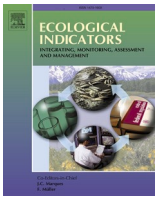
Ecological Indicators 122 (2021) 107247

Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind



Intestine of invasive fish Prussian carp as a target organ in metal exposure assessment of the wastewater impacted freshwater ecosystem

Tatjana Mijošek *, Vlatka Filipović Marijić, Zrinka Dragun, Dušica Ivanković, Nesrete Krasnići, Zuzana Redžović, Marijana Erk

Ruđer Bošković Institute, Division for Marine and Environmental Research, Laboratory for Biological Effects of Metals, Bijenička Cesta 54, 10000 Zagreb, Croatia

ARTICLE INFO

Keywords:
Metal contamination
Wastewaters
Biomarkers
Oxidative stress
Subcellular metal distribution
Monitoring

ABSTRACT

The application of invasive fish Prussian carp (*Carassius gibelio* Bloch, 1782) as bioindicator organism, using intestine as bioindicator tissue of anthropogenic influence in the lowland Ilova River was estimated. Intestinal tissue enables the investigation of dietborne metal uptake, so the first record on intestinal metal levels in Prussian carp was presented, as total and cytosolic fraction, which indicates the proportions of potentially toxic and bioavailable metals. Pollution impact was also estimated by analyses of biomarkers of oxidative stress (malondialdehyde), antioxidative capacity (catalase and glutathione) and of metal exposure (metallothioneins). All analyzed parameters were compared in the intestine of fish from the reference site and contaminated site impacted by technological and municipal wastewaters in two seasons. Both total and cytosolic As, Ca, Cd, Cs, Cu, Mg, Na and Rb levels were significantly higher at contaminated than the reference site in at least one season, whereas Mn and V had higher concentrations at the reference site. Despite differences in concentrations, average proportions of total metal levels in cytosolic fraction were comparable at two sites, i.e. over 70% for Na, K, Rb, Se, Cd, Cs, As and Mo, indicating their high possibility of binding to important biomolecules. In addition, higher levels of malondialdehyde in both seasons and enhanced catalase activity in spring, indicated disturbed environmental conditions near the contaminated site and need of continuous monitoring of this region. Finally, our research represents successful application of widely distributed invasive species in ecotoxicological studies, whereas intestine was shown as a suitable bioindicator tissue, clearly reflecting dietary metal uptake.

1. Introduction

Prussian carp (*Carassius gibelio* Bloch, 1782), a cyprinid fish species nowadays widely distributed in Europe and Asia, has a high invasion potential and can tolerate unfavorable environmental conditions including low oxygen levels, variable temperatures and high levels of anthropogenic pollution (De Boeck et al., 2004). Accordingly, it is an appropriate bioindicator in pollution assessment studies (De Boeck et al., 2004; Falfushynska et al., 2011; Tsangaris et al., 2011), including metal contamination. As a highly dominant fish species in the Ilova River, it was chosen as a bioindicator organism to evaluate the extent of existing anthropogenic impact on the biota of that ecosystem. Ilova River is a lowland river in the continental part of the Republic of Croatia, significant as a part of protected wetland area Lonjsko Polje Nature Park but it is under the influence of municipal (Town of Kutina) and industrial wastewaters (fertilizer factory) (Radić et al., 2013; Mijošek et al., 2020a). Effluents of industrial and municipal wastewaters contain a wide variety of pollutants depending on the type of activities, but high concentrations of trace metals have been often reported in wastewaters of those types (Mendiguchía et al., 2007).

Monitoring of trace and macro elements accumulation in Prussian carp in existing studies was usually carried out by measuring their total concentrations in commonly used target organs such as muscle, liver, kidney and gills (Andreji et al., 2006; Has-Schön et al., 2008; Falfushynska et al., 2011; Yabanli et al., 2014; Milošković and Simić, 2015; Đikanović et al., 2016; Zhelyazkov et al., 2018), but to our knowledge, the intestinal tissue of Prussian carp has not yet been applied as indicator tissue in metal exposure assessment. Despite its crucial role in fish digestion and nutrient absorption, as well as dietborne metal uptake (Clearwater et al., 2000), intestinal tissue is generally rarely applied as a bioindicator tissue. Existing studies mostly reported on only total metal concentrations in the intestine of different fish species (Dallinger and

* Corresponding author.
E-mail address: tmijosek@irb.hr (T. Mijošek).

https://doi.org/10.1016/j.ecolind.2020.107247
Received 5 August 2020; Received in revised form 10 November 2020; Accepted 2 December 2020
Available online 22 December 2020

Kautzky, 1985; Staniskiene et al., 2006; Filipović Marijić and Raspor, 2010, 2012; Jarić et al., 2011; Nachev and Sures, 2016; Yeltekin and Sağlamer, 2019), with only few exceptions by Filipović Marijić and Raspor (2012) and Mijošek et al. (2019a,b) who reported cytosolic metal concentrations in European chubs and brown trouts, respectively. Total concentrations, however, do not reflect biologically and metabolically available metal content since in organisms, metals are involved in many metabolic processes and end up incorporated in different cellular components (Wallace et al., 2003), such as metal-rich granules or metallothioneins (detoxified metal forms) and sensitive biomolecules (nondetoxified metal forms) (Urien et al., 2018). Therefore, in the present study, to get more information on subcellular partitioning of metals and their potentially toxic levels and effects, we have measured both total and cytosolic concentrations in the intestine of invasive fish Prussian carp. Metals in cytosolic fraction can bind and interact with either biologically available part containing microsomes and heat sensitive proteins (e.g., enzymes) or detoxified part involving heat-stable proteins (e.g., metallothioneins) (Bonneris et al., 2005; Urien et al., 2018).

Moreover, many environmental contaminants, including organic compounds and metals lead to extensive formation of reactive oxygen species (ROS) which consequently cause oxidative damage to cellular biomolecules including DNA, proteins and unsaturated lipids in cell membranes of organisms (Martínez-Álvarez et al., 2005). To overcome adverse effects of ROS, fish have an efficient antioxidant defense system involving both non-enzymatic compounds (vitamins E and C, glutathione (GSH) and other thiols) and enzymatic compounds (catalase, CAT; superoxide dismutase, SOD; and glutathione-S-transferase, GST). Besides by directly increasing the cellular concentration of ROS, metals promote oxidative damage also by lowering the cellular antioxidant capacity (Pinto et al., 2003). Thus, in order to evaluate the extent of oxidative stress and efficiency of antioxidant system in fish from the Ilova River, levels of malondialdehyde (MDA) as biomarker of oxidative damage and CAT activity as enzymatic antioxidant and GSH as non-enzymatic antioxidant, as two biomarkers of antioxidative capacity, were measured in their intestine. Additionally, biomarkers of metal exposure, metallothioneins (MTs), were used, because their induction is considered as a direct response to the elevated intracellular metal concentrations. Since levels of most biomarkers are known to be affected by various biotic and abiotic factors (size, age, feeding behavior, oxygen levels, pH, temperature, presence of contaminants) and organisms are almost always exposed to multiple contaminants, using the multi-biomarker approach, as in our research, better reflects real environmental state and presence of certain contaminants (Martínez-Álvarez et al., 2005).

Thus, the overall aim of the present study was to evaluate the potential threats for the organisms and for the protected area of Lonjsko Polje Nature Park by presenting, for the first time, metal cytosolic concentrations and proportions of potentially toxic metal fractions in the intestine of Prussian carp, and by applying multi-biomarker approach to assess the oxidative stress levels. In addition, we have estimated the potential and benefits of applying intestinal tissue as a target bioindicator organ and dietary uptake site and invasive fish species Prussian carp as bioindicator in metal risk assessment.

2. Materials and methods

2.1. Study area and fish sampling

Samplings of Prussian carps (*C. gibelio*) were conducted in the Ilova River in the continental part of Croatia. Lower part of the river watercourse is nowadays known to be threatened by technological (petrochemical processing in fertilizer factory) and municipal (Town of Kutina) wastewaters. Study was performed at two sites (reference and contaminated) and two seasons (autumn 2017 and spring 2018) in order to evaluate the application of fish intestine as a bioindicator tissue in the real environmental conditions. Reference site was located near the Ilova village and upstream of the Town of Kutina and pollution sources. Contaminated site was located near the Trebež village, about 8 km downstream of the confluence of the Kutinica River that discharges industrial wastewater originating mostly from a fertilizer factory (Radić et al., 2013), and is a part of protected area of Lonjsko Polje Nature Park. Detailed description of the sampling sites was given by Mijošek et al. (2020a). Radić et al. (2013) previously investigated the water contamination at one sampling site of the Ilova River which was located immediately downstream of the Town of Kutina, near the fertilizer factory. They recorded higher values of Fe, Cd, Pb, Cr, Hg, Zn, Cu, and Ni at this site near the factory compared to the reference site, but only concentrations of Pb and Hg were above limits set by WHO (Radić et al., 2013). Mijošek et al. (2020a) revealed that, during the same sampling campaigns as in this study, majority of measured elements were significantly elevated in water near the Trebež village, with Al, As, Cd, Ni and V being the most concerning elements as their concentrations were several times higher in comparison to the Ilova village. Similar trends were confirmed for sediments, where average level of Cd was about 20 times higher at the Trebež village compared to the Ilova village. 2–3 times higher levels at the Trebež village were observed for As, Cu, Ni, Pb, U, V and Zn while other elements were either up to 2 times higher at that site or comparable between the two investigated sites (Mijošek et al., 2020a). Further, contamination and enrichment factors, as well as pollution load indices, calculated for sediment samples, also indicated at least slightly disrupted environmental conditions at the contaminated site near the Trebež village (Mijošek et al., 2020a).

Chosen bioindicator organism, due to the highest abundance in the Ilova River, was the invasive cyprinid fish species Prussian carp (*Carassius gibelio* Bloch, 1782). In autumn, 20 fish specimens were sampled at each site, while in spring 23 and 20 fish individuals were sampled from the reference and the contaminated site, respectively. As a standard procedure, electro-fishing was used for the fish sampling, following the Croatian standard HRN EN 14011 (2005). Captured fish were kept alive in an aerated water tank for about 2–3 h before further processing.

2.2. Dissection and biometric parameters

All fish were euthanized using freshly prepared anesthetic tricaine methane sulphonate (MS 222, Sigma Aldrich, USA) according to the Ordinance on the protection of animals used for scientific purposes (European Union, 2010). Following the fish sacrifice, total masses and lengths were recorded, and the liver, gonads and posterior part of the intestine were dissected and weighed. Posterior part of the intestinal tissue of each fish was stored at − 80 °C until further analyses. Biometric calculations involved different indices: hepatosomatic index (HSI = (LM/M) × 100; Heidinger and Crawford, 1977), gonadosomatic index (GSI = (GM/M) × 100; Wootton, 1990) and Fulton condition index (FCI = (M/L^3) × 100; Ricker, 1975), where M is the body mass (g), L is the total length (cm), LM is the liver mass (g) and GM is the gonad mass (g).

