Multielement analysis in the fish hepatic cytosol as a screening tool in the monitoring of natural waters

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Abstract

The possibility of direct measurement of trace elements in hepatic cytosol of European chub (*Squalius cephalus*) by HR ICP-MS after cytosol dilution with Milli-Q water and subsequent acidification was investigated. Due to low detection limits of this procedure, determination of 13 elements (As, Cd, Co, Cu, Fe, Mn, Mo, Pb, Sb, Sn, Sr, V, Zn) was possible in the chub hepatic cytosol, exhibiting excellent measurement repeatability in duplicates. Some of these elements were also measured by HR ICP-MS in acid digested cytosols (Cd, Co, Cu, Fe, Mn, Mo, Sr, V, Zn). Good agreement of the results obtained after sample dilution and sample digestion indicated that complex organic matrix of hepatic cytosol did not affect measurement reliability. Cytosolic concentrations of 13 trace elements in the chub liver were quantified in the following order: Fe,Zn>Cu>Mn>Mo>Sr,V,Cd>Co>As,Pb>Sn>Sb. Unlike Cd, Cu, Fe, Mn and Zn for which the cytosolic concentrations were previously reported after measurement by AAS, cytosolic concentrations of eight additional trace elements characteristic for the liver of chubs inhabiting the low contaminated river water were reported here for the first time (ng g^{-1}): Mo 136.8-183.6, Sr 32.7-63.0, V 17.5-69.0, Co 24.3-30.7, As 9.9-29.5, Pb 5.8-35.6, Sn 5.5-12.4 and Sb 0.9-2.6. The simultaneous measurement of large number of trace elements in the cytosolic fractions of fish tissues, which comprise potentially metal-sensitive sub-cellular pools, could be beneficial as a screening tool in the monitoring of natural waters, because it would enable timely recognition of increased fish exposure to metals.

Key words: HR ICP-MS; cytosol; European chub; liver; trace elements

1. Introduction

Metals and metalloids are considered as serious pollutants of the aquatic environment because of their environmental persistence and tendency to be concentrated in aquatic organisms (Harte et al. 1991; Schüürmann and Markert 1998; Fidan et al. 2008). Their accumulation in the tissues of organisms can result in chronic illnesses and even cause potential damage to the population (Holcombe et al. 1976; Barlas 1999). Dissolved metal and metalloid levels, which are usually measured as indicators of water pollution, are not completely bioavailable. Therefore, one of the most effective ways to evaluate metal and metalloid bioavailability is to monitor their concentrations in tissues of sentinel species (Kraemer et al. 2006), among which fish are considered as one of the most indicative organisms for the estimation of trace metal pollution potential (Papagiannis et al. 2004). The liver is often chosen as a target organ for trace element analysis due to its importance in accumulation of different metals (e.g. Cu and Cd; Giguère et al. 2004), and because its response time to short-term fluctuations in ambient metal concentrations is slower than for organs in direct contact with the external environment, such as the gills and the gut (Kraemer et al. 2005). In addition, metabolically active organs are particularly susceptible to toxic effects (Fidan et al. 2008).

To obtain information about the metal and metalloid levels which are presumably metabolically available our research mainly focused on metal and metalloid analyses in the soluble hepatic fraction. To our knowledge, the information on subcellular distribution of various metals and metalloids and their variability in the cytosolic fraction of fish tissues is scarce. Research on this topic most commonly refer to Cd, Cu and Zn in various fish species (e.g. in yellow perch (Kraemer et al. 2006; Campbell et al. 2008); rainbow trout (Kamunde and MacPhail 2008; Sappal et al. 2009); zebrafish and roach (Paris-Palacios and Biagianti-Risbourg 2006)), and only sporadically to other elements such as Pb (e.g. in mummichog (Goto and Wallace 2010); brown trout and European eel (Linde et al. 1999)), Ni and Tl (e.g. in yellow perch (Campbell et al. 2008); fathead minnow (Lapointe and Couture 2009)) and Se (e.g. in grunt (Zhang and Wang 2006)). The benefit of metal and metalloid determination in the soluble tissue fraction over determination of total tissue load lays in the fact that accumulated metals and metalloids, as described for Cd, fractionate into metabolically reactive and detoxified pools. The potential for effects is often not related to total metal burden, because a considerable proportion of the bioaccumulated load will be in a detoxified form (McGeer et al. 2012). For example, metals could be detoxified in a form of metal rich

granules, which will be separated within the pellets already after centrifugation of tissue homogenate at 10 000×g, together with cellular debris and mitochondria (Campbell et al. 2005; Giguère et al. 2006). Centrifugation of liver homogenate at 50 000×g, as used in our study for isolation of soluble hepatic fraction (SOP 1999), therefore enables determination of metals and metalloids in potentially metal-sensitive sub-cellular pools (Giguère et al. 2006). In addition, trace elements present in the soluble tissue fraction, i.e. within organelles, or associated with enzymes and metallothioneins, can be considered as metals/metalloids trophically available to predators (Wallace and Luoma 2003).

