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Accumulation of metals relevant for agricultural contamination in gills of European chub (*Squalius cephalus*)

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Abstract

The study of metal bioaccumulation in the gills of European chub (*Squalius cephalus*) was conducted in September 2009 at medium-size rural river Sutla, characterized by agricultural and municipal type of water contamination. The concentration ranges were established for the first time in the soluble, metabolically available fractions of chub gills for 12 metals, which are environmentally extremely relevant and yet only seldom studied, as follows in a decreasing order: K, 225-895 mg L^{-1}; Na, 78-366 mg L^{-1}; Ca, 19-62 mg L^{-1}; Mg, 13-47 mg L^{-1}; Rb, 164-1762 µg L^{-1}; Sr, 24-81 µg L^{-1}; Ba, 13-67 µg L^{-1}; Mo, 1.3-16 µg L^{-1}; Co, 0.7-2.7 µg L^{-1}; Li, 0.4-2.2 µg L^{-1}; Cs, 0.2-1.9 µg L^{-1}; and V, 0.1-1.8 µg L^{-1}. The concentrations of Fe (1.6-6.4 mg L^{-1}) and Mn (16-69 µg L^{-1}) were also determined and were in the agreement with previous reports. By application of general linear modeling, the influence of different abiotic (metal exposure level) and biotic parameters (fish sex, age, size and condition) on metal bioaccumulation was tested. It was established that bioaccumulation of many metals in fish depended on various physiological conditions, wherein Ba could be singled out as metal exhibiting the strongest association with one of biotic parameters, being significantly higher in smaller fish. However, it was also undoubtedly demonstrated that the concentrations of three metals can be applied as reliable indicators of metal exposure even in the conditions of low or moderate water contamination, such as observed in the Sutla River, and those were nonessential elements Li and Cs, and essential element Fe. The results of our study present an important contribution to maintenance of high ecological status of European freshwaters, through enrichment of knowledge on the bioaccumulation of various metals in gills of European chub as frequently applied bioindicator species in monitoring of water pollution.

Keywords: bioaccumulation, contamination, European chub, gills, metals, river

1. Introduction

Due to metal toxicity, persistence and tendency for bioaccumulation, metal contamination presents an important environmental issue, especially for aquatic ecosystems. Increased input of metals in natural waters can origin from different natural or anthropogenic sources, among which diffuse agricultural pollution presents one of the most serious threats to water quality (Environment Agency 2006). In small rural rivers across the world, which are vital for the life and the economy of the areas they are flowing through, agricultural runoff is often main source of water contamination, followed by small industrial facilities and municipal wastewater outlets. Although total waste input from such sources does not have to be immense, the fact that small watercourses also have small dilution capacity can lead to considerable water and sediment contamination (Dragun et al. 2011). The example can be found in the Sutla River, a medium sized rural watercourse on the border of Croatia and Slovenia, which was proven as more contaminated compared with many major rivers under significant industrial impact, due to their higher water discharge and thus also higher dilution capacity (Dragun et al. 2011). In the Sutla River, a high number of metals were found in increased concentrations, and among them it is important to point out metals which are characteristic for agricultural and municipal water contamination: Na and K, which are characteristic for sewage (Dautović 2006), Li, which is characteristic for thermal waters (Fiket et al. 2007), as well as Ba, Co, K, Rb, Sr, and V, which can be found increased near the corn fields and cultivated land due to application of fertilizers (Senesi et al. 1983; Vachirapatama et al. 2002; Schrauzer 2004). All these elements can be commonly found in high concentrations in the water of small rural watercourses, and yet their bioaccumulation and possible effects on aquatic organisms are studied quite rarely. The studies of metal toxicity and bioaccumulation in aquatic organisms more often refer to metals defined as priority toxic pollutants, such as Cd, Pb, Ni, and Hg (EPCEU 2008, 2013), as well as to important essential metals, such as Cu, Fe, Mn, and Zn, and only sporadically to the other metals.

In our study on the Sutla River, metal bioaccumulation was investigated using a bioindicator organism common for European freshwaters, namely European chub (*Squalius cephalus*), and the gills were selected as a target organ due to their direct contact with the river water and fast response to changes in the exposure level (Barišić et al. 2015). At this point, a number of reports on bioaccumulation of different metals in the chub gills can be found, namely report on Cd and Zn accumulation in chub from the Lot River in France (Andres et al. 2000), report on Cd, Cr, Hg, and Pb accumulation in chub from lake Beyşehir in Turkey (Altındağ and Yiğit 2005), report on Cd, Cr, Cu, Hg, Ni, Pb, and Zn accumulation in chub from Jihlava River in Czech Republic (Spurný et al. 2009), report on B, Co, Cr, Cu, Fe, Mg, Mn, Ni, and Zn accumulation in chub from Enne Dame Lake in Turkey (Uysal et al. 2009), report on Al, As, Cd, Fe, Mn, Ni, Se, and Si accumulation in chub from the Delice River in Turkey (Akbulut and Tuncer 2011), and the most recent report on Cd, Cr, Cu, Mn, Ni, Pb, and Zn accumulation in chub from Yamula Dam lake in Turkey (Duman and Kar 2012).

However, all these published data refer to total tissue metal concentrations in acid digested gill tissues, whereas our research was focused on metals in soluble gill fraction. The benefit of our approach can be ascribed to the following facts: (1) cytosolic metal concentrations in organs of aquatic organisms are often more responsive to contamination than whole body burdens and, therefore, more representative of the bioavailable concentrations in the water (Langston et al. 1998); (2) metal toxicity is often not related to total metal burden in the specific organ, because large proportions of bioaccumulated metals can be present in a detoxified form (McGeer et al. 2012), for example within metal rich granules, which can be removed in the process of isolation of soluble tissue fraction, together with cellular debris and mitochondria (Campbell et al. 2005; Giguère et al. 2006). As a confirmation, metal concentrations in soluble fractions of different fish organs have been repeatedly proven as reliable indicators of water contamination with metals (Campbell et al. 2005, 2008; Giguère et al. 2006). In addition, metals present in the soluble tissue fractions of aquatic organisms, i.e. within lisosomes and microsomes, or associated with enzymes and metallothioneins, can be considered as metals trophically available to predators (Wallace and Luoma 2003). But, to our knowledge, we have provided the only data on metal concentrations in soluble fractions of several organs (gills, liver and intestine) of European chub, which are indispensable for monitoring of metal contamination in European freshwaters (Dragun et al. 2015). And, specifically, we have so far provided the information for only six elements in soluble gill fractions, namely for Cd, Cu, Fe, Mn, and Zn in chub from the Sava River (Dragun et al. 2007, 2009) and for Cd, Cu, Pb, and Zn in chub from the Sutla River (Dragun et al. 2012, 2013b).

