gills and the liver that can occur due to the variable functions of those organs, as well as to check for possible variations of cytosolic metal/nonmetal distributions that can be caused by other factors, such as the seasonal changes in fish physiology or different

95 water quality at different sites associated to other parameters in addition to metal
96 contamination.
97
98 **2. Materials and methods**
99 *2.1. Study period and area*
100 The study was performed at two sites at the Ilova River (Fig. 1) in two seasons, autumn 2017
101 (October 5th) and spring 2018 (May 3rd and 4th). Location near the Ilova village (site 1;
102 45°26'45.08'' N 16°49'43.34'' E) was chosen as a reference site, since it is situated upstream
103 of known sources of pollution, such as municipal and industrial wastewater outlets. Location
104 near the Trebež village (site 2; 45°21'21.21'' N 16°46'26.16'' E), situated 16 km downstream
105 from the reference site and approximately 8 km downstream of the site where the Kutinica
106 River inflows into the Ilova River, was chosen as the potentially contaminated site, since the
107 Kutinica River receives the municipal wastewaters of the Kutina town and industrial
108 wastewaters of the fertilizer factory. The Trebež site was located in the vicinity of the Ilova
109 River mouth into the Sava River (De Coninck et al., 2018). Dissolved concentrations of six
110 studied trace elements in the river water at the reference location (the Ilova village) in October
111 2017 were the following (in μg L^{-1}): Se, 0.786±0.019; Mo, 0.561±0.027; Cd, 0.011±0.006; Fe,
112 17.9±2.17; Cu, <0.4; and Zn, <7.3 (De Coninck et al., 2018). Dissolved concentrations of the
113 studied trace elements in the river water at the contaminated location (the Trebež village) in
114 October 2017 were the following (in μg L^{-1}): Se, 1.01±0.112; Mo, 0.981±0.062; Cd,
115 0.053±0.003; Fe, 21.6±1.52; Cu, 0.716±0.030; and Zn, <7.3 (De Coninck et al., 2018). Based
116 on the presented dissolved metal/nonmetal concentrations in the Ilova river water at two
117 sampling sites, somewhat increased water metal contamination referred only to Cd at the
118 Trebež site, as a possible sign of the influence of fertilizer production.
119 *2.2. Fish sampling and dissection of the gills and liver*
120 This study was performed using fish species Prussian carp (*Carassius gibelio* Bloch, 1782).
121 The sampling by electrofishing using electrofisher Hans Grassl (EL63 II GI, 5.0 KW, 137
122 Honda GX270, 300/600V max., 27/15A max.) was carried out in accordance with the Croatian

123 standard HRN EN 14011 (2005). Several studies have shown electrofishing to be among the
124 most effective techniques for fish sampling in freshwater habitats, as well as much less
125 disturbing than many other invasive techniques of sampling (e.g. netting, long-lining, etc.)
126 (IMBRIW, 2013; Jenkins, 2014; Yoder and Smith, 1998). The applied electrofisher uses direct
127 current (it has also pulse current output, but it was not used), which is shown to be less harmful
128 than alternate current output (Snyder, 2003). Moreover, we have used the appropriate
129 electrofishing protocol (2-4 A, duration few seconds) to achieve only the short-term fish
130 stunning and thus minimize the potential for electrofishing injury to fishes, as recommended by
131 many authors (Dean and Temple, 2011; IMBRIW, 2013). During transportation, the captured
132 fish were kept alive in an opaque plastic tank filled with aerated river-water, which was taken
133 from each respective sampling site. In the laboratory, the fish were anesthetized using
134 unbuffered tricaine methane sulphonate (MS 222, Sigma Aldrich) in accordance with the
135 Ordinance on the protection of animals used for scientific purposes (NN 55/2013) and applying
136 the dosage suggested by Topić Popović et al. (2012) and Xu et al. (2008) (~50 mg L^{-1}, duration
137 under 10 minutes), and then euthanized. Although some authors recommend MS-222 buffering,
138 due to its ability to lower water pH as a result of the formation of methanesulfonic acid, some
139 studies suggest that MS-222 has a minimal effect on the water acidity (Alpharma, 2001).
140 Specifically, Xu et al. (2008) have reported that unbuffered MS-222 lowers pH of water in the
141 fish tank below neutral values (~7) only if applied in concentrations above 50 mg L^{-1}. We have
142 then recorded fish total masses and lengths, as well as their sex by gonad examination at
143 macroscopic level. The gills and the liver were then isolated and stored at -80°C for further
144 analyses. During two sampling campaigns, in total 83 fish were sampled for the assessment of
145 metal bioaccumulation and biomarker responses, 20 to 23 at each site in each sampling
146 campaign. Out of all caught fish, we have selected in total 11 fish specimens for the analyses of
147 the cytosolic metal distributions in the gills, and 12 fish specimens for the analyses in the liver,
148 two or three per each site in each season. Biometric characteristics of these fish are presented in
149 Table 1 (referring to gill analyses) and Table 2 (referring to hepatic analyses).

2.3. The preparation of gill and hepatic samples for the analyses

The preparation of gill and hepatic samples for the analyses, with an aim to isolate the cytosolic fractions, was comprised of homogenization and centrifugation steps described in detail in our previous papers (Dragun et al., 2018a,b). According to described procedure, the samples of gills and liver were shreded in ice-cold glass vessels. A volume of cooled homogenization buffer [100 mM Tris-HCl/Base (Sigma, pH 7.5 at 4°C) supplemented with reducing agent (1 mM dithiotreitol, Sigma)] was added into each vessel (w/v 1:5) and the obtained suspensions were homogenized in an ice cooled tube by 10 strokes of Potter-Elvehjem homogenizer (Glas-Col, USA) set at 6,000 rpm. The homogenates were centrifuged for 2 h at 50,000×*g* in the Avanti J-E centrifuge (Beckman Coulter, USA) cooled at +4°C. Obtained supernatants (S50) represented cytosolic tissue fractions, which, according to procedure described by Bonneris et al. (2005), futher contained microsomes. The cytosolic fractions isolated from the gills and the liver of Prussian carp were immediately stored at -80°C until further analyses. The applied sample preparation procedure has followed the recommendations of Szpunar et al. (2003), to use buffers at the physiological pH and antioxidant in homogenization step, refrigerated centrifuge in the centrifugation step, and immediate freezing of the obtained cytosols, in order to avoid dissociation of the complexes, i.e. to limit the change of the equilibrium of trace elements and biomacromolecules in the cells and tissues.

2.4. Chromatographic fractionation of gill and hepatic cytosols of Prussian carp

To obtain information on cytosolic distributions of six trace elements (Cd, Cu, Zn, Fe, Mo and Se) among metal-binding biomolecules of various molecular masses, cytosols isolated from the gills and liver of Prussian carp were fractionated using SEC-HPLC system (Perkin Elmer, series 200, USA). According to de la Calle Guntiñas et al. (2002), SEC-HPLC is possibly the best chromatographic option for the separation of metal-binding proteins, because it is a mild method with theoretically no chemical reactions, which secures that the conformation and activity of the protein do not alter. Prepacked size exclusion column Tricorn™ Superdex 200 10/300 GL (GE Healthcare Biosciences, USA) for globular proteins, with a separation range

177 from 10 to 600 kDa, was applied under conditions which were previously described by Krasnići
178 et al. (2013, 2014, 2018, and 2019) and Dragun et al. (2018b). Applied mobile phase was 20
179 mM Tris-HCl/Base (Sigma, pH 8.1 at 22°C), a flow rate was 0.5 mL min^{-1}, whereas mode was
180 isocratic, in accordance with the recommendation to use weak alkaline eluents for separation of
181 metal-binding proteins to avoid dissociation of the metals (de la Calle Guntiñas et al., 2002).
182 Each sample was run two times consecutively through the column, using a volume of 100 µL
183 per each run, or a total volume of 200 µL. One-minute fractions were collected in the plastic
184 tubes using a fraction collector FC 203B (Gilson, USA), starting at 13^{th} minute and ending at
185 52^{nd}. The void volume of the column was determined by use of blue dextran (Table 3). The
186 equation of column calibration straight line was calculated using elution times of seven protein
187 standards (thyroglobulin, apoferritin, β-amylase, alcohol dehydrogenase, bovine albumin,
188 superoxide dismutase and carbonic anhydrase; Sigma, USA) (Table 3), which were run through
189 the column under the same conditions as the samples. The elution time was also determined for
190 MT standard, MT-2 (Enzo Life Sciences, USA) (Table 3).
191 *2.5. Measurement of trace element concentrations in the gill and hepatic cytosols of Prussian*
192 *carp, and in the SEC-HPLC fractions of gill and hepatic cytosols*
193 The concentrations of six trace elements (essential elements Cu, Zn, Fe, Mo, and Se;
194 nonessential element Cd) were measured in the cytosols of Prussian carp gills and liver, as well
195 as in one-minute fractions obtained by SEC-HPLC separation of cytosols, as previously
196 described (Dragun et al., 2018a,b; Krasnići et al., 2013; 2014; 2018; 2019). Prior to
197 measurement, gill and hepatic cytosols were digested in duplicate in the laboratory dry oven at
198 85°C for 3.5 h, after the addition of concentrated HNO_3 (225 µL; *Rotipuran® Supra* 69%, Carl
199 Roth GmbH + Co. KG, Germany) and 30% H_2O_2 (75 µL; *Suprapur®*, Merck, Germany) into
200 each sample (300 µL). Digested samples were afterwards five times diluted with Milli-Q water
201 (Dragun et al., 2018a). SEC-HPLC fractions were only acidified with HNO_3 (*Suprapur*, Merck,
202 Germany, final acid concentration in the samples 0.16%) prior to measurement. To all the
203 samples, In (Fluka, Germany) was added as an internal standard (1 µg L^{-1}).

