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Metal-binding biomolecules in the liver of northern pike (*Esox lucius* Linnaeus, 1758): the first data for the family Esocidae

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Abstract

Metal-handling strategies of various fish species are known to vary significantly in association with their intracellular metal behaviour. Thus, to better understand the possible consequences of increased metal exposure in fish it is important to perform comparative studies on metal-binding biomolecules in organs of different species. This study was the first of this kind on a liver of an esocid fish (northern pike, *Esox lucius*), and the gathered information were compared to fish belonging to three other families, Leuciscidae, Cyprinidae and Salmonidae. Distributions of ten elements among cytosolic biomolecules of different molecular masses were studied by size exclusion HPLC combined offline with high resolution ICP-MS. The results indicated predominant association of Co, Fe and Mo to high molecular mass biomolecules (>100 kDa), of Zn and Bi to both high and medium molecular mass biomolecules (>30 kDa), of Mn and Se to medium molecular mass biomolecules (30-100 kDa), and Ag, Cd and Cu to low molecular mass biomolecules (10-30 kDa), presumably metallothioneins. Evident binding to metallothioneins was also detected for Zn and Bi. For several metals, distinct differences were observed when cytosolic metal distributions of northern pike were compared to leuciscids, salmonids and cyprinids. More pronounced Zn binding to metallothioneins was recorded in leuciscids and cyprinids than both esocids and salmonids, whereas cytosolic Mn and Se distributions clearly differed between all studied fish families. Accordingly, in assessment of metal pollution it is vital to consider the exposed species, which requires prior comprehensive comparative research on numerous aquatic organisms.

Key words: cytosol, freshwater fish, green liver syndrome, HPLC, ICP-MS

1. Introduction

Nowadays, the pollution of aquatic systems is evergrowing, and among various pollutants metals can be regarded as a serious threat to the water quality and aquatic life (Dragun et al., 2009, 2011; Ramani et al., 2012; Filipović Marijić et al., 2018; Mijošek et al., 2020). Although many metals have important functions in the living organisms (e.g. Zn, Cu, Fe), they can also be toxic when present in the environment in high concentrations. In addition, many metals (e.g. Ag, Bi, Cd) have no known physiological functions, and thus can be linked to confirmable toxicity (Wong et al., 2017). Metals can cause toxic effects through variety of mechanisms, but their binding to physiologically relevant biomolecules within the cells can be regarded as one of the most important modes of their toxicity (Mason and Jenkins, 1995; Wallace et al., 2003; Van Campenhout et al., 2008; Wang, 2013; Caron et al., 2018; Urien et al., 2018).

On the other hand, some cytosolic biomolecules, such as metallothioneins (MT) or glutathione (GSH), can bind and thus detoxify or eliminate the metal in/from the cell (Lange et al., 2002). To be able to predict or even just to recognize the possible effects of specific metals at specific exposure levels, it is therefore of utmost importance to know their fate within different organisms, tissues and cells, as well as to have information on the metal-binding biomolecules which are included in metabolism, detoxification and toxicity of certain metals in specific aquatic species.

With that in mind, at the beginning of the 21st century a new scientific field was developed, named metallomics, aiming at systematic and comprehensive approach to study of metals or metalloids in a biological context (Lobinski et al., 2010). Various methods can be used within this field, but the most common starting point is the application of a combination of different high performance liquid chromatography (HPLC) techniques for fractionation of diverse metal forms (e.g. size exclusion (SEC), ion exchange) and inductively coupled plasma mass spectrometry (ICP-MS) for multielement concentration measurement in thus obtained fractions, with the final aim to investigate and characterize metal-binding biomolecules (Montes-Bayón et al., 2003; Van Campenhout et al., 2004, and the references cited therein).

For aquatic organisms, especially fish, there are not many information on metal-binding biomolecules. Usually the studies are aimed at analysis of one specific biomolecule, and very often that is MT and its association with various metals within the cell (Goenaga Infante et al., 2003, 2006; Van Campenhout et al., 2004, 2008; Huang et al., 2007; Hauser-Davis et al., 2014), or at analysis of one metal and all the biomolecules that bind it (e.g. uranium; Bucher et al., 2014; Frelon et al., 2020). More systematic approaches, which would provide deeper insight in behaviour of higher number of metals within the organs and cells of various fish species, are not often encountered. In recent years, our research group have initiated a series of investigations on few organs (liver, gills, intestine) of several fish species (European and Vardar chub (*Squalius cephalus* and *Squalius vardarensis;* Krasnići et al., 2013, 2014, 2018, 2019), Prussian carp (*Carassius gibelio;* Dragun et al., 2020; Mijošek et al., 2021), brown trout (*Salmo trutta;* Dragun et al., 2018)), to describe the distribution of a number of metals among the cytosolic biomolecules of different molecular masses by use of SEC-HPLC and high resolution (HR) ICP-MS. Similar type of studies was so far conducted by only few researchers, and encompassed analyses on fish organs (liver of juvenile yellow perch (*Perca flavescens;* Caron et al. 2018); liver and gonads of white suckers (*Catostomus commersonii;* Urien et al., 2018)) and on the other aquatic organisms (*Perna perna* mussels: gills (Hauser-Davis et al., 2021) and muscle and digestive gland (Lavradas et al., 2016); *Mytilus galloprovincialis* mussels – digestive gland (Strižak et al., 2014); marine crustaceans (Li et al., 2005)). Such studies will contribute to expansion of knowledge on metal behaviour in fish and on metal-binding biomolecules, thus providing the higher understanding of potential threats coming from metal exposure of the fish and the other aquatic organisms, as well as the basis for development and application of adequate biomarkers of metal exposure/effects.

The aim of our current study was to extend the investigation to liver of northern pike (*Esox lucius*) as a representative of the family Esocidae. For this species, even the data on metal bioaccumulation in its liver are very scarce and limited to few elements (e.g. Đikanović et al., 2016; Łuczyńska et al., 2019; Nikolić et al., 2021.), whereas, to our knowledge, the data on metal-binding biomolecules in northern pike are nonexistent. Thus, the information provided in

this paper are the first of this kind for northern pike. In addition, the comparison of the results obtained for northern pike liver to previously gathered data for European and Vardar chub as the representatives of the family Leuciscidae, Prussian carp as the representative of the family Cyprinidae, and brown trout as a representative of the family Salmonidae, will enable to more closely establish the extent of the variability in metal distributions among various cytosolic biomolecules that can be expected among different fish families. We have previously determined that there is evident difference in intracellular behaviour of several metals between fish belonging to families Leuciscidae, Cyprinidae and Salmonidae, probably due to specificities of their physiology, life habits and feeding (Dragun et al., 2020). Our results will thus show to which of these families northern pike, as an esocid fish, resembles more closely regarding the metal handling strategies. And, finally, an interesting phenomenon was observed in northern pike in spring periods of two consecutive years, namely the appearance of intensely green liver in smaller fish specimens. Such occurrence was previously reported in the literature under the name of green liver syndrome (GLS) (Takagi et al., 2006; Cai et al., 2020). Our additional aim was to determine if such distinct difference in liver colour, probably as a sign of certain metabolic processes, have caused the changes in metal intracellular distributions in the liver of northern pike.

2**. Materials and methods**

2.1. Fish sampling and liver dissection

The fish used in this study (northern pike, *E. lucius*) were caught at the lowland section of the Mrežnica River (Croatia) near the Town of Duga Resa (~2 km in both directions; Fig. 1) in two spring periods, 2020 and 2021. The samplings were performed by electrofishing device Hans Grassl (EL63 II GI, 5.0 KW, 137 Honda GX270, 300/600V max., 27/15A max.) in accordance with the Croatian standard HRN EN 14011 (2005), as described by Dragun et al. (2020). The fish were immediately euthanized at the location of the sampling, using unbuffered tricaine methane sulphonate (MS 222, Sigma Aldrich) and following the Ordinance on the protection of animals used for scientific purposes (NN 55/2013), as well as the previously reported exposure

conditions (Dragun et al., 2020). Prior to liver dissection, the total fish masses and lengths were measured, their condition indices were calculated, their sex was determined by gonad examination at macroscopic level, and colour of their liver were registered (Table 1). For the study of hepatic trace element distributions among cytosolic biomolecules of different molecular masses in northern pike, we have used the liver of seven specimens which were stored in liquid nitrogen and later on in the freezer at -80°C. During the defined sampling periods, the environmental exposure levels (i.e. dissolved concentrations in the river water) of ten trace elements analyzed within this study varied as follows (in μg L^{-1}): Ag, <0.001; Bi, <0.001; Cd, 0.006±0.002; Co, 0.028±0.008; Cu, 0.101±0.067; Fe, 8.45±5.31; Mn, 2.14±0.50; Mo, 0.558±0.145; Se, 0.138±0.072; Zn, <0.519 (Čerkez, 2021; *unpublished data*).

2.2. The isolation of northern pike hepatic cytosols for SEC-HPLC and HR ICP-MS analyses

In order to isolate the cytosolic fractions from northern pike liver, we have cut liver samples to small pieces and then added cooled homogenization buffer [100 mM Tris-HCl/Base (Sigma, pH 7.5 at 4°C) supplemented with reducing agent (1 mM dithiotreitol, Sigma)] (m_{liver}/v_{buffer} 1:5). The obtained suspensions were homogenized in an ice cooled tube by 10 strokes of Potter-Elvehjem homogenizer (Glas-Col, USA) at 6,000 rpm. The homogenates were then centrifuged at 50,000×*g* for 2 h at +4°C in the Avanti J-E centrifuge (Beckman Coulter, USA), and supernatants were afterwards stored at -80°C. Thus obtained supernatants (S50) represented soluble tissue fractions, i.e. hepatic cytosols, additionally containing only microsomes (Bonneris et al., 2005). The entire procedure was performed under ice-cold conditions, and all the necessary preconditions were applied to avoid/limit the equilibrium change among trace elements and biomacromolecules in the cells/tissues (Szpunar et al., 2003; Dragun et al., 2020).