Therefore, during the study on the Sutla River, our goal was to extend the research on the other metals (Ba, Ca, Co, Cs, Fe, K, Li, Mg, Mn, Mo, Na, Rb, Sr, and V), which are especially important for small rural and agriculturally impacted watercourses, and to increase the knowledge on their bioaccumulation in the gills of European chub, as an important bioindicator species in European freshwaters. By application of general linear modeling, we have tested the association of several abiotic and biotic factors with metal concentrations in soluble fraction of chub gills, namely metal exposure in the river water, fish sex, age and size, condition index, gonadosomatic index and the size of the gills, and established which factor had the predominant influence on bioaccumulation of each particular metal. Finally, the applied statistical approach gave us the opportunity to identify the metals which concentrations in soluble fraction of chub gills could be applied as credible indicators of metal exposure in monitoring of natural freshwaters.

2. Materials and methods

2.1 Study period and area

The study was performed in the late summer of 2009 (from September 14th to 16th). It was carried out at the Sutla River, which is 91 km long left tributary to the Sava River. The Sutla River mostly forms the state border between Croatia and Slovenia. It has a catchment area of 581 km^2, and its water discharge in 2009 was in the range from 0.73 to 68.8 m^3 s^{-1} and (Dragun et al. 2011). This river is considered as moderately contaminated, and the known contamination sources refer to small industrial facility (glass factory), municipal wastewaters, agricultural runoff, and thermal bath discharges (Dragun et al. 2011). Fish were sampled at five locations, starting from the source of the Sutla River to its confluence with the Sava River. The map of study area with marked sampling sites is presented in Fig 1. and was previously published by Dragun et al. (2012, 2013b). Detailed physico-chemical and microbiological characterization of the river water quality at the selected sampling sites in September/October of 2009 was also previously published (Dragun et al. 2011). The samples of surface river water for dissolved metal analysis were taken simultaneously with fish sampling, and the measured concentrations of 14 elements discussed in this study, as well as pH and dissolved oxygen values are presented in Table 1 (data were extracted from previously published detailed study on river water quality; Dragun et al. 2011).

2.2 Fish sampling

The selected indicator organism for this study was European chub (*S. cephalus* L.), wide spread fish species in European freshwaters. At each sampling site, 15 chub specimens were caught, or in total 75 fish. The sampling was performed by electro fishing, according to the Croatian standard HRN EN 14011:2005. The captured fish were kept alive in aerated water tank till further processing in the laboratory. Prior to sacrifice and dissection, the fish were anesthetized with Clove oil (Sigma Aldrich). Total mass and total length were recorded, then the gills and the gonads were isolated and weighed, and the gills were stored at -80°C for further analyses. Gill index and gonadosomatic index (GSI) were calculated based on the ratio of gill and gonad mass, respectively, to total chub mass (Şaşi 2004). Fulton condition indices (FCI) were calculated according to Rätz and Lloret (2003). The age was determined by counting growth zones on scales, which were taken dorsolaterally below the dorsal fin; for purpose of age determination optical microscope BH-2 (Olympus) was used (Ognev and Fink 1956, Treer et al. 1995). Sex was determined by both macroscopic and microscopic examination of gonads. For microscopic identification of sex, a section of gonad tissue from each fish was placed on a glass microscope slide, and the slides were viewed under a 40 and 100 times amplification using optical microscope BH-2 (Olympus). Basic characteristics of sampled fish are presented in Table 2, whereas detailed analysis of spatial variability of biometric parameters was published previously (Dragun et al. 2013b).

2.3 Isolation of soluble fractions from chub gills

The samples of gill tissue were first cut into small pieces. Then cooled homogenization buffer [20 mM Tris-HCl/Base (Sigma, pH 8.6 at 4°C) supplemented with reducing agent (2 mM dithiotreitol, Sigma)] was added (w/v 1:5). It was followed by homogenization by 10 strokes of Potter-Elvehjem homogenizer (Glas-Col, USA) in ice cooled tube at 6,000 rpm. For better separation, the homogenates were centrifuged subsequently two times in the Avanti J-E centrifuge (Beckman Coulter) at 50,000×g for 2 h at 4°C. Supernatant obtained after second centrifugation (S50), which represents water soluble cytosolic tissue fraction containing lysosomes and microsomes (Bonneris et al. 2005), was separated and stored at -20°C for subsequent metal analyses.

2.4 Determination of metal concentrations in the soluble gill fractions

Preparation for metal measurements in soluble fractions of chub gills included only sample dilution with Milli-Q water and acidification (Dragun et al. 2013a). Dilution factor was 100 for Na, K, Mg and Ca, and 10 for the remaining elements. Each sample was acidified with HNO_3 (*Suprapur*, Merck, Germany) to a final acid concentration in the samples of 0.65%. Measurement of 14 elements presented in this paper was performed on a high-resolution inductively coupled plasma mass spectrometer (HR ICPMS Element 2, Thermo Finnigan, Germany) equipped with an autosampler ASX 510 (CETAC Technologies, USA) and sample introduction kit consisting of a SeaSpray nebulizer and cyclonic spray chamber Twister. Typical instrumental conditions and measurement parameters used throughout the work were reported previously (Fiket et al. 2007). Measurements of ^{7}Li, ^{85}Rb, ^{98}Mo, and ^{133}Cs were operated in low-resolution mode; of ^{23}Na, ^{24}Mg, ^{42}Ca, ^{51}V, ^{55}Mn, ^{56}Fe, ^{59}Co,

^{86}Sr, and ^{138}Ba in medium resolution mode; and of ^{39}K in high resolution mode. Two external calibrations were performed, one using standard containing Na, K, Mg, and Ca (Fluka, Germany) and the other using multielement stock standard solution for trace elements (Analitika, Czech Republic). All standards were prepared in 1.3% HNO_3 (Suprapur; Merck, Germany). Prior to measurement, In (1 µg L^{-1}; indium atomic spectroscopy standard solution, Fluka, Germany) was added to all samples and standards as an internal standard (Fiket et al. 2007).