The measurements were performed using high resolution (HR) ICP-MS (Element 2, Thermo Finnigan, Germany), equipped with an autosampler SC-2 DX FAST (Elemental Scientific, USA) and sample introduction kit, which consisted of cyclonic spray chamber Twister and SeaSpray nebulizer. Measurements were performed in low-resolution mode for ^{82}Se, ^{98}Mo, and ^{111}Cd, and in medium-resolution mode for ^{56}Fe, ^{63}Cu, and ^{66}Zn. External calibration was performed using adequately diluted multielement standard solution for trace elements (Analitika, Czech Republic), prepared in 1.3% HNO_3 (*Suprapur*; Merck, Germany), in which In (1 μg L^{-1}; Fluka, Germany) was added as an internal standard. Limits of detection (LOD) for trace elements measured in cytosol were determined as three standard deviations of ten consecutively measured trace element concentrations in the blank samples, consisting of Tris-HCl/Base buffer, dithiothreitol, H_2O_2 and HNO_3, which were prepared for measurement in the same way as the samples. LODs for trace elements measured in the digested cytosols were the following (in ng g^{-1}): Cd, 0.430; Cu, 13.5; Fe, 141; Mo, 0.680; Se, 2.93; and Zn, 635 (Dragun et al., 2018a). LODs for trace elements measured directly in the SEC-HPLC fractions were the following (in μg L^{-1}): Cd, 0.005; Cu, 0.037; Fe, 0.084; Mo, 0.004; Se, 0.138; and Zn, 2.40 (Dragun et al., 2018b; Krasnići et al., 2013; 2014; 2018; 2019). The accuracy verification of HR ICP-MS measurements was based on 14 independent analyses in quality control sample for trace elements (catalog no. 8072, lot no. 146142-146143, UNEP GEMS, Canada), and the recoveries were the following (average±standard deviation): Cd, 92.5±3.1%; Cu, 95.3±3.2%; Fe, 101.1±7.4%; Mo, 96.9±2.9%; Se, 92.8±8.8%; and Zn, 96.9±4.1%.

2.6. Data processing and statistical analyses

Required calculations were performed in Microsoft Excel 2007, whereas graphs were created in statistical program SigmaPlot 11.0 for Windows.

3. Results and discussion

Prussian carps (*C. gibelio*) used for the analyses of metal/nonmetal distributions in the gills were 14.9-23.7 cm long, and their masses ranged from 50.1 to 239.3 g; they included seven female and four male specimens (Table 1). Fish used for the analyses of metal/nonmetal

232 distributions in the liver were 14.8-27.2 cm long, and their masses ranged from 43.0 to 339.2 g;
233 they included nine female and three male specimens (Table 2). Fish chosen for the analyses in
234 both organs were, therefore, of similar biometric characteristics, and of uniform size
235 distribution among seasons and sites, with female predominance. The exception from size
236 uniformity referred only to generally smaller fish at the Ilova village in autumn 2017, whereas
237 the female predominance in the group of fish selected for chromatographic analyses was in
238 accordance with the whole sampled population, which contained ~60% of female Prussian
239 carps. Female predominance in Prussian carp populations was already reported by several
240 authors (e.g., Ergüden, 2015; Uysal et al., 2015). For the analyses presented in this paper we
241 have deliberately chosen the fish specimens with variable metal/nonmetal cytosolic
242 concentrations in the gills and liver, with the aim to enable detection of differences in cytosolic
243 metal/nonmetal distributions at different bioaccumulation levels. Thus, the concentrations
244 presented in Tables 1 and 2 cannot be considered as indicators of the exposure level in the river
245 water.
246 Distribution profiles of six analyzed elements (Cu, Zn, Cd, Fe, Mo, and Se) among cytosolic
247 metal/nonmetal-binding biomolecules of different molecular masses in the gills and liver of
248 Prussian carp, which will be discussed below, are presented in Figs. 2-7, separately for each
249 organ and each season, to observe the possible differences that could occur due to physiological
250 variability caused by different functions of gills and liver or by different metabolic statuses in
251 different periods of the year. The spatial origin of the analyzed fish is also indicated within the
252 figures, with the aim to record the possible influence of the variable living conditions on the
253 cytosolic metal/nonmetal distributions.
254 The elution times of metal/nonmetal-containing chromatographic peaks presented in the Figs.
255 2-7 were associated to molecular masses of specific metal/nonmetal-binding cytosolic
256 biomolecules (Table 4), using the calibration straight line of the applied chromatographic
257 column (Table 3). As already described in all our previous studies of cytosolic metal/nonmetal
258 distributions characteristic for various fish species, for the purposes of easier discussion we
259 always use the categorization of cytosolic biomolecules in the following four classes, according

to their molecular masses (MM) (Table 4) (Dragun et al., 2018b; Krasnići et al., 2013, 2014, 2018, 2019): 1) HMM class – containing the biomolecules of high molecular masses (>100 kDa); 2) MMM class – containing the biomolecules of medium molecular masses (>30-100 kDa); 3) LMM class – containing the biomolecules of low molecular masses (10-30 kDa); and 4) VLMM class – containing the biomolecules of very low molecular masses (<10 kDa). As SEC-HPLC has an inherent drawback of low resolution, providing in most cases no more than 6 to 8 peaks (de la Calle Guntiñas et al., 2002), it is reasonable to expect that many biomolecules will coelute, especially in the complex samples, such as cytosols of fish organs. Therefore, the obtained peaks for six metals/nonmetal in gills and liver of Prussian carp, which will be discussed below, in many cases probably reflected the binding of analyzed metals/nonmetal to more than one cytosolic biomolecule.

3.1. Copper, zinc and cadmium distribution profiles

Both Cu and Zn play an important role in numerous aspects of cellular metabolism and allow many critical enzymes to function properly (Classen et al., 2011; Harris, 2001; Osredkar and Sustar, 2011). Copper proteins have diverse roles in biological electron transport and oxygen transportation (Lippard and Berg, 1994; Osredkar and Sustar, 2011). Zinc functions in biology can be separated into three main categories: catalytic, regulatory, and structural (Osredkar and Sustar, 2011; Stipanuk, 2006). In blood plasma, Zn can be bound to and transported by albumin and transferrin (Whitney and Rolfes, 2010), whereas carbonic anhydrase and carboxypeptidase, which are vital to the processes of carbon dioxide regulation and digestion of proteins, respectively (Lindskog, 1997; Zundahl, 1998), represent the examples of Zn-containing enzymes (Osredkar and Sustar, 2011). Both Cu and Zn can also act as antioxidants, either as essential components of Cu/Zn superoxide dismutase, an enzyme that detoxifies superoxides by converting them to oxygen and hydrogen peroxide, or through induction of the synthesis of metallothioneins (MT) which can bind metals with pro-oxidant activity, such as Cd, and scavenge hydroxyl radicals and singlet oxygen with their thiol groups (Banni et al., 2011; Dondero et al., 2005; Osredkar and Sustar, 2011). Cadmium, on the other hand, is a

287 highly toxic element that has no known biological function and can cause adverse effects to all
288 living organisms (Banni et al., 2011).
289 The common characteristics of Cd, Cu and Zn in both gills and liver of Prussian carp (Figs. 2-
290 4) was their elution in the region of LMM biomolecules (t_e 31th-33rd minute) with the maximum
291 at MM of 8-14 kDa (Table 4). These peaks corresponded well with the peak of MT standard,
292 which was, under the same conditions, eluted in 31st minute (Table 3). MTs are a family of low
293 molecular mass, cysteine-rich proteins (up to 30% of their constituent aminoacids), which have
294 the capacity to bind both essential metals, like Cu and Zn, and nonessential metals, like As and
295 Cd, by the thiol groups of their cysteine residues (Paris and Usher, 2019). In the liver of
296 Prussian carp, the elution in MT region was very clear, with sharp peaks, for all three metals
297 (Figs. 2c,d; 3c,d; 4c,d). The association of Cd, Cu and Zn with biomolecules of MM in the
298 range from ~10-30 kDa, presumably MTs, was previously observed in the liver of European
299 and Vardar chub (*S. cephalus* and *S. vardarensis*), as well as of brown trout (*S. trutta*) (Dragun
300 et al., 2018; Krasnići et al., 2013, 2018). Contrary, in the gills of Prussian carp only Cd elution
301 associated to MTs was clear and evident (Fig. 2a,b), same as observed for the gills of European
302 and Vardar chub (Krasnići et al., 2014, 2018), whereas it was not the case for the other two
303 metals (Figs. 3a,b; 4a,b). In the case of Cu, an indication of its binding to MTs in the gills of
304 Prussian carp was observed (Fig. 3a,b), but due to much lower cytosolic Cu concentrations in
305 the gills compared to the liver (10-100 times lower; Tables 1 and 2), Cu elution peaks could not
306 be clearly defined, same as previously reported for the gills of European and Vardar chub
307 (Krasnići et al., 2014, 2018). On the other hand, for Zn, which was present in both organs of
308 Prussian carp either in comparable cytosolic concentrations or even higher in the gills (Tables 1
309 and 2), the elution in the Prussian carp gills was much more pronounced in the protein regions
310 other than MTs, and its presence in the MT region was observed only as a slight uplift from the
311 baseline (Fig. 4a,b). Van Campenhout reported similar finding for the kidney of common carp
312 (*C. carpio*), where only 2% of cytosolic Zn was bound to MTs, despite much higher renal than
313 hepatic Zn concentrations. The absence of clear Zn binding to MTs in the gills of Prussian carp
314 was consistent with previous findings on the gills of European and Vardar chub, where the

