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Biochemical, haematological and oxidative stress responses of common carp (*Cyprinus carpio* L.) after sub-chronic exposure to copper

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ABSTRACT: The aim of the present study was to investigate the impact of copper-based pesticides (at concentrations of copper of 20, 30, 40 and 70 µg/l) on one-year-old common carp (*Cyprinus carpio* L.) during 28 days of exposure. Abnormal behaviour was observed in fish exposed to 70 µg/l from Day 14. Histological alterations were noticed only in liver in the groups exposed to 40 and 70 µg/l. Significant changes ($P < 0.05$) in almost all haematological indices were found, especially in the group exposed to the highest concentration of copper (70 µg/l). Biochemical analysis revealed various significant ($P < 0.05$) differences among the tested groups. Significant differences in copper tissue concentration ($P < 0.05$) among groups were found in liver, gills and kidney. Among antioxidative enzymes, significant changes were revealed mainly in catalase and glutathione-S-transferase activity ($P < 0.05$). In gills, metallothionein content increased significantly ($P < 0.05$) in the group exposed to the highest copper concentration (70 µg/l) compared with the other tested groups, including the control. A significant ($P < 0.05$) change in total glutathione content was recorded in liver and gills, although the reduced/oxidised ratio was not affected. Oxidative damage to lipids increased significantly ($P < 0.05$) with increasing copper concentration in liver and kidney. The results demonstrate the deleterious influence of copper on common carp even at low, environmentally relevant concentrations.

Keywords: metals; fish; antioxidant defence; pesticides

Copper is a transition metal with a high abundance in aquatic and terrestrial environments. Copper, as an essential nutrient, plays an important role in various functions in cellular biochemistry, especially as a cofactor for many enzymes and as a constituent of the non-enzymatic antioxidants ceruloplasmin and the metallothioneins (Amiard et al. 2006). Through these mechanisms, copper participates in antioxidative defence against various deleterious substances (Ahmad et al. 2000; Pandey et al. 2001; Parvez and Raisuddin 2006). Despite its protective effect copper may be very toxic to fish, in a manner which is strongly dependent on its concentration and the physicochemical parameters of the water (Carvalho and Fernandes 2006; Abdel-Tawwab et al. 2007). Copper naturally occurs in fresh water systems in the concentration range from 0.5 to 24 µg/l (An and Kampbell 2003; Hansen et al. 2006; Ruas et al. 2008). However, its concentration may increase due to an anthropogenic input of up to 100 µg/l or more (Mansour and Sidky 2002; Bervoets and Blust 2003; Oliva et al. 2012). Mining activities, the leather industry, domestic waste, and the application of fertilisers and pesticides, especially algicides and fungicides, are the main sources of aquatic copper pollution (WHO 1998).

One of the known mechanisms of copper toxicity to fish is the promotion of oxidative stress

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(Lushchak 2011). Direct oxidative damage to cells can be the result of the participation of copper in the production of reactive oxygen species through the Fenton reaction. Copper can interact with various antioxidant enzymes, particularly in acute exposures, or at the beginning of chronic exposure. Copper can also be bound to thiol-containing molecules such as glutathione (Stohs and Bagchi 1995; Maracine and Segner 1998) and metallothioneins (Roesijadi 1996; Amiard et al. 2006), and thereby interfere with antioxidant defence.

The aim of the present study was to investigate the impact of two copper-based pesticide compounds (copper oxychloride and copper sulphate) at slightly higher concentrations than environmental ones on one-year-old common carp (*Cyprinus carpio* L.) during 28 days of exposure. Furthermore, the study also focused on the difference in their effects on fish. The toxic impact was evaluated on the basis of results of haematological, biochemical and histopathological examinations and indices of oxidative stress.

MATERIAL AND METHODS

Experimental protocol. One-year-old common carp (*Cyprinus carpio* L.) (body weight 161 ± 32 g) were obtained from a commercial fish farm. They were randomly distributed into ten 200 l glass aquaria, with 12 specimens in each. The fish were supplied twice a day with a commercial feed at a rate of 1.5% body weight both before and during the experiment. After two weeks of acclimation to laboratory conditions (water temperature 19–23 °C, photoperiod 12 h/12 h), the fish were exposed to the commercial formulation Kuprikol 50 (84% copper oxychloride) at copper concentrations of 20.0 and 40.0 µg/l and to copper sulphate ($CuSO_4 \cdot 5H_2O$) at copper concentrations of 30.0 and 70.0 µg/l for 28 days. Each treated group, including the control, was tested in duplicate.

The exposure concentrations were selected in order to be representative of environmental concentrations encountered in polluted freshwaters (De Lafontaine et al. 2000; Lebrun et al. 2014). Copper oxychloride and copper sulphate are compounds commonly used in agriculture as well as in many industries. These two compounds represent the most frequently used copper-based pesticides; therefore, they were chosen for present study. The actual copper concentrations were measured on three different sampling days during the experiment, each time before and after bath renewal. The copper concentrations determined by atomic absorption spectrometry were 20.2 ± 1.6 and 43.1 ± 1.9 µg/l for the groups exposed to Kuprikol 50, and 32.3 ± 2.2 and 68.7 ± 2.1 µg/l for the groups exposed to copper sulphate (mean ± SEM). The actual copper concentration in the control group was found to be 1.5 ± 1.1 µg/l (mean ± SEM). The experiment was conducted in a semi-static system with complete bath renewal every 12 h. The control groups were subjected to dechlorinated tap water. For all groups, the parameters of water quality had the following values: water temperature: 22 ± 1 °C; oxygen saturation: above 60%; pH: 7.5–8.0; Ca + Mg: 3.4 ± 0.3 mmol/l; COD_{Mn}: 6.2 ± 7.5 mg/l O_2; BOD_5: 1.8 ± 1.7 mg/l O_2; $ANC_{4.5}$: 5.1 ± 0.6 mmol/l; and NH_3: 0.01 ± 0.01 mg/l. The photoperiod was 12 h/12 h.

Experimental procedures were in compliance with national legislation (Act No. 246/1992 Coll., on the Protection of Animals Against Cruelty, as amended and Decree No. 419/2012 Coll., on the Protection, Breeding, and Use of Experimental Animals, as amended).

Fish sampling and biometrical data. After blood sampling at the end of the experiment (Day 28), the fish were stunned with a blow to the head and sacrificed by spinal transection, then measured and weighed. Tissue samples were removed (liver, gills, caudal kidney, brain, and muscle) and stored at –85 °C until further analyses were performed. Whole livers were weighed prior to freezing. Selected organs (liver, gill, caudal kidney, spleen, and skin) were taken for histopathological examination. Condition factor [CF = (weight × 100)/ length^3] and hepatosomatic index [HSI = (liver weight/body weight) × 100] were calculated.