2.3. Homogenization procedure

Homogenization was performed as already described by Mijošek et al. (2019a,b). Prior to GSH analyses, a piece of intestinal tissue was homogenized in 5 volumes of ice-cold 5% sulfosalicylic acid (SSA) using Potter-Elvehjem homogenizer (Glas-Col, USA) and then centrifuged at 10,000 × g for 10 min at 4 °C (Biofuge Fresco, Heraeus, Germany). Another piece of fish intestine used for the analyses of metals and other biomarkers was homogenized in 5 volumes of ice-cold 100 mM Tris-HCl/base (Merck, Germany, pH 8.1 at 4 °C) supplemented with 1 mM dithiothreitol (DTT, Sigma, USA), 0.5 mM phenylmethylsulfonyl fluoride (PMSF, Sigma, USA) and 0.006 mM leupeptin (Sigma, USA). Samples were homogenized by Potter-Elvehjem homogenizer (Glas-Col, USA) in an ice cooled tube. Appropriate aliquot of each homogenate was separated and set aside for subsequent digestion and analyses of total metal levels (includes insoluble and soluble tissue fractions). The

remaining part of homogenate was centrifuged by Avanti J-E centrifuge (Beckman Coulter, USA) in few steps: supernatant obtained by centrifugation at 3000 × g for 10 min at 4 °C was used for MDA analyses, at 10,000 × g for 30 min at 4 °C for analyses of CAT activity and lastly at 50,000 × g for 2 h at 4 °C for the determination of cytosolic metal concentrations (includes soluble tissue fraction) and MT analyses. Obtained supernatants were all stored at − 80 °C until analyses.

2.4. Digestion of intestinal tissue fractions and determination of total and cytosolic trace and macro elements concentrations

To prepare the samples for the metal quantitation, intestinal homogenates and cytosols were digested by adding the oxidation mixture (v/v 1:3 for homogenates and v/v 1:1 for cytosols) of concentrated HNO_3 (Rotipuran® Supra 69%, Carl Roth, Germany) and 30% H_2O_2 (Suprapur®, Merck, Germany) in the volume ratio of 3:1. Homogenization buffer represented a blank sample and was digested in the same procedure as samples. Digestion procedure was performed in the dry oven (FN 055, Nuve, Turkey) at 85 °C for 3.5 h. Following digestions, samples were diluted with Milli-Q water, 1:20 prior to Na, K and Mg analyses and 1:5 prior to Ca and trace element analyses (Dragun et al., 2018; Mijošek et al., 2019b).

High resolution inductively coupled plasma mass spectrometer (HR ICP-MS, Element 2; Thermo Finnigan, Germany), equipped with an autosampler SC-2 DX FAST (Elemental Scientific, USA) was used to determine the trace and macro elements concentrations. Determination of 5 elements (^{82}Se, ^{85}Rb, ^{98}Mo, ^{111}Cd and ^{133}Cs) was operated in low resolution mode; of 11 elements (^{23}Na, ^{24}Mg, ^{42}Ca, ^{51}V, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn and ^{86}Sr) in medium resolution mode; and of 2 elements (^{39}K and ^{75}As) in high resolution mode. To correct changes in peak intensities, In (1 μg L^{-1}, Indium Atomic Spectroscopy Standard Solution, Fluka, Germany) was used as an internal standard.

Two external calibrations were performed, one for the macro elements using multielement standard containing Na (1.0 g L^{-1}), K (2.0 g L^{-1}), Mg 0.4 g L^{-1}) and Ca (2.0 g L^{-1}) (Fluka, Germany) and the second calibration using multielement stock standard solution for trace elements (Analitika, Czech Republic) in which standard solution of Cs (Fluka, Germany) and Rb (Sigma-Aldrich, Germany) were added. All standards were prepared in 1.3% HNO_3 (Suprapur; Merck, Germany).

Two quality control samples (QC) obtained from UNEP/GEMS were used to check the accuracy and precision of HR ICP-MS measurements: QC for trace metals (QC trace metals, catalogue no. 8072, lot no. 146142–146143; Burlington, Canada) and QC sample for macro elements (QC minerals, catalogue no. 8052, lot no. 146138–146139; Burlington, Canada). Following recoveries were obtained (%) (based on three measurements in control sample for trace elements and Ca and two measurements for K, Mg and Na): As (94.0 ± 3.7), Ca (95.6 ± 1.2), Cd (94.0 ± 0.8), Co (96.0 ± 1.9), Cu (97.2 ± 2.2), Fe (93.1 ± 4.7), K (95.8 ± 1.2), Mg (90.4 ± 2.5), Mn (93.5 ± 3.7), Na (97.7 ± 1.1), Ni (96.1 ± 0.1), Se (93.9 ± 1.9), Sr (98.2 ± 1.1), V (96.6 ± 1.0) and Zn (97.2 ± 3.6).

Limits of detection (LOD) were calculated as three standard deviations of ten consecutive metal measurements in the blank (homogenization buffer) digested according to the procedure for cytosols. LOD for trace and macro elements were already published by Dragun et al. (2018) and Mijošek et al. (2019b).

Results obtained by measurement in digested homogenates present total metal/metalloid concentrations, while the results obtained for cytosolic fractions represent soluble, cytosolic metal/metalloid concentrations. All concentrations obtained in this study are presented either as μg kg^{-1} or mg kg^{-1} of wet tissue (w.w.) depending on the element. Proportions of total metal/metalloid present in the cytosolic fractions of the intestine of *C. gibelio* were calculated as ratios of cytosolic to total metal concentrations and finally expressed as percentages (%).

2.5. Biomarkers determination

2.5.1. Determination of the MDA concentration – biomarker of oxidative stress

MDA levels were measured by spectrophotometrical method adapted from Botsoglou et al. (1994) and Ringwood et al. (2003). Mixture of 1% butylated hydroxytoluene (BHT, Sigma-Aldrich, USA) dissolved in ethanol and 10% trichloroacetic acid (TCA, Kemika, Croatia) dissolved in Milli-Q water (BHT/TCA = 1:100) was added to the supernatants (S3), which were then put in a refrigerator at 4 °C for 15 min and centrifuged at 4000 × g for 15 min at 4 °C. In thus obtained supernatants 2-thiobarbituric acid (TBA, Alfa Aesar, Germany) was added and the samples were then heated at 100 °C for 30 min. After period of cooling, the absorbance was read at 535 nm wavelength using the spectrophotometer/fluorometer microplate reader Infinite M200 (Tecan, Switzerland). Calibration curve was constructed using 8 different concentrations of MDA (Aldrich, USA) dissolved in 1 N HCl (Kemika, Croatia). Values were expressed as nmol of MDA per gram of wet tissue mass. Detailed description was given by Mijošek et al. (2019b).

2.5.2. Determination of CAT activitiy and GSH levels – biomarkers of antioxidative capacity

CAT activity was measured according to the spectrophotometrical method of Claiborne (1985). 15.8 mM H_2O_2, prepared of sodium phosphate buffer (50 mM, pH 7.0) and hydrogen peroxide (30%), was added to the ten times diluted samples. Absorbance was read at 240 nm wavelength at 25 °C using the spectrophotometer/fluorometer microplate reader Infinite M200 (Tecan, Switzerland). Final CAT activity was expressed as μmol of degraded H_2O_2 per min per mL and calculated using a molar extinction coefficient of 43.6 M^{-1} cm^{-1}.

Spectrophotometric DTNB-GSSG reductase recycling assay (Tietze, 1969) was used for the determination of total GSH levels. The procedure for the microtiter plate assay was adapted from Rahman et al. (2006). 0.1 M potassium phosphate buffer supplemented with 1 mM EDTA disodium salt, pH 7.5, was used for the preparation of all solutions. Solution containing DTNB (3.79 mM) and glutathione reductase (6 U mL^{-1}) was added to the sample and the mixture was then vortexed and kept in dark for 5 min. Next, NADPH (0.192 mM) solution was added and the absorbance was read for 5 min in 1-min intervals at 412 nm. Calibration curve, used to calculate final GSH concentrations, was made using GSH standards (3.125–25 nmol mL^{-1}) which were prepared in 0.5% SSA. The results were expressed as nmol of GSH per g of wet tissue mass.

2.5.3. Determination of MT levels – biomarker of metal exposure

Prior to the electrochemical MT determination, cytosols (S50 fraction) were heat-treated to avoid possible interferences of thermosensitive high molecular mass cytosolic proteins with the electrochemical MT determination. As thermostable proteins, MTs remain in the solution after the heat-treatment. To obtain heat treated supernatants (HT S50), cytosolic fractions were firstly 10 times diluted with 0.9% NaCl (Suprapur®, Merck, Germany) and then heated at 85 °C for 10 min in the Dri Block (Techne, UK). Following, samples were placed on the ice for 30 min at 4 °C and then centrifuged at 10,000 × g for 15 min at 4 °C using Biofuge Fresco centrifuge (Kendro, USA) to get this MT rich fraction (Erk et al., 2002).

MT concentrations were measured in HT S50 by differential pulse voltammetry following the modified Brdička procedure (Raspor et al., 2001). 797 VA Computrace voltammetric measuring stand (Metrohm, Switzerland) was used, equipped with a three-electrode system (hanging mercury drop electrode, HMDE, as a working electrode, an Ag/AgCl/saturated KCl reference electrode and a platinum counter electrode). Electrolyte solution consisted of 2 M NH_4Cl/NH_4OH and 1.2 × 10^{-3} M $Co(NH_3)_6Cl_3$ (v/v 1:1), pH = 9.5, and was thermostated to 20 °C and purged with the pure nitrogen. Applied measurement parameters were adapted from Mijošek et al. (2018). Straight calibration line, constructed

with the commercially available standard rabbit liver MT-2 (Enzo, USA), dissolved in 0.25 M NaCl, was used for the calculation of MT concentrations which were presented as mg MT g^{-1} of wet tissue (w.w.).

2.6. Statistical analyses

Main statistical analyses were made in SigmaPlot 11.0 (Systat Software, USA), while Microsoft Office Excel 2007 was used for regular calculations. Considering that assumptions of normality and homogeneity of variance were not always met, Mann-Whitney *U* test was applied to test the significance of differences in metal concentrations and biomarker values in the fish intestine between two seasons and two sites. Differences were regarded as significant at $p < 0.05$. Following the nonparametric analyses, correlation between parameters was tested using Spearman correlation analysis. Data are presented as mean ± standard deviation (S.D.).

3. Results

3.1. Fish biometry

Spatial differences were evident as higher values of all biometric parameters in fish from the Trebež village compared to the reference site, except of comparable HSI levels in autumn. Trend was even statistically significant for total length in autumn, body mass and FCI in both seasons and GSI in spring (Table 1). Seasonal differences pointed to higher levels of almost all biometric parameters in autumn than spring, being especially striking and significant for HSI at both locations, as well as significant for body mass and FCI in fish from the Ilova village. Exception was GSI which showed higher levels in spring at both locations, but statistically significant only at Trebež village (Table 1).

3.2. Total and cytosolic trace and macro elements concentrations in the fish intestine

The results on total and cytosolic trace and macro element levels in the intestine of *C. gibelio* represent the first data of this kind for this invasive fish species. They are presented in three categories: a) elements with higher concentrations at the contaminated site (Trebež village) (Fig. 1); b) elements with higher concentrations at the reference site (Ilova village) (Fig. 2); c) elements with mostly comparable concentrations at both locations (Fig. 3).

