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Distribution of Co, Cu, Fe, Mn, Se, Zn and Cd among cytosolic proteins of different molecular masses in gills of European chub (*Squalius cephalus* L.)

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Abstract

The distribution of essential elements Co, Cu, Fe, Mn, Se, and Zn, and nonessential element Cd among cytosolic proteins of different molecular masses in the gills of European chub (*Squalius cephalus*) sampled in the moderately contaminated Sutla River in September of 2009, was studied after the protein separation by size exclusion high performance liquid chromatography (SEC-HPLC), and the metal determination in the obtained fractions by high resolution inductively coupled plasma mass spectrometry (HR ICP-MS). The aims of the study were to characterize the distribution profiles of metals within different protein categories in gills in the conditions of low metal exposure in the river water, and to compare them with the previously published hepatic profiles. The distribution profiles of analyzed metals were mainly characterized with several peaks. However, some observations could be emphasized: both Cu and Cd were eluted near metallothionein elution time; elution time of one of Co peaks could be associated with Co-containing compound cobalamin; increasing cytosolic Fe concentrations resulted in possible Fe binding to storage protein ferritin; both Mn and Zn had poorly resolved peaks covering wide ranges of molecular masses and indicating their binding to various proteins; both Zn and Se increased in protein fractions of molecular masses <5 kDa following their concentration increase in the gill cytosol; expected clear metallothionein peak was not observed for Zn. Comparison of gill profiles with previously published hepatic profiles revealed similar and in case of some elements (e.g. Co, Fe, Mn and Se) almost identical distributions in both organs regarding elution times. Contrary, heights of obtained peaks were different, indicating possible metal binding to the same proteins in the gills and liver, but in different proportions. The results obtained in this study can be used as a basis for comparison in monitoring studies, for identification of changes that would occur after exposure of chub to increased metal concentrations.

Keywords: cytosolic proteins, European chub, gills, HR ICP-MS, metals, SEC-HPLC

1. Introduction

In aquatic environment, the degree of metal pollution is often evaluated by establishing the effects of increased metal exposure on aquatic organisms, such as fish, specifically by measuring metal concentrations in the liver and gills (Kamaruzzaman et al. 2010). Gills have a large surface area that is continuously in contact with the external medium, and thus present the main uptake route of contaminants from aqueous phase (Playle 1998). In addition, through blood circulation gills can also accumulate chemicals that were taken up by other exposure routes (Levine and Oris 1999). The study of metal effects on gills is important because gills play a key role in fish physiology, for example in respiration, osmotic and ionic regulation, and acid-base balance (Ahmad et al. 2008). Metal ions can interfere with these gill functions by causing cellular damage to gill cells (Evans 1987; De Boeck et al. 2001). Although some metals, such as Cu, Co, Fe, Mn, Se and Zn, are essential micronutrients which are required for numerous physiological processes, they can also be toxic. The ability to induce toxic effects is not only a feature of metals with no known functions in the organism (e.g. Cd), but also of the essential elements, when they are present in organisms in concentrations above their threshold. They can all induce toxic effects by different modes of action, for example some of them can generate reactive oxygen species (ROS); as a result of an effort to maintain ROS levels within physiological limits, the activity of biotransformation and antioxidant enzymes, such as glutathione S-transferase (GST), catalase (CAT), and superoxide dismutase (SOD), increases (Formigari et al. 2007). Therefore, to assess biological effects of metals, it is insufficient to only measure metal concentrations in the gills and other tissues (Lehtonen and Schiedek 2006), but it is necessary to detect and characterize protein molecules which bind metals, as well. Next to the identification of metalloproteins, for the understanding of protein processes it is also necessary to identify nonproteinaceous molecules of a relatively small size, which deliver metals to metalloproteins (Outten and O'Halloran 2001). Separation of proteins by size exclusion high performance liquid chromatography (SEC-HPLC) combined with metal detection techniques such as inductively coupled plasma mass spectrometry (ICP-MS) has been previously described as a valuable tool for accomplishing such a goal (Prange and Schaumlöffel 2002; Krasnići et al. 2013; Strižak et al. 2014).

The focus of this study was the investigation of distribution of essential elements Cu, Co, Fe, Mn, Se, and Zn, and nonessential metal Cd among SEC-HPLC separated cytosolic proteins of different molecular masses from the gills of European chub (*Squalius cephalus*) sampled in the moderately contaminated Sutla River (Dragun et al. 2011). Similar study was previously performed on the liver of the same chub specimens (Krasnići et al. 2013), but to our knowledge there is no such information available for gills either of chub or other freshwater fish. Therefore, the main aim of the current study was to define the basal metal distributions of seven selected elements among different protein categories, i.e. the distributions characteristic for the conditions of low metal exposure in the water. An additional aim was to compare metal distribution profiles in gills with previously published profiles in liver (Krasnići et al. 2013), and to define the similarities and differences of cytosolic metal allocation within these two functionally different organs.

2. Materials and methods

2.1. Fish selected for analyses

For this study, we have selected seven specimens out of 75 European chub (*S. cephalus*) caught in the Sutla River in September of 2009. The fish were caught by electrofishing and then dissected, as previously described in detail by Dragun et al. (2011; 2012; 2013) and Krasnići et al. (2013). They were 20.1 to 29.7 cm long, with masses ranging from 94.5 to 260.6 g, and age from 2 to 4 years (Table 1). Sex composition of chub specimens selected for analyses was 86% females (Table 1). The selection of the samples for analyses was based on two criteria: the sample availability and cytosolic metal concentrations in the chub gills. For smaller chub specimens, the gills were not large enough to obtain sufficient volume of cytosol for HPLC separation. Among the remaining samples, the basic criteria for selection were the cytosolic metal concentrations, which were mainly rather low in the gills, for example much lower than in the liver. Therefore, we have chosen for this study gill cytosols with the highest metal concentrations, to ensure the best possible resolution of obtained peaks. Consequently, number of fish selected for this study (n=7) was smaller compared to study performed on liver (n=28, Krasnići et al. 2013).

