Characterization and identification of selected metal-binding biomolecules from hepatic and gill cytosols of Vardar chub (*Squalius vardarensis* Karaman, 1928) by various techniques of liquid chromatography and mass spectrometry

Nesrete Krasnići[1], Zrinka Dragun[1*], Snježana Kazazić[2], Hasan Muharemović[2], Marijana Erk[1], Maja Jordanova[4], Katerina Rebok[4], Vasil Kostov[5]

[1]Ruđer Bošković Institute; Division for Marine and Environmental Research; Laboratory for Biological Effects of Metals; P.O. Box 180, 10002 Zagreb, Croatia

[2]Ruđer Bošković Institute; Division of Physical Chemistry; Laboratory for mass spectrometry and functional proteomics; P.O. Box 180, 10002 Zagreb, Croatia

[4]Faculty of Natural Sciences and Mathematics, Ss. Cyril and Methodius University in Skopje, Arhimedova 3, 1000 Skopje, Macedonia

[5]Institute of Animal Sciences, Ile Ilievski 92a, 1000 Skopje, Macedonia

* Corresponding author

Phone: +385-1-4680216;

Fax: +385-1-4680242;

E-mail: zdragun@irb.hr

Krasnići, Nesrete; Dragun, Zrinka; Kazazić, Snježana; Muharemović, Hasan; Erk, Marijana; Jordanova, Maja; Rebok, Katerina; Kostov, Vasil (2019) Characterization and identification of selected metal-binding biomolecules from hepatic and gill cytosols of Vardar chub (Squalius vardarensis Karaman, 1928) by various techniques of liquid chromatography and mass spectrometry. Metallomics, 11 . pp. 1060-1078. ISSN 1756-5901.

Abstract

Metals play crucial physiological roles, but they can also cause irreparable toxic effects through binding to important cellular biomolecules in aquatic organisms. The aim of this study was to determine the exact molecular masses and to identify several selected metal-binding biomolecules in hepatic and gill cytosols of Vardar chub (*Squalius vardarensis* Karaman, 1928). Methods applied for the achievement of this goal were SEC-AEC-HPLC for two-dimensional separation of cytosolic biomolecules, HR ICP-MS for metal measurements, and mass spectrometry (MALDI-TOF-MS and LC-MS/MS) for biomolecule mass determination and identification. Analyzed biomolecules reffered to: Fe-binding biomolecules, which were identified as hemoglobin subunits β in the liver (molecular masses of ~15 kDa), and hemoglobin subunits α and β in the gills (molecular masses of ~11 kDa, ~13 kDa and ~15 kDa); heat-stable Cd-binding biomolecules, which were identified as MT isoforms MT-I and MT-II (molecular mass of ~6.0 kDa in both liver and gills, and an additional 4.9 kDa isoform in the gills); heat-stable Mo-binding biomolecules of molecular masses equal to 3.3 kDa (in the gills) and 8.5 kDa (in the liver). An important finding of this study was obvious presence of different isoforms of the same biomolecules in the liver and gills. It was, among others, manifested through absence of Zn binding to MTs only in the gills, indicating that the same type of biomolecule can be responsible for different functions in different organs. Thus, for better understanding of metal behaviour in aquatic organisms, it is crucial to know cellular metal-binding biomolecules and their functions.

Key words: fish, hemoglobin, liver, MALDI-TOF-MS, metallothioneins, SEC-AEC-HPLC

Significance to metallomics:

This study has brought us one step closer to the understanding of the fate of metals bioaccumulated in the organs of an important aquatic bioindicator species, namely Vardar chub. Application of two-dimensional chromatographic separation of cytosolic biomolecules according to their sizes and charges, and of subsequent metal and biomolecule analyses by various techniques of mass spectrometry (HR ICP-MS, MALDI-TOF-MS, LC-MS/MS) enabled recognition of important Fe, Cd, and Mo-binding biomolecules in Vardar chub liver and gills. Differences in biomolecule characteristics observed between two organs have confirmed the importance of the studies of organ-specific isoforms of various proteins within the cells.

Introduction

As a consequence of the human activities (such as mining, industry, traffic, and agriculture), environmental contamination with metals, especially contamination of aquatic systems, is steadily increasing and affecting living organisms[1]. For evaluation of the degree of metal contamination and possible pollution of the aquatic ecosystems, fish play an important role due to their key position in many food webs, thus being useful for the assessment of the bioaccumulation and effects of metals originating from water, food and sediment[2-5]. Metals bioaccumulated in fish organs bind to or interact with cytosolic molecules and can interfere with the cell functions, leading to various deleterious effects[6-7]. On the other hand, metals also play an important physiological role in the living organisms, since many biological functions critically depend on the interaction with certain metals within the cell; for example, several proteins require a metal ion to be able to perform their catalytic activities, to stabilize their structures and to properly perform their functions[8-10]. Therefore, metal physiological functions, as well as their possible toxic effects on metabolism and homeostasis of fish organisms can be assessed by analyses of various metal binding biomolecules within the cells, such as various metalloenzymes and other metalloproteins[11-14]. A large number of studies on metal-binding biomolecules was carried out in the frame of various metal speciation projects, but only a few fish metalloproteins were so far identified and their functions clarified, allowing better insight into the intracellular fate of bioaccumulated metals, as well as toxic and defensive mechanisms that are triggered by metal exposure[15-17].

To obtain the valuable information on the identity and functions of metal-binding biomolecules in the living systems it is necessary to apply adequate state of the art analytical equipment[18]. The researchers in this field generally use combination of high-sensitivity atomic detectors, such as inductively coupled plasma mass spectrometry (ICP-MS), different techniques of high performance liquid chromatography (HPLC), and various types of mass spectrometry (MS), to enable metal/nonmetal analyses within the cells, as well as characterization and possible identification of specific metal-binding biomolecules[19-24]. Association of multidimensional separation approaches (e.g. size exclusion (SEC)-HPLC and anion exchange (AEC)-HPLC)) with various techniques of mass spectrometry (e.g. matrix assisted laser desorption/ionization (MALDI) time-of-flight (TOF) MS, or liquid chromatography (LC) MS) further improves the reliable identification of so far unknown and undescribed metal binding biomolecules, that will in return provide a possibility of precise assessment of hazardous environmental situations[25-26].

In our previous work we have implemented offline coupling of SEC-HPLC and high resolution (HR) ICP-MS to determine distributions of several elements (Cd, Co, Cu, Fe, Mn, Mo, Pb, Se, Tl and Zn) among cytosolic biomolecules of the gills and/or liver of three fish species,

European chub (*Squalius cephalus*)[27-28], Vardar chub (*Squalius vardarensis*)[29] and brown trout (*Salmo trutta*)[30]. In our studies, the liver were chosen as a key metabolic and detoxification organ of fish, containing a high amount of various metalloproteins which are characterized by a wide range of molecular masses[7, 31]. The gills were chosen as an important entry route of contaminants, such as metals; they are known for their good reflection of the current environmental situations, through metal bioaccumulation and oxidative stress responses[32]. However, the complete separation and a precise determination of molecular masses of all metal-binding biomolecules contained within hepatic or gill cytosols of selected fish species would be rather difficult, and probably not even possible, based solely on the SEC-HPLC-HR-ICP-MS measurements. The resolution and sensitivity of SEC-HPLC method is such that it offers a rough separation of studied metal-binding biomolecules, and obtained elution peaks of each studied element generally cover rather wide ranges of molecular masses, which can include several metalloproteins. Furthermore, elution peaks of different metals can partially or completely overlap, thus needing a further separation before any kind of additional analyses[27-30].

Hence, in this study, which presents the continuation of our previous study on the liver and the gills of Vardar chub[29], our first goal was to implement anion-exchange chromatography as an additional separation technique following the size-exclusion chromatography, to isolate purified fractions that contain, whenever possible, individual metal-binding biomolecules. Our second goal was to implement two mass spectrometry techniques into our research (MALDI-TOF-MS and LC-MS/MS), to more precisely determine the molecular masses of several metal-binding biomolecules from hepatic and gill cytosols of Vardar chub, and, when possible, to identify them. Our attention was directed towards analyses of several hepatic and gill metal-binding biomolecules, which were roughly determined in our previous study on Vardar chub[29], and those were: (1) medium molecular mass (~25-50 kDa) Fe-binding biomolecules; (2) heat-stable low molecular mass (~7-25 kDa) Cd-, Cu- and Zn-binding biomolecules, presumably metallothioneins (MTs); and (3) heat-stable very low molecular mass (~5-10 kDa) Mo-binding biomolecules. By application of two-dimensional HPLC separation followed by HR ICP-MS analyses of metals and MS analyses of above listed metal-binding biomolecules, our general aim in this study was to extend the knowledge and understanding of metal handling strategies in Vardar chub, as an important bioindicator species.

Experimental data

Fish sampling and organ dissection

As a bioindicator species in this study we have used Vardar chub (*Squalius vardarensis* Karaman, 1928), a member of a Cyprinidae family, because it is a widespread fish species in the Macedonian rivers, and closely related to European chub (*Squalius cephalus*), fish species abundant in the Europe freshwaters. Fish sampling was performed in the spring period (June 2015) in two rivers in north-eastern Macedonia, Bregalnica (N 41°43.57' E 22°10.27') and Zletovska (N 40°58.54' E 21°39.45'), and the map of the sampling area was previously published[1]. Fish were caught by electro fishing, using electrofisher Samus 725G, according to the guidelines described in the standard CEN EN 14011:2003. The captured fish were transported to the laboratory, and during transportation they were kept alive in tank containing aerated river water, which was collected at each sampling site. In the laboratory, fish were anesthetized with Clove Oil (Sigma Aldrich, USA) and euthanized, and then liver, gills and gonads were dissected. Fish total lengths (cm) and total masses (g) were measured, Fulton condition indices (FCI) were calculated using the formula provided by Rätz and Lloret[33], whereas fish sex was determined histologically[34]. The liver and gills were put in liquid nitrogen immediately upon the dissection, and afterwards they were stored in the freezer at -80°C. In total, five fish were analyzed in the course of this study, and their biometric parameters are given in Table 1.

Isolation of cytosolic fractions from Vardar chub liver and gills and their heat-treatment

Frozen Vardar chub livers and gills were put on ice and cut in small pieces, and then cooled homogenization buffer [100 mM Tris-HCl/Base (Sigma, pH 8.1 at 4°C) supplemented with reducing agent (1 mM dithiotreitol (DTT), Sigma)], w/v 1:5, was poured in. Obtained mixture was homogenized in a glass tube, which was put on ice, applying 10 strokes of PTFE-coated pestle at 6,000 rpm (Potter-Elvehjem homogenizer, Glas-Col, USA). Hepatic and gill cytosols were obtained by homogenate centrifugation at 50,000×g for 2 h at 4 °C, using Avanti J-E centrifuge (Beckman Coulter), i.e. the supernatants (S50) obtained after centrifugation corresponded to water-soluble cytosolic tissue fractions[35] containing lysosomes and microsomes[36]. Supernatants were aliquoted and stored in the freezer at -80°C.

