The influence of the season and the biotic factors on the cytosolic metal concentrations in the gills of the European chub (*Leuciscus cephalus* L.)

Zrinka Dragun*, Biserka Raspor, Marijana Podrug

Ruđer Bošković Institute, Division for Marine and Environmental Research, POBox 180, 10002 Zagreb, Croatia

* corresponding author
Phone: xx385-1-4680216;
Fax: xx385-1-4680242;
E-mail: zdragun@irb.hr

Abstract

The influence of the season and the biotic factors (age and gill mass) on metal and protein levels in the gill cytosol of the young chubs (2 and 3 years old) was studied in Sava River in autumn 2005 and spring 2006. The obtained results are the first reported cytosolic concentrations of five metals and proteins for the chub gills. The average levels in autumn and spring, respectively, for total cytosolic proteins were 11.2 and 19.9 mg mL^{-1}, for Zn 6.3 and 10.3 μg mL^{-1}, for Fe 3.9 and 9.6 μg mL^{-1}, for Cu 68.4 and 79.0 ng mL^{-1}, for Mn 55.0 and 63.5 ng mL^{-1}, and for Cd 2.9 and 3.6 ng mL^{-1}. The influence of the gill mass on both the protein and the metal levels was observed, but it was seasonally dependent. In autumn, positive correlations were obtained between the gill mass and four parameters (total proteins, Mn, Zn and Fe), and negative with Cu. Contrary, in spring, even negative correlations of total proteins

and some metals with the gill mass were observed. The proposed explanation for the different dependence of metal levels on the gill mass in autumn and spring was the seasonal difference in feeding intensity and metabolic rate, with presumably faster metabolism and water filtration through gills in spring. This hypothesis was further supported by the statistically significantly higher concentrations of the total proteins, Zn and Fe, as well as the Fulton condition indices in the spring period.

Keywords: bioindicator, fish, gill cytosol, metals, river

Introduction

To obtain more complete information on river water quality, it is not enough to determine the quantity of the chemical constituents in water. It is also crucial to define the effects that exposure to contaminants can induce in the aquatic organisms selected as bioindicators. Due to the several reasons, fish are often used as bioindicators. From the ecological point of view, fish are at the top of the aquatic food chain, and therefore mirror the combination of the biotic and abiotic conditions in the particular aquatic environment. In addition, their size and mass of their organs enable numerous analyses, while their long life span results in more pronounced effects, such as metal accumulation. Furthermore, since fish are used in human diet, it is also of primary importance to determine fish health status (Chovanec et al., 2003).

In freshwater fish, the main uptake routes for metals are water filtering through the gills and food consumption through the digestive tract (Heath, 1995). Since the gill and the intestine are in direct contact with the ambient water and ingested food, these organs are expected to respond quickly to changes in metal exposure (Kraemer et al., 2005). The metal

concentrations in the bioindicator tissues are usually determined in the completely digested tissue samples. However, Ferrarello et al. (2000) emphasized the significance of the cytosol fraction, because the metal ions, upon entering the cell, first react with the specific high molecular proteins, functioning as intracellular transporters. Therefore, in this study the metal concentrations were determined in the cytosol of the gill tissue of the European chub (*Leuciscus cephalus* L.), as the fish species widespread in European freshwater.

Prior investigations conducted on different fish species indicated that tissue metal concentrations are directly or indirectly influenced by a very large set of abiotic and biotic factors (Andres et al., 2000). Large percentage of tissue metal concentration variability can be, for example, ascribed to the differences in the fish size or age (Phillips, 1980; Linde et al., 1998). Therefore, it is important first to establish the relationship between the biotic factors and the cytosolic metal concentrations in two seasons, which are characteristic for the biota: autumn and spring. Only then the metal bioavailability in water, based on the metal accumulation in the fish tissues, could be assessed with more certainty and the appropriate sampling period could be selected. In our investigation, we have used the comprehensive data set obtained by sampling the fish in two seasons at five sites along the Sava River water-course, to study the dependence of the cytosolic metal levels in the chub gills on the season, as well as on the fish age and size.

Materials and methods

Fish sampling

Approximately 10-20 specimens of European chub (*Leuciscus cephalus* L.) per site were caught at five sites in Sava River (Croatia), in two seasons. The autumn sampling was accomplished within 10 days, at the end of September 2005. Due to the unfavourable weather conditions, the spring sampling, conducted during April and May 2006, lasted over one month. The selected sites (Figure 1) enclose the sector of Sava River water-course from Otok Samoborski (Croatian-Slovenian state border) to Jasenovac (the state border between Croatia and Bosnia and Herzegovina). The basic physico-chemical parameters of Sava river-water at selected sampling sites and the site coordinates recorded with GPSMAP 76CS (Garmin International, USA) are given in Table 1. 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. First, the biometric data were recorded, including the chub total length and total mass, while Fulton condition index was later calculated according to Rätz and Lloret (2003). The scales were taken dorsolaterally below the dorsal fin, and secluded for subsequent age determination. The age was determined by counting growth zones, which appear on calcified structures of fish body (scales), using optical microscope BH-2 (Olympus) (Ognev and Fink, 1956, Treer et al., 1995). After the fish were anesthetized with MS 222 (tricaine methane sulphonate, Sigma Aldrich) and sacrificed, the gills were isolated, weighted and stored at -80°C until further analyses. Sex was determined by histological examination of chub gonads.