The measurement of large number of elements in soluble fractions of fish tissues, both essential and nonessential, could be advantageous for monitoring of natural waters, because it would enable timely recognition of increased fish exposure to metals or disturbance in metal homeostasis. Due to rather high detection limits of atomic absorption spectrometry (AAS) - the analytical method so far used in our studies, our research was limited to five metals, four essential (Cu, Fe, Mn and Zn) and one nonessential (Cd), in the cytosolic fraction of different tissues of marine and freshwater fish (Filipović and Raspor 2003; Dragun et al. 2007; Filipović Marijić and Raspor 2008; Podrug and Raspor 2009; Podrug et al. 2009). To simultaneously obtain information on greater number of trace elements present in the soluble fraction of fish tissues sometimes in very low concentrations, we have applied high resolution inductively coupled plasma mass spectrometry (HR ICP-MS) in our studies, which enabled multielement analyses in fish tissue cytosols, as well as much lower detection limits compared to AAS. Here we present for the first time the cytosolic concentrations of additional eight trace elements in the soluble fraction of chub liver (As, Co, Mo, Pb, Sb, Sn, Sr and V), among which some were proven as essential or potentially essential (Co, Mo, As and V), whereas the remaining (Pb, Sb, Sn and Sr) have no known physiological functions. Lead and Sb were even defined as pollutants of priority interest by European Water Framework Directive (EPCEU 2008) and USEPA (1999), respectively. However, even essential and potentially essential elements, such as Co, As and V, can be highly toxic if excessively present in the fish tissues.

The aims of this study, accordingly, can be summarized as follows:

- our primary aim was to investigate the possibility of trace element measurement by HR ICP-MS in chub hepatic cytosol after simple dilution and acidification, without prior acid digestion;

- the additional aims were to compare the results obtained by above described procedure with the results obtained by additional analytical procedure, i.e. measurement by HR ICP-MS after cytosol acid digestion; and
- to provide the preliminary data set for As, Co, Mo, Pb, Sb, Sn, Sr and V concentrations in the hepatic cytosol of European chub living in the aquatic environment with low metal contamination, such as the Sava River in Croatia (Dragun et al. 2009a; Milačič et al. 2010), which could be roughly considered as "constitutive" metal/metalloid levels.

2. Experimental

2.1 Fish sampling

The selected bioindicator organism for this study was European chub (*Squalius cephalus* L.), a fish species from the family of carps (*Cyprinidae*), widespread in the European freshwater and tolerant to chemical and physical pollution (Gandolfi et al. 1991). European chub is an omnivorous fish species, which feeds on algae, plants and various seeds (Vostradovsky 1973), as well as worms, molluscs, crayfish and insect larvae. Larger chub specimens also eat different species of small fish (Maitland and Campbell 1992). The chubs were caught in the Sava River in Croatia. For the present study, 150 km long section of the Sava River, starting at the Croatian-Slovenian state border and ending at the town of Jasenovac (the state border between Croatia and Bosnia and Herzegovina), was chosen (Krča et al. 2007). The dissolved concentrations of several elements (As, Cd, Co, Cu, Fe, Mn, Mo, Pb, Sr, V, Zn) in the water of the Sava River from Zagreb to Jasenovac could be considered as not significantly above the natural level (Dautović et al. 2007; Dragun et al. 2009a), based on comparison with two other rivers in Croatia, the pristine river Una (Dautović et al. 2007) and the reference site on the Sutla River (Dragun et al. 2011). The sediment contamination of the selected Sava river section was also considered as negligible (Milačič et al. 2010). The map of the study area with selected sampling sites was previously published (Dragun et al. 2007). The samplings were performed with electro fishing device (EL63 II GI 5kw, Honda GX270), according to the Croatian standard (HRN EN 14011 2005), in two periods: one regarded as reproductive (either pre-spawning or spawning period in April/May 2006) and one regarded as non-reproductive (post-spawning period in September 2006). During the few hours of sampling and transportation, the captured fish were kept alive in tank filled with river water taken from their respective sampling sites, and the water was aerated. In the laboratory, the

fish were first anesthetized with MS 222 (tricaine methane sulphonate, Sigma Aldrich) and then sacrificed; the livers were isolated, weighed and stored at -80°C until further analyses.

2.2 Isolation of cytosolic fraction from chub liver

Isolation of the cytosolic fraction from the chub hepatic tissue was performed according to standard operating procedure (SOP), developed for metallothionein isolation from fish tissues at Norwegian Institute for Water Research (NIVA) in the frame of the BEQUALM programme (Biological Effects Quality Assurance in Monitoring Programmes) (SOP 1999), which will be hereafter described. The samples of hepatic tissue were first diluted 6 times with cooled homogenization buffer, consisting of 100 mM Tris-HCl/Base (pH 8.1 at 4°C) and the reducing agent 1 mM dithiotreitol (both by Sigma). Potassium chloride (150 mM) was added because the tissue fractions used for elemental analyses and for EROD activity determination were prepared from the same liver homogenates (Krča et al. 2007). The diluted tissue samples were homogenized by Potter-Elvehjem homogenizer (Glas-Col, USA) in ice cooled tube at 6,000 rpm. The homogenates were centrifuged in the Sorval RC28S centrifuge (Kendro, USA) at 50 000×g for 2 h at 4°C. Next, the supernatant obtained after centrifugation at 50 000×g or S50, which represents water soluble cytosolic tissue fraction, was separated from the pellet and stored at -80°C for subsequent trace element analyses (Podrug et al. 2009).