2.5 Analytical quality control

All measurements were performed in duplicate. For checking the accuracy of measurements by HR ICP-MS, quality control samples obtained from UNEP GEMS (QC trace metals, catalog no. 8072, lot no. 146142-146143; QC minerals, catalog no. 8052, lot no. 146138-146139; Burlington, Canada) were used. A generally good agreement was observed between our data and certified values, with obtained recoveries generally in the range of 99±4% for Na, 117±4% for K, 96±6% for Mg, 98±4% for Ca, 111±2% for Sr, 110±4% for Ba, 114±4% for V, 112±7% for Mn, 99±2% for Fe, 112±3% for Co and 104±6% for Mo. Limits of detection (LOD) and limits of quantification (LOQ) were calculated as three and ten standard deviations, respectively, of ten consecutive trace element determinations in the blank sample (2 mM Tris-HCl/Base, 0.2 mM dithiotreitol, 0.65% HNO_3). LODs and LOQs for diluted samples, as well as corresponding concentrations which could be detected and quantified in undiluted soluble gill fractions are presented in Table 3.

2.6 Data processing and statistical analyses

Statistical program SigmaPlot 11.0 for Windows was applied for creation of graphs. The data analysis was performed using SAS/STAT 13.2 software, Version 9.4 of the SAS System for Windows. The logarithmic transformation (3+ln(x)) was used to achieve normality and variance homogeneity due to data deviation from the normal distribution. The GLMSELECT procedure with stepwise option (both ways), which applies traditional approach, in which the sequence of additions and deletions is determined by significance level (95%) as selection criteria, was used for finding model specification for each element. The final models were obtained as general linear models (GLM procedure with intercept, SS4 option on model statement, including main effects and their interactions, where appropriate). Group means testing was conducted by LSMEANS option with Tukey-Kramer multiple comparison adjustment. To explore estimated sizes of the effects for model variables and their interactions for each analysis, option EFFECTSIZE was used. Comparison of metal concentration in soluble gill fractions between sites was performed by analysis of variance with Tukey-Kramer multiple comparison adjustment *post-hoc* test. The significance level in all statistical analyses was set at $p<0.05$.

3. Results and discussion

Fourteen elements, which have been analyzed during this study, could be categorized by different criteria:

- according to their concentrations in the water, as well as in the organisms, as major (Na, K, Mg, and Ca) and trace (Li, Rb, Cs, Sr, Ba, V, Mn, Fe, Co, and Mo) elements;
- according to their functions in living organisms, as essential (Na, K, Mg, Ca, V, Mn, Fe, Co, and Mo) and nonessential (Li, Cs, Rb, Sr, and Ba) elements;
- and finally, according to their chemical properties, as alkali metals (Li, Na, K, Rb, and Cs), alkaline earth metals (Mg, Ca, Sr, and Ba) and transition metals (V, Mn, Fe, Co, and Mo).

Metal concentration ranges in soluble tissue fractions, as well as the causes of their variability within fish organs probably depend on the very nature of the studied elements and thus also on these three classifications.

If chub from all five sampling sites were considered together, the highest concentration ranges in the soluble gill fractions were determined, as expected, for four major elements and Fe (mg L^{-1}): K (225-895) > Na (78-366) > Ca (19-62) > Mg (13-47) > Fe (1.6-6.4). The concentrations of the remaining elements were found in the following ranges (µg L^{-1}): Rb (164-1762) > Sr (24-81) > Mn (16-69) > Ba (13-67) > Mo (1.3-16) > Co (0.7-2.7) > Li (0.4-2.2) > Cs (0.2-1.9) > V (0.1-1.8). Amongst measured elements, concentrations in soluble fractions of chub gills were previously reported only for Fe and Mn. Those concentrations, referring to chub caught in the Sava River in autumn seasons of two consecutive years, 2005 (Dragun et al. 2007) and 2006 (unpublished results), amounted on average to 3.9 mg L^{-1} and 5.4 mg L^{-1} for Fe and 55 µg L^{-1} and 42 µg L^{-1} for Mn, respectively, and corresponded well with the results obtained in the present study.

When metal concentrations in soluble gill fractions at five sampling sites were compared, notable and statistically significant between-site concentration variability was observed for all elements, but it was the least pronounced for four major elements (Na, Fig. 2b; K, Fig. 2c; Mg, Fig. 3a; and Ca, Fig. 3b). As seen from Table 1, five sampling sites differed in the level of metal contamination of the river water, and the lowest concentrations were found at site 1 (Hum na Sutli) for Li, Mg, Sr, Ba, V, Co, and Mo, at site 4 (Klanjec) for Na, K, Rb, Cs, and Ca, and at site 5 (Drenje Brdovečko) for Mn and Fe (Dragun et al. 2011). The highest water metal concentrations, on the other hand, were generally found at only one site, the site 2 (Donje Brezno), for all metals, except for Ba which was increased in the water at sites 2-5 compared to site 1. Contrary to water metal concentrations, tissue concentrations exhibited diversified patterns of variability, which did not seem to reflect solely the level of metal exposure. To establish which factors could be mainly associated with the variability of each particular analyzed element, we have created complex univariate model for each element separately (Tables 4-6). The factors which were considered as possible sources of metal variability in chub gills were the following: (1) metal exposure in the river water, (2) fish sex, age, and size expressed as total chub mass, and (3) fish condition, specifically FCI, GSI and gill index. Rather good explanation of metal variability in chub gills by created models was obtained for Li (87%), Co (64%), Ba (61%), Fe (54%), and V (53%), whereas it was somewhat weaker for Ca (42%), Mn (41%), Sr (39%), Cs (33%), Rb (27%) and Mo (21%). For all the listed metals, except for Rb, one or several factors (effects) could be distinguished as being significantly associated with their variability. Only in the case of Rb, none of the tested factors had statistically significant influence, although their combination (interaction) explained 27% of Rb variability. For Na, K and Mg, the percentage of variability explained by the proposed models was extremely low (<10%), which was consistent with their generally very low concentration variability.

