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Biological effects of multimetal (Ni, Cd, Pb, Cu, Cr, Zn) mixture in rainbow trout *Oncorhynchus mykiss*: Laboratory exposure and recovery study

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ABSTRACT

The present study tested the biological consequences of exposure to a multimetal mixture as a multiple chemical stressor on *Oncorhynchus mykiss* at molecular, cellular, physiological and whole-organism levels and on biomarker responses of this fish during the depuration period. To represent environmentally relevant multiple chemical stressors, in our study, we used the mixture of Zn, Cu, Ni, Cr, Pb and Cd at the concentrations corresponding to Maximum-Permissible-Concentrations (MPCs) acceptable for the EU inland waters. This study was undertaken with a view to elucidate if changes in the MPC of the test mixture components (Ni, Pb, Cd) could cause significantly different biomarker responses in *O. mykiss* from those previously determined in the carnivorous and omnivorous fishes exposed to the mixture of the same metals but at different MPCs of Ni, Pb and Cd. This study has revealed that exposure to mixtures of metals at MPC produces genotoxic effects in fish blood erythrocytes and a lethargic effect on *O. mykiss* behaviour, and, also, significantly increases the levels of Cd, Cr and Ni accumulated in the gills tissue. *O. mykiss* successfully depurated Cr and Ni in less than 28 days, however, the level of Cd decreased by only approximately 40% over the same period. A significant capacity of *O. mykiss* to restore its DNA integrity (Comet assay) after exposure to metal mixtures was revealed. However, the 28-day recovery