Protein and enzymatic markers. Tissue samples (liver, gills, caudal kidney, and brain) were homogenised in 50mM potassium phosphate buffer with 1mM EDTA (pH 7.4). One part of the homogenate (non-centrifuged) was used to estimate LPO (lipid peroxidation). The remaining part of the homogenate was centrifuged at 11 200 *g* for 20 min at 4 °C and the supernatant was used for the determination of GR (glutathione reductase), GPx (glutathione peroxidase), CAT (catalase) and GST (glutathione-S-transferase) activity, and protein concentration.

Total protein concentration was quantified using the method described by Smith et al. (1985) using a Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) with bovine serum albumin as a standard.

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The total catalytic concentration of GST was determined by measuring the conjugation of 1-chloro-2,4-dinitrobenzene with reduced glutathione at 340 nm (Habig et al. 1974). The specific activity was expressed as nmol NADPH/min/mg of protein.

The catalytic concentration of GR was determined by measuring NADPH (nicotinamidadeninedinucleotide phosphate-reduced) oxidation at 340 nm (Carlberg and Mannervik 1975). The catalytic concentration of GPx was calculated from the rate of NADPH oxidation in the reaction with GR at 340 nm (Flohe and Gunzler 1984). The specific activities of GR and GPx were expressed as nmol NADPH/min/mg of protein.

The catalytic concentration of CAT was determined by measuring H_2O_2 breakdown at 240 nm. The specific activity was expressed as μmol/H_2O_2/min/mg of protein (Aebi 1984). The activity of CAT was not determined in brain samples due to the lack of tissue.

All these spectrophotometric methods were performed using a Varioskan Flash Spectral Scanning Multimode Reader (Thermo Scientific).

Lipid peroxidation. Lipid peroxidation was determined using the TBARS (thiobarbituric acid reactive substances) method at 535 nm with a molar extinction coefficient of 1.56×10^5 M/cm (Lushchak et al. 2005). The concentration was expressed as nmol of TBARS per gram of wet weight tissue. The method was performed using a Varioskan Flash Spectral Scanning Multimode Reader (Thermo Scientific).

Glutathione. Tissue concentrations of GSH (reduced glutathione) and GSSG (glutathione disulphide) in liver, gill, and caudal kidney samples were measured using high performance liquid chromatography with electrochemical detection. The system consisted of two solvent delivery pumps operating in the range of 0.001–9.999 ml/min (Model 582 ESA Inc., Chelmsford, MA), a Zorbax eclipse AAA C18 (150 × 4.6; 3.5 μm particle size; Agilent Technologies, USA), and a CoulArray electrochemical detector (Model 5600A, ESA, USA). Supernatants from the centrifuged homogenates (15 000 *g*, 20 min, 4 °C) were used for the measurements. Total GSH was calculated as the sum of GSH and GSSG, and expressed as μmol per g of wet weight.

Metallothioneins. Levels of metallothioneins in liver, gills, and caudal kidney were determined using the differential pulse voltammetry Brdicka reaction. The samples (app. 0.2 g) were frozen with liquid nitrogen and spread in a mortar, and then exactly 1000 μl of 0.2M phosphate buffer (pH 7.2) was added to the homogenised sample. The obtained homogenate was transferred to a test-tube and vortexed for 15 min at 4 °C (Vortex Genie, USA). The supernatant was subsequently heat-treated. Briefly, the sample was kept at 99 °C in a thermomixer (Eppendorf 5430, USA) for 15 min with occasional stirring, and then cooled to 4 °C. The denatured homogenates were centrifuged at 4 °C, 15 000 *g* for 30 min. (Eppendorf 5402, USA). Heat treatment effectively denatures and removes high molecular weight proteins from samples. Electrochemical measurements were performed with a 747 VA Stand instrument connected to a 693 VA Processor and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes and a cooled sample holder (4 °C). A hanging mercury drop electrode with a drop area of 0.4 mm^2 was used as the working electrode. An Ag/AgCl/3M KCl electrode was used as the reference, and a platinum electrode as the auxiliary. Metallothionein concentrations in fish tissues are given as ng/mg of protein. Samples were processed according to Fabrik et al. (2008).

Haematology profile. Blood samples were taken from each fish by puncturing the caudal vein and were stabilised with sodium heparin (50 IU/ml of blood). Heparinised blood samples were used for the evaluation of haematological indicators, including erythrocyte count (RBC), haemoglobin concentration (Hb), haematocrit (HCT), mean erythrocyte volume (MCV), mean erythrocyte haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), leukocyte count (WBC), and differential leukocyte count. Samples were processed according to Svobodova et al. (2012).

Biochemical profile. Plasma samples were obtained after the centrifugation of blood (800 *g*, 10 min, 4 °C) and stored at –85 °C until further analyses were performed. The analysed biochemical parameters included albumin, total proteins, glucose, ammonia, triacylglycerols, lactate, cholesterol, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), calcium, and phosphorus. Plasma biochemical indicators were measured using a Konelab 20i biochemical automatic analyser (ThermoScientific, Czech Republic) and commercial test kits (BioVendor, Czech Republic).

Ceruloplasmin. Plasma samples (15 μl) were mixed with 100 μl of *p*-phenylenediamine (PPD)

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solution (41 mg PPD/25 ml of sodium acetate buffer – pH 5.2) and incubated at 37 °C. The absorbance at 550 nm was recorded every 60 s for 30 min (Ceron and Martinez-Subiela 2004). The results were expressed as the amount of absorbance increase per minute × 10 000. The analysis was performed using a Varioskan Flash Spectral Scanning Multimode Reader (Thermo Scientific).

FRAP. The ferric-reducing ability of plasma (FRAP) was determined using a Konelab 20i biochemical analyser. Five µl of plasma were added to 150 µl of fresh FRAP reagent (pH 3.6) and the absorbance was measured at 600 nm. Final FRAP values were obtained by comparing the absorbances of test reaction mixtures with those containing ferrous ions in known concentrations (Benzie and Strain 1996).

Copper concentration in tissues. Tissue samples (liver, caudal kidney, gills, and muscle) (10 mg) were placed into glass vials and 700 µl of nitric acid (65%, w/w) and 300 µl of hydrogen peroxide (30%, w/w) were added. The digestion of samples took place in a microwave system Multiwave 3000 (Anton-Paar GmbH, Austria). Sample preparation for subsequent electrochemical measurements was as follows: 100 µl mineralised sample were pipetted into Eppendorf tubes with 1900 µl acetate buffer (pH = 5.00). A blank digestion was simultaneously carried out in the same way. The determination of copper concentration by differential pulse voltammetry was performed with a 797 VA Computrace instrument connected to an 813 Compact Autosampler (Metrohm, Switzerland) using a standard cell with three electrodes. A hanging mercury drop electrode with a drop area of 0.4 mm^2 was used as the working electrode. An Ag/AgCl/3M KCl electrode was used as the reference and a platinum electrode as the auxiliary. Differential pulse voltammetric measurements were carried out under the following parameters: time of accumulation 120 s, deposition potential – 1.15 V, modulation time 0.04 s, interval time 0.3 s, initial potential – 1.3 V, end potential 0.2 V, step potential 5 mV, modulation amplitude 25 mV. The copper concentrations in fish tissues are given as µg per g of wet weight.