3.1. Association with the level of exposure

Out of 14 analyzed elements, only three could be directly associated with the level of exposure in the Sutla River water: Li (Fig. 2a, Table 4), Cs (Fig. 2e, Table 4) and Fe (Fig. 4c, Table 6). For these three elements, the main effect in the model, which was 3-20 times stronger than the influence of any other tested factor (according to Semipartial Eta-Square, Tables 4 and 6), referred to higher metal concentrations in gills found at the sites with higher water metal concentrations. Such behavior could be expected for nonessential elements, such as Li and Cs, since their concentrations are generally not submitted to strict homeostatic control in living organisms, unlike the concentrations of essential elements. It was previously reported that one of the main factors affecting Cs bioaccumulation is its concentration in the water (Rowan and Rasmussen 1994; Bird et al. 1999). Pinder et al. (2009) also reported that although Cs concentration accumulated in fish muscle could be affected by numerous factors, such as K concentration in the water, it is principally determined by its initial concentration in the water column. Contrary to Cs, Rb in chub gills did not exhibited association with the level of exposure at the concentration in the river water lower than 10 $\mu g\ L^{-1}$ (Fig. 2d, Table 4). Explanation can be found in the fact that, although Rb and Cs are chemically similar and both are chemical analogues of K, Rb is more rapidly depurated than Cs from freshwater biota (Campbell et al. 2005). Among these three chemical analogues, Cs has the longest biological half-life time followed by Rb (~100 days and 15-16 days, respectively, in channel catfish, *Ictalurus punctatus*), whereas the half-life time of K is measurable in hours (Peters et al. 1999). In addition, according to existing literature both K and Rb do not tend to accumulate in any specific organ and tissue, contrary to Cs which was already repeatedly reported to be accumulated in skeletal muscle (Peters et al. 1999). In our study, Cs accumulation in the gills was evident already at rather low Cs concentration in the river water of 58 ng L^{-1} (Table 1). This was further confirmed by the study on Vardar chub (*Squalius vardarensis*), where Cs accumulation was observed in both the gills and the liver, but at much higher dissolved Cs concentrations in the water column of 1 μg L^{-1} or even higher (Ramani et al. 2014a, b).

The remaining analyzed nonessential elements, Sr and Ba, have not reflected the exposure level in the water (Fig. 3c-d, Table 5). Similar chemical properties of Sr and Ba to Ca could have caused them to have similar patterns of variability as that essential element. For example, in the gills of chub from the Sutla River Sr and Ca were positively associated (R^2=0.356, p<0.0001). This finding was opposite to previous reports that Sr and Ca inhibit each other's uptake completely competitively on the level of the whole organism, as well as in the gills of common carp (*Cyprinus carpio*) (Chowdhury et al. 2000; Chowdhury and Blust 2001), due to uptake of both elements through Ca transport systems located in the chloride cells of gills (Flik et al. 1995). In the natural aquatic systems, metals are present in different chemical forms, and, often, metal complexation with water-soluble ligands decreases metal uptake by aquatic organisms. For example, natural organic matter can form complexes with dissolved metals, thereby reducing bioavailability and/or toxicity of some metals to aquatic organisms (Smith et al. 2015). However, it has been demonstrated for Sr and Ca uptake that in the presence of certain complexing ligands it is not always a mere function of the free water metal concentrations, and that certain complex species may contribute significantly to their overall uptake (Chowdhury and Blust 2002). Therefore, different chemical composition of the river water at different sampling sites of the Sutla River, and not only dissolved Sr concentrations, could have influenced the observed Sr bioaccumulation in chub gills.

Contrary, Fe as an essential element reflected the level of metal exposure (Fig. 4c, Table 6), as was previously reported for gills of European chub (*S. cephalus*) from the Sava River (Dragun et al. 2009). Among essential elements, Fe is well known for its tendency to accumulate in living organisms in a form of ferritin (e.g. in fish liver and gills; Krasnići et al. 2013, 2014), as storage in a case of nutritional deficiency. Our results are in accordance with the demonstration made by Bury and Grosell (2003) that Fe can be absorbed from the water by the gills in teleost fish. Contrary to Fe, the most of the essential elements are subjected to rather strict homeostatic control in fish organism, and only the conditions of high metal exposure, much higher compared to naturally found water metal concentrations, would result in metal bioaccumulation in fish organs. Accordingly, although significant association with water metal concentrations was also observed for essential elements Ca (Table 5), Mo and V (Table 6), it was of opposite direction, i.e. lower gill concentrations were observed at the sites with higher metal exposure, indicating that they were probably the reflection of some other biotic or abiotic factor, negatively associated with metal contamination of the river water. It is in accordance with the findings of other authors about essential elements. Richards and Playle (1998) reported that no significant accumulation of essential element Co by gills of trout (*Oncorhynchus mykiss*) occurs after exposure to natural waters supplemented by Co (~0.5 mg L^{-1}), probably due to Co competition with Ca and complexation by dissolved organic matter. Only after exposure to much higher and environmentally less realistic Co concentration (6.8 mg L^{-1}), Co was found 30 times increased in the gills of a cyprinid fish *Capoeta fusca* compared to control group (Mansouri et al. 2013). Similarly, only after exposure to very high Mo concentrations in the water (5-250 mg L^{-1}), Mo was accumulated in a dose-dependent manner in the gills of juvenile kokanee salmon (*Oncorhynchus nerka*; Reid 2002).

3.2. Association with fish sex, age and size

Only two elements exhibited significant association with chub sex, Ca (Table 5) and Co (Table 6). Calcium was significantly higher in males and Co in females. The fish from sites 1, 3 and 5 were either all or predominantly female (Table 2), and therefore it could be expected for Ca to be lower, and for Co to be higher at those three sampling sites. However, Ca variability between sites was generally very low and did not follow the suggested pattern of lower concentrations at sites inhabited predominantly by female fish (Fig. 3b), whereas the spatial distribution of Co followed only partially the suggested pattern of higher concentrations at those sites (Fig. 4d). It can be concluded that the variability of both elements was predominantly caused by some other factors, as also seen from the created models for these elements, in which the sex was either among the least significant influences (for Ca) or did not stand out from other factors (for Co).

The association of chub age and total mass with metal concentrations in gills was also observed, however, mainly representing one of the less significant effects. Age was significantly associated with the variability of five elements: Li (Table 4), Sr (Table 5), Fe, Co, and Mn (Table 6), with higher concentrations characteristic for older fish. Positive association with fish size was obtained only for Cs (Table 4), whereas all the other observed metal associations with total chub mass were negative, i.e. the concentrations of several metals seemed to be increased in smaller fish (K, Mg, Ca, Sr, Ba, and Mo; Tables 4-6). Among the elements associated to chub age or size, only Ba stands out, since large portion of its variability actually could be attributed to changes in chub mass; among tested factors total chub mass had 15 times stronger influence on Ba concentration in chub gills compared to all the other tested factors (Table 5).