315 majority of Zn was bound to biomolecules of higher MM (Krasnići et al., 2014, 2018, 2019).
316 Additional analyses of MT fractions from Vardar chub liver and gills by anion-exchange HPLC
317 and MALDI-TOF-MS revealed the presence of two MT isoforms in each organ, MT-I and MT-
318 II; molecular masses of both hepatic isoforms were identical (6.0 kDa), but differed from gill
319 isoforms (4.9 kDa) (Krasnići et al., 2019). This finding, together with confirmation that MT
320 isoforms from the gills, unlike the hepatic ones, did not bind Zn, indicated the possibility of
321 different MT functions in these two organs (Krasnići et al., 2019).
322 Futhermore, Cd, as nonessential metal, was in both organs of Prussian carp eluted only within a
323 single sharp peak associated to MTs, and no additional Cd-protein associations were observed
324 (Fig. 2). That was consistent with predominant Cd elution with MTs in the livers of other fish
325 species, such as white suckers (*C. commersonii*) (Urien et al., 2018), common carp (Van
326 Campenhout et al., 2004) and European eel (*A. anguilla*) (Goenaga Infante et al., 2003).
327 Contrary, Cu and Zn, as essential metals, were observed to sometimes bind to proteins from
328 other MM regions (Figs. 3 and 4). In the liver of Prussian carp, Cu was, comparable to Cd,
329 associated solely to MT region (Fig. 3c,d), same as in the liver of European and Vardar chub,
330 and of white suckers (Krasnići et al., 2013, 2018; Urien et al., 2018). However, in the gills of
331 Prussian carp an indication of Cu binding to MMM proteins (maximum elution at 30 kDa) was
332 also observed (Fig. 3a,b; Table 4), which, for example, encompassed the MM of Cu-containing
333 cytosolic enzyme superoxide dismutase (32 kDa; Pedrajas et al., 1993). It was interesting that
334 the liver of brown trout from the Krka River exhibited higher similarity to the gills than to the
335 liver of Prussian carp, with observable Cu elution reported within the range of biomolecules of
336 higher MM (above 85 kDa; Dragun et al., 2018). Binding of Cu to biomolecules of higher MM
337 was previously also described for the gills of European chub, and covered MM up to 500 kDa
338 (Krasnići et al., 2014). Zinc in the liver of Prussian carp was also predominantly associated to
339 MT region, same as Cd and Cu, but some elution was also observed in two HMM regions
340 (maximum elutions at MMs of 100-200 kDa and above 600 kDa) (Fig. 4c,d; Table 4). Elution
341 of Zn in those same regions of higher MM was also observed in the liver of both chub species
342 and of brown trout (Dragun et al., 2018; Krasnići et al., 2013; 2018). Contrary to liver, in the

343 gills of Prussian carp predominant Zn elution was found precisely in those two HMM protein
344 regions (Fig. 4a,b; Table 4), again comparable to the gills of European and Vardar chub
345 (Krasnići et al., 2014, 2018). The elution of Zn in the mentioned regions of higher MM could
346 refer to a number of Zn-containing proteins and enzymes, such as Zn transport proteins albumin
347 (66 kDa, Table 3) and transferrin (70-80 kDa; Sun et al., 2012), and cytosolic enzymes carbonic
348 anhydrase (29.7 kDa in the kidney of Black Sea trout (*Salmo trutta labrax*); Kucuk and Gulcin,
349 2016), superoxide dismutase (32 kDa; Pedrajas et al., 1993), and alcohol dehydrogenase (80
350 kDa in vertebrates, Thompson et al., 2018). Fish gills are, furthermore, known as carbonic
351 anhydrase rich tissue (Gilmour and Perry, 2009). In the gills of Prussian carp, Zn was further
352 eluted within a sharp peak in VLMM biomolecule region (maximum elution at MM below 2
353 kDa) (Fig. 4a,b; Table 4), whereas no sign of such Zn elution was seen in the samples of
354 hepatic cytosols (Fig. 4c,d). Zinc elution with biomolecules of MM below 5 kDa was
355 previously reported only for the gills of European chub (Krasnići et al., 2014), including MM of
356 reduced glutathione (GSH, ~307 Da,
357 https://pubchem.ncbi.nlm.nih.gov/compound/Glutathione). GSH is the intracellular thiol
358 compound, i.e. tripeptide composed of cysteine, glutamic acid and glycine, which plays a major
359 role in the protection of cells from oxidative injury, and can be present within the cells free or
360 bound to proteins (Iwasaki et al., 2009). GSH was also reported as capable of complexing with
361 metal cations and detoxifying them soon after they enter the cells, thus being the first line of
362 defense against metal toxicity (e.g., Cd), preceding even MT induction (Canesi et al., 1999;
363 Lavradas et al., 2016; Saad et al., 2016).
364 Increases of the bioaccumulation levels of all three metals in both organs were reflected in the
365 increases of already existing peaks, and no new metal-protein associations were observed as a
366 consequence of seasonal or spatial impact. For all three metals in the liver and Cd in the gills of
367 Prussian carp, the increases of their cytosolic concentrations were followed by increased elution
368 in MT region (Figs. 2a-d, 3c,d, 4c,d). Caron et al. (2018) also observed Cd and Cu increase in
369 MM pool containing MTs following the increase of total cytosolic concentrations of Cd and Cu
370 in the liver of juvenile yellow perch (*P. flavescens*), the same as Goenaga Infante et al. (2003)

observed for Zn, Cu and Cd in the liver of European eel. In the case of Cu and Zn in the gills of Prussian carp, the significant and clear changes of their profiles as a consequence of incresed cytosolic concentrations in the gills of Prussian carp were not observed (Figs. 3a,b, 4a,b).

In a conclusion, MTs have been shown to play an important role in the detoxification of metals in fish (Paris and Usher, 2019). And, based on our results, both studied organs obviously participated in the detoxification of toxic element Cd, gills as a site of direct contact and uptake of pollutants, and thus the first line of defense, and the liver as the main storage and detoxification organ, preventing the long term toxic effects. On the other hand, MTs also have important role in essential metal ion homeostasis (Paris and Usher, 2019), such as Cu and Zn (Olsson, 1996). It is known that Zn can be held in MT reserves and that Zn-binding by MTs contribute to maintainance of intracellular Zn levels (Osredkar and Sustar, 2011). Involvement of MTs in Zn and Cu regulation have been, among others, reported for the eel liver (Goenaga Infante et al., 2003). So, the fact that binding of both essential metals, Cu and Zn, to MTs, was increased following the increase of their cytosolic concentrations only in the liver of Prussian carp, indicated that the liver was the principal organ for storage of those metals, and thus also their source for metabolic requirements of the organism, which pointed to important role of the liver more than the gills in the preservation of Cu and Zn homeostasis. It is possible that gills have mainly the uptake and transitory role in Zn and Cu metabolism, and that they do not participate in their storage. This can be especially corroborated for Zn, by its binding to VLMM biomolecules observed only in the gills, which possibly reflected Zn association to metallochaperones. The binding of metals to soluble proteins of low molecular masses, called metallochaperones, which deliver metals to specific sites within the cell was best described for Cu, but it is likely that analogous trafficking pathways exist as well for the other metals (Loutet et al., 2015; O'Halloran and Cizewski Culotta, 2000; Portnoy et al., 2001). In addition to transport function, metallochaperones can also bind the excess metal ions, thus contributing to metal detoxification (Regvar and Vogel-Mikuš, 2011). The role of both Cu and Zn in antioxidative defense in the gills, as the site of direct contact with pollutants, can also be presumed, based on their observed binding to biomolecules which MM corresponded to GSH

(~307 Da, https://pubchem.ncbi.nlm.nih.gov/compound/Glutathione) in the case of Zn, and to superoxide dismutase (32.5 kDa; Pedrajas et al., 1993) in the case of both Cu and Zn.

3.2. Iron

In fish, Fe is essential for the function of the heme-based oxygen-binding proteins, hemoglobin and myoglobin (Kuhn et al., 2016). Hemoglobin (64 kDa) is a tetrameric red blood cell protein with two pairs of identical subunits that transport oxygen through the organism; myoglobin, on the other hand, is the single chain cytosolic hemoprotein of 17 kDa located primarily in muscles, whose role is to increase the diffusion rate of dioxygen from capillary red blood cells to cytoplasm and mitochondria (Beard et al., 1996). Furthermore, Fe has an important role in the redox chemistry and mitochondrial cellular respiration (Hirst, 2013), as a part of cytochromes and FeS proteins (Beard et al., 1996). It also participates in DNA synthesis, production of neurotransmitters, and metabolism of tyrosine, collagen, fatty acids, and carnitine (Kuhn et al., 2016). In the cell cytosol it can be present as a part of various peroxidase enzymes, such as catalase, which degrades hydrogen peroxide formed as a byproduct of some oxidative reactions (Beard et al., 1996). It also can be bound to transferrin (70-80 kDa; Sun et al., 2012), which is an Fe transporter protein, and transferrin mRNA level was reported to be higher in Fe deficiency conditions, to promote Fe uptake by the cells (Kamińska-Gibas et al., 2018). Since excess of Fe can lead to production of reactive oxygen species, free Fe level is tightly controlled in the organisms (Beard et al., 1996; Kuhn et al., 2016).

In both studied organs of Prussian carp, the gills and the liver, Fe was eluted within two peaks, one HMM peak with the maxima in the range from 300-400 kDa, and one MMM peak with the maxima at 30-40 kDa (Table 4, Fig. 5). However, the striking difference between two organs was that one peak was predominant in one, and the other in the other organ. Namely, in the gills the predominant Fe peak was MMM peak (Fig. 5a,b; Table 4), which encompassed the molecular masses of some well known Fe-containing proteins which could be found in the soluble tissue fraction (e.g., hemoglobin; 64 kDa; Beard et al., 1996). Previous study on Vardar chub confirmed the presence of monomers (~15.5 kDa), dimers (~31.5 kDa) and trimers (~47

kDa) of hemoglobin α and β subunits in MMM Fe-peak in both the gills and the liver (Krasnići et al., 2019), and their predominant presence in the gills is consistent with high blood supply of that organ. In the gills, HMM Fe-peak was almost negligible (Fig. 5a,b).