2.3. SEC-HPLC separation of cytosolic biomolecules from northern pike liver

Separation of metal-binding biomolecules of various molecular masses from hepatic cytosols of northern pike was done by SEC-HPLC system (Perkin Elmer, series 200, USA) with prepacked

column Tricorn™ Superdex 200 10/300 GL (GE Healthcare Biosciences, USA) for globular proteins (separation range: 10 to 600 kDa) and a diode array UV/VIS detector. Separation conditions were previously reported in Dragun et al. (2020) and references cited therein. A buffer, 20 mM Tris-HCl/Base (Sigma, pH 8.1 at 22°C), was applied as a mobile phase, with a flow rate of 0.5 mL min^{-1}, using isocratic mode. Total volume of 200 µL per sample was applied on the column (two runs of 100 µL). Fraction collector (FC 203B) with the thermostated cuvette holder (FC SPL-2085B-HDW) (Gilson, USA) and recirculating chiller (RC3000G; Grant, UK), was used for collection of one-minute fractions in the plastic tubes (from 13^{th} to 52^{nd} minute). Blue dextran was used for determination of the void volume of the column (Table 2), while the equation of column calibration straight line was calculated based on the elution times (t_e) of seven protein standards (thyroglobulin, apoferritin, β-amylase, alcohol dehydrogenase, bovine albumin, superoxide dismutase and carbonic anhydrase; Sigma, USA) (Table 2). The obtained calibration straight line was applied to relate the elution times of chromatographic peaks containing specific metals/nonmetal to molecular masses of cytosolic biomolecules that presumably bind them (Table 3). We have additionally determined t_e for metallothionein standard (MT-1, Enzo Life Sciences, USA). All the standards were run through the column applying the same conditions as used for the samples. All the precautions were made to avoid dissociation of the metals from metal-binding proteins (according to de la Calle Guntiñas et al., 2002). Recoveries from the column for the analyzed elements, based on the analyses of four samples (No. 1, 2, 5, and 9), were higher than 75% for Ag, Bi, Cd, Co, Cu, Se, and Zn, whereas they were below 75%, but higher than 50% for Fe, Mn, and Mo.

2.4. Metal/nonmetal measurements by HR ICP-MS

We have measured the concentrations of ten trace elements (essential elements Co, Cu, Fe, Mn, Mo, Se, and Zn; nonessential elements Ag, Bi, and Cd) in the digested hepatic cytosols of northern pike, and in SEC-HPLC-obtained fractions of hepatic cytosols, using HR ICP-MS (Element 2, Thermo Finnigan, Germany), equipped with an autosampler SC-2 DX FAST (Elemental Scientific, USA) and sample introduction kit (cyclonic spray chamber Twister and

SeaSpray nebulizer). Measurements of ^{82}Se, ^{98}Mo, ^{109}Ag, ^{111}Cd, and ^{209}Bi were performed in low-resolution mode, and of ^{55}Mn, ^{56}Fe, ^{59}Co, ^{63}Cu, and ^{66}Zn in medium resolution mode. Applied calibration mode was external, and it was based on the use of adequate dilutions of multielement standard solution for trace elements (Analitika, Czech Republic) in 2% (vol.) HNO_3 (*Normatom® 67*-69% *for trace element analysis*, VWR Chemicals, UK).

Prior to measurement, hepatic cytosols were digested in duplicate in acid/peroxide digestion mixture ($v_{cytosol}/v_{mixture}$ 1:1). The digestion mixture contained concentrated HNO_3 (*Normatom® 67*-69% *for trace element analysis*, VWR Chemicals, UK) and 30% H_2O_2 (*Suprapur®*, Merck, Germany) (v_{HNO3}/v_{H2O2} 3:1). The procedure was carried out at 85°C in the laboratory dry oven, and lasted for 3.5 h. After digestion, the samples were further diluted five times with Milli-Q water. SEC-HPLC fractions, on the other hand, were not digested, but only acidified with HNO_3 (*Normatom® 67*-69% *for trace element analysis*, VWR Chemicals, UK, final concentration in the samples: 1% (vol.)). Indium (Fluka, Germany) was added to all the samples and calibration standards, as an internal standard (1 $\mu g\ L^{-1}$).

Limits of detection (LOD) for trace elements measured in cytosols were determined on the basis of three standard deviations of ten successively measured trace element concentrations in the blank samples (Tris-HCl/Base buffer, dithiothreitol), which were prepared for measurement by the same procedure as the hepatic cytosols. The determined LODs were the following (in ng g^{-1}): Ag, 0.369; Bi, 0.241; Cd, 0.080; Co, 0.220; Cu, 16.3; Fe, 75.2; Mn, 1.70; Mo, 0.205; Se, 1.53; and Zn, 22.9. LODs for trace elements measured in the SEC-HPLC fractions of hepatic cytosols, according to our previously published reports (Krasnići et al., 2013), were the following (in $\mu g\ L^{-1}$): Bi, 0.002; 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. The accuracy check of HR ICP-MS measurements was based on analyses of selected trace element concentrations in quality control samples (UNEP GEMS, Canada) on six separate occasions. The following recoveries (average±standard deviation) were achieved: Cd, 102.2±1.2%; Co, 103.2±3.0%; Cu, 101.5±2.1%; Fe, 98.4±4.0%; Mn, 101.3±1.9%; Mo, 99.5±3.0%; and Zn, 105.6±4.5%.

210

211 *2.6. Calculations and graphical data presentation*

212 All the calculations were done in Microsoft Office Excel – version 16. The graphs were drawn
213 by use of statistical program SigmaPlot 11.0 for Windows.

214

215 **3. Results and discussion**

216 The setting of our study enabled the determination of basal cytosolic distributions of ten trace
217 elements among biomolecules of various masses in the liver of northern pike, since the
218 exposure conditions in the Mrežnica River, where pike were caught, indicated low level of
219 water contamination (Čerkez, 2021). Under such conditions of sublethal chronic metal
220 exposure, the use of liver, an internal, metal-accumulating organ, as target organ for metal
221 analyses is especially important due to its adaptive capacity (Stubblefield et al., 1999; Lange et
222 al., 2002). Moreover, the information that we have obtained in this study allowed the first
223 deeper insight in the metal handling strategies of any member of the family Esocidae.
224 For the sake of simpler comparison with previously published reports for the liver of several
225 other fish species (Krasnići et al., 2013, 2018; Dragun et al., 2018; Krasnići et al., 2019;
226 Dragun et al., 2020), we have applied biomolecule categorization in four groups of molecular
227 masses (MM) (Table 3): 1) HMM, which contains the biomolecules of high molecular masses
228 (>100 kDa); 2) MMM – medium molecular masses (>30-100 kDa); 3) LMM – low molecular
229 masses (10-30 kDa); and 4) VLMM – very low molecular masses (<10 kDa) which refer to
230 peaks outside of the separation range of the column. As the separation method applied in this
231 study has rather limited resolution possibilities, it can be expected that many of metal-binding
232 biomolecules coelute, and, therefore, the obtained chromatographic peaks have possibly
233 reflected the binding of analyzed elements to one or more cytosolic biomolecules (de la Calle
234 Guntiñas et al., 2002; Dragun et al., 2020). An example of SEC-HPLC chromatograms is given
235 in the Figure SI-1 in supplementary information, recorded at four different wavelenghts: two in
236 UV region, at 254 nm characteristic for metal-thiolate bond absorption (e.g. metallothioneins;
237 Rodríguez-Cea et al., 2006) and at 280 nm characteristic for aromatic ring (e.g. tyrosine and

tryptophan; Amarowicz and Shahidi, 1997); and two in visible region, at 415 nm characteristic for heme moiety and at 540 nm characteristic for oxyhemoglobin (McDevitt et al., 2020). Further in the text we will discuss in more detail each group of biomolecules and the elements that they tend to bind in the liver of northern pike.

3.1. Metals that are predominantly distributed within HMM biomolecule region (Co, Fe, and Mo)

The high content of cytosolic proteins was observed in HMM region (t_e ≤23 min), as seen from the chromatograms recorded at 254 and 280 nm (absorption of metal-thiolate bond and aromatic ring; Fig. SI-1 a and b). Three of ten analyzed elements in this study were predominantly eluted with HMM biomolecules, namely essential elements Co, Fe and Mo (Fig. 2). The elution of Co (Fig. 2a, Table 3) was recorded in two HMM peaks, minor elution in the column void volume with maximum at t_e of 15 min (>600 kDa) and major elution at 20-21 min (~170-230 kDa). High Co elution in HMM region was previously found in the liver of European and Vardar chub (Krasnići et al., 2013, 2018), brown trout (Dragun et al., 2018) and juvenile yellow perch (Caron et al., 2018).

Iron (Fig. 2b) and Mo (Fig. 2c) were eluted in the peaks located between two Co peaks, i.e. with maxima at t_e of 18 min (~400 kDa) and 19 min (~300 kDa), respectively (Table 3). Major Fe elution within HMM region was reported for the liver of European and Vardar chub (380-400 kDa; Krasnići et al., 2013, 2018), brown trout (380 kDa; Dragun et al., 2018) and Prussian carp (300-400 kDa), corresponding to elution time of standard protein apoferritin (17.41 min; 443 kDa; Table 2), and probably indicating Fe binding to storage protein ferritin (450 kDa; Aisen et al., 2001; Carriquiriborde et al., 2004), thus confirming important and well-known function of liver in Fe storage and metabolism (Kamińska-Gibas et al., 2018). Predominant Mo elution within HMM region (maxima at 230-240 kDa) was reported for the liver of European and Vardar chub (Krasnići et al., 2013, 2018), brown trout (Dragun et al., 2018) and Prussian carp (Dragun et al., 2020), possibly indicating association with cytosolic enzymes such as

xanthine oxidoreductase (290 kDa; Battelli et al., 2016) and/or aldehyde oxidase (132 kDa; Uchida et al., 2003).

Although the major quantities of these metals were associated to HMM biomolecules, all three of them were also eluted in smaller quantities with biomolecules of lower molecular masses. Both Co and Fe (Fig. 2a,b) were eluted in MMM region with maxima at t_e of 26 min (46 kDa). The minor elution of Co in the MMM biomolecule region was also observed in the liver of Vardar chub (31-85 kDa; Krasnići et al., 2013) and brown trout (20-85 kDa; Dragun et al., 2018). In the case of Fe, somewhat lower elution in MMM compared to HMM region was observed in the liver of European and Vardar chub (35-40 kDa; Krasnići et al., 2013, 2018) and brown trout (31-85 kDa; Dragun et al., 2018), whereas it was almost negligible in the liver of Prussian carp (30-40 kDa; Dragun et al., 2020). Further analysis of Fe-binding MMM biomolecules in the liver of Vardar chub confirmed the presence of monomers (~15.5 kDa), dimers (~31.5 kDa) and trimers (~47 kDa) of hemoglobin subunits (Krasnići et al., 2019). In this study, high protein peak was observed at t_e of 26 min in chromatogram recorded at wavelength of 415 nm confirming the presence of heme (Fig. SI-1 c), whereas rather small peak was observed at 540 nm indicating partial presence of hemoglobin in the form of oxyhemoglobin (Fig. SI-1 d).

Moreover, both Co and Mo (Fig. 2a,c) were eluted in VLMM region (Table 3).

Very small Co peaks in the VLMM biomolecule region were previously reported for the liver of European and Vardar chub (Krasnići et al., 2013, 2018), brown trout (Dragun et al., 2018), and juvenile yellow perch (Caron et al., 2018), possibly reflecting Co association with cobalamine (vitamin B12) structure (1.3 kDa; Kirschbaum, 1981; Blust, 2012). Elution of Mo in VLMM region was previously reported for the liver of European and Vardar chub (Krasnići et al., 2013, 2018), brown trout (Dragun et al., 2018) and Prussian carp (Dragun et al., 2020), possibly indicating Mo binding to metallochaperones or nonprotein cofactors, such as family of low molecular mass pterin-based cofactors, Moco (Loutet et al., 2015; Mendel, 2013).