Total metal levels, which represent the combination of both soluble and insoluble metal fraction, as well as cytosolic levels, which only refer to soluble metal fraction, showed various spatial and temporal patterns among measured metals/metalloids. Total concentrations of Cd, Cs and Cu were significantly higher at the contaminated site in both seasons, and of As, Ca, Mg, Na and Rb in only one season (Fig. 1). In addition, cytosolic Ca, Cd, Cs, Cu, Fe and Rb were significantly higher at the contaminated compared to the reference site in both seasons and As, Mg and Na in one season. Manganese and V were significantly higher at the reference site in autumn and spring considering both fractions (Fig. 2), whereas other elements (Co, K, Mo, Ni, Se, Sr and Zn) had mostly comparable concentrations in both locations (Fig. 3). Seasonal trends were not so clear, but concentrations of total and cytosolic Cd and Cs, with the addition of total K were significantly higher in autumn compared to the spring season in both locations, while the opposite trend was shown significant for total and cytosolic As, Co and Sr, total Mn and Zn and only cytosolic V. Other elements did not show clear pattern (Figs. 1–3).

3.3. Proportions of intestinal trace and macro elements present in the cytosolic fractions

Average proportions of total metal levels present in cytosolic intestinal fraction, i.e. soluble tissue fraction where metals are capable of binding to biologically important molecules, are presented in Table 2. The ratio over 70% was found for Na, K, Rb, Se, Cd, Cs, As and Mo, between 50% and 70% for Mg, Co, Zn, and Sr, while the average proportions of Cu, Ca, Mn, Fe, V and Ni were below 50%.

3.4. Biomarker responses

3.4.1. MDA - biomarker of oxidative stress

MDA levels were significantly higher in fish caught at the location near the Trebež village, showing approximately 2 to 3 times higher average values at contaminated compared to the reference site in autumn and spring, respectively (Fig. 4a). In both seasons, average MDA concentrations were around 40 nmol g^{-1} w.w. at the Ilova village site and 87–105 nmol g^{-1} w.w. at the Trebež village (Fig. 4a). Seasonal differences were not significant and did not show clear trend.

3.4.2. CAT and GSH - biomarkers of antioxidative capacity

Regarding CAT activity, significant spatial difference was observed in spring with elevated enzyme activity in fish from the contaminated site. Seasonally, significant difference was observed at the reference site with higher average activity in autumn (258.8 ± 45.1 μmol H_2O_2 min^{-1} mL^{-1}) compared to spring (200.8 ± 51.3 μmol H_2O_2 min^{-1} mL^{-1}) (Fig. 4b). There were no significant seasonal differences at the contaminated site, although average CAT activity was slightly higher in spring (255.5 ± 63.6 μmol H_2O_2 min^{-1} mL^{-1}) compared to autumn (239.8 ± 51.6 μmol H_2O_2 min^{-1} mL^{-1}).

GSH levels did not show significant and unique season- or site-specific differences. Average value was slightly higher at the reference site compared to the contaminated site in autumn, whereas opposite pattern was visible in spring with higher values at the contaminated location (Fig. 4c). Average values of GSH concentrations ranged from 1109.9 ± 174.0 nmol g^{-1} w.w. to 1329.7 ± 299.1 nmol g^{-1} w.w. when both locations and seasons are considered (Fig. 4c).

Table 1
Biometric parameters (mean ± S.D.) of Prussian carp (*Carassius gibelio*) from the Ilova River at the reference (Ilova village) and contaminated site (Trebež village) in two sampling campaigns (autumn and spring). Statistically significant differences (Mann-Whitney *U* test) at $p < 0.05$ level between two seasons at each sampling site are marked with asterisk (*) and between two sampling sites within the same season are assigned with different superscript letters (A and B).

Location	Season	Total length (cm)	Body mass (g)	GSI (%)	HSI (%)	FCI (g cm^{-3}*100)
Ilova village	**Autumn 2017** n = 20	16.2 ± 1.6^{A}	69.82 ± 23.17$^{*, A}$	3.11 ± 1.44	5.87 ± 1.78*	1.59 ± 0.09$^{*, A}$
	Spring 2018 n = 23	15.9 ± 2.2	54.57 ± 21.43$^{*, A}$	5.25 ± 3.60^{A}	1.44 ± 0.53*	1.31 ± 0.10$^{*, A}$
Trebež village	**Autumn 2017** n = 20	18.8 ± 2.9^{B}	122.34 ± 58.13^{B}	4.67 ± 2.68*	5.44 ± 1.52*	1.70 ± 0.12^{B}
	Spring 2018 n = 20	17.5 ± 3.9	103.03 ± 83.00^{B}	7.63 ± 4.67$^{*, B}$	2.36 ± 0.77*	1.67 ± 0.15^{B}

GSI –gonadosomatic index; HSI –hepatosomatic index; FCI – Fulton condition index.

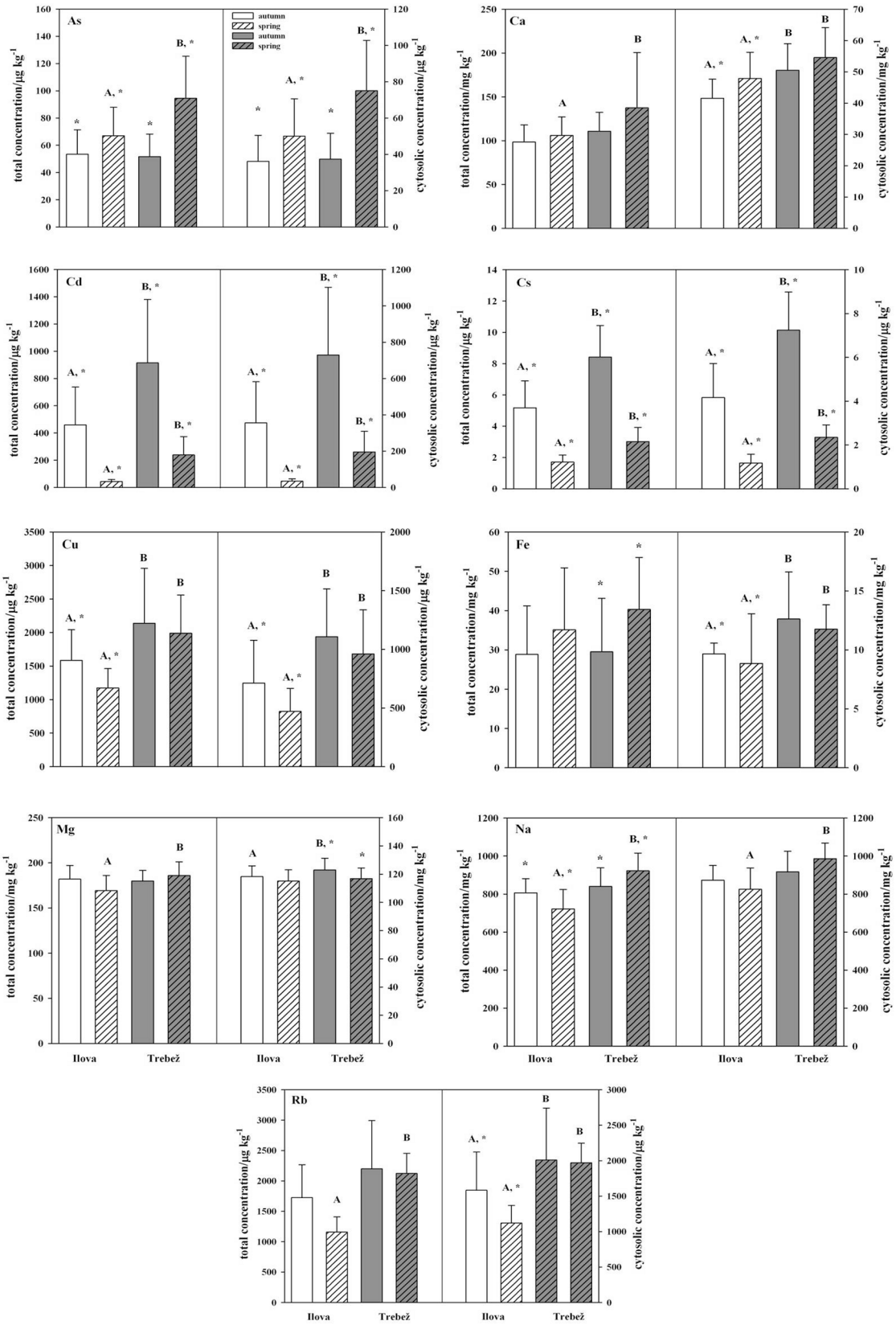


Fig. 1. Total and cytosolic concentrations of nine metals/metalloids in the intestine of Prussian carp from the Ilova River at two sampling sites (reference: Ilova village; contaminated: Trebež village) and two seasons that were elevated at the contaminated site. Statistically significant differences (Mann-Whitney *U* test) at $p < 0.05$ levels between two seasons at each sampling site are marked with asterisk (*) and between two sampling sites within the same season are assigned with different superscript letters (A and B).

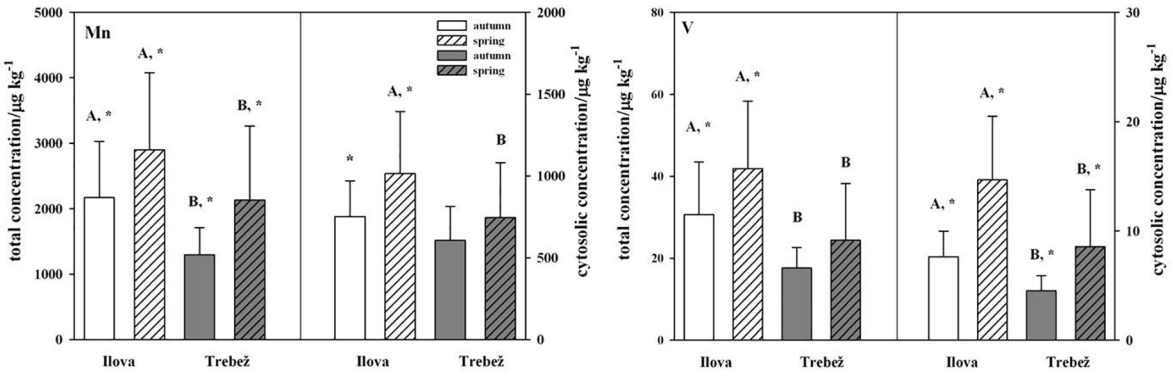


Fig. 2. Total and cytosolic concentrations of two metals in the intestine of Prussian carp from the Ilova River at two sampling sites (reference: Ilova village; contaminated: Trebež village) and two seasons that were elevated at the reference site. Statistically significant differences (Mann-Whitney *U* test) at $p < 0.05$ level between two seasons at each sampling site are marked with asterisk (*) and between two sampling sites within the same season are assigned with different superscript letters (A and B).

3.4.3. *MT- biomarker of metal exposure*

Significant differences in MT concentrations were not observed regarding site or season. However, slightly higher MT induction was evident at the contaminated compared to the reference site in both seasons and in autumn compared to spring at both investigated locations (Fig. 4d). Average values of MT concentrations ranged from 2.13 ± 0.80 mg g^{-1} w.w. to 2.57 ± 0.93 mg g^{-1} w.w. when both locations and seasons are considered.