Contrary, in the liver HMM Fe-peak was predominant, whereas MMM Fe-peak was almost negligible (Fig. 5c,d). According to the literature data and the elution time of standard protein apoferritin (Table 3), HMM Fe-peak most probably referred to Fe-storage protein ferritin (450 kDa; Aisen et al., 2001; Carriquiriborde et al., 2004), confirming liver as a key organ in Fe metabolism, and the primary site of Fe storage, as already stated by many authors (e.g., Kamińska-Gibas et al., 2018). Specifically, Van Dijk et al. (1975) have indicated liver as the main storage pool for Fe in tench (*Tinca tinca*) and Walker and Fromm (1976) in rainbow trout (*Oncorhynchus mykiss*).

Almost identical Fe distributions as those observed in Prussian carp organs were previously reported for European and Vardar chub (Krasnići et al., 2013, 2014, 2018). In both organs of both chub species, Fe was eluted in two peaks, HMM with the maxima at 380-400 kDa, and MMM with the maxima at 35-40 kDa, with the HMM peak being predominant in the liver and the MMM peak in the gills (Krasnići et al., 2013, 2014, 2018). Similar elution patterns were also observed in the liver of brown trout, but in addition to two already mentioned peaks, Fe was also eluted within VLMM biomolecule region (5-10 kDa) (Dragun et al., 2018), which could have referred to Fe bound to citrates, nucleotides, pyrophosphates, amino acids, and/or protein chelates or complexes of iron (Beard et al., 1996). It was previously described that Fe binds to biomolecules of low molecular masses, belonging to so-called transit iron pool, which contains small soluble complexes that help in intracellular Fe transport from one Fe-binding protein to the other (Fontecave and Pierre, 1991: Jacobs, 1977). This finding indicated a species-specific variability in Fe distribution, and obviously higher degree of similarity of Prussian carp to two chub species (all species in the Cyprinidae family) than to brown trout (species in the Salmonidae family), as was already observed in the case of Cu distribution in the liver. This was in accordance with the fact that brown trout inhabits clear, cold mountain streams with high dissolved oxygen concentrations (Stauffer et al., 1995), whereas chub species

454 are more common in slow lowland rivers and lakes (Kottelat and Freyhof, 2007), similar to
455 Prussian carp, which dominates in stagnant and slow-flowing waters (Ergüden, 2015).
456 Same as observed for Cd, Cu and Zn, the changes in cytosolic Fe distributions in both Prussian
457 carp organs were associated to different levels of Fe bioaccumulation, and could not be
458 specifically connected to either seasonal or spatial effect. Cytosolic Fe concentrations in the
459 gills varied less than in the liver (Tables 1 and 2), and thus the changes in the distribution
460 profiles were also less obvious and reflected only in the slight increase of MMM peak (Fig.
461 5a,b), which was consistent with the previous reports for the European and Vardar chub
462 (Krasnići et al., 2014, 2018). According to Carriquiriborde et al. (2004), gills do not have a
463 major role in Fe metabolism, and thus possibly the majority of Fe found in the soluble fractions
464 of the gills of Prussian carp could be ascribed to blood protein hemoglobin. Contrary, Fe
465 bioaccumulation in the liver varied pronouncedly among the analyzed samples (Table 2), and
466 its higher values were reflected in the strong increase of HMM peak (Fig. 5c,d), further
467 confirming the storage of Fe in the liver. The same was also observed not only in both chub
468 species, but in brown trout, as well (Dragun et al., 2018; Krasnići et al., 2013; 2018). Such
469 findings were consistent with previous reports about increase of ferritin expression in the
470 hepatic cells when Fe accumulation was high, and about decrease associated to Fe release into
471 the bloodstream, when Fe concentration in blood was lower (Kamińska-Gibas et al., 2018).
472 This is further consistent with theoretical possibility of storage of up to 4500 ferric iron atoms
473 in one ferritin molecule, although it is usually only 20% saturated (Aisen et al., 2001; Beard et
474 al., 1996).
475 *3.3. Molybdenum*
476 The trace element Mo is essential for nearly all organisms and forms the catalytic centre of a
477 large variety of enzymes, such as mitochondrial enzyme sulphite oxidase and cytosolic
478 enzymes xanthine oxidoreductase and aldehyde oxidase (Montefiori et al., 2017; Schwarz et al.,
479 2009). Molybdenum enzymes catalyze reactions that involve the two-electron transfer from or
480 to a substrate, accompanied by the transfer of an oxygen atom, which is derived from water or
481 incorporated into it (Hille et al., 2011; Mendel, 2012).

In the gills of Prussian carp, Mo was eluted within a single sharp VLMM peak with a maximum at 5 kDa, encompassing the molecular masses from ~2-8 kDa (Table 4, Fig. 6a,b). Contrary, previous SEC-HPLC studies on the gills of two chub species (*S. cephalus* and *S. vardarensis*) using the same Superdex 200 column failed to clearly detect the biomolecules that bind Mo, even though the cytosolic Mo concentrations in the gills of those fish were comparable to concentrations in the gills of Prussian carp (Krasnići et al., 2014, 2018). The fact that VLMM Mo-binding biomolecules in the gills could be more clearly detected in Prussian carp than in either chub species possibly indicates better developed uptake or perhaps defense mechanisms in the Prussian carp as the invasive fish species. However, the use of the Superdex 75 column enabled the detection of Mo-binding VLMM biomolecules in the gills of Vardar chub, which were proven to be heat-stable and their exact mass determined by MALDI-TOF-MS was 3.3 kDa (Krasnići et al., 2019), which corresponds well with the estimated mass of Mo-binding biomolecules in the gills of Prussian carp.

Although in the liver small amount of Mo was also eluted within VLMM peak, it was predominantly distributed within HMM peak with a maximum at 235 kDa (Table 4, Fig. 6c,d). Similar results were previously obtained for the liver of European chub (Krasnići et al., 2013), Vardar chub (Krasnići et al., 2018) and brown trout (Dragun et al., 2018b), with the maxima of predominant Mo elutions at 230-240 kDa, and the maxima of minor Mo elutions observed at 5-7 kDa. HMM Mo-peak encompassed MM of cytosolic enzymes xanthine oxidoreductase (290 kDa; Battelli et al., 2016) and aldehyde oxidase (132 kDa; Uchida et al., 2003). As for hepatic VLMM Mo-binding biomolecule, the MALDI-TOF-MS analysis revealed its exact mass in Vardar chub liver to be 8.5 kDa (Krasnići et al., 2019). Furthermore, its presence increased after the heat-treatment of the cytosols, indicating that it originated from some heat-sensitive biomolecule of somewhat higher MM (Krasnići et al., 2019).

The observed variability of distribution profiles in both organs was associated to variability in bioaccumulation levels, as also observed for the other metals, and followed the basic characteristics of Mo distributions. Namely, the increase of cytosolic Mo concentrations in the gills (Table 1) was reflected in the clear increase of VLMM peak, as especially evident for

several fish sampled in autumn (Fig. 6a). Contrary, the increase of cytosolic Mo concentrations in the liver (Table 2) was mainly reflected in the increase of HMM peak, as seen for the samples with the highest cytosolic concentrations in the liver in both seasons (Fig. 6c,d), and as already reported for Mo in the liver of Vardar chub (Krasnići et al., 2018) and brown trout (Dragun et al., 2018b).

The observed differences in Mo distributions between two organs possibly reflected the different Mo roles in each one of them. Involvement of Mo in metabolic processes can be presumed in the liver, thus explaining its predominant presence in the protein pool of higher molecular masses (>100 kDa). Contrary, the presence of Mo in the gills probably reflects its recent uptake, and thus the binding to VLMM biomolecules (<10 kDa) could indicate Mo binding to small metallochaperones or even partly to nonprotein cofactors. It was described that certain metals in cytoplasm can be present within free nonprotein cofactors; this is especially characteristic for Mo which is bound by family of related low molecular mass pterin-based cofactors, Moco (Loutet et al., 2015; Mendel, 2012). For example, molecular mass of free Mo cofactor from *Escherichia coli* was previously estimated to be 700-5000 Da using Sephadex G-10 and G25 gel filtration chromatography (Amy and Rajagopalan, 1979). The newer calculations estimate the mass of molybdopterin to be around 500 Da (http://ecmdb.ca/compounds/M2MDB000446).

3.4. Selenium

According to Mariotti et al. (2012), until now 41 selenoproteins have been characterized in teleost fish. Selenoproteins refer to proteins that contain covalently bonded selenocysteine, but not all of them have known functions (Lopez Heras et al., 2011). On the other hand, Se can be nonspecifically bound to proteins in a form of selenomethionine in a place of methionine, and those proteins are referred to as proteins containing selenium (Janz, 2012; Lopez Heras et al., 2011). In the gills of Prussian carp, Se was eluted within four peaks, one HMM (maximum at 109 kDa), one MMM peak (maximum at 30 kDa), and two VLMM peaks (maxima at 3.9 kDa and 0.8 kDa) (Table 4, Fig. 7a,b). HMM and MMM peaks, which were better resolved in the autumn than spring samples and which altogether covered the MM region from 20-400 kDa