2.2. Isolation of cytosolic fraction from European chub gills

The isolation of cytosol from chub gill tissue was previously described (Dragun et al. 2012 and 2013; Krasnići et al. 2013). In brief, gill tissues were homogenized by Potter–Elvehjem homogenizer (Glas-Col), using 20 mM Tris-HCl/Base (Sigma, pH 8.6 at 4 °C) supplemented with reducing agent 2 mM dithiotreitol (Sigma) as homogenization buffer, and then centrifuged subsequently two times in the Avanti J-E centrifuge (Beckman Coulter) at 50,000×g for 2 h at 4°C. Supernatant (S50) obtained after second centrifugation, which represents water soluble cytosolic tissue fraction containing lysosomes and microsomes (Bonneris et al. 2005), was separated for further analyses.

2.3. SEC-HPLC fractionation of chub gill cytosol

For the fractionation of chub gill cytosol we have used size exclusion column Tricorn™Superdex™ 200 10/300 GL (GE Healthcare Biosciences) and Perkin-Elmer HPLC system (series 200), as previously described in detail by Krasnići et al. (2013). Column exclusion limit was defined as molecular mass (MM) of 1,300 kDa for globular proteins, whereas the optimal separation range was given as 10-600 kDa. The void volume was determined by use of blue dextran (defined MM: 2,000 kDa), which was eluted at 16.31 minute. For column calibration, six protein standards were used (thyroglobulin, apoferritin, β-amylase, alcohol dehydrogenase, bovine serum albumin, and carbonic anhydrase, Sigma), dissolved in 20 mM Tris–HCl/Base (Sigma, pH 8.1 at 22 °C), which was also used as mobile phase at a flow rate of 0.5 mL min^{-1} (isocratic mode). Calibration straight line was created based on known MM of protein standards and their respective elution times (t_e; Table 2; presented in detail by Krasnići et al. 2013). Metallothionein (MT) standard Zn-MT95 (Ikzus) was also run through the column and narrow and well-defined double peak was obtained with maxima at t_e 29.85 and 30.90 minutes. For MTs, more intense peak at longer retention time is characteristic for the monomers, whereas the peak at shorter retention time is characteristic for dimers or other complexes (Wang et al. 2001). The injection volume for gill cytosol samples was 100 μL (except for fish No. 1: 50 μL), and for each sample, four consecutive

chromatographic runs were performed (total sample volume: 400 μL; fish No. 1: 200 μL). The fractions were collected at 1 minute intervals in the plastic tubes using a fraction collector (FC 203B, Gilson). The resolution of these fractions with respect to molecular mass is given by the equation of the calibration straight line ($y=0.1172x + 7.7195$; y = MM; $x = t_e$; Krasnići et al. 2013).

2.4. Determination of trace element concentrations

Trace element concentrations were determined in ten times diluted gill cytosols and in SEC-HPLC-separated cytosolic fractions, both acidified by HNO_3 (Suprapur, Merck; final acid concentrations: 0.65% and 0.16%, respectively). Indium (Fluka) was added to all samples as an internal standard (1 μg L^{-1}). The measurements were performed on high resolution ICP-MS (Element 2, Thermo Finnigan), using an autosampler (ASX 510, Cetac Technologies) and sample introduction kit consisting of SeaSpray nebulizer and cyclonic spray chamber Twister. Due to low cytosolic metal concentrations in the gills, the analyses were performed for only six essential (Co, Cu, Fe, Mn, Se, and Zn) and one nonessential metal (Cd) and did not include Mo and Pb, which were previously analyzed in the liver. Measurements of ^{82}Se 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 calibration was performed using standards prepared in 2% HNO_3 (Suprapur, Merck) by appropriate dilutions of 100 mg L^{-1} multielement stock standard solution (Analytika). For quality control, QC sample for trace elements was used (UNEP GEMS/Water PE Study No. 7), and generally good agreement was observed between our data and the certified values. Limits of detection were as follows (in μg L^{-1}): Cd, 0.005; Co, 0.002, Cu, 0.037; Fe, 0.084; Mn, 0.002; Se, 0.138; and Zn, 2.40 (Krasnići et al. 2013).

2.5. Determination of total cytosolic protein concentrations

The concentrations of total proteins in the gill cytosol were measured according to Lowry et al. (1951). The Bio-Rad DC Protein Assay was applied according to manufacturer's instructions. The measurements were performed on the spectrophotometer/fluorometer (Tecan, Infinite M200) at 750 nm wavelength. Calibration curve was constructed with five different concentrations (0.25-2.0 mg mL^{-1}) of bovine serum albumin (Serva, Germany) dissolved in the homogenization buffer. Total protein concentrations are presented in Table 1, separately for each of the analyzed samples of chub gill cytosol.

2.6. Data processing and statistical analyses

Chromatographic results were processed using Totalchrom Version 6.3.1 software (Perkin-Elmer). Graphs were created using the statistical program SigmaPlot 11.0 for Windows.