Isolation of cytosolic fraction

The gill tissue samples were homogenized by homogenizer (Glas-Col, USA) in ice cooled tube at 6,000 rpm. The samples were first diluted 6 times with cooled homogenizing buffer (100 mM Tris-HCl/Base (Sigma), pH 8.1 at 4°C) supplemented with reducing agent (1 mM

dithiotreitol (Sigma)). The homogenates were then centrifuged in the Sorval RC28S centrifuge (Kendro, USA) at 50,000×g for 2 h at 4°C. Next, supernatant (S50) was separated and stored at -80°C for subsequent metal and protein analyses. The supernatant (S50) represents water soluble cytosolic tissue fraction.

Determination of total cytosolic protein concentration

Total cytosolic protein concentrations were measured according to Lowry (Lowry et al., 1951) in S50 tissue fraction. In the wells of microplate, 5 μL of 10× diluted gill cytosols were added, followed by 25 μL of Reagent A (copper tartrate, Bio-Rad) and 200 μL of Reagent B (Folin reagent, Bio-Rad). The measurement was performed after appearance of the blue colour, on photometer Anthos Microplate Reader HT3 (Austria) at 750 nm wavelength. Calibration curve was created using five different concentrations (0.2-2 mg mL^{-1}) of Bovine Serum Albumin (Serva) diluted in homogenizing buffer.

Determination of cytosolic metal concentrations

The concentrations of five metals (Zn, Fe, Cu, Mn and Cd) were measured in a duplicate in 5× diluted S50 fraction of chub gills by Varian SpectrAA 220 atomic absorption spectrometer. Flame technique (air/acetylene) was applied for Zn (at 213.9 nm) and Fe (at 248.3 nm) measurement, while graphite furnace with universal platforms (Varian GTA-100) was used for measurement of Cu (at 324.8 nm), Mn (at 279.5 nm) and Cd (at 228.8 nm). Deuterium lamp was used for background correction. External calibration was performed for each metal using the appropriate dilutions of respective metal stock solutions (1000 mg l^{-1} by Merck) in 5× diluted homogenizing buffer. The detection limits for five measured metals

were following: Zn 0.007 mg L^{-1}; Fe 0.015 mg L^{-1}; Cu 0.354 μg L^{-1}; Mn 0.224 μg L^{-1}; and Cd 0.023 μg L^{-1}.

Statistical analyses

Statistical analyses were performed using standard statistical package SigmaStat 1.0, and included descriptive statistics, Pearson correlation and linear regression analysis, as well as Two-way ANOVA followed by Bonferroni t-test. The graphs were created using the statistical program SigmaPlot 8.02 for Windows.

Results

The biometric parameters, as well as the cytosolic concentrations of total proteins and of five metals (Zn, Fe, Cu, Mn, Cd) in the chub gills obtained at five sites along Sava River water-course in two seasons are presented in Table 2. The total number of chub specimens caught per season was 78 in autumn (10-22 per site), and 73 in spring (12-18 per site). The ratio between females and males in both seasons was 1.5:1. The chub age was mostly in the range from two to three years (95% of all analyzed fish), with only few older specimens (four or five years old).

The influence of the season

Two-way ANOVA, with season and site as independent factors, was performed on the entire data set (Table 2) to establish if the measured parameters differed between two seasons. Total length, total mass, gill mass and Cd concentrations were comparable in specimens caught in

both seasons. Contrary, the statistically significantly higher levels of Fulton condition index, total proteins, Zn, Fe, Cu and Mn were obtained in spring period ($p<0.05$). Fulton condition index was 10% higher in spring compared to autumn, and the total protein concentration as much as 75%. Among four essential metals (Zn, Fe, Cu and Mn) whose concentrations were higher in spring, the most pronounced seasonal impact was obtained for iron and zinc (140% and 60% higher spring concentrations, respectively). Subsequently, the Bonferroni t-test was applied to establish if the seasonal differences were site dependent. Statistically significantly higher spring concentrations at all five sites were obtained for total cytosolic proteins, Zn and Fe ($p<0.05$), while Fulton condition index, although higher at all five sites in spring, was statistically significantly higher at only one site (Lukavec Posavski: $p<0.05$). Bonferroni t-test revealed that the seasonal differences in Cu level were not statistically significant at any site, while Mn level was statistically significantly higher in spring at two sites (Otok Samoborski and Lukavec Posavski: $p<0.05$). At some sites, as seen from Table 2, the concentrations of Cu and Mn were somewhat higher in autumn, indicating that seasonal impact on Cu and Mn level is not so significant as for Zn and Fe.

The influence of the chub age and size

Two-way ANOVA, with age and sampling site as independent factors, was performed to establish the age dependence of all measured parameters, for autumn and spring season (Table 3). The statistically significant age influence was observed for four biometric parameters in both seasons, with increase towards the older age. The Bonferroni t-test revealed that the differences between all three age groups (2, 3 and 4 year old fish) in length, total mass and gill mass were statistically significant ($p<0.05$). Furthermore, the significant age dependence was recorded in autumn for total proteins, Fe and Mn, and in spring for Fe. However, except

the statistically significant differences in the level of total cytosolic proteins between all three age groups obtained in autumn, the Bonferroni t-test pointed out only to the 4-year old fish as the specimens with statistically significantly ($p<0.05$) higher Fe and Mn concentrations compared to juvenile, 2-3 year old, fish. The differences in Fe and Mn levels within the group of juvenile fish were not observed. The age influence was not observed for Zn, Cu and Cd in autumn, and for total proteins, Zn, Cu, Cd and Mn in spring (Table 3). Although only a small number of older fish (>3 years) was included in this analysis, the results indicate that the age may have a significant influence even on the cytosolic metal concentrations, if sexually mature fish should be used as bioindicators. However, our results have shown that, irrespective of the season, in the group of juvenile chub the age has a significant positive effect only on the fish size.