Increase of cytosolic concentrations of Co and Fe was reflected in the height increase of their HMM peaks (t_e at 20-21 min and 18 min, respectively), as seen for Co in Vardar chub and

293 brown trout liver (Dragun et al., 2018; Krasnići et al., 2018) and for Fe in liver of European and
294 Vardar chub, brown trout and Prussian carp (Krasnići et al., 2013, 2018; Dragun et al., 2018,
295 2020). Increase of cytosolic Mo concentrations was reflected in the increase of both HMM (t_e
296 at 19 min) and VLMM (t_e at 34-35 min) Mo peaks, whereas in the Vardar chub, brown trout
297 and Prussian carp liver it was reflected mainly in the increase of HMM peak (Krasnići et al.,
298 2018; Dragun et al., 2018, 2020).
299 High similarity of northern pike with two leuciscid species (European and Vardar chub), one
300 cyprinid (Prussian carp) and one salmonid species (brown trout) was observed considering Co,
301 Fe and Mo elution profiles, indicating generally comparable intracellular behaviour of these
302 metals in all studied fish (Krasnići et al., 2013; Dragun et al., 2018; Krasnići et al., 2018;
303 Dragun et al., 2020). Only difference referred to Fe distribution profiles, namely most of the
304 other studied fish generally had much higher hepatic levels of MMM Fe-binding biomolecules,
305 presumably hemoglobin, compared to northern pike. That was an indication of lower blood
306 perfusion of northern pike liver, which was moreover very easily observed based on its pale
307 liver colour. In addition, in northern pike liver, similarly to all studied leuciscid and cyprinid
308 fish, Fe elution was not recorded within VLMM biomolecule region (<10 kDa), which was
309 observed in brown trout (Dragun et al., 2018, 2020).
310
311 *3.2. Metals that are comparably distributed between HMM and MMM biomolecule regions (Bi*
312 *and Zn)*
313 The observed Zn binding within HMM and MMM biomolecule regions probably reflected its
314 essential function in several metabolic processes and its significant role as a cofactor in
315 numerous enzymes and metalloproteins in living organisms (Hauser-Davis et al., 2014),
316 whereas in the case of nonessential element Bi it could have indicated the possibility of toxic
317 effects. Accordingly, the large portions of both Bi and Zn (Fig. 3, Table 3) were eluted within
318 several HMM peaks, namely both metals were eluted in the column void volume at t_e of 15 min
319 (maxima at >600 kDa), same as Co, and at 18-19 min (maxima at ~300-400 kDa), same as Fe
320 and Mo. The predominant portion of Zn (Fig. 3b) was eluted at t_e of 22 min (maximum at 135

kDa, Table 3), again comparable to Co. High Zn elution in the region of HMM was previously also reported for the liver of brown trout (Dragun et al., 2018) and European eel (*Anguilla anguilla*, Van Campenhout et al., 2008).

The predominant portion of Bi (Fig. 3a) was eluted in MMM region at t_e of 24-25 min (maximum at ~60-80 kDa, Table 3). In soluble fraction of Indonesian tuna muscles, beta-actin (protein with an important role in wound healing and tissue morphogenesis, and containing five cysteine residues for metal interaction) was identified as a common target protein for binding multiple metals (e.g. Hg, Cu, Ag, and Bi) (Nong et al., 2021, and the references cited therein). Nong et al. (2021) estimated molecular mass of beta-actin to be 50-75 kDa, corresponding to estimated molecular mass of Bi-binding biomolecule in the liver of northern pike. Contrary, only small portion of Zn (Fig. 3b) was eluted in that region at t_e of 26 min (maximum at 46 kDa, Table 3), including molecular masses of various Zn-containing proteins and enzymes, such as transport proteins albumin (66 kDa, Table 2) and transferrin (70–80 kDa; Sun et al., 2012), as well as several cytosolic enzymes (carbonic anhydrase, 29.7 kDa, Kucuk and Gulcin, 2016; superoxide dismutase, 32 kDa, Pedrajas et al., 1993; and alcohol dehydrogenase, 80 kDa, Thompson et al., 2018).

Considerable portions of both Bi and Zn (Fig. 3a,b) were also eluted within LMM region at t_e of 30-31 min (maxima at 12-16 kDa, Table 3), which corresponded to elution time of MT standard (31.24 minute; Table 2). MTs are low molecular mass proteins with approximately 30% of cysteinyl residues in their primary structure (Hamer, 1986). It is well known that they are involved in homeostasis of essential metals (such as Cu and Zn), as well as in detoxification of nonessential metals (such as Cd and Ag) by lowering the intracellular level of free metal ions through their binding to abundant SH groups (Kägi and Schäffer, 1988; Roesijadi and Robinson, 1994; Roesijadi, 1996; Urien et al., 2018). MTs can bind a number of metals other than Cd, Cu and Zn, including Bi (Wong et al., 2017). Morover, in the soluble fraction of Indonesian tuna muscles, an unidentified Bi-binding protein was observed using separation by SEC-HPLC, and its molecular mass was approximately 14 kDa (Nong et al., 2021), thus possibly corresponding to MTs.

Finally, Zn (Fig. 3b) was eluted in two more peaks within VLMM region (Table 3).

The binding of Zn to biomolecules of MM below 10 kDa is usually more common for the profiles of fish uptake organs than of the liver (e.g. gills of European chub, Krasnići et al., 2014; gills and intestine of Prussian carp, Dragun et al., 2020, Mijošek et al., 2021) and could refer to Zn association with reduced glutathione (GSH, 307 Da), or to metallochaperones (Dragun et al., 2020), both included in metal detoxification by binding the excess metal ions (Regvar and Vogel-Mikuš, 2011). GSH acts as a "first line defense" against metals (Singhal et al., 1987). It has the ability to modify toxicity of metals by changing metal uptake and elimination rates, as well as by chelating the metal ions immediately after they enter the cell (Lange et al., 2002, and references therein). Accordingly, its possible involvement in rapid hepatic turnover of Zn in the form of GSH-conjugates was previously reported for rainbow trout (*Oncorhynchus mykiss*) (Lange et al., 2002).

Increase of cytosolic Bi and Zn concentrations was reflected in the height increase of all the observed peaks. However, for Bi it was especially evident in MMM region (t_e at 24-25 min), and, following pronounced concentration increase, it was also eluted in high quantity in HMM region (t_e at 15 min and 19 min). The most pronounced increase of Zn peaks, following concentration increase, was observed in HMM region (t_e at 15 min and 22 min), as well as in LMM, i.e. MT, region (t_e at 30-31 min). In the liver of Vardar chub, European eel and Prussian carp, the increases of Zn cytosolic concentrations were mainly reflected in the increased elution in MT region (Goenaga Infante et al., 2003; Van Campenhout et al., 2008; Krasnići et al., 2018; Dragun et al., 2020), whereas in the liver of brown trout it was reflected in the increased elution in HMM region (Dragun et al., 2018).

Comparison with two leuciscid species (European and Vardar chub), one cyprinid (Prussian carp) and one salmonid fish (brown trout) indicated higher similarity between northern pike and brown trout (i.e. esocid and salmonid fish) considering Zn elution profiles. Although most of the observed peaks were seen in all fish species, both northern pike and brown trout had higher quantity of Zn eluted in HMM and MMM regions, whereas in all leuciscid and cyprinid fish the Zn elution was much more pronounced in the MT region indicating higher detoxification

potential (Krasnići et al., 2013; Dragun et al., 2018; Krasnići et al., 2018; Dragun et al., 2020). So far, there is no information for Bi cytosolic distribution in leuciscid, cyprinid and salmonid fish, making that interspecies comparison impossible.

3.3. Metal/nonmetal that are predominantly distributed within MMM biomolecule region (Mn and Se)

Selenium is an essential element which is mostly covalently bound within selenoproteins that are required for biochemical processes in the cells; however, it has a relatively narrow range of concentrations beneficial for the cell and when it is surpassed toxic effects can appear (Urien et al., 2018, and the references cited therein). On the other hand, Mn is a metal with an essential role in the functioning of many enzymes (Mogobe et al., 2015).

Only small quantities of Mn were eluted in three peaks within HMM region (Fig. 4a), with maxima at t_e of 15 min (>600 kDa), 18-19 min (~300-400 kDa) and 22 min (135 kDa) (Table 3). Contrary, high Mn elution in HMM region was observed in the liver of European and Vardar chub (at 140 kDa; Krasnići et al., 2013, 2018), and brown trout (at ~600 kDa and 180 kDa; Dragun et al., 2018), whereas the information on Prussian carp is not available.

In northern pike, however, the main portions of both Mn and Se (Fig. 4a,b) were eluted within MMM region, with maxima at t_e of 25-27 min (35-60 kDa, Table 3), overlapping with minor elutions of Co, Fe and Zn, and major elution of Bi. Again, contrary to northern pike, Mn elution in MMM region was only minor in European and Vardar chub (maxima at 47-66 kDa; Krasnići et al., 2013, 2018), whereas in brown trout it was rather high (at 85 kDa), but comparable to HMM peak (Dragun et al., 2018). The MMM peak comprises molecular masses of Mn transport proteins albumin (66 kDa, Table 2) and transferrin (80 kDa; Martin-Antonio et al., 2009). The elution range of Se, on the other hand, included molecular masses of some well known cytosolic Se-compounds, such as thioredoxin reductase which participates in the defense against oxidative stress (64.1 kDa; Lopez Heras et al., 2011; Akyol and Kuzu, 2017) and selenoprotein P which participates in the transport of Se from liver to remote tissues (SelP; ~50 kDa; Kryukov and Gladyshev, 2000; Papp et al., 2007). The elution of Se in European and

405 Vardar chub, brown trout and Prussian carp was only minor or negligible in MMM region
406 (Krasnići et al., 2013, 2018; Dragun et al., 2018, 2020).
407 Small quantities of both Mn and Se were further eluted in VLMM region, Se (Fig. 4b) in two
408 clear peaks (and Mn (Fig. 4a) within a single small peak. Elution of Mn in VLMM region was
409 not observed or was negligible in the liver of European and Vardar chub, and brown trout,
410 whereas Se elution in VLMM region was previously recorded in the liver of the other fish
411 species (Krasnići et al., 2013, 2018; Dragun et al., 2018, 2020). In European chub (Krasnići et
412 al., 2013) and Prussian carp (Dragun et al., 2020) it was only minor, in Vardar chub it was more
413 pronounced (Krasnići et al., 2018), whereas in brown trout it was predominant with almost
414 negligible Se elution in regions of higher molecular masses (Dragun et al., 2018). The
415 association of Se with biomolecules of molecular mass <2 kDa was also reported for white
416 suckers (Urien et al., 2018). Selenium binding to compounds that participate in a defence
417 against oxidative stress is well known, and thus observed Se elution within VLMM region
418 could indicate association with small antioxidative compounds like selenomethionine (~0.2
419 kDa; Klotz et al., 2003) or selenoneine (~0.5 kDa;Yamashita and Yamashita, 2010; Yamashita
420 et al., 2012).
421 Both Mn and Se did not show the clear pattern of peak increase following the increase of their
422 cytosolic concentrations. However, generally certain increase of peak height for both elements
423 was observed in the MMM region (t_e of 25-27 min). As Mn is regulated within a narrow range,
424 the clear information on distribution changes due to its increased bioaccumulation were also not
425 reported for the other fish (Krasnići et al., 2013, 2018; Dragun et al., 2018). As for Se, in the
426 liver of European and Vardar chub, and Prussian carp, its increased presence was associated
427 either to LMM, MMM or HMM biomolecules, whereas in the brown trout higher Se hepatic
428 bioaccumulation was reflected in the height increase of Se-VLMM peaks (Dragun et al., 2018).
429 The most evident feature of Mn distribution in the liver of northern pike, which distinguishes it
430 from European and Vardar chub, and brown trout, was sharp and narrow elution in MMM
431 region, whereas Mn elution in leuciscid fish was limited to HMM region, and in salmonid fish

covered much wider regions, encompassing, with the comparable intensity, the molecular masses from high to low values.