Histopathological examination. Selected fish tissues (liver, gills, cranial and caudal kidney, spleen, and skin) were prepared for histopathological examination, fixed in buffered 10% neutral formalin, dehydrated, embedded in paraffin wax, sectioned on a microtome at a thickness of 4 µm, and stained with haematoxylin and eosin. All affected tissues were histopathologically compared with tissue sections from the control group.

Statistical analysis. Statistical assessment was carried out using Unistat 5.6 for Excel software (Czech Republic). Data were tested using the Shapiro-Wilk test, and one way analysis of variance (ANOVA) was applied to test the differences in measured indices among experimental groups. Individual differences among the groups were tested successively using the multiple comparison Tukey HSD test. If the condition of a normal distribution was not satisfied, a non-parametric Kruskal-Wallis test was applied. Significance was accepted at $P < 0.05$. All data are reported as mean ± standard error of mean (SEM).

RESULTS

Mortality and fish behaviour

Mortality was observed only at the highest concentration of copper (70 µg/l). In total, three fish died at this concentration (after 14, 21 and 26 days

Table 1. Biometric indices of common carp after 28-day waterborne exposure to copper

Indices	Control	20	30	40	70
		(µg/l)			
Total length (cm)	22.8 ± 0.2^{a,b}	23.3 ± 0.2^{a}	22.9 ± 0.3^{a,b}	22.7 ± 0.3^{a,b}	22.2 ± 0.2^{b}
Standard length (cm)	19.0 ± 0.2^{a}	19.0 ± 0.2^{a}	18.7 ± 0.3^{a,b}	18.4 ± 0.2^{a,b}	18.1 ± 0.2^{b}
Body weight (g)	175.0 ± 5.4^{a}	175.9 ± 5.3^{a}	161.8 ± 7.6^{a}	161.1 ± 6.0^{a}	130.2 ± 3.5^{b}
Liver weight (g)	4.24 ± 0.18	4.21 ± 0.17	3.85 ± 0.21	3.67 ± 0.17	3.90 ± 0.26
HSI	2.44 ± 0.09^{a}	2.40 ± 0.08^{a}	2.39 ± 0.08^{a}	2.31 ± 0.11^{a}	2.97 ± 0.17^{b}
CF	2.55 ± 0.03^{a}	2.56 ± 0.04^{a}	2.45 ± 0.04^{a}	2.54 ± 0.03^{a}	2.20 ± 0.03^{b}

HSI = hepatosomatic index; CF = condition factor. Data are expressed as mean ± SEM. Significant differences ($P < 0.05$) are indicated by different superscripts

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of exposure). Behavioural changes were observed at the highest concentration from Day 14 to the end of the experiment. Abnormal behaviour included reduced food intake and reduced swimming activity.

Biometric indices

The mean values of biometric indices are presented in Table 1. A significant increase ($P < 0.05$) in HSI was found in fish exposed to a copper concentration of 70 µg/l. Condition factor, body weight, and standard length were significantly decreased ($P < 0.05$) at the copper concentration of 70 µg/l. No significant changes were found in liver weight.

Haematological profile

Results of the haematological examination of blood samples are presented in Table 2. It is obvious that sub-chronic exposure to copper resulted in significant changes in almost all haematological indices, especially in the group exposed to the highest concentration of copper. Erythrocyte count increased with copper concentration, with a significant ($P < 0.05$) difference between the highest concentration and control; haematocrit values exhibited a similar tendency. Leukocyte count decreased non-significantly with increasing copper concentration. Significantly less ($P < 0.05$) lymphocytes were found at the highest copper concentration (70 µg/l) compared with the control and with the 40 µg/l concentration. On the other hand, we obtained a significantly higher amount of monocytes, segmented and band neutrophile granulocytes, metamyelocytes, and myelocytes at the 70 µg/l concentration ($P < 0.05$) compared with the control and the other tested copper concentrations.

Biochemical profile

The results of blood plasma biochemical indices are shown in Table 3 and Figure 1. Significant ($P < 0.05$) differences in total protein, albumin, phosphorus, calcium, glucose, triacylglycerols, cholesterol, LDH, ammonia, ALT (Table 3), FRAP, and ceruloplasmin (Figure 1) were found among the tested groups. The activities of AST and ALP were not significantly affected by copper exposure.

Table 2. Results of haematological examination of common carp after 28-day waterborne exposure to copper

Indices	Control	20	30	40	70
		(µg/l)			
RBC (T/l)	1.56 ± 0.05^{a}	1.73 ± 0.04^{a,b}	1.92 ± 0.05^{b,c}	1.66 ± 0.05^{a,b}	2.02 ± 0.05^{c}
Hb (g/l)	77.17 ± 2.46^{a,b}	78.14 ± 1.98^{a,b}	81.49 ± 1.49^{a,b}	75.05 ± 1.92^{a}	86.59 ± 4.72^{b}
HCT (l/l)	0.27 ± 0.01^{a}	0.28 ± 0.01^{a,b}	0.30 ± 0.01^{b}	0.27 ± 0.01^{a,b}	0.33 ± 0.01^{c}
MCV (fl)	172.30 ± 3.79^{a}	164.63 ± 4.20^{a,b}	154.72 ± 2.88^{b}	166.07 ± 4.11^{a,b}	163.40 ± 5.11^{a,b}
MCH (pg)	52.73 ± 2.96^{a}	45.56 ± 1.47^{a,b}	42.86 ± 0.94^{b}	45.76 ± 1.21^{a,b}	45.46 ± 1.31^{a,b}
MCHC (g/l)	292.82 ± 7.46^{a}	276.29 ± 3.90^{b}	277.09 ± 3.30^{a,b}	275.61 ± 2.70^{b}	280.27 ± 6.36^{a,b}
WBC (G/l)	36.47 ± 4.73	30.06 ± 2.77	24.28 ± 2.81	33.47 ± 2.23	25.67 ± 3.58
Lymphocytes (G/l)	35.21 ± 4.76^{a}	29.04 ± 2.66^{a,b}	24.54 ± 2.55^{a,b}	31.13 ± 2.15^{a}	18.06 ± 2.38^{b}
Monocytes (G/l)	0.02 ± 0.02^{a}	0.02 ± 0.02^{a}	0.18 ± 0.09^{a,b}	0.02 ± 0.02^{a}	0.19 ± 0.05^{b}
NG-Segments (G/l)	0.32 ± 0.10^{a}	0.37 ± 0.10^{a,b}	0.49 ± 0.09^{a,b}	0.72 ± 0.021^{a,b}	1.98 ± 0.68^{b}
NG-Bands (G/l)	0.13 ± 0.08^{a}	0.14 ± 0.07^{a}	0.44 ± 0.14^{a,b}	0.12 ± 0.05^{a}	1.49 ± 0.59^{b}
Metamyelocytes (G/l)	0.69 ± 0.16^{a,b}	0.39 ± 0.09^{a,b}	1.07 ± 0.50^{a}	0.66 ± 0.17^{a,b}	4.03 ± 1.83^{b}
Myelocytes (G/l)	0.05 ± 0.03^{a}	0.09 ± 0.04^{a,b}	0.14 ± 0.11^{a}	0.16 ± 0.05^{a,b}	0.57 ± 0.26^{b}
Plasma cells (G/l)	0.05 ± 0.05	–	0.08 ± 0.08	–	0.59 ± 0.57