3. Results and discussion

In living organisms, most of metal ions are bound to specific proteins or enzymes, and could act as active or structural centers of proteins (Garcia et al. 2006). Therefore, it is essential to expand the knowledge on specific proteins to which trace metals are associated in different fish tissues, such as gills and liver, to be able to understand their essential functions, their potential role in detoxification processes, as well as possible

undesirable impacts on fish. The analysis of distribution of trace elements in the gill cytosol of European chub (*S. cephalus*) presents a supplementation of the similar study recently performed on the hepatic cytosol of the same fish species (Krasnići et al. 2013). The distributions of selected metals among four protein categories, as previously defined by Krasnići et al. (2013) (high molecular mass proteins, HMM: >100 kDa; medium molecular mass proteins, MMM: 30-100 kDa; low molecular mass proteins, LMM: 10-30 kDa; and very low molecular mass proteins, VLMM: <10 kDa), which were established in this study, represent the first step towards identification of specific metal binding proteins in the chub gills. Protein separation of better quality could not be obtained in this phase of the study due to the limitation imposed by the applied column (Superdex™ 200 10/300 GL), as seen from a chromatogram presented in the Fig. 1. However, when comparison was made with hepatic chromatogram (Krasnići et al. 2013), it can be seen that sharper and better distinguished protein peaks were obtained in the gills (Fig. 1), possibly due to approximately 25% lower total cytosolic protein concentrations in the gills of all sampled chub (n=75; median: 14.5 mg mL^{-1}; range: 6.5-17.8 mg mL^{-1}) compared to liver (n=75; median: 19.1 mg mL^{-1}; range: 12.0-24.7 mg mL^{-1}) (Dragun et al. 2013).

In addition, the variations in metal allocation in different fish organs were established by comparison between gill profiles presented in this paper (Fig. 2-3) and previously published hepatic profiles (Krasnići et al. 2013). In general, the cytosolic concentrations of majority of metals in gills of all sampled chub (e.g., Cd: 0.68±0.36 ng mL^{-1}, Cu: 42.6±10.4 ng mL^{-1}, Dragun et al. 2013; Pb 5.3±9.3 ng mL^{-1}, Dragun et al. 2012) were much lower compared to the liver (e.g., Cd: 19.4±11.6 ng mL^{-1}, Cu: 1.5±0.7 μg mL^{-1}, Dragun et al. 2013; Pb 6.6±16.1 ng mL^{-1}, Dragun et al. 2012), which could be expected considering that gills can transfer absorbed metals by blood to the liver as main detoxification organ (Souza et al. 2013). However, low metal concentrations in the gills were in many cases the cause of rather undefined metal distribution profiles in this tissue. Therefore, only several representative distribution profiles, with clear and distinguishable peaks, are presented in Fig. 2 and 3, whereas the profiles for all seven chub specimens are presented as supplementary information (Fig. SI-1 – SI-7). Since the chub specimens analyzed in this study originated from moderately contaminated Sutla River (dissolved metal concentrations in the river water: Cd, 0.01-0.31 μg L^{-1}; Co, 0.06-0.42 μg L^{-1}; Cu, 0.17-3.74 μg L^{-1}; Fe, 3.1-80.5 μg L^{-1}; Mn, 0.4-261.1 μg L^{-1}; Zn, <5.0 μg L^{-1}; Dragun et al. 2011), the profiles presented in this paper could be regarded as characteristic for fish non-exposed to metals and can serve as a basis for comparison in the future studies of metal distribution in the gills with higher cytosolic metal concentrations.

3.1. Distribution profiles of essential elements

3.1.1. Cobalt

Cobalt was found in protein fractions covering wide range of MMs, with three distinguished peaks (Fig. 2a). The first one occurred within HMM protein category, with maximum corresponding to protein MM of 80 kDa (Table 3). The other two smaller peaks occurred within VLMM protein category, with maxima corresponding to protein MMs of 4 and 2 kDa, respectively (Table 3). Cobalt profiles obtained for six chub with cytosolic Co concentrations in the gills ranging from 1.02 to 1.60 ng mL^{-1} were comparable. Only in one fish the first VLMM peak (4 kDa) was approximately 10 times higher, which could not be explained by higher cytosolic Co

concentration in the liver of that chub (cytosolic Co concentration: 1.06 ng mL^{-1}; Fig. SI-1). The distribution profile of gill Co was almost identical to previously published hepatic profile (Krasnići et al. 2013), with the exception that Co peaks in the hepatic cytosol were higher, narrower and sharp, which could be explained by 2.5 times higher cytosolic Co concentration in presented hepatic sample (3.96 ng mL^{-1}) compared to the gills (1.60 ng mL^{-1}). The association of Co with VLMM protein fraction in gills can be explained as possible binding to known Co-containing compound, cobalamin (1.3 kDa; Kirschbaum 1981), as already observed in liver (Krasnići et al. 2013). However, in the liver, HMM peak was considerably higher than VLMM peak, indicating almost negligible portion of Co possibly associated to cobalamin. Contrary, in the gills all three peaks were nearly equal. It can be hypothesized that Co starts to bind to HMM proteins when present in cytosol in higher concentrations. As already pointed out for the liver (Krasnići et al. 2013), it would be beneficial to identify these Co-binding proteins in the gills, too, because waterborne metal cations, like Co^{2+}, can interfere with normal function of gills in ionic regulation, acid base balance, gas transfer, and nitrogenous waste excretion (Richards and Playle 1998), for example, by disrupting Ca transport (Hille 1992).