The heat-treatment of hepatic and gill cytosols was performed according to slightly modified procedure described by Erk et al.[37]. The cytosols were heated at 70 °C for 10 minutes using the DB-2D Dri-Block (Techne, UK). After the heat-treatment, samples were kept on ice for 30 min at 4°C, and then centrifuged at 10,000×*g* for 15 min at 4°C (Heraeus Biofuge Fresco, Kendro, USA). The supernatants (S10), containing heat-stable biomolecules, were separated from pellets and stored at -80°C.

Determination of total cytosolic protein concentrations

Total protein concentrations were measured in the hepatic and gill cytosols of Vardar chub according to Lowry, as previously described by Krasnići et al.[28]. For protein determination we have used the Bio-Rad DC Protein Assay and the measurements were carried out on the spectrophotometer/fluorometer (Tecan, Infinite M200) at wavelength of 750 nm. The concentrations were read from the calibration curve, which was constructed using five concentrations (0.25-2.0 mg mL^{-1}) of bovine serum albumin (Serva, Germany) dissolved in the homogenization buffer. Total protein concentrations in the gills and liver are presented separately for each fish in Table 1.

SEC-HPLC fractionation of cytosols and heat-treated cytosols from Vardar chub liver and gills

Distribution of metals/nonmetal among biomolecules of different molecular masses in the cytosols and heat-treated cytosols from Vardar chub livers and gills was studied using SEC-HPLC (Perkin Elmer HPLC system, series 200, USA), as previously described in details by Krasnići et al.[27-29]. Two types of SEC columns for globular proteins (GE Healthcare Biosciences, USA) were used: prepacked Tricorn™ Superdex 200 10/300 GL with a separation range of 10-600 kDa and Tricorn™ Superdex 75 10/300 GL column with a separation range of 3-70 kDa. For each sample two chromatographic runs were carried out, and in each run volume of 100 μL was applied on the column. The separation was achieved using 20 mM Tris-HCl/Base (Sigma, pH 8.1 at 22°C, flow 0.5 mL min^{-1}) as a mobile phase (isocratic mode) in both types of SEC columns. The fractions were collected in the plastic tubes at one-minute intervals using a fraction collector (FC 203B, Gilson, USA). For the column calibration, several protein standards (thyroglobulin, apoferritin, β-amylase, alcohol dehydrogenase, conalbumin, bovine albumin, ovalbumin, carbonic anhydrase, cytochrome C, vitamin B12; Sigma, USA) were run through the column under the same conditions as the samples. The equation of the calibration straight line for Superdex 200 was: $y = -0.281x + 1.6473$; and for Superdex 75: $y = -0.3343x + 1.6664$ ($y = Kav$; $x = \log MM$). The void volume was determined by use of blue dextran (defined MM: 2,000 kDa), which was eluted in column Superdex 200 at 16.3 min and in Superdex 75 at 15.6 min.

AEC-HPLC separation of fractions of interest collected after SEC_{200}-HPLC and SEC_{75}-HPLC fractionation of cytosols and heat-treated cytosols from Vardar chub liver and gills

Fractions of interest collected from Vardar chub hepatic and gill cytosols using fractionation by SEC_{200}-HPLC or SEC_{75}-HPLC were pooled and preconcentrated to a volume of ~200 μL by ultrafiltration using centrifugal filters Amicon Ultra (cut off at 3 kDa, Merck, Millipore, Ireland) and centrifugation at 14,000×*g* at 4°C in centrifuge Heraeus Biofuge Fresco (Kendro,

USA). Concentrated samples were then applied in volumes of 100 µL on anion-exchange column mono Q 5/50 GL (5×50 mm; GE Healthcare Bio-Sciences, Sweden) to perform AEC-HPLC separation. The separation and elution were achieved using a 4 mM Tris-HCl buffer (pH 7.4) as a mobile phase A, and 250 mM ammonium acetate buffer prepared in 10 mM Tris-HCl (pH 7.4) as a mobile phase B. The mobile phase flow was 1 mL min^{-1} and the gradient elution protocol was adjusted according to the procedure described by Rodríguez-Cea et al.[38]. The protein absorbances were monitored at 254 and 280 nm using a Diode Array Detector (DAD) (Perkin Elmer, series 200, USA). For each sample, two consecutive chromatographic runs were performed, and the fractions containing metals and biomolecules of interest were collected at 0.5 min intervals in the plastic tubes using a fraction collector (FC 203B, Gilson, USA).

Measurements of metal/nonmetal concentrations in Vardar chub hepatic and gill cytosols, as well as in fractions obtained by SEC-HPLC and AEC-HPLC separations

The concentrations of Cd, Co, Cu, Fe, Mn, Mo, Se, and Zn were measured in the hepatic and gill cytosols of Vardar chub, which were ten times diluted with Milli-Q water and acidified with HNO_3 (*Suprapur*, Merck, Germany; final acid concentration in the samples: 0.65 %) prior to measurements. Fractions of hepatic and gill cytosols collected after SEC-HPLC and AEC-HPLC separations were only acidified with HNO_3 (*Suprapur*, Merck, Germany, final acid concentration in the samples: 0.16 %) prior to offline metal/nonmetal measurements. Indium (Fluka, Germany) was added as an internal standard (1 µg L^{-1}) in all samples[39]. The measurements were performed by HR ICP-MS (Element 2, Thermo Finnigan, Germany), equipped with an autosampler SC-2 DX FAST (Elemental Scientific, USA) and sample introduction kit consisting of a SeaSpray nebulizer and cyclonic spray chamber Twister. Measurements of ^{82}Se, ^{98}Mo, and ^{111}Cd were operated in low-resolution mode, whereas ^{55}Mn, ^{56}Fe, ^{59}Co, ^{63}Cu, and ^{66}Zn were measured in medium resolution mode. External calibrations were performed using multielement standard solution for trace elements (Analitika, Czech Republic). All standards were prepared in 1.3 % HNO_3 (Suprapur; Merck, Germany) and supplemented with In (1 µg L^{-1}; Fluka, Germany). Limits of detection (LOD) were calculated as three standard deviations of ten consecutive measurements of trace elements in the blank solution (Tris-HCl/Base, dithiotreitol, HNO_3). The LODs for trace elements measured within this study were as follows (in µg L^{-1}): Cd, 0.005; Co, 0.002, Cu, 0.037; Fe, 0.084; Mn, 0.002; Mo, 0.004; Se, 0.138; and Zn, 2.40[27-29]. The accuracy of metal/nonmetal measurements by HR ICP-MS was checked by analysis of quality control sample (QC for trace metals, catalog no. 8072, lot no. 146142-146143, Burlington, Canada). A generally good agreement was observed between our data and the certified values, as can be seen from the obtained recoveries: Cd (96.2±2.0 %), Co (98.5±2.7 %), Cu (94.8±4.0 %), Fe (94.5±4.3 %), Mn (97.3±2.9 %), Mo (96.4±13.4 %), Se (98.2±5.3 %), and Zn (104.4±15.5 %).

Determination of exact molecular masses of selected cytosolic biomolecules from Vardar chub liver and gills and their identification by MALDI-TOF-MS and LC-MS/MS

The fractions, containing specific metal-binding biomolecules, were collected after one-dimensional (SEC-HPLC) and two-dimensional chromatographic separations (SEC-AEC-HPLC). They were further on pooled and preconcentrated by ultrafiltration using centrifugal filters Amicon Ultra (cut off at 3 kDa, Merck, Millipore, Ireland) and centrifugation at 14,000×*g* at 4°C in centrifuge Heraeus Biofuge Fresco (Kendro, USA). Thus prepared samples were then analysed by MALDI TOF-MS (Bruker Daltonik Microflex series, Germany) and LC-MS/MS (Bruker Daltonik amaZon ETD ion trap system, Germany).

Analyses on MALDI-TOF-MS were used to determine exact molecular masses of isolated Vardar chub hepatic and gill biomolecules of interest. For this purpose, 1 μL of preconcentrated samples were spotted in two replicates onto a 96-spot steel target plate (Bruker Daltonik, Germany) and allowed to visibly dry at room temperature. Subsequently, 1 μL of α-cyano-4-hydroxycinnamic acid (10 mg mL^{-1}; Bruker Daltonik, Germany) in 50 % acetonitrile/2.5 % trifluoroacetic acid was added on top of the samples as matrix.

The analyses on LC-MS/MS were performed with an aim to identify, whenever possible, isolated cytosolic biomolecules from Vardar chub liver and gills. For that purpose, preconcentrated samples were first dried and then redissolved in 20 mM ammonium bicarbonate. In this solution DTT was added to a final concentration of 10 mM in order to reduce possible disulfide bonds in the proteins. After 1 hour at room temperature, iodoacetamide (IAA) was added to the reaction mixture to a final conncentration of 54 mM and left in the dark at room temperature for another hour. Finally, trypsin (Trypsin Gold, Promega, USA) was added (1μg per 100 μg of proteins) and the mixture was incubated overnight at room temperature. The reaction was stopped by addition of formic acid.

Analyses of obtained samples of tryptic peptides were performed by low resolution ion trap LC-MS/MS. Peptides were loaded onto a Dionex Ultimate 3000 RSLC trap colum (C18 resin, Acclaim "pepmap", 100 Å, 5 μm, 1 mm×5 mm) in 1 μL of solution containing 0.1 % formic acid and 2 % acetonitrile, and desalted for 2 min with 0.1 % formic acid at a flow rate of 20 μL/min. Peptides were separated on cappilary column (C18 resin, Acclaim "pepmap", 100 Å, 2 μm, 0.3 mm×150 mm at the flow rate of 1 μL/min. Mobile phase A consisted of 0.1 % formic acid in water and mobile phase B consisted of 0.1 % formic acid in acetonitrile. The 45 min multistep gradient consisted of mobile phase B: 1 min 5 %, 30 min linear gradient to 45 %, 1 min linear gradient to 90 %, 4 min hold on 90 %, 3 min linear gradient to 5 %, 6 min hold on 5 %. The Amazon ETD ion trap (Bruker Daltonik, Germany) mass spectrometer was operated at

ESI capillary voltage of -4500 V, while high voltage end plate offset was -500 V. Nebulizer was set on 10 psi. The temperature of dry gas was set at 200 °C with flow of 5 L/min. Helium was used as a collision gas. The fragmentation amplitude was set at 0.60 V and ramped between 30 % and 300 % of this value. Product ion spectra were sequentially recorded for each selecter precursor. The acquisition software was set up in autoMSMS mode using to three precursor ions with active exclusion on (precursor exclusion after two MSMS spectra for 2 min). MS and MSMS spectra were acquired within a scan range from 300 to 1500 *m/z* using averages from five/two spectra and scan rate of 8100 (*m/z*)/sec. DataAnalysis software 4.0 (Bruker Daltonik GmbH, Germany) was used to extract MS and MS/MS data and to create Mascot (Matrix Science, UK) file for database search. Search parameters were set to: database NCBInr all entries, enzyme trypsin 1 miss cleavage, carbamidomethyl as global and methionine oxidation as variabile modification, mass tolerance for MS/MS spectra was 0.5 Da.

