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A study of toxicity and genotoxicity of copper, zinc and their mixture to rainbow trout (*Oncorhynchus mykiss*)

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The aim of the study was to evaluate the toxicity and genotoxicity of heavy metals (HM) (Cu and Zn) and their mixture to rainbow trout (*Oncorhynchus mykiss*). No significant alterations in the erythrocyte count and haemoglobin concentration were found after exposure to Cu, Zn and their mixture at all three concentrations studied. The most significant decrease ($P < 0.001$) in leukocyte count was determined in the blood of fish exposed to 0.25 LC50 of HM and their mixture, 0.125 and 0.0625 LC50 of HM mixtures. A slight, but significant increase ($P < 0.05$) in hematocrit level was found in the blood of fish exposed to a 0.125 LC50 concentration of Cu and HM mixture.

The frequency of micronucleated erythrocytes (MNE) increased after exposure to HM and their mixture at all three concentrations studied (0.25, 0.125 and 0.06 LC 50) ($P < 0.0001$), but there were no significant differences in MNE levels among the concentrations studied ($P = 0.136$). After a 96-hour recovery (in clean water) of the fish exposed to a HM mixture, the levels of MNE significantly decreased at the highest and lowest concentrations studied ($P = 0.0001$ and $P = 0.004$, respectively).

Key words: fish, toxicity, genotoxicity, copper, zinc, micronuclei, haemotological parameters

INTRODUCTION

Most studies that evaluate the toxicity of aquatic system pollutants to organisms and their systems have investigated exposures to individual toxicants. Separate effects of copper and zinc on aquatic organisms are widely studied, as low concentrations of these metals are normal constituents of all natural waters, they are essential minerals and cofactors of many enzymes in fish [1, 2]. However, both metals are toxic to animals and plants at the levels close to ones found in many unpolluted water bodies. Acute lethal and sublethal copper effects on adult fish physiological parameters [2–4] or copper hazards to invertebrates [5] were intensively studied as levels of dissolved copper are often increased by anthropogenic impact such as direct application in agriculture. Zinc toxic effects on plants, invertebrates and fish were and are studied also for a wide versatile use of this metal (e.g., zinc sulphate is added to salmon feed to help the fish to avoid some diseases) [6, 7].

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However, aquatic ecosystems are usually exposed simultaneously to a mixture of toxic substances, where different interactions among metals are possible. There are very few studies of the acute toxicity of mixtures of heavy metals [8, 9], responses of different functional systems or changes in physiological parameters of fish caused by heavy metals [10–12]. Comparative studies demonstrated that the effects of heavy metal mixtures could differ by their toxicity to living organisms from the effects of single components [13, 14]. Mixtures of some metals are characterized by some antagonistic effects [13, 15] while others by synergetic ones [16]. Copper in animals interacts with essential trace elements such as iron, zinc and also with nonessential elements. Interactions may be either beneficial or harmful to the organism [17, 18]. There are some studies to show the genotoxicity of copper on aquatic organisms [19, 20], but actual mechanisms of its genotoxicity are poorly discussed. One of the possible paths of Cu genotoxicity is induction of oxidative stress and production of DNA damaging reactive oxygen species [21]. In our previous study (unpublished data) we didn't find any increase in DNA damage in copper-and zinc-

exposed trout erythrocytes by means of the Comet assay. Therefore we applied a micronucleus test capable of assessing both clastogenic and aneugenic effects. Both the haematological parameters and the micronucleus test are sensitive tools in ecotoxicological studies. Therefore we chose them for evaluation of toxic and genotoxic effects of copper, zinc and their mixture to rainbow trout.

MATERIALS AND METHODS

Rainbow trout adults were obtained from the Žeimena hatchery and kept in holding tanks of about 3000 l capacity supplied with flow-through artesian aerated water. For experiments, the fishes were transferred from holding tanks to aquaria of 40 l capacity and kept in the new medium until acclimation, i.e. till they started to swim freely and feed well. The length of fish under study ranged from 15.5 ± 1.1 to 18.0 ± 1.2 cm and weight from 32.7 ± 1.0 to 43.1 ± 2.3 g, respectively. Artesian water of high quality was used for dilution. The average hardness of dilution water was approximately 284 mg/l as $CaCO_3$, alkalinity was 244 mg/l as HCO_3^-, the mean pH was 8.0, the temperature was equal to 12 ± 0.5 °C and the oxygen concentration ranged from 8 to 10 mg/l. Water in the aquarium was changed daily and fish were fed until satiety with the commercial DANA FEED fish food.