The dependence of the cytosolic metal and protein concentrations on the chub size will be discussed only with the reference to the gill mass, due to the high positive and statistically significant ($p<0.01$) correlations between three biometric parameters associated to the chub size (length, total mass and gill mass). Figures 2 and 3 illustrate the dependence of the concentrations of total proteins and five metals on the chub gill mass in two seasons, autumn 2005 and spring 2006, respectively. In autumn period (Figure 2), the positive correlations of different significance were obtained at all five sampling sites between the gill mass and four parameters: total cytosolic protein, Mn, Zn and Fe concentrations. The correlations were statistically significant ($p<0.05$) at three sites for total proteins, Mn and Zn (Figure 2a-c), and at one site for Fe (Figure 2d). As could be expected, the strongest correlations were obtained at two sites, Otok Samoborski and Jasenovac, where the specimens had the widest ranges of the gill masses (0.2-1.6 g and 0.2-2.0 g, respectively). Contrary, negative correlations between Cu concentrations and the gill mass were obtained at all five sites, being statistically

significant at Otok Samoborski ($p<0.01$) (Figure 2e). For the nonessential metal Cd, it was not possible to establish clearly the dependence on the chub size, and the correlations with the gill mass varied from positive to negative. As seen on Figure 3, the positive influence of the gill mass on several measured parameters obtained in autumn have attenuated in spring period, leading to the absence of correlation or even to the appearance of negative correlations with the gill mass. The statistically significant negative correlations with the gill mass were obtained only for total cytosolic proteins at two sites, Otok Samoborski and Oborovo ($p<0.05$) (Figure 3a).

Discussion

There are only few reports in the literature regarding the metal bioaccumulation in the gills of the European chub (*Leuciscus cephalus* L.), and all of them refer to the metal levels in the whole gill tissue. The metal concentrations in the chub gills reported so far for the reference river sites are the Cd and Zn concentrations (0.02 μg g^{-1} and 90 μg g^{-1} wet weight, respectively) obtained in the unpolluted section of the river Lot, France (Andres et al., 2000) and the concentrations of several metals in river Saricay, Turkey (Cd: 0.04 μg g^{-1}; Cu: 0.69 μg g^{-1}; Fe: 87.3 μg g^{-1}; Mn: 3.32 μg g^{-1}; Zn: 28.6 μg g^{-1} wet weight) (Yilmaz et al., 2007). Our results (Table 2), therefore, represent the first data on the cytosolic metal concentrations in the gills of the chub, and the basis for the future studies. The highest cytosolic concentrations in the chub gills were obtained for essential metal Zn, followed by Fe. Copper and Mn mass concentrations were approximately 100 times lower, and Cd as much as 5000 times lower than Zn in the gill cytosol, which is consistent with the previous findings that high tissue concentrations of Zn in comparison to other metals are characteristic for fish (Moiseenko and Kudryavtseva, 2001).

To achieve better insight in the natural changes of cytosolic metal concentrations in the chub gills, the influence of season, age and size was studied. The chub specimens used in this study can be regarded as sexually immature, since chub (*Leuciscus cephalus*) males become sexually mature at the age of four, and females at the age of five (Arlinghaus and Wolter, 2003). The influence of sex was, therefore, not considered. The statistical analysis indicated that the age was not an adequate parameter for the explanation of metal variability in juvenile fish, either. Only the chub size was significantly influenced by age. However, we have found that in the group of young chubs, even the specimens of the same age can vary greatly in size at different sites, but also within the same sampling site (Table 2). Since chub lifetime amounts to 10-15 years, 2-3 year old fish should be considered as one age group of juvenile fish. The common parameter for expressing the individual growth variability, i.e. the fish size, described by length or mass, should be also a better indicator of the variability of the cytosolic metal concentrations in juvenile fish than the age. Giguère et al. (2004) recommended to consider the age as a relevant factor only for the studies conducted using the adult fish, while, similarly to our study, 1-2 year old perches were defined as the one group of young fish.

The studies on the various fish species, as well as on different types of tissues lead to the contradictory conclusions regarding the metal concentration dependence on the fish size. The size dependence could not be clearly established for gill concentrations of Cd and Zn in the study on chub (*Leuciscus cephalus*), roach (*Rutilus rutilus*), perch (*Perca fluviatilis*) and bream (*Abramis brama*) in river Lot, France (Andres et al., 2000). The positive dependence was obtained for concentrations of Fe, Zn and Mn in gills of chub (*Leuciscus cephalus*) and pike-perch (*Stizostedion lucioperca*) in Beysehir Lake, Turkey (Tekin-Özan and Kir, 2006), while the negative dependence was reported for Cu and Zn in the gills of bream (*Abramis*