4. Discussion

4.1. *Fish biometry*

Significantly higher biometric parameters (TL, TM and FCI) at the contaminated site indicated higher bioavailability of food and nutrients (Lambert and Dutil, 1997) at that site, possibly connected with organic matter sourcing from wastewaters discharged into the river watercourse near the contaminated location. Contrary, in many studies, FCI decline was observed at highly contaminated locations (Laflamme et al., 2000; Rajotte and Couture, 2002; Zhelev et al., 2018). Thus, contamination of the Ilova River evidently did not induce additional defense mechanisms that might cause decreased FCI values. Additionally, these three parameters were higher in autumn than in spring at both investigated locations. Opposite trend was observed for GSI, with higher values in spring which coincides with the spawning period of Prussian carp, occurring from April to July (Sasi, 2008). Due to the high energy demands during the fish reproductive development (Maddock and Burton, 1998), FCI and HSI often show the opposite trend than GSI, resulting in mostly lower values in the spawning periods of different fish species (Farkas et al., 2003; Sabrah et al., 2016; Mijošek et al., 2019b) and of Prussian carp (Leonardos et al., 2008; De Giosa et al., 2014), as also confirmed in our research.

4.2. *Total and cytosolic trace and macro elements concentrations in the fish intestine*

Intestinal metal levels in fish cytosol represent elements in the soluble tissue fraction which might bind to biologically important biomolecules and therefore potentially can cause toxic effects, whereas total metal concentrations refer to total metal tissue load, including both metabolically available and detoxified fractions. Our results pointed to the differences in total and cytosolic metal concentrations, i.e. higher total concentrations for several metals, but their quite similar spatial patterns (Figs. 1–3).

As studies including fish intestine as target organ in metal pollution assessments are still rare, data on total and cytosolic metal concentrations in the intestinal tissue of Prussian carp represent the first data of such kind for this invasive fish. Descending order of total trace elements in our research mostly followed the trend: Zn > Fe > Mn ≥ Rb ≥ Cu > Se ≥ Cd > Ni > Sr > Mo > As > Co > V > Cs (Figs. 1–3). The highest total concentrations of Fe, Zn, Mn and Cu have already been observed in the intestine of other investigated freshwater fish species: rainbow trout (Dallinger and Kautzky, 1985), perch (Sures et al., 1999), starlet (Jarić et al., 2011), European chub (Filipović Marijić and Raspor, 2012), barbel (Nachev and Sures, 2016), brown trout (Mijošek et al., 2019a) and *Salmo trutta macrostigma* (Yeltekin and Sağlamer, 2019). The descending order of cytosolic elements in the intestine of Prussian carp in our research was Zn > Fe > Rb > Cu ≥ Mn > Se > Cd > Sr > Ni > Mo ≥ As > Co > V ≥ Cs (Figs. 1–3), therefore mostly following the same trend as the total metal concentrations and confirming the pattern of Zn > Fe > Cu > Cd for cytosolic intestinal metals recorded in European chub from the Sava River (Filipović Marijić and Raspor, 2012) and brown trout from the Krka River (Mijošek et al., 2019a, 2019b), both in Croatia. Considering macro elements, descending concentration order was K > Na > Mg > Ca in all mentioned freshwater fish species.

In order to consider our results in the wider context, we made comparison with the data on metal accumulation in liver, muscle and gills of Prussian carp reported in other studies. Andreji et al. (2006) investigated total metal concentrations in muscles of five fish species from Nitra River (Slovakia), impacted by sewage waters, power plant, chemical factory and lignite mines, and observed pattern for Prussian carp was Fe > Zn > Cu > Mn > Cd > Ni > Co. In gills, liver and muscle of Prussian carp from the agriculturally impacted Marmara Lake (Turkey) descending order of investigated metals was Cu > Cd > Ni > Cr > Pb > Al > As > Hg (Yabanli et al., 2014). Finally, Đikanović et al. (2016) compared metal accumulation in liver, muscle and gills of nine fish species from the Međuvršje Reservoir (Serbia) which receives untreated industrial and communal waters, and Prussian carp was shown as the most effective accumulator of most metals and the highest concentrations of Fe and Zn were observed in all tissues.

Metal accumulation in the intestine of Prussian carps was compared with environmental metal concentrations in the water and sediments of the Ilova River and their spatial patterns indicated higher levels of several metals/metalloid in the area near the Trebež village. Cadmium, Cs, Cu and Rb were 2–5 times higher in fish from the contaminated than reference site depending on the season (Fig. 1), while in the water and sediment samples Cd, Cs and Rb were also considerably higher near the Trebež village (Mijošek et al., 2020a). It is already known that Cs and Cd concentration in water is one of the main factors that affect their bioaccumulation in organisms (Rowan and Rasmussen, 1994; Pinder et al., 2011; Dragun et al., 2019). However, concentrations of V in water and sediment samples were also significantly higher at the Trebež village, but that trend was not observed in fish in the present research. Quite the

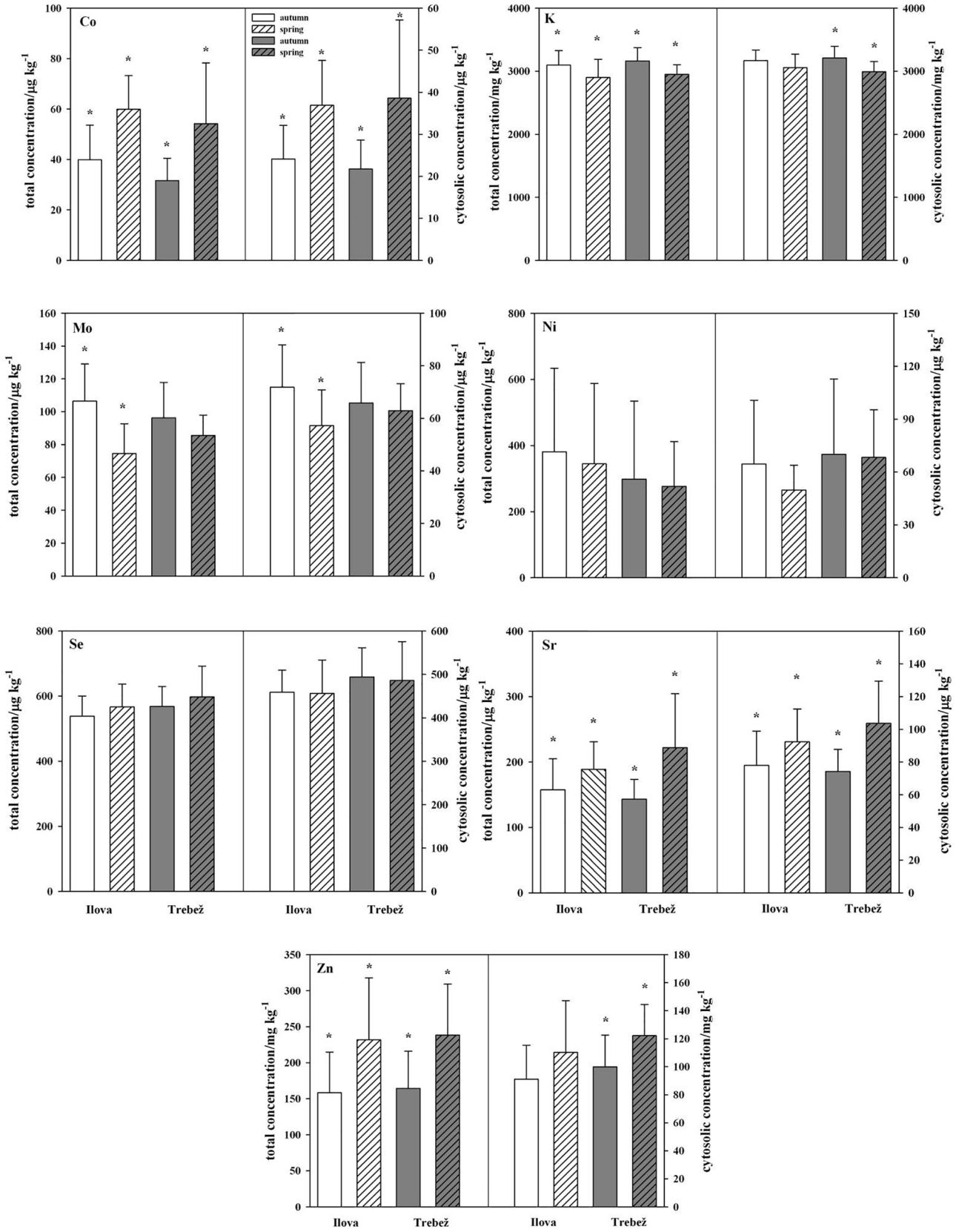


Fig. 3. Total and cytosolic concentrations of seven metals/metalloids in the intestine of Prussian carp from the Ilova River at two sampling sites (reference: Ilova village; contaminated: Trebež village) and two seasons with comparable concentrations in both sites. Statistically significant differences (Mann-Whitney *U* test) at $p < 0.05$ level between two seasons at each sampling site are marked with asterisk (*) and between two sampling sites within the same season are assigned with different superscript letters (A and B).

Table 2
Average proportions of total metal/metalloid amounts present in the cytosolic fractions (%) of the intestine of *C. gibelio* from two sampling sites (reference site: Ilova village and contaminated site: Trebež village) and two sampling campaigns (autumn 2017 and spring 2018). The results are presented as mean ± S.D. of all sites and campaigns, with average range (minima and maxima) within brackets. Metals/metalloids are listed in the descending order.

	Average proportion in cytosols
Na	**109.9 ± 7.2** (107.2–114.9)
K	**102.8 ± 5.7** (101.4–105.9)
Rb	**92.9 ± 5.2** (91.1–96.8)
Se	**84.1 ± 10.2** (80.8–87.2)
Cd	**79.9 ± 5.9** (77.5–82.2)
Cs	**79.0 ± 11.8** (69.3–86.3)
As	**72.8 ± 11.7** (67.4–78.6)
Mo	**71.5 ± 8.0** (67.6–77.4)
Mg	**66.3 ± 4.7** (63.0–68.5)
Co	**65.4 ± 10.7** (61.1–70.6)
Zn	**57.1 ± 12.4** (49.8–62.8)
Sr	**50.2 ± 6.0** (48.4–52.4)
Cu	**46.1 ± 12.4** (38.8–54.0)
Ca	**44.2 ± 5.4** (42.4–46.1)
Mn	**39.0 ± 8.2** (35.5–47.1)
Fe	**34.3 ± 14.1** (22.1–45.5)
V	**30.7 ± 8.8** (26.1–36.2)
Ni	**24.7 ± 13.8** (19.0–30.7)

opposite, V concentrations were even 1.5–2 times higher in the intestine of fish from the reference site (Fig. 2). As Ilova village is also located near the agricultural area, enhanced V accumulation in fish might be the consequence of using fertilizers, herbicides and insecticides which were reported as possible metal sources of variety of metals, including V (Dragun et al., 2011; Ramani et al., 2014). Additionally, V concentrations in fish might reflected their feeding behavior as dietborne metal uptake can be of equal or even higher importance than the waterborne metal uptake (Clearwater et al., 2000). Manganese levels were also significantly higher in fish from the reference site (Fig. 2), but in water samples that difference was observed only in autumn (Mijošek et al., 2020a). Hence, further research should involve determination of metal concentrations in fish food as other possible metal source, especially due to intestinal role in food digestion and nutrient absorption. Cobalt, K, Mo, Ni, Se, Sr and Zn had comparable concentrations in the fish intestine from both sites (Fig. 3), which could also not be associated to the environmental exposure from the water and sediments, indicating strong regulative role of essential elements in fish, as already reported for different species in many studies (Olsvik et al., 2000; Monna et al., 2011; Dragun et al., 2019) and possible additional impact of feeding habits on metal levels.