When Se is considered, its distribution profiles in northern pike also differed from all the other studied fish. Its predominant MMM elution, which possibly indicated binding to thioredoxin reductase or selenoprotein P, was not observed either in leuciscid and cyprinid or salmonid fish. The predominant Se elution in the European and Vardar chub liver was associated to the biomolecules in the LMM region of 10-60 kDa (Krasnići et al., 2013, 2018), whereas in the liver of Prussian carp the major part of Se was eluted within HMM region (maximum at 141 kDa; Dragun et al., 2020). As previously suggested (Dragun et al., 2020), it is possible that Se elution in leuciscid and cyprinid fish, either with HMM proteins in Prussian carp liver or LMM proteins in chub liver, indicated Se association to the same enzyme, i.e. glutathione peroxidase, either in the form of intact enzyme (96 kDa, homotetramer with four subunits; Bastos et al., 2007) or enzyme subunits (~23 kDa; Bastos et al., 2007). On the other hand, Se elution in salmonid fish was predominantly present in VLMM region, corresponding to only minor elution in northern pike, and confirming Se association to small antioxidative molecules. All of those Se-binding compounds, glutathione peroxidase, thioredoxin reductase, selenomethionine and selenoneine, contribute to the defense of oxidative stress, but they were evidently differently expressed in the fish from different families, indicating specific defense strategies of each species.

3.4. Metals that are predominantly distributed within LMM biomolecule region (Ag, Cd, and Cu)

The major quantities of three metals, Ag, Cd and Cu (Fig. 5), were eluted within a single sharp peak in LMM region, with maxima at t_e of 30-31 min (12-16 kDa, Table 3), overlapping with significant elutions of Bi and Zn, as well as MT standard (31.24 min; Table 2), thus indicating binding to MTs for all five metals, and predominant in the case of Ag, Cd and Cu. Despite this, MT peak was not clearly seen in the chromatogram recorded at 254 nm (absorption of metal-thiolate bond; Fig. SI-1a), but was rather small in comparison to other protein peaks, which can

460 be explained by the fact that MT in northern pike liver comprises less than 0.5% of total
461 cytosolic proteins (this study, unpublished results). We have already observed elution of Cd, Cu
462 and Zn in MT region in the liver of several fish species (e.g. European and Vardar chub,
463 Krasnići et al., 2013, 2018; brown trout; Dragun et al., 2018; and Prussian carp, Dragun et al.,
464 2020). MT fractions from Vardar chub liver were additionally analyzed by anion-exchange
465 HPLC and MALDI-TOF-MS, revealing the presence of two MT isoforms of identical
466 molecular masses (6.0 kDa) but varying in total charge (Krasnići et al., 2019). Furthermore, for
467 some of those elements predominant elution with MTs was also observed in the liver of few
468 other fish species (e.g. common carp (*Cyprinus carpio*; Cd, Cu, Zn), Van Campenhout et al.,
469 2004, Huang et al., 2007; European eel (Cd, Cu), Goenaga Infante et al., 2003; European eel
470 (Cd, Cu, Zn), Van Campenhout et al., 2008; Prussian carp (Cd, Cu), Van Campenhout et al.,
471 2010; juvenile yellow perch (Ag, Cd, Cu), Caron et al., 2018; and white suckers (Cd, Cu),
472 Urien et al., 2018).
473 Almost negligible quantities of Cd and Cu were additionally eluted in regions of higher
474 molecular masses. Namely, only a small quantity of Cd (Fig. 5b) was eluted in HMM region in
475 the column void volume with maximum at t_e of 15 min (>600 kDa, Table 3). Cadmium peak in
476 HMM region (500-1000 kDa) was also observed for the liver of brown trout (Dragun et al.,
477 2018), whereas small Cd elution with biomolecules of medium molecular masses (35-100 kDa)
478 was observed in European chub, and was more obvious in the samples with higher cytosolic Cd
479 concentrations (Krasnići et al., 2013). In nothern pike liver, Cu (Fig. 5c) was also eluted in
480 HMM and MMM regions with maxima at t_e of 23 min (~100 kDa, Table 3) and 26 min (46
481 kDa, Table 3), respectively, including, for example, the molecular mass of Cu-containing
482 cytosolic enzyme superoxide dismutase (32 kDa; Pedrajas et al., 1993). Copper elution within
483 the range of biomolecules of higher molecular masses was further recorded in the liver of
484 European chub (27-60 kDa; Krasnići et al., 2013), brown trout (above 85 kDa; Dragun et al.,
485 2018), juvenile yellow perch (~44 kDa, Caron et al., 2018) and European eel (>60 kDa; Van
486 Campenhout et al., 2008).

For Ag, Cd and Cu in the liver of northern pike, increases of their cytosolic concentrations were clearly and rather proportionally reflected in the increases of their LMM peak heights. Increased elution in MT region following the increase of cytosolic concentrations was previously observed for Cd and Cu in the liver of European and Vardar chub (Krasnići et al., 2013, 2018), brown trout (Dragun et al., 2018), Prussian carp (Van Campenhout et al., 2010; Dragun et al., 2020), juvenile yellow perch (Caron et al., 2018), and European eel (Goenaga Infante et al., 2003; Van Campenhout et al., 2008), as well as for Cd in white suckers (Urien et al., 2018) and common carp (Huang et al., 2007).

High similarity of northern pike with two leuciscid species (European and Vardar chub), one cyprinid (Prussian carp) and one salmonid fish (brown trout) was observed considering Cd and Cu elution profiles, indicating comparable Cd and Cu handling strategies, i.e. predominant binding to MTs (Krasnići et al., 2013; Dragun et al., 2018; Krasnići et al., 2018; Dragun et al., 2020). For Ag, data on intracellular distribution profiles in both chub species, brown trout and Prussian carp were not reported, and thus comparison between four fish families could not be made.

3.5. Comparison of cytosolic metal/nonmetal distributions in the liver of northern pike (family Esocidae) to fish from families Leuciscidae, Cyprinidae and Salmonidae

Disturbances caused by metal exposure in fish, such as osmoregulatory disorders or oxidative stress caused by Cu, are reported to be species-specific (de Paula et al., 2021). Currently existing information suggest that such differences occur because different fish species significantly vary in the subcellular handling of metals; species-specific differences in transporters, chaperones, metal-binding proteins and other targets could be important factors in metal toxicity and metal sensitivity of specific fish species (Eyckmans et al., 2012; Shekh et al., 2021). Shekh et al. (2021) recently confirmed this hypothesis by finding that greater sensitivity of rainbow trout than white sturgeon (*Acipenser transmontanus*) to Cd exposure could be explained by the fact that, compared to rainbow trout, white sturgeon divert a higher amount of

514 Cd towards biologically inactive metal pool and a lower amount towards the biologically active
515 metal pool (Shekh et al., 2021).
516 In our study, as described in detail above, many differences, but also some similarities, were
517 observed between the fish belonging to different families regarding the intracellular metal
518 distributions, which could also affect their abilities to cope with the conditions of increasing
519 metal exposure. In our previous work, we have demonstrated several obvious differences
520 between fish belonging to families Leuciscidae, Cyprinidae and Salmonidae (Dragun et al.,
521 2020). Including another family, Esocidae, in that comparison, the importance of considering
522 the species-specific differences when studying the metal-handling strategies of fish was only
523 further accentuated.
524 Still, it is interesting to observe that for several elements the members of all studied families
525 have the exact same cellular responses. For Cd and Cu, the predominant binding to MT was
526 recorded in all five fish species (esocid northern pike, salmonid brown trout, leuciscids
527 European and Vardar chub, and cyprinid Prussian carp) (Krasnići et al., 2013; 2018; Dragun et
528 al., 2018; 2020). Furthermore, for Co, Fe and Mo, almost identical cytosolic profiles were also
529 observed in all the fish, with only somewhat lower Fe elution in the hemoglobin region of
530 northern pike liver.
531 However, for the rest of the elements, striking differences were observed among fish species. In
532 line with our aim to establish if northern pike gravitates more towards salmonids, leuciscids or
533 cyprinids regarding metal intracellular behaviour, only in the case of Zn we have observed
534 stronger resemblance between this esocid fish and salmonid brown trout. Both these fish had
535 much higher Zn elution in HMM and MMM regions, and lower elution in LMM (i.e. MT)
536 region compared to leuciscid and cyprinid fish, probably reflecting differences in their
537 detoxification potentials. De Boeck et al. (2003) previously reported that significant differences
538 can appear in the expression and induction of MT among different fish species.
539 When Mn and Se were considered, northern pike differed equally from all the other studied
540 families; namely Mn was bound to MMM biomolecules in esocids, to several molecular regions
541 in salmonids, and to HMM biomolecules in leuciscids; and Se was bound to MMM

biomolecules in esocids, to VLMM biomolecules in salmonids, to LMM biomolecules in leuciscids and to HMM biomolecules in cyprinids. The observed distinctions in intracellular metal distributions could be a sign of different modes and abilities of these four fish families to handle the same levels of metal exposure and thus should be further explored.