brama) (Farkas et al., 2003) and Mn in the gills of moggel (*Labeo umbratus*) (Nussey et al., 2000). A possible cause for this kind of opposite observations could be the sampling season. In our study, we have noticed striking difference between two seasons, autumn and spring, regarding the metal concentration size dependence in the chub gills (Figures 2 and 3). As opposed to autumn, when 30-50% of total protein, Mn and Zn variability could be attributed to differences in chub size (Figure 2), in spring this marked positive size dependence have decreased or even changed the sign (Figure 3). Only 12% of total protein variability could be associated to the changes of the gill mass in spring, and even less for the other parameters. The opposite correlation sign between the gill mass and several parameters in two seasons could be explained by the possible differences in feeding habits and metabolic rates in autumn and spring. It is well known that fish metabolism can vary depending on abiotic conditions (e.g. temperature), food availability, as well as on the phase of the reproductive cycle (McCoy et al., 1995; Köck et al., 1996; Olsson et al., 1996; Clements and Raubenheimer, 2006). Since the water temperature was mainly comparable in autumn and spring samplings (13-15°C and 12-19°C, respectively), and the sampled fish were sexually immature, the observed seasonal differences were most likely the result of the higher food supply in the spring period, which lead to the increase of the water filtration through gills, as well as of the chub metabolic rate. Therefore, it can be hypothesized that the established autumn size dependences (positive for total proteins, Mn, Zn and Fe, and negative for Cu) reflect the basal relations in the period of the slower metabolism, while in spring an additional factor arises, and that is the metabolic rate. The ratio between the gill surface and the fish size decreases with the increase of the fish size (Pauly, 1981), leading to decreased ventilation and metabolic rate in the bigger fish (Neely, 1979). Consequently, the smaller fish, having faster filtration and metabolic rates, should also have higher metal and protein concentrations, i.e. metal and protein negative correlations with the size are the result of homeostatic regulation (Wiener and Giesy, 1979).

Finally, in the period of the intense feeding and water filtration, the combination of two opposite influences, the basal positive dependence on the gill mass and the negative dependence on the metabolic rate, could result in very week or even negative correlations with the gill mass, as was the case in our study in spring.

The hypothesis that the metabolic and feeding rates of the chubs caught in the Sava River during the spring period were higher compared to the autumn period can be further supported by the obtained seasonal differences for several measured parameters. The Fulton condition indices, which reflect the energy reserves and give the information about the recent feeding activity (Lambert and Dutil, 1997), were higher in spring than in autumn at all sampling sites (Table 2). The higher condition factors in the feeding months were also reported for the chubs in the İkizcetepeler dam lake in Turkey (Koç et al., 2007). Furthermore, the cytosolic concentrations of total proteins, Zn and Fe in the gills of the chubs sampled in April/May 2006 in river Sava, were significantly higher compared to the sampling performed in September 2005 (Table 2). The seasonal changes of metal concentrations in fish tissues can arise due to the changes of the feeding and growth rate, as well as the result of the changes in the fish condition (McCoy et al., 1995; Farkas et al., 2002). The metal concentrations, especially for essential metals like Zn, increase following the increase of the metabolic activity (Andres et al., 2000).

Conclusions

The study on the gills of chub (*Leuciscus cephalus*) revealed that cytosolic concentrations of five metals (Zn, Fe, Cu, Mn and Cd) and total proteins in young, sexually immature fish (2-3 years old) depend on the fish size. However, due to different metabolic rates in two seasons,

characteristic for the biota, the size influence is seasonally dependent, as well as the levels of the cytosolic proteins and some essential metals (Zn and Fe). In the conclusion, due to the significant influence of the fish size and season on the cytosolic metal levels in the chub gills, these parameters should be taken in consideration when interpreting the results of the field studies, to obtain more reliable information on water pollution.

Acknowledgements

The financial support by the Ministry of Science, Education and Sport of Republic Croatia (project No. 098-0982934-2721) is acknowledged. This study was carried out as a part of the European FP6 project **SA**va **RI**ver **B**asin: Sustainable Use, Management and Protection of Resources (INCO-CT-2004-509160), in the cooperation of the four laboratories from Division for Marine and Environmental Research, Ruđer Bošković Institute: Laboratory for Aquaculture, Laboratory for Organic Biogeochemistry, Laboratory for Molecular Ecotoxicology and Laboratory for Biological Effects of Metals. The authors are thankful to all SARIB project participants for the help in the field work. The special thanks are due to M.Sc. Damir Valić and Dr. Božidar Kurtović for the histological examination of the chub gonads.

References

Andres, S., Ribeyre, F., Tourencq, J-N., Boudou, A., 2000. Interspecific comparison of cadmium and zinc contamination in the organs of four fish species along a polymetallic pollution gradient (Lot River, France). Sci. Total Environ. 248, 11-25.

Arlinghaus, R., Wolter, C., 2003. Amplitude of ecological potential: chub *Leuciscus cephalus* (L.) spawning in an artificial lowland canal. J. Appl. Ichthyol. 19, 52-54.

Chovanec, A., Hofer, R., Schiemer, F., 2003. Fish as bioindicators. In: Markert, B.A., Breure, A.M., Zechmeister, H.G. (Eds.). Bioindicators & Biomonitors: Principles, Concepts and Applications. Elsevier Science Ltd., Amsterdam, Netherlands, pp. 639-676.

Clements, K.D., Raubenheimer, D., 2006. Feeding and Nutrition. In: Evans, D.H., Claiborne, J.B. (Eds.). The Physiology of Fishes, 3rd edition. Taylor & Francis, Boca Raton, USA, pp. 47-82.

Farkas, A., Salánki, J., Specziár, A., 2002. Relation between growth and the heavy metal concentration in organs of bream *Abramis brama* L. populating Lake Balaton. Arch. Environ. Con. Tox. 43, 236-243.