Data processing and statistical analyses

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

Results and discussion

SEC_{200}-HPLC analyses of trace element distributions in hepatic and gill cytosols of Vardar chub

SEC_{200}-HPLC analyses of trace element distributions in hepatic and gill cytosols of Vardar chub were previously done using specimens sampled in spring and autumn seasons of 2012 from three Macedonian rivers, Bregalnica, Zletovska and Kriva[29]. That study made it possible to define the molecular masses of cytosolic biomolecules that bind essential elements Co, Cu, Fe, Mn, Mo, Se and Zn, and nonessential and very toxic element Cd in the conditions of low and high metal exposure[29]. Our further goal was to characterize more specifically several of those biomolecules, which was the reason to repeat SEC_{200}-HPLC analyses on hepatic and gill cytosols of the same fish species following the new sampling campaign in June 2015 at Bregalnica and Zletovska rivers. We were, thus, able to confirm previously obtained results, and then to isolate the metal-containing fractions of interest which we needed for further analyses. The graphical presentation of SEC_{200}-HPLC analyses for Co, Cu, Fe, Mn, Mo, Se, Zn, and Cd in the hepatic cytosols and for Fe, Se, Zn, and Cd in the gill cytosols of Vardar chub are presented within electronic supplementary information (Fig. SI-1 and SI-2), whereas the information on elution times and corresponding molecular masses of metal-binding biomolecules are presented in Table 2. Four elements (Co, Cu, Mn, and Mo) were not measured

in the gill cytosols due to their very low cytosolic concentrations. Same as in our previous studies[27-29], metal-binding biomolecules were defined as belonging to high molecular mass region (HMM, >100 kDa), medium molecular mass region (MMM, 30-100 kDa), low molecular mass region (LMM, 10-30 kDa), and very low molecular mass region (VLMM, <10 kDa).

Hepatic Co (Fig. SI-1a, Table 2) was distributed between two peaks, with higher Co quantity bound to HMM biomolecules (141-299 kDa) and lower Co quantity bound to MMM biomolecules (40-109 kDa). Hepatic Mn (Fig. SI-1d, Table 2) was distributed between two peaks, with higher Mn quantity bound to HMM biomolecules (85-233 kDa) and much lower Mn quantity bound to MMM biomolecules (15-66 kDa). Hepatic Mo (Fig. SI-1e, Table 2) was distributed between four peaks covering wide region of molecular masses, but a majority of Mo was bound to HMM biomolecules (66-385 kDa). Appreciable quantities of Mo were also bound to MMM biomolecules (11-51 kDa) and VLMM biomolecules (3-9 kDa). Predominant binding of Co, Mn, and Mo to HMM biomolecules in the liver of Vardar chub was consistent with previous observations on Vardar chub[29] and can be explained by the essential function of these metals in the activities of numerous enzymes[40-41]. Furthermore, both hepatic (Fig. SI-1f, Table 2) and gill Se (Fig. SI-2b, Table 2) in this study were mainly distributed between two VLMM peaks, with the major Se quantity bound to VLMM biomolecules of 0.3-1.5 kDa, which encompassed small peptides and free amino acids, such as selenomethionine[42]. High Se affinity for VLMM biomolecules in hepatic and gill cytosols of Vardar chub was already reported in our previous study, and could be associated to Se binding to small biomolecules involved in the defense against oxidative stress[29,43-44].

Hepatic Cu (Fig. SI-1b, Table 2) in this study was distributed within one peak, containing LMM biomolecules (5-24 kDa). The same was observed for hepatic (Fig. SI-1h, Table 2) and gill Cd (Fig. SI-2d, Table 2). This was consistent with the previous study on Vardar chub, where Cu- and Cd-binding LMM biomolecules were eluted within a single and sharp peak at the elution time of MT standard, indicating high affinity of Cu and Cd for binding to MTs[29]. Such binding, suggesting a detoxification response of the organism, was previously reported for white sucker (*Catostomus commersonii*)[7], juvenile yellow perch (*Perca flavescens*)[45], and European eel (*Anguilla anguilla*)[46]. Both hepatic (Fig. SI-1g, Table 2) and gill Zn (Fig. SI-2c, Table 2) were also found bound to LMM biomolecules (7-24 kDa), but this binding was more obvious and more intense in the liver, and indicated hepatic Zn association to MTs. It was consistent with observations for both Cu and Cd, and was also in agreement with the previous reports on pearl cichlid (*Geophagus brasiliensis*) and sea catfish (*Netuma barba*)[38], as well as on European eel (*A. anguilla*)[46]. However, notable quantities of hepatic and gill Zn were additionally found bound to HMM biomolecules (>495 kDa) and to MMM biomolecules (24-

299 kDa). Large quantities of Zn eluted within HMM and MMM protein regions were also observed in our previous study in both Vardar chub organs[29], indicating Zn essential role in the function of many proteins and enzymes[47-48], such as transport protein albumin (66 kDa) and enzymes Cu–Zn superoxide dismutase (SOD, 32 kDa) and carboanhydrase (29 kDa)[49]. Furthermore, potential Zn binding to MTs, which was more pronounced and clear in the liver than in the gills, was also observed in our former study on Vardar chub[29].

In this study, hepatic Fe (Fig. SI-1c, Table 2) was distributed between two comparable peaks, the first one referring to HMM biomolecules (181-637 kDa) and the second one to MMM biomolecules (24-51 kDa). In the gills (Fig. SI-2a, Table 2), however, the majority of Fe was distributed within one peak containing MMM biomolecules (19-51 kDa), which coincided with the second Fe peak of hepatic cytosol. This was in agreement with the previous finding on Fe distribution in liver and gills of Vardar chub, with much more pronounced Fe presence in the HMM protein region in the liver[29], most likely referring to Fe binding to ferritin (~450 kDa)[49], the primary iron storage protein[50]. The suggested explanation was that the absence of HMM Fe-binding biomolecules in the gills was the reflection of known function of the liver and not the gills in the process of Fe storage[29]. Similarly, Neves et al.[50] found ferritin expression in both the liver and brain of sea bass (*Dicentrarchus labrax*), but higher concentrations were measured in the liver, as the major organ of iron storage. On the other hand, we have found comparable Fe quantities eluted within MMM protein region in both organs of Vardar chub, which possibly reflected Fe binding to transport or enzymatic proteins, such as hemoglobin (~65 kDa)[49], transferrin (~80 kDa)[49], myoglobin (~17 kDa)[49], catalase (subunits of ~60 kDa)[49], or some other.

Since it is usually expected to find transport protein transferrin in the hepatic samples, as described by Neves et al.[50] in the study on the liver of sea bass (*D. labrax*), we have run the standard protein transferrin (76-81 kDa) through the SEC_{200}-column and recorded its elution time at 22nd to 27th minute, with a maximum at approximately 24th minute, corresponding to MM of 85 kDa (Fig. SI-3). However, the corresponding peak was not observed in either hepatic or gill SEC_{200} Fe profiles of Vardar chub (Figs. SI-1c and SI-2a). We have, furthermore, collected the MMM Fe-fractions from both liver and gills after SEC_{200}-HPLC separation (t_e 25-30 min), and then recorded MALDI-TOF-MS spectra for collected samples (Fig. SI-4a-b), as well as for standard proteins transferrin (Sigma-Aldrich Co., USA) and Zn,Cu-SOD (Sigma-Aldrich Co., USA) (Fig. SI-4c-d). We have included SOD because MMM Fe-fractions have partly overlapped with the tails of LMM Cu-fractions and MMM Zn-fractions (Table 2). Comparison of MALDI-TOF-MS spectra obtained for the samples and standards clearly indicated the absence of transferrin in our samples, but confirmed the probable presence of

SOD, based on concurrent appearance of similar peaks in spectra of both hepatic and gill samples and of SOD standard (peaks at 31 kDa, 15.5 kDa (2+), and 7.75 kDa (4+)). Since hemoglobin subunits have molecular mass of approximately 15 kDa[51-52], peaks at 15.5 kDa could have additionally indicated the presence of hemoglobin in our samples, especially taking in consideration the reddish colour of the collected fractions. Several peaks observed below 10 kDa in the MALDI-TOF spectra of hepatic and gill samples have possibly indicated Fe-binding to biomolecules belonging to a low-molecular-mass iron pool, sometimes called the transit iron pool[53], which contains small and soluble complexes that serve as intracellular Fe transport from one Fe-binding protein to the other[54].

Analysis of Vardar chub hepatic and gill MMM Fe-binding biomolecules (isolated by SEC_{200}-HPLC) using AEC-HPLC and MS techniques

Since likely presence of non-Fe-binding proteins was established in MMM Fe-fractions obtained by SEC_{200}-HPLC separation, in the next step of our research we have collected those fractions from liver and gills (t_e 26-29 min, Figs. SI-1c and SI-2a), purified them using AEC-HPLC (Fig. 1), and then analyzed Fe-binding proteins present in hepatic and gill samples using MALDI-TOF-MS and LC-MS/MS (Fig. 2, Table 3 and 4). AEC-separation was often applied after SEC-fractionation in the course of isolation and characterization of Fe-binding biomolecules, e.g. in human serum[55-57] and in fish (rainbow trout, *Oncorhynchus mykiss*)[52]. During AEC-separation, Fe-binding biomolecules were eluted within single clear and sharp peaks, with elution time from 10.0 to 14.5 min for liver (Fig. 1 a-c) and from 9.5 to 13.5 min for gills (Fig. 1 d-f), whereas the concentration of ammonium acetate (mobile phase B) needed for their elution generally ranged from 32.5 to 43.8 mM (Table 3). Small Zn and Cu peaks separated from Fe-peaks were observed (t_e 15.0-17.5 min), especially in the profiles of hepatic samples (Fig. 1a-c), showing the advantage of AEC-HPLC as useful protein purification procedure.

To further characterize purified Fe-binding biomolecules, we have collected Fe-fractions eluted during AEC separation (liver: t_e 11-14 min; gills: t_e 10-13 min) and recorded their mass spectra by MALDI-TOF-MS. The MALDI spectra obtained for the hepatic Fe-fractions always showed one major peak at 15.4 kDa, and sometimes two additional smaller peaks at 31.5 kDa and 46.9 kDa (Fig. 2a). Recorded peaks most likely corresponded to hemoglobin (Hb) spectra, with characteristic Hb monomers (~15 kDa), dimers (~30 kDa) and trimers (~45 kDa). Hemoglobin, although a large protein of molecular mass of approximately 64.5 kDa, has four subunits each containing polypeptide chain (α or β) and a heme group[58]. In the rainbow trout (*O. mykiss*) blood, five major α chains and four major β chains have been identified, with the mass range from 15.1 to 16.0 kDa[52], corresponding well to our results. In the study on human blood, Yu et

al.[51] have also observed the simultaneous occurrence of monomers and dimers after separation of Hb on SDS-PAGE mini-gels. Accordingly, peak at ~15 kDa most theoretically corresponded to either an α-chain, or β-chain, peak at ~30 kDa probably corresponded to covalent product of α-α, β-β, or α-β crosslinking, whereas peak at 45 kDa probably corresponded to a covalently linked trimer[51]. The MALDI-TOF spectra obtained for the gill Fe-fractions also showed clear peaks at ~15 kDa, which could be attributed to Hb, but additional peaks were observed at lower molecular masses, indicating multiple charge on proteins (Fig. 2b), whereas the signs of multimers were not recorded. For example, in fish No.1 (Fig. 2b), the other two peaks were probably double charged (peak 2: 7.7 kDa; 2+) or triple charged (peak 1: 5.1 kDa; 3+).