3.6. Specificity of metal/nonmetal cytosolic distributions profiles in northern pike specimens with green liver

In the course of the project, the sampling of northern pike was performed in two spring periods (2020 and 2021) and in the autumn of 2021. Only in spring periods the specimens with green liver were observed, and they were mainly the smallest among sampled fish (maximum mass 381 g, but mostly below 150 g). Buccal or generalized green discolouration of the tissues of northern pike was previously reported from Sweden and Canada (Evensen, 2006). Green liver syndrome was, on the other hand, observed in several fish species (e.g., red sea bream *Pagrus major*, Takagi et al., 2006; yellowtail *Seriola quinqueradiata,* Maita et al., 1997; Takagi et al., 2005; yellow catfish *Pelteobagrus fulvidraco*, Cai et al., 2020), and was associated to various factors, such as diets low in fish meat due to deficiency of specific aminoacids (taurine) (Takagi et al., 2006), diets with high levels of plant ingredients (Cai et al., 2020), or even physiological effects of low water temperature in winter (Sakaguchi and Hamaguchi, 1979). In the study on yellowtail, it was explained that green liver occurs as a result of a hemolytic biliverdin overproduction and reduction in the excretion of bile pigments from the liver into the bile (Takagi et al., 2005). Biliverdin is a degradation product of heme catabolism, rapidly converted to bilirubin by biliverdin reductase (Dimitrijević et al., 2018). Absorption maxima of green compounds biliverdin (molecular mass 582.6 Da) at neutral pH and its immediate precursor verdohemochrome (molecular mass 794.7 Da) are at 670 nm and 656 nm, respectively (Yoshida and Kikuchi, 1974). To confirm biliverdin as a cause of the green colour of the liver of northern pike in our study, we have comparatively scanned the cytosols of yellow and green liver in the wavelength range of 230-900 nm (spectrophotometer Infinite M200, Tecan, Switzerland), and observed clear differences (Fig. 6). The yellow/brown liver revealed the

absorption profile with three peaks characteristic for hemoglobin: the first and the highest peak at 415 nm, characteristic for heme moiety and called Soret peak; the other two smaller peaks at 540 and 570 nm, characteristic for oxyhemoglobin (according to McDevitt et al., 2020). The green liver, on the other hand, revealed reducement of the hemoglobin peaks and occurrence of the wide peak in the wavelength range of 620-690 nm, with maximum at 650-670 nm, corresponding to biliverdin and its precursor.

Complexes of biliverdin, as well as the other bile pigment bilirubin, with proteins can serve in protection against metal poisoning (Goncharova and Urbanova, 2009, and the references therein), and both pigments form monomeric complexes with Cd, Cu, and Zn ions and dimeric complexes with Mn ions (Goncharova and Urbanova, 2009). Goncharova and Urbanova (2009) further proposed that they, as good chelating agents, can probably inactivate Zn-metalloenzymes by binding to their Zn atoms. Moreover, Dimitrijević et al. (2018) reported that, at physiological pH, biliverdin can build a complex with Cu ions in 1:1 stoichiometry. Thus, we wanted to establish if probable increased presence of biliverdin in some specimens of northern pike have caused changes in the cytosolic hepatic distributions of elements analyzed in this study. Three fish with green liver were used for this analysis, and the results presented in Fig. 7 indicate that clear differences between metal/nonmetal distributions in yellow/brown and green liver of northern pike were not obvious. Further studies should be conducted to establish if increased presence of biliverdin causes the changes in enzyme/protein activities in fish liver.

4. Conclusions

Combined SEC-HPLC and HR ICP-MS analyses of the cytosolic metal/Se distributions in northern pike liver provided the first ever information on the intracellular metal/Se behaviour in an esocid fish. Considering that this study was conducted in the weakly contaminated environment of the Mrežnica River in Croatia, the distributions within the hepatic cytosol established for ten elements corresponded to fish basic metabolism, and can serve as the basis for further research of the metal handling strategies of northern pike, but also for comparative study of the fish in general. The obtained results indicated more pronounced tendency of the

majority of analyzed essential elements (Co, Fe, Mo, Zn, Mn and Se) towards the cytosolic biomolecules of higher molecular masses, which is in agreement with their structural and functional roles in many proteins and enzymes. Contrary, all three analyzed nonessential metals were found to bind to low molecular mass detoxification protein metallothionein, either predominantly (Ag and Cd) or partly (Bi). The first exception to this rule was essential element Cu, which was predominantly bound to low molecular mass MT in accordance with the function of that protein in homeostasis of both Zn and Cu. The other exception was nonessential metal Bi; although partly detoxified by MT, its predominant binding to biomolecules of higher molecular masses could have indicated its potential for toxic effects. Comparison of the information gathered for the liver of an esocid fish northern pike with the published information on several leuciscid, cyprinid and salmonid species revealed a number of species-specific differences in their intracellular metal-handling which is important when evaluating the impact of pollution on different fish species. On the other hand, the occurrence of the green liver in the smaller specimens of northern pike in the spring periods, most probably due to increased hepatic level of biliverdin, have not shown to be the cause of any differences in cytosolic metal/Se distributions compared to the specimens with the yellow/brown liver, which is still not a final proof that there are not any differences in enzyme/protein activities. Accordingly, the next step of our research on northern pike will be identification of specific hepatic metal-binding biomolecules by application of additional chromatographic separations and various techniques of mass spectrometry.

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Figure captions

Figure 1. The map of the Mrežnica River course in Croatia with marked sampling area in the vicinity of the Town of Duga Resa.

Figure 2. The hepatic distribution profiles of a) Co, b) Fe, and c) Mo among cytosolic biomolecules of different molecular masses in the northern pike (*Esox lucius*) with yellow/brown liver from the Mrežnica River (caught in the vicinity of the Town of Duga Resa in spring 2020). The results are presented as nanograms of metals eluted at the specific elution times (t_e).

Figure 3. The hepatic distribution profiles of a) Bi and b) Zn among cytosolic biomolecules of different molecular masses in the northern pike (*Esox lucius*) with yellow/brown liver from the Mrežnica River (caught in the vicinity of the Town of Duga Resa in spring 2020). The results are presented as nanograms of metals eluted at the specific elution times (t_e).

Figure 4. The hepatic distribution profiles of a) Mn and b) Se among cytosolic biomolecules of different molecular masses in the northern pike (*Esox lucius*) with yellow/brown liver from the Mrežnica River (caught in the vicinity of the Town of Duga Resa in spring 2020). The results are presented as nanograms of metals eluted at the specific elution times (t_e).

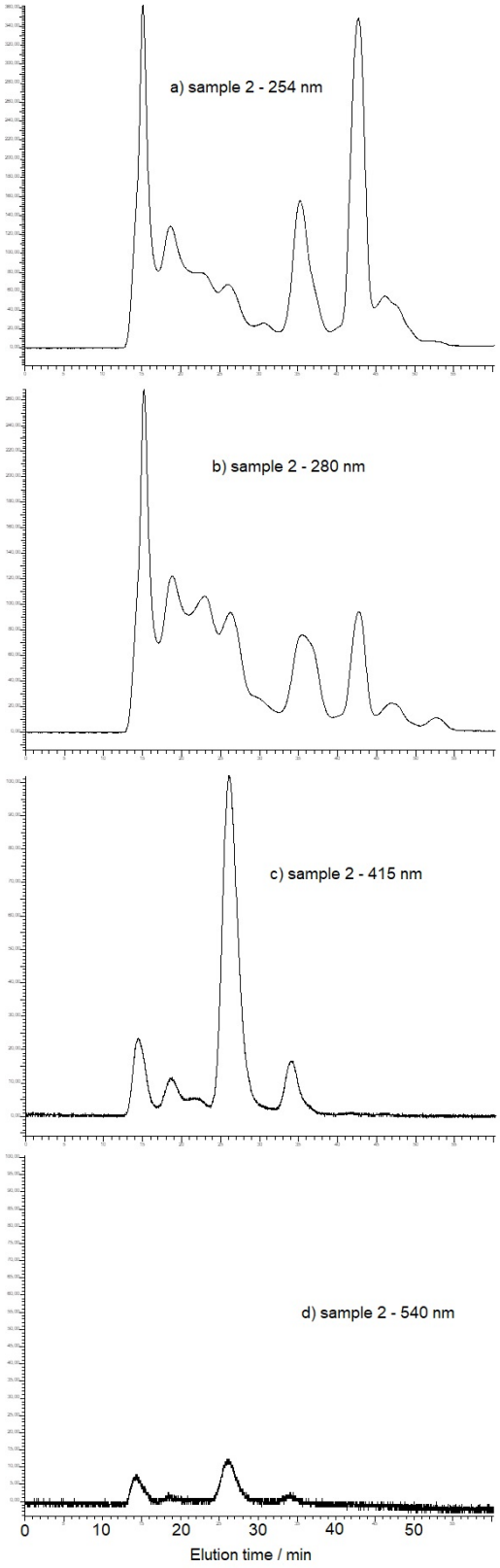
Figure 5. The heaptic distribution profiles of a) Ag, b) Cd, and c) Cu among cytosolic biomolecules of different molecular masses in the northern pike (*Esox lucius*) with yellow/brown liver from the Mrežnica River (caught in the vicinity of the Town of Duga Resa in spring 2020). The results are presented as nanograms of metals eluted at the specific elution times (t_e).

Figure 6. Absorption spectra of hepatic cytosols from yellow and green liver of northern pike (*Esox lucius*) from the Mrežnica River (caught in the vicinity of the Town of Duga Resa in spring 2020 and 2021).

Figure 7. The hepatic distribution profiles of a) Ag, b) Cd, c) Cu, d) Mn, e) Se, f) Zn, g) Co, h) Fe, and i) Mo among cytosolic biomolecules of different molecular masses in the northern pike (*Esox lucius*) with green liver from the Mrežnica River (caught in the vicinity of the Town of

937 Duga Resa in spring 2021). The results are presented as nanograms of metals eluted at the
938 specific elution times (t_e).

Figure SI-1.


a) sample 2 - 254 nm
b) sample 2 - 280 nm
c) sample 2 - 415 nm
d) sample 2 - 540 nm
0
10
20
30
40
50
Elution time / min

Caption of Figure SI-1. An example of SEC-HPLC chromatogram profiles of hepatic cytosol of northern pike (sample No. 2; 100 μL) after separation on Tricorn™ Superdex 200 10/300 GL column, with UV/VIS detection at four wavelengths: a) λ=254 nm; b) λ=280 nm; c) λ=415 nm; d) λ=540 nm.

Table 1. Biometric information on seven specimens of northern pike (*Esox lucius*) used for hepatic metal distribution analyses by SEC-HPLC and HR ICP-MS. The fish were caught in spring periods of 2020 and 2021 from the lower section of the Mrežnica River (Croatia), in the vicinity of Duga Resa town.

Fish ID	Total length / cm	Total mass / g	FCI	Sex*	Liver colour
1	54.4	1155	1.057	F	yellow/brown
2	66.8	1778	0.870	F	yellow/brown
5	41.0	463	0.968	F	yellow/brown
9	65.0	2217	1.197	F	yellow/brown
22	22.5	80.0	0.702	M	green
26	22.5	60.0	0.750	M	green
36	22.5	81.0	1.013	M	green

*M – male; F – female

Table 2. Concentrations, elution times (t_e) and molecular masses of blue dextran (for determination of void volume), seven proteins used for calibration of Superdex™ 200 10/300 GL size exclusion column, and metallothionein and glutathione standards. Equation of calibration straight line was: Kav = -0.264×log MM + 1.555.