Farkas, A., Salánki, J., Specziár, A., 2003. Age- and size-specific patterns of heavy metals in the organs of freshwater fish *Abramis brama* L. populating a low-contaminated site. Water Res. 37, 959-964.

Ferrarello, C.N., de la Campa, M.D.F., Muniz, C.S., Sanz-Medel, A., 2000. Metal distribution patterns in the mussel *Mytilus edulis* cytosols using size-exclusion chromatography and double focusing ICP-MS detection. Analyst 125, 2223-2229.

Giguère, A., Campbell, P.G.C., Hare, L., McDonald, D.G., Rasmussen, J.B., 2004. Influence of lake chemistry and fish age on cadmium, copper, and zinc concentrations in various

organs of indigenous yellow perch (*Perca flavescens*). Can. J. Fish. Aquat. Sci. 61, 1702-1716.

Heath, A.G., 1995. *Water pollution and fish physiology*. Lewis Publishers, Boca Raton, USA.

HRN EN 14011, 2005. Fish sampling by electric power [Uzorkovanje riba električnom strujom].

Koç, H.T., Erdogan, Z., Tinkci, M., Treer, T., 2007. Age, growth and reproductive characteristics of chub, *Leuciscus cephalus* (L., 1758) in the İkizcetepeler dam lake (Balikesir), Turkey. J. Appl. Ichthyol. 23, 19-24.

Köck, G., Triendl, M., Hofer, R., 1996. Seasonal patterns of metal accumulation in Arctic char (*Salvelinus alpinus*) from an oligotrophic Alpine lake related to temperature. Can. J. Fish. Aquat. Sci. 53, 780-786.

Kraemer, L.D., Campbell, P.G.C., Hare, L., 2005. Dynamics of Cd, Cu and Zn accumulation in organs and sub-cellular fractions in field transplanted juvenile yellow perch (*Perca flavescens*). Environ. Pollut. 138, 324-337.

Lambert, Y., Dutil, J.-D., 1997. Can simple condition indices be used to monitor and quantify seasonal changes in the energy reserves of Atlantic cod (*Gadus morhua*)? Can. J. Fish. Aquat. Sci. 54 (Suppl. 1), 104-112.

Linde, A.R., Sanchez-Galan, S., Izquierdo, J.I., Arribas, P., Maranon, E., Garcia-Vazquez, E., 1998. Brown trout as biomonitor of heavy metal pollution: effect of age on the reliability of the assessment. Ecotox. Environ. Safe. 40, 120-125.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin Phenol reagent. J. Biol. Chem. 193, 265-275.

McCoy, C.P., O'Hara, T.M., Bennett, L.W., Boyle, C.R., Lynn, B.C., 1995. Liver and kidney concentrations of zinc, copper and cadmium in channel catfish (*Ictalurus punctatus*): variations due to size, season and health status. Vet. Hum. Toxicol. 37, 11-15.

Moiseenko, T.I., Kudryavtseva, L.P., 2001. Trace metal accumulation and fish pathologies in areas affected by mining and metallurgical enterprises in the Kola Region, Russia. Environ. Pollut. 114, 285-297.

Neely, W.B., 1979. Estimating rate constant for the uptake and clearance of chemicals by fish. Environ. Sci. Technol. 13, 1506-1510.

Nussey, G., van Vuren, J.H.J., du Preez, H.H., 2000. Bioaccumulation of chromium, manganese, nickel and lead in the tissues of the moggel, *Labeo umbratus* (Cyprinidae), from Witbank Dam, Mpumalanga. Water SA 26, 269-284.

Ognev, S.I., Fink, N., 1956. The Biology of Vertebrae. [Biologija kralješnjaka]. Grafički zavod Hrvatske, Zagreb.

Olsson, P.E., Larsson, A., Haux, C., 1996. Influence of seasonal changes in water temperature on cadmium inducibility of hepatic and renal metallothionein in rainbow trout. Mar. Environ. Res. 42, 41-44.

Pauly, D., 1981. The relationship between gill surface area and growth performance in fish: a generalization of von Bertalanffy's theory of growth. Meeresforschung 28, 251-282.

Phillips, D.J.H., 1980. Quantitative aquatic biological indicators. Applied Science Publishers, London, UK, pp. 488-493.

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

1 Tekin-Özan, S., Kir, I., 2006. Concentrations of some heavy metals in organs of two fish
2 species from the Beysehir Lake, Turkey. Fresen. Environ. Bull. 15, 530-534.
3 Treer, T., Safner, R., Aničić, I., Lovrinov, M., 1995. Fishery. [Ribarstvo]. Globus, Zagreb.
4 Wiener, J.G., Giesy, Jr. J.P., 1979. Concentrations of Cd, Cu, Mn, Pb and Zn in fishes in a
5 highly organic softwater pond. J. Fish. Res. Board Can. 36, 270-279.
6 Yilmaz, F., Özdemir, N., Demirak, A., Levent Tuna, A., 2007. Heavy metal levels in two fish
7 species *Leuciscus cephalus* and *Lepomis gibbosus*. Food Chem. 100, 830-835.
8
9

Figure captions

Figure 1. The map of the section of Sava River selected for the sampling of chubs (*Leuciscus cephalus*), with denoted sampling sites. The site legend: OS - Otok Samoborski, SZ – Sava-Zagreb, OB - Oborovo, LP - Lukavec Posavski, JAS - Jasenovac.