Although seasonal differences in our research were not so pronounced and clear, for more elements elevated total and cytosolic levels were recorded in spring at both investigated locations (Figs. 1–3). Seasonal changes of metal concentrations in fish may result from factors such as fish growth and reproductive cycle, changes in water temperature, pH or seasonal variations of metal exposure from water, food and sediments. The link of metal concentrations and reproductive stage might be due to increased metabolic needs for essential metals such as Fe, Mn and Zn as constitutive part of important biomolecules (Miramand et al., 1991; Filipović Marijić and Raspor, 2010). Therefore, our results on mostly higher metal levels in spring, the spawning period of Prussian carp, might be related to the physiological changes during reproductive period. Similar to our results, during the spawning periods of European chub mostly higher essential metal levels were observed in the gill (Dragun et al., 2007) and intestinal cytosols (Filipović Marijić and Raspor, 2010), as well as in the intestinal cytosols of brown trouts (Mijošek et al., 2019b).

Therefore, the cause of variability of metal content in the fish

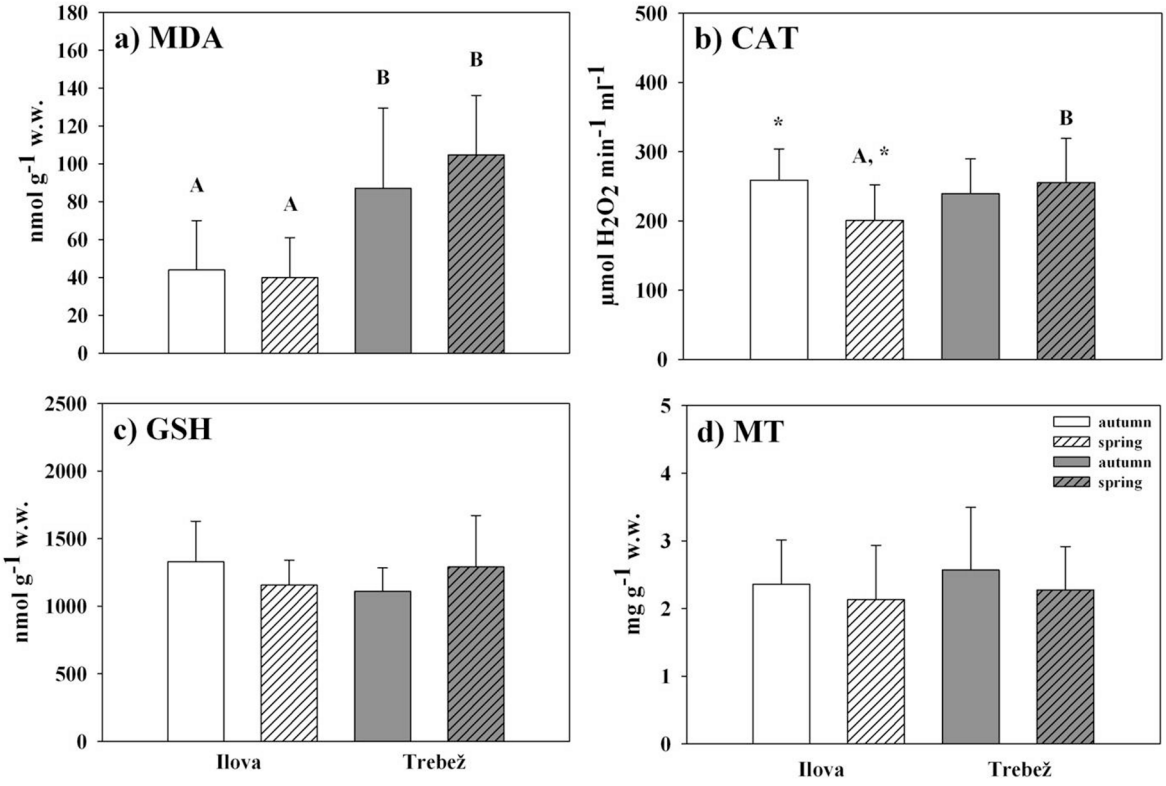


Fig. 4. Biomarker levels (a) MDA; b) CAT; c) GSH; d) MT) in the intestine of Prussian carp from the Ilova River at two sampling sites (reference: Ilova village; contaminated: Trebež village) and two seasons (autumn and spring). Statistically significant differences (Mann-Whitney *U* test) at $p < 0.05$ level between two seasons at each sampling site are marked with asterisk (*) and between two sampling sites within the same season are assigned with different superscript letters (A and B).

intestine cannot be completely explained by waterborne metal uptake or by sediments as alternative metal source so further research should involve determination of metal concentrations in fish food as other possible source since significance of dietary metal uptake has already been shown in variety of freshwater fish species (Clearwater et al., 2000; Lapointe and Couture, 2009; Filipović Marijić and Raspor, 2012; Rajkowska and Protasowicki, 2013).

4.3. Proportions of intestinal trace and macro elements present in the cytosolic fractions of C. gibelio intestine

Trace and macro elements cytosolic fraction represent their portions available to bind to physiologically important biomolecules, and therefore, might be potentially toxic (Wallace et al., 2003; Urien et al., 2018). Despite the differences in metal concentrations between the sites, the portions of metabolically available metal contents in the intestine of Prussian carps were mostly comparable at both locations. For Ca, Cu, Fe, Mn, Ni and V < 50% of total metal levels were present in the soluble cytosolic fraction, indicating their higher presence in tissue fraction which is not considered as metabolically available. We have already reported the proportion of Tl in the intestine of the same specimens to be around 40% (Mijošek et al., 2020b). Proportions of the other investigated metals (As, Cd, Co, Cs, K, Mg, Mo, Na, Rb, Se, Sr and Zn) were > 50% in cytosol, where metals have a potential to become toxic by binding to enzymes (metal-sensitive fraction), but can also be detoxified by binding to cytosolic biomolecules, such as metallothioneins (Wallace et al., 2003; Urien et al., 2018). Thus, although As, Cd, Cs, Mo, Rb and Se were present in proportions even higher than 70% (Table 2), such result does not necessarily reflect their toxic levels in organisms because it can also indicate metals detoxified by metallothioineins, which cannot cause harmful effects (Bonneris et al., 2005). As seen in Table 2, K and Na as the main cations responsible for maintaining normal cytosolic osmolarity were completely present in the cytosols.

Cytosolic proportions of intestinal metal levels in other fish species were reported only for brown trout from the karst Krka River (Mijošek et al., 2019a, 2019b) and European chub from the lowland Sava River (Filipović Marijić and Raspor, 2012). The average percentages of analyzed elements present in the soluble, cytosolic intestinal fraction of Prussian carp were comparable to the order found in brown trout: K, Na (>99%) > Se (88%) > Cs (86%) > Cd (84%) > Rb (82%) > Mo (73%) > As (69%) > Mg (67%) > Tl (60%) > Co (56%) > Fe (50%) > Sr (48%) > Zn (47%) > Ca (43%) > V (41%) > Mn (40%) > Cu (38%) > Ni (36%) (Mijošek et al., 2019a, 2019b); and in European chub: Cd (90–100%), Zn (70–80%), Cu (50–80%), Fe and Mn (30–40%) (Filipović Marijić and Raspor, 2012). Therefore, in the intestinal cytosolic fractions of different fish species metals were present in similar proportions, despite considerable differences in their concentrations. Some smaller differences in proportions are probably associated with different physiological characteristics and biology of specific fish species, as well as with different environmental conditions.

The information on cytosolic metal proportions in the intestinal tissues of some other fish species is not available, so we made the short overview of subcellular compartmentalization of metals investigated in other fish tissues. Van Campenhout et al. (2010) investigated cytosolic metal distribution in liver and kidneys of Prussian carps from metal-impacted habitats and revealed high proportion (60–70%) of the total tissue Cd, Cu and Zn concentrations in hepatic, and 50% of total Cd and 30% of total Cu and Zn concentrations in renal cytosols. For other fish species, Rosabal et al. (2015) reported the organelles and metal-sensitive fraction with heat-denaturated proteins (HDP) as main binding site for As in the liver of *Anguilla anguilla* and *Anguilla rostrata,* which indicated high probability of toxic effects. Although present in cytosol in very high proportion, as in our research, it is known that Cd mostly binds to the heat-stable proteins (HSP), metallothioneins, which therefore indicates its high detoxification level and much decreased risk of harmful effects (Kraemer et al., 2006; Rosabal et al., 2015). Although it was recorded that Se was mostly present in cytosols in the liver of Arctic char *Salvelinus alpines*, the same amount of Se was found in HSP and fraction containing lysosomes, microsomes and HDP, which suggested that significant part of Se is actually present in detoxified form (Barst et al., 2016). Further, Urien et al. (2018) reported results on subcellular partitioning of As, Cd, Cu, Se and Zn in liver and gonads of wild white suckers (*Catostomus commersonii*) and concluded that As, Cd, and Cu mostly bind to HSPs, contrary to Se and Zn which mostly bind to HDPs, although Zn was also found to be distributed in all the other subcellular fractions, as expected due to its essential role as a co-enzyme in many metabolically important processes (Mason and Jenkins, 1995). Low proportions of Ni in cytosols found in our research are in accordance with results from Rosabal et al. (2015) who reported that granule-like structures have the main role in detoxifying Ni in the liver of European eels. However, opposite results for Ni were also reported, specifically for wild yellow perch, in which hepatic Ni concentrations were the highest in HDP fraction within metal-sensitive fraction (Giguere et al., 2006), which enables possible interaction with physiologically important biomolecules and potential toxic effects of this element.

Hence, elements present in cytosols might only be partially toxic, due to the existing detoxification mechanisms involving their binding to HSPs (MTs and MT-like proteins), especially in conditions of only moderate metal contamination. However, some amount of metals is always found to be bound to metal sensitive HDPs showing that mechanisms of detoxification are not completely efficient. Their presence in cytosols indicates potential threats and possible toxic effects of metals/metalloids for organisms which include blocking functional groups, substitution of essential metals or modification of active sites of important biomolecules (Mason and Jenkins, 1995).

4.4. Biomarker responses

In order to estimate the application of intestine as bioindicator tissue and provide a comprehensive assessment of the environmental quality, multi-biomarker approach was applied. The impact of metal contamination on fish from the Ilova River was investigated to assess the level of oxidative stress in fish by the measurement of MDA, as a manifestation of lipid peroxidation, and of antioxidants (CAT and GSH), as components of antioxidant defense system (van der Oost et al., 2003). In addition, MTs were used as a direct link with metal contamination since these low molecular mass cysteine and metal-rich proteins have significant roles in maintaining the homeostasis of essential trace metals (Zn and Cu), removal of toxic metals (Cd, Ag and Hg) and protection against oxidative damage (Vašák, 2005).