Negative association with fish size could possibly be attributed to faster metabolism and rate of uptake in younger and thus smaller fish (Wiener and Giesy 1979). It could be observed that such association was obtained for all studied alkaline earth elements (Mg, Ca, Sr, and Ba), whereas only one alkali (K) and one transition metal (Mo) exhibited such behavior. On the other hand, positive association with fish age and size means that higher concentrations could be expected in older and thus bigger fish, and indicates time-related accumulation. We have already reported positive dependence on fish age and size for Fe and Mn in gills of chub (*S. cephalus*) from the Sava River (Dragun et al. 2007). Previously, positive association with fish size was also reported for V in gills of marine fish *Tylosurus crocodiles* (Yazdanabad et al. 2014), and for Fe in gills of pike (*Esox lucius*; Rajkowska and Protasowicki 2013). Accordingly, such association seem to be more common for transition elements (Fe, Co, Mn, V), as well as for elements which exhibit tendency to accumulate in fish gills after increased environmental exposure, such as Li and Cs.

3.3. Association with fish condition

Evaluation of metal association with fish condition indicated that few elements had comparable spatial distribution as tested indices, namely FCI, GSI, or gill index. In our previous paper we have pointed out that the changes of these three indices were at least partially a consequence of changes in water saturation with oxygen (Dragun et al. 2013b). Both GSI and gill index were increased at two upstream sites, which were characterized by low oxygen saturation (dissolved oxygen on average 48-60%), whereas FCI had opposite trend, with the highest values at downstream, highly oxygenated, sites (dissolved oxygen on average 84-99%) (Dragun et al. 2011, 2013b). Therefore, the increased or decreased metal bioaccumulation associated to changes in FCI, GSI or gill index could have occurred due to changes in fish physiology, but also due to changes in metal speciation in the river water as a result of varying water oxygenation. As example, Fe (Fig. 4c), Mo (Fig. 4e) and Ca (Fig. 3b) showed spatial distribution opposite to FCI and comparable to GSI and gill index (Table 2), with decreasing concentration trend towards downstream sites. This was confirmed by significant negative association of Fe with FCI (Table 6), and positive of Ca (Table 5) and Mo (Table 6) with GSI. For Ca and Mo, the association with GSI even presented the strongest influence in the model. In other words, these three metals were increased in chub caught at the sites characterized by low oxygen level (Table 1). For Fe, which participates in oxygen transport as a part of hemoglobin, it could be hypothesized that concentration increase was a result of fish need to enhance oxygen uptake. However, it is also possible that the reason was of purely chemical nature. It is well known that Fe enters the gills in the ferrous (Fe^{2+}) state (Bury and Grosell 2003), and therefore low oxygen saturation in the Sutla River water at two upstream sites could lead to Fe reduction from Fe^{3+} to Fe^{2+}, and consequently to its higher availability for uptake by chub gills, only confirming Fe concentration in chub gills as a good indicator of Fe bioavailability. Contrary, it cannot be expected for Mo bioavailability to increase under the conditions of low oxygen, because it is commonly known that a soluble Mo form, molybdate ion MoO_4^{2-}, is formed only in contact with oxygen, whereas the most of the other molybdenum compounds have low solubility in water. Therefore, it is more likely to presume that increased Mo concentration in chub gills at low oxygenated sites could have resulted from chemical similarity with Fe and concurrent accumulation of both metals in gills.

Opposite trend was observed for Mn and Co in chub gills with lower concentrations at upstream sites compared to downstream sites. However, only for Mn this was confirmed by negative association with GSI, which even had the strongest influence on Mn out of all analyzed parameters in the model (Table 6). Although the association of Co with fish condition could not be established by obtained model, similar behavior of Mn and Co was confirmed by their significant positive association (R^2=0.220, p<0.0001). Both of these metals were present in lower concentrations in the gills of chub with more developed gonads. It could be related to their possible role in fish reproductive cycle, since Mn is important in fish embryonic development (Rajkowska and Protasowicki 2013), and possibly its transport to gonads could result in decreased concentrations in other tissues, such as the gills. Furthermore, since Mn spatial distribution was partly opposite to Ca spatial distribution, competition of Ca^{2+} ions with Mn^{2+} ions for absorption through the gill surface can also be presumed, as a cause of decreased Mn bioavailability to fish at the sites with higher Ca concentrations (Seymore et al. 1995), which is similar to finding of Birungi et al. (2007) for *Oreochromis niloticus*. There was another possible explanation for lower metal concentrations in soluble gill fractions, especially of Mn, at low oxygenated upstream sites. It was previously observed that under conditions of moderate hypoxia (~50% oxygen saturation) fish could develop mechanisms of adaptation, which could cause lower bioaccumulation of some metals (Dolci et al. 2013). It was specifically observed for Mn in silver catfish, *Rhamdia quelen* (Dolci et al. 2013), despite the fact that reducing conditions in the river water generally result in increased Mn bioavailability, due to its transfer from particulate to dissolved metal fraction.

4. Conclusion

Study of accumulation of 14 environmentally relevant metals (alkali metals: Li, Na, K, Rb, Cs; alkaline earth metals: Mg, Ca, Sr, Ba; transition metals: V, Mn, Fe, Co, Mo) in the soluble gill fractions of European chub (*S. cephalus*) revealed that only 3 of these elements could be used as reliable indicators of moderate exposure in the river water, namely two nonessential elements, Li and Cs, and one essential element, Fe. All the other elements either exhibited small differences between sites indicating strict homeostatic control in the fish organism (like major essential elements Na, K, Mg, Ca) or were at least partially associated to changes in fish physiology. Although, according to applied general linear modeling, chub sex, age and size accounted only for a small part of metal variability in chub gills, some patterns still could be observed. Negative association was obtained with fish age or size in the group of alkaline earth elements, and it was especially strong for Ba. Contrary, positive association of these two parameters was obtained with transition elements, such as Fe, Mn and Co. Positive association with age or size was further established for metals exhibiting clear tendency for accumulation in chub gills under the conditions of increased exposure, namely Li and Cs. The influence of the other abiotic and biotic factors on metal variability in fish organs was also considered, from variability in water composition (e.g. in oxygen saturation) to consequent changes in fish physiology (e.g. differences in oxygen demand, varying gonad maturity). Such factors possibly could affect metal speciation and their availability in the water, as well as fish physiological need for specific metals, which altogether could finally influence metal bioaccumulation in fish organs, as specifically presumed for Fe, Mn, Mo, Co, and Ca. The results presented in this paper represent the first data on the concentrations of 12 studied elements, with the only exception of previously studied Fe and Mn, in the soluble fraction of chub gills. Since European chub, due to its wide geographical distribution, is an important bioindicator organism, increasing knowledge and understanding of patterns of metal bioaccumulation in its organs, specifically in metabolically available soluble fractions, could present an important contribution to the monitoring and preservation of European freshwaters.