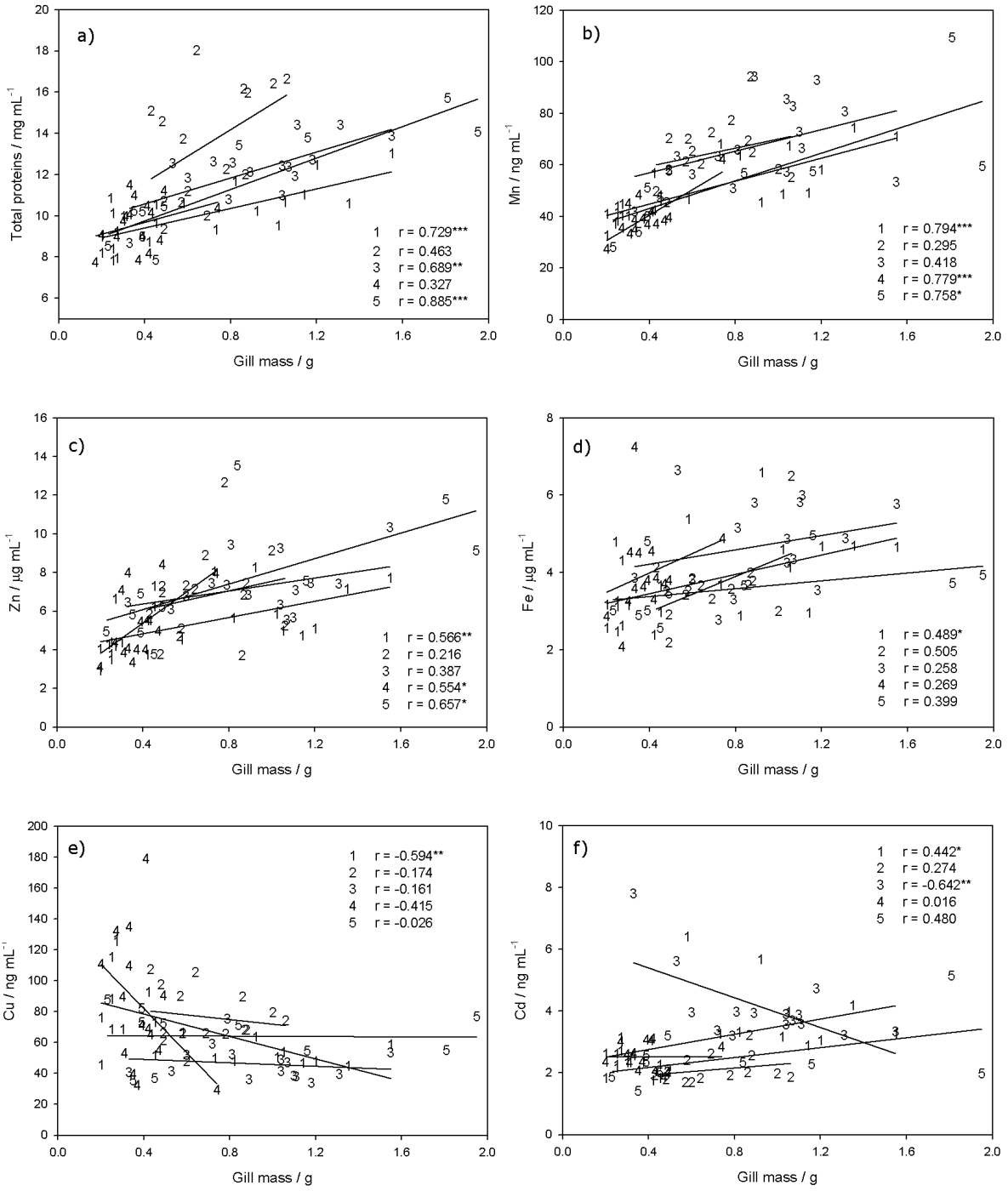
Figure 2. Linear regression analysis: the dependence on the gill mass established for several parameters measured in the chub gill cytosol at five sites along Sava River in autumn 2005: a) total proteins, b) Mn, c) Zn, d) Fe, e) Cu, f) Cd. The site legend: 1-Otok Samoborski; 2-Sava-Zagreb; 3-Oborovo; 4-Lukavec Posavski; 5-Jasenovac. The level of significance for Pearson correlation coefficients (r): ***$p<0.001$; **$p<0.01$; *$p<0.05$.

Figure 3. Linear regression analysis: the dependence on the gill mass established for several parameters measured in the chub gill cytosol at five sites along Sava River in spring 2006: a) total proteins, b) Mn, c) Zn, d) Fe, e) Cu, f) Cd. The site legend: 1-Otok Samoborski; 2-Sava-Zagreb; 3-Oborovo; 4-Lukavec Posavski; 5-Jasenovac. The level of significance for Pearson correlation coefficients (r): ***$p<0.001$; **$p<0.01$; *$p<0.05$

Figure 1.


Slovenia
Hungary
river Sava
OS
ZAGREB
S Z
OB
SISAK
Croatia
LP
JAS
river Sava
Bosnia and Herzegovina

1 Figure 2.
2



3
4

Figure 3.

