Biological Trace Element Research (2019) 188:494–507
https://doi.org/10.1007/s12011-018-1415-x

Trace Metals in the Freshwater Fish *Cyprinus carpio*: Effect to Serum Biochemistry and Oxidative Status Markers

Anton Kovacik[1] · Eva Tvrda[1] · Michal Miskeje[2] · Julius Arvay[3] · Marian Tomka[4] · Katarina Zbynovska[1] · Jaroslav Andreji[5] · Lukas Hleba[6] · Eva Kovacikova[2] · Martin Fik[5] · Peter Cupka[1] · Jozef Nahacky[7] · Peter Massanyi[1]

Received: 7 May 2018 / Accepted: 10 June 2018 / Published online: 2 July 2018

Abstract

Interactions between trace metals, serum biochemical parameters, and oxidative status markers were observed. Freshwater fish *Cyprinus carpio* blood samples ($n = 38$) were collected at the beginning of May ($n = 19$) and at the end of July ($n = 19$) of 2015. The concentrations of metals (As, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Se, Sr, and Zn) were analyzed in blood serum samples of fishes by inductively coupled plasma optical emission spectrometry (ICP-OES), and Hg was determined by cold-vapor atomic absorption spectroscopy (CV-AAS). The general scheme of descending concentrations of metals in blood serum samples was as follows: Zn > Fe > Cu > Sr > Cr > Ni > Mn > Pb > Se > As > Cd > Hg. Zn was the most accumulated element (4.42–119.64 mg/L) in both seasons. Overall, the trace element content was higher in spring season, except Hg, Ni, Se, and Sr. The seasonal effect was confirmed for Mn, Zn, Mg, Glu, AST, and Chol levels and for most oxidative status markers. The gender effect was confirmed for Sr, GPx, PC, Chol, and CK concentrations. Trace metals (especially Cd, Cr, Cu, Fe, Hg, Mn, Ni, Sr, Zn, As) significantly affected some blood serum chemistry parameters. The correlation analysis between oxidative status markers (ROS, TAC, MDA, SOD, GSH, UA, BHB, and Alb) and trace metal (Cd, Cu, Ni, Sr, Hg, Pb, Fe, Mn) content confirmed statistically significant interactions in both seasons. Obtained results indicate specific actions of trace metals.

Keywords Metals · Blood biochemistry · Oxidative stress · Antioxidant enzymes · Bio-monitoring

Introduction

Biomarkers such as serum chemistry and parameters of oxidative balance are good indicators of an overall biological status providing information on the effects of contaminants on the organism [1]. One of the most important environmental pollutants affecting health are trace metals [2–10], pesticides [11–14], pharmaceuticals [15–17], and endocrine disruptors [18, 19]. Monitoring of the presence and/or levels of contaminants in the organism under natural conditions and testing their associations to the physiological status is important for understanding their possible toxic effects and health risk on animals and humans. Particularly, trace metal investigation is still one of the main focuses of toxicological studies over the past years. Environmental contamination by trace metals is a consequence of anthropogenic activities connected to the industry, agriculture, and waste management [12, 20–23].

✉ Anton Kovacik
anton.kovacik@yahoo.com; anton.kovacik@uniag.sk

[1] Department of Animal Physiology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic

[2] AgroBioTech Research Centre, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic

[3] Department of Chemistry, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic

[4] Department of Biochemistry and Biotechnology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic

[5] Department of Poultry Science and Small Animal Husbandry, Faculty of Agrobiology and Food Resources, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic

[6] Department of Microbiology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic

[7] Department of Food Hygiene and Safety, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic

Measurement of blood serum chemistry parameters is necessary for the diagnosis of the health status in aquatic animals living in potentially polluted areas. Toxic effects of trace metals are generally connected to changes in the levels of biochemical parameters monitored in many studies [4, 21, 24–27].

A variety of chemicals may cause oxidative stress as a consequence of increased levels of reactive oxygen species (ROS) affecting the mitochondrial function followed by alterations to the enzymatic and/or endogenous antioxidants in blood [10] and tissues [8, 28–30]. Oxidative stress is a sensitive endpoint for metal toxicity due to alterations of target tissues or inhibition of enzymes containing thiol groups. As such, a combined exposure to trace metals can affect the antioxidant ability of blood [1]. Trace metals accumulated in aquatic animals are potentially redox active, which may suggest an imbalance between ROS production and antioxidant mechanisms of the fish [23, 31]. The main antioxidant enzymes are superoxide dismutase, glutathione peroxidase, and catalase [10, 23, 32]. Glutathione as endogenous antioxidant is also an integral part of the defenses against oxidative stress [33, 34] and primarily prevents the oxidation of water soluble proteins [35]. Furthermore, non-enzymatic antioxidants include uric acid and albumin. Biomarkers of oxidative stress (malondialdehyde as an index of lipid peroxidation and protein carbonyls as product of protein oxidation) are used as indicators of oxidative damage [14, 22, 36, 37].

The aim of the present study was to investigate seasonal and sex interactions between trace element/metal content in the blood serum and serum biochemical parameters as well as oxidative status markers in freshwater fish common carp.

Material and Methods

Experimental Design, Animal Management, and Blood Sampling

This study was realized during spring and summer of 2015. Fishes were bred by semi-intensive method of farming (university experimental pond in Kolíňany—West Slovak Lowland—Slovak Republic; 48°21′14.6″N 18°13′03.2″E) (Fig. 1). Fish stocking was realized at the beginning of March 2015. Catching of the fish was realized at beginning of May (spring season) and at the end of July (summer season) 2015. The freshwater fish (*Cyprinus carpio*) were caught by seine net. In total, 38 fishes were collected. After catching, the animals were transferred in polyethylene bags to the laboratory in 20 min for blood collection. Fish were manipulated by a competent person in accordance with the provisions of the

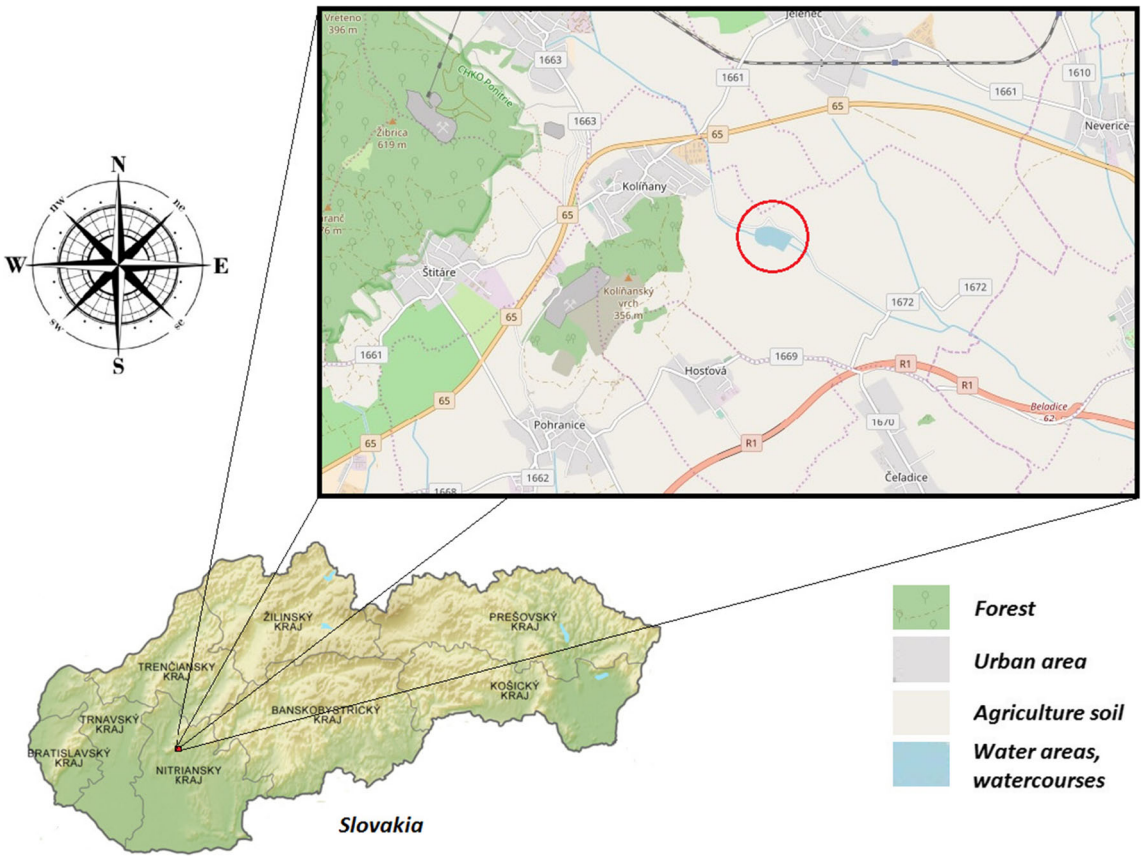

Fig. 1 The University fish pond Kolíňany (West Slovak Lowland—Slovak Republic; 48°21′14.6″N 18°13′03.2″E)

national law, approved by the Ethics Committee of the Slovak University of Agriculture in Nitra, protocol number 48/2013. After standard ichthyology evaluation (standard length and weight measurements, age determination by scales—Table 1), blood sampling was realized. For comparison with other authors, which presented length of fish as total length (TL), the transformation equation is given: $TL = 9.28564 + 1.16322 \times SL$ ($r = 0.9876$, $r^2 = 95.76\%$, $p < 0.001$).