3.1.2. Copper

The distribution profile of Cu in chub gills is presented for two samples with different cytosolic Cu concentrations (Fig. 2b). The sample with lower Cu concentration (42.1 ng mL^{-1}) was characterized with two Cu-peaks (Fig. 2b, Table 3). The first peak occurred within HMM region with maximum associated to protein MM of about 500 kDa (Table 3). The second peak appeared in MMM region and had maximum at t_e of 27 minutes, which could be associated to protein MM of 35 kDa (Table 3). The range of molecular masses covered by this peak also included MTs, with t_e of 30.9 minutes (Table 2). Such profile was obtained for six chub specimens, with cytosolic Cu concentrations ranging from 40.7 to 49.9 ng mL-1 (Fig. SI-2). In the other profile presented in Fig. 2b, originated from the sample with almost twice higher cytosolic Cu concentration (76.0 ng mL^{-1}), both of these peaks were higher. Also, an additional HMM peak which was not observed at lower Cu concentrations was present in that profile. It had maximum at t_e of 22 minutes, and covered the range of protein MMs from 60 to 310 kDa, which could possibly indicate Cu binding to several well-known Cu-containing proteins, such as albumin (66 kDa; Table 2), ceruloplasmin (151 kDa; Boivin et al. 2001), β-amylase (200 kDa; Table 2), or transcuprein (270 kDa; Liu et al. 2007). Similar feature of Cu profiles in gills and liver was Cu elution within MT peak which increased with increasing cytosolic Cu concentration (Fig. 2b; Krasnići et al. 2013). However, this association was more evident in the hepatic cytosol, probably due to significantly higher Cu concentrations (0.45-3.87 μg L^{-1}) compared to gill cytosol. On the other hand, in gill cytosol Cu was found in HMM region indicating possible presence of blood protein ceruloplasmin in the sample, whereas in the hepatic cytosol blood proteins were not recorded (Krasnići et al. 2013), or Cu association with them was negligible compared to its association with MTs.

3.1.3. Iron

The distribution profile of Fe in chub gills is presented, same as for Cu, for two samples with different Fe concentrations, and in both samples it was characterized with two Fe-containing peaks (Fig. 2c). The predominant peak was found in MMM region and covered the range of molecular masses from 10-80 kDa. The

smaller peak was found in HMM region with maximum corresponding to protein MM of 405 kDa, and it became more evident in the sample with higher Fe concentration (Table 3). The MMM peak was observed in all seven analyzed chub specimens, whereas HMM peak was more evident in gills of two specimens with cytosolic Fe concentrations above 5 μg mL^{-1} (Fig. SI-3). The position of Fe peaks in gill Fe profile was identical as in the hepatic profile (Krasnići et al. 2013). The predominant MMM peak was explained as possible binding of Fe to certain Fe-containing proteins (Krasnići et al. 2013), like enzyme catalase (60 kDa) or transport protein myoglobin (17 kDa) (Wolf et al. 2007). The HMM peak, on the other hand, was attributed to possible Fe storage in a form of ferritin (450 kDa; Szpunar and Lobinski 1999) (Krasnići et al. 2013). Assumed binding to ferritin was easier to observe in liver than in the gills due to higher cytosolic Fe concentrations in the liver, indicating more pronounced role of the liver than gills in Fe storage.

3.1.4. Manganese

Manganese distribution profile in chub gills included two poorly resolved peaks (Fig. 2d) covering a wide range of MMs from HMM to LMM region (310-2 kDa; Table 3). The first maximum corresponded to protein MM of about 105 kDa which could involve binding to one of known Mn containing proteins, such as superoxide dismutase or arginase, both having MM about 100 kDa (Wolf et al. 2007), or even transferrin (80 kDa; Martin-Antonio et al. 2009). The range of cytosolic Mn concentrations in chub gills was rather narrow (33.7-69.1 ng mL^{-1}), and therefore almost identical distribution profiles were obtained in the gills of all seven chub (Fig. SI-4). In hepatic cytosol, sharper Mn peaks were observed, with the predominant peak within HMM region (Krasnići et al. 2013). However, Mn concentration in the gill cytosol was 3.5-8 times lower compared to hepatic Mn concentration (250 ng mL^{-1}; Krasnići et al. 2013). Therefore, gill distribution profile actually corresponded only to the wide baseline of the hepatic profile, without clear peaks which were found in liver at higher Mn concentrations.

3.1.5. Selenium

Selenium profile was characterized by three peaks (Fig. 2e). The first two were joined and poorly resolved, and covered wide range of molecular masses from 2 to 310 kDa, with maxima in HMM region (180 kDa) and LMM region (15 kDa), respectively (Table 3). The third peak was detached and sharp. It became evident in the sample with Se concentration higher than 100 ng mL^{-1}, and further increased with increasing cytosolic Se concentration. In two samples with cytosolic Se concentrations lower than 100 ng mL^{-1}, that peak was still rather indistinct and had a maximum at t_e of 37 minutes (Fig. SI-5a). However, in other five samples with cytosolic Se concentrations in the range from 103.4 to 147.2 ng mL^{-1}, the peak height increased 4-7 times and the maximum shifted to lower molecular masses (t_e of 39 minutes; Fig. SI-5b and SI-5c). It corresponded to VLMM proteins in the range of molecular masses below 2 kDa (Table 3). Selenium cytosolic concentrations in the gills (67-147 ng mL^{-1}) and the liver (45-171 ng mL^{-1}; Krasnići et al. 2013) were similar, which enabled objective profile comparison. Both Se profiles in the gills and in the liver had three peaks at the same locations. However, Se increase in the gill cytosol was mainly reflected in the sharp increase of VLMM peak (Fig. 2e), which could be associated to low molecular mass selenocompounds effective in the defense against oxidative stress, for example by acting as a strong free radical scavenger, such as newly identified organic Se species in bluefin tuna (*Thunnus orientalis*),

selenoneine (~0.5 kDa; Yamashita and Yamashita 2010; Yamashita et al. 2012) or selenomethionine (~0.2 kDa; Klotz et al. 2003). Contrary, increase of cytosolic Se concentrations in the liver resulted with sharp increase of LMM peak (Krasnići et al. 2013), which could be associated to several selenoproteins catalytically active in redox processes, such as glutathione peroxidases, iodothyronine deiodinases, thioredoxin reductases (Hauser-Davis et al. 2012), whereas in the gills HMM and LMM peaks increased only slightly.