2.3 Determination of trace element concentrations in chub hepatic cytosols

The determination of trace element concentrations in chub hepatic cytosols comprised of two steps: sample preparation and trace element measurement. The primary and preferential analytical procedure tested in this study was based on the measurement by HR ICP-MS in diluted and acidified chub hepatic cytosols (ICP-DIL). Additional procedure applied for the sake of the result comparison was based on the measurement by HR ICP-MS in acid digested cytosols (ICP-DIG). ICP-DIL procedure offers several advantages over ICP-DIG procedure. It is beneficial in terms of time and cost efficiency, because much less time is needed for the completion of simple cytosol dilution in comparison to prolonged digestion process, and the costly equipment is not required, such as for example microwave oven. In addition, possible sample contamination and overdilution would be reduced by this procedure, due to decreased sample manipulation and lower chemical usage, as can be seen from the description of each procedure. A brief overview of these procedures is given in Table 1.

2.3.1 Measurements by HR ICP-MS in diluted and acidified cytosols (ICP-DIL)

Prior to measurements, cytosols were ten times diluted with Milli-Q water to obtain larger volume of sample for the analysis, and to diminish the influence of complex cytosol matrix as much as possible without addition of chemicals. The samples were also acidified: 20 μL of 65% HNO_3 (*Suprapur*, Merck, Germany) was added per 2 mL of diluted cytosol. Measurements of trace element concentrations were performed by HR ICP-MS (Element 2, Thermo, Bremen, Germany), equipped with a double focusing mass analyser using reverse Nier-Johnson geometry. An autosampler (ASX 510, Cetac Technologies, USA) and sample introduction kit consisting of a SeaSpray nebulizer and cyclonic spray chamber Twister were employed to transport the analytes into the plasma of the ICP-MS. Measurements of ^{98}Mo, ^{111}Cd, ^{120}Sn, ^{121}Sb and ^{208}Pb were operated in low resolution mode, ^{51}V, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{63}Cu, ^{66}Zn and ^{86}Sr in medium resolution mode, whereas ^{75}As was measured in high resolution mode. Standard solutions were prepared by appropriate dilutions of 100 mg L^{-1} multielement stock standard solution (Analytika, Prague, Czech Republic) in which single element standard solutions (1 g L^{-1}) of Sb (Analytika, Prague, Czech Republic) and Sn (Analytika, Prague, Czech Republic) were added. Indium (1 μg L^{-1}; Indium Atomic Spectroscopy Standard Solution, Fluka, Germany) was added to samples as an internal standard, thus enabling the use of external calibration with the standards prepared in 2% HNO_3 (S*uprapur*, Merck, Germany).

2.3.2 Measurements by HR ICP-MS in acid digested cytosols (ICP-DIG)

Due to limited quantity of hepatic cytosol, for the analyses by ICP-DIG we have used the samples remained after previously performed analyses by AAS, which were already five times diluted (Podrug et al. 2009). The step of sample preparation in ICP-DIG consisted of cytosol digestion in laboratory drying oven (ST-05, Instrumentaria, Croatia). The volumes of 0.6 mL of five times diluted cytosols were added to PFA (perfluoroalkoxy polymer resin) vials (30 mL) with threaded caps, and mixed with 0.45 mL of HNO_3 (65%, *Suprapur*, Merck, Germany) and 0.15 mL of H_2O_2 (30%, *Suprapur*, Merck, Germany). The caps were additionally wrapped with PTFE-teflon tape (0.075 mm) to prevent evaporation, and then put into the oven for 3.5 h at 85°C. After digestion, cytosols were five times diluted with Milli-Q water to lower the acid level in the samples below 5%, which resulted with final dilution factor in ICP-DIG amounting to 50 times. To prevent even higher sample dilutions which might lead to inability to measure low concentrations of some elements in the cytosol, we

have used laboratory drying oven for digestion and not the microwave oven, because the latter requires use of larger volumes of chemicals. The measurements in acid digested cytosols were performed by HR ICP-MS, under the same conditions as described in the section 2.3.1. Due to high dilution factor, the measurements by ICP-DIG could be performed only for Cd, Co, Cu, Fe, Mn, Mo, Sr, V and Zn, whereas As, Pb, Sb and Sn could not be measured.

2.4. Validation of trace element measurements in diluted and acidified cytosols by HR ICP-MS (ICP-DIL)

The validation of trace element measurements in diluted and acidified cytosols by HR ICP-MS (ICP-DIL) included the following: determination of the detection limits, determination of measurement repeatability in duplicates, and the comparison with the results obtained by ICP-DIG.

2.4.1. Detection limits

The detection limits were calculated as three standard deviations of 9-10 measurements in the blank sample. The blank sample for ICP-DIL was ten times diluted homogenization buffer with the addition of HNO_3 (10 mM Tris-HCl/Base, 0.1 mM dithiotreitol, 15 mM KCl and 0.65% HNO_3). For the sake of comparison, the detection limits were also established for ICP-DIG. The applied blank sample for ICP-DIG was five times diluted digestion mixture (2 mM Tris-HCl/Base, 0.02 mM dithiotreitol, 3 mM KCl, 4.9% HNO_3 and 0.75% H_2O_2). The detection limits for ICP-DIL and ICP-DIG are presented in the Table 2.