Blood samples ($n = 38$) were taken in spring ($n = 19$) and summer ($n = 19$) seasons from male ($n = 17$) and female ($n = 21$) individuals. Blood was collected from *aorta ventralis* (*Nomina Anatomica*) from each fish. The samples were allowed to coagulate at room temperature. Subsequently, the samples were centrifuged for 20 min at 3000 rpm. Blood serum was collected and stored at − 20 °C until analyses at the Department of Animal Physiology.

Clinical Biochemistry Analyses

Blood serum concentrations of calcium (Ca), magnesium (Mg), total protein (TP), glucose (Glu), urea, cholesterol (Chol), triglycerides (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatine kinase (CK), total bilirubin (Bili), and total protein (TP) were measured using DiaSys (Diagnostic Systems GmbH, Holzheim, Germany) commercial kits and the semi-automated clinical chemistry analyzer Randox RX Monza (Randox Laboratories, Crumlin, UK) [6]. Sodium (Na), potassium (K), and chloride (Cl) ions were analyzed using an EasyLite analyzer (Medica, Bedford, MA, USA) provided with an ion-selective electrode [7, 38].

Assessment of the Oxidative Status

ROS production in each sample was assessed by the chemiluminescence assay using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma-Aldrich) as the probe [39]. The test samples consisted of luminol (2.5 μL, 5 mmol/L) and 100 μL of sample. Negative controls were prepared by replacing blood with 100 μL of PBS (Dulbecco's Phosphate Buffer Saline without calcium chloride and magnesium chloride; Sigma-Aldrich). Positive controls included 100 μL PBS, 2.5 μL luminol, and 50 μL hydrogen peroxide (H_2O_2, 30%; 8.8 M; Sigma-Aldrich). Chemiluminescence was measured using the Glomax Multi$^+$ Combined Spectro-Fluoro Luminometer (Promega Corporation, Madison, WI, USA) [36, 37]. The results are expressed as relative light units (RLU)/s/g protein.

An improved enhanced chemiluminescence antioxidant assay using horseradish peroxidase conjugate and luminol was used to study the total antioxidant capacity (TAC) of the sample [40]. Five to one hundred micromoles per liter of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Sigma-Aldrich) was used as the standard, while a signal reagent consisting of 0.1 mol/L Tris-HCl (Sigma-Aldrich), 12 mol/L H_2O_2 (Sigma-Aldrich), 41.8 mmol/L 4-iodophenol (Sigma-Aldrich), and 282.2 mmol/L luminol (Sigma-Aldrich) was used to induce the chemiluminescent reaction. Chemiluminescence was measured on 96-well plates in 10 cycles of 1 min using the Glomax Multi$^+$ Combined Spectro-Fluoro Luminometer (Promega Corporation). The results are expressed as micromoles of Trolox Eq. per gram of protein.

Superoxide dismutase (SOD), glutathione peroxidase (GPx), and D-3-hydroxybutyrate (BHB) activity was measured using the Randox commercial kits (Randox Laboratories, Crumlin, Great Britain) and the semi-automated analyzer Randox RX Monza (Randox Laboratories, Crumlin, UK) [36]. The results are expressed as units per gram of protein (SOD, GPx) and micromoles per gram of protein (BHB).

Catalase (CAT) activity was quantified according to Beers and Sizer [41] by monitoring the decrease of hydrogen peroxide (H_2O_2) at 240 nm [36]. The values are expressed as units per milligram of protein.

Reduced glutathione (GSH) was evaluated by the Ellman method [42]. GSH concentration is expressed as milligrams per gram of protein.

Table 1 Characteristics of analyzed fish

Date of catching	*n*	Age (year)	SL (mm)		BW (g)	
			Mean ± SD	Range	Mean ± SD	Range
Spring season	19	7–9	388.2 ± 27.0	335–440	1926.8 ± 327.1	1260–2610
Summer season	19	6–9	407.9 ± 30.8	350–485	2218.5 ± 369.9	1710–2902
Male	17	6–9	387.7 ± 21.4	345–420	1910.7 ± 313.1	1260–2362
Female	21	6–9	406.4 ± 34.2	335–485	2203.9 ± 411.6	1580–2902
Total	38		398.0 ± 30.3	335–485	2072.68 ± 394.7	1260–2902

SL standard length, *BW* body weight, *SD* standard deviation, *n* number of individuals

Albumin (Alb) concentration was measured using the ALB BioLa Test (PLIVA-Lachema, Brno, Czech Republic) commercial kit. Albumin binds with Bromo Cresol Green (BCG) at pH 4.2 causing a shift in absorbance of the yellow BCG dye. The blue-green color formed is proportional to the concentration of albumin, when measured photometrically at 578 nm with the help of the Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc.). Albumin concentration is expressed as grams per gram of protein.

Uric acid quantification was characterized by the oxidation of the substance leading to H_2O_2 and allantoin formation. The resulting H_2O_2 was detected by reacting with *N*-ethyl-*N*-(2-hydroxy-3-sulphopropyl)-m-toluidine and 4-aminoantipyrine. The absorbance is subsequently measured at 550 nm. BioLa Uric Acid commercial kit (PLIVA-Lachema, Brno, Czech Republic) and the Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc.) were used for the assay. Uric acid concentration is expressed as micromoles per gram of protein.

Carbonyl group quantification was performed through the traditional 2,4-dinitrophenylhydrazine (DNPH) method [43]. Protein carbonyls are expressed as nanomoles per gram of protein [44].

Lipid peroxidation (LPO) expressed through malondialdehyde (MDA) production was measured with the help of the TBARS assay, modified for a 96-well plate and ELISA reader [36, 37]. MDA concentration is expressed as micromoles per gram of protein.

Protein concentration was quantified using the DiaSys Total Protein (Diagnostic Systems GmbH, Holzheim, Germany) commercial kit and the semi-automated clinical chemistry photometric analyzer Randox RX Monza (Randox Laboratories, Crumlin, UK) [6]. The measurement is based on the Biuret method, according to which copper sulfate reacts with proteins to form a violet blue color complex in alkaline solution, and the intensity of the color is directly proportional to the protein concentration when measured at 540 nm.

Detection of Trace Metals

The concentrations of trace metals (arsenic [As], cadmium [Cd], chromium [Cr], copper [Cu], iron [Fe], manganese [Mn], nickel [Ni], lead [Pb], selenium [Se], strontium [Sr], and zinc [Zn]) were analyzed in blood serum samples of fishes by inductively coupled plasma optical emission spectrometry (ICP-OES) and mercury (Hg) was determined by cold-vapor atomic absorption spectroscopy (CV-AAS).

Pre-analytical Procedure for ICP-OES Analysis

High-purity chemicals were used for all operations. For elemental analysis, the fish serum samples were kept at − 20 °C until analysis. The defrosted samples (1 mL) were mineralized (wet mineralization) in the high-performance microwave digestion system Ethos UP (Milestone Srl, Sorisole, BG, Italy) in a solution of 5 mL HNO_3 (TraceSELECT®, Honeywell Fluka, Morris Plains, USA) and 1 mL of H_2O_2 (30%, for trace analysis, Merck Suprapur®). Samples, and blank sample, were digested according to preloaded method "animal tissue" developed by manufacturer for assuring the best result. The method consists of 15-min heating to 200 °C, keeping this temperature for 15 min and 15 min of active cooling. The digests cooled to 50 °C were filtered through the Sartorius filter discs (grade 390) (Sartorius AG, Goettingen, Germany) into the volumetric flask and filled up with ultrapure water to a volume of 50 mL.

ICP-OES Analysis

Analysis of the elements (As, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Se, Sr, and Zn) was carried out using inductively coupled plasma optical emission spectrophotometer (ICP-OES 720, Agilent Technologies Australia (M) Pty Ltd.). Detections limits (μg/L) of measured elements were follows: As 1.50, Cd 0.05, Cr 0.15, Cu 0.30, Fe 0.10, Mn 0.03, Ni 0.30, Pb 0.80, Se 2.00, Sr 0.01, Zn 0.20. Details of the instrumental operating conditions are listed in Table 2. In the experiment, Multielement standard solution V for ICP (Sigma-Aldrich Production GmbH, Switzerland) was used. The validity of the whole procedure was checked by processing of duplicate samples against the certified reference material (CRM–ERM CE278K, Sigma-Aldrich Production GmbH, Switzerland).

CV-AAS Analysis

Total mercury content (Hg) was determined directly in the defrosted blood serum samples by a selective mercury analyzer AMA-254 (Altec, Praque, Czech Republic) based on CV-AAS.

The detection limit for mercury was 1.5 ng/L [6].

Statistical Analyses

Obtained data were checked for normality using a Kolmogorov-Smirnov test before statistical analyses. Mann-Whitney non-parametric test was used to assess the differences in the investigated parameters and metal concentrations between seasons and genders. The Spearman *R* correlation coefficient was used to measure the association between the trace elements concentrations and all investigated parameters in the blood serum. The minimum significance level was $P < 0.05$. Statistical analyses were performed using STATGRAPHICS Centurion (© StatPoint Technologies, Inc., USA) and GraphPad Prism 3.02 (GraphPad Software Incorporated, San Diego, California, USA).