3.1.6. Zinc

Zinc profile in chub gills was characterized by several peaks covering wide range of protein MMs from HMM to VLMM region (Fig. 2f). The first Zn peak was the widest and had two maxima within HMM protein region, but extended from ~10-900 kDa. The first maximum was within t_e of void volume and could be associated with protein MM of ~500 kDa, whereas the second one could be associated with protein MM of ~100 kDa (Table 3). The second and third peak were better resolved and appeared within VLMM protein category, the second one with maximum at 3 kDa, and the third one below 1 kDa (Table 3). However, the second peak (maximum at 3 kDa) was distinctly observed only in two gill samples with cytosolic Zn concentrations above 21 μg mL^{-1}, whereas it could not be clearly distinguished in five samples with cytosolic Zn in the range from 9.6-15.2 μg mL^{-1} (Fig. SI-6). Similarly to gills, the hepatic Zn profile was also characterized by wide and poorly resolved peaks covering broad range of MMs (Krasnići et al. 2013). This was an indication of Zn binding to large number of proteins both in the liver and in the gills, for example transport protein albumin (66 kDa, Table 2), and enzymes alcohol dehydrogenase (150 kDa, Table 2), Cu-Zn superoxide dismutase (32.5 kDa) or carbonic anhydrase (29 kDa, Table 2) (Sanz-Medel et al. 2003). In the gills, MM of MTs (16.6 and 12.5 kDa, Table 2) was also encompassed by the right tail of the first wide peak, but clear Zn-MT peak was not observed. Contrary, hepatic Zn peak which presumably indicated association to MTs was sharp and could be clearly differentiated from the other Zn peaks (Krasnići et al. 2013). However, it should be emphasized, that contrary to other metals, Zn concentrations in the gills (14.4-21.2 μg mL^{-1}) were two to three times higher than in the liver (7.2 μg mL^{-1}, Krasnići et al. 2013), and possibly Zn binding to various HMM and MMM proteins masked Zn-MT association. On the other hand, the gill Zn profile was distinguished by two high VLMM peaks, which increased following the increase of Zn cytosolic concentration (Fig. 2f), whereas hepatic Zn profile had only few small, barely visible Zn peaks in the VLMM region (Krasnići et al. 2013).

3.2. Distribution profile of nonessential metal cadmium

The most prominent peak of nonessential element Cd in the gills was found in the LMM protein region (Fig. 3), with maximum at elution time of MTs (t_e ~29 minutes; Table 2 and 3), same as previously described for chub hepatic cytosol (Krasnići et al. 2013). It was observed in all seven analyzed chub specimens, but somewhat higher in the gills of two specimens with cytosolic Cd concentrations above 1 ng mL^{-1} (Fig. SI-7b) compared to five specimens with cytosolic Cd concentration in the range from 0.48-0.88 ng mL^{-1} (Fig. SI-7a). Some indication of Cd distribution within HMM and MMM proteins was also observed (Fig. 3), especially in two samples with cytosolic Cd concentrations above 1 ng mL^{-1} (Fig. SI-7b), which could point to association to various proteins, such as, for example, transferrin (801 kDa), which is recently recognized as a major Cd binding

protein in fish blood plasma (De Smet et al. 2001). Next to association with LMM fractions, a small portion of cytosolic Cd was found associated with MMM fractions (35-105 kDa) even in the hepatic cytosol of chub (Krasnići et al. 2013), whereas in the hepatic cytosol of squid (*Todarodes pacificus*) a large portion was bound to species with MM >70 kDa (Tanaka et al. 1983). However, at low cytosolic Cd concentration, as found in the presented gill sample (0.88 ng mL^{-1}; Fig. 3), such association could not be clearly established. Prevailing Cd allocation within MT peak was indicated both by Cd distribution profile in the gills at low cytosolic Cd concentration (Fig. 3, Table 2 and 3) and in the liver at 8-67 times higher cytosolic Cd concentrations (7-59 ng mL^{-1}; Krasnići et al. 2013). It was a confirmation of known high affinity of Cd for MTs, as a mechanism of protection against toxicity (Roesijadi 1992; Park et al. 2001).

4. Conclusions

Based on metal determination by HR ICP-MS after fractionation of chub gill cytosol by SEC-HPLC, distribution profiles of several essential and nonessential trace elements (Co, Cu, Fe, Mn, Se, Zn, and Cd) among cytosolic proteins of different molecular masses were determined. Comparison of gill profiles with previously published hepatic profiles (Krasnići et al. 2013) revealed almost identical distributions of Co, Fe, Mn and Se in both organs. The obtained peaks had similar or identical t_e, but different heights, indicating possible binding to same proteins in the gills and liver, but in different proportions. For example, with increasing cytosolic Fe concentration, a peak appeared at t_e of Fe-storage protein ferritin (t_e 18 minutes; MM ~400 kDa), but much smaller compared to hepatic Fe profile, indicating more important function of liver in Fe storage. Selenium, on the other hand, increased in the VLMM region in the range of MM below 2 kDa following the increase of cytosolic Se in the gills, contrary to hepatic Se which was allocated mainly with LMM or MMM proteins (10-60 kDa). Furthermore, for both Cu and Cd, a peak was obtained near t_e of MTs (27 and 29 minutes, respectively), same as in the hepatic cytosol. However, an additional Cu peak in HMM region (>100 kDa) was obtained in the gills, which was not previously observed in the chub liver. Zinc had wide and poorly resolved peaks in both organs, but unlike hepatic cytosol, expected clear MT peak was not observed in the gills, possibly due to binding of Zn in higher proportion to other proteins of higher molecular masses. Similar to Se, as a result of increase of cytosolic Zn concentration in the gills, Zn increase was observed in the VLMM region at MM <5 kDa, which was not registered in the chub liver. The obtained profiles were mainly characteristic for fish non-exposed to metals, i.e. for low total cytosolic metal concentrations, and thus could be used as a basis for comparison in monitoring studies, as well as for detection of changes in the profiles of the fish exposed to increased metal concentrations.