2.4.2. Repeatability of measurement in duplicates

Due to the limited quantity of the analysed samples, it was not possible to determine commonly applied indicator of method validity, i.e. the repeatability of ten consecutive measurements in several adequately prepared samples of different metal concentrations. Instead, we have determined the repeatability of trace element measurements in duplicates in diluted and acidified cytosols by HR ICP-MS, for as many samples as possible (n=34-39; Table 2). For the sake of comparison, the same was done for ICP-DIG (n=100-102; Table 2). The results are presented in a form of relative standard deviations (RSD, %).

2.4.3. Comparison of the results obtained by ICP-DIL and by ICP-DIG

The comparison of the results obtained by ICP-DIL with the results obtained by ICP-DIG was based on the comparison of detection limits and repeatability in duplicates (Table 2), as well as on calculations of ratios (%) between the obtained trace element concentrations (ICP-DIL:ICP-DIG; Fig. 1).

Since there was no adequate liquid certified reference material available on the market with corresponding composition to cytosols, we were not able to determine recoveries for ICP-DIL. Therefore, we have used the certified reference material for trace metals (DOLT-3: dogfish liver; distributed by National Research Council Canada) which has to be digested prior to measurement, and determined the recoveries for ICP-DIG for 4 certified elements (Table 3). With the aim to obtain liquid samples comparable to cytosols, the weights of 0.136-0.176 g of DOLT-3 were suspended in 1 mL of Milli-Q water, and then digested in the same way as the chub hepatic cytosol. The digestion of certified reference material was repeated five times, to enable the assessment of the reproducibility. The calculation of the ratios of ICP-DIL against ICP-DIG allowed the relative evaluation of accuracy for ICP-DIL for those four elements (Cd, Cu, Fe, and Zn).

In addition, the relationships between trace element concentrations obtained by ICP-DIL and ICP-DIG were established by correlation analysis (Fig. 1). The comparison of the results obtained by different procedures could enable better insight in the quality of trace element measurement in diluted and acidified cytosols by HR ICP-MS and its applicability as the fast and reliable screening tool in the monitoring studies.

2.5. Data processing and the statistical analyses

All the calculations were performed with the Microsoft Office Excel 2007. The measured concentrations in diluted or digested cytosols were multiplied with the appropriate dilution factors (10 for ICP-DIL, 50 for ICP-DIG; Table 1), and expressed as ng or μg of trace element per mL of S50 fraction. The descriptive statistics (mean, standard deviation, median, minimum, maximum, Q1, Q3) and the calculation of Spearman correlation coefficients were performed by SigmaStat 3.5.

For calculation of constitutive trace element ranges, the mean values and standard deviations were not used, to avoid the influence of several extreme values of some elements, which could be possibly caused by other factors and not by natural variability. Lower (Q_1)

and upper quartiles (Q_3) of complete data sets were therefore set as lower and upper limits of the constitutive ranges.

3. Results and discussion

3.1. Validation of trace element measurement by HR ICP-MS in diluted and acidified chub hepatic cytosols

In our previous studies, we have used AAS for trace element analyses, which enabled the measurement of limited number of trace elements (Cd, Cu, Fe, Mn and Zn) in the cytosols of different chub tissues (in the gills (Dragun et al. 2007; Dragun et al. 2009b,c); liver (Podrug and Raspor 2009; Podrug et al. 2009); intestine (Filipović Marijić and Raspor 2010)). The other elements could not be measured because of high detection limits of that technique: the lower limits of optimum working ranges of FAAS for elements included in this study amount from 20 to 1000 ng mL^{-1} (Varian 1989), whereas ETAAS is typically up to 100 times more sensitive (Varian 1988). The introduction of HR ICP-MS in future research, therefore, would offer an opportunity for simultaneous measurement of larger number of elements present in the tissue cytosols of wild-caught fish in concentrations too low to be measured by AAS. It could eventually provide more complete insight in the metal exposure of organisms inhabiting natural waters.

The detection limits of ICP-DIL (trace element determination by HR ICP-MS in cytosols which are not acid digested, but only diluted and acidified) for the 13 elements selected in this study were mostly much lower than their concentrations in the chub hepatic cytosol, which is a good basis for reliable measurements (Table 2). These LODs were also noticeably lower compared to LODs of ICP-DIG (Table 2), as well as compared to previously published LODs of AAS (Dragun et al., 2007), as obvious benefit of ICP-DIL procedure. The repeatability of the measurement in duplicate of this procedure was excellent for majority of measured elements, with RSD mostly below 3% (Table 2). RSD was somewhat higher only for few elements present in the cytosol in very low concentrations, often below 1 ng mL^{-1} (5-10% for As, Sb, Sn; Table 2). The repeatability of measurement in duplicate of ICP-DIL was, therefore, either equal or even better compared to acid digestion based procedure ICP-DIG (Table 2).