Table 2 Operating parameters for the determination of elements by ICP-OES

Method parameters	
RF power (kW)	1.30
Plasma flow (L/min)	15.0
Auxiliary flow (L/min)	1.50
Nebulizer flow (L/min)	0.85
Replicated read time (s)	5.00
Instrument stabilization (s)	15
Sample uptake delay (s)	25
Pump rate (rpm)	15
Rinse time (s)	10
Element (λ/nm)	As 188.980, Cd 226.502, Cr 267.716, Cu 324.754, Fe 234.350, Mn 257.610, Ni 231.604, Pb 220.353, Se 196.026, Sr 407.771, Zn 206.200

Results

In the present study, we assessed the relationship between trace metal content and biochemical/oxidative status markers in freshwater fish blood. Mean and median seasonal/gender concentrations of obtained trace metals in the blood serum are summarized in Fig. 2 (mean, median, 25–75%, min-max) and *P* values of Mann-Whitney test are shown in Table 3. The general scheme of descending concentrations of trace metals in blood serum samples was follows: Zn > Fe > Cu > Sr > Cr > Ni > Mn > Pb > Se > As > Cd > Hg. Zn was the most accumulated element (4.42–119.64 mg/L). Significant differences among season and sex were not detected for concentrations of As, Cd, Cr, Cu, Fe, Hg, Ni, Pb, and Se. Mn ($P < 0.05$) and Zn ($P < 0.01$) were significantly higher in spring season. Mean concentration of Sr was significantly higher in female fish ($P < 0.01$). Overall, the trace element content was higher in spring season, except Hg, Ni, Se, and Sr, but non-significantly.

Mean and median seasonal/gender concentrations of oxidative status markers are presented in Fig. 3. Compared to fishes collected during different seasons, we observed that the activities of ROS, SOD, CAT, GPx, PC, MDA, and bilirubin were significantly increased in spring season and the activities of TAC, GSH, UA, and albumin were significantly higher in summer season. GPx and PC activities were significantly higher in male fish (Table 3).

Median concentrations of serum chemistry parameters are presented in Fig. 4. Glucose, AST, and cholesterol contents were significantly higher in summer season ($P < 0.05$). Cholesterol level was significantly higher in female fish ($P < 0.05$). Finally, higher significant value of CK was found in male fish ($P < 0.05$).

The Spearman *R* correlation coefficients were used to assess the relationships between the serum chemistry/oxidative stress parameters and the trace metal concentrations in the blood serum. Statistically significant correlations during seasons are shown in Table 4.

In the spring season, positive statistically significant correlations between the Co, Sr, and Zn concentrations and Ca and Mg were found. Moreover, a statistically significant positive correlation was detected between K and Fe resp. Mn; Cd, Fe, and Mn were negatively correlated with Na and Cl. Positive (statistically significant) correlations were found between Ni and ALP, Hg and Chol, As negatively correlated with bilirubin. Concentration of ROS negatively correlated with Cd, Cu, Ni, and Sr in spring season. Ni was also positively correlated with TAC and UA and was negatively correlated with SOD and MDA. Hg was negatively correlated (statistically significant) with TAC and was positively correlated with MDA and BHB. The analysis also revealed significant negative associations between Cu and MDA resp. SOD and positive association between Cu and GSH. Negative and significant relationships occurred between Cd and SOD, and Pb and BHB. Significant, negative correlation was seen between Fe and albumin in spring season.

The correlation analysis in summer season showed the significant positive relationship between Mg and Cr; Cu, Fe, and Hg; Ca and Zn; Na and Cu; K and As; Cl and Ni; and Mn and Urea resp. ALP, Cu, and cholesterol. Concentrations of CK were positively correlated with Cr and Hg; TG was also positively correlated with Cu and Hg. Negative significant correlations between Ni and Na, TP, Chol, and TG were found in summer season. Glucose level showed a negative correlation with Cr and Hg. During summer season, the concentration of Cu positively correlated with ROS, MDA, and BHB activities; Sr concentrations positively correlated with both TAC and GSH activities; Hg concentration positively correlated with ROS activity; Mn concentration positively correlated with GSH activity. The negative correlations during summer season in oxidative status markers were obtained between the concentration of Fe and bilirubin, Sr and MDA, and Cu and UA.

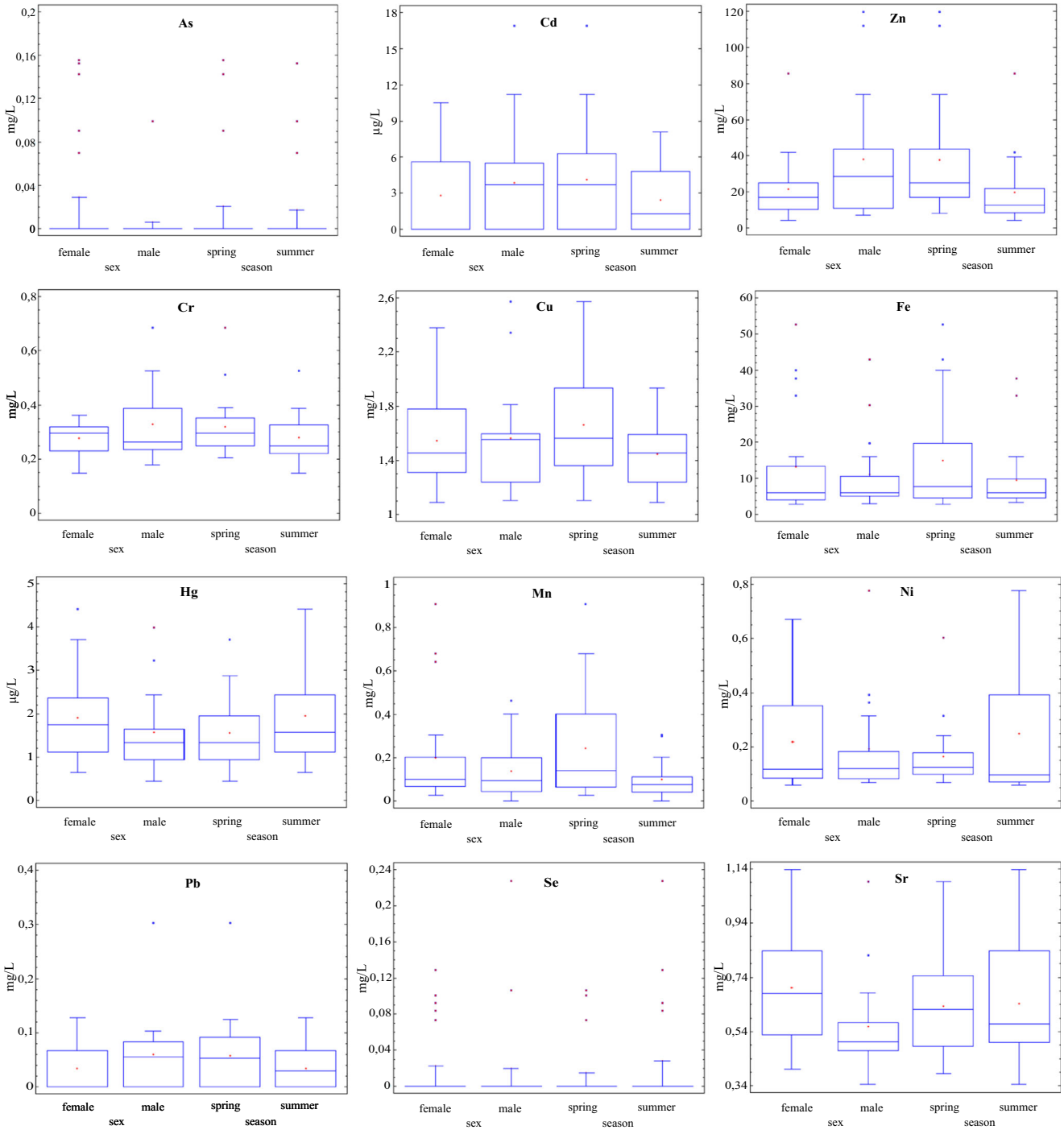

Fig. 2 Trace elements concentrations (mg/L resp. µg/L) measured in blood serum of *C. carpio*. Data are presented using box-whisker plot (mean, median, 25–75%, min.-max.)

Discussion

Findings obtained in the present study describe the interactions between environmental pollutants and physiological status of freshwater fish during spring and summer season. Various trace elements in polluted environments may have different effects on living organisms; therefore, it is necessary to monitor and test such associations between them. Most of the studies demonstrate bioaccumulation of metals in different tissues [9, 22, 45–52] of aquatic animals. Still, there are very few blood studies of aquatic animals [24, 26]. Reports have demonstrated ecotoxicology interactions between trace metals and serum chemistry parameters [21, 53], or oxidative stress markers [5, 8, 29, 54] in aquatic animals.