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Table 1. The values of pH, dissolved oxygen level and dissolved macro and trace element concentrations in the Sutla River water at five sampling sites (1 – Hum na Sutli; 2 – Donje Brezno; 3 – Kumrovec; 4 – Klanjec; 5 – Drenje Brdovečko) in September of 2009 (extracted from Dragun et al. 2011). The results of dissolved metal concentrations are presented in a form of mean and standard deviation of two parallel measurements.

	Ratio*	1	2	3	4	5
pH		7.79	7.94	8.20	8.18	8.24
Dissolved oxygen / %		51.2	53.7	94.1	91.7	86.6
Li / µg L^{-1}	13.3:1	2.19±0.02	29.14±0.58	5.17±0.32	4.21±0.20	4.18±0.09
Na / mg L^{-1}	7.6:1	14.20±0.39	89.43±0.66	14.59±0.37	11.70±0.12	11.76±0.03
K / mg L^{-1}	3.1:1	3.84±0.02	10.78±0.33	3.84±0.10	3.45±0.15	3.74±0.11
Rb / µg L^{-1}	5.6:1	2.40±0.14	9.77±0.01	1.91±0.05	1.76±0.09	1.84±0.02
Cs / µg L^{-1}	58:1	0.002±0.001	0.058±0.000	0.002±0.001	0.001±0.000	0.001±0.000
Mg / mg L^{-1}	1.2:1	20.09±0.51	25.01±0.33	22.33±0.59	22.19±0.27	20.94±0.17
Ca / mg L^{-1}	1.1:1	68.73±1.27	78.13±1.21	70.54±1.27	68.19±1.48	70.36±0.16
Sr / µg L^{-1}	1.7:1	222.5±43.0	388.0±20.0	328.0±12.7	325.5±7.3	339.2±8.5
Ba / µg L^{-1}	2.5:1	18.90±2.35	36.59±0.02	41.06±0.19	46.84±0.58	44.07±0.66
V / µg L^{-1}	3.4:1	0.220±0.013	0.736±0.003	0.568±0.007	0.502±0.029	0.341±0.000
Mn / µg L^{-1}	36.9:1	2.11±0.04	16.31±3.76	2.41±0.47	3.30±0.63	0.442±0.045
Fe / µg L^{-1}	6.2:1	12.26±1.45	19.58±1.06	4.16±0.09	6.70±2.70	3.14±0.02
Co / µg L^{-1}	5.2:1	0.066±0.007	0.342±0.016	0.092±0.005	0.088±0.004	0.084±0.002
Mo / µg L^{-1}	15.0:1	0.562±0.036	8.41±0.23	1.53±0.06	1.26±0.04	1.43±0.013

*The ratio between the highest and the lowest measured dissolved concentration of each metal in the Sutla River.

Table 2. Basic characteristics of European chub caught at five sampling sites in the Sutla River (1 – Hum na Sutli; 2 – Donje Brezno; 3 – Kumrovec; 4 – Klanjec; 5 – Drenje Brdovečko) in September of 2009 (discussed in detail in Dragun et al. 2012, 2013).

	1	**2**	**3**	**4**	**5**
n	15	15	14[a]	15	15
Total body mass / g	132.1±63.8	77.1±23.8	139.1±113.3	127.6±49.7	122.3±82.0
Total body length / cm	23.4±3.8	19.9±2.1	22.6±6.1	23.0±3.0	21.8±3.9
FCI / (g cm^{-3})*100	0.96±0.07	0.95±0.09	0.99±0.06	1.00±0.06	1.09±0.08
GSI / %	2.86±0.40	1.82±0.68	0.86±0.35	0.69±0.32	0.49±0.13
Gill index / %	1.24±0.13	1.43±0.25	1.07±0.16	1.08±0.14	0.98±0.15
Sex (n – F/M)	15/0	8/7	14/0	7/8	14/1
Age	3.0±0.7	2.0±0.5	2.5±1.3	2.5±0.5	2.2±0.6

Legend: n – number of samples; F – females; M - males

[a] One fish was to small to obtain sufficient sample volume for all analyses.

Table 3. Limits of detection and quantification (LOD and LOQ, respectively; expressed as $\mu g\ L^{-1}$) for trace elements in diluted samples and soluble gill fraction of European chub gills.

	Diluted samples		**Soluble gill fraction**	
	LOD / $\mu g\ L^{-1}$	**LOQ / $\mu g\ L^{-1}$**	**LOD / $\mu g\ L^{-1}$**	**LOQ / $\mu g\ L^{-1}$**
Li	0.004	0.012	0.040	0.120
Rb	0.003	0.008	0.030	0.080
Cs	0.001	0.003	0.010	0.030
Sr	0.017	0.055	0.170	0.550
Ba	0.108	0.359	1.08	3.59
V	0.002	0.005	0.020	0.050
Mn	0.002	0.007	0.020	0.070
Fe	0.084	0.282	0.84	2.82
Co	0.002	0.007	0.020	0.070
Mo	0.004	0.012	0.040	0.120

Table 4. Univariate test statistics for models, main effects and their interactions, as well as size of the effects for natural logarithm transformed concentrations of alkali metals.

	R^2 / *p*-value of model	Main effects and interactions	*p*-value[a]	Semipartial Eta-Square[b]	Trend of changes
Li	0.869 / <0.0001	Intercept	<0.0001		
		Gill index	0.0013	0.0233	negative association
		Age	0.0092	0.0148	3 year old > 2 year old
		Sex	0.4309	0.0013	
		Li in water	<0.0001	0.6092	positive association
		TM	0.3986	0.0015	
		TM*Age	0.0050	0.0173	
		Age*Sex	0.0108	0.0141	
Na	0.063 / <0.0001	Intercept	<0.0001		
		FCI	0.0327	0.0626	negative association
K	0.059 / <0.0001	Intercept	<0.0001		
		TM	0.0386	0.0589	negative association
Rb	0.265 / <0.0001	Intercept	<0.0001		
		Age	0.0823	0.0341	
		FCI	0.1030	0.0300	
		GSI	0.8834	0.0002	
		FCI*Age	0.0485	0.0443	
		GSI*Age	0.0376	0.0494	
Cs	0.329 / <0.0001	Intercept	0.9014		
		Cs in water	<0.0001	0.3115	positive association
		TM	0.0029	0.0914	positive association

[a] *p*-value of main effects and interactions
[b] semipartial Eta-square of main effects and interactions - the proportion of total variation explained by each main effect and interaction from the corresponding model
Legend: R^2 – determination coefficient; TM - total chub mass; FCI - Fulton condition index; GSI - gonadosomatic index

Table 5. Univariate test statistics for models, main effects and their interactions, as well as size of the effects for natural logarithm transformed concentrations of alkaline earth metals.