The cytosolic concentrations of five metals (Cd, Cu, Fe, Mn and Zn) in liver were previously measured by AAS and published for the same chub specimens as used in this study (Podrug and Raspor 2009; Podrug et al. 2009). The results obtained by ICP-DIL procedure exhibited excellent agreement with those published results, as well as with the results obtained for acid digested cytosols by ICP-DIG procedure. The latter was confirmed by positive, statistically significant, correlation coefficients, mostly above 0.9 (Figure 1a-e). On average, the results obtained by ICP-DIL for all five metals were mainly within ±10% of the results obtained by ICP-DIG (Figure 1a-e).

The results obtained by ICP-DIL were also compared with the results obtained by ICP-DIG for additional four trace elements (Co, Mo, Sr and V; Fig. 1f-i). The concentrations of Co and V obtained by both methods were mainly comparable (Fig. 1f and 1i), whereas somewhat higher concentrations of Mo were obtained by ICP-DIL compared to ICP-DIG (Fig. 1g). The most pronounced deviations between ICP-DIL and ICP-DIG were observed for Sr (Fig. 1h). The probable reason was the low repeatability of Sr measurements in duplicate by ICP-DIG (Table 2), which eventually lead to suspiciously high Sr values obtained by ICP-DIG in four samples (Fig. 1h). The correlations between the results obtained by ICP-DIL and ICP-DIG were positive and statistically significant, and mainly above 0.7 (Fig. 1f-i), which could be perceived as satisfactory, taking into consideration rather low concentrations of these elements present in the chub hepatic cytosol (Table 2).

Since the recoveries could not be determined directly for ICP-DIL, due to inability to find adequate certified reference material, we have determined the recoveries for ICP-DIG for four metals present in the applied certified reference material (Cd, Cu, Fe and Zn), and evaluated the accuracy of ICP-DIL relative to ICP-DIG. The recoveries of all four elements were within ±5% from certified values (Table 3), with high reproducibility of five repeated measurements (RSD<3%; Table 3). As described above, agreement between the concentrations obtained by ICP-DIL and ICP-DIG, with latter confirmed as accurate, was excellent (Fig. 1). This was an indirect confirmation that measurements of Cd, Cu, Fe and Zn by ICP-DIL were also accurate.

In a conclusion, the ICP-DIL procedure, i.e. the determination of trace elements by HR ICP-MS after cytosol dilution and acidification, exhibited a good repeatability in duplicates, agreement with measurements in acid digested cytosols by HR ICP-MS, and has been proven

accurate for several elements (Cd, Cu, Fe and Zn). These findings, in addition to low detection limits, as well as fast and easy sample preparation with minimal chances of sample contamination and overdilution, make this procedure advantageous for environmental studies.

3.2. Preliminary data for eight trace elements in the chub hepatic cytosol

The preliminary set of data for eight trace elements (As, Co, Mo, Pb, Sb, Sn, Sr, V) in the chub hepatic cytosol obtained by ICP-DIL procedure is given in Table 4. Since only the remaining samples were used for these analyses, the collected data refer to approximately half of the total number of fish sampled in this study. The results were presented for a total number of 36 and 23 chubs in the spring and autumn periods of 2006, respectively, in contrast to 81 and 59 fish that were actually sampled from the Sava River. A partial data set was a limiting factor, preventing more detailed analysis of physiological variability of the concentrations of As, Co, Mo, Pb, Sb, Sn, Sr and V in the soluble fraction of the chub liver. Therefore, constitutive ranges were defined for both studied periods together, and included both female and male chub specimens 2-5 years old (length: 22.1±3.8 cm; body mass: 122.7±59.6 g).

The constitutive ranges of trace elements in the chub hepatic cytosol defined in this study were as follows, in the decreasing order: Mo 22.8-30.6 ng mL^{-1}; Sr 5.45-10.5 ng mL^{-1}; V 2.92-11.5 ng mL^{-1}; Co 4.05-5.11 ng mL^{-1}; As 1.65-4.91 ng mL^{-1}; Pb 0.97-5.93 ng mL^{-1}; Sn 0.91-2.07 ng mL^{-1}; Sb 0.15-0.43 ng mL^{-1}. To enable the comparison of our results with total loads of trace elements in the fish liver commonly reported in the monitoring studies, we have additionally presented cytosolic constitutive ranges in ng g^{-1}, after multiplication by 6, which was a factor of tissue dilution during homogenization: Mo 136.8-183.6; Sr 32.7-63.0; V 17.5-69.0; Co 24.3-30.7; As 9.9-29.5; Pb 5.8-35.6; Sn 5.5-12.4; and Sb 0.9-2.6. These ranges should be, however, solely comprehended as the preliminary information required for differentiation between metal exposed and unexposed specimens, which would be impossible without at least an approximate idea on the ranges of trace elements present in the tissues of metal unexposed aquatic organisms. Combining our data with the previous reports (Podrug et al. 2009), it can be seen that metal/metalloid concentrations in the chub hepatic cytosol were present in the following order: Fe,Zn>Cu>Mn>Mo>Sr,V,Cd>Co>As,Pb>Sn>Sb. Comparable concentration order for several metals was reported for the whole liver tissue of cyprinid fish *Barbus xanthopterus* (Fe>Zn>Cu>Pb>Mn>Co>Cd) (Alhas et al. 2009) and *Tor grypus* (Fe>Zn>Cu>Pb>Mn>Co) (Oymak et al. 2009).