Seasonal effect was confirmed for Mn and Zn and sex effects were observed only in case of Sr. Seasonal variations

Table 3 Results (*P* values) of Mann-Whitney test for the concentrations of trace metals, oxidative status markers, and serum chemistry parameters among season and gender

Metal	As	Cd	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Se	Sr	Zn		
Factor														
Season	0.4817	0.2155	0.1274	0.0763	0.1827	0.1604	*0.0289*	0.3414	0.1958	0.3174	0.5000	*0.0055*		
Gender	0.0695	0.2934	0.1892	0.4186	0.3845	0.1323	0.3193	0.3845	0.0879	0.2302	*0.0038*	0.0711		
Redox marker	ROS	TAC	SOD	CAT	GPx	PC	MDA	BHB	GSH	UA	Alb	Bili		
Factor	*P* value													
Season	*0.0330*	*0.0020*	*0.0043*	*0.0028*	*0.0026*	*0.0007*	*0.0016*	0.0855	*0.0342*	*0.0068*	*0.0056*	*0.0365*		
Gender	0.1523	0.1376	0.1240	0.1024	*0.0314*	*0.0178*	0.0667	0.4422	0.1284	0.0606	0.2964	0.0886		
Serum marker	Ca	Mg	Na	K	Cl	Urea	TP	Glu	AST	ALT	ALP	Chol	TG	CK
Factor	*P* value													
Season	0.1428	0.0123	0.4825	0.3414	0.3630	0.1056	0.4767	*0.0100*	*0.0104*	0.2581	0.2616	*0.0205*	0.0558	0.3699
Gender	0.4100	0.1201	0.1007	0.1932	0.0598	0.2209	0.0634	0.4817	0.4742	0.1448	0.4041	*0.0246*	0.2405	*0.0146*

Italicized values are significant at $P < 0.05$, resp. $P < 0.01$

of trace metals bioaccumulation in aquatic animals were confirmed in many studies [3, 5, 55, 56]. Other studies showed sex differences in metal tissue bioaccumulation, such as Zn in the liver and skin of *Lethrinus lentjan* [57] or Hg in the muscle of *Silurus glanis* [58]. Secondly, we observed seasonal and gender differences for serum chemistry and oxidative stress markers. The serum biochemical values were consistent with values found in other fresh water fish [12, 13, 59]. Giarratano et al. [5] emphasized on a significant seasonal effect on oxidative stress markers (lipid radical content, MDA, α-tocopherol, total thiol groups, and metallothioneins) and trace metals content (Cd, Pb, Cu, Zn, Ni, Cr, Al) in the tissue of *Neohelice granulata*.

Health Status Observation

Serum chemistry parameters were within the reference range [60], except for decreased values of Cl^- and increased contents of K^+, TP, Glu, Chol, and ALP. Changes in the levels of serum glucose and total protein can be observed in case of liver failure [27] and nephrotoxicity [1]. Decreased chlorides are associated with stress, extreme temperatures, and infection as well as trace metal toxicity [60]. Gopal et al. [26] described the effects of Cu, Hg, Ni, and Pb on the blood protein biochemistry of *C. carpio*. Their results revealed a same tendency for all metals: an initial increased mobilization followed by a steady depletion. However, it should be noted that lethal and sub-lethal concentrations of respective trace metals were used. Increased blood glucose content (hyperglycemic conditions; enhanced glycogenolysis) influenced by trace metals (Cu, Ni, Fe, Mn, Zn) was observed in fish (*Mastacembelus armatus*) living in water contaminated by wastewater from a thermal power plant [61]. On the other hand, Fırat and Kargın [4] tested individual and combined effects of Cd and Zn on the serum chemistry parameters of freshwater fish (*Oreochromis niloticus*), but their results had an opposite tendency for glucose, for TP, and partly for cholesterol (decreasing content against higher levels of Cd and Zn in spring season). Fırat and Kargın [4] as well as Öner et al. [27] also confirmed associations between metals (Cd and Zn) and liver enzymes ALT and AST (increasing after exposure) which is comparable with our levels of ALT, but not for AST. We observed positive correlation between Ni levels as well as an increased ALP content in spring season and between Mn and ALP in summer season. Increased blood levels of liver enzymes are the main indicator to inform us about the liver and cellular damage caused by metal poisoning [62, 63]. ALT and AST activities were comparable to other studies on common carp [64]; however, the ALP activity was several-fold higher in our study, which could be explained by pathological processes such as liver impairment, kidney dysfunction, and bone disease [63]. The enzymes ALT and AST are used as biomarkers to detect hepatotoxicity, while ALP indicates bile duct epithelial damage [65].

Interactions between blood serum minerals (Ca, Mg, Na, K, Cl) and trace metals were confirmed in spring (Sr, Zn, Cd, Fe, Mn) and summer (Zn, Cr, Cu, Fe, Hg, Ni, As) seasons. Relation between Pb and Ca is probably the best known. Lead as a potential neurotoxicant is able to mimicry calcium. Lead competes with calcium for binding sites on calcium-regulated proteins [66, 67]; and it can also start the disruption of calcium transport [68]. The physiological investigation of Cd poisoning in fish showed a slight decrease of blood potassium and an increase of blood plasma magnesium [69], while copper exposure reflected a decrease in the plasma sodium, potassium, calcium, and chloride concentrations [70], though these relationships were not confirmed in our study, except Cu and Na relationship in summer season.

The correlation analysis also showed some other relationships between the tested parameters and trace metals. Blood urea concentration was significantly affected by manganese in summer season. Öner et al. [27] confirmed a significant

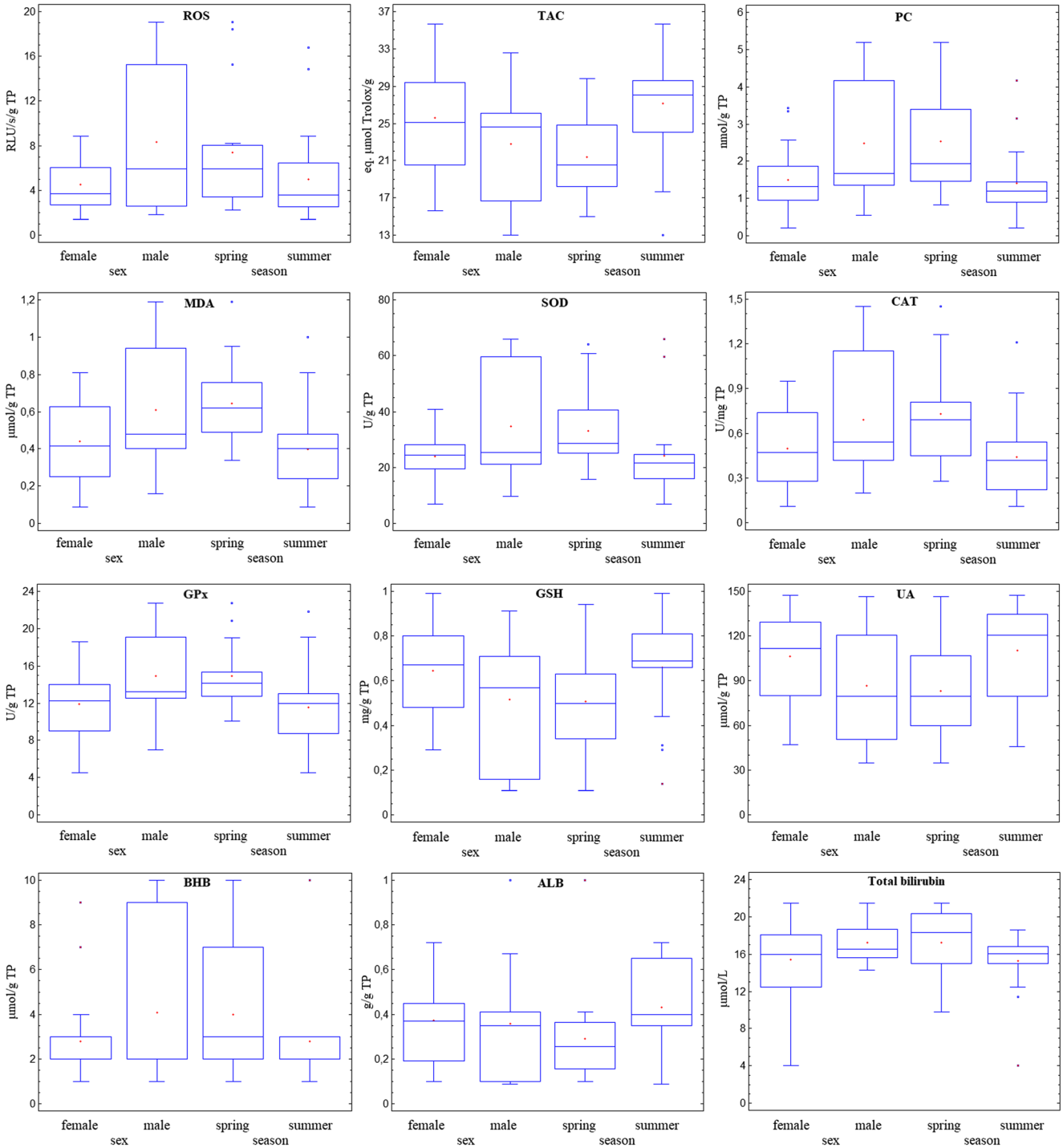

Fig. 3 Oxidative status markers measured in blood serum of *C. carpio*. Data are presented using box-whisker plot (mean, median, 25–75%, min.-max.)

increase of blood urea nitrogen (BUN) in Cd, Cu, and Cr exposed fish (*Oreochromis niloticus*). Increased BUN is associated with gill and kidney disease or failure [21, 71].