538 (Table 4), represented only minor part of eluted Se. The major part of eluted Se in the gills was
539 contained within two VLMM peaks, covering MM region from 0.4 to 14 kDa (Table 4). Similar
540 results were previously reported for European and Vardar chub, with the major gill Se elution
541 in the molecular mass region below 2 kDa (Krasnići et al., 2014; 2018). Since Se in fish
542 primarily accumulates as selenomethionine and selenocysteine, as reported for rainbow trout
543 fed Se-supplemented diets (Godin et al., 2015), observed association to VLMM compounds
544 could refer to Se present in the cytosol in the form of free selenocysteine (167 Da;
545 https://pubchem.ncbi.nlm.nih.gov/compound/Selenocysteine) or selenomethionine (196 Da;
546 https://pubchem.ncbi.nlm.nih.gov/compound/Selenomethionine). As previously suggested by
547 Krasnići et al. (2014), such Se elution could also indicate that in the gills Se binds to low
548 molecular mass selenocompounds that act as strong free radical scavengers and thus participate
549 in the antioxidative defense. The antioxidative compounds that scavenge free radicals are
550 generally of low molecular mass, they reduce free radicals, getting oxidized themselves
551 (Bragadóttir, 2001). An example of such compound is selenoneine (2-selenyl-*N,N,N*-trimethyl-
552 L-histidine), which is recently identified selenometabolite in various tissues of bluefin tuna
553 (*Thunnus orientalis*) (~0.5 kDa; Yamashita and Yamashita 2010; Yamashita et al. 2012), as
554 well as in the liver of sea turtles (Anan et al., 2011a). Another low molecular mass
555 selenoprotein is *SelW* (~10 kDa), a cytosolic protein that possibly participates in antioxidant
556 function, but its exact physiological function and enzymatic activity is largely unknown (Kim
557 and Jeong, 2011; Lopez Heras et al., 2011).
558 Contrary, in the liver of Prussian carp, the major part of Se was eluted within one HMM peak
559 with a maximum at 141 kDa (range ~40-400 kDa), whereas minor part was eluted within two
560 VLMM peaks with maxima at 1.1 kDa and 3.9 kDa (Table 4, Fig. 7c,d). Similarly, in the liver
561 of brown trout and rainbow trout from lakes in Argentina only up to 13% of total soluble Se
562 was associated to compounds of very low molecular masses, selenocysteine and
563 selenomethionine, the first one being predominant (Kristan et al., 2013). On the other hand,
564 several well-known selenoproteins have MM within the range of HMM peak, such as cytosolic

565 isoforms of antioxidative enzymes glutathione peroxidase that catalyzes conversion of H_2O_2
566 into water (96 kDa homotetramer with four subunits, as identified in the liver of pacu,
567 *Piaractus mesopotamicus*; Bastos et al., 2007) and thioredoxin reductase that reduces
568 thioredoxin with electrons from NADPH, which then participate in defense against oxidative
569 stress (64.1 kDa monomer, as identified in the gills of rainbow trout; Akyol and Kuzu, 2017)
570 (Lopez Heras et al., 2011). Furthermore, an important selenoprotein in the fish liver is
571 selenoprotein P (*SelP;* ~50 kDa, as identified in zebrafish (*Danio rerio*); Kryukov and
572 Gladyshev, 2000) which is primarily synthesized in liver and secreted to plasma to function in
573 the transport and delivery of Se to remote tissues (Papp et al., 2007). Accordingly, in the liver
574 of rainbow trout, Wang et al. (2018) reported a much higher abundance of *SelP* than of the
575 other selenoprotein genes. The comparison of hepatic Se distribution in Prussian carp with the
576 results previously published for two chub species revealed some similarities, but also some
577 differences (Krasnići et al., 2013; 2018). The similar finding referred to minor hepatic Se
578 elution in VLMM region below 5 kDa, especially for European chub and in some samples of
579 Vardar chub (Krasnići et al., 2013; 2018). The evident difference, however, reffered to Se
580 elution within the regions of higher molecular masses. Although in both chub species hepatic
581 Se was eluted within both HMM (~140 kDa) and LMM/MMM regions (20-30 kDa), HMM
582 elution was rather small, and the predominant elution in the chub liver was associated to the
583 biomolecules in the MM region of 10-60 kDa (Krasnići et al., 2013; 2018). The Se elution in
584 the regions of 40-400 kDa in Prussian carp liver and 10-60 kDa in chub liver, however, could
585 possibly indicate the presence of the same enzyme, i.e. glutathione peroxidase, in the first case
586 in the form of intact enzyme and in the latter one in the form of enzyme subunits (~23 kDa, as
587 determined for the liver of pacu; Bastos et al., 2007). This can be further corroborated by the
588 report of Wang et al. (2018), who found that glutathione peroxidase gene in rainbow trout liver
589 was included among the selenoprotein genes that responded the most to increasing dietary Se
590 levels.

Cytosolic Se concentrations in the gills of Prussian carp were kept within a rather narrow range (Table 1), and their only slight increase in some fish was reflected in the increase of VLMM peaks, especially of the one bellow 2 kDa (Fig. 7a,b), which was consistent with the findings for the gills of both chub species (Krasnići et al., 2014; 2018). In the liver of Prussian carp, broader range of Se concentrations was observed (Table 2), as well as initially higher concentrations than in the gills, which was similar to the reports for Atlantic salmon (*Salmo salar*) (Betancor et al., 2016). Accordingly, the strong increase of HMM peak (up to 400 kDa) was recorded as a consequence of the pronounced increase of Se concentrations in the hepatic cytosol in the fish sampled near the Ilova village in the spring period (Table 2, Fig. 7d). Unlike the Prussian carp and two chub species, in which increased presence of Se in the liver was associated either to biomolecules of high (up to 400 kDa) or medium molecular masses (up to 60 kDa), respectively, in the brown trout from the Krka River, as well as in the white suckers, higher Se bioaccumulation in the liver was reported to lead to the increase of Se elution in the VLMM regions (<2 kDa or 2.5-5 kDa) (Dragun et al., 2018; Urien et al., 2018), which was more similar to Se behaviour in the gills than in the liver of all three aforementioned species. For Se, the same as in the case of Fe and Cu, the hepatic elution profiles of Prussian carp more closely resembled to the profiles of the chub species than to those of the brown trout, and in the case of Se, to those of the white suckers. The possibility of such species-specific differences were previously indicated by Onning (2000), who stated that the differences in Se distribution between fish species could be associated to differences between active and passive species, to the variability of the feed (Maher, 1987), and to differences in selenium metabolism among species.

As in the case of all above discussed metals, different Se distribution among cytosolic biomolecules in two organs can also be explained by different roles of gills and liver in Se metabolism. All Se species ingested via food or drinking water are first transformed into selenide (Se^{2-}), and then selenide is utilized for the biosynthesis of selenocysteine for incorporation into selenoproteins (Anan et al., 2011b). Although Se inorganic forms (selenate and selenite) dissolved in the water are oxyanions that are not absorbed considerably through

gill membranes (Pedersen et al., 1998), the information on potential uptake of dissolved Se via gills in freshwater fish is still insufficient and should be further inveastigated (Janz, 2012). Thus, the higher presence of low molecular mass selenocompounds, which function as intermediaries in the synthesis of selenoproteins (Akesson and Srikumar, 1994; Ganther, 1984) can be expected at the sites of Se uptake, such as gills. On the other hand, the liver is the primary organ for Se accumulation in fish and other vertebrates (Sato et al., 1980), being the dominant site of selenoprotein synthesis and catabolism (Burk and Hill, 2009), which can explain higher presence of selenocompounds of higher molecular masses in that organ of Prussian carp.

4. Conclusions

The study of metal/nonmetal cytosolic distributions in the gills and liver of Prussian carp from the Ilova River using SEC-HPLC and HR ICP-MS enabled to shed some light on the metal handling strategies of that invasive freshwater fish species under the conditions of moderate contamination of aquatic environment. Binding of nonessential and very toxic metal Cd solely to biomolecules of MM of 8-14 kDa (presumably MTs), in both organs of Prussian carp, indicated efficient Cd detoxification, both in the gills as the uptake site and in the liver as main metabolic centre of the organism. The liver was, further, confirmed as the main site of detoxification, storage and metabolism for the remaining five elements, based on the metal/nonmetal binding to biomolecules of higher MM, probably enzymes and other components of metabolic reactions, or storage and detoxification proteins. Specifically, the predominant binding of Cu and Zn to MTs, of Fe to biomolecules of MM of 300-400 kDa (presumably ferritin), of Mo to biomolecules of MM of ~100-300 kDa (possibly cytosolic enzymes xanthine oxidoreductase or aldehyde oxidase), and of Se to biomolecules of MM of ~100-200 kDa (possibly cytosolic enzymes glutathione peroxidase or thioredoxin reductase), was observed in the liver, and was almost negligible in the gills. On the other hand, in the gills binding of metals/nonmetal to biomolecules of rather low MM, even below 5 kDa (possibly

antioxidants, metallochaperones, or nonprotein cofactors), was observed, indicating the gill involvement in antioxidative and detoxification processes, as well as in metal transfer, as an organ which presents the first contact with the contaminated environment. Specifically, in the gills we have observed Zn binding to biomolecules of MM below 2 kDa (possibly reduced antioxidant glutathione), sole Mo binding to biomolecules of MM of ~2-8 kDa (possibly small metallochaperones or nonprotein cofactors), predominant Se binding to biomolecules of MM of 0.4-14 kDa (possibly antioxidative selenocompounds, such as selenoneine, or, intermediaries in the synthesis of selenoproteins, such as selenocysteine or selenomethionine). The differences in hepatic and gill metal/nonmetal distributions among cytosolic proteins and peptides, thus, have reflected different functions of the liver and the gills, whereas the comparison with other fishes additionally indicated species-specific variability. The obtained results present a significant contribution to better understanding of the metal/nonmetal fate and behaviour in Prussian carp, but have also indicated the necessity to consider the differences between organs, as well as between organisms, during the assessment of metal pollution of aquatic systems. Finally, from the environmental point of view, it is evident that qualitative changes of metal/nonmetal intracellular distributions in two organs of Prussian carp characteristic for specific season or site were not observed, i.e. the occurrence of different peaks reflecting metal/nonmetal associations with different cytosolic biomolecules in two sampling seasons or at two differently contaminated sampling sites was not recorded. The changes in metal/nonmetal distributions were generally of quantitative nature, namely they revealed that specific metals/nonmetal are always binding to the same biomolecules within the same organ/species, but with the increasing cytosolic metal/nonmetal concentrations within the cells they exhibit higher tendency to bind to certain biomolecules. Since the application of SEC-HPLC enables only approximate mass determination, it would be useful in further research to identify the biomolecules in question by use of additional separation techniques, as well as mass spectrometry. Such approach would enable to establish more precisely whether the metal/nonmetal intracellular distributions observed in this study have indicated detoxification mechanisms or expression of metal/nonmetal toxicity potentials, which at this point can be only hypothesized. In addition,

such information will be useful in the process of the development of new biomarkers of metal/nonmetal exposure and effects, based on the molecular masses of the metal/nonmetal-binding biomolecules provided in this study, which are crucial in the monitoring of metal contamination of freshwater ecosystems.