However, it is interesting to observe that Pb concentrations in the whole liver tissue of these species appear to be rather high (2-3 μg g^{-1}, on wet mass basis), in the range of essential element Cu. In contrast, in the cytosol of chub liver Pb was present in considerably lower concentrations, in the range of nonessential metal Cd (Pb: 6-36 ng g^{-1}, on wet mass basis, which is 1-2% or even less compared to total Pb present in the liver of two above discussed species). There is a possibility that such difference could be associated to interspecies variability, since, for example, whole liver burdens of Pb, Cd and As reported for another cyprinid fish, common carp (*Cyprinus carpio*), were rather low and comparable (on wet mass basis, approximately 30 ng g^{-1}, 20 ng g^{-1}, 50 ng g^{-1}, respectively (Čelechovská et al. 2007)), and similar to the concentrations of these elements in the chub hepatic cytosol measured in our study (on wet mass basis, <40 ng g^{-1}, <60 ng g^{-1}, <30 ng g^{-1}, respectively). However, there is also a possibility of Pb storage mainly in the non-soluble tissue fraction (e.g. granules). The study on salmonid fish, brown trout (*Salmo trutta*), and anguillid fish, European eel (*Anguilla anguilla*), in unpolluted Pigueña River in Spain revealed that in contrast to Cd, Cu and Zn, which were found in high percentages in hepatic cytosol, Pb was mainly located in the non-cytosolic fraction (Linde et al. 1999). Although whole tissue burdens can be good indicators of overall accumulation of metals in specific tissues, they are often not conclusive in understanding metal pollution impacts on aquatic organisms (Clements and Rees 1997; Farag et al. 1999). The fact that portions of certain metals present in the soluble tissue fraction and thus available for possible interactions and effects on the metal-sensitive cell components, such as various proteins and enzymes, could comprise only few percent of the complete tissue metal quantity, accentuates the need to further study the variability of metals present in that specific fraction, both as a result of the natural causes and the changes in the environmental exposure.

4. Conclusions

The measurements of trace elements in diluted and acidified chub hepatic cytosols by HR ICP-MS exhibited excellent repeatability in duplicates and comparability with measurements by HR ICP-MS in acid digested cytosols, indicating that complex cytosol matrix did not affect the quality of obtained results. Therefore, the application of HR ICP-MS - a multielement, low detection limit analytical technique - in monitoring studies for simultaneous determination of large number of environmentally relevant trace elements in

diluted and acidified fish tissue cytosols could present a valuable contribution to fast and reliable early recognition of increased fish exposure to metals in natural aquatic systems.

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Figure captions

Figure 1. The relationships of metal concentrations measured by HR ICP-MS in diluted and acidified hepatic cytosols of European chub (ICP-DIL) with metal concentrations measured by HR ICP-MS in acid digested cytosols (ICP-DIG), presented as scatter plots; a) Cd; b) Cu; c) Fe; d) Mn; e) Zn; f) Co; g) Mo; h) Sr; i) V; r – Spearman correlation coefficient; ratio (%) – the mean ± standard deviation of the ratios obtained between the concentrations measured by two procedures; n – number of samples

Figure 1.