Increased concentrations of MDA reflect oxidative stress in organisms induced by enhanced production of ROS (Banerjee et al., 1999). In our research, significantly higher intestinal values of MDA were observed in fish at the contaminated site compared to the reference location in both seasons (Fig. 4a). Obtained MDA values ranged from 39.87 to 104.73 nmol g^{-1} w.w., which is, especially at the reference site of the Ilova River, much lower than MDA levels reported for brown trout from the Krka River (Mijošek et al., 2019b). Although concentrations were not as high as in brown trouts, significant difference between the two sites of the Ilova River suggested that fish were exposed to higher levels of oxidative stress near the Trebež village, which might be linked to mostly elevated metal levels at that site (Mijošek et al., 2020a).

Efficiency of fish antioxidant defense system was tested by analyzing CAT activity and GSH levels. Various responses of CAT activity were observed in animals exposed to metallic contaminants in either field or laboratory experiments depending on the dose, element, the species or the route of exposure (Atli et al., 2006; Tsangaris et al., 2011; Greani et al., 2017; Mijošek et al., 2019b). CAT activity in the intestine of Prussian carp in our research was significantly elevated in fish from the contaminated compared to the reference site in spring, while there were no differences in autumn, possibly associated with the trend of higher metal levels in spring. Although average GSH levels followed the same

patterns as CAT activity, significant differences were not observed (Fig. 4b and 4c). Obtained values of CAT and GSH were either comparable or slightly lower than reported for the intestine of the brown trouts from the Krka River (Mijošek et al., 2019b). To our knowledge, there is no other available literature data on GSH levels in the intestinal tissue of any fish species.

Antioxidants responses induced by pollution vary for different species, enzymes, single or mixed contaminants. The literature data reported on either higher, unchanged or lower activities of antioxidants as the response to pollutant exposure in both laboratory and field studies (van der Oost et al., 2003) and metal caused formation of ROS and damaging tissues (DNA, proteins and lipids) has already been well investigated. For example, Berntssen et al. (2001) showed that exposure of Atlantic salmon to dietary Cu had a direct effect on lipid peroxidation of the intestine even at low concentrations, while Cd induced additional MDA synthesis only at its highest concentration. In the same research, significant differences in GSH levels in the intestine or liver were not observed among fish fed diets containing different Cd concentrations. Considering studies on Prussian carps, Tsangaris et al. (2011) investigated oxidative stress biomarkers in toxicity testing of Ukrainian polluted river waters. They measured oxidative stress biomarkers in *C. gibelio* liver after the 96 h exposure to the river water samples and found out that antioxidant enzymes, including CAT, mostly increased after the exposure, while there were no differences in MDA levels between the exposed and control fish. Further, Liu et al. (2005) reported significant induction of GSH in liver of *Carassius auratus* after Cu exposure. Considering CAT activity, Atli et al. (2006) investigated response to Ag, Cd, Cr, Cu and Zn among five tissues of freshwater fish *Oreochromis niloticus* and increasing concentrations of all elements, except Cu, caused considerable enzyme inhibition in fish intestine. Radić et al. (2013) cage-exposed *Cyprinus carpio* to the water of the Ilova River for 7- and 21-day period and observed a significant increase in lipid peroxidation in both gills and liver as bioindicator tissues, as well as decline in CAT and glutathione reductase (GR) activity in the gills, which suggested a higher sensitivity of gills and earlier failure of the antioxidant system. Correlation analysis in our research confirmed significantly positive relation of As with MDA levels ($r = 0.484$, $p < 0.05$) and with CAT activity ($r = 0.474$, $p < 0.05$) in fish from the contaminated site in autumn, as well as of CAT activity and As at the reference site in spring ($r = 0.535$, $p < 0.05$). Similar relation of oxidative stress with As exposure had already been documented by Bhattacharya and Bhattacharya (2007) who reported induced tissue lipid peroxidation and increased activity of CAT in the liver of *Clarias batrachus* after exposure to As. Greani et al. (2017) also reported significant increase in MDA concentrations in muscles, liver, kidney and fins of exposed fish, as well as an enhanced antioxidants (CAT and SOD) activities, when investigating the effect of chronic As exposure under environmental conditions on oxidative stress in wild trout (*Salmo trutta*). In our research, some of these elements such as Cd, Cu and As were significantly elevated in fish from the contaminated site, therefore, also pointing to their possible role in similar oxidative stress responses.

In addition to biomarkers related to the oxidative stress, metallothioneins (MTs) were used as widely recognized biomarkers of metal exposure. Due to their high affinity to specific metals, they are considered as efficient scavengers of ROS and contribute to the protection against oxidative injuries and many other environmental stressors (Viarengo et al., 1999). Significant MT induction was not observed in our research at any site (Fig. 4d). Comparison to other fish species revealed that average values of 2.0–2.5 mg g^{-1} w.w. in Prussian carp were higher than in the intestine of the brown trout from the Krka River (0.8–1.5 mg g^{-1} w.w.) (Mijošek et al., 2019a) and slightly lower than in the intestine of European chub from the Sava River (2.9–3.1 mg g^{-1} w.w.) (Filipović Marijić and Raspor, 2010). Concentrations of the main MT inducers, Cd, Cu and Zn, were higher in fish from the contaminated than the reference site of the Ilova River (Fig. 1), possibly causing slightly higher average MT levels at that site. However, significant correlation of MT levels was confirmed only with Zn at the reference site in autumn ($r = 0.509$, $p < 0.05$) and at the contaminated site in spring ($r = 0.506$, $p < 0.05$). MT levels are known to be dependent on numerous factors including season, temperature, pH, age and size, gender, reproductive status (Hylland et al., 1998), making it difficult to distinguish the exact cause of MT induction.

Thus, all biomarkers, to a higher or lesser degree, indicated exposure to higher levels of oxidative stress at the contaminated site, but cell antioxidant system seems to work in fish from both locations without significant decrease in levels of CAT, GSH or enhanced induction of MTs, which are usually caused by high metal concentrations in highly polluted environments. Therefore, biomarker responses in the intestinal tissue confirmed only moderate level of contamination of the studied freshwater system and indicated that fish intestine reflects environmental conditions (Mijošek et al., 2020a).

5. Conclusions

Prussian carp itself was shown as a suitable bioindicator species in aquatic environmental pollution assessment and the intestine as suitable bioindicator organ, which reflects responses to contaminants from dietary pathways. Special significance of the presented data is that they are the first data on distribution of 18 elements and on the levels of oxidative stress biomarkers in the intestine of Prussian carp, which can serve as a basis for comparison in future monitoring programmes. Total and cytosolic concentrations of many trace and macro elements, as well as some biomarker responses in the intestine of Prussian carp pointed to more disturbed environmental conditions at the contaminated site of the investigated ecosystem, the Ilova River. Presence of many analyzed elements in the cytosolic intestinal fractions was over 50%, therefore pointing that majority of metals/metalloids in the intestine can potentially cause toxic effects. Although a portion of metals present in the cytosol is still expected to be detoxified by MTs, there is a significant possibility of harmful effects of these elements for the organisms by binding to biologically important molecules such as enzymes.

Although obtained biological changes mostly indicated moderate pollution impact, significantly higher concentrations of elements such as As, Cd, Cs, Cu and Fe in combination with higher levels of oxidative stress (MDA) in fish from the contaminated site near the Trebež village highlighted the need of regular monitoring of the water quality and aquatic organisms of this region, especially knowing that this location is a part of protected wetland area of Lonjsko Polje Nature Park.

Due to its high spread potential and the fact that Prussian carp is already, naturally or introduced, a widely spread species in Europe, this data present an important contribution to the future monitoring and preservation of European freshwater systems and serve as an good example of using invasive, instead of and along with native fish species in ecotoxicological and biomonitoring studies.

CRediT authorship contribution statement

Tatjana Mijošek: Investigation, Resources, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Vlatka Filipović Marijić:** Investigation, Writing - review & editing, Validation, Resources, Funding acquisition, Supervision. **Zrinka Dragun:** Investigation, Validation, Writing - review & editing. **Dušica Ivanković:** Investigation, Writing - review & editing. **Nesrete Krasnići:** Investigation. **Zuzana Redžović:** Investigation, Resources. **Marijana Erk:** Funding acquisition, Project administration, Resources, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the Croatian Science Foundation, within the project "Accumulation, subcellular mapping and effects of trace metals in aquatic organisms" AQUAMAPMET (IP-2014-09-4255). Authors are also grateful for the help in the sampling to the members of the Laboratory for Aquaculture and Pathology of Aquatic Organisms from the Ruđer Bošković Institute.

References

Andreji, J., Stránai, I., Massányi, P., Valent, M., 2006. Accumulation of some metals in muscles of five fish species from lower nitra river. J. Environ. Sci. Health, Part A 41 (11), 2607–2622. https://doi.org/10.1080/10934520600928003.

Atli, G., Alptekin, Ö., Tükel, S., Canli, M., 2006. Response of catalase activity to Ag+, Cd2+, Cr^{6+}, Cu^{2+} and Zn^{2+} in five tissues of freshwater fish *Oreochromis niloticus*. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 143 (2), 218–224. https://doi.org/10.1016/j.cbpc.2006.02.003.

Banerjee, B.D., Seth, V., Bhattacharya, A., Pasha, S.T., Chakraborty, A.K., 1999. Biochemical effects of some pesticides on lipid peroxidation and free-radical scavengers. Toxicol. Lett. 107 (1-3), 33–47. https://doi.org/10.1016/S0378-4274(99)00029-6.

Barst, B.D., Rosabal, M., Campbell, P.G.C., Muir, D.G.C., Wang, X., Köck, G., Drevnick, P. E., 2016. Subcellular distribution of trace elements and liver histology of landlocked Arctic char (*Salvelinus alpinus*) sampled along a mercury contamination gradient. Environ. Pollut. 212, 574–583. https://doi.org/10.1016/j.envpol.2016.03.003.

Berntssen, M.H.G., Aspholm, O.Ø., Hylland, K., Wendelaar Bonga, S.E., Lundebye, A.-K., 2001. Tissue metallothionein, apoptosis and cell proliferation responses in Atlantic salmon (*Salmo salar* L.) parr fed elevated dietary cadmium. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 128 (3), 299–310. https://doi.org/10.1016/S1532-0456(00)00204-0.

Bhattacharya, A., Bhattacharya, S., 2007. Induction of oxidative stress by arsenic in *Clarias batrachus*: involvement of peroxisomes. Ecotoxicol. Environ. Saf. 66 (2), 178–187. https://doi.org/10.1016/j.ecoenv.2005.11.002.

Bonneris, E., Giguère, A., Perceval, O., Buronfosse, T., Masson, S., Hare, L., Campbell, P. G.C., 2005. Sub-cellular partitioning of metals (Cd, Cu, Zn) in the gills of a freshwater bivalve, *Pyganodon grandis*: role of calcium concretions in metal sequestration. Aquat. Toxicol. 71 (4), 319–334. https://doi.org/10.1016/j.aquatox.2004.11.025.