To identify Fe-binding biomolecules with certainty, we additionally analyzed AEC-collected hepatic and gill Fe-fractions by LC-MS/MS with subsequent Mascot database search, which confirmed Hb presence in soluble fractions of both liver and gills of Vardar chub (Table 4). In both liver and gills, subunit β of Hb (corresponding to subunits originating from various fish species) was identified, whereas subunit α was only found in one gill sample (fish No. 2, Table 4). The observation of subunit α can explain the MALDI spectra obtained for the gills of fish No. 2, which next to peak of 15.3 kDa also contained two additional peaks of lower molecular masses (at 11.3 kDa and 13.5 kDa) which possibly corresponded to Hb subunit α. Lower molecular mass of α-chain (15.1 kDa) compared to β-chain (15.9 kDa) was also recorded in human Hb[51], as well as in rat Hb (α: 15.3 kDa; β: 16.0 kDa)[59]. Our results, therefore, undoubtedly confirmed the presence of blood in the samples of Vardar chub liver and gills, which was not surprising considering that organ perfusion was not performed prior to liver and gills dissection and isolation of soluble tissue fractions. In addition, major binding of Fe to hemoglobin instead of transferrin is consistent with finding by Fernández-Menéndez et al.[59], who showed that in rat red blood cells less than 13 % of the total Fe was bound to transferrin, i.e. 87-93 % of Fe was found bound to hemoglobin. Combination of SEC- and AEC-HPLC with MS analyses was proven to be sensitive and optimal tool for detection of Hb variants, as also pointed out by Kleinert et al.[60].

Analysis of Vardar chub hepatic and gill LMM Cd-binding biomolecules (isolated by SEC_{200}-HPLC) using AEC-HPLC

Our further aim was to better characterize the potential MT fraction from Vardar chub liver and gills, i.e. Cd, Cu, and Zn-binding LMM biomolecules observed after SEC_{200}-HPLC separation. Hyphenated techniques, such as combination of SEC-HPLC and AEC-HPLC with ICP-MS, allow us to distinguish different MT isoforms, which could bring much more insightful information on regulation of essential elements such as Cu and Zn, and on detoxification of metals such as Cd in various organisms[61]. Thus, we have collected hepatic and gill Cd-

containing LMM fractions produced by SEC_{200}-HPLC separation (t_e 30-34 min) and analysed them by AEC-HPLC (Fig. 3a-d).

In general, two well resolved Cd-peaks were obtained by AEC-separation of Cd-containing LMM fractions from both Vardar chub liver (Fig. 3 a,b) and gills (Fig. 3 c,d), with elution times from 9.0-12.0 minutes and 12.5-16.5 minutes, whereas the concentrations of ammonium acetate (mobile phase B) needed for their elution generally ranged from 32.5 to 43.8 mM and 43.8 to 62.5 mM, respectively (Table 3). The elution of these biomolecules did not require hard ionic strength conditions in the anion-exchange column, which is consistent with the previous studies on MTs from several other fish species (*Cyprinus carpio*, *A. anguilla*, *G. brasiliensis*), which reported MT elution at 30-45 mM ammonium acetate[38,62-63]. In addition to AEC-separation of hepatic and gill samples, we have also analyzed MT standards from rabbit liver (isoforms MT-I and MT-II) using AEC-HPLC (Fig. 3 e,f). Examination of their absorbance profiles at 254 nm revealed that MT-I standard eluted at 9.5-11.5 minutes, whereas MT-II standard eluted at 13.0-16.0 minutes. Evidently, elution time of MT-I standard coincided with the first Cd-containing peak, whereas elution time of MT-II standard coincided with the second Cd-containing peak of Vardar chub organs, further confirming that analyzed LMM Cd-containing fractions indeed contained MTs, specifically MT isoforms MT-I and MT-II. The Cd-binding biomolecules were previously investigated in the liver of two species of flatfish (*Limanda limanda* and *Microstomus kitt*) by Duquesne and Richard[64], who also confirmed the existence of two isoforms of hepatic MT, with MT-II as a predominant isoform. Several other authors also reported Cd-binding to two MT isoforms, MT-I and MT-II, in organs of various fish species: in the liver of eel (*A. anguilla*)[46] and dab (*L. limanda*)[65] and in the liver and gills of goldfish (*Carassius auratus*), yellow catfish (*Pelteobagrus fulvidraco*), stone moroko (*Pseudorasbora parva*) and barbel chub (*Squaliobarbus curriculus*)[66]. The obtained results in this study further demonstrated the importance of multidimensional chromatographic approach in MT analyses, since sole application of SEC-HPLC separation revealed Cd-binding to MTs, but was not able to reveal the presence of various isoforms.

The obtained AEC-peaks contained all three analyzed metals (Cd, Cu, and Zn) in hepatic samples (Fig. 3a,b), whereas in the gills they referred solely to Cd (Fig. 3c,d), since Cu was not measured due to its low cytosolic concentrations in the gills, and Zn was not detected in MT region after AEC-separation. This was an indication of the absence of Zn binding to MTs in Vardar chub gills, as was already anticipated based on the results of SEC_{200}-HPLC analysis of gill cytosols (Fig. SI-2). Furthermore, it was in agreement with the absence of clear Zn peak at the elution time of MTs in the gills of European chub (*S. cephalus*) from the Sulta River[28]. Observed differences between Zn distribution in liver and in gills suggested a tissue-dependent physiological role of MT isoforms. Although both organs evidently contained MT-I and MT-II

isoforms, it is possible that each isoform differed in amino acid sequence between organs, presenting gill and hepatic subisoforms of MT-I and MT-II. The hepatic Zn-containing MT isoforms are therefore considered, not only as a means of detoxification, but also a storage for essential metals, which can be donated to the other proteins when needed[35,67]. The gill Cd-containing MT isoforms, on the other hand, can be hypothesized to serve predominantly as a detoxification tool.

It was previously reported by Goenaga Infante et al.[2] that Cd was preferably bound to a major MT-I isoform in rabbit liver standard and that the increase of the Cd amount bound to MTs in the liver of gibel carp (*Carassius auratus gibelio*) mainly reflected the induction of a MT-I isoform. Such predominant binding to MT-I was observed in our study only in one liver sample (fish No. 3, Fig. 3b), whereas in the same fish, Cd binding to MT-II was more pronounced in the gills (fish No. 3, Fig. 3d). Similarly, Li et al.[66] reported Cd binding only to MT-I in the liver of bighead carp (*Aristichthys nobilis*) and white amur bream (*Parabramis pekinensis*), whereas in the gills of the same fish species Cd was bound to both MT-I and MT-II. Goenaga Infante et al.[2] further reported that, in contrast to Cd, the excess of intracellular Cu and Zn appears to be sequestered by four MT isoforms in the liver of gibel carp (*C. auratus gibelio*), including MT-I and MT-II isoforms, which were also detected using AEC-HPLC. This phenomenon of differential metal-binding by specific MT isoforms, which has been reported in a various types of organisms (mammals, snails, mussels)[68-70], might be related to different role of each MT isoform in the metal detoxification and regulation[38].

Further characterization of MT fractions by application of MALDI-TOF-MS was done after heat-treatment and SEC_{75}-HPLC fractionation of hepatic and gill cytosols, which are presented in the next subsection.

SEC_{75}-HPLC analyses of trace element distributions in hepatic and gill cytosols before and after the heat-treatment

According to Rosabal et al.[71], heat-stable cytosolic fraction contains biomolecules, such as MTs, which are consistently involved in the detoxification of trace metals. MTs are heat-stable proteins, owing to high content of cysteine residues (30 % of their amino acids) which give heat-stability to peptides[7]. However, in addition to MTs, we have previously detected the presence of the other heat-stable biomolecules in Vardar chub liver and gills, namely VLMM biomolecules (<10 kDa) that bind Mo and Se[29]. Our aim, therefore, was to further purify cytosolic samples to obtain the fractions which contain solely heat-stable peptides and proteins characteristic for Vardar chub liver and gills, i.e. to remove by the heat-treatment at 70°C high molecular mass proteins that could interfere with the characterization of heat-stable

biomolecules[16,37]. We have performed SEC_{75}-HPLC analyses on both untreated and heat-treated hepatic and gill cytosols of Vardar chub to define the changes in the cytosolic distributions of several elements (Cd, Cu, Zn, Mo and Se) that occur after the cytosol heat-treatment. As already stated above in the introductory part of the section *SEC_{200}-HPLC analyses of trace element distributions in hepatic and gill cytosols of Vardar chub*, by performing this procedure we were able to confirm the results obtained during the 2012 campaign[29], and to isolate the heat-stable metal-containing fractions that we wanted to further characterize. Distribution profiles of five mentioned elements in the hepatic and gill cytosols of Vardar chub before and after the heat-treatment are presented within supplemetary information (Fig. SI-5 and SI-6), whereas their elution times and molecular masses of corresponding biomolecules are given in Table 5.

In our previous study, we have reported binding to heat-stable LMM biomolecules (10-30 kDa), presumably MTs, for Cd and Cu in the liver and Cd in the gills of Vardar chub[29]. In this study, elution within LMM peak (9-31 kDa, Table 5) was again observed for both hepatic Cd (Fig. SI-5a,d) and Cu (Fig. SI-5c,f), as well as for the gill Cd (Fig. SI-5g,i). The observed LMM peaks remained mostly unchanged after the heat-treatment in the case of the liver (changes within 10 %, Table 6) and slightly decreased in the case of the gills (changes up to 30 %, Table 6), confirming the presence of Cd,Cu-binding heat-stable biomolecules, likely MTs, in the Vardar chub liver and gills. As the heat-treatment causes a partial removal (~50 % after 10 minutes at 70 °C) of MT20 fraction (MT dimers) from the samples due to the presence of disulphide bonds within MT20 molecules[37], observed 30 % decrease of gill LMM peak after the heat-treatment most likely referred to the slight reduction of MT dimers[37]. MT monomers (MT10), on the other hand, are resistant to any physical or chemical treatment and do not change under the influence of increased temperature[37]. In the case of the Vardar chub gills, a small part of Cd was additionally eluted within MMM peak (38-137 kDa, Fig. SI-5g,i, Table 5), which visibly decreased (up to 65 %, Table 6) after the heat-treatment, indicating heat-sensitive nature of Cd-binding MMM biomolecules in the gills. This was a confirmation of the previous finding of Cd binding to MMM biomolecules only in the Vardar chub gills (60-110 kDa), which was hypothesized to refer to undetoxified portion of gill Cd[29].