RBC = erythrocyte count; Hb = haemoglobin concentration; HCT = haematocrit value; MCV = mean erythrocyte volume; MCH = mean erythrocyte haemoglobin; MCHC = mean corpuscular haemoglobin concentration; WBC = leucocyte count; NG = neutrophil granulocytes. Data are expressed as mean ± SEM. Significant differences ($P < 0.05$) are indicated by different superscripts

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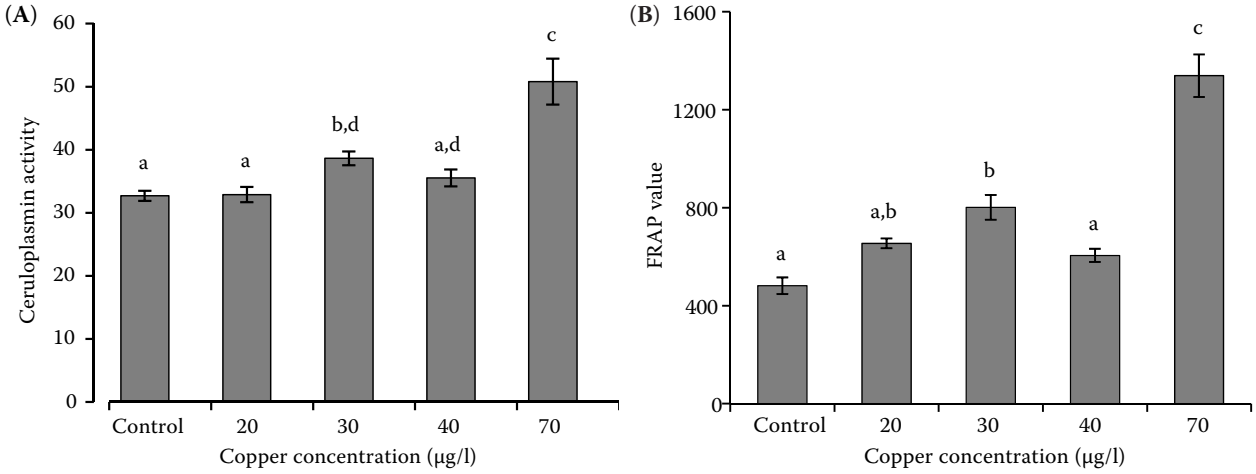

Figure 1. Ceruloplasmin activity **(A)** (increase in absorbance per min ×10 000) and ferric-reducing ability of plasma (FRAP) value **(B)** (Fe^{2+} equivalent µmol/l) in plasma of common carp after 28-day waterborne exposure to copper. Data are expressed as mean ± SEM. Significant differences ($P < 0.05$) are indicated by different superscripts

Copper concentration in tissues

Copper distribution in carp tissues is shown in Table 4. The highest copper content was measured in liver and the lowest in muscle in all tested groups, including the control. Significant differences ($P < 0.05$) between groups were found with respect to copper concentrations in liver, gills and kidney. No significant changes were found in muscle.

Enzymatic antioxidants

The enzymatic activities of GR and GPx are shown in Figure 2A and 2B, respectively. Significant ($P < 0.05$) changes in GPx and GR activity in brain and kidney were found in the tested groups. GR and GPx activity in liver and gill did not change significantly after copper exposure. GPx activity in kidney increased significantly ($P < 0.05$) with increasing

Table 3. Results of biochemical examination of common carp after 28-day waterborne exposure to copper

Indices	Control	20	30	40	70
		(µg/l)			
Glucose (mmol/l)	3.51 ± 0.13^{a}	4.44 ± 0.21^{b}	5.12 ± 0.29^{b,c}	4.51 ± 0.23^{b}	7.97 ± 0.74^{c}
Ammonia (µmol/l)	2.13 ± 0.02^{a}	2.26 ± 0.02^{b}	2.32 ± 0.02^{b}	2.29 ± 0.02^{b}	2.27 ± 0.02^{b}
Total protein (g/l)	1.32 ± 0.01^{a}	1.34 ± 0.01^{a,b}	1.36 ± 0.01^{a,b}	1.36 ± 0.01^{b}	1.35 ± 0.01^{a,b}
Albumin (g/l)	7.85 ± 0.27^{a}	7.96 ± 0.35^{a,b}	9.25 ± 0.43^{b}	7.74 ± 0.30^{a}	9.32 ± 0.54^{b}
ALT (µkatal/l)	0.57 ± 0.04^{a}	0.63 ± 0.04^{a}	0.68 ± 0.05^{a,b}	0.61 ± 0.04^{a}	0.89 ± 0.08^{b}
AST (µkatal/l)	1.77 ± 0.13	1.64 ± 0.07	1.59 ± 0.08	1.57 ± 0.10	1.51 ± 0.10
ALP (µkatal/l)	0.51 ± 0.09	0.55 ± 0.08	0.53 ± 0.11	0.56 ± 0.10	0.91 ± 0.20
Cholesterol (mmol/l)	3.34 ± 0.15^{a}	3.79 ± 0.11^{a,b}	4.81 ± 0.17^{c}	3.99 ± 0.15^{b}	4.81 ± 0.18^{c}
Triacylglycerols (mmol/l)	1.48 ± 0.10^{a}	1.49 ± 0.09^{a,b}	2.00 ± 0.12^{a,b}	1.51 ± 0.11^{b}	2.15 ± 0.22^{a,b}
LDH (µkatal/l)	0.38 ± 0.06^{a}	0.47 ± 0.05^{a}	0.44 ± 0.07^{a}	0.35 ± 0.09^{a}	0.85 ± 0.08^{b}
Lactate (mmol/l)	1.47 ± 0.15	1.35 ± 0.15	1.55 ± 0.20	0.89 ± 0.09	1.46 ± 0.32
Phosphorus (mmol/l)	2.06 ± 0.09^{a}	2.29 ± 0.09^{b}	2.24 ± 0.07^{a,b}	2.19 ± 0.12^{a,b}	2.06 ± 0.08^{a}
Calcium (mmol/l)	2.08 ± 0.05^{a}	2.15 ± 0.03^{b}	2.38 ± 0.04^{b}	2.14 ± 0.01^{b}	3.51 ± 0.13^{b}

ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; LDH = lactate dehydrogenase. Data are expressed as mean ± SEM. Significant differences ($P < 0.05$) are indicated by different superscripts

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Table 4. Copper concentrations (µg/g of wet weight) in tissues (liver, kidney, gills and muscle) of common carp after 28-day waterborne exposure to copper

Tissue	Control	20	30	40	70
			(µg/l)		
Liver	136.3 ± 21.8^{a}	167.8 ± 19.2^{a,c}	192.6 ± 22.2^{a,b}	262.5 ± 26.2^{b}	234.0 ± 11.8^{b,c}
Gills	37.0 ± 3.4^{a}	62.0 ± 8.1^{a,b}	53.7 ± 6.7^{a,b}	61.9 ± 7.2^{a,b}	65.4 ± 8.6^{b}
Kidney	37.3 ± 4.0^{a}	44.5 ± 4.3^{a,c}	65.5 ± 5.2^{b}	57.8 ± 3.1^{b,c}	53.9 ± 3.1^{a,b}
Muscle	24.0 ± 3.2	33.5 ± 4.5	30.1 ± 2.4	36.3 ± 5.6	32.6 ± 3.2

Data are expressed as mean ± SEM. Significant differences ($P < 0.05$) are indicated by different superscripts

copper concentration. GST activity in all analysed tissues was significantly ($P < 0.05$) influenced by copper exposure (Figure 2C). CAT activity in liver and kidney decreased significantly ($P < 0.05$) at the copper concentration of 70 µg/l, but remained unchanged in gills (Figure 2D).

Non-enzymatic antioxidants

Metallothionein content in kidney and liver did not respond to copper exposure (Figure 3A). Its content increased significantly ($P < 0.05$) in gills after exposure to the highest copper concentration (70 µg/l) compared with all other tested concentrations and the control. A significant ($P < 0.05$) decrease in total GSH content in liver was recorded at the highest copper concentration (70 µg/l) compared with the control and the concentrations of 20 and 40 µg/l (Figure 3A). The opposite trend was revealed in total GSH content in gills, which was significantly ($P < 0.05$) higher at the highest copper concentration (70 µg/l) compared with the control and the copper concentration of 40 µg/l. Neither total GSH content in kidney nor the reduced/oxidized glutathione (GSH/GSSG) ratio in all analysed tissues (liver, kidney, gills) was significantly affected by copper exposure (Figure 3C).

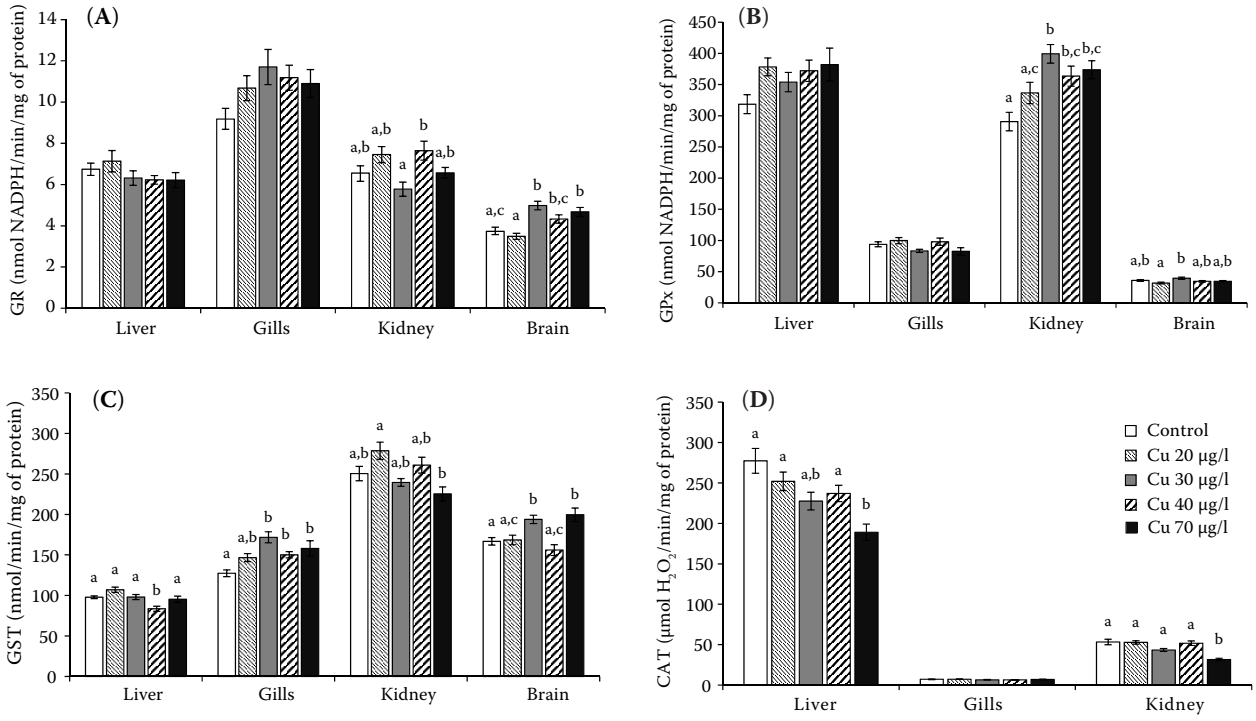

Figure 2. Activity of glutathione reductase (GR) (**A**); glutathione peroxidase (GPx) (**B**); glutathione S-transferase (GST) (**C**); and catalase (CAT) (**D**) in tissues of common carp after 28-day waterborne exposure to copper. Data are expressed as mean ± SEM. Significant differences ($P < 0.05$) are indicated by different superscripts