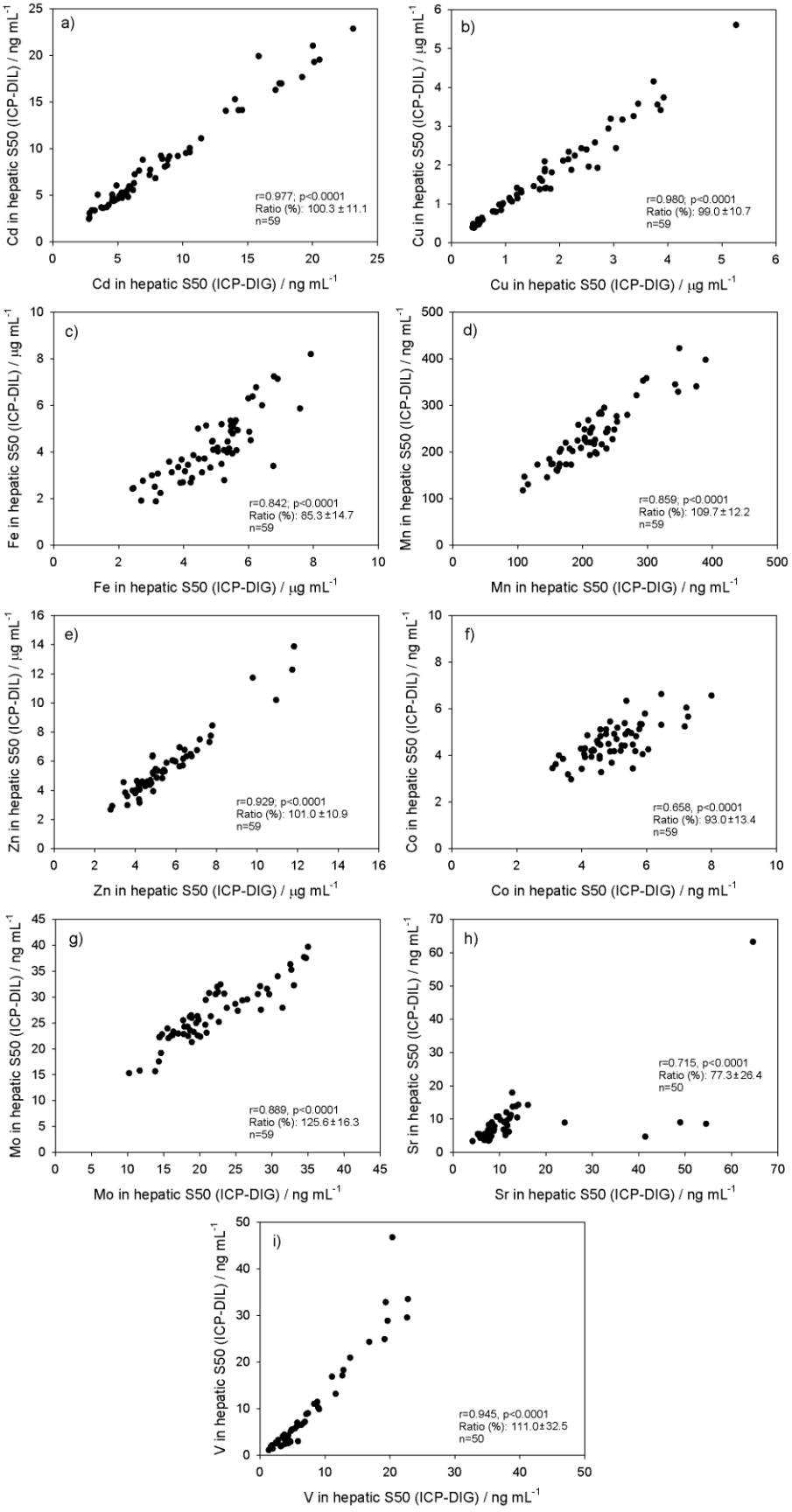
a)
Cd in hepatic S50 (ICP-DIL) / ng mL-1
Cd in hepatic S50 (ICP-DIG) / ng mL-1
r=0.977; p<0.0001
Ratio (%): 100.3 ± 11.1
n=59
b)
Cu in hepatic S50 (ICP-DIL) / µg mL-1
Cu in hepatic S50 (ICP-DIG) / µg mL-1
r=0.980; p<0.0001
Ratio (%): 99.0 ± 10.7
n=59
c)
Fe in hepatic S50 (ICP-DIL) / µg mL-1
Fe in hepatic S50 (ICP-DIG) / µg mL-1
r=0.842; p<0.0001
Ratio (%): 85.3 ± 14.7
n=59
d)
Mn in hepatic S50 (ICP-DIL) / ng mL-1
Mn in hepatic S50 (ICP-DIG) / ng mL-1
r=0.859; p<0.0001
Ratio (%): 109.7 ± 12.2
n=59
e)
Zn in hepatic S50 (ICP-DIL) / µg mL-1
Zn in hepatic S50 (ICP-DIG) / µg mL-1
r=0.929; p<0.0001
Ratio (%): 101.0 ± 10.9
n=59
f)
Co in hepatic S50 (ICP-DIL) / ng mL-1
Co in hepatic S50 (ICP-DIG) / ng mL-1
r=0.658, p<0.0001
Ratio (%): 93.0 ± 13.4
n=59
g)
Mo in hepatic S50 (ICP-DIL) / ng mL-1
Mo in hepatic S50 (ICP-DIG) / ng mL-1
r=0.889, p<0.0001
Ratio (%): 125.6 ± 16.3
n=59
h)
Sr in hepatic S50 (ICP-DIL) / ng mL-1
Sr in hepatic S50 (ICP-DIG) / ng mL-1
r=0.715, p<0.0001
Ratio (%): 77.3 ± 26.4
n=50
i)
V in hepatic S50 (ICP-DIL) / ng mL-1
V in hepatic S50 (ICP-DIG) / ng mL-1
r=0.945, p<0.0001
Ratio (%): 111.0 ± 32.5
n=50

Table 1. Brief overview of analytical procedures used for sample preparation and analysis of trace elements in the hepatic cytosol of European chub (*Squalius cephalus*).

	ICP-DIL	**ICP-DIG**
Sample preparation	Dilution with Milli-Q water and acidification	Digestion of diluted cytosols with HNO_3 and H_2O_2
Measurement method	HR ICP-MS	HR ICP-MS
Overall dilution factor	10	50
Trace elements measured	As, Cd, Co, Cu, Fe, Mn, Mo, Pb, Sn, Sb, Sr, V, Zn	Cd, Co, Cu, Fe, Mn, Mo, Sr, V, Zn

Table 2. Analytical characteristics of trace element determination by HR ICP-MS in diluted and acidified hepatic cytosols (ICP-DIL) and in acid digested hepatic cytosols (ICP-DIG): the range of the measured trace element concentrations in the chub hepatic cytosol (ng mL^{-1}); limits of detection (LOD; ng mL^{-1}); repeatability of trace element measurements in duplicate, expressed as median of relative standard deviations (RSD)