The concentrations of Cu and Zn were chosen based on previous studies, which indicated that the 96-hour LC50 of copper was 0.65 mg/l and the 96-hour LC50 of zinc was 3.79 mg/l [3, 22]. Chemically pure salts of copper sulphate ($CuSO_4 \cdot 5H_2O$) and zinc sulphate ($ZnSO_4 \cdot 7H_2O$) dissolved in distilled water were used as toxicants. The final concentrations were recalculated according to the amount of copper and zinc ions. Expressions of the three metal concentrations studied are shown in Table 1. Fish

Table 1. **Concentrations of Cu, Zn and their mixture studied**

Metals	Concentrations	
	Part of LC50	mg/l
Copper	0.25	0.16
Zinc	0.25	0.948
Mixture of Cu and Zn	0.25 + 0.25	0.16 + 0.948
Copper	0.125	0.08
Zinc	0.125	0.474
Mixture of Cu and Zn	0.125 + 0.125	0.08 + 0.474
Copper	0.0625	0.04
Zinc	0.0625	0.238
Mixture of Cu and Zn	0.0625 + 0.0625	0.04 + 0.238

were divided into exposure groups for every concentration studied: fish in tens were exposed to copper and zinc, twenty fish to the mixture of metals, and ten fish were kept in clean water as control. After a 96-hour exposure, ten fish from the metal mixture exposed group were transferred to clean (metal-free) water for recovery for the next 96 hours.

After a 96-hour exposure fish under study and control ones were caught and blood was taken for the measurement of haematological parameters and for the micronucleus test. Blood samples were collected by incision of the caudal blood vessels. Heparin sodium salt was used for stabilization of the fish blood. Erythrocyte count (RBC, $10^6 \times mm^3$), haemoglobin concentration (Hb, g/l), hematocrit level (Hct, L/l), leukocyte count (WBC, $10^3 \times mm^3$) were determined using routine methods [12]. For the micronucleus test blood was taken from each fish, smeared on slides and air-dried. After fixation in methanol for 10 min, the slides were stained with the 10% Giemsa solution for 8 min, rinsed with distilled water and dried. The frequency of micronucleated erythrocytes (MNE) was evaluated by scoring at 1250× magnification. A total of 10000 erythrocytes were examined for each fish. Only cells with intact cellular and nuclear membrane were scored. Round or ovoid-shaped non-refractory particles with their colour and structure similar to chromatin, their diameter equal to 1/3–1/50 of the main nucleus and clearly detached from it were interpreted as micronuclei (MN).

The statistical analysis of the data was performed with SPSS 10.0 software and the significance of the data was determined by using one-way ANOVA and the Post Hoc LSD test.

RESULTS

Haematological parameters

Haematological parameters of rainbow trout exposed to Cu, Zn and their mixture at three different concentrations are represented in Table 2.

Erythrocyte count in the blood of control fish ranged from 0.88 ± 0.06 to 0.95 ± 0.03 × 10^6 mm^3. Exposure to separate Cu and Zn did not induce significant changes in this parameter. A slight but significant increase ($p < 0.01$) of this parameter was found only in fish exposed to a mixture of 0.25 96-hour LC50 of Cu and 0.25 96-hour LC50 of Zn. A significant reduction of erythrocyte count to 0.81 ± 0.05 × 10^6 mm^3 ($p < 0.005$) was observed after a 96-hour recovery period.

Haemoglobin concentration in the blood of control fish ranged from 95.6 ± 6.4 to 99.6 ± 4.4 g/l. No significant changes were observed in the haemoglobin concentration of exposed fish.

The hematocrit of the control fish ranged from 0.40 ± 0.02 to 0.42 ± 0.03 L/l. A marginal increase in this parameter was found in fish exposed to 0.25

Table 2. **Haematological parameters of rainbow trout exposed to Cu, Zn, and their mixture (mean ± S.E.M.)**