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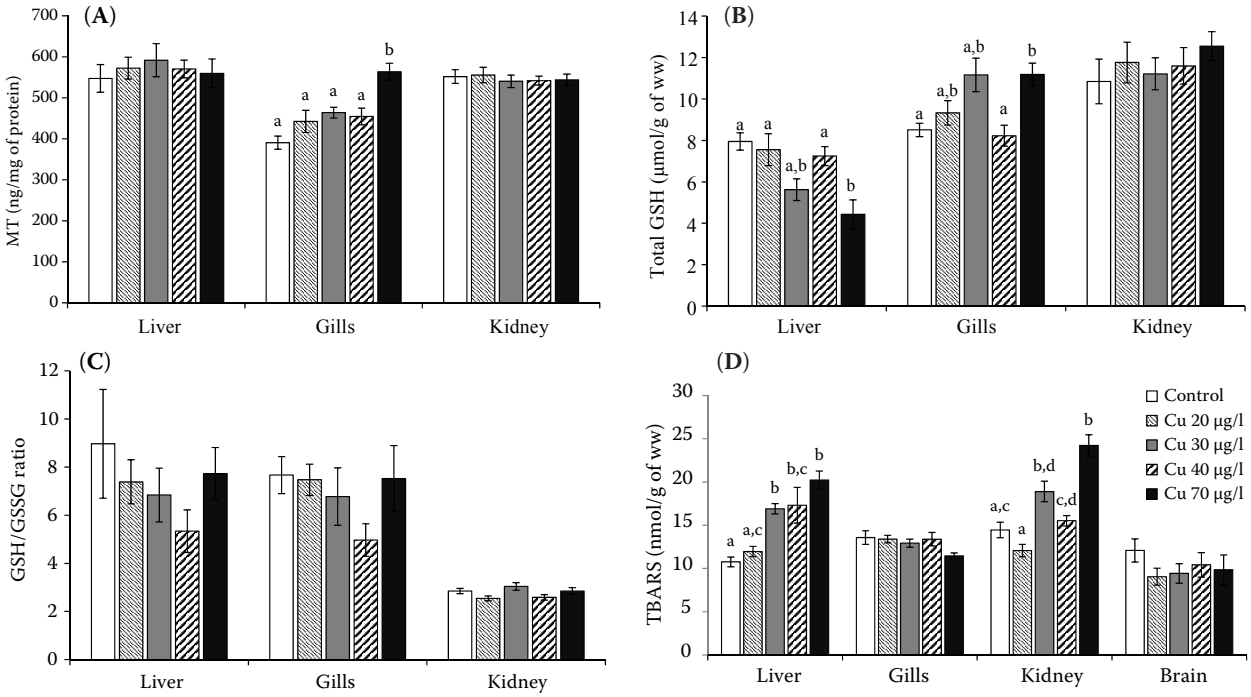

Figure 3. Content of metallothioneins (MT) (**A**); total glutathione (Total GSH) (**B**); reduced/oxidized glutathione ratio (GSH/GSSG) (**C**); and thiobarbituric acid reactive substances (TBARS) (**D**) in tissues of common carp after 28-day waterborne exposure to copper. Data are expressed as mean ± SEM. Significant differences ($P < 0.05$) are indicated by different superscripts

Lipid peroxidation

Figure 3D shows lipid peroxidation levels in all analysed tissues. Oxidative damage to lipids in liver and kidney increased significantly ($P < 0.05$) with increasing copper concentration. The peroxidation of lipids in gills and brain was not influenced by copper exposure.

Histopathological examination

Histopathological examination revealed pathological lesions in liver of fish after exposure to copper concentrations of 40 and 70 µg/l. Morphological changes were represented by marked dystrophic lesions of hepatocytes. Morphological signs of cell injury included the hydropic-to-vacuolar degen-

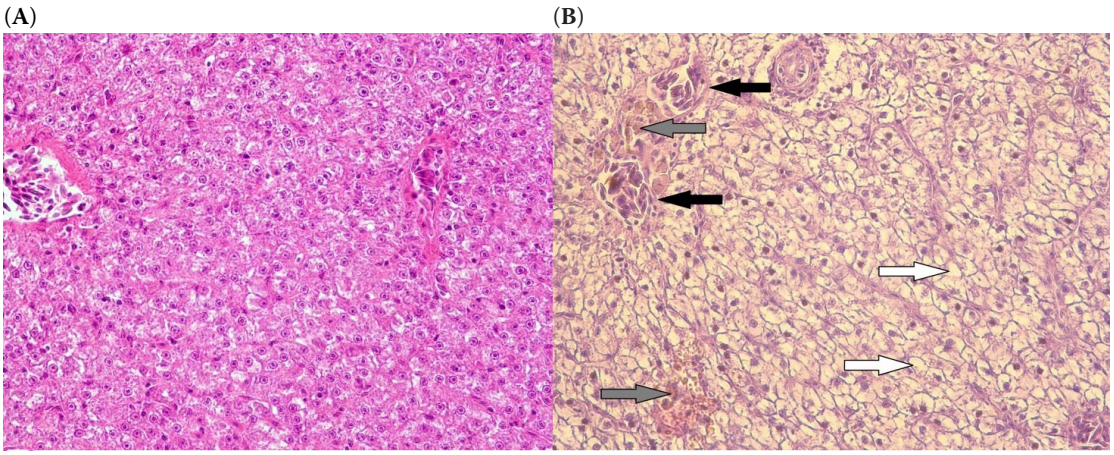

Figure 4. Results of histopathological examination of liver of common carp after 28-day waterborne exposure to copper. (**A**) Control; (**B**) copper concentration of 70 µg/l (white arrows = diffuse vacuolar degeneration of hepatocytes; black arrows = dilatations of capillaries; grey arrows = cholestasis); magnification × 400

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eration of hepatocytes, the dilatation of capillaries, mild hyperaemia, and cholestasis (Figure 4). The tissues and organs of fish in the experimental groups exposed to 20 and 30 µg/l of copper exhibited no pathomorphological changes.

DISCUSSION

Despite its important biological functions, copper can be harmful to fish. The present study showed that chronic waterborne exposure to slightly higher copper concentrations than environmental ones could have negative effects on the health status of common carp. Such effects include behavioural changes, the impairment of haematological and biochemical indices, an imbalance in antioxidant defence, and also pathological lesions in tissues.

Behavioural changes in aquatic organisms have been frequently observed as a consequence of copper exposure (Pyle and Mirza 2007). Copper is able to cause direct degeneration of the olfactory epithelium (Julliard et al. 1993) and damage to the neuromasts of the lateral line (Hernandez et al. 2006). Alterations to the olfactory organ and mechanoreception may lead to reduced food intake and reduced swimming activity, as observed at the highest copper concentration (70 µg/l) in the present study. Behavioural changes during the exposure period were also reported by Sanchez et al. (2005), who evaluated the toxic effect of copper on three-spined stickleback (*Gasterosteus aculeatus* L.). They documented reduced mobility and loss of appetite during 21 days of exposure to copper at a concentration of 200 µg/l. Similar findings such as apathy, slow movement, and decreased food intake were described in juvenile common carp during the first two weeks of exposure to copper at a concentration of 0.80 µmol/l (50 µg/l) (De Boeck et al. 1997). An impairment in feeding behaviour was also observed in common carp exposed to 1 µmol/l (64 µg/l) of copper (Kuzmina 2011).

In connection with reduced food intake, in our study both body weight and condition factor were reduced in fish exposed to copper at a concentration of 70 µg/l. Lower body weight together with unchanged liver weight led to a reduced HSI value at the highest copper concentration (70 µg/l).

The liver is the main organ responsible for the maintenance of copper homeostasis and is also a possible target for copper-induced damage in fish (Grosell et al. 1998; Abdel-Tawwab et al. 2007; Kamunde and McPhail 2008). In our study, we observed hydropic and vacuolar degeneration of hepatocytes, the dilatation of capillaries, mild hyperaemia, and cholestasis. Vacuolar degeneration in liver of common carp as a consequence of the toxic effect of $CuSO_4$ (intraperitoneally injected as 10 mg/kg Cu of body weight) was also reported (Varanka et al. 2001). The authors also documented swollen nuclei and mitochondria in hepatocytes.