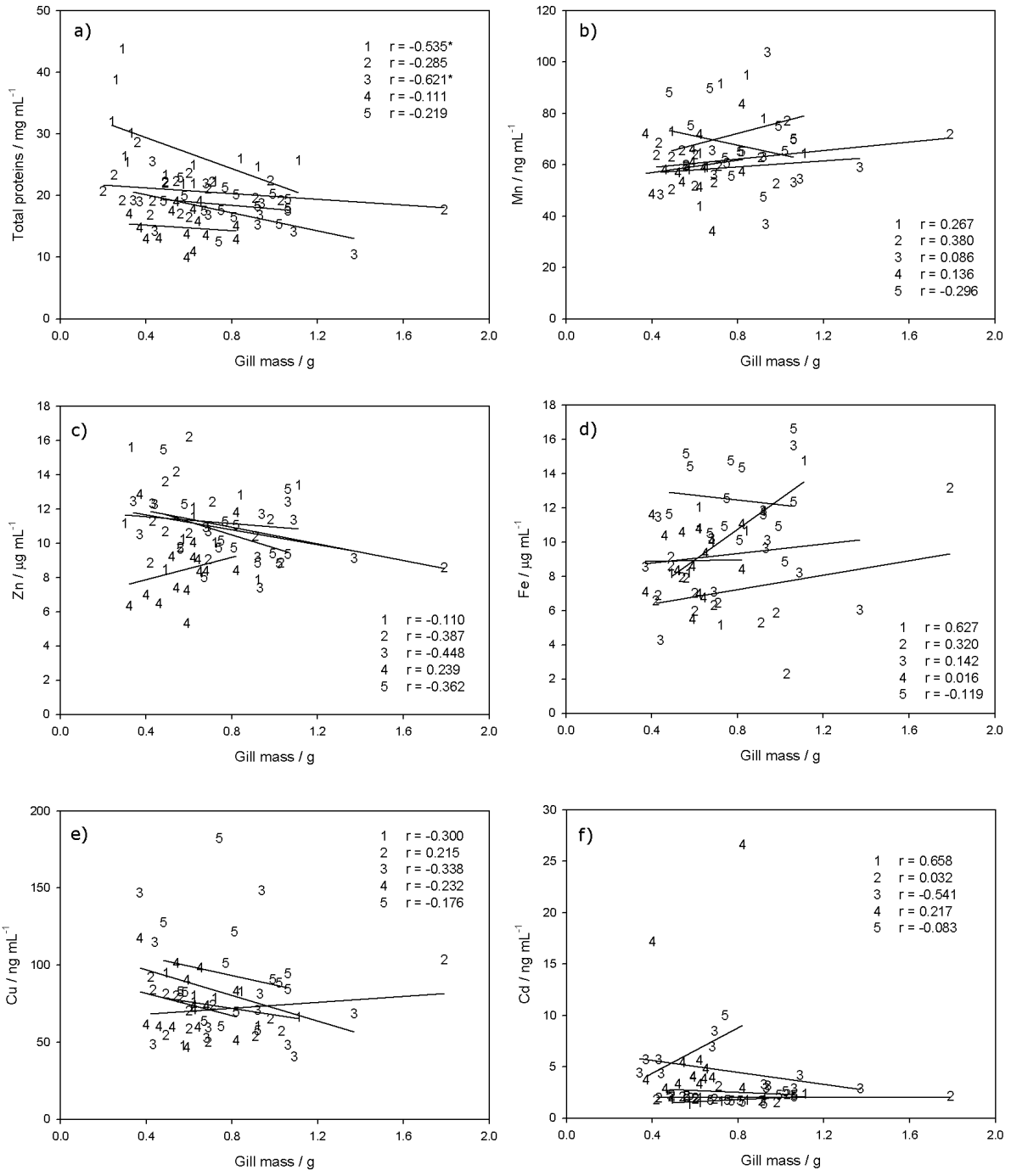

a)
Total proteins / mg mL-1
Gill mass / g
1 r = -0.535*
2 r = -0.285
3 r = -0.621*
4 r = -0.111
5 r = -0.219
b)
Mn / ng mL-1
Gill mass / g
1 r = 0.267
2 r = 0.380
3 r = 0.086
4 r = 0.136
5 r = -0.296
c)
Zn / µg mL-1
Gill mass / g
1 r = -0.110
2 r = -0.387
3 r = -0.448
4 r = 0.239
5 r = -0.362
d)
Fe / µg mL-1
Gill mass / g
1 r = 0.627
2 r = 0.320
3 r = 0.142
4 r = 0.016
5 r = -0.119
e)
Cu / ng mL-1
Gill mass / g
1 r = -0.300
2 r = 0.215
3 r = -0.338
4 r = -0.232
5 r = -0.176
f)
Cd / ng mL-1
Gill mass / g
1 r = 0.658
2 r = 0.032
3 r = -0.541
4 r = 0.217
5 r = -0.083

Table 1. Basic physico-chemical parameters of Sava river-water at the moment of fish catchments at five sampling sites in two seasons, autumn 2005 (A-05) and spring 2006 (S-06). The autumn samplings were performed from September 19th to 28th, and spring samplings from April 18th to May 25th.