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445

446 **Figure captions**

447 **Figure 1.** An example of SE-HPLC chromatogram profile of chub gill cytosol (50 μL) after separation on
448 Superdex™ 200 10/300 GL column, with UV detection at two wavelengths: a) λ = 280 nm (characteristic for
449 peptide bond); b) λ = 254 nm (characteristic for Cd-mercaptide bond)

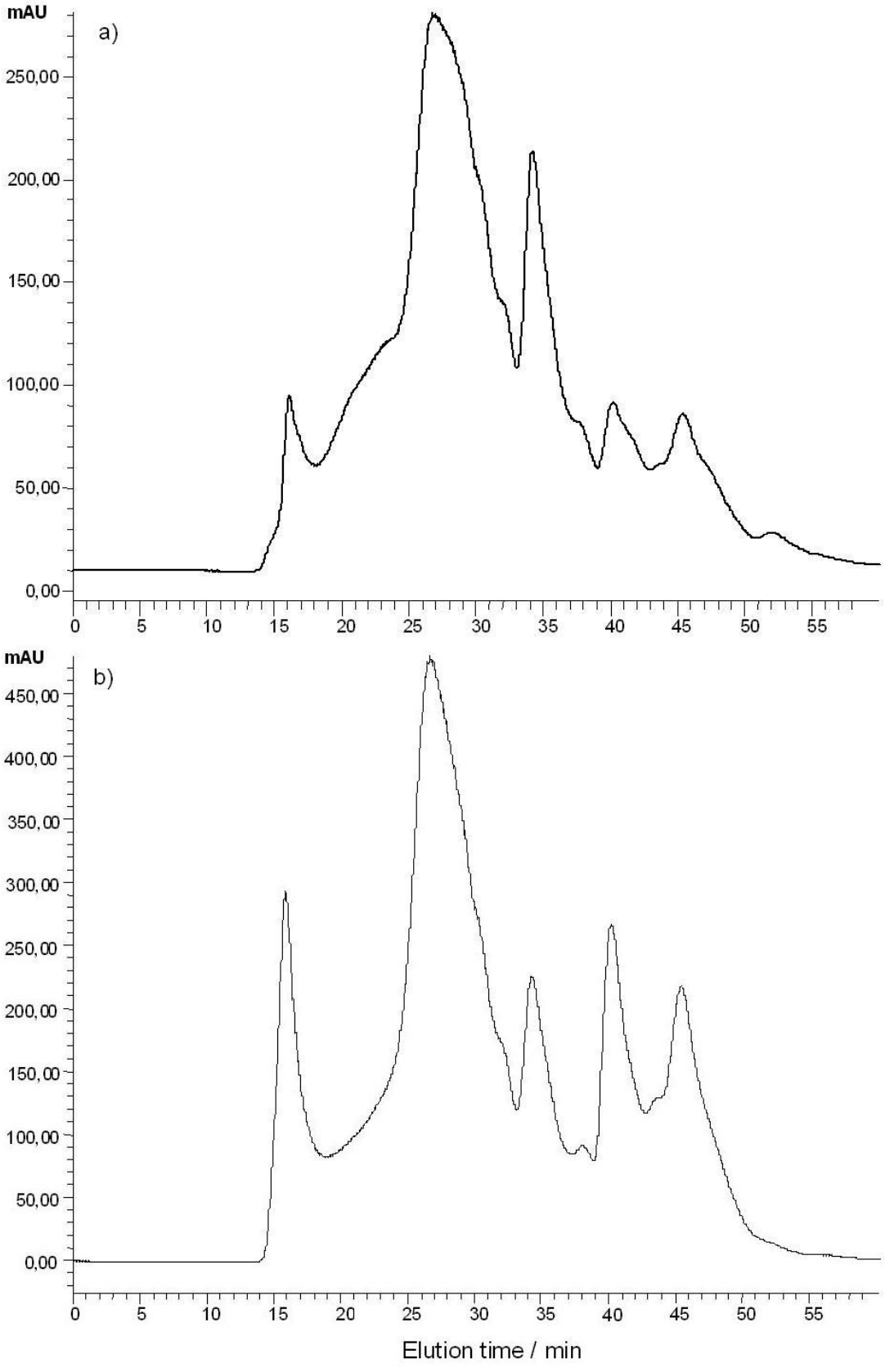
450 **Figure 2.** Distribution profiles of essential trace elements among cytosolic proteins of different molecular
451 masses from European chub gills, separated by SE-HPLC with Superdex™ 200 10/300 GL column a) Co; b) Cu;
452 c) Fe; d) Mn; e) Se; and f) Zn; the results are presented as ng of trace element eluted at specific elution times,
453 after passing 400 μL of gill cytosol through the chromatographic column; the results obtained for fish No. 1 were
454 multiplied by 2, because they were obtained by passing 200 μL of gill cytosol through the chromatographic
455 column

456 **Figure 3.** Distribution profile of nonessential trace element Cd among cytosolic proteins of different molecular
457 masses from European chub gills, separated by SE-HPLC with Superdex™ 200 10/300 GL column; the results
458 are presented as described in the caption of Fig. 2

459

Krasnići, Nesrete; Dragun, Zrinka; Erk, Marijana; Raspor, Biserka. Distribution of Co, Cu, Fe, Mn, Se, Zn and Cd among cytosolic proteins of different molecular masses in gills of European chub (Squalius cephalus L.). // Environmental science and pollution research. 21 (2014) , 23; 13512-13521.

Figure 1.



Krasnići, Nesrete; Dragun, Zrinka; Erk, Marijana; Raspor, Biserka. Distribution of Co, Cu, Fe, Mn, Se, Zn and Cd among cytosolic proteins of different molecular masses in gills of European chub (Squalius cephalus L.). // Environmental science and pollution research. 21 (2014) , 23; 13512-13521.