	Concentration / mg mL^{-1}	t_e / min	MM / kDa
Blue dextran	2	15.04	2000
Thyroglobulin	8	15.77	669
Apoferritin	10	17.41	443
β-amilase	4	20.05	200
Alcohol dehydrogenase	5	21.40	150
Bovine albumin	10	22.45	66
Superoxide dismutase	1.25	27.04	32.5
Carbonic anhydrase	3	28.70	29
Metallothionein 1	1	31.24	6.15

Table 3. Elution times (t_e) and molecular masses (MM) of cytosolic biomolecules that bind specific elements in the liver of northern pike (*Esox lucius*), based on separation by SEC-HPLC (Superdex 200 10/300 GL column). Table provides peaks maxima for each analyzed element (i.e., t_e and corresponding MM for the chromatographic fractions with the highest content of specific trace elements), as well as peaks widths which are presented within the brackets.

	[a]HMM 1		[a]HMM 2		[a]HMM 3		[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	t_e / min	MM / kDa
Ag									30, 31 (29-34)	16, 12 (21-<10)				
Bi	15 (14-16)	>600	19 (16-22)	301 (>600-135)			24, 25 (22-27)	79, 60 (135-35)	30, 31 (28-33)	16, 12 (27-<10)				
Cd	15 (13-16)	>600							30, 31 (29-33)	16, 12 (21-<10)				
Co	15 (14-16)	>600	20, 21 (17-24)	230, 176 (515-79)			26 (24-27)	46 (79-35)			36 (34-38)	<10		
Cu					23 (22-25)	103 (135-60)	26 (25-28)	46 (60-27)	30, 31 (29-33)	16, 12 (21-<10)				
Fe			18 (16-21)	394 (>600-176)			26 (24-28)	46 (79-27)						
Mn	15 (14-16)	>600	18, 19 (17-20)	394, 301 (515-230)	22 (20-23)	135 (230-103)	25, 27 (23-30)	60, 35 (103-16)					40 (39-42)	<10
Mo			19 (17-22)	301 (515-135)							34, 35 (33-36)	<10		
Se							25 (23-28)	60 (103-27)			36 (35-38)	<10	40 (38-42)	<10
Zn	15 (13-16)	>600	18 (17-20)	394 (515-230)	22 (21-25)	135 (176-60)	26 (25-28)	46 (60-27)	30, 31 (29-32)	16, 12 (21-<10)	34 (33-36)	<10	46 (44-48)	<10

[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 (10-30 kDa)
[d]VLMM peak – trace element peak with a maximum within very low molecular mass protein region (<10 kDa)

Figure 1.

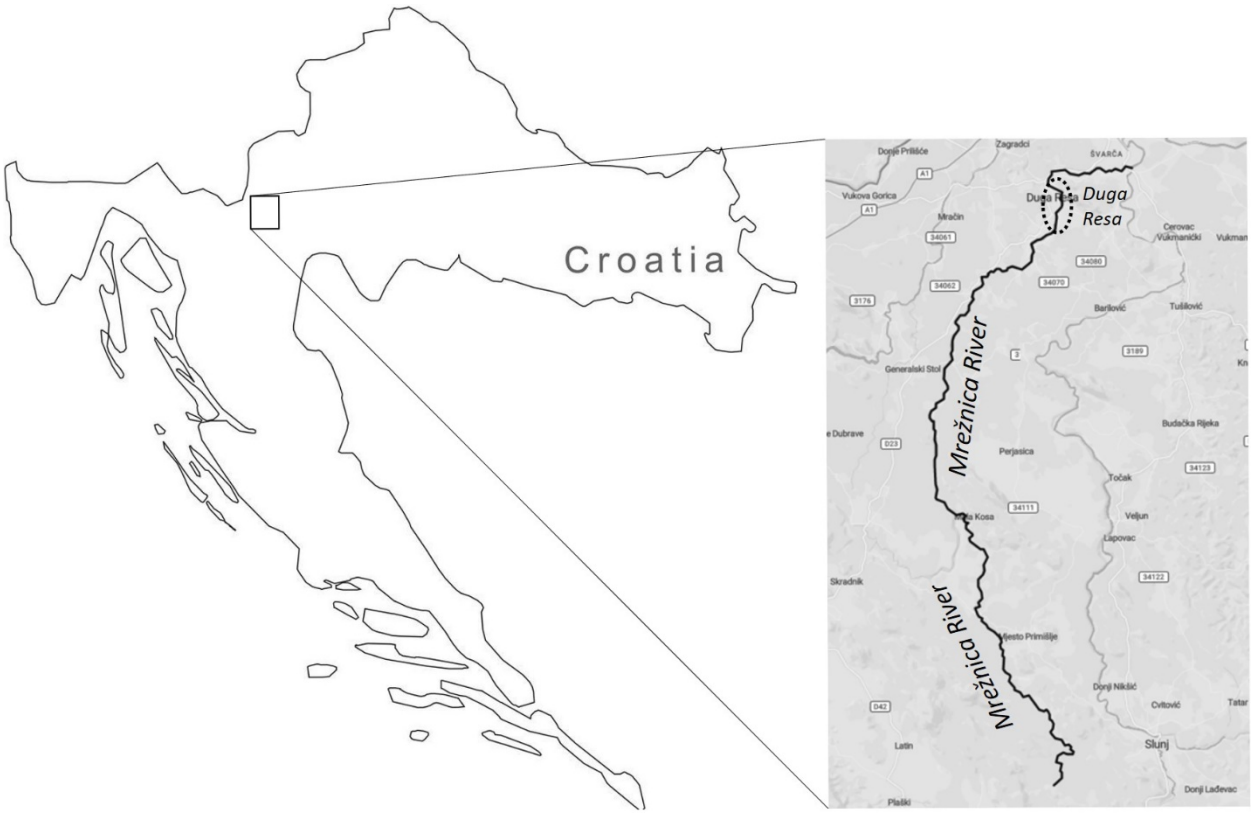

Croatia
Duga Resa
Mrežnica River
Mrežnica River
Donji Pribić
Zagradci
ŠVARČA
Vukova Gorica
Mračin
Cerovac Vukmanički
Barilović
Tušilović
Generalski Stol
Budačka Rijeka
Perjasica
Točak
Veljun
Lapovac
Skradnik
Donji Nikšić
Cvitović
Slunj
Latin
Plaški
Donji Lađevac

Figure 2.

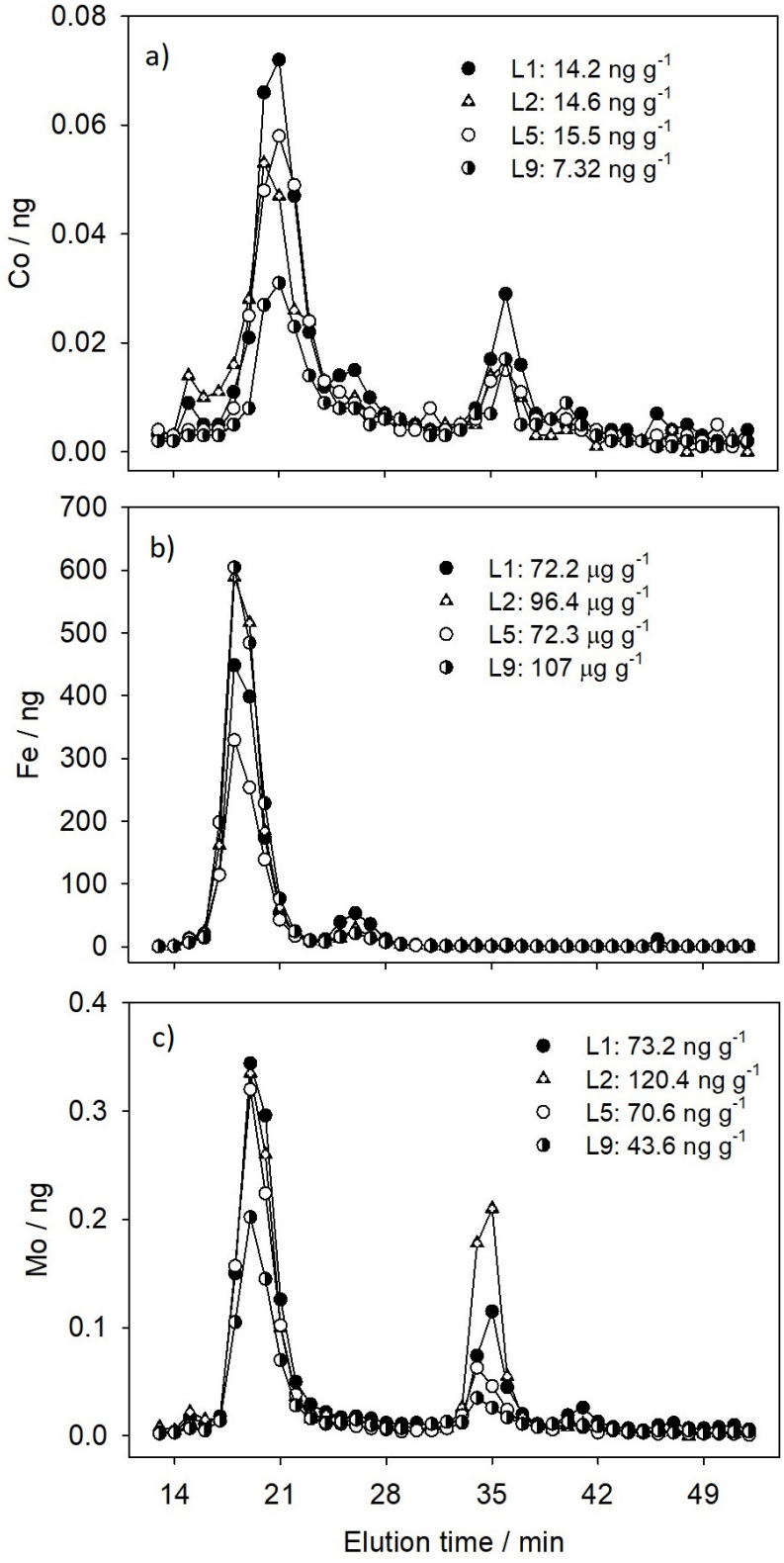

a)
L1: 14.2 ng g-1
L2: 14.6 ng g-1
L5: 15.5 ng g-1
L9: 7.32 ng g-1
Co / ng
0.08
0.06
0.04
0.02
0.00
b)
L1: 72.2 µg g-1
L2: 96.4 µg g-1
L5: 72.3 µg g-1
L9: 107 µg g-1
Fe / ng
700
600
500
400
300
200
100
0
c)
L1: 73.2 ng g-1
L2: 120.4 ng g-1
L5: 70.6 ng g-1
L9: 43.6 ng g-1
Mo / ng
0.4
0.3
0.2
0.1
0.0
14
21
28
35
42
49
Elution time / min

Figure 3.