Sampling site	Season	Water temperature / °C	pH	Dissolved oxygen / %	Conductivity / μS cm^{-1}
Otok Samoborski N 45° 50,543' E 15° 43,497'	A-05 S-06	14.4-15.6 12.8	7.84 7.87	78-83 98	410 465
Sava-Zagreb N 45° 46,572' E 15° 56,524'	A-05 S-06	13.2 11.5	7.92 7.86	78 94	377 473
Oborovo N 45° 41,286' E 16° 14,875'	A-05 S-06	13.7 12.1	7.84 7.76	80 85	346 507
Lukavec Posavski N 45° 24,081' E 16° 32,337'	A-05 S-06	13.9-15.1 11.7-17.1	7.83 7.59-7.85	70-76 81-83	346-408 415-495
Jasenovac N 45° 15,825' E 16° 53,658'	A-05 S-06	13.3 19.5	7.89 7.59	80 76	352 403

Table 2. The biometric parameters and the concentrations of the total proteins and metals in the gill cytosol of the European chub (*Leuciscus cephalus* L.) caught at five sites in Sava River, in autumn 2005 (A-05) and spring 2006 (S-06) (average±standard deviation)

Site	Season	No. of samples*	Total length / cm	Total mass / g	Gill mass / g	Fulton condition index / %	Total proteins / mg mL^{-1}	Zn / μg mL^{-1}	Fe / μg mL^{-1}	Cu / ng mL^{-1}	Mn / ng mL^{-1}	Cd / ng mL^{-1}
Otok Samoborski	A-05	22	18.5±3.8	67.6±43.8	0.63±0.43	0.94±0.04	9.96±1.38	5.31±1.59	3.75±1.09	69.4±26.1	50.0±12.0	3.07±1.19
	S-06	14	17.6±2.2	57.3±25.1	0.54±0.28	1.00±0.07	27.6±6.5	11.3±2.3	10.2±2.9	73.3±14.7	70.9±17.1	1.75±0.31
Sava-Zagreb	A-05	15	19.5±1.4	72.5±17.4	0.70±0.20	0.97±0.05	13.5±2.8	6.79±2.31	3.66±0.92	76.6±17.4	64.7±11.9	2.10±0.42
	S-06	18	18.7±2.8	72.5±40.6	0.63±0.37	1.03±0.07	20.7±3.0	11.1±2.3	7.08±2.40	71.5±16.1	61.6±7.9	2.03±0.37
Oborovo	A-05	15	21.0±2.1	96.4±29.0	0.94±0.32	1.01±0.05	12.3±1.5	7.28±1.39	4.71±1.14	46.3±10.9	68.4±15.9	4.10±1.19
	S-06	12	20.4±2.8	97.1±40.1	0.77±0.33	1.07±0.09	17.4±3.9	10.8±1.6	9.34±3.09	79.8±39.1	59.9±18.4	4.54±1.80
Lukavec Posavski	A-05	16	16.2±1.3	39.3±10.4	0.37±0.13	0.90±0.06	9.62±1.16	5.29±1.77	3.96±1.16	83.8±42.8	39.9±7.9	2.52±0.42
	S-06	15	17.8±1.8	68.3±23.6	0.58±0.15	1.16±0.12	14.8±2.8	8.45±2.02	8.93±1.87	74.7±20.7	59.3±11.9	6.52±6.81
Jasenovac	A-05	10	19.9±4.3	91.1±66.9	0.81±0.63	0.99±0.09	11.3±2.7	7.43±3.16	3.59±0.79	64.3±18.2	54.0±22.3	2.50±1.04
	S-06	14	20.7±1.5	98.5±25.8	0.80±0.19	1.09±0.07	18.4±2.6	10.5±2.0	12.5±2.3	93.2±32.9	67.7±11.6	2.51±2.17
All sites	A-05	78	18.9±3.2	71.3±40.8	0.67±0.40	0.96±0.07	11.2±2.4	6.25±2.16	3.94±1.10	68.4±28.3	55.0±17.0	2.90±1.15
	S-06	73	19.0±2.6	77.8±35.1	0.66±0.29	1.07±0.10	19.9±5.8	10.3±2.3	9.55±3.04	79.0±27.1	63.5±13.3	3.60±3.87

* In both seasons, due to the small volume of cytosol, the concentrations of metals were not measured in all samples (number of samples for metal analyses in autumn: Zn, Fe, Mn - 77; Cu and Cd - 76; in spring: Zn - 65; Cd - 62; Fe, Cu - 61; Mn - 59).

Table 3. The influence of the chub age on the biometric parameters, total cytosolic protein and metal concentrations in two seasons: Two-way ANOVA with age and sampling site as independent factors. The parameters exhibiting the significant age dependence ($p<0.05$) are presented with bold numbers.

	Autumn, 2005.			Spring, 2006.		
	2 years	3 years	4 years	2 years	3 years	4 or 5 years
n	42	31	5	27	42	4
Length / cm	**17.2**	**19.6**	**25.8**	**17.1**	**19.9**	**22.4**
Total mass / g	**51.1**	**77.7**	**172.7**	**52.8**	**89.6**	**135.1**
Gill mass / g	**0.46**	**0.76**	**1.59**	**0.45**	**0.76**	**1.06**
Condition index / %	**0.95**	**0.96**	**1.03**	**1.01**	**1.10**	**1.14**
Total proteins / mg mL^{-1}	**10.5**	**11.6**	**14.8**	21.2	19.0	19.9
Zn / μg mL^{-1}	6.19	6.34	8.07	10.2	10.5	10.7
Fe / μg mL^{-1}	**3.61**	**4.05**	**5.11**	**9.08**	**9.48**	**13.2**
Cu / ng mL^{-1}	70.4	66.3	64.3	83.7	77.0	73.3
Mn / ng mL^{-1}	**52.2**	**54.7**	**75.2**	65.6	63.3	63.5
Cd / ng mL^{-1}	2.87	2.73	3.39	3.77	3.39	3.07