Elution within LMM peak (11-31 kDa, Table 5) was also observed for the hepatic Zn (Fig. SI-5b,e), but, as expected, not for the gill Zn (Fig. SI-5h,j), again confirming the absence of Zn-MT binding in the gills of Vardar chub. Hepatic Zn-LMM peak was proven to be relatively heat-stable, taking in consideration rather low decrease of LMM peak after the heat-treatment (up to 22 %, Table 6). This small decrease of Zn-LMM peak was probably related to the reduction of MT20 fraction[37], as already discussed above for the gill Cd-LMM fraction. The

findings of this study are consistent with the previously reported Zn binding to heat-stable LMM biomolecules (10-30 kDa) in the liver and its absence in the gills of Vardar chub[29]. In this study, in both organs of Vardar chub, Zn was also eluted within MMM peak (~20-137 kDa), which represented the minor part of cytosolic Zn in the liver (Fig. SI-5b,e), and the major part in the gills (Fig. SI-5h,j). After the heat-treatment, both hepatic and gill Zn-MMM peaks were almost completely removed (decrease up to 80 %, Table 6), pointing to the heat-sensitivity of Zn-binding MMM proteins. Zinc binding to MMM biomolecules (30-140 kDa) and their almost complete removal by the heat-treatment was also previously reported for Vardar chub liver and gills[29].

In the untreated hepatic cytosols of Vardar chub, Mo was eluted within two peaks (Fig.SI-6a,c), with the majority of Mo eluted within the first MMM peak (31-137 kDa, Table 5), and only small amount eluted within the second VLMM peak (5-11 kDa, Table 5). However, after the cytosol heat-treatment, hepatic Mo-MMM peak was almost completely removed (decrease of ~80 %, Table 6), whereas Mo-VLMM peak has markedly increased (146-272 %, Table 6), suggesting that a part of Mo has shifted from MMM region to VLMM biomolecule region. The same finding was previously reported for Vardar chub liver, and hypothesized as possible decomposition of heat-sensitive Mo-MMM-proteins to smaller, heat-stable, Mo-binding biomolecules[29]. In contrast, both before and after the heat-treatment, gill Mo (Fig.SI-6e,g) was eluted almost completely within VLMM peak (5-11 kDa, Table 5) which coincided with the second peak of hepatic cytosol, again confirming the results of our previous study[29] and proving the heat-stability of gill Mo-VLMM biomolecules. Gill Mo-VLMM peak also slightly increased after the heat-treatment, but only up to 30 % (Table 6). Since small part of Mo was eluted in the MMM protein region, and was removed after the heat-treatment (Fig.SI-6e,g), it was possible that portion of Mo also shifted from MMM to VLMM region, as observed for the liver, thus explaining slight increase of VLMM peak.

In the untreated hepatic (SI-6b and d) and gill cytosols (SI-6f and h) of Vardar chub, Se eluted within one or two peaks in the MMM protein region (~10-140 kDa, Table 5), as well as within two VLMM peaks (3-6 kDa and 0.5-1.6 kDa, Table 5). After the cytosol heat treatment, Se-MMM peaks of both organs were almost completely removed (decrease up to 80 %, Table 6), confirming their heat-sensitivity. In contrast, Se-VLMM peaks exhibited evident heat-stability, with hepatic Se-VLMM peaks having slightly decreased (up to 27 %, Table 6), and gill Se-VLMM peaks having remained almost unchanged (increase up to 6 %, Table 6) after the heat-treatment. These results were consistent with our previous report on Se in Vardar chub organs, indicating partial Se binding to small, heat-stable compounds within the hepatic and gill cytosols of Vardar chub[29].

Our further aim in this study was to more closely characterize the heat-stable biomolecules that bind Cd (probable MT fractions), as well as those that bind Mo in the liver and gills of Vardar chub. To achieve this goal, we have collected the fractions of interest obtained by the above described SEC_{75}-HPLC fractionation of heat-treated Vardar chub hepatic and gill cytosols, and then analyzed them by AEC-HPLC and MALDI-TOF-MS.

Analysis of hepatic MT fractions of Vardar chub (isolated by heat-treatment and SEC_{75}-HPLC) using AEC-HPLC and MALDI-TOF-MS

We have collected LMM fractions containing Cd, Cu and Zn, which were produced by SEC_{75}-HPLC separation of heat-treated hepatic cytosols (t_e 21-25 min), and analysed them by AEC-HPLC. The distribution profiles of Cu, Zn and Cd obtained by combination of AEC-HPLC separation and subsequent measurement on HR ICP-MS are presented in Fig. 4 for the samples of hepatic cytosols purified by the heat-treatment, and they confirmed the results already presented for the untreated hepatic cytosols (Fig. 3 a,b) in the section *Analysis of Vardar chub hepatic and gill LMM Cd-binding biomolecules (isolated by SEC_{200}-HPLC) using AEC-HPLC*. As can be seen, in both analyzed fish Cd, Cu, and Zn were eluted within two peaks, first one from 9.5 to 12.5 min (concentration of ammonium acetate as a mobile phase B from 32.5 to 43.8 mM) and second one from 12.5 to 15 min (concentration of ammonium acetate as a mobile phase B from 43.8 to 62.5 mM) (Fig. 4 a,b). This was a confirmation of the above discussed Cd, Cu and Zn association to two different MT isoforms in the liver of Vardar chub, where the elution time of standard MT-I (9.5-11.5 min; Fig. 3e) coincided with the first Cd, Cu, and Zn peak and the elution time of standard MT-II (13-16 min; Fig. 3f) coincided with the second Cd, Cu, and Zn peak.

Further on, we have collected these two hepatic fractions obtained by AEC-HPLC separation, marked as L-Cd1 fraction (t_e 9.5-12 min) and L-Cd2 fraction (t_e 12.5-15 min) and analyzed them by MALDI-TOF-MS, to determine their exact molecular masses and define if there are observable differences in masses of MT-I and MT-II isoforms of Vardar chub liver. First, we have recorded mass spectra for each one of MT standards (Fig. 5 a,b) and established that molecular masses of MT-I (6.2 kDa) and MT-II (6.1 kDa) differed by only 100 Da. Molecular masses of major peaks in L-Cd1 and L-Cd2 fractions were the same (6.0 kDa; Table 7; Fig. 5c,d) and only 100-200 Da lower than molecular masses of MT standards. Considering the accuracy of the MALDI-TOF-MS instrument, as well as the possibility of some differences in MT saturation or in the content of specific metals bound to MTs, which both can result with the mass differences of few hundred Da[72], our findings suggested that the mass of two MT isoforms isolated from Vardar chub liver corresponded to the masses of analyzed MT-I and MT-II standards. MT isoforms I and II commonly differ from one another very little

considering their molecular masses[73]. Accordingly, rabbit liver standards MT-I and MT-II without Zn (Enzo, Switzerland), which we have used in this study, were declared as both having the same molecular masses, amounting to 6.145 kDa, whereas their fully saturated forms (including seven Zn ions) have a mass of 6.603 kDa. Carpenè and Vašák[73] identified two MT isoforms (MT-1 and MT-2) in the liver of goldfish (*C. auratus*) using ion exchange chromatography and amino acid sequencing. Two isoforms differed in only one aminoacid residue, with MT-1 containing 61 and MT-2 containing 62 residues[73]. So, it was evident that it is not possible to distinguish these two isoforms based solely on their masses, since molecular masses of some MT isoforms are too close for successful separation by SEC-HPLC and mass spectrometry[72]. The application of AEC-HPLC as an intermediate step can be helpful in such cases. MT isoforms differ in aminoacid composition other than cystein residues, which causes them to have different isoelectric points and different hydophobicities[74], and consequently their distinct separation by AEC-HPLC following SEC-HPLC can be expected. Twodimensional approach of MT separation using SEC-AEC-HPLC was often applied in various aquatic organisms, such as flatfish (*L. limanda*)[65], gibel carp (*C. auratus gibelio*)[2], eel (*A. anguilla*)[62], sea catfish (*N. barba*) and pearl cichlid (*G. brasiliensis*)[38], and molluscs[75]. Further similarity between two MT isoforms from Vardar chub liver and two MT standards referred to the fact that in all four samples we have also detected smaller peaks probably corresponding to MT dimers, with the molecular masses equal to 12.0 kDa for the liver and 12.3-12.4 kDa for the standards. Concurrent presence of MT-monomers and MT-dimers was previously reported by Ivanković et al.[76] in both control and Cd-exposed mussels (*Mytilus galloprovincialis*).

In addition to described MT peaks, in MALDI-TOF-MS spectra of L-Cd1 and L-Cd2 several other minor peaks were observed, as shown in Figs. 5c and 5d, which could present new molecular signatures that bind to Cd in fish liver. Minor peaks observed within L-Cd1 fraction had molecular masses equal to 4.3 kDa, 4.4 kDa, 8.6 kDa and 9.3 kDa, whereas within L-Cd2 fraction the following minor peaks were observed: 4.3 kDa, 4.4 kDa, 7.9 kDa, 8.2 kDa and 8.6 kDa. Since low resolution ion trap LC-MS/MS applied in this study could not reliably determine listed Cd-binding biomolecules, their investigation will be included in our future studies.

Analysis of gill MT fractions of Vardar chub (isolated by heat-treatment and SEC_{75}-HPLC) using MALDI-TOF-MS

To further characterize gill MT, we have collected Cd-containing LMM fractions, which were produced by SEC_{75}-HPLC separation of heat-treated gill cytosols (t_e 21-25 min), and tried to analyse them by AEC-HPLC, same as we have done for the liver. However, due to the low

concentrations of analyzed metal and proteins in the samples, we were not able to obtain the detectable results. Therefore, we have analyzed the LMM fractions collected after SEC_{75}-HPLC separation (t_e 21-25 min) directly on MALDI-TOF-MS. The obtained spectrum presented in Fig. 5e showed three peaks, with a major peak at mass of 4.9 kDa (Table 7). This peak did not seem as it referred to MTs. However, Maltez et al.[75] also found major Cd-containing peak at mass of 5.1 kDa in the snail *Marisa cornuarietis*. Although they initially inferred that this mass was too low if compared with already classified metallothionein-like proteins (MLPs), they eventually came to conclusion, based on sequencing analysis, that MLP isoform at mass 5.1 kDa could correspond to MT[75]. In our study, two smaller peaks were also obtained, one at 6.2 kDa which corresponded to masses of MT standards (6.1-6.2 kDa; Fig. 5a,b), and another at 3.1 kDa which probably corresponded to double charged MT molecule. Using such approach of MS analysis directly after SEC-HPLC separation obviously has its disadvantages, as it did not provide the possibility to reliably discern two isoforms, which presence was observed after AEC-HPLC separation of SEC_{200}-HPLC fractions from untreated gill cytosols (Fig. 3c,d; Table 3) and discussed in the section *Analysis of Vardar chub hepatic and gill LMM Cd-binding biomolecules (isolated by SEC_{200}-HPLC) using AEC-HPLC*. However, our analyses did indicate that there were differences between MTs from two organs. Our finding that gill MTs, unlike hepatic MTs, do not bind Zn, is consistent with the report by Noël-Lambot et al.[77] who claimed that gill MT of eels (*A. anguilla*), contrary to liver, binds very small amounts of Zn and Cu. They even asserted that MT does not seem to be a normal constituent of the gills, but rather that its synthesis in the gills is induced as a consequence of Cd exposure[77]. Van Campenhout et al.[78] found similar type of difference between liver and kidneys of carp (*C. carpio*), with only 2 % of cytosolic Zn bound to MTs in the kidneys, and over 30 % in the liver. This difference between gill and hepatic MT was further confirmed by the fact that gills, in addition to small amount of commonly found 6 kDa MT, contained predominant MT isoform of lower molecular mass (~5 kDa).