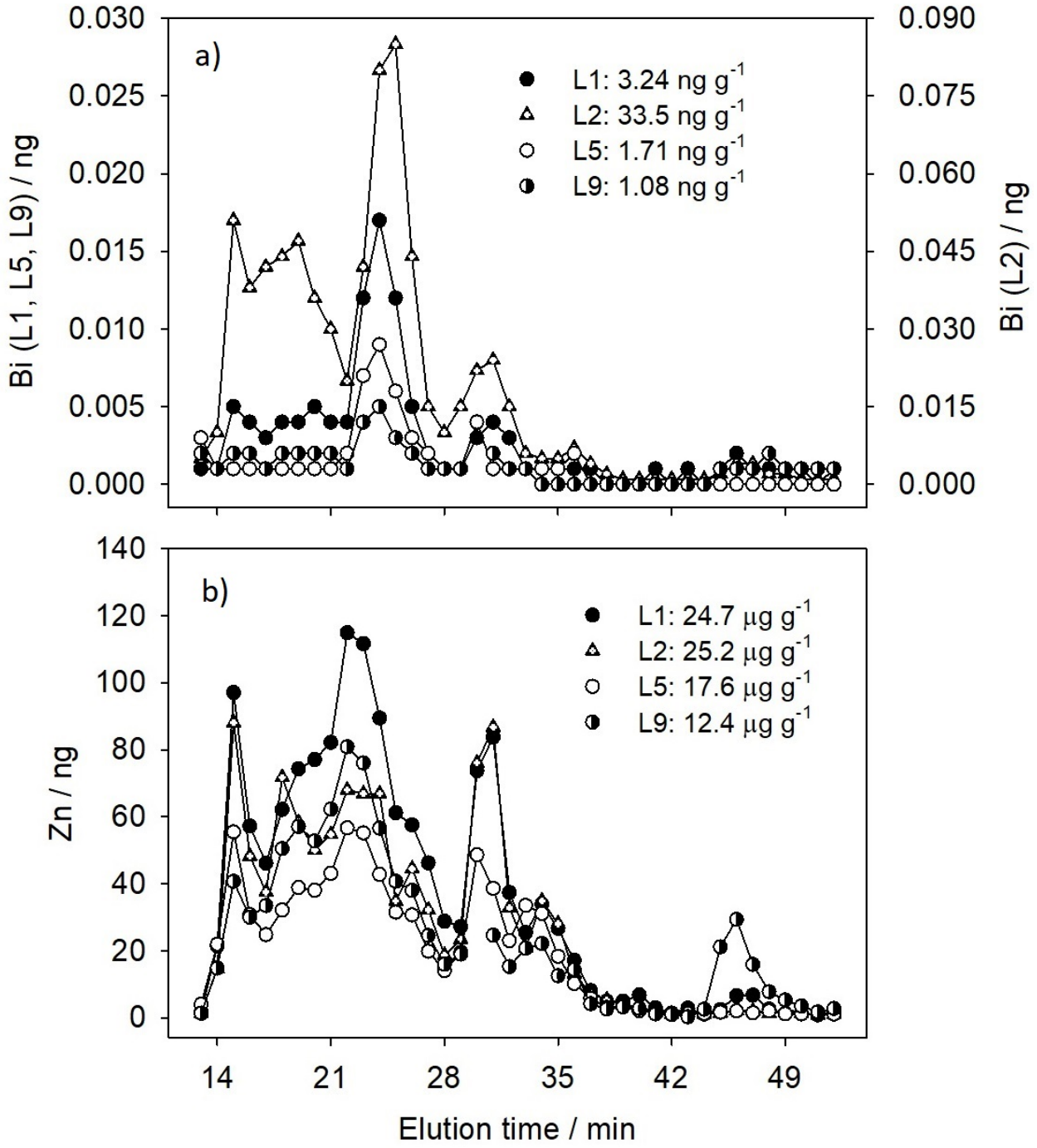

a)
L1: 3.24 ng g-1
L2: 33.5 ng g-1
L5: 1.71 ng g-1
L9: 1.08 ng g-1
Bi (L1, L5, L9) / ng
Bi (L2) / ng
0.030
0.025
0.020
0.015
0.010
0.005
0.000
0.090
0.075
0.060
0.045
0.030
0.015
0.000
b)
L1: 24.7 µg g-1
L2: 25.2 µg g-1
L5: 17.6 µg g-1
L9: 12.4 µg g-1
Zn / ng
140
120
100
80
60
40
20
0
14
21
28
35
42
49
Elution time / min

Figure 4.

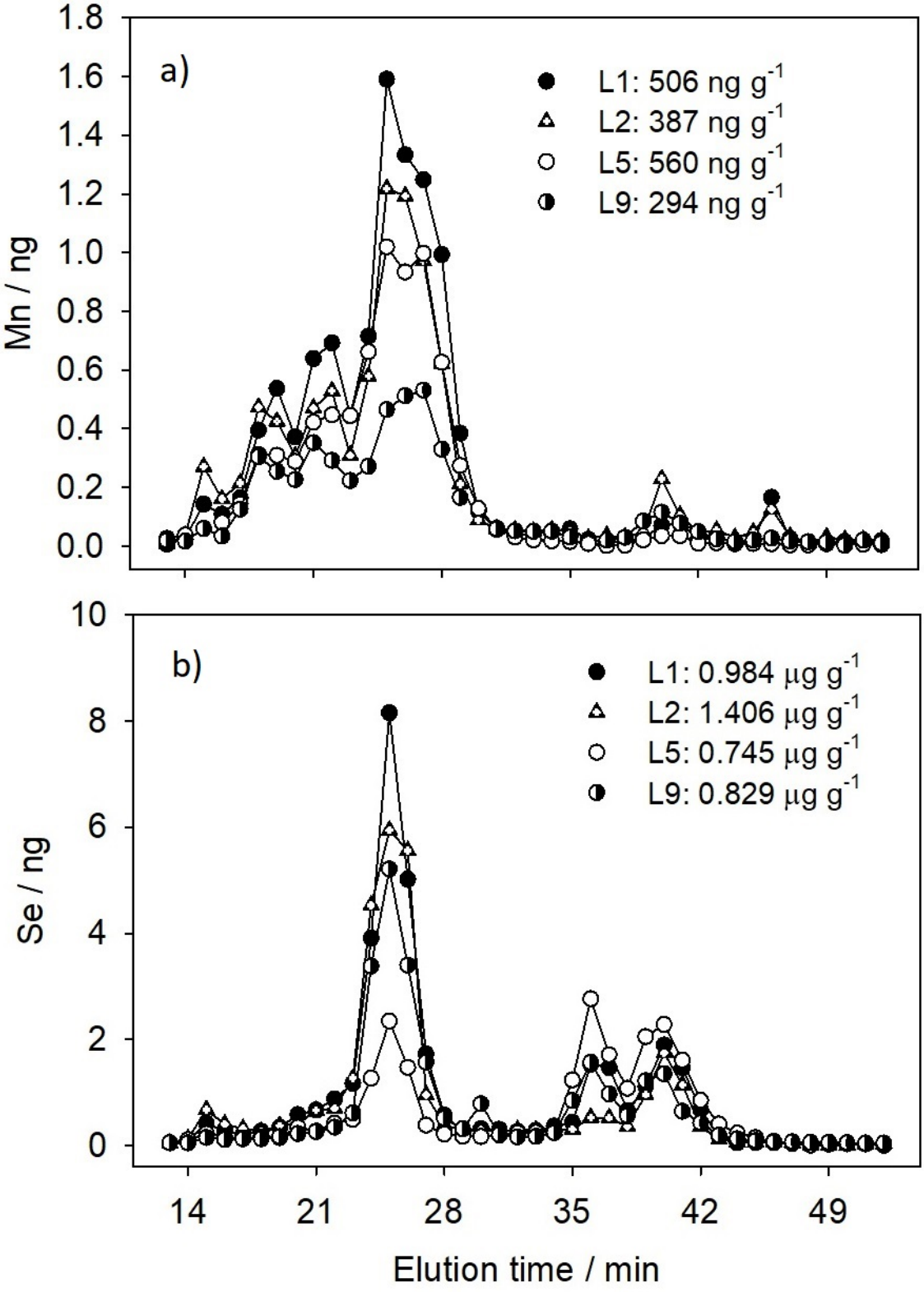

a)
L1: 506 ng g-1
L2: 387 ng g-1
L5: 560 ng g-1
L9: 294 ng g-1
Mn / ng
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
b)
L1: 0.984 µg g-1
L2: 1.406 µg g-1
L5: 0.745 µg g-1
L9: 0.829 µg g-1
Se / ng
10
8
6
4
2
0
14
21
28
35
42
49
Elution time / min

Figure 5.