		ICP-DIL			ICP-DIG		
	Trace elements in the hepatic cytosol / ng mL^{-1}	**[a]LOD / ng mL^{-1}**	**[b]RSD / %**	**[c]RSD> 30%**	**[a]LOD / ng mL^{-1}**	**[b]RSD / %**	**[c]RSD> 30%**
Cd	2.5-22.9	0.022	1.7	0	0.079	1.7	0
Cu	385-5609	0.186	1.3	0	1.12	2.9	0
Fe	1882-8200	1.54	1.8	0	27.47	3.2	1
Mn	117.5-422.8	0.040	1.4	0	0.239	3.2	0
Zn	2681-13881	28.2	1.6	0	192.8	2.5	2
Co	3.0-6.6	0.040	1.7	0	0.047	5.4	3
Mo	15.3-39.7	0.026	1.7	0	0.433	2.3	0
Sr	3.4-63.3	0.149	2.4	0	1.95	11.0	15
V	1.1-46.8	0.010	2.0	0	0.047	5.7	11
As	<12.2	3.12	6.0	0	-	-	-
Pb	<122.9	0.115	2.9	2	-	-	-
Sb	<0.94	0.032	7.9	4	-	-	-
Sn	0.3-12.9	0.028	5.7	0	-	-	-

[a] based on three standard deviations of measurements in 9-10 blank samples, and multiplied with the factor of dilution (10 for ICP-DIL; 50 for ICP-DIG)

[b] total number of samples analyzed in duplicate by ICP-DIL was 39, except for As, Sr, V (n=34); and by ICP-DIG it was 100-102

[c] number of samples with RSD higher than 30%

Table 3. Quality control for ICP-DIG using DOLT-3, dogfish liver certified reference material for trace metals, distributed by National Research Council Canada.

	Certified values / μg g^{-1}	Obtained values[a] / μg g^{-1}	Reproducibility[a] / %	Recovery[b] / %
Cd	19.4±0.6	18.6±0.2	1.1	96
Cu	31.2±1.0	32.7±0.3	0.8	105
Fe	1484±57	1562±38	2.4	105
Zn	86.6±2.4	88.5±1.2	1.3	102

[a] based on 5 independently digested and measured subsamples
[b] based on the ratio of average obtained value and average certified value

Table 4. The preliminary data set (median, minimum-maximum) for eight trace elements in the hepatic cytosol of European chub caught in Sava River at five selected sites (1 – Otok Samoborski; 2 – Sava in Zagreb; 3 – Oborovo; 4 – Lukavec Posavski; 5 - Jasenovac) in two periods.

Period	Site	n	Co / ng mL^{-1}	Mo / ng mL^{-1}	Sr / ng mL^{-1}	V / ng mL^{-1}	As / ng mL^{-1}	Pb / ng mL^{-1}	Sb / ng mL^{-1}	Sn / ng mL^{-1}
April/May 2006	1	2	4.57 4.18-4.96	28.13 24.27-31.99	6.81 4.69-8.93	3.87 2.51-5.23	0.92 0.68-1.16	1.94 0.95-2.93	0.135 0.080-0.190	1.02 0.98-1.05
	2	[a]12	4.40 3.42-6.63	24.81 22.21-30.54	9.02 8.09-14.31	18.28 2.19-29.57	4.67 1.98-5.27	3.66 0.19-29.69	0.180 0.020-0.400	0.72 0.31-1.99
	3	[b]8	4.44 3.86-6.57	23.79 15.78-29.44	7.52 3.70-63.25	2.54 1.93-4.09	4.71 3.99-10.11	2.15 ≤4.66	0.273 0.035-0.940	0.96 0.51-3.12
	4	7	4.29 2.97-6.05	22.65 15.30-26.48	5.51 4.20-6.87	2.82 1.44-3.24	8.83 1.84-12.19	2.85 0.38-4.25	0.420 0.150-0.570	1.98 1.35-3.07
	5	7	4.30 3.62-5.11	26.27 22.96-32.43	12.03 8.30-17.95	28.87 11.05-46.79	2.28 1.28-3.13	1.36 0.18-6.15	0.140 0.070-0.290	1.51 0.48-2.22
September 2006	1	8	4.87 3.19-5.33	29.62 21.31-39.70	7.30 3.37-14.25	4.03 1.12-7.01	0.79 ≤5.93	1.47 0.82-3.73	0.170 0.125-0.465	1.02 0.73-1.33
	2	2	4.65 4.06-5.24	28.94 22.61-35.28	5.21 3.65-6.78	2.80 2.67-2.92	0.85 0.68-1.03	3.72 1.05-6.40	0.268 0.205-0.330	1.23 1.04-1.43
	3	2	4.65 3.85-5.45	26.30 23.26-29.35	6.26 3.49-9.03	7.28 5.53-9.04	2.59 1.95-3.24	9.29 5.28-13.31	0.227 0.215-0.240	7.04 1.18-12.91
	4	3	4.70 3.97-6.34	30.53 27.53-30.66	6.77 5.51-10.74	7.01 4.28-7.20	1.95 1.65-2.25	1.58 0.19-6.70	0.360 0.225-0.475	1.83 1.08-3.61
	5	8	4.18 3.28-5.66	31.94 15.66-37.72	5.94 4.73-11.23	7.68 3.52-13.17	2.50 1.85-5.35	32.85 0.62-122.9	0.450 0.405-0.510	2.67 1.37-3.80

[a] for As, Sr and V, n=7; [b] for As, Sr and V, n=4