Analysis of heat-stable Mo-binding biomolecules from hepatic and gill cytosols of Vardar chub (isolated by heat-treatment and SEC_{75}-HPLC) using MALDI-TOF-MS

Since there is no information available about heat-stable biomolecules that bind Mo, or about Mo-binding biomolecules in fish in general, it was interesting to further characterize such molecules in the hepatic and gill cytosols of Vardar chub. As stated in the literature, molecular characterization of Mo transporters, storage proteins and chaperones in fish, as well as genomic and proteomic studies concerning Mo-exposed fish have not yet been carried out[79]. Thus, we have collected Mo-containing VLMM fractions, which were produced by SEC_{75}-HPLC separation of heat-treated hepatic and gill cytosols (t_e 26-31 min),

and tried to analyse them and purify by AEC-HPLC. The same as happened in the case of the gill MT, also happened during analyses of Mo fractions, i.e. we were not able to obtain the detectable results due to the low concentrations of analyzed metal and proteins in the samples of both liver and gills. Therefore, we have also analyzed Mo-containing VLMM fractions collected after SEC_{75}-HPLC separation (t_e 26-31 min) directly on MALDI-TOF-MS. This fraction, however, was partly overlapping with the minor Se peak (Fig. SI-6b,d,f,h; Table 5), and our initial aim was to separate Mo-binding biomolecules from the traces of Se-binding biomolecules using AEC-HPLC. Since this was not successfully done, we could expect to find some minor peaks referring to small Se-binding biomolecules on MALDI spectra. And, indeed, on the recorded spectra we have found few unresolved small peaks (in the liver: 6.9 kDa and 9.6 kDa; Fig. 6a) or even background noise (in the gills; Fig. 6b) which could possibly be associated to either Mo or Se (Fig. 6). The obtained MALDI spectra presented in Figs. 6a and 6b showed the presence of two clear peaks in both organs. However, in the liver major peak was observed at mass of 8.5 kDa, whereas another, much lower, peak was obtained at mass of 4.2-4.4 kDa, probably referring to double charged species (Table 7). In the gills, on the other hand, the major peak was observed at mass of 3.3 kDa, whereas smaller peak was recorded at mass of 8.5 kDa, which corresponded to the mass of major hepatic peak (Table 7). This could be associated to the results of SEC_{75}-HPLC distribution (Fig. SI-6a,c,e,g; Table 5), where evident differences were seen between two organs. Thus, it is possible that biomolecule with a mass of ~8 kDa, which was predominant in the liver, and only minor in the gills, reffers to Mo-binding species which appeared after the heat-treatment of the cytosols, by degradation of MMM heat-sensitive biomolecules (~30-130 kDa). In contrast, smaller biomolecule at ~3 kDa possibly reffers to heat-stable Mo-binding species which was initially present in the cytosol, even before the heat-treatment, and which was predominat in the gills and negligible in the liver. Since LC-MS/MS analysis with subsequent Mascot search did not resulted with definite recognition of two Mo-binding biomolecules, due to the use of low resolution ion trap mass spectrometer, we are currently performing sequencing with the aim of their final characterization.

Conclusions

Application of two-dimensional fractionation of cytosolic biomolecules from Vardar chub liver and gills by a combination of SEC-HPLC and AEC-HPLC, followed by analyses of isolated fractions by two mass spectrometry techniques, MALDI-TOF-MS and LC-MS/MS, with an aim to better characterize and identify several selected metal-binding biomolecules, resulted with the following findings: 1) MMM Fe-binding biomolecules from Vardar chub liver and gills, defined by SEC-HPLC to have molecular masses of ~20-50 kDa,

were identified as hemoglobin subunits β in the liver, with the molecular masses of ~15 kDa according to MS, and hemoglobin subunits α and β in the gills, with the molecular masses of ~11 kDa, ~13 kDa and ~15 kDa according to MS; 2) AEC-HPLC separation of LMM Cd-binding biomolecules from both hepatic and gill cytosols of Vardar chub indicated Cd binding to two MT isoforms, MT-I and MT-II, in both studied organs; 3) heat-stable hepatic LMM Cd-binding biomolecules, defined by SEC-HPLC to have molecular masses of ~10-30 kDa, were identified as MTs of molecular masses equal to 6.0 kDa, according to MS, whereas gill LMM Cd-binding biomolecules were identified as MTs of molecular masses equal to 4.9 kDa, according to MS; 4) heat-stable VLMM Mo-binding biomolecules from Vardar chub liver and gills, defined by SEC-HPLC to have molecular masses of 5-11 kDa, were identified as biomolecules of molecular masses equal to 3.3 kDa and 8.5 kDa, according to MS, with smaller biomolecule being predominat in the gills and the larger one in the liver. Our study, thus, revealed the differences between two studied organs regarding the characteristics of metal-binding biomolecules (different molecular masses of hemoglobin and MTs in liver and gills) and regarding their prevalence (in the case of Mo). In addition, it was found that gill MT, unlike MT hepatic isoform, does not bind Zn, indicating the possibility of different MT functions in these two organs. The need for simultaneous application of several analytical techniques in the research of cellular metal-binding biomolecules should be emphasized, to enable adequate separation and characterization of studied biomolecules. SEC-HPLC, which can separate the biomolecules according to their masses, is not able to separate the isoforms which differ in charge, whereas the masses recorded by that technique are in general higher than the masses obtained by MS analyses. Therefore, use of multidimensional separations, as well as subsequent application of MS, as a method of higher sensitivity and accuracy in mass determination, is crucial for more reliable characterization of metal-binding proteins with the purpose of better understanding of metal behaviour within the cells.

Live subject statement

The research presented in this paper complied with the Croatian legislation (Ministry of Agriculture, Directive on protection of animals used for scientific purposes, Official Gazette 55/2013) and EU legislation (Directive 2010/63/EU of the European Parliament and of the

Council of 22 September 2010 on the protection of animals used for scientific purposes, Official Journal of the European Union, 276/33) relevant for animal use for scientific purposes. Laboratory for Biological Effects of Metals, where the study was performed, is registered for killing the fish and work on the isolated organs, tissues and carcases of the animals that are killed for that purpose at the Administration for Veterinary Medicine and Food Safety of the Ministry of Agriculture (No. of the decision HR-POK-025).

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This study was carried out as a part of bilateral project between Macedonia and Croatia, titled "The assessment of the availability and effects of metals on fish in the rivers under the impact of mining activities". The financial support of the Ministry of Science and Education of the Republic of Croatia for institutional funding of Laboratory for Biological Effects of Metals and the Croatian Science Foundation for project no. IP-2014-09-4255 is acknowledged.

References

1 S. Ramani, Z. Dragun, D. Kapetanović, V. Kostov, M. Jordanova, M. Erk, Z. Hajrulai-Musliu, Surface water characterization of three rivers in the lead/zinc mining region of northeastern Macedonia, *Arch. Environ. Contam. Toxicol*., 2014, **66**, 514-528.

2 H. Goenaga Infante, K. Van Campenhout, R. Blust and F.C. Adams, Anion-exchange high performace liquid chromatography hyphenated to inductively coupled plasma-isotope dilution-time-of-flight mass spectrometry for speciation analysis of metal complexes with metallothionein isoforms in gibel carp (*Carassius auratus gibelio*) exposed to environmental metal pollution, *J. Chromatogr. A*, 2006, **1121**, 184-190.

3 Z. Dragun, N. Krasnići, Ž. Stržak and B. Raspor, Lead concentration increase in the hepatic and gill soluble fractions of European chub (*Squalius cephalus*) - an indicator of increased Pb exposure from the river water, *Environ. Sci. Pollut. Res.,* 2012, **19**, 2088-2095.

4 Z. Dragun, N. Tepić, N. Krasnići and E. Teskeredžić, Accumulation of metals relevant for agricultural contamination in gills of European chub (*Squalius cephalus*), *Environ. Sci. Pollut. Res*., 2016, **23**, 16802-16815.

5 R.A. Hauser-Davis, R.C. de Campos and R.L. Ziolli, Fish metalloproteins as biomarkers of environmental contamination, *Rev. Environ. Contam. Toxicol*., 2012, **218,** 101-123.

6 W.X. Wang, Prediction of metal toxicity in aquatic organisms, *Chin. Sci. Bull*., 2013, **58**, 194-202.

7 N. Urien, S. Jacob, P.Couture, and P.G.C. Campbell, Cytosolic distribution of metals (Cd, Cu) and metalloids (As, Se) in livers and gonads of field-collected fish exposed to an environmental contamination gradient: an SEC-ICP-MS analysis, *Environments*, 2018, **5**, 102.

8 R. van der Oost, J. Beyer and N.P.E. Vermeulen, Fish bioaccumulation and biomarkers in environmental risk assessment: a review, *Environ. Toxicol. Pharmacol*., 2003, **13**, 57-149.

9 D. Webb, M.M. Gagnon and T. Rose, Metabolic enzyme activities in black bream (*Acanthopagrus butcheri*), *Comp. Biochem. Physiol. Part C*, 2005, **141**, 356-365.

10 M. Acosta, S. Torres, L. Mariño-Repizo, L.D. Martinez and R.A. Gil, Novel method for metalloproteins determination in human breast milk by size exclusion

chromatography coupled to inductively coupled plasma mass spectrometry, *J. Pharm. Biomed. Anal*., 2018, **158**, 209-213.

11 T.J. Griffin, S.P. Gygi, B. Rist, R. Aebersold, A. Loboda, A. Jilkine, W. Ens and K.G. Standing, Quantitative proteomic analysis using a MALDI quadrupole time-of-flight mass spectrometer, *Anal. Chem*., 2001, **73**, 978-986.

12 A. Vioque-Fernandez, E.A. de Almeida, J. Ballesteros, T. Garcia-Barrera, J.L. Gomez-Ariza and J. Lopez-Barea, Doñana National Park survey using crayfish (*Procambarus clarkii*) as bioindicator: esterase inhibition and pollutant levels, *Toxicol. Lett*., 2007, **168**, 260-268.