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Figure 1. A map of the sampling area: site 1 (location near the Ilova village representing a reference site) and site 2 (location near the Trebež village representing a contaminated site).

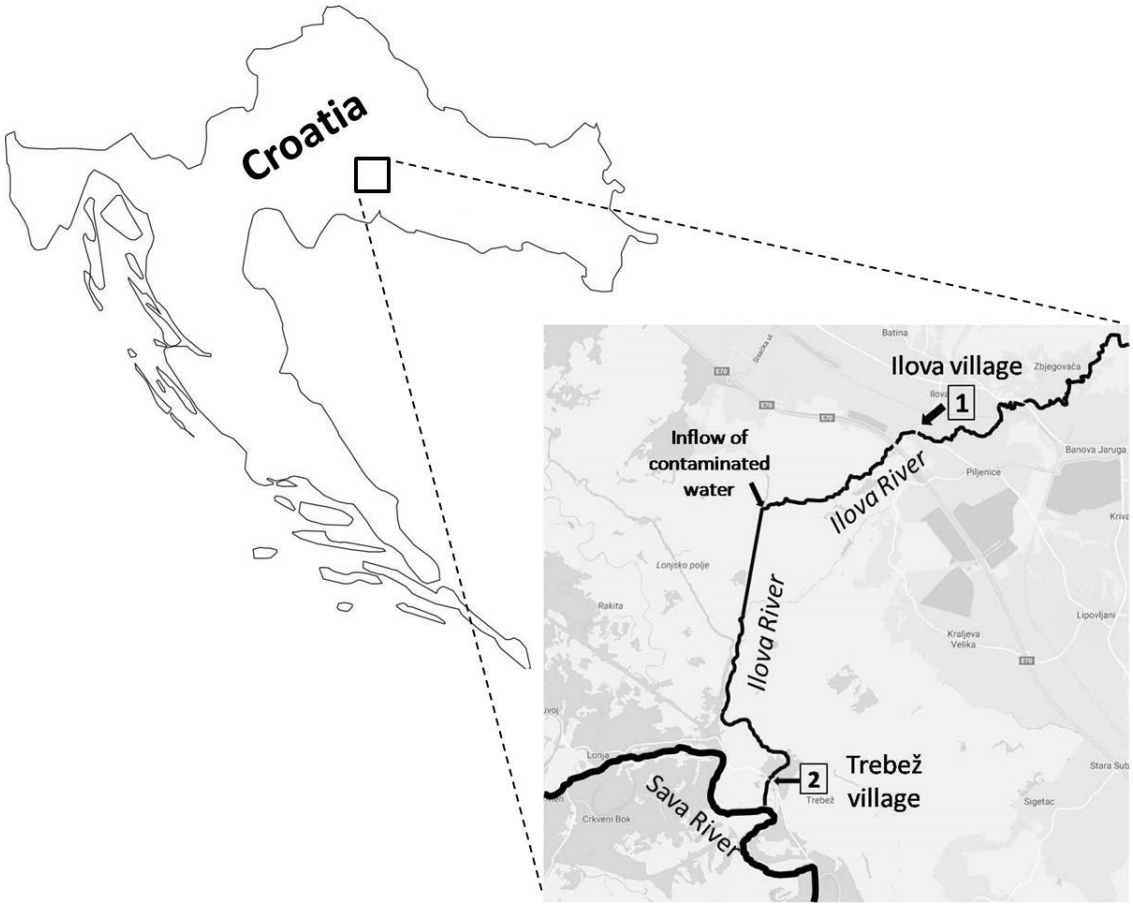

Figure 2. Cadmium distribution profiles among cytosolic biomolecules of different molecular masses in the gills (a, b) and liver (c, d) of Prussian carp (*Carassius gibelio*) from two sites at the Ilova River (Ilova village and Trebež village) in two sampling campaigns (autumn: October 2017; spring: May 2018). The results are presented as nanograms of metals eluted at the specific elution times (t_e).

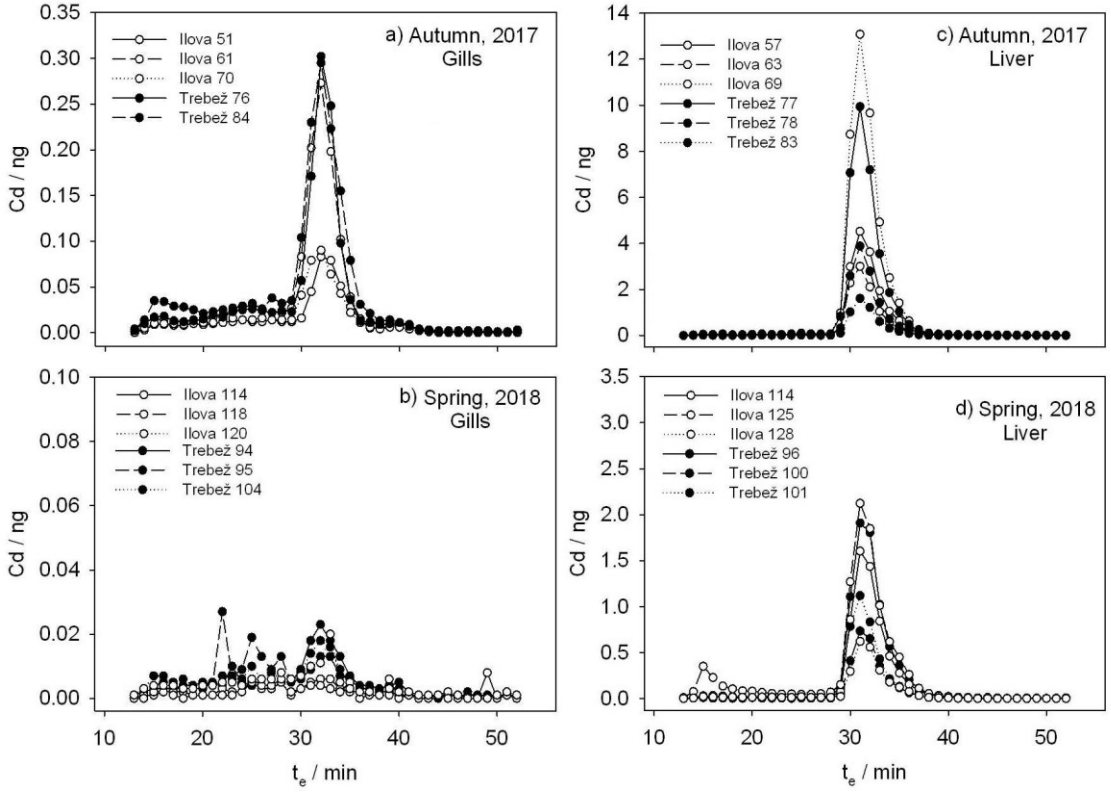

Figure 3. Copper distribution profiles among cytosolic biomolecules of different molecular masses in the gills (a, b) and liver (c, d) of Prussian carp (*Carassius gibelio*) from two sites at the Ilova River (Ilova village and Trebež village) in two sampling campaigns (autumn: October 2017; spring: May 2018). The results are presented as nanograms of metals eluted at the specific elution times (t_e).

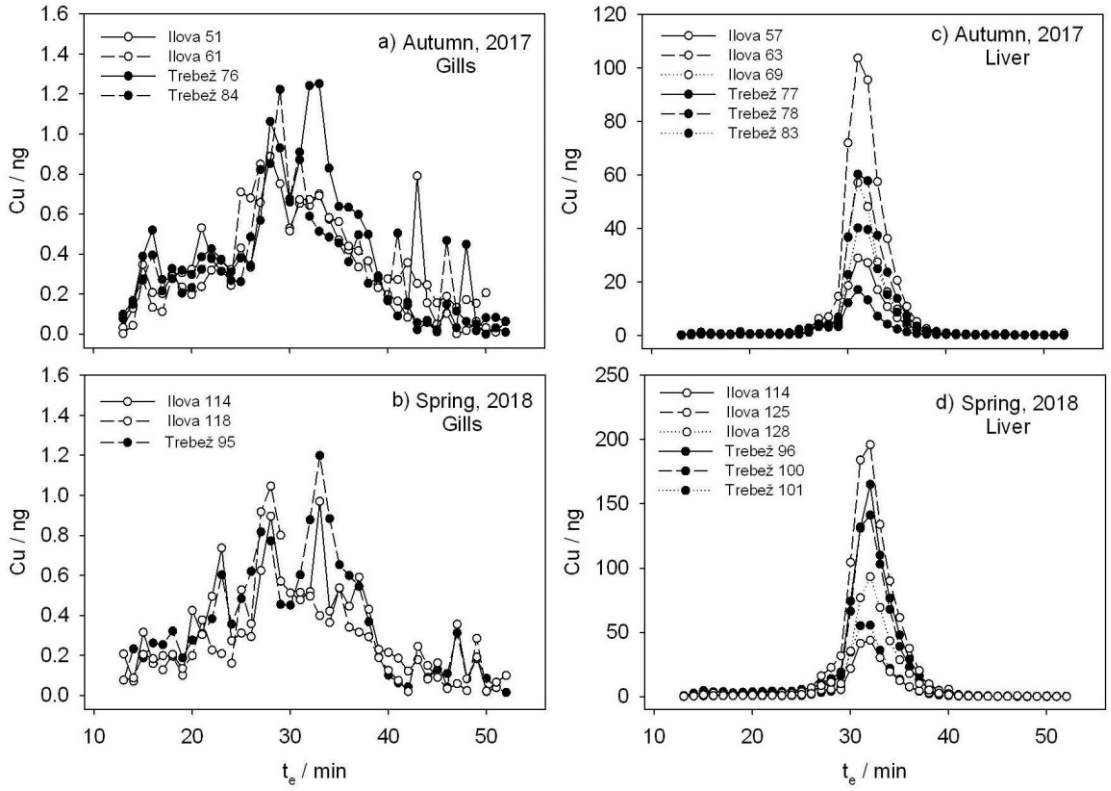

Figure 4. Zinc distribution profiles among cytosolic biomolecules of different molecular masses in the gills (a, b) and liver (c, d) of Prussian carp (*Carassius gibelio*) from two sites at the Ilova River (Ilova village and Trebež village) in two sampling campaigns (autumn: October 2017; spring: May 2018). The results are presented as nanograms of metals eluted at the specific elution times (t_e).