Metals	RBC, 1×10^6 mm^3	Hb, g/l	Hct, L/l	WBC, 1×10^3 mm^3
		0.25 96-hour LC50		
Control	0.88 ± 0.06	95.6 ± 6.4	0.42 ± 0.03	20.4 ± 1.7
Cu	0.95 ± 0.05	94.2 ± 3.9	0.48 ± 0.02	10.7 ± 0.8**
Zn	0.84 ± 0.06	98.7 ± 3.5	0.45 ± 0.03	9.5 ± 2.3**
Cu + Zn	1.10 ± 0.05**	89.6 ± 5.6	0.46 ± 0.02	10.1 ± 1.1**
96-hour recovery	0.81 ± 0.05‡‡	88.2 ± 3.8	0.39 ± 0.01	13.0 ± 2.5**
		0.125 96-hour LC50		
Control	0.95 ± 0.03	99.6 ± 4.4	0.40 ± 0.02	19.3 ± 1.2
Cu	1.12 ± 0.06	99.4 ± 4.9	0.47 ± 0.03*	15.7 ± 2.5
Zn	0.81 ± 0.06	97.5 ± 3.7	0.42 ± 0.04	14.8 ± 1.8
Cu + Zn	0.96 ± 0.09	101.2 ± 3.0	0.45 ± 0.02*	10.1 ± 1.2*
96-hour recovery	0.88 ± 0.09	93.1 ± 3.0	0.38 ± 0.01	15.8 ± 1.2
		0.0625 96-hour LC50		
Control	0.91 ± 0.01	95.6 ± 8.6	0.42 ± 0.03	19.1 ± 2.0
Cu	0.92 ± 0.06	88.8 ± 4.7	0.43 ± 0.04	17.9 ± 1.7
Zn	0.82 ± 0.04	83.8 ± 3.3	0.37 ± 0.02	15.1 ± 0.8*
Cu + Zn	0.91 ± 0.03	88.4 ± 4.4	0.44 ± 0.03	14.3 ± 1.4*
96-hour recovery	1.00 ± 0.04	96.6 ± 4.4	0.41 ± 0.02	18.4 ± 1.1

The levels of significance: * – $p < 0.05$, ** – $p < 0.01$; ‡‡ – significant decrease ($p < 0.005$) as compared with the erythrocyte count after 96-hour exposure to a Cu and Zn mixture. **RBC** (red blood cell count) – erythrocyte count, **Hb** – haemoglobin, **Hct** – hematocrit, **WBC** (white blood cell count) – leukocyte count.

96-hour LC50 of Cu. A slight, however, significant increase in hematocrit level was found also in fish exposed to 0.125 96-hour LC50 concentration of Cu and the mixture of metals.

The leukocyte count of control fish ranged from 19.3 ± 1.2 to 20.4 ± 1.7 × 10^3 mm^3. At a concentration of 0.25 96-hour of LC50, both separate metals as well as their mixture (0.25 96-hour of LC50 of Cu + 0.25 96-hour of LC50 Zn) induced a significant almost 2-fold drop in leukocyte count (Table 2). A similar low leukocyte count was found in the blood of fish after a 96-hour recovery in metal-free water. Reduction of this parameter was found after exposure to lower concentrations of separate metals (0.125 96-hour LC50). However, the mixture of metals (0.125 96-hour of LC50 of Cu + 0.125 96-hour of LC50 Zn) significantly diminished leukocyte count. A significant decrease in leukocyte count was found also in fish exposed to 0.06 96-hour of LC50 of Zn and to a corresponding mixture of metals (0.06 96-hour of LC50 of Cu + 0.06 96-hour of LC50 Zn). Meanwhile, leukocyte count in the blood of fish after a 96-hour recovery in clean water did not significantly differ from control ones (Table 2).

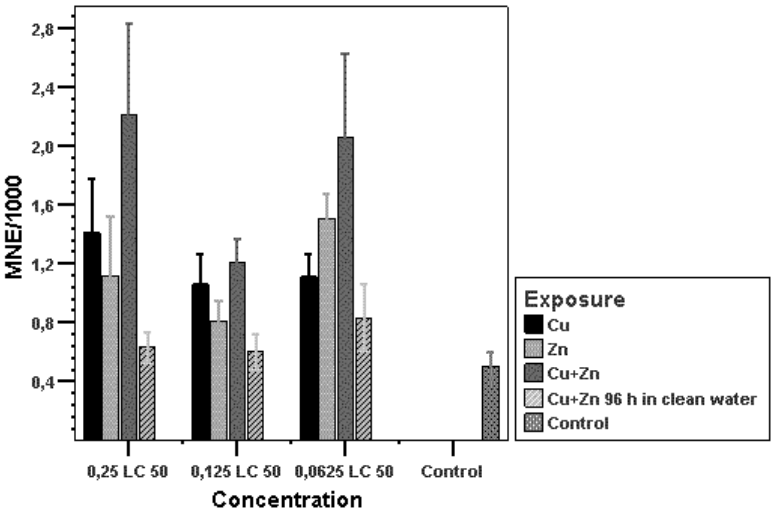

Figure. Frequency of micronucleated erythrocytes in blood of rainbow trout exposed to three concentrations of Cu, Zn and their mixture. Bar shows mean ± S.E.M