13 M.A. Garcia-Sevillano, M. Gonzalez-Fernandez, R. Jara-Biedma, T. Garcia-Barrera, J. Lopez-Barea, C. Pueyo and J.L. Gomez-Ariza, Biological response of free-living mouse *Mus spretus* from Doñana National Park under environmental stress based on assessment of metal-binding biomolecules by SEC-ICP-MS, *Anal. Bioanal. Chem*., 2012, **404**, 1967-1981.

14 M.A. García-Sevillano, T. García-Barrera, F. Navarro, N. Abril, C. Pueyo, J. López-Barea and J.L. Gómez-Ariza, Use of metallomics and metabolomics to assess metal pollution in Doñana National Park (SW Spain), *Environ. Sci. Technol*., 2014, **48**, 7747-7755.

15 D.H. Petering, M. Goodrich, W.Hodgman, S. Krezoski, D. Weber, C.F. Shaw, R. Spieler and L. Zettergren, in *Biomarkers of Environmental Contamination*, ed. J.F. McCarthy, L.R. Shugart, Lewis Publishers, USA, 1990, Metal-binding proteins and peptides for the detection of heavy metals in aquatic organisms, pp. 239-254.

16 R.T. Lavradas, R.C. Chávez Rocha, T. Dillenburg Saint' Pierre, J.M. Godoy and R.A. Hauser-Davis, Investigation of thermostable metalloproteins in *Perna perna* mussels from differentially contaminated areas in Southeastern Brazil by bioanalytical techniques, *J. Trace Elem. Med. Biol.*, 2016, **34**, 70-78.

17 A. Caron, M. Rosabal, O. Drevet, P. Couture and P.G.C. Campbell, Binding of trace elements (Ag, Cd, Co, Cu, Ni, and Tl) to cytosolic biomolecules in livers of juvenile yellow perch (*Perca flavescens*) collected from lakes representing metal contamination gradients, *Environ. Toxicol. Chem.*, 2018, **37**, 576-586.

18 J. Szpunar and R. Łobiński, Multidimensional approaches in biochemical speciation analysis, *Anal. Bioanal. Chem*., 2002, **373**, 404-411.

19 J.A. Caruso and M. Montes-Bayón, Elemental speciation studies - new directions for trace metal analysis, *Ecotoxicol. Environ. Saf.*, 2003, **56**, 148-163.

20 J.L. Gomez-Ariza, T. Garcia-Barrera, F. Lorenzo, V. Bernal, M.J. Villegas and V. Oliveira, Use of mass spectrometry techniques for the characterization of metal bound to proteins (metallomics) in biological systems, *Anal. Chim. Acta*, 2004, **524**, 15-22.

21 J. Bettmer, M. Montes-Bayón, J. Ruiz Encinar, M.L. Fernández Sánchez, M.R.F. de la Campa and A. Sanz Medel, The emerging role of ICP-MS in proteomic analysis, *J. Proteomics*, 2009, **72,** 89-1005.

22 M. González-Fernández, T. García-Barrera, A. Arias-Borrego, J. Jurado, C. Pueyo, J. López-Barea and J.L. Gómez-Ariza, Metallomics integrated with proteomics in deciphering metal-related environmental issues, *Biochimi*e, 2009, **91**, 1311-1317.

23 S. Mounicou, J. Szpunar and R. Łobiński, Metallomics, the concept and methodology, *Chem. Soc. Rev.*, 2009, **38**, 1119-1138.

24 M. Montes-Bayón, M. Sharar, M. Corte-Rodriguez, Trends on (elemental and molecular) mass spectrometry based strategies for speciation and metallomics, *Trends Analyt. Chem*., 2018, **104**, 4-10.

25 M. Montes-Bayón, K. De Nicola and J.A. Caruso, Liquid chromatography-inductively coupled plasma mass spectrometry. *J. Chromatogr. A*, 2003, **1000**, 457-476.

26 J.L. Gomez-Ariza, T. Garcia-Barrera, F.Lorenzo and A. Arias, Analytical characterization of bioactive metal species in the cellular domain (metallomics) to simplify environmental and biological proteomics, *Intern. J. Environ. Anal. Chem*. 2005, **85**, 255-266.

27 N. Krasnići, Z. Dragun, M. Erk and B. Raspor, Distribution of selected essential (Co, Cu, Fe, Mn, Mo, Se, Zn) and nonessential (Cd, Pb) trace elements among protein fractions from hepatic cytosol of European chub (*Squalius cephalus* L.), *Environ. Sci. Pollut. Res*., 2013, **20**, 2340-2351.

28 N. Krasnići, Z. Dragun, M. Erk and B. Raspor, Distribution of Co, Cu, Fe, Mn, Se, Zn, and Cd among cytosolic proteins of differenet molecular masses in gills of European chub (*Squalius cephalus* L.), *Environ. Sci. Pollut. Res.*, 2014, **21**, 13512-13521.

29 N. Krasnići, Z. Dragun, M. Erk, S. Ramani, M. Jordanova, K. Rebok and V. Kostov, Size-exclusion HPLC analysis of trace element distributions in hepatic and gill cytosol of Vardar chub (*Squalius vardarensis* Karaman) from mining impacted rivers in north-eastern Macedonia. *Sci. Total Environ*., 2018, **613-614**, 1055-1068.

30 Z. Dragun, N. Krasnići, N. Kolar, V. Filipović Marijić, D. Ivanković and M. Erk, Cytosolic distributions of highly toxic metals Cd and Tl and several essential elements in the liver of brown trout (*Salmo trutta* L.) analyzed by size exclusion chromatography and inductively coupled plasma mass spectrometry, *Chemosphere*, 2018, **207**, 162-173.

31 V. Nischwitz, B. Michalke and A. Kettrup, Identification and quantification of metallothionein isoforms and superoxide dismutase in spiked liver extracts using HPLC-ESI-MS offline coupling and HPLC-ICP-MS online coupling, *Anal. Bioanal. Chem.*, 2003, **375**, 145-156.

32 P. Pereira, H. De Pablo, C. Vale and M. Pachecho, Combined use of environmental data and biomarkers in fish (*Liza aurata*) inhabiting a eutrophic and metal-contaminated coastal system - gills reflect environmental contamination, *Mar. Environ. Res.*, 2010, **69**, 53-62.

33 H.J. Rätz and J. Lloret, Variation in fish condition between Atlantic cod (*Gadus morhua*) stocks, the effect on their productivity and management implications, *Fish. Res.*, 2003, **60**, 369-380.

34 M. Jordanova, K. Rebok, Z. Dragun, S. Ramani, L. Ivanova, V. Kostov, D. Valić, N. Krasnići, V. Filipović Marijić and D. Kapetanović, Histopathology investigation on the Vardar chub (*Squalius vardarensis*) populations captured from the rivers impacted by mining activities, *Ecotoxicol. Environ. Saf.*, 2016, **129**, 35-42.

35 K. Van Campenhout, H. Goenaga Infante, P.T. Hoff, L. Moens, G. Goemans, C. Belpaire, F. Adams, R. Blust and L. Bervoets, Cytosolic distribution of Cd, Cu and Zn, and metallothionein levels in relation to physiological changes in gibel carp (*Carassius auratus gibelio*) from metal-impacted habitats, *Ecotoxicol. Environ. Saf.*, 2010, **73**, 296-305.

36 E. Bonneris, A. Giguère, O. Perceval, T. Buronfosse, S. Masson, L. Hare and P.G.C. Campbell, Sub-cellular partitioning of metals (Cd, Cu, Zn) in the gills of a freshwater bivalve, *Pyganodon grandis*: role of calcium concretions in metal sequestration, *Aquat. Toxicol.*, 2005, **71,** 319-334.

37 M. Erk, D. Ivanković, B. Raspor and J. Pavičić, Evaluation of different purification procedures for the electrochemical quantification of mussel metallothioneins, *Talanta*, 2002, **57**, 1211-1218.

38 A. Rodríguez-Cea, A.R. Linde Arias, M.R. Fernández de la Campa, J. Costa Moreira and A. Sanz-Medel, Metal speciation of metallothionein in white sea

catfish, *Netuma barba*, and pearl cichlid, *Geophagus brasiliensis*, by orthogonal liquid chromatography coupled to ICP-MS detection, *Talanta*, 2006, **69**, 963-969.

39 Ž. Fiket, V. Roje, N. Mikac and G. Kniewald, Determination of arsenic and other trace elements in bottled waters by high resolution inductively coupled plasma mass spectrometry, *Croat. Chem. Acta*, 2007, **80**, 91-100.

40 M. Kobayashi and S. Shimizu, Cobaltproteins, *Eur. J. Biochem.*, 1999, **261**, 1-9.

41 G. Schwarz, R.R. Mendel and M.W. Ribbe, Molybdenum cofactors, enzymes and pathways, *Nature*, 2009, **460**, 839-847.

42 L.O. Klotz, K.D. Kröncke, D.P. Buchczyk and H. Sies, Role of copper, zinc, selenium and tellurium in the cellular defense against oxidative and nitrosative stress, *J. Nutr*., 2003, **133**, 1448S-1451S.

43 Y. Yamashita and M. Yamashita, Identification of a novel selenium containing compound, selenoneine, as the predominant chemical form of organic selenium in the blood of a bluefin tuna, *J. Biol. Chem*., 2010, **285**, 18134-18138.

44 Y. Yamashita, T. Yabu, K. Touhata and M. Yamashita, Purification and characterization of glutathione peroxidase 1 in the red muscle of Pacific bluefin tuna *Thunnus orientalis*, *Fish. Sci*., 2012, **78**, 407-413.

45 A. Giguère, P.G.C. Campbell, L. Hare and P. Couture, Sub-cellular partitioning of cadmium, copper, nickel and zinc in indigenous yellow perch (*Perca flavescens*) sampled along a polymetallic gradient, *Aquat. Toxico*l., 2006, **77**, 178-189.

46 K. Van Campenhout, H. Goenaga Infante, G. Goemans, C. Belpaire, F. Adams, R. Blust and L. Bervoets, A field survey of metal binding to metallothionein and other cytosolic ligands in liver of eels using an on-line isotope dilution method in combination with size exclusion (SE) high pressure liquid chromatography (HPLC) coupled to inductively coupled plasma time-of-flight mass spectrometry (ICP-TOF-MS), *Sci. Total. Environ*., 2008, **394**, 379-389.

47 M.B. de la Calle Guntińas, G. Bordin and A.R. Rodriguez, Identification, characterization and determination of metal binding proteins by liquid chromatography, A review, *Anal. Bioanal. Chem.*, 2002,**374**, 369-378.

48 C. Andreini, L. Banci, I. Bertini and A. Rosato, Counting the zinc-proteins encoded in the human genome, *J. Proteome Res.*, 2006, **5**, 196-201.

49 C. Wolf, N. Wenda, A. Richter and A. Kyriakopoulos, Alteration of biological samples in speciation analysis od metalloproteins, *Anal. Bioanal. Chem*., 2007, **389**, 799-810.

50 J.V. Neves, J.M. Wilson and P.N.S. Rodrigues, Transferrin and ferritin response to bacterial infection: the role of the liver and brain in fish, *Dev. Comp. Immunol.*, 2009, **33**, 848-857.