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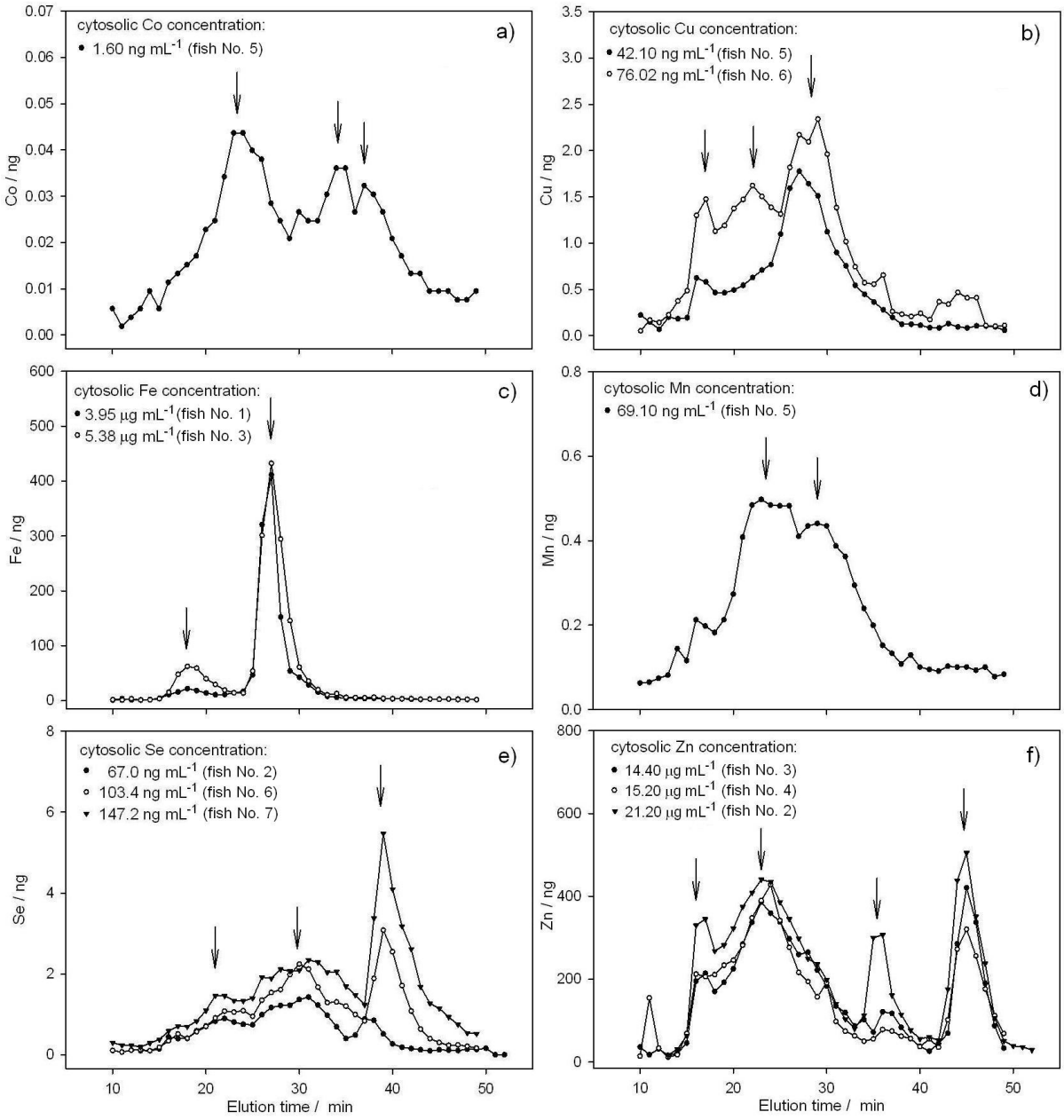
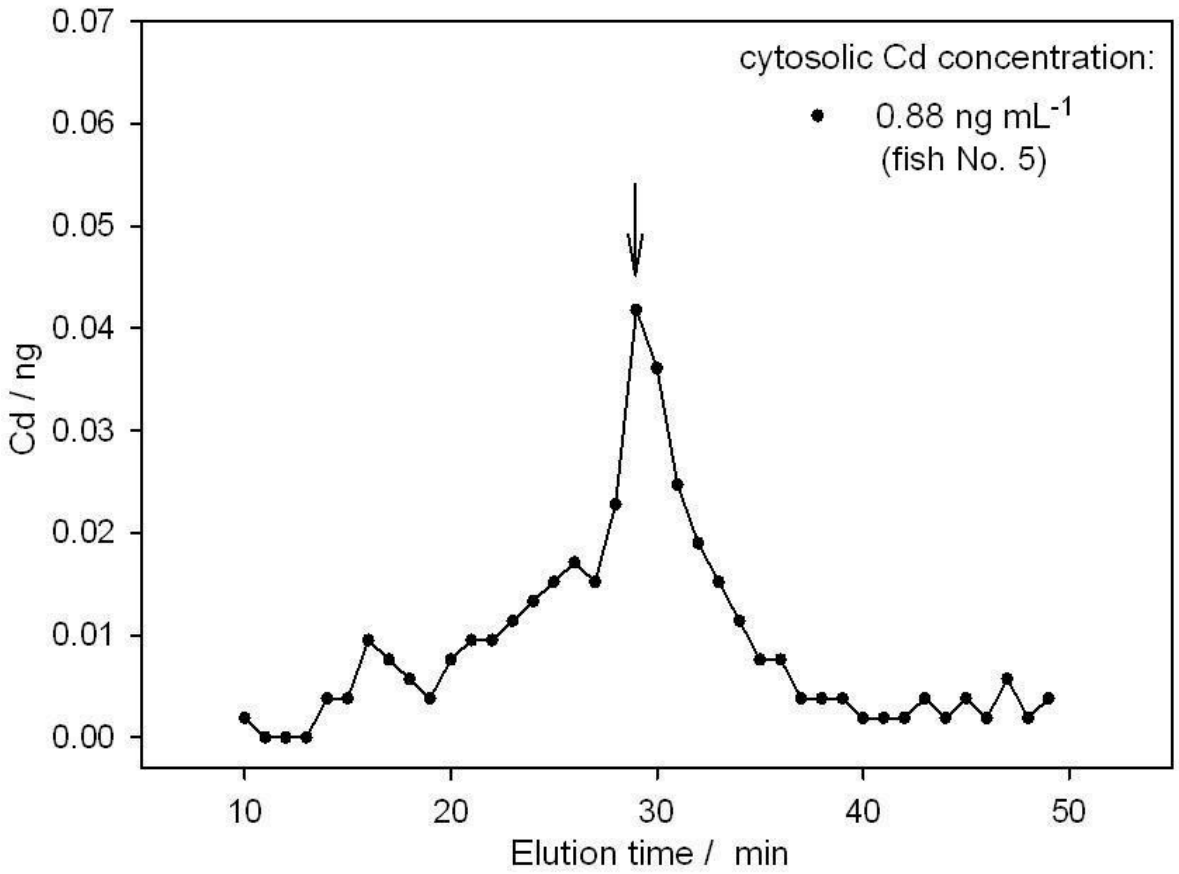