	R^2 / *p*-value of model	Main effects and interactions	*p*-value[a]	Semipartial Eta-Square[b]	Trend of changes
Mg	0.072 / <0.0001	Intercept	<0.0001		
		TM	0.0214	0.0724	negative association
Ca	0.419 / <0.0001	Intercept	<0.0001		
		TM	<0.0001	0.1824	negative association
		GSI	<0.0001	0.2955	positive association
		Gill index	0.0111	0.0600	negative association
		Sex	0.0228	0.0478	males > females
		Ca in water	0.0310	0.0427	negative association
		Sex*Ca in water	0.6697	0.0016	
Sr	0.387 / <0.0001	Intercept	<0.0001		
		TM	<0.0001	0.2583	negative association
		Age	<0.0001	0.1658	3 year old > 2 year old
		TM*Age	<0.0001	0.1765	
Ba	0.614 / <0.0001	Intercept	<0.0001		
		TM	<0.0001	0.6138	negative association
		GSI	0.0277	0.0283	positive association
		Gill index	0.0079	0.0418	negative association

[a] *p*-value of main effects and interactions
[b] semipartial Eta-square of main effects and interactions - the proportion of total variation explained by each main effect and interaction from the corresponding model
Legend: R^2 – determination coefficient; TM - total chub mass; GSI - gonadosomatic index

Table 6. Univariate test statistics for models, main effects and their interactions, as well as size of the effects for natural logarithm transformed concentrations of transition metals.

	R^2 / *p*-value of model	Main effects and interactions	*p*-value[a]	Semipartial Eta-Square[b]	Trend of changes
V	0.530 / <0.0001	Intercept	0.0053		
		FCI	0.0282	0.0376	negative association
		GSI	0.1760	0.0140	
		Sex	0.7294	0.0009	
		V in water	<0.0001	0.1901	negative association
		GSI*V in water	<0.0001	0.1655	
		Sex*V in water	0.0028	0.0965	
Mn	0.408 / <0.0001	Intercept	<0.0001		
		GSI	<0.0001	0.3393	negative association
		Gill index	0.0002	0.1335	positive association
		Age	0.0010	0.1041	3 year old > 2 year old
		Sex	0.4186	0.0058	
		Age*Sex	0.0405	0.0385	
Fe	0.544 / <0.0001	Intercept	<0.0001		
		FCI	0.0073	0.0530	negative association
		Age	0.0161	0.0422	3 year old > 2 year old
		Fe in water	<0.0001	0.1746	positive association
		Age*Fe in water	0.0698	0.0383	
Co	0.640 / <0.0001	Intercept	0.1123		
		TM	0.0746	0.0201	
		Gill index	0.0658	0.0214	
		FCI	0.9175	0.0001	
		Age	0.0096	0.0437	3 year old > 2 year old
		Sex	0.0015	0.0673	females > males
		Co in water	0.3642	0.0125	
		FCI*Age	0.0106	0.0425	
		TM*Sex	0.3529	0.0053	
		FCI*Sex	0.0065	0.0486	
		Gill index*Co in water	0.4914	0.0088	
		Age*Sex	0.4323	0.0038	
Mo	0.208 / <0.0001	Intercept	<0.0001		
		GSI	0.0003	0.1660	positive association
		TM	0.0100	0.0806	negative association
		Mo in water	0.0183	0.0671	negative association

[a] *p*-value of main effects and interactions
[b] semipartial Eta-square of main effects and interactions - the proportion of total variation explained by each main effect and interaction from the corresponding model
Legend: R^2 – determination coefficient; TM - total chub mass; FCI - Fulton condition index; GSI - gonadosomatic index

Figure legends

Figure 1. The map of the Sutla River with marked sampling sites. The site legend: 1 – Hum na Sutli, 2 – Donje Brezno, 3 – Kumrovec, 4 – Klanjec, 5 – Drenje Brdovečko.

Figure 2. The concentrations (on wet mass basis) of 5 alkali metals in the soluble gill fractions of European chub (*Squalius cephalus*) caught at five sampling sites in the Sutla River in September of 2009: a) Li, b) Na, c) K, d) Rb, and e) Cs. The results are presented as box-plots. The boundaries of box-plot indicate 25th and 75th percentiles; a line within the box marks the median value; whiskers above and below the box indicate 10th and 90th percentiles, whereas the black dots present all outliers. Differences between sites are indicated with different letters (a, b, c), based on analysis of variance (p<0.0001 for all metals) with Tukey-Kramer multiple comparison adjustment *post-hoc* test (p<0.05). Number of samples per site was 14-15, as indicated in Table 2. Site legend: 1 – Hum na Sutli; 2 – Donje Brezno; 3 – Kumrovec; 4 – Klanjec; 5 – Drenje Brdovečko.