Botsoglou, N.A., Fletouris, D.J., Papageorgiou, G.E., Vassilopoulos, V.N., Mantis, A.J., Trakatellis, A.G., 1994. A rapid, sensitive, and specific thiobarbituric acid method for measuring lipid peroxidation in animal tissues, food, and feeds tuff samples. J. Agric. Food Chem. 42, 1931–1937. https://doi.org/10.1021/jf00045a019.

Claiborne, A., 1985. Catalase activity. In: Greenwald, R.A. (Ed.), CRC Handbook of Methods for Oxygen Radical Research. CRC Press, Boca Raton FL, pp. 283–284.

Clearwater, S.J., Baskin, S.J., Wood, C.M., McDonald, D.G., 2000. Gastrointestinal uptake and distribution of copper in rainbow trout. J. Exp. Biol. 203, 2455–2466.

Dallinger, R., Kautzky, H., 1985. The importance of contaminated food for the uptake of heavy metals by rainbow trout (*Salmo gairdneri*): a field study. Oecologia 67 (1), 82–89. https://doi.org/10.1007/BF00378455.

De Boeck, G., Meeus, W., Coen, W.D., Blust, R., 2004. Tissue-specific Cu bioaccumulation patterns and differences in sensitivity to waterborne Cu in three freshwater fish: rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), and gibel carp (*Carassius auratus gibelio*). Aquat. Toxicol. 70 (3), 179–188. https://doi.org/10.1016/j.aquatox.2004.07.001.

De Giosa, M., Czerniejewski, P., Rybczyk, A., 2014. Seasonal changes in condition factor and weight-length relationship of invasive *Carassius gibelio* (Bloch, 1782) from Leszczynskie Lakeland, Poland. Adv. Zool. 2014, 1–7. https://doi.org/10.1155/2014/678763.

Dragun, Z., Raspor, B., Podrug, M., 2007. The influence of the season and the biotic factors on the cytosolic metal concentrations in the gills of the European chub (*Leuciscus cephalus* L.). Chemosphere 69 (6), 911–919. https://doi.org/10.1016/j.chemosphere.2007.05.069.

Dragun, Z., Kapetanović, D., Raspor, B., Teskeredžić, E., 2011. Water quality of medium size watercourse under baseflow conditions: the case study of River Sutla in Croatia. Ambio 40 (4), 391–407. https://doi.org/10.1007/s13280-010-0119-z.

Dragun, Z., Filipović Marijić, V., Krasnići, N., Ivanković, D., Valić, D., Žunić, J., Kapetanović, D., Smrzlić, I.V., Redžović, Z., Grgić, I., Erk, M., 2018. Total and cytosolic concentrations of twenty metals/metalloids in the liver of brown trout *Salmo trutta* (Linnaeus, 1758) from the karstic Croatian river Krka. Ecotoxicol. Environ. Saf. 147, 537–549. https://doi.org/10.1016/j.ecoenv.2017.09.005.

Dragun, Z., Tepić, N., Ramani, S., Krasnići, N., Filipović Marijić, V., Valić, D., Kapetanović, D., Erk, M., Rebok, K., Kostov, V., Jordanova, M., 2019. Mining waste as a cause of increased bioaccumulation of highly toxic metals in liver and gills of Vardar chub (*Squalius vardarensis* Karaman, 1928). Environ. Pollut. 247, 564–576. https://doi.org/10.1016/j.envpol.2019.01.068.

Đikanović, V., Skorić, S., Gačić, Z., 2016. Concentrations of metals and trace elements in different tissues of nine fish species from the Međuvršje Reservoir (West Morava River Basin, Serbia). Arch. Biol. Sci. 68 (4), 811–819.

Erk, M., Ivanković, D., Raspor, B., Pavičić, J., 2002. Evaluation of different purification procedures for the electrochemical quantification of mussel metallothioneins. Talanta 57 (6), 1211–1218. https://doi.org/10.1016/S0039-9140(02)00239-4.

European Union, 2010. Directive 2010/63/EU of the European parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal of the European Union No. L 276/33 (20.10.2010).

Falfushynska, H.I., Gnatyshyna, L.L., Stoliar, O.B., Nam, Y.K., 2011. Various responses to copper and manganese exposure of *Carassius auratus gibelio* from two populations. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 154 (3), 242–253. https://doi.org/10.1016/j.cbpc.2011.06.001.

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 (5), 959–964. https://doi.org/10.1016/S0043-1354(02)00447-5.

Filipović Marijić, V., Raspor, B., 2010. The impact of the fish spawning on metal and protein levels in gastrointestinal cytosol of indigenous European chub. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 152, 133–138. https://doi.org/10.1016/J.CBPC.2010.03.010.

Filipović Marijić, V., Raspor, B., 2012. Site-specific gastrointestinal metal variability in relation to the gut content and fish age of indigenous European chub from the Sava River. Water Air Soil Pollut. 223 (8), 4769–4783. https://doi.org/10.1007/s11270-012-1233-2.

Giguere, A., Campbell, P., Hare, L., Couture, P., 2006. Sub-cellular partitioning of cadmium, copper, nickel and zinc in indigenous yellow perch (*Perca flavescens*) sampled along a polymetallic gradient☆. Aquat. Toxicol. 77 (2), 178–189. https://doi.org/10.1016/j.aquatox.2005.12.001.

Greani, S., Lourkisti, R., Berti, L., Marchand, B., Giannettini, J., Santini, J., Quilichini, Y., 2017. Effect of chronic arsenic exposure under environmental conditions on bioaccumulation, oxidative stress, and antioxidant enzymatic defenses in wild trout *Salmo trutta* (Pisces, Teleostei). Ecotoxicology 26 (7), 930–941. https://doi.org/10.1007/s10646-017-1822-3.

Has-Schön, E., Bogut, I., Rajković, V., Bogut, S., Čačić, M., Horvatić, J., 2008. Heavy Metal Distribution in Tissues of Six Fish Species Included in Human Diet, Inhabiting Freshwaters of the Nature Park "Hutovo Blato" (Bosnia and Herzegovina). Arch. Environ. Contam. Toxicol. 54 (1), 75–83. https://doi.org/10.1007/s00244-007-9008-2.

Heidinger, R.C., Crawford, S.D., 1977. Effect of temperature and feeding rate on the liversomatic index of largemouth bass, *Micropterus salmoides*. J. Fish. Res. Board Can. 34, 633–638. https://doi.org/10.1139/f77-099.

HRN EN 14011, 2005. Fish sampling by electric power (In Croatian). Croatian Standard Institute, Zagreb.

Hylland, K., Nissen-Lie, T., Christensen, P.G., Sandvik, M., 1998. Natural modulation of hepatic metallothionein and 584 cytochrome P4501A in flounder, *Platichthys flesus* L. Mar. Environ. Res. 46, 51–55.

Jarić, I., Višnjić-Jeftić, Ž., Cvijanović, G., Gačić, Z., Jovanović, L.J., Skorić, S., Lenhardt, M., 2011. Determination of differential heavy metal and trace element accumulation in liver, gills, intestine and muscle of starlet (*Acipenser ruthenus*) from the Danube River in Serbia by ICP OES. Microchem. J. 98, 77–81. https://doi.org/10.1016/J.MICROC.2010.11.008.

Kraemer, L.D., Campbell, P.G.C., Hare, L., 2006. Seasonal variations in hepatic Cd and Cu concentrations and in the sub-cellular distribution of these metals in juvenile yellow perch (*Perca flavescens*). Environ. Pollut. 142 (2), 313–325. https://doi.org/10.1016/j.envpol.2005.10.004.

Laflamme, J.S., Couillard, Y., Campbell, P.G.C., Hontela, A., 2000. Interrenal metallothionein and cortisol secretion in relation to Cd, Cu, and Zn exposure in yellow perch, Perca flavescens, from Abitibi lakes. Can. J. Fish. Aquat. Sci. 57, 1692–1700. https://doi.org/10.1139/f00-118.

Lambert, Y., Dutil, J.-D., 1997. Can simple condition indices be used to monitor and quantify seasonal changes in the energy reserves of cod (*Gadus morhua*)? Can. J. Fish. Aquat. Sci. 54, 104–112. https://doi.org/10.1139/cjfas-54-S1-104.

Lapointe, D., Couture, P., 2009. Influence of the route of exposure on the accumulation and subcellular distribution of nickel and thallium in juvenile fathead minnows (*Pimephales promelas*). Arch. Environ. Contam. Toxicol. 57, 571–580. https://doi.org/10.1007/s00244-009 9298-7.

Leonardos, I.D., Tsikliras, A.C., Eleftheriou, V., Cladas, Y., Kagalou, I., Chortatou, R., Papigioti, O., 2008. Life history characteristics of an invasive cyprinid fish (*Carassius gibelio*) in Chimaditis Lake (northern Greece). J. App. Ichtyol. 24 (7), 213-217. https://doi.org/10.1111/j.1439-0426.2007.01031.x.

Liu, H., Zhang, J.F., Shen, H.,Wang, X.R.,Wang,W.M., 2005. Impact of copper and its EDTA complex on the glutathione-dependent antioxidant system in freshwater fish (*Carassius auratus*). Bull. Environ. Contam. Toxicol. 74, 1111–1117. https://doi.org/10.1007/s00128-005-0696-x.

Maddock, D.M., Burton, M.P.M., 1999. Gross and histological observations of ovarian development and related condition changes in American plaice. J. Fish Biol. 53, 928–944. https://doi.org/10.1111/j.1095-8649.1998.tb00454.x.

Martínez-Álvarez, R.M., Morales, A.E., Sanz, A., 2005. Antioxidant defenses in fish: biotic and abiotic factors. Rev. Fish Biol. Fisheries 15 (1-2), 75–88. https://doi.org/10.1007/s11160-005-7846-4.

Mason, A.Z., Jenkins, K.D., 1995. Metal detoxification in aquatic organisms. In: Tessier, A., Turner, D. (Eds.), Metal Speciation and Bioavailability in Aquatic Systems. Wiley & Sons, Chichester, UK, pp. 479–608.

Mendiguchía, C., Moreno, C., García-Vargas, M., 2007. Evaluation of natural and anthropogenic influences on the Guadalquivir River (Spain) by dissolved heavy metals and nutrients. Chemosphere 69 (10), 1509–1517. https://doi.org/10.1016/j.chemosphere.2007.05.082.

Mijošek, T., Erk, M., Filipović Marijić, V., Krasnići, N., Dragun, Z., Ivanković, D., 2018. Electrochemical determination of metallothioneins by the modified Brdička procedure as an analytical tool in biomonitoring studies. Croat. Chem. Acta 91 (4), 475–480. https://doi.org/10.5562/cca3444.

Mijošek, T., Filipović Marijić, V., Dragun, Z., Ivanković, D., Krasnići, N., Erk, M., Gottstein, S., Lajtner, J., Sertić Perić, M., Matoničkin Kepčija, R., 2019a. Comparison of electrochemically determined metallothionein concentrations in wild freshwater salmon fish and gammarids and their relation to total and cytosolic metal levels. Ecol. Ind. 105, 188–198. https://doi.org/10.1016/j.ecolind.2019.05.069.