Oxidative Status Markers

In response to environmental pollutants, oxidative stress can provide valuable information regarding the internal environment. The production of reactive oxygen species (ROS) can cause oxidative damage at the cellular level. Generally, trace metals generate and promote ROS (such as hydrogen peroxide or the peroxide radical, superoxides, and

Fig. 4 Serum chemistry parameters measured in blood serum of *C. carpio*. Data are presented using box-whisker plot (mean, median, 25–75%, min.-max.) ▶

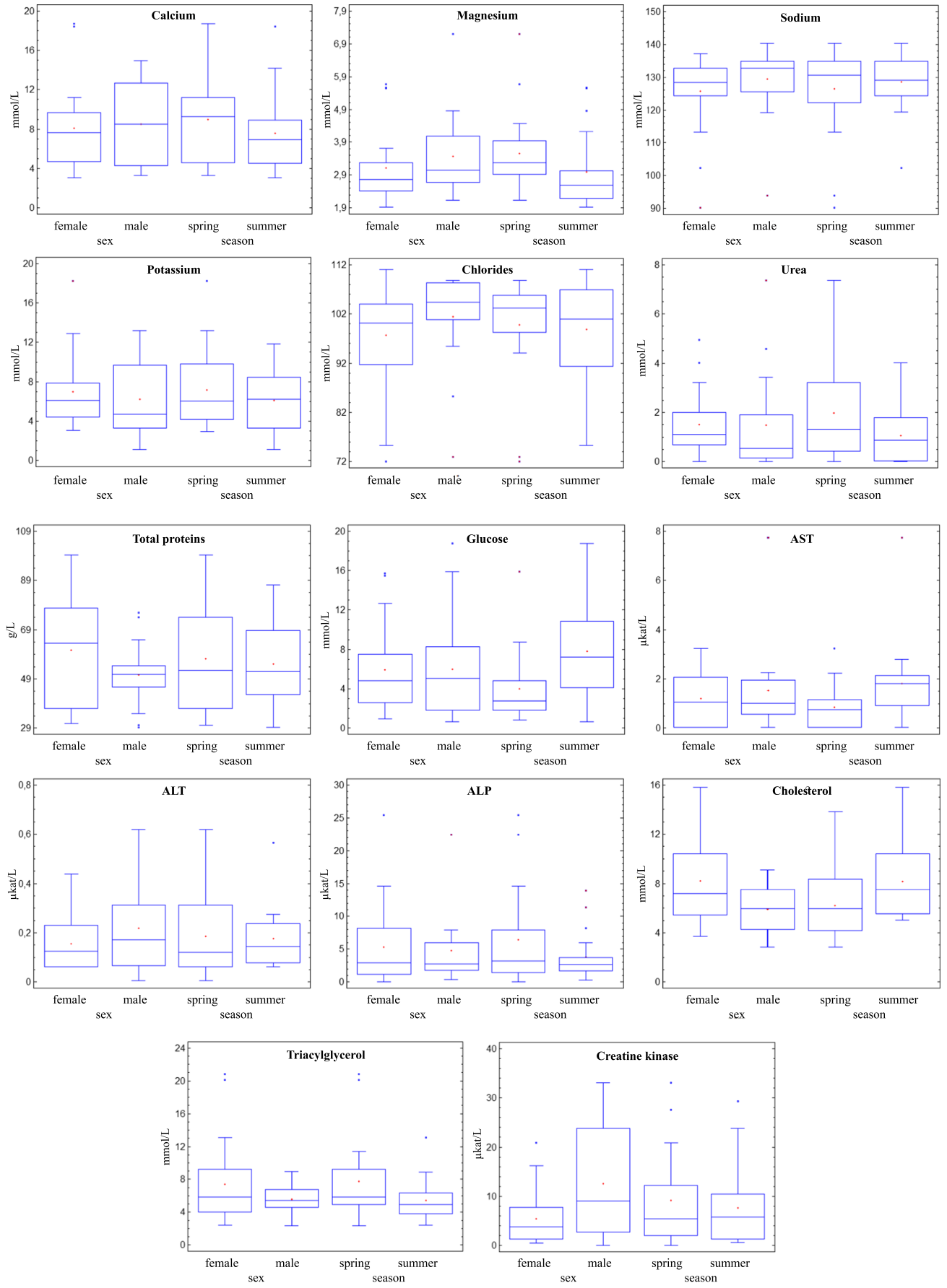
Calcium
mmol/L
Magnesium
mmol/L
Sodium
mmol/L
Potassium
mmol/L
Chlorides
mmol/L
Urea
mmol/L
Total proteins
g/L
Glucose
mmol/L
AST
µkat/L
ALT
µkat/L
ALP
µkat/L
Cholesterol
mmol/L
Triacylglycerol
mmol/L
Creatine kinase
µkat/L
female
male
spring
summer
sex
season

Table 4 Statistically significant correlations between trace metal concentrations and investigated blood serum parameters (oxidative status markers and serum chemistry parameters) in different seasons

Spring season			Summer season		
Investigated parameter	Metal	Spearman *R* (*P* value)	Investigated parameter	Metal	Spearman *R* (*P* value)
Ca	Sr	0.5833 (0.0196)	Ca	Zn	0.9167 (0.0002)
Ca	Zn	0.6691 (0.0074)	Mg	Cr	0.5281 (0.0251)
Mg	Sr	0.6719 (0.0044)	Mg	Cu	0.5491 (0.0198)
Mg	Zn	0.6263 (0.0079)	Mg	Fe	0.5105 (0.0303)
Na	Cd	− 0.5214 (0.0270)	Mg	Hg	0.7175 (0.0023)
Na	Fe	− 0.7635 (0.0012)	Na	Cu	0.4860 (0.0390)
Na	Mn	− 0.7407 (0.0017)	Na	Ni	− 0.6649 (0.0048)
K	Fe	0.7389 (0.0017)	K	As	0.5620 (0.0171)
K	Mn	0.5230 (0.0265)	Cl	Ni	0.5309 (0.0243)
Cl	Cd	− 0.5045 (0.0323)	Urea	Mn	0.6802 (0.0039)
Cl	Fe	− 0.6632 (0.0049)	TP	Ni	− 0.6228 (0.0082)
Cl	Mn	− 0.6175 (0.0088)	Glu	Cr	− 0.5754 (0.0146)
ALP	Ni	0.6147 (0.0173)	Glu	Hg	− 0.6246 (0.0081)
Chol	Hg	0.5930 (0.0119)	ALP	Mn	0.5253 (0.0303)
Bili	As	− 0.5737 (0.0180)	Chol	Cu	0.4982 (0.0345)
TG	Hg	0.4667 (0.0477)	Chol	Ni	− 0.4632 (0.0494)
ROS	Cd	− 0.5628 (0.0244)	Bili	Fe	− 0.4625 (0.0497)
ROS	Cu	− 0.7721 (0.0020)	TG	Cu	0.7105 (0.0026)
ROS	Ni	− 0.5760 (0.0212)	TG	Ni	− 0.6667 (0.0047)
ROS	Sr	− 0.6201 (0.0131)	TG	Hg	0.4702 (0.0461)
TAC	Ni	0.5049 (0.0434)	CK	Cr	0.4923 (0.0424)
TAC	Hg	− 0.5025 (0.0445)	CK	Hg	0.5046 (0.0375)
MDA	Cu	− 0.6150 (0.0172)	ROS	Cu	0.5509 (0.0194)
MDA	Ni	− 0.5693 (0.0275)	ROS	Hg	0.4667 (0.0477)
MDA	Hg	0.5133 (0.0468)	TAC	Sr	0.5930 (0.0119)
SOD	Cd	− 0.4981 (0.0463)	MDA	Cu	0.4831 (0.0411)
SOD	Cu	− 0.6789 (0.0066)	MDA	Sr	− 0.4822 (0.0408)
SOD	Ni	− 0.5343 (0.0326)	GSH	Mn	0.5075 (0.0313)
GSH	Cu	0.5420 (0.0358)	GSH	Sr	0.4749 (0.0439)
UA	Ni	0.5971 (0.0208)	UA	Cu	− 0.4667 (0.0477)
BHB	Pb	− 0.5381 (0.0441)	BHB	Cu	0.4856 (0.0349)
BHB	Hg	0.5868 (0.0281)			
Alb	Fe	0.5911 (0.0221)			

nitric oxide) overproduction in the cell [72]. Inversely, cells own specific defense mechanisms to protect against ROS-mediated oxidative damage. The antioxidant systems are classified into two major groups. Enzymatic antioxidants (SOD, CAT, GPx) that act as the body's first line of defense by catalyzing ROS conversion to less reactive or inert species, and non-enzymatic (endogenous) antioxidants (GSH, UA, Alb), which provide a secondary defense against ROS ([10, 23, 31–34]). Environmentally induced oxidative stress in aquatic animals was described in several studies, specifically in relation to the trace metal content [3, 5, 10, 22].