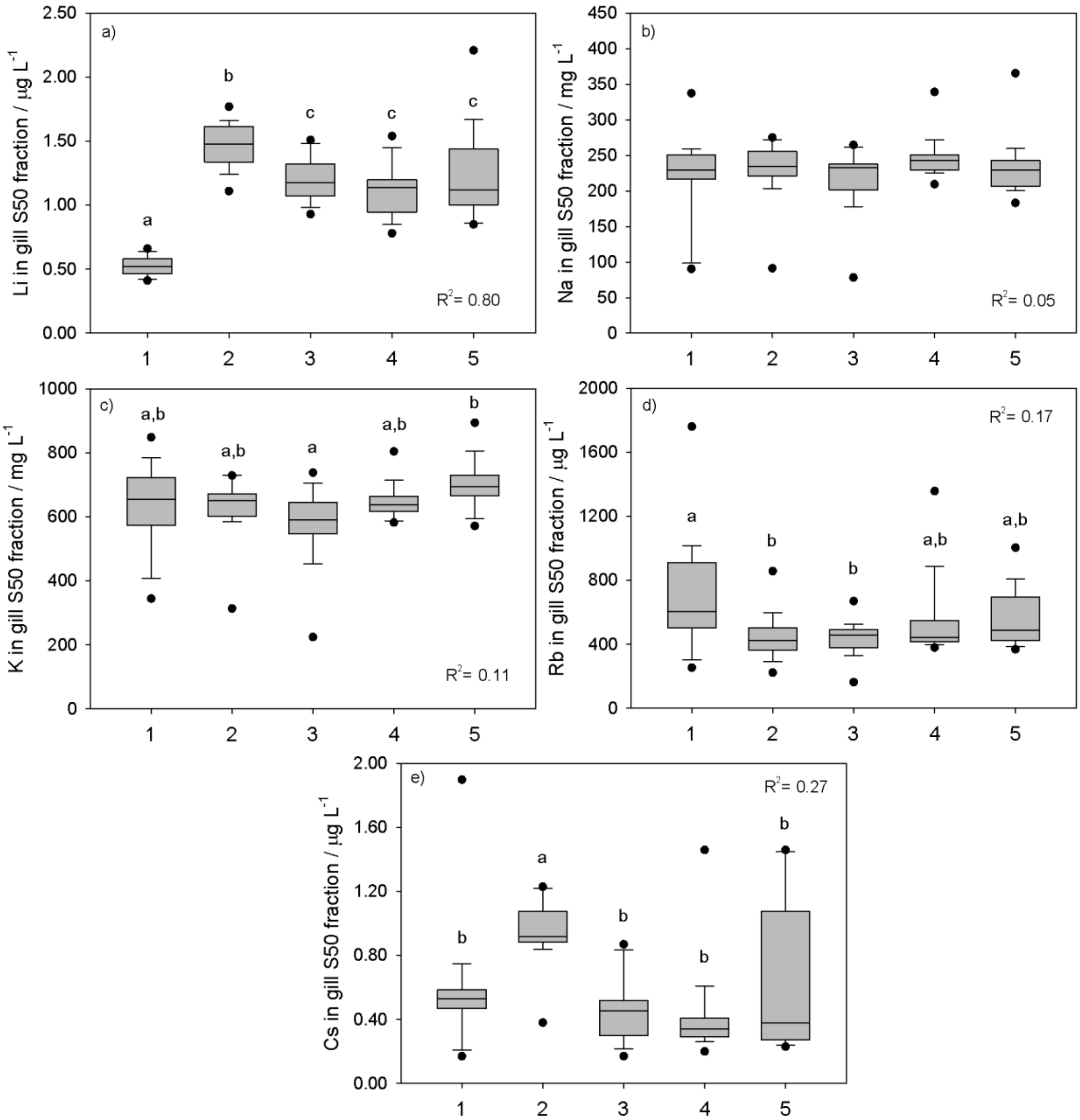

Figure 3. The concentrations (on wet mass basis) of 4 alkaline earth metals in the soluble gill fractions of European chub (*Squalius cephalus*) caught at five sampling sites in the Sutla River in September of 2009: a) Mg, b) Ca, c) Sr, and d) Ba. The results are presented as described in the caption of Fig. 1.

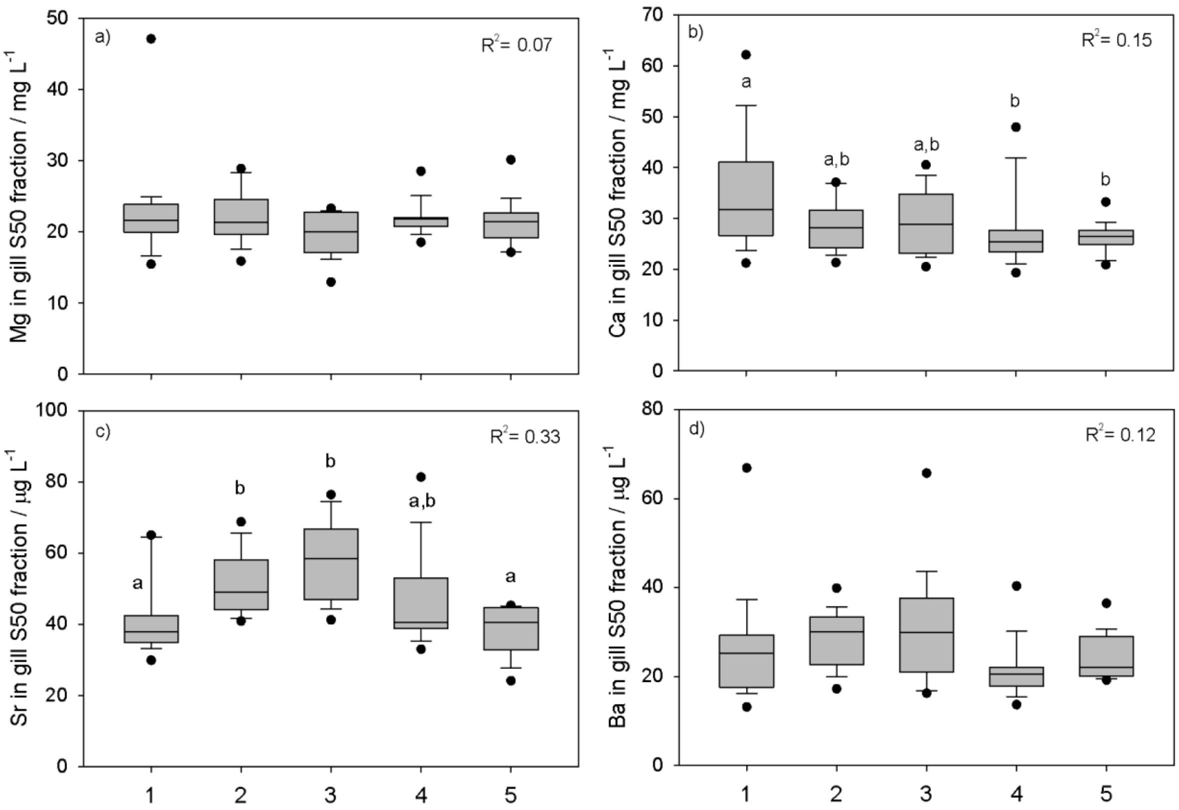

Figure 4. The concentrations (on wet mass basis) of 5 transition metals in the soluble gill fractions of European chub (*Squalius cephalus*) caught at five sampling sites in the Sutla River in September of 2009: a) V, b) Mn, c) Fe, d) Co, e) Mo. The results are presented as described in the caption of Fig. 1.