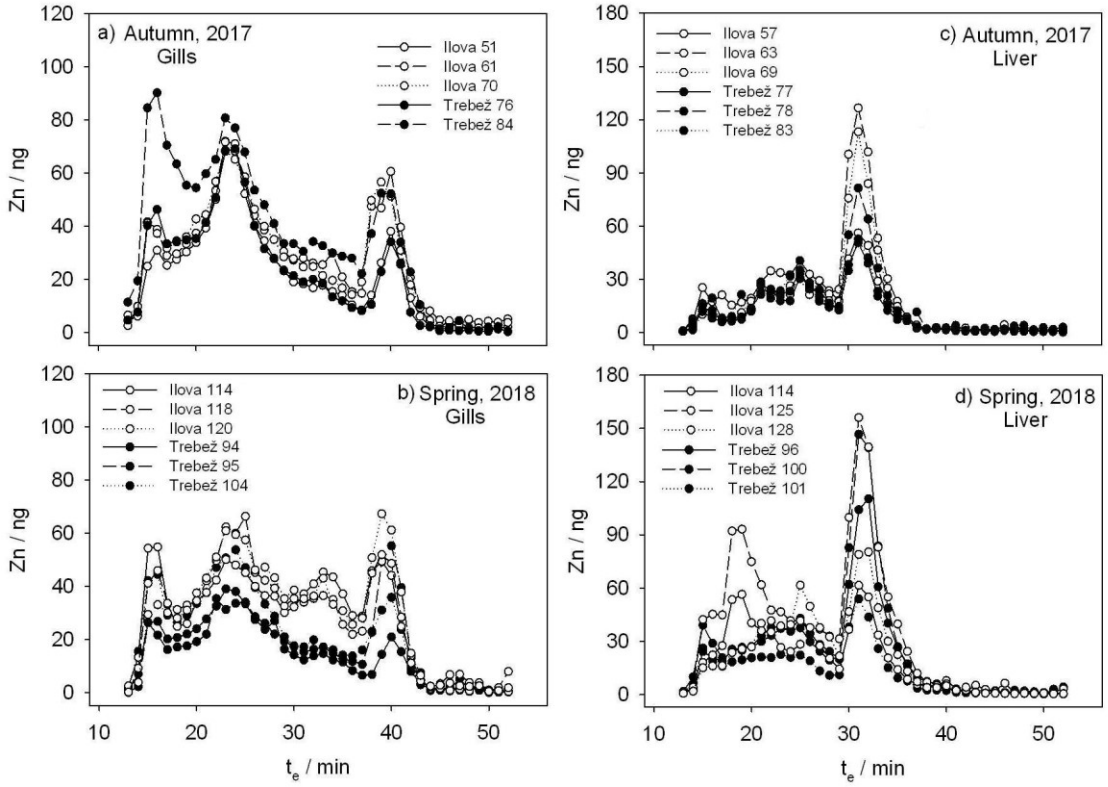

Figure 5. Iron distribution profiles among cytosolic biomolecules of different molecular masses in the gills (a, b) and liver (c, d) of Prussian carp (*Carassius gibelio*) from two sites at the Ilova River (Ilova village and Trebež village) in two sampling campaigns (autumn: October 2017; spring: May 2018). The results are presented as nanograms of metals eluted at the specific elution times (t_e).

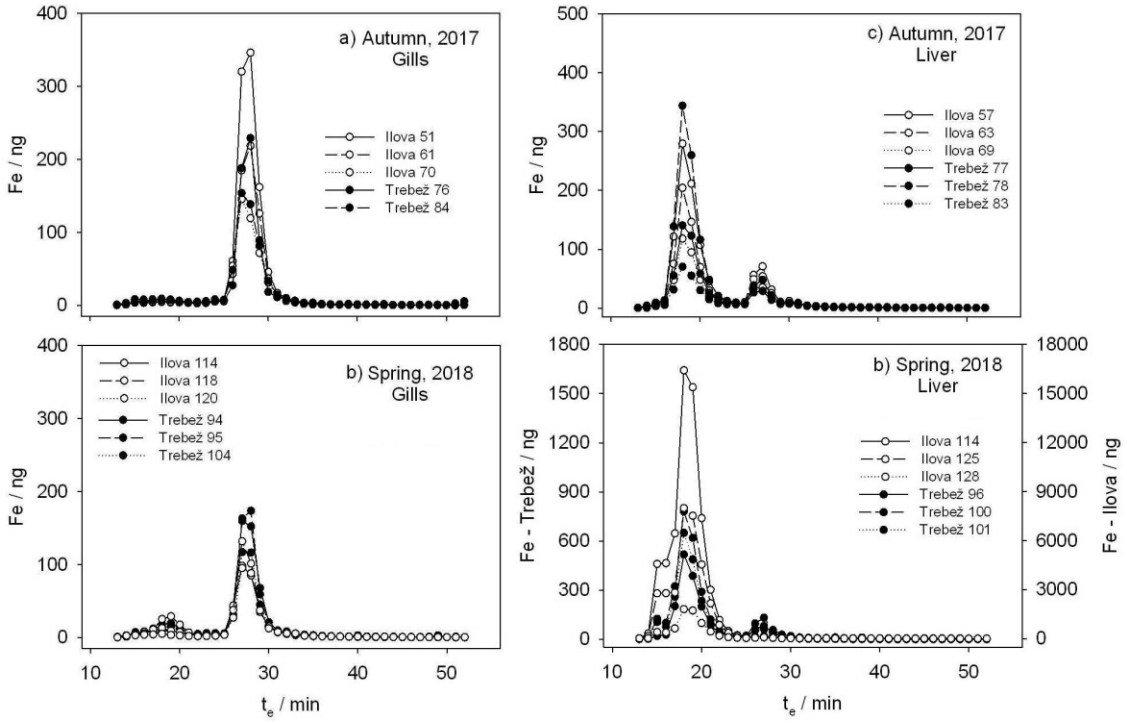

Figure 6. Molybdenum distribution profiles among cytosolic biomolecules of different molecular masses in the gills (a, b) and liver (c, d) of Prussian carp (*Carassius gibelio*) from two sites at the Ilova River (Ilova village and Trebež village) in two sampling campaigns (autumn: October 2017; spring: May 2018). The results are presented as nanograms of metals eluted at the specific elution times (t_e).

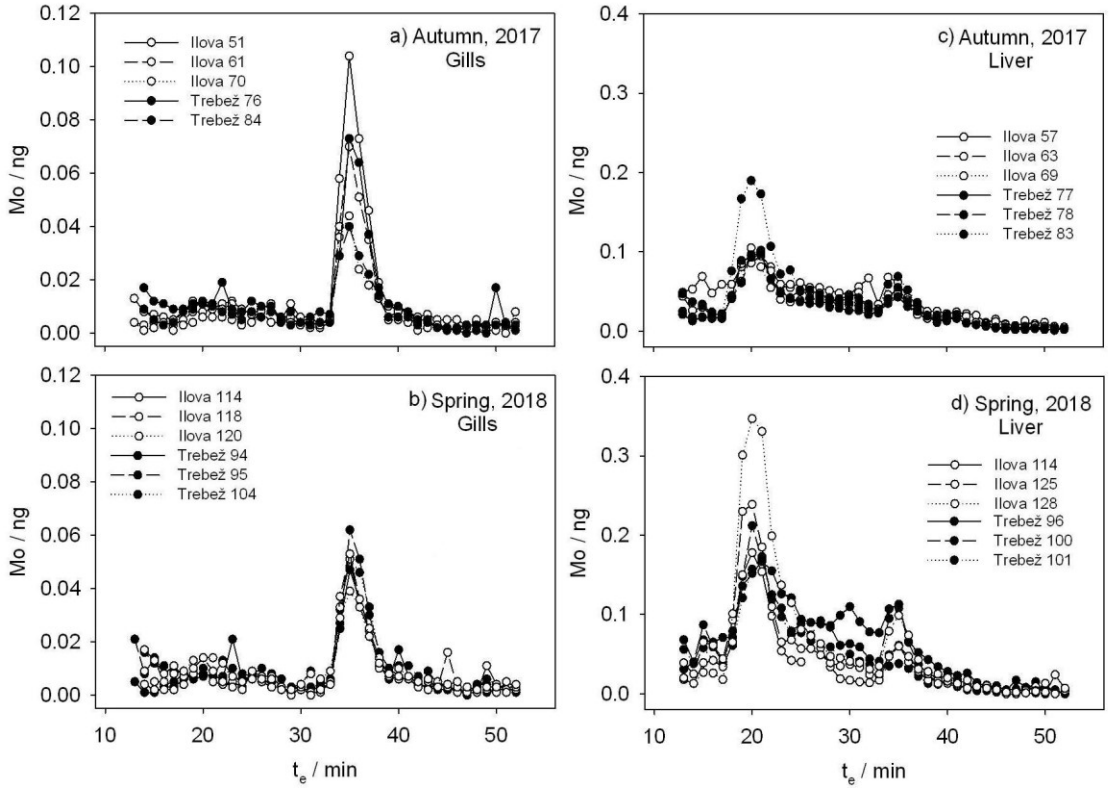

Figure 7. Selenium distribution profiles among cytosolic biomolecules of different molecular masses in the gills (a, b) and liver (c, d) of Prussian carp (*Carassius gibelio*) from two sites at the Ilova River (Ilova village and Trebež village) in two sampling campaigns (autumn: October 2017; spring: May 2018). The results are presented as nanograms of metals eluted at the specific elution times (t_e).