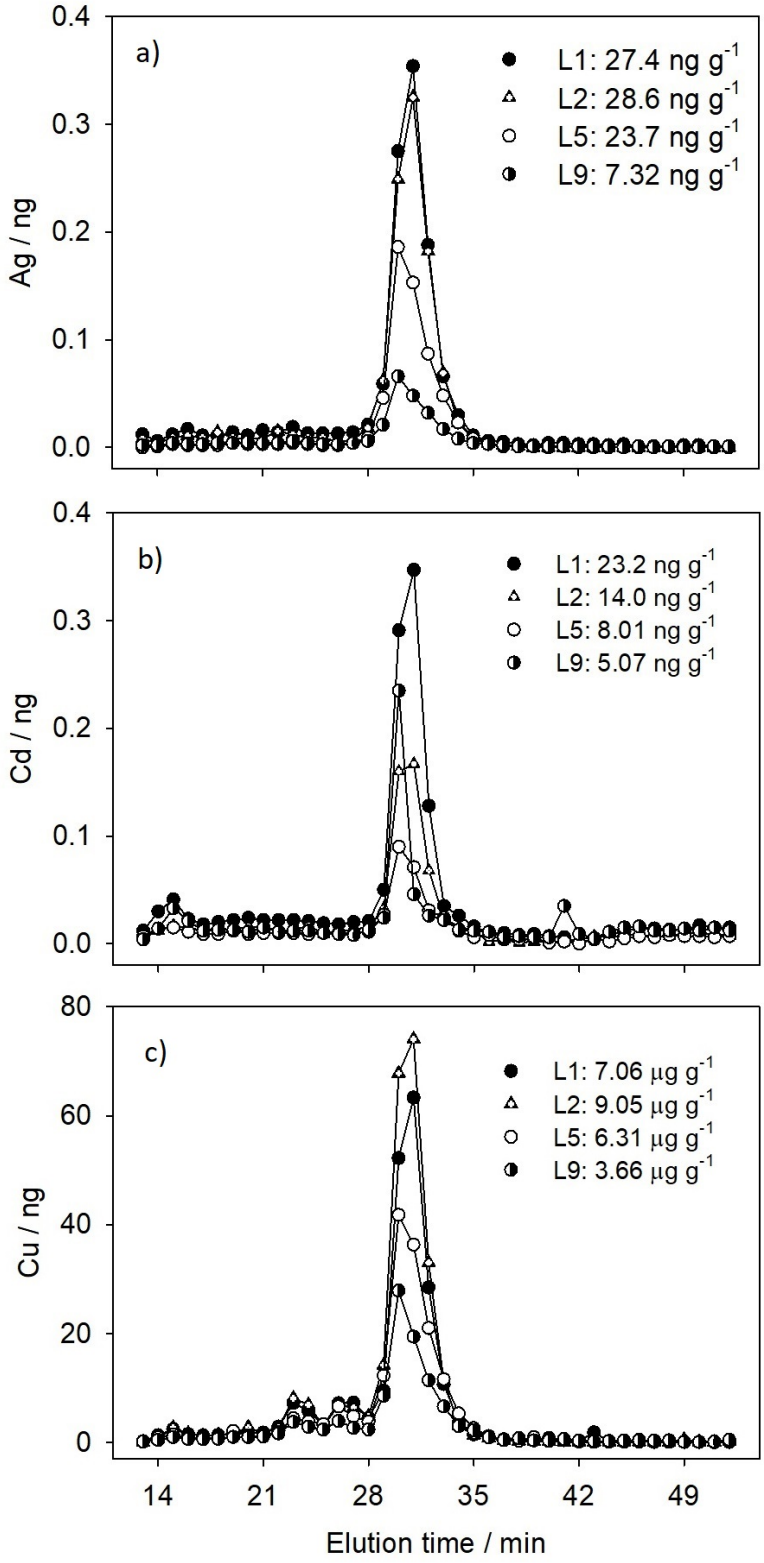

a)
L1: 27.4 ng g-1
L2: 28.6 ng g-1
L5: 23.7 ng g-1
L9: 7.32 ng g-1
Ag / ng
b)
L1: 23.2 ng g-1
L2: 14.0 ng g-1
L5: 8.01 ng g-1
L9: 5.07 ng g-1
Cd / ng
c)
L1: 7.06 µg g-1
L2: 9.05 µg g-1
L5: 6.31 µg g-1
L9: 3.66 µg g-1
Cu / ng
Elution time / min

Figure 6.

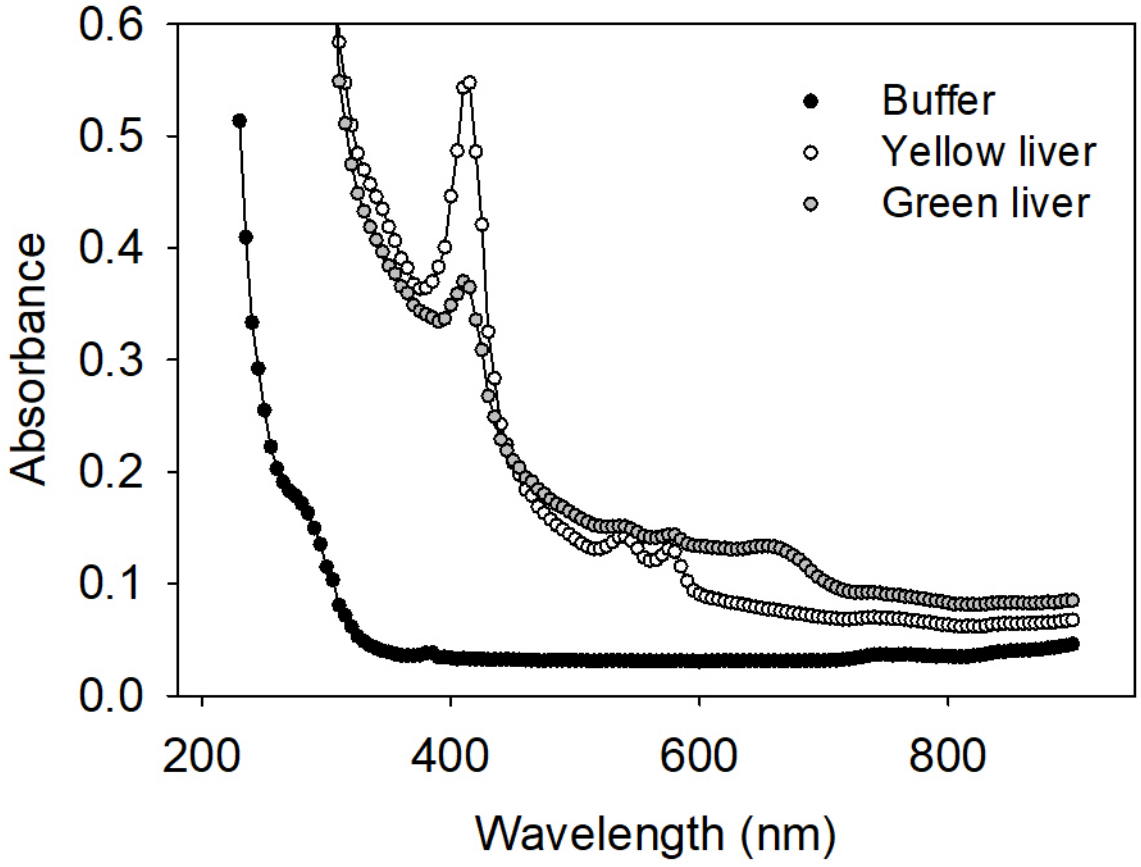
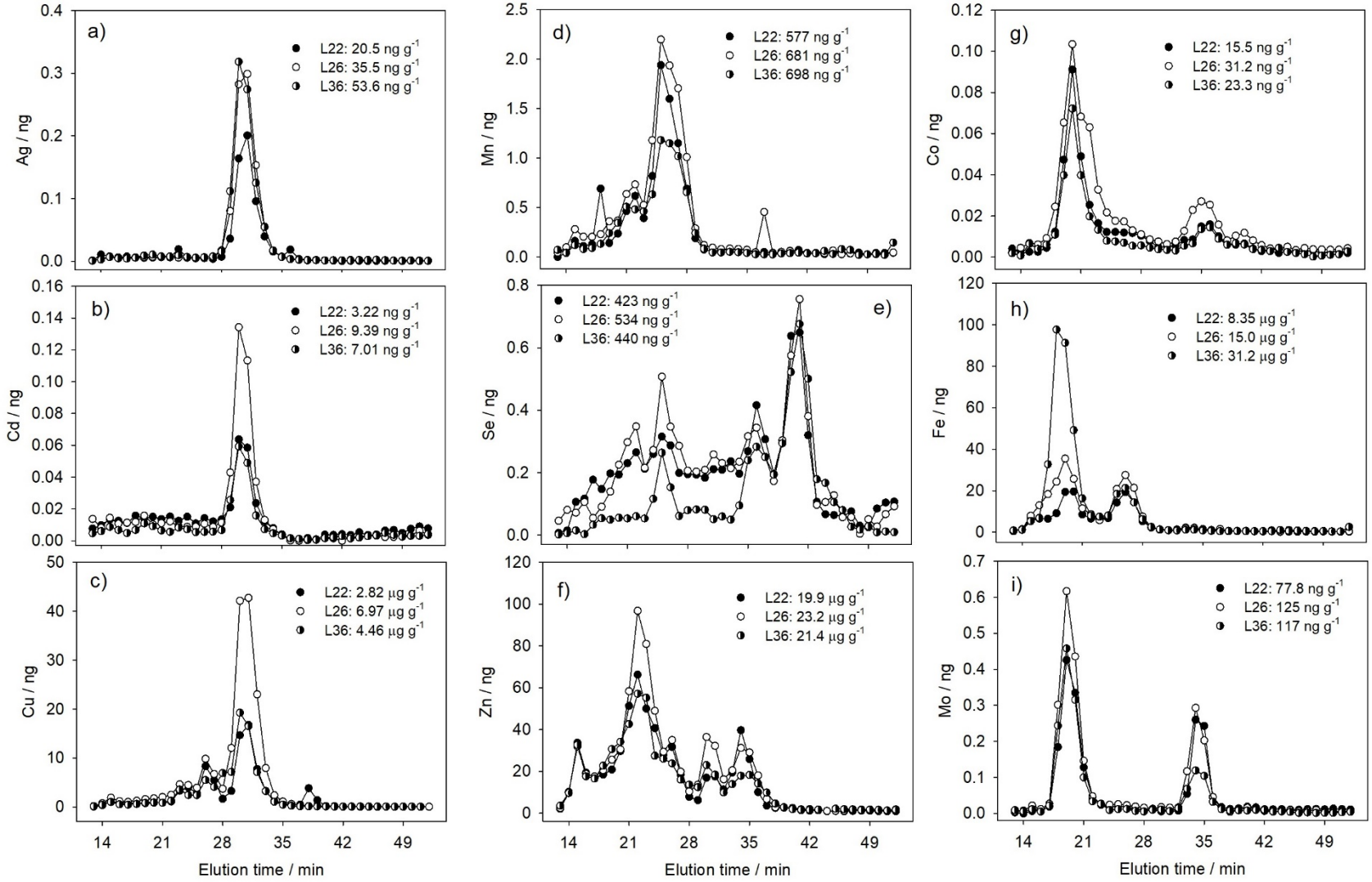

0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance
200
400
600
800
Wavelength (nm)
Buffer
Yellow liver
Green liver

Figure 7.


a)
L22: 20.5 ng g-1
L26: 35.5 ng g-1
L36: 53.6 ng g-1
Ag / ng
b)
L22: 3.22 ng g-1
L26: 9.39 ng g-1
L36: 7.01 ng g-1
Cd / ng
c)
L22: 2.82 µg g-1
L26: 6.97 µg g-1
L36: 4.46 µg g-1
Cu / ng
d)
L22: 577 ng g-1
L26: 681 ng g-1
L36: 698 ng g-1
Mn / ng
e)
L22: 423 ng g-1
L26: 534 ng g-1
L36: 440 ng g-1
Se / ng
f)
L22: 19.9 µg g-1
L26: 23.2 µg g-1
L36: 21.4 µg g-1
Zn / ng
g)
L22: 15.5 ng g-1
L26: 31.2 ng g-1
L36: 23.3 ng g-1
Co / ng
h)
L22: 8.35 µg g-1
L26: 15.0 µg g-1
L36: 31.2 µg g-1
Fe / ng
i)
L22: 77.8 ng g-1
L26: 125 ng g-1
L36: 117 ng g-1
Mo / ng
Elution time / min
Elution time / min
Elution time / min

Highlights

- the first comprehensive study of metal-binding biomolecules in an esocid fish
- metal distributions in northern pike hepatic cytosol determined by SEC-HPLC-ICP-MS
- binding to metallothioneins established for Ag, Bi, Cd, Cu, and Zn
- essential metals tend to bind biomolecules of higher molecular masses (>100 kDa)
- differences between several freshwater fish species established for Zn, Mn, and Se