Bocchetti et al. [73] tested the effect of various sampling periods to oxidative stress in aquatic organisms (*Tapes philippinarum* and *Mytilus galloprovincialis*) and showed a significant seasonal impact on the activities of CAT, GST, CAT, and GPx, which is comparable with our results (TAC, CAT, and GPx activities exhibited significant differences among seasons). Moreover, the ROS, SOD, GSH activity, and concentrations of PC, MDA, UA, albumin, and bilirubin in the blood were also significantly affected by season.

We observed many significant correlations between metals and oxidative status markers in both seasons (Table 4). The effect of Cu and Pb content on the CAT activity, Al, and Ni

content on higher MDA concentrations in burrowing crab (*Neohelice granulate*) were confirmed by Giarratano et al. [5]. Shi et al. [74] also found that nickel toxicity is associated with ROS generation with a subsequent lipid peroxidation, and alkyl and alkoxyl radical production.

Ruas et al. [10] have observed oxidative status biomarkers, such as changes in the glutathione (GSH) content, activity of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) and the levels of lipid peroxidation (LPO), in the blood of three cichlid fish (*Oreochromis niloticus*, *Tilapia rendalli*, and *Geophagus brasiliensis*) during two seasons (autumn, spring) from unpolluted and polluted (Cr, Cd, Cu, Zn, Mn, Fe pollution) sites. Their results showed a significantly increased activity of SOD, LPO, GSH, and LPO/CAT+GPx ratio in the polluted site, which may indicate the induction of oxidative stress. According to Javed et al. [23], antioxidant enzymes (SOD, CAT, GST) and lipid peroxidation in different tissues (liver and kidney) of fish (*Channa punctatus*) exposed to metals (Cu, Ni, Fe, Mn, Cr, Zn) showed a significant increase of their activity; however, the level of non-enzymatic parameters (GSH) decreased. In another study [28], the authors recorded a decreased activity of SOD and CAT and an increased MDA (lipid peroxidation) content in the liver of metal-exposed catfish. As such, we may suppose that if the first line of defense is activated immediately, the activity of antioxidant enzymes will increase. If the first antioxidant protection line is not rapidly involved, oxidative stress and subsequent lipoperoxidation can occur, resulting in an increased concentration of MDA in blood, cells, and tissues. In addition, oxidative stress can be the cause of protein oxidation, which can be expressed through an increased content of PC in the blood or tissues.

Correlation analysis of Abarikwu et al. [28] showed comparable results to ours, particularly the effect of nickel and cadmium on the activity of superoxide dismutase. Lead affected the function of various antioxidant enzymes; decreased activity of SOD, CAT, GPx, and GSH; and increased lipid peroxidation [75], which demonstrate induced oxidative injury. GSH is a sulfhydryl-rich tripeptide that is generally involved in the protection of cells against toxicants and in the metabolism of xenobiotics [76]. [33]) showed that the exposure of a fish population (*Oreochromis niloticus*) to polluted water (Cd, Cu, Cr, Pb, Zn) caused oxidative stress followed by the response of the glutathione metabolism. Glutathione molecules were depleted after metal exposure, which caused increased glutathione S-transferase activity and may reflect on the deterioration cell protection ability. Gopal et al. [26] observed a decreasing tendency of albumin content in blood serum of common carp following exposure of different concentrations of mercuric chloride, lead nitrate, copper sulfate, and nickel sulfate, which refers to liver disease and stress situations. De Oliveira et al. [22] evaluated several biomarkers in *Anodontites trapesialis* after 96 h of confinement downstream of a coal mine. Increased bioaccumulation of metals (Al and Fe) resulted in an increased lipid peroxidation and protein oxidation in gills, while SOD was not affected. Low level of uric acid as an endogenous antioxidant is less of a health concern; however, its increased levels may refer to several disturbances of the kidney [77].

Conclusions

Taken together, obtained data on *C. carpio* indicate that trace metals (especially Cd, Cr, Cu, Fe, Hg, Mn, Ni, Sr, Zn, As) affect blood serum chemistry parameters (Ca, Mg, Na, K, Cl, Urea, TP, Glu, ALP, Chol, TG, CK); however, there was not serious damage to health status, except for ALP which may indicate bile duct epithelial damage. The correlation analysis between oxidative status markers (ROS, TAC, MDA, SOD, GSH, UA, BHB, Alb) and trace metal (Cd, Cu, Ni, Sr, Hg, Pb, Fe, Mn) content confirmed statistically significant interactions in both seasons.

Nevertheless, the results indicate many other associations between monitored contaminants and physiological parameters. When aquatic ecosystems are polluted with contaminants such as trace metals, aquatic animals, especially fish, should be also contaminated through bioaccumulation, as these are in continual contact with polluted environment, suggesting that trace metals negatively influence the fish physiology.

Further studies are necessary for the bio-monitoring of environmental, ecological, and pollutant stress factors as ecological risk assessment in association of biomarkers in living organisms.

Funding Information This work was supported by the Grant Agency of SUA in Nitra, project no. 06-GA SPU-16 (0.20), VEGA 1/0625/15 (0.10) and by the Slovak Research and Development Agency under the contract No. APVV-16-0289 (0.50). This work was also supported by AgroBioTech Research Centre built in accordance with the project Building "AgroBioTech" Research Centre ITMS 26220220180 (0.20).

Compliance with Ethical Standards

The study was approved by the Ethics Committee of the Slovak University of Agriculture in Nitra, protocol number 48/2013.

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Gupta, R. C. (2014) Biomarkers in toxicology, first ed. Academic Press. pages
2. Binkowski ŁJ, Sawicka-Kapusta K, Szarek J, Strzyżewska E, Felsmann M (2013) Histopathology of liver and kidneys of wild living Mallards Anas platyrhynchos and Coots Fulica atra with

considerable concentrations of lead and cadmium. Sci Total Environ 450:326–333. https://doi.org/10.1016/j.scitotenv.2013.02.002