Mijošek, T., Filipović Marijić, V., Dragun, Z., Krasnići, N., Ivanković, D., Erk, M., 2019b. Evaluation of multi-biomarker response in fish intestine as an initial indication of anthropogenic impact in the aquatic karst environment. Sci. Total Environ. 660, 1079–1090. https://doi.org/10.1016/j.scitotenv.2019.01.045.

Mijošek, T., Filipović Marijić, V., Dragun, Z., Ivanković, D., Krasnići, N., Redžović, Z., Sertić Perić, M., Vdović, N., Bačić, N., Dautović, J., Erk, M., 2020a. The assessment of metal contamination in water and sediments of the lowland Ilova River (Croatia) impacted by anthropogenic activities. Environ. Sci. Pollut. Res. 27 (20), 25374–25389. https://doi.org/10.1007/s11356-020-08926-7.

Mijošek, T., Filipović Marijić, V., Dragun, Z., Ivanković, D., Krasnići, N., Redžović, Z., Veseli, M., Gottstein, S., Lajtner, J., Sertić Perić, M., Matoničkin Kepčija, R., Erk, M., 2020b. Thallium accumulation in different organisms from karst and lowland rivers of Croatia under wastewater impact. Environ. Chem. 17 (2), 201–212. https://doi.org/10.1071/EN19165.

Milošković, A., Simić, V., 2015. Arsenic and other trace elements in five edible fish species in relation to fish size and weight and potential health risks for human consumption. Pol. J. Environ. Stud. 24, 199–206. https://doi.org/10.15244/pjoes/24929.

Miramand, P., Lafaurie, M., Fowler, S.W., Lemaire, P., Guary, J.C., Bentley, D., 1991. Reproductive cycle and heavy metals in the organs of red mullet, *Mullus barbatus* (L), from the northwestern Mediterranean. Sci. Total Environ. 103, 47–56. https://doi.org/10.1016/0048-9697(91)90352-F.

Monna, F., Camizuli, E., Revelli, P., Biville, C., Thomas, C., Losno, R., Scheifler, R., Bruguier, O., Baron, S., Chateau, C., Ploquin, A., Alibert, P., 2011. Wild brown trout affected by historical mining in the Cévennes National Park, France. Environ. Sci. Technol. 45 (16), 6823–6830. https://doi.org/10.1021/es200755n.

Nachev, M., Sures, B., 2016. Seasonal profile of metal accumulation in the acanthocephalan *Pomphorhynchus laevis*: a valuable tool to study infection dynamics and implications for metal monitoring. Parasit. Vectors 9, 300–308. https://doi.org/10.1186/s13071-016-1576-4.

Olsvik, P.A., Gundersen, P., Andersen, R.A., Zachariassen, K.E., 2000. Metal accumulation and metallothionein in two populations of brown trout, *Salmo trutta*, exposed to different natural water environments during a run-off episode. Aquat. Toxicol. 50, 301–316.

Pinder III, J.E., Hinton, T.G., Taylor, B.E., Whicker, F.W., 2011. Cesium accumulation by aquatic organisms at different trophic levels following an experimental release into a small reservoir. J. Environ. Radioact. 102 (3), 283–293. https://doi.org/10.1016/j.jenvrad.2010.12.003.

Pinto, E., Sigaud-Kutner, T.C.S., Leitão, M.A.S., Okamoto, O.S., Morse, D., Colepicolo, P., 2003. Heavy metal-induced oxidative stress in algae. J. Phycol. 39, 1008–1018.

Radić, S., Gregorović, G., Stipaničev, D., Cvjetko, P., Šrut, M., Vujčić, V., Oreščanin, V., Vinko Klobučar, G.I., 2013. Assessment of surface water in the vicinity of fertilizer factory using fish and plants. Ecotoxicol. Environ. Saf. 96, 32–40. https://doi.org/10.1016/j.ecoenv.2013.06.023.

Rahman, I., Kode, A., Biswas, S.K., 2006. Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. Nat. Protoc. 1 (6), 3159–3165. https://doi.org/10.1038/nprot.2006.378.

Rajkowska, M., Protasowicki, M., 2013. Distribution of metals (Fe, Mn, Zn, Cu) in fish tissues in two lakes of different trophy in Northwestern Poland. Environ. Monit. Assess. 185 (4), 3493–3502. https://doi.org/10.1007/s10661-012-2805-8.

Rajotte, J.W., Couture, P., 2002. Effects of environmental metal contamination on the condition, swimming performance, and tissue metabolic capacities of wild yellow perch (*Perca flavescens*). Can. J. Fish. Aquat. Sci. 59, 1296–1304. https://doi.org/10.1139/F02- 095.

Ramani, S., Dragun, Z., Kapetanović, D., Kostov, V., Jordanova, M., Erk, M., Hajrulai-Musliu, Z., 2014. Surface water characterization of three rivers in the lead/zinc mining region of Northeastern Macedonia. Arch. Environ. Contam. Toxicol. 66 (4), 514–528. https://doi.org/10.1007/s00244-014-0012-z.

Raspor, B., Paić, M., Erk, M., 2001. Analysis of metallothioneins by the modified Brdička procedure. Talanta 55, 109–115. https://doi.org/10.1016/S0039-9140(01)00399-X.

Ricker, W.E., 1975. Computation and interpretation of biological statistics of fish populations. B. Fish. Res. Board Can. 191, 1–382.

Ringwood, A.H., Hoguet, J., Keppler, C.J., Gielazyn, M.L., Ward, B.P., Rourk, A.R., 2003. Cellular biomarkers (lysosomal destabilization, glutathione & lipid peroxidation). Three common estuarine species: a methods handbook. Marine Resources Research Institute South Carolina Department of Natural Resources, pp. 1–45.

Rosabal, M., Pierron, F., Couture, P., Baudrimont, M., Hare, L., Campbell, P.G., 2015. Subcellular partitioning of non-essential trace metals (Ag, As, Cd, Ni, Pb, Tl) in livers of American (*Anguilla rostrata*) and European (*Anguilla anguilla*) yellow eels. Aquat. Toxicol. 160, 128–141.

Rowan, D.J., Rasmussen, J.B., 1994. Bioaccumulation of radiocesium by fish: the influence of physiochemical factors and trophic structure. Can. J. Fish. Aquat. Sci. 51, 2388–2410.

Sabrah, M.M., Mohamedein, L.I., El-Sawy, M.A., Abou El-Naga, E.H., 2016. Biological characteristics in approaching to biochemical and heavy metals of edible fish *Terapon puta*, Cuvier, 1829 from different fishing sites along the Suez Canal, Egypt. J. Fish Aquat. Sci. 11, 147-162. https://doi.org/10.3923/jfas.2016.147.162.

Sasi, H., 2008. The length and weight relations of some reproduction characteristics of Prussian carp, *Carassius gibelio* (Bloch, 1782) in the South Aegean Region (Aydın-Turkey). Turk. J. Fish. Aquat. Sc. 8, 87–92.

Staniskiene, B., Matusevicius, P., Budreckiene, R., Skibniewska, K.A., 2006. Distribution of heavy metals in tissues of freshwater fish in Lithuania. Pol. J. Environ. Stud. 15, 585–591.

Sures, B., Steiner, W., Rydlo, M., Taraschewski, H., 1999. Concentrations of 17 elements in the zebra mussel (*Dreissena polymorpha*), in different tissues of perch (*Perca fluviatilis*), and in perch intestinal parasites (*Acanthocephalus lucii*) from the subalpine lake Mondsee, Austria. Environ. Toxicol. Chem. 18 (11), 2574–2579. https://doi.org/10.1002/etc.5620181126.

Tietze, F., 1969. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal. Biochem. 27 (3), 502–522. https://doi.org/10.1016/0003-2697(69)90064-5.

Tsangaris, C., Vergolyas, M., Fountoulaki, E., Goncharuk, V.V, 2011. Genotoxicity and oxidative stress biomarkers in *Carassius gibelio* as endpoints for toxicity testing of Ukrainian polluted river waters. Ecotoxicol. Environ. Saf. 74 (8), 2240–2244. https://doi.org/10.1016/j.ecoenv.2011.08.010.

Urien, N., Cooper, S., Caron, A., Sonnenberg, H., Rozon-Ramilo, L., Campbell, P.G.C., Couture, P., 2018. Subcellular partitioning of metals and metalloids (As, Cd, Cu, Se and Zn) in liver and gonads of wild white suckers (*Catostomus commersonii*) collected downstream from a mining operation. Aquat. Toxicol. 202, 105–116. https://doi.org/10.1016/j.aquatox.2018.07.001.

Van Campenhout, K., Goenaga Infante, H., Hoff, P.T., Moens, L., Goemans, G., Belpaire, C., Adams, F., Blust, R., Bervoets, L., 2010. Cytosolic distribution of Cd, Cu and Zn, and metallothionein levels in relation to physiological changes in gibel carp (*Carassius auratus gibelio*) from metal-impacted habitats. Ecotoxicol. Environ. Saf. 73 (3), 296–305. https://doi.org/10.1016/j.ecoenv.2009.10.007.

van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13 (2), 57–149. https://doi.org/10.1016/S1382-6689(02)00126-6.

Vašák, M., 2005. Advances in metallothionein structure and functions. J. Trace Elem. Med Biol. 19 (1), 13–17. https://doi.org/10.1016/j.jtemb.2005.03.003.

Viarengo, A., Burlando, B., Dondero, F., Marro, A., Fabri, R., 1999. Metallothionein as a tool in biomonitoring programmes. Biomarkers 4, 455–466. https://doi.org/10.1080/135475099230615.

Wallace, W.G., Lee, B.G., Luoma, S.N., 2003. Subcellular compartmentalization of Cd and Zn in two bivalves. I. Significance of metal-sensitive fractions (MSF) and biologically detoxified metal (BDM). Mar. Ecol. Prog. Ser. 249, 183–197. https://doi.org/10.3354/meps249183.

Wootton, R.J., 1990. Ecology of teleost fishes. Chapman and Hall, Fish and Fisheries Series 1. London, New York. https://doi.org/10.1007/978-94-009-0829-1_9 (404 pp).

Yabanli, M., Yozukmaz, A., Sel, F., 2014. Bioaccumulation of heavy metals in tissues of the gibel carp *Carassius gibelio*: example of Marmara Lake, Turkey. Russ. J. Biol. Invasions 5 (3), 217–224. https://doi.org/10.1134/S2075111714030126.

Yeltekin, A.C., Sağlamer, E., 2019. Toxic and trace element levels in *Salmo trutta macrostigma* and *Oncorhynchus mykiss* trout raised in different environments. Pol. J. Environ. Stud. 28 (3), 1613–1621. https://doi.org/10.15244/PJOES/90620.

Zhelev, Zh.M., Tsonev, S.V., Boyadziev, P.S., 2018. Significant changes in morpho-physiological and haematological parameters of *Carassius gibelio* (Bloch, 1782) (Actinopterygii: Cyprinidae) as response to sporadic effusions of industrial wastewater into the Sazliyka River, Southern Bulgaria. Acta Zool. Bulg. 70 (4), 547–556.

Zhelyazkov. G. I., Georgiev, D.M., Peeva, S.P., Kalcheva, S.E., Georgieva, K.Y., 2018. Chemical composition and levels of heavy metals in fish meat of the Cyprinidae family from Zhrebchevo Dam, Central Bulgaria. Ecol. Balk. 10, 133-140.