Because the liver is the main storage organ for essential metals, the accumulation of copper in liver tissue is related to its concentration in the environment and the exposure time. Therefore, copper concentration in fish liver can be used as an indicator of water contamination (Jezierska and Witeska 2001). Quantitatively speaking, food taken up through the intestine is the most important source of copper in fish. In addition, fish possess the ability to absorb copper through the gills, but this covers only 10% of physiological demands (Grosell 2012). Although gill absorption was the main source of copper in fish in the present study, we found the highest copper concentration in the liver confirming the key role of the liver in copper metabolism. We registered a concentration-dependent increase in copper content in liver, gills, and kidney. In contrast, copper content in muscle was not affected by exposure time; also, the copper concentration was lowest in muscle tissue compared to the other samples. Overall, we observed a copper distribution in tissues comparable to the findings of other studies (De Boeck et al. 2004; Celechovska et al. 2007; Kandemir et al. 2010).

In general, haematological indices can be influenced by a wide range of factors, both endogenous and exogenous (Nespolo and Rosenmann 2002). The haematological response is non-specific towards chemical stressors; however, it can indicate that fish are exposed to environmental stress (Cazenave et al. 2005). Changes in red blood cell profile are probably an adaptive response to the impairment of gas exchange in copper-exposed gills and increased energetic demands on the fish (Witeska et al. 2010). In the blood of fish under stress, an increase in erythrocyte counts, haemoglobin concentrations, and haematocrit levels are frequently observed. In our study, the increased erythrocyte counts and haematocrit values could be due to enhanced erythropoiesis as a result of chronic metal exposure (Kondera and Witeska 2013).

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Immunosuppression induced by copper exposure, expressed through a decreased leukocyte count in fish, was described (Svobodova et al. 1994; Witeska et al. 2009). However, the tendency towards a decreasing leukocyte count was not significant in the present study. The decrease in white blood cell count, as a sign of the suppression of non-specific immunity, can be transient and gradually followed by a return to control levels, indicating an adaptive reaction of the organism. A decrease in lymphocytes together with a relative increase in neutrophil count is considered a common finding in the leukocyte profile of fish following exposure to copper and other metals (Jezierska and Witeska 2001). We observed an increase in neutrophils, both band and segmented, and also an increase in monocytes – thus, an increase in the number of cells with phagocytic activity. The activation of phagocytosis was also documented in goldfish (*Carassius auratus*) after acute exposure to copper sulphate (100 µg/l as copper) (Muhvich et al. 1995). Changes in the production of reactive oxygen species in macrophages was demonstrated in experimental studies with fish exposed to copper (Rougier et al. 1994; Bennani et al. 1996; Dethloff and Bailey 1998).

The plasma biochemical profile provides important information about the internal environment of the organism. An increased plasma glucose concentration, a commonly observed effect caused by various stress stimuli, was apparent in all tested copper concentrations. Initially, metals, including copper, activate glycogenolysis via increased secretion of catecholamines. Later, the gluconeogenic action of cortisol is apparent (Takei and Loretz 2006). Reduced insulin secretion, causing hyperglycaemia, was also described in fish after long-term exposure to copper (Van Vuren et al. 1994; Moon 2001). An increase in plasma glucose levels was documented by several authors in similar studies after the chronic exposure of various fish species to low copper concentrations (Gill et al. 1992; Heydarnejad et al. 2013; Pretto et al. 2014).

Disturbances in carbohydrate metabolism caused by metal intoxication may also be represented by changes in the accumulation of lactate and LDH activity (Teodorescu et al. 2012; Perumalsamy and Arumugam 2013). Increased LDH activity was revealed at the highest copper concentration (70 µg/l); however, it was not accompanied by an increase in lactate level. Increased LDH activity is known to be a sign of anaerobic metabolism and is often a consequence of slight tissue damage – in our study, the consequence of liver damage. On the other hand, the liver still retains the ability to scavenge lactate from plasma (Gill et al. 1992; De Boeck et al. 1995).

An elevated plasma ammonia level, found at all tested copper concentrations, is a commonly observed effect of sub-lethal copper exposure in fish (Grosell 2012). Both increased ammonia production and the inhibition of its excretion could explain such observations. Cortisol, produced in fish under stress, stimulates protein metabolism, resulting in increased ammonia plasma levels (De Boeck et al. 1995; De Boeck et al. 2001). The mechanism behind the decreased excretion of ammonia via the gills, caused by copper, could be connected with the inhibition of the branchial enzyme carbonic anhydrase and the reason why ammonia in the form of NH_4^+ remains trapped in gill cells (Wilson et al. 1994). Another possible explanation could be related to the inhibition of the branchial Na^+/ K^+-ATPase pump, involved in ammonia excretion through the gills (Grosell et al. 2002).

Transaminases play an important role in protein metabolism, especially in the liver. Large amounts of ALT and AST are released into blood as a consequence of liver damage. In our study, the increase in ALT activity at the highest copper concentration (70 µg/l) might have been due to hepatocellular damage, which was revealed by histopathological examination of the tissue. These results are in accordance with those reported by many authors (Karan et al. 1998; Varanka et al. 2001; Hassaan et al. 2014). Hepatobiliary disorder may also lead to decreased cholesterol clearance from blood and its elevated concentration in plasma, as was revealed at copper concentrations of 30, 40, and 70 µg/l. Accordingly, it is likely that copper exposure resulted in damage to the hepatobiliary system and its function in exposed fish.

Copper is able to interfere with osmoregulation in fish, which leads to ionic imbalance in plasma (Pelgrom et al. 1995). Several mechanisms of copper-induced osmotic disturbance have been suggested, such as the altered permeability of the gill epithelium resulting from direct cell damage or an effect on active transport via Na^+/ K^+/ATPase (Grosell and Marshall 2006). Calcium metabolism in fish differs from the mammalian kind, because of the lack of parathyroid glands in fish. Also, calcitonin probably plays a less important role in

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calcium balance in fish than in mammals. In contrast, prolactin and somatolactin, released from the pituitary gland, contribute to calcium regulation in fish. Moreover, in fish, a unique glycoprotein, stanniocalcin, is produced by the so-called Stannius corpuscules, which participates in the regulation of calcium levels (Clark et al. 2002). The increased calcium levels observed in our study could be due to the activation of pituitary gland hormones initiated by copper exposure. Prolactin is known to activate calcium uptake via a reduction in epithelium permeability in the gills, the digestive tract, and renal tubules (Kawauchi et al. 2009).

Ceruloplasmin serves as a copper transporter in blood and represents 90% of the total amount of copper in plasma (Harris 1991; Di Giulio and Meyer 2008). Besides its transport function, ceruloplasmin possesses antioxidant properties through its ferroxidase activity and is involved in the homeostasis of iron (Luza and Speisky 1996). It modulates the coagulation cascade, participates in the inactivation of the production of biogenic amines, and is associated with cardiovascular diseases (Shukla et al. 2006). Our findings on the dose-dependent increase in ceruloplasmin levels confirmed the important role of ceruloplasmin in copper metabolism. Our results are consistent with several other studies (Parvez et al. 2003; Tang et al. 2013).