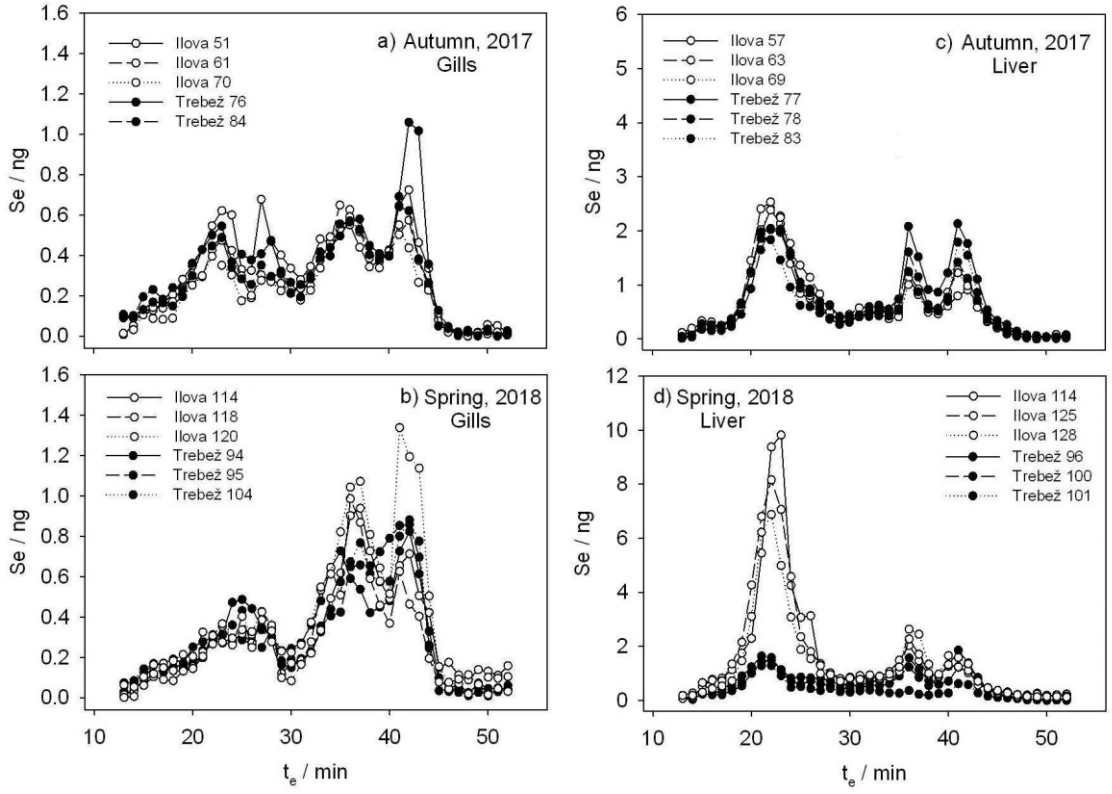

Table 1. Biometric characteristics and cytosolic trace element concentrations in the gills of eleven specimens of Prussian carp (*Carassius gibelio* Bloch, 1782) used in this study for analyses of gill trace element distributions.

		Fish ID	Total length / cm	Total mass / g	Sex*	Cd / ng g^{-1}	Cu / µg g^{-1}	Fe / µg g^{-1}	Mo / ng g^{-1}	Se / ng g^{-1}	Zn / µg g^{-1}
Autumn 2017	**Ilova village**	**51**	16.0	61.37	M	16.8	0.225	48.2	15.9	349.1	23.6
		61	16.5	75.40	F	44.6	0.220	35.2	10.9	328.6	23.7
		70	16.2	68.41	F	20.0	-	29.8	8.37	322.6	20.0
	Trebež village	**76**	23.7	239.3	F	45.8	0.253	38.8	10.1	360.9	18.5
		84	20.3	137.8	F	57.7	0.192	36.3	7.62	340.6	35.1
Spring 2018	**Ilova village**	**114**	22.0	125.6	F	1.62	0.158	25.9	6.42	264.0	42.2
		118	18.7	82.07	M	1.50	0.129	28.2	8.46	286.4	23.7
		120	17.3	60.67	M	2.97	-	20.1	6.51	339.6	21.9
	Trebež village	**94**	17.1	96.22	F	5.64	-	33.8	8.34	349.6	28.8
		95	14.9	50.06	M	3.84	0.204	36.2	8.40	325.3	21.9
		104	21.0	157.0	F	4.95	-	28.8	7.77	318.7	20.9

*M – male; F – female

Table 2. Biometric characteristics and cytosolic trace element concentrations in the liver of twelve specimens of Prussian carp (*Carassius gibelio* Bloch, 1782) used in this study for analyses of hepatic trace element distributions.

		Fish ID	Total length / cm	Total mass / g	Sex*	Cd / ng g^{-1}	Cu / µg g^{-1}	Fe / µg g^{-1}	Mo / ng g^{-1}	Se / ng g^{-1}	Zn / µg g^{-1}
Autumn 2017	**Ilova village**	**57**	17.9	99.32	F	463.6	4.94	52.5	66.5	653.2	8.96
		63	16.2	67.22	M	289.5	15.6	46.6	56.7	691.8	14.0
		69	18.6	95.63	M	1193.2	8.50	34.9	48.8	673.7	12.5
	Trebež village	**77**	23.4	238.1	F	921.4	2.50	32.4	49.2	697.5	8.84
		78	20.3	165.2	F	374.8	9.91	62.3	53.9	708.2	11.7
		83	18.5	101.1	F	160.7	6.79	28.3	80.6	649.0	11.4
Spring 2018	**Ilova village**	**114**	22.0	125.6	F	194.4	7.81	3840.7	90.5	1964.3	17.2
		125	18.0	77.34	F	247.3	33.4	1817.3	107.3	1662.0	22.7
		128	14.8	43.03	M	70.3	14.8	326.1	161.7	1504.3	18.4
	Trebež village	**96**	26.6	316.7	F	234.6	27.1	91.0	151.4	702.7	18.6
		100	27.2	339.2	F	81.7	24.6	153.3	140.7	752.5	24.8
		101	20.8	124.6	F	119.5	9.48	123.1	118.3	448.0	9.67

*M – male; F – female

Table 3. Molecular masses (MM), concentrations and elution times (t_e) of blue dextran, metallothionein standard and seven proteins used for calibration of Superdex™ 200 10/300 GL size exclusion column. Equation of calibration straight line was: Kav=-0.277×log MM+1.627.

	MM / kDa	Concentration / mg mL^{-1}	t_e / min
Blue dextran	2000	2	15.41
Metallothionein standards			
Metallothionein 2	6.15	1	30.98
Protein standards for column calibration			
Carbonic anhydrase	29	3	29.60
Superoxide dismutase	32.5	1.25	27.71
Bovine albumin	66	10	23.06
Alcohol dehydrogenase	150	5	21.80
β-amilase	200	4	20.55
Apoferritin	443	10	17.88
Thyroglobulin	669	8	16.12

Table 4. Elution times (t_e) and molecular masses (MM) of cytosolic biomolecules that bind specific elements, which were isolated from Prussian carp (*Carassius gibelio* Bloch, 1782) gills (G) and liver (L) and separated by SEC-HPLC (Superdex 200 10/300 GL column). Table provides peak maxima for biomolecules that bind each analyzed element in both organs (i.e., t_e and MM for the chromatographic fractions with the highest content of specific trace elements), as well as peak widths which are presented within the brackets.

	[a]HMM 1		[a]HMM 2		[b]MMM		[c]LMM		[d]VLMM 1		[d]VLMM 2	
	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
Cd G							32 (29-36)	11 (24-3.9)				
Cd L							31 (29-35)	14 (24-5)				
Cu G					28 (25-30)	30 (65-18)	33 (30-38)	8 (18-2.4)				
Cu L							31, 32 (27-38)	14, 11 (39-2.4)				
Zn G	16 (14-19)	653 (1088-303)	23 (20-28)	109 (235-30)			32, 33 (30-36)	11, 8 (18-3.9)			40 (37-43)	1.4 (3.0-0.7)
Zn L	16 (14-18)	653 (1088-392)	21, 25 (19-28)	182, 65 (303-30)			31 (29-36)	14 (24-3.9)				
Fe G	19 (17-21)	303 (506-182)			28 (25-31)	30 (65-14)						
Fe L	18 (15-22)	392 (843-141)			27 (25-29)	39 (65-24)						
Mo G									35 (33-39)	5 (8-1.8)		
Mo L			20 (17-24)	235 (506-85)					35 (33-38)	5 (8-2.4)		
Se G			23 (18-25)	109 (392-65)	28 (26-30)	30 (51-18)			36 (31-39)	3.9 (14-1.8)	42 (40-45)	0.8 (1.4-0.4)
Se L			22 (18-27)	141 (392-39)					36 (34-38)	3.9 (7-2.4)	41 (39-44)	1.1 (1.8-0.5)

[a]HMM peak – trace element peak with a maximum within high molecular mass protein region (>100 kDa)
[b]MMM peak – trace element peak with a maximum within medium molecular mass protein region (30-100 kDa)
[c]LMM peak – trace element peak with a maximum within low molecular mass protein region (10-29 kDa)
[d]VLMM peak – trace element peak with a maximum within very low molecular mass protein region (<10 kDa)