Micronucleus test

The levels of micronucleated erythrocytes (MNE) are shown in Figure. The highest levels of MN were induced in fish exposed to Cu and Zn metal mixtures, especially in groups exposed to 0.25 and 0.0625 96-hour LC50 concentrations (Post Hoc LSD test, P = 0.0001). Significantly increased levels of MNE were also found in fish exposed to the highest concentration of Cu and the lowest concentration of Zn (Post Hoc LSD test, $P < 0.05$). A comparison of MNE levels of all concentrations studied did not reveal any significant difference (ANOVA test, F = 2.03, P = 0.136). After a 96-hour recovery of trout exposed to a mixture of metals there was a significant decre-

ase in MNE level in fish exposed to highest and lowest concentrations (Post Hoc LSD test, P = 0.0001 and P = 0.004, respectively).

DISCUSSION

No significant alteration in erythrocyte count and haemoglobin concentration was found in fish exposed separately to copper and zinc. These data suggest an insignificant effect of Cu and Zn alone on rainbow trout erythrocytes. However, a temporary increase in erythrocyte count in fish exposed to a mixture of metals (0.25 96-hour LC50 Cu + 0.25 96-hour LC50 Zn) indicated an augmentation of the adverse effect of these pollutants (Table 2). This increase in erythrocyte count was found in the blood of 40% of fish (to 1.22×10^6 mm^3) and could be explained by haemoconcentration. Haemoconcentration was observed in the blood of rainbow trout during an acutely lethal copper exposure [23]. The authors explained such an increase in red cell count by a release of erythrocytes from the spleen, caused by high catecholamine levels. A significant drop in erythrocyte count after a 96-hour recovery (0.25 96-hour LC50 concentration) could be explained by findings of Witeska and Kosciuk [7]. They suggested that waterborne heavy metals, initially bound to the gills and subsequently deposited in other tissues, might affect the fish, even if toxic agents were removed from the water. An increase in hematocrit levels could be explained as a typical stress response in metal-exposed fish. A significant drop in leukocyte count, especially in fish exposed to a mixture of metals, indicated the high sensitivity of the immune system to metal impact. It has been known that copper and zinc induce a decrease in white blood cell count in fish [7, 24–27]. A mixture of Cu and Zn (0.25 96-hour LC50 + 0.25 96-hour LC50) induced a significant drop in leukocyte count of 60% of the fish studied down to the concentration below 11×10^3 mm^3. The elevation of leukocyte count was observed in blood of fish after recovery in clean water. However, this level was significantly lower than in controls. Lower concentrations of metals in the mixture caused a similar drop in leukocyte count, but after recovery in clean water the leukocyte level reached the control level. The immunotoxic effect of the mixture of metals was most obvious at lower mixture concentrations studied, where separate Cu and/or Zn did not cause significant alterations in leukocyte count.

Our previous data demonstrated the more-than-additive toxic effects of metal mixture towards embryos and larvae of rainbow trout and a partial additive toxic effect on adult fish [9]. Mixtures of copper and zinc salts in marine or freshwater fishes were more-than-additive in toxicity, producing more deaths in 96 hours than expected on the basis of individual components [28, 29], and were generally acknowledged to be more-than-additive in toxicity to a variety of aquatic organisms [30]. But mixtures of copper (0 to 90 μg/l) and zinc (0 to 1.200 μg/l) were only additive in action to a marine bacterium (*Photobacterium phosphoreum*), and sometimes mixtures of Cu and Zn salts were less-than-additive in action, as judged by DNA, RNA and protein contents of newly hatched fathead minnows (*Pimephales promelas*) exposed for 4 days [31]. Significant changes in the leukocyte count of fish from our study indicated an enhanced adverse effect and additive toxicity of Cu and Zn mixture on fish as compared with the effects of Cu and Zn alone.

Our study confirmed a different sensitivity of the haematological parameters studied as well. No significant changes found in the haemoglobin concentration of fish exposed to metal and a slightly significant alteration in hematocrit indicate that the red blood cell parameters studied were less sensitive to intoxication and probably more easily compensated. These data are in agreement with the data of Witeska and Kosciuk [7] who studied the changes in the common blood parameters of carp after a short-term exposure to zinc.