3. Duarte CA, Giarratano E, Amin OA, Comoglio LI (2011) Heavy metal concentrations and biomarkers of oxidative stress in native mussels (Mytilus edulis chilensis) from Beagle Channel coast (Tierra del Fuego, Argentina). Mar Pollut Bull 62(8):1895–1904. https://doi.org/10.1016/j.marpolbul.2011.05.031
4. Fırat Ö, Kargın F (2010) Individual and combined effects of heavy metals on serum biochemistry of Nile tilapia Oreochromis niloticus. Arch Environ Contam Toxicol 58(1):151–157. https://doi.org/10.1007/s00244-009-9344-5
5. Giarratano E, Gil MN, Marinho CH, Malanga G (2016) Metals from mine waste as potential cause of oxidative stress in burrowing crab Neohelice granulata from San Antonio bay. Ecotoxicol Environ Saf 132:68–76. https://doi.org/10.1016/j.ecoenv.2016.05.029
6. Kovacik A, Arvay J, Tusimova E, Harangozo L, Tvrda E, Zbynovska K, Cupka P, Andrascikova S, Tomas J, Massanyi P (2017) Seasonal variations in the blood concentration of selected heavy metals in sheep and their effects on the biochemical and hematological parameters. Chemosphere 168:365–371. https://doi.org/10.1016/j.chemosphere.2016.10.090
7. Massanyi P, Stawarz R, Halo M, Formicki G, Lukac N, Cupka P, Schwarz P, Kovacik A, Tusimova E, Kovacik J (2014) Blood concentration of copper, cadmium, zinc and lead in horses and its relation to hematological and biochemical parameters. J Environ Sci Health, Part A: Tox Hazard Subst Environ Eng 49(8):973–979 https://doi.org/10.1080/10934529.2014.894322
8. Mohanty D, Samanta L (2016) Multivariate analysis of potential biomarkers of oxidative stress in Notopterus notopterus tissues from Mahanadi River as a function of concentration of heavy metals. Chemosphere 155:28–38. https://doi.org/10.1016/j.chemosphere.2016.04.035
9. Pilote M, André C, Turcotte P, Gagné F, Gagnon C (2018) Metal bioaccumulation and biomarkers of effects in caged mussels exposed in the Athabasca oil sands area. Sci Total Environ 610:377–390. https://doi.org/10.1016/j.scitotenv.2017.08.023
10. Ruas CBG, dos Santos Carvalho C, de Araújo HSS, Espíndola ELG, Fernandes MN (2008) Oxidative stress biomarkers of exposure in the blood of cichlid species from a metal-contaminated river. Ecotoxicol Environ Saf 71(1):86–93. https://doi.org/10.1016/j.ecoenv.2007.08.018
11. Falandysz J, Wyrzykowska B, Warzocha J, Barska I, Garbacik-Wesołowska A, Szefer P (2004) Organochlorine pesticides and PCBs in perch Perca fluviatilis from the Odra/Oder river estuary, Baltic Sea. Food Chem 87(1):17–23. https://doi.org/10.1016/j.foodchem.2003.10.011
12. Kaya H, Çelik EŞ, Yılmaz S, Tulgar A, Akbulut M, Demir N (2015) Hematological, serum biochemical, and immunological responses in common carp (Cyprinus carpio) exposed to phosalone. Comp Clin Pathol 24(3):497–507. https://doi.org/10.1007/s00580-014-1930-x
13. Sepici-Dinçel A, Benli AÇK, Selvi M, Sarıkaya R, Şahin D, Özkul IA, Erkoç F (2009) Sublethal cyfluthrin toxicity to carp (Cyprinus carpio L.) fingerlings: biochemical, hematological, histopathological alterations. Ecotoxicol Environ Saf 72(5):1433–1439. https://doi.org/10.1016/j.ecoenv.2009.01.008
14. Slaninova A, Smutna M, Modra H, Svobodova Z (2009) Oxidative stress in fish induced by pesticides. Neuroendocrinol Lett 30(1):2
15. Corcoran J, Winter MJ, Tyler CR (2010) Pharmaceuticals in the aquatic environment: a critical review of the evidence for health effects in fish. Crit Rev Toxicol 40(4):287–304. https://doi.org/10.3109/10408440903373590
16. Nunes B, Carvalho F, Guilhermino L (2006) Effects of widely used pharmaceuticals and a detergent on oxidative stress biomarkers of the crustacean Artemia parthenogenetica. Chemosphere 62(4):581–594. https://doi.org/10.1016/j.chemosphere.2005.06.013
17. Rodrigues S, Antunes SC, Correia AT, Nunes B (2016) Acute and chronic effects of erythromycin exposure on oxidative stress and genotoxicity parameters of Oncorhynchus mykiss. Sci Total Environ 545:591–600. https://doi.org/10.1016/j.scitotenv.2015.10.138
18. Jambor T, Tvrdá E, Tušimová E, Kováčik A, Bistáková J, Forgács Z, Lukáč N (2017) In vitro effect of 4-nonylphenol on human chorionic gonadotropin (hCG) stimulated hormone secretion, cell viability and reactive oxygen species generation in mice Leydig cells. Environ Pollut 222:219–225. https://doi.org/10.1016/j.envpol.2016.12.053
19. Mills LJ, Chichester C (2005) Review of evidence: are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? Sci Total Environ 343(1):1–34. https://doi.org/10.1016/j.scitotenv.2004.12.070
20. Árvay J, Tomáš J, Hauptvogl M, Kopernická M, Kováčik A, Bajčan D, Massányi P (2014) Contamination of wild-grown edible mushrooms by heavy metals in a former mercury-mining area. J Environ Sci Heal B 49(11):815–827. https://doi.org/10.1080/03601234.2014.938550
21. Bernet D, Schmidt H, Wahli T, Burkhardt-Holm P (2001) Effluent from a sewage treatment works causes changes in serum chemistry of brown trout (Salmo trutta L.). Ecotoxicol Environ Saf 48(2):140–147. https://doi.org/10.1006/eesa.2000.2012
22. de Oliveira LF, Cabral MT, Vieira CED, Antoniazzi MH, Risso WE, dos Reis Martinez CB (2016) Metals bioaccumulation and biomarkers responses in the Neotropical freshwater clam Anodontites trapesialis: implications for monitoring coal mining areas. Sci Total Environ 571:983–991. https://doi.org/10.1016/j.scitotenv.2016.07.086
23. Javed M, Ahmad I, Usmani N, Ahmad M (2016) Bioaccumulation, oxidative stress and genotoxicity in fish (Channa punctatus) exposed to a thermal power plant effluent. Ecotoxicol Environ Saf 127:163–169. https://doi.org/10.1016/j.ecoenv.2016.01.007
24. Brumbaugh WG, Schmitt CJ, May TW (2005) Concentrations of cadmium, lead, and zinc in fish from mining-influenced waters of northeastern Oklahoma: sampling of blood, carcass, and liver for aquatic biomonitoring. Arch Environ Contam Toxicol 49(1):76–88. https://doi.org/10.1007/s00244-004-0172-3
25. Canli EG, Canli M (2015) Low water conductivity increases the effects of copper on the serum parameters in fish (Oreochromis niloticus). Environ Toxicol Pharmacol 39(2):606–613. https://doi.org/10.1016/j.etap.2014.12.019
26. Gopal V, Parvathy S, Balasubramanian PR (1997) Effect of heavy metals on the blood protein biochemistry of the fish Cyprinus carpio and its use as a bio-indicator of pollution stress. Environ Monit Assess 48(2):117–124. https://doi.org/10.1023/A:1005767517819
27. Öner M, Atli G, Canli M (2008) Changes in serum biochemical parameters of freshwater fish Oreochromis niloticus following prolonged metal (Ag, Cd, Cr, Cu, Zn) exposures. Environ Toxicol Chem 27(2):360–366. https://doi.org/10.1897/07-281R.1
28. Abarikwu SO, Essien EB, Iyede OO, John K, Mgbudom-Okah C (2017) Biomarkers of oxidative stress and health risk assessment of heavy metal contaminated aquatic and terrestrial organisms by oil extraction industry in Ogale, Nigeria. Chemosphere 185:412–422. https://doi.org/10.1016/j.chemosphere.2017.07.024
29. Eyckmans M, Celis N, Horemans N, Blust R, De Boeck G (2011) Exposure to waterborne copper reveals differences in oxidative stress response in three freshwater fish species. Aquat Toxicol 103(1):112–120. https://doi.org/10.1016/j.aquatox.2011.02.010
30. Lushchak VI (2011) Environmentally induced oxidative stress in aquatic animals. Aquat Toxicol 101(1):13–30. https://doi.org/10.1016/j.aquatox.2010.10.006

31. Livingstone DR (2001) Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar Pollut Bull 42(8):656–666
32. Zbynovska K, Petruska P, Kalafova A, Ondruska L, Jurcik R, Chrastinova L, Tusimova E, Kovacik A, Capcarova M (2016) Antioxidant status of rabbits after treatment with epicatechin and patulin. Biologia 71(7):835–842
33. Eroglu A, Dogan Z, Kanak EG, Atli G, Canli M (2015) Effects of heavy metals (Cd, Cu, Cr, Pb, Zn) on fish glutathione metabolism. Environ Sci Pollut Res 22(5):3229–3237. https://doi.org/10.1007/s11356-014-2972-y
34. Sies H (1997) Oxidative stress: oxidants and antioxidants. Exp Physiol 82(2):291–295. https://doi.org/10.1113/expphysiol.1997.sp004024
35. Sedlak TW, Saleh M, Higginson DS, Paul BD, Juluri KR, Snyder SH (2009) Bilirubin and glutathione have complementary antioxidant and cytoprotective roles. Proc Natl Acad Sci 106(13):5171–5176. https://doi.org/10.1073/pnas.0813132106
36. Tvrdá E, Tušimová E, Kováčik A, Paál D, Greifová H, Abdramanov A, Lukáč N (2016a) Curcumin has protective and antioxidant properties on bull spermatozoa subjected to induced oxidative stress. Anim Reprod Sci 172:10–20. https://doi.org/10.1016/j.anireprosci.2016.06.008
37. Tvrdá E, Tušimová E, Kováčik A, Paál D, Libová Ľ, Lukáč N (2016b) Protective effects of quercetin on selected oxidative biomarkers in bovine spermatozoa subjected to ferrous ascorbate. Reprod Domest Anim 51(4):524–537. https://doi.org/10.1111/rda.12714
38. Kolesarova, A., Slamecka, J., Jurcik, R., Tataruch, F., Lukac, N., Kovacik, J., Capcarova, M., Valent, M., Massanyi, P. (2008). Environmental levels of cadmium , lead and mercury in brown hares and their relation to blood metabolic parameters. J Environ Sci Health, Part A: Tox Hazard Subst Environ Eng, 43, 646–650. https://doi.org/10.1080/10934520801893741
39. Kashou, A. H., Sharma, R., & Agarwal, A. (2013). Assessment of oxidative stress in sperm and semen. Spermatogenesis: Methods and Protocols, 351–361. doi https://doi.org/10.1007/978-1-62703-038-0_30
40. Muller, C. H., Lee, T. K. Y., & Montaño, M.A. (2013). Improved chemiluminescence assay for measuring antioxidant capacity of seminal plasma. In Carrell, D. A., & Aston, K. I. (2013). Spermatogenesis. Methods and Protocols (1st ed.). New York, NY: Springer Science+Business Media. pp. 363–376. https://doi.org/10.1007/978-1-62703-038-0_31
41. Beers RF, Sizer IW (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J Biol Chem 195(1):133–140
42. Ellman GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82(1):70–77. https://doi.org/10.1016/0003-9861(59)90090-6
43. Tvrda E, Mackovich A, Greifova H, Hashim F, Lukac N (2017) Antioxidant effects of lycopene on bovine sperm survival and oxidative profile following cryopreservation. Vet Med 62(8). https://doi.org/10.17221/86/2017-VETMED
44. Weber D, Davies MJ, Grune T (2015) Determination of protein carbonyls in plasma, cell extracts, tissue homogenates, isolated proteins: focus on sample preparation and derivatization conditions. Redox Biol 5:367–380. https://doi.org/10.1016/j.redox.2015.06.005
45. Andreji J, Stranai I, Massanyi P, Valent M (2006) Accumulation of some metals in muscles of five fish species from lower Nitra River. J Environ Sci Heal A 41(11):2607–2622. https://doi.org/10.1080/10934520600928003
46. Canli M, Atli G (2003) The relationships between heavy metal (Cd, Cr, Cu, Fe, Pb, Zn) levels and the size of six Mediterranean fish species. Environ Pollut 121(1):129–136. https://doi.org/10.1016/S0269-7491(02)00194-X
47. Jarić I, Višnjić-Jeftić Ž, Cvijanović G, Gačić Z, Jovanović L, Skorić S, Lenhardt M (2011) Determination of differential heavy metal and trace element accumulation in liver, gills, intestine and muscle of sterlet (Acipenser ruthenus) from the Danube River in Serbia by ICP-OES. Microchem J 98(1):77–81. https://doi.org/10.1016/j.microc.2010.11.008
48. Karadede H, Ünlü E (2000) Concentrations of some heavy metals in water, sediment and fish species from the Atatürk Dam Lake (Euphrates), Turkey. Chemosphere 41(9):1371–1376. https://doi.org/10.1016/S0045-6535(99)00563-9
49. Malik N, Biswas AK, Qureshi TA, Borana K, Virha R (2010) Bioaccumulation of heavy metals in fish tissues of a freshwater lake of Bhopal. Environ Monit Assess 160(1):267–276. https://doi.org/10.1007/s10661-008-0693-8
50. Mendil D, Ünal ÖF, Tüzen M, Soylak M (2010) Determination of trace metals in different fish species and sediments from the River Yeşilırmak in Tokat, Turkey. Food Chem Toxicol 48(5):1383–1392. https://doi.org/10.1016/j.fct.2010.03.006
51. Tóth T, Andreji J, Tóth J, Slávik M, Árvay J, Stanovic R (2012) Cadmium, lead and mercury content in fishes—case study. J Microbiol, Biotechnol Food Sci 1:837–847
52. Vinodhini R, Narayanan M (2008) Bioaccumulation of heavy metals in organs of fresh water fish Cyprinus carpio (common carp). Int J Environ Sci Technol 5(2):179–182. https://doi.org/10.1007/BF03326011
53. Adams DH, Sonne C, Basu N, Dietz R, Nam DH, Leifsson PS, Jensen AL (2010) Mercury contamination in spotted seatrout, Cynoscion nebulosus: an assessment of liver, kidney, blood, and nervous system health. Sci Total Environ 408(23):5808–5816. https://doi.org/10.1016/j.scitotenv.2010.08.019
54. Qu R, Feng M, Wang X, Qin L, Wang C, Wang Z, Wang L (2014) Metal accumulation and oxidative stress biomarkers in liver of freshwater fish Carassius auratus following in vivo exposure to waterborne zinc under different pH values. Aquat Toxicol 150:9–16. https://doi.org/10.1016/j.aquatox.2014.02.008
55. Çoğun H, Yüzereroğlu TA, Kargin F, Firat Ö (2005) Seasonal variation and tissue distribution of heavy metals in shrimp and fish species from the Yumurtalik coast of Iskenderun Gulf, Mediterranean. Bull Environ Contam Toxicol 75(4):707–715. https://doi.org/10.1007/s00128-005-0809-6
56. Rotchell JM, Clarke KR, Newton LC, Bird DJ (2001) Hepatic metallothionein as a biomarker for metal contamination: age effects and seasonal variation in European flounders (Pleuronectes flesus) from the Severn Estuary and Bristol Channel. Mar Environ Res 52(2):151–171. https://doi.org/10.1016/S0141-1136(00)00270-1
57. Al-Yousuf MH, El-Shahawi MS, Al-Ghais SM (2000) Trace metals in liver, skin and muscle of Lethrinus lentjan fish species in relation to body length and sex. Sci Total Environ 256(2):87–94. https://doi.org/10.1016/S0048-9697(99)00363-0
58. Squadrone S, Prearo M, Brizio P, Gavinelli S, Pellegrino M, Scanzio T, Abete MC (2013) Heavy metals distribution in muscle, liver, kidney and gill of European catfish (Silurus glanis) from Italian rivers. Chemosphere 90(2):358–365. https://doi.org/10.1016/j.chemosphere.2012.07.028
59. Borges, A., Scotti, L. V, Siqueira, D. R., Jurinitz, D. F., & Wassermann, G. F. (2004). Hematologic and serum biochemical values for jundiá (Rhamdia quelen). Fish Physiol Biochem, 30(2004), 21–25
60. Groff JM, Zinkl JG (1999) Hematology and clinical chemistry of cyprinid fish: common carp and goldfish. Vet Clin North Am Exot Anim Pract 2(3):741–776. https://doi.org/10.1016/S1094-9194(17)30120-2
61. Javed M, Usmani N (2013) Assessment of heavy metal (Cu, Ni, Fe, Co, Mn, Cr, Zn) pollution in effluent dominated rivulet water and

their effect on glycogen metabolism and histology of Mastacembelus armatus. SpringerPlus 2(1):390. https://doi.org/10.1186/2193-1801-2-390
62. Datta S, Saha DR, Ghosh D, Majumdar T, Bhattacharya S, Mazumder S (2007) Sub-lethal concentration of arsenic interferes with the proliferation of hepatocytes and induces in vivo apoptosis in Clarias batrachus L. Comp Biochem Physiol C: Toxicol Pharmacol 145(3):339–349
63. Yang JL, Chen HC (2003) 2003. Serum metabolic enzyme activities and hepatocyte ultrastructure of common carp after gallium exposure. Zool Stud 42(3):455–461
64. Li XY, Chung IK, Kim JI, Lee JA (2004) Subchronic oral toxicity of microcystin in common carp (Cyprinus carpio L.) exposed to Microcystis under laboratory conditions. Toxicon 44(8):821–827. https://doi.org/10.1016/j.toxicon.2004.06.010
65. Wallace, A. D., Meyer, S. A. 2010. Hepatotoxicity, in: Hodgson. (2010). *A textbook of modern* Toxicology 4th ed. John Wiley & Sons, Inc., Hoboken, New Jersey, pp. 277–289
66. Bressler J, Kim KA, Chakraborti T, Goldstein G (1999) Molecular mechanisms of lead neurotoxicity. Neurochem Res 24(4):595–600. https://doi.org/10.1023/A:1022596115897
67. Prozialeck WC, Grunwald GB, Dey PM, Reuhl KR, Parrish AR (2002) Cadherins and NCAM as potential targets in metal toxicity. Toxicol Appl Pharmacol 182(3):255–265. https://doi.org/10.1006/taap.2002.9422
68. Bradbury SP, Carlson RW, Henry TR, Padilla S, Cowden J (2008) Toxic responses of the fish nervous system. In: Di Giulio RT, Hinton DE (Eds.). (2008). The toxicology of fishes. Crc Press, pp 417–455
69. Larsson Å, Haux C, Sjöbeck ML (1985) Fish physiology and metal pollution: results and experiences from laboratory and field studies. Ecotoxicol Environ Saf 9(3):250–281. https://doi.org/10.1016/0147-6513(85)90045-4
70. Nussey, G., Van Vuren, J. H. J., & Du Preez, H. H. (1995). Effect of copper on the haematology and osmoregulation of the Mozambique tilapia, Oreochromis mossambicus (Cichlidae). Comp Biochem Physiol, Part C: Pharmacol, Toxicol Endocrinol, 111(3), 369–380. https://doi.org/10.1016/0742-8413(95)00063-1
71. Nelson K, Jones J, Jacobson S, Reimschuessel R (1999) Elevated blood urea nitrogen (BUN) levels in goldfish as an indicator of gill dysfunction. J Aquat Anim Health 11(1):52–60 https://doi.org/10.1577/1548-8667(1999)011<0052:EBUNBL>2.0.CO;2
72. Parris WE, Adeli K (2002) In Vitro Toxicological assessment of heavy metals and intracellular mechanisms of toxicity. In: Sarkar B (ed) Heavy Metals in the Environment. Marcel Dekker, Inc., New York, pp 69–93
73. Bocchetti R, Lamberti CV, Pisanelli B, Razzetti EM, Maggi C, Catalano B, Sesta G, Martuccio G, Gabellini M, Regoli F (2008) Seasonal variations of exposure biomarkers, oxidative stress responses and cell damage in the clams, Tapes philippinarum, and mussels, Mytilus galloprovincialis, from Adriatic Sea. Mar Environ Res 66(1):24–26. https://doi.org/10.1016/j.marenvres.2008.02.013
74. Shi X, Kasprzak KS, Dalal NS (1993) Generation of free radicals in reactions of Ni (II)-thiol complexes with molecular oxygen and model lipid hydroperoxides. J Inorg Biochem 50(3):211–225. https://doi.org/10.1016/0162-0134(93)80026-6
75. Flora SJS (2014) Metals. In: Gupta, R. C., Biomarkers in toxicology, first ed. Academic Press, pp. 485-519
76. Bols NC, Dayeh VR, Lee LEJ, Schirmer K (2005) Use of fish cell lines in the toxicology and ecotoxicology of fish. Piscine cell lines in environmental toxicology. In: Mommsen TP, Moon TW (eds) Biochemistry and Molecular Biology of Fishes, 6. Elsevier Science, pp. 43–84. https://doi.org/10.1016/S1873-0140(05)80005-0
77. Timbrell J (2000) Principles of biochemical toxicology, third edn. CRC Press, London