51 Z.Yu, G. Friso, J.J. Miranda, M.J. Patel, T. Lo-Tseng, E.G. Moore and A.L. Burlingame, Structural characterization of human hemoglobin crosslinked by bis(3,5-dibromosalicyl) fumarate using mass spectrometric techniques, *Protein Sci.*, 1997, **6**, 2568-2577.

52 A.Fago, E. Forest and R.E. Weber, Hemoglobin and subunit multiplicity in the rainbow trout (*Oncorhynchus mykiss*) hemoglobin system, *Fish Physiol. Biochem.*, 2002, **24**, 335-342.

53 A. Jacobs, An intracellular transit iron pool, *CIBA Fdn. Symp.*, 1977, **51**, 91-106.

54 M. Fontecave and J.L. Pierre, Iron metabolism: the low-molecular-mass iron pool, *Biol. Metals*, 1991, **4**, 133-135.

55 M. Montes-Bayoń, A.B.S. Cabezuelo, E.B. Gonzalez, J.I.G. Alonso and A. Sanz-Medel, Capabilities of fast protein liquid chromatography coupled to a double focusing inductively coupled plasma mass spectrometer for trace metal speciation in human serum, *J. Anal. At. Spectrom.*, 1999, **14**, 947-951.

56 C.S. Muniz, J.M.M. Gayon, J.I.G. Alonso and A. Sanz-Medel, Speciation of essential elements in human serum using anion-exchange chromatography coupled to post-column isotope dilution analysis with double focusing ICP-MS, *J. Anal. At. Spectrom.*, 2001, **16**, 587-592.

57 O. Mestek, J. Komínková, R. Koplík, M. Kodíček and T. Zima, Quantification of trace elements in human serum fractions by liquid chromatography and inductively coupled plasma mass spectrometry, *Appl. Organomet. Chem.*, 2007, **21,** 5-14.

58 M.F. Perutz, M.G. Rossman, A.F. Cullins, H. Muirhead, G.Will and A.C.T. North, The structure of haemoglobin. A three dimensional Fourier synthesis at 5.5 Å resolution, obtained by X-ray analysis, *Nature*, 1960, **185**, 416-422.

59 S. Fernández-Menéndez, M.L. Fernández-Sánchez, R.R. Alves Peixoto, B. Fernández-Colomer and A. Sanz-Medel, In vivo study of the effect of lactoferrin on iron metabolism and bioavailability from different iron chemical species for formula milkfortification, *Electrophoresis*, 2018, **39**, 1702-1713.

60 P. Kleinert, M. Schmid, K. Zurbriggen, O. Speer, M. Schmugge, B. Roschitzki, S.S. Durka, U. Leopold, T. Kuster, C.W. Heizmann, H. Frischknecht and H. Troxler,

963 Mass spectrometry: a tool for enhanced detection of hemoglobin variants, *Clin.*
964 *Chem*., 2008, **54**, 69-76.
965 61 J. Szpunar and R. Łobiński, Species-selective analysis for metal biomacromolecular
966 complexes using hyphenated techniques, *Pure Appl. Chem*., 1999, **71**, 899-918.
967 62 A. Rodríguez-Cea, M.R. Fernández de la Campa, E. Blanco González, B. Andón
968 Fernández and A. Sanz-Medel, Metal speciation analysis in eel (*Anguilla anguilla*)
969 metallothioneins by anionic exchange-FPLC-isotope dilution-ICP-MS, *J. Anal. At.*
970 *Spectrom.*, 2003, **18**, 1357-1364.
971 63 H. Goenaga Infante, K. Van Campenhout, R. Blust and F.C. Adams, Inductively
972 coupled plasma time-of-flight mass spectrometry coupled to high-performance
973 liquid chromatography for multi-elemental speciation analysis of metalloproteins in
974 carp cytosols, J. *Anal. At. Spectrom*., 2002, **17**, 79-87.
975 64 S. Duquesne and A. Richard, Isolation and characterization of naturally and
976 artificially induced hepatic metallothioneins from two species of flatfish: *Limanda*
977 *limanda* and *Microstomus kitt*, *Mar. Biol*., 1994, **119**, 461-470.
978 65 M. Lacorn, A. Lahrssen, N. Rotzoll, T.J. Simat and H. Steinhart, Quantification of
979 metallothionein isoforms in fish liver and its implications for biomonitoring,
980 *Environ. Toxicol. Chem*., 2001, **20**, 140-145.
981 66 S. Li, Z. Yang, J. Cao, B. Qiu and H. Li, Determination of metallothionein isoforms
982 in fish by cadmium saturation combined with anion exchange HPLC-ICP-MS,
983 *Chromatographia*, 2018, **81**, 881-889.
984 67 A.Z. Mason, N.Perico, R. Moeller, K. Thrippleton, T. Potter, D. Lloyd and V.E.
985 Kagan, Metal donation and apo-metalloenzyme activation by stable isotopically
986 labeled metallothionein, *Mar. Environ. Res.*, 2004, **58**, 371-375.
987 68 R. Dallinger, B. Berger, P.Hunziker and J.H.R. Kagi, Metallothionein in snail Cd
988 and Cu metabolism, *Nature*, 1997, **388**, 237-238.
989 69 M. Vašák and D.W. Hasler, Metallothioneins: new functional and structural insights,
990 *Curr. Opin. Chem. Biol*., 2000, **4**, 177-183.
991 70 F. Geret and R.P. Cosson, Induction of specific isoforms of metallothionein in
992 mussel tissues after exposure to cadmium or mercury, *Arch. Environ. Contam.*
993 *Toxicol*., 2002, **42**, 36-42.
994 71 M. Rosabal, S. Mounicou, L.Hare and P.G.C. Campbell, Metal (Ag, Cd, Cu, Ni, Tl,
995 Zn) binding to cytosolic biomolecules in field-collected larvae of the insect
996 *Chaoborus*, *Environ. Sci. Technol*., 2016, **50**, 3247-3255.

997 72 H. Chassaigne and R. Łobiński, Polymorphism and identification of metallothionein
998 isoforms by reversed-phase HPLC with on-line ion-spray mass spectometric
999 detection, *Anal.Chem.*, 1998, **70**, 2536-2543.
1000 73 E. Carpenè and M. Vašák, Hepatic metallothioneins from goldfish (*Carassius*
1001 *auratus* L.), *Comp. Biochem. Physiol. Part B*, 1989, **92**, 463-468.
1002 74 K.T. Suzuki, in *Metallothioneins: Synthesis, Structure and Properties of*
1003 *Metallothioneins, Phytochelatins, and Metal-Thiolate Complexes*, ed. M.J. Stillman,
1004 C.F. Shaw, K.T. Suzuki, VCH Publishers, New York, 1992, Preparation od
1005 metallothioneins, pp. 14-30.
1006 75 H.F. Maltez, M.V. Tagle, M.R. Fernández de la Campa and A. Sanz-Medel, Metal-
1007 metallothioneins like proteins investigation by heteroatom-tagged proteomics in
1008 two different snails as possible sentinel organisms of metal contamination in
1009 freshwater ecosystmes, *Anal. Chim. Acta*, 2009, **650**, 234-240.
1010 76 D. Ivanković, J. Pavičić, S. Kozar and B. Raspor, Multiple forms of metallothionein
1011 from the digestive gland of naturally occurring and cadmium-exposed mussels,
1012 *Mytilus galloprovincialis*, *Helgol. Mar. Res.*, 2002, **56**, 95-101.
1013 77 F. Noël-Lambot, C. Gerday and A. Disteche, Distribution of Cd, Zn and Cu in liver
1014 and gills of the eel *Anguilla anguilla* with special reference to metallothioneins.
1015 *Comp. Biochem. Physiol. Part C*, 1978, **61**, 177-187.
1016 78 K. Van Campenhout, H. Goenaga Infante, F. Adams and R. Blust, Induction and
1017 binding of Cd, Cu and Zn to metallothionein in carp (*Cyprinus carpio*) using
1018 HPLC-ICP-TOFMS, *Toxicol. Sci*., 2004, **80**, 276-287.
1019 79 S.D. Reid, in *Fish Physiology: Homeostasis and Toxicology od Essential Metals*,
1020 vol. 31A, ed. C. Wood, A. Farrell, C. Brauner, Academic Press, 2011, Molybdenum
1021 and chromium, pp. 375-415.
1022

Figure captions

Figure 1. AEC-HPLC analyses of hepatic and gill MMM Fe-peaks, which were obtained by SEC_{200}-HPLC separations (t_e 26-29 min). The obtained AEC distribution profiles of Fe, Zn, and Cu in three samples of liver (a, b, c) and Fe and Zn in three samples of gills (d, e, f) are presented in this figure as nanograms of metals eluted every half minute. The major Fe-containing peaks (liver: t_e 11-14 min; gills: t_e 10-13 min), which are marked by dotted lines, were further used for MS analyses.

Figure 2. Mass spectra obtained by MALDI-TOF-MS for MMM Fe-binding biomolecules, which were separated by SEC_{200}-HPLC followed by AEC-HPLC from hepatic cytosols (a) and gill cytosols (b) of Vardar chub (the spectra are presented for fish No. 2).

Figure 3. AEC-HPLC analyses of hepatic and gill LMM Cd-peaks (presumably containing MTs), which were obtained by SEC_{200}-HPLC separations (t_e 30-34 min). The obtained AEC distribution profiles of Cd, Cu, and Zn in two samples of liver (a, b) and Cd and Zn in two samples of gills (c, d) are presented in this figure as nanograms of metals eluted every half minute. AEC-HPLC chromatographs recorded by UV detection at 254 nm are presented for standard proteins MT-I (e) and MT-II (f).

Figure 4. AEC-HPLC analyses of hepatic LMM Cd-peaks, which were obtained by SEC_{75}-HPLC separations of heat-treated hepatic cytosols (t_e 21-25 min). The obtained AEC distribution profiles of Cd, Zn, and Cu in two samples of heat-treated hepatic cytosols (a, b) are presented in this figure as nanograms of metals eluted every half minute. The L-Cd1 peaks (t_e 9.5-12 min) and L-Cd2 peaks (t_e 12.5-15 min), which are marked by dotted lines, were further used for MS analyses.

Figure 5. Mass spectra obtained by MALDI-TOF-MS for the following samples: metallothionein standards MT-I (a) and MT-II (b); two heat stable LMM hepatic peaks, L-Cd1 (c) and L-Cd2 (d), which were separated by SEC_{75}-HPLC and AEC-HPLC from heat-treated hepatic cytosols of Vardar chub (the spectra are presented for fish No. 5); heat stable LMM Cd-containing peak, G-Cd (e), which was separated by SEC_{75}-HPLC from heat-treated gill cytosol of Vardar chub (the spectra are presented for fish No. 2).

Figure 6. Mass spectra obtained by MALDI-TOF-MS for heat-stable VLMM Mo-binding biomolecules, which were separated by SEC_{75}-HPLC from heat-treated hepatic (a) and gill (b) cytosols of Vardar chub (the spectra are presented for fish No. 2).