Little is known about Cu and Zn genotoxicity. Guecheva et al. [19] elucidated the genotoxicity of copper sulphate to planaria by means of the Comet assay. After Cu exposure, they found elevated levels of DNA strand breakage and inhibition of DNA repair of planaria preexposed to methylmethan sulfonate. The authors have suggested that genotoxicity could appear via the action of induced reactive oxygen species, while the inhibition of DNA repair enzymes could be caused by a non-specific binding of Cu^{2+} cations to essential sites in the enzyme molecule. As reported by Gabbianelli et al. [21], Cu induced a slight increase of comet parameters in the erythrocytes of *Sparus aurata* exposed for 20 days *in vivo*. In our study, the micronucleus test showed the potential genotoxicity of Cu, Zn and their mixture to rainbow trout erythrocytes, but there were no dose-dependant changes in MNE levels. After a 96-hour recovery of the trout exposed to a mixture of metals, there was a significant decrease in MNE level. This could be due to an efficient metal detoxication and excretion from the organism. Taking into account the micronucleus test data, we could make an assumption that Cu and Zn act as aneugens: they induce aneuploidy resulting in micronucleus formation. It is known that Pb and Hg interact with tubulin and disturb chromosome segregation [32] as well as zinc, copper and cobalt reduce the number of free sulfhydryl groups on tubulin, which may be responsible for their effects on tubulin polymerisation [33].

The results of our study demonstrated that even a short-term exposure of fish to metals could have genotoxic effects and higher concentrations of a bi-

nary mixture of metals might induce significant changes in the immune system. The concentrations studied were 4 times for copper and 2.4 times for zinc higher as compared with Maximum Permissible Concentrations for copper (0.010 mg/l) and zinc (0.1 mg/l) in water reservoir – receiver [34]. However, sometimes these concentrations may be exceeded in natural water bodies. Our study gives a possibility, by simple and reliable methods, to evaluate and predict the effects of a short-term exposure of fish to a mixture of toxicants.

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VARIO, CINKO IR JŲ MIŠINIO TOKSINIO IR GENOTOKSINIO POVEIKIO VAIVORYKŠTINIAM UPĖTAKIUI (*ONCORHYNCHUS MYKISS*) TYRIMAI

S a n t r a u k a

Nustatant toksinį ir genotoksinį sunkiųjų metalų poveikį buvo pasirinkti hematologiniai rodikliai ir mikrobranduolių testas. Paveikus vaivorykštinius upėtakius trimis skirtingomis vario, cinko ir jų mišinio koncentracijomis, eritrocitų skaičius ir hemoglobino koncentracija kraujyje ženkliai nepakito. Leukocitų skaičius statistiškai patikimai sumažėjo ($p < 0,001$) tų upėtakių kraujyje, kurie buvo paveikti didžiausia vario, cinko ir jų mišinio koncentracija (0,25 LC50), taip pat 0,125 ir 0,0625 LC50 metalų mišinio koncentracijomis. Hematokrito vertė statistiškai patikimai padidėjo ($p < 0,05$) tik vario ir metalų mišinio 0,125 LC50 koncentracija paveiktose žuvyse.

Aukščiausiais mikrobranduolių (EMB) dažniais pasižymėjo žuvys, paveiktos vario ir cinko mišiniu: statistiškai patikimai nuo kontrolės skyrėsi 0,25 ir 0,0625 LC50 koncentracijomis paveiktų upėtakių EMB dažniai ($p = 0,0001$). Kiek mažiau

EMB susiformavo variu, cinku paveiktų upėtakių eritrocituose, o patikimai nuo kontrolės skyrėsi tik 0,25 LC50 vario ir 0,0625 LC50 cinko koncentracijomis paveiktų upėtakių EMB dažniai (LSD testas, $p < 0,05$). Ištyrus trijų skirtingų koncentracijų metalų poveikį, EMB dažnių priklausomybė nuo dozės nenustatyta (ANOVA, $F = 2,03$, $p = 0,136$). Vario ir cinko mišiniu paveiktus upėtakius keturioms paroms įleidus į švarų vandenį, EMB dažniai statistiškai patikimai sumažėjo tik didžiausia ir mažiausia koncentracijomis paveiktų upėtakių (atitinkamai $p = 0,0001$ ir $p = 0,004$).