Krasnići, Nesrete; Dragun, Zrinka; Erk, Marijana; Raspor, Biserka. Distribution of Co, Cu, Fe, Mn, Se, Zn and Cd among cytosolic proteins of different molecular masses in gills of European chub (Squalius cephalus L.). // Environmental science and pollution research. 21 (2014) , 23; 13512-13521.

Figure 3.



Krasnići, Nesrete; Dragun, Zrinka; Erk, Marijana; Raspor, Biserka. Distribution of Co, Cu, Fe, Mn, Se, Zn and Cd among cytosolic proteins of different molecular masses in gills of European chub (Squalius cephalus L.). // Environmental science and pollution research. 21 (2014) , 23; 13512-13521.

Table 1. Basic biometric characteristics and total cytosolic protein concentrations in the gills of seven European chub (*Squalius cephalus*) specimens caught in the Sutla River in September of 2009, which were used for this study.

Fish No.	Length / cm	Mass / g	Age / years	Sex	Total cytosolic proteins mg mL^{-1}
1	29.7	260.6	4	F	14.4
2	25.3	140.0	3	F	14.7
3	23.1	120.4	3	F	16.1
4	20.1	94.5	2	F	13.6
5	24.3	153.5	3	F	16.8
6	26.5	174.9	3	M	15.7
7	25.0	156.5	4	F	16.1

Krasnići, Nesrete; Dragun, Zrinka; Erk, Marijana; Raspor, Biserka. Distribution of Co, Cu, Fe, Mn, Se, Zn and Cd among cytosolic proteins of different molecular masses in gills of European chub (Squalius cephalus L.). // Environmental science and pollution research. 21 (2014) , 23; 13512-13521.

Table 2. Elution times (t_e) and molecular masses (MM) of six protein standards for Superdex 200 10/300 GL size exclusion column calibration, and of rabbit metallothionein standard.

Protein	**t_e / min**	**MM / kDa**	**Concentration / mg mL^{-1}**
Thyroglobulin	16.7	669	8
Apoferritin	18.0	443	10
β-amylase	20.7	200	4
Alcohol dehydrogenase	21.0	150	5
Bovine albumin	22.9	66	10
Carbonic anhydrase	28.7	29	3
Metallothionein (1st peak)	29.9	16.6[a]	5
Metallothionein (2nd peak)	30.9	12.5[a]	5

[a]MM of metallothionein was calculated from calibration equation.

Table 3. Distribution of trace elements among cytosolic fractions of chub gill containing proteins of different molecular masses, separated by size exclusion HPLC with Superdex 200 10/300 GL column. Elution times (t_e) and molecular masses (MM) of proteins contained in the fractions in which respective elements were eluted are given in the table. Presented numbers refer to maxima of trace element peaks (i.e. the fractions with the highest trace element concentrations), whereas the numbers within the brackets refer to the beginnings and the ends of trace element peaks. The results presented in this table are based on analyses of gill cytosol of seven chub specimens.

	Element	[a]HMM peak 1		[a]HMM peak 2		[b]MMM peak		[c]LMM peak		[d]VLMM peak 1		[d]VLMM peak 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
Essential elements	Co	-	-	24 (19-29)	80 (310-20)	-	-	-	-	34 (32-36)	4 (9-2)	37 (36-40)	2 (2-1)
	Cu	17 (15-18)	530 (915-410)	22 (19-25)	140 (310-60)	27 (25-34)	35 (60-5)	-	-	-	-	-	-
	Fe	18 (16-22)	405 (700-140)	-	-	27 (24-31)	35 (80-10)	-	-	-	-	-	-
	Mn	-	-	23 (19-27)	105 (310-35)	-	-	29 (27-38)	20 (35-2)	-	-	-	-
	Se	-	-	21 (19-24)	180 (310-80)	-	-	30 (25-37)	15 (60-2)	-	-	39 (37-44)	1 (≤2)
	Zn	17 (15-18)	530 (915-405)	23 (19-31)	105 (310-10)	-	-	-	-	36 (34-40)	3 (5-1)	45 (43-48)	<1 (<1)
Non-essential element	Cd	-	-	-	-	-	-	29 (27-35)	20.9 (35-4)	-	-	-	-

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

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