The Ferric-reducing ability of plasma is a simple method which determines the antioxidant power of plasma mediated through generally non-protein antioxidants. The major antioxidants contributing to FRAP in human plasma are uric acid (60%) and ascorbate (15%); the minor ones are proteins (10%), alpha-tocopherol (5%) and bilirubin (5%) (Benzie and Strain 1996). Because uric acid is a minor product of nitrogen metabolism in fish, and ceruloplasmin and albumin are not very active in FRAP due to the low pH during the measurement, the increase in FRAP values in our study is more likely to be the result of the elevation of plasma ascorbate, alpha-tocopherol, and bilirubin levels. However, although the total value of uric acid in fish plasma is usually low, increased levels have been recorded in fish exposed to copper (Abdel-Tawwab et al. 2007; Hassaan et al. 2014). An increase in FRAP was observed in fish after exposure to terbuthylazine (Mikulikova et al. 2011), but a decline in FRAP seems to be a more frequent response in fish exposed to stress stimuli (Dabrowski et al. 2004; Haluzova et al. 2010).

In the present study, copper concentration played a greater role than the type of copper-based compound in the majority of analysed parameters. However, in some cases, the type of tested substance also appears to be important. It seems evident that copper sulphate is more toxic than copper oxychloride, particularly in the case of cholesterol, FRAP and ceruloplasmin activity.

Metallothioneins are metal-binding proteins with an important function in the homeostasis of essential metals, such as copper and zinc, and in the protection of the organism against toxic metals, particularly cadmium and mercury (Roesijadi 1996; Amiard et al. 2006). Metallothionein induction occurred only in the gills at the highest copper concentration (70 µg/l), while in other organs it was relatively uniform. Surprisingly, we did not observe metallothionein induction in the liver, despite the fact that this is the usual result of copper exposure. De Boeck et al. (2003) found a clear correlation between copper and metallothionein levels in the liver (but not in the gills) of common carp exposed to a sub-lethal copper concentration. Similar results have been described in various fish species (Parvez et al. 2003; Jebali et al. 2008). However, the induction of metallothionein gene expression in cyprinid fish species following copper exposure was also detected in liver, spleen, kidney, and gills by (Wang et al. 2014), and in liver and kidney by Cho et al. (2008). Metallothionein induction in gills could be explained as a response to the direct contact of gill tissue with copper. We did not observe any oxidative damage to lipids in gills, in contrast with liver and kidney (discussed below), which could be interpreted as a protective effect of metallothioneins on gill tissue.

Glutathione is believed to detoxify copper via two mechanisms. Firstly, it scavenges reactive oxygen species created by copper in the Fenton reaction through GPx activity. Secondly, its cysteinyl group has been implicated in the sequestration of copper, thereby reducing its intracellular concentration (Stohs and Bagchi 1995). The GSH/GSSG ratio is usually used as a biomarker of the thiol status of aquatic organisms (Van der Oost et al. 2003). Although the GSH/GSSG ratio was not affected in our study, differences in total GSH were detected in liver and gills. Glutathione produced in the liver could be transported to the gills according to need, which could explain the elevated total GSH levels in gills found in our study. Later, there may be disruption of the synthesis of GSH directly in the liver

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with a subsequent reduction in total GSH in this tissue (Kretzschmar 1996).

In common carp, antioxidant enzymes have been shown to be either activated or inhibited by copper, depending on the dose, duration, and route of exposure. Dautremepuits et al. (2002) found decreased GST activity in cranial kidney and decreased GST and CAT activities in liver in common carp after 96 h of waterborne exposure to copper at concentrations of 100 and 250 µg/l. Furthermore, these authors found no changes in GR activity. Decreased CAT and GPx activities in liver were found in common carp 24 and 48 h after the intra-peritoneal injection of copper (10 mg/kg of body weight) (Varanka et al. 2001). Eyckmans et al. (2011) described an increase in CAT activity in gills of common carp after 24 h of exposure to copper at a concentration of 65 µg/l followed by a return to initial values within three days. In the present study, substantial changes were revealed in GST and CAT activities. A decline in CAT activity at the highest copper concentration might be related to the inhibition of enzyme synthesis, or the direct binding of copper to –SH groups of this enzyme (Vutukuru et al. 2006), and/or the exhaustion of its activity due to the flux of superoxide radicals (Kono and Fridovich 1982).

Glutathione-S-transferase is an important enzyme detoxifying a wide variety of chemicals via conjugation with GSH in the liver (Kretzschmar 1996). Moreover, GST possesses antioxidant properties including Se-independent glutathione peroxidase activity and its function as a scavenger of oxidative stress products arising from the oxidation of lipids, nucleic acids, and proteins (Leaver and George 1998). We detected altered GST activity in all tissues, but with diverse tendencies. Several GST isozymes have been found in fish, some of them specific to fish species (Borvinskaya et al. 2013). To accurately elucidate the involvement of GST in copper toxicity, the analysis of different isozymes would be necessary. We can only assume that because other antioxidant enzymes were not greatly affected, the function of GST might be more related to the detoxification mechanism than to direct antioxidant defence.

Lipid peroxidation is frequently observed and used as a biomarker of the oxidative damage to fish caused by copper (Varanka et al. 2001; Pretto et al. 2014). Increased LPO at higher concentrations of copper was evident in liver and kidney and could be related to the higher copper levels detected in these tissues. In the case of liver, it might be related to the failure/exhaustion of antioxidant defence, both enzymatic and non-enzymatic, as manifested by a decreased level of total GSH as well as reduced CAT activity. In our study, oxidative damage to lipids was probably a major cause of the morphological changes to hepatocytes. Evidence of LPO was also found in kidney. However, antioxidative defence was still much more effective in kidney than in liver, as evidenced by increased GPx activity and an increased level of total GSH. There were no histopathological changes, but the biochemistry profile revealed disturbances in osmoregulation in the kidney.

CONCLUSIONS

In summary, this study provides further evidence that copper has a potentially deleterious effect on the fish organism and is able to cause changes in biochemical and haematological parameters and elements of antioxidant defence. The present study demonstrates the influence of copper on common carp, even at slightly increased concentrations compared to those typically found in the environment. The clearest changes were found at the highest tested copper concentration, which still represented environmentally relevant levels. Liver was the most affected tissue, with disturbances even at the morphological level. The most sensitive biochemical markers in plasma in the present study appeared to be glucose, ammonia, phosphorus and calcium, which were already affected at the lowest concentration (20 µg/l), whereas haematological indices, copper level in kidney, and selected oxidative parameters were influenced at concentrations of 30 µg/l and above. Overall, we can conclude that total copper concentration played a greater role than the type of copper-based pesticide formulation, although in some cases pesticide-specific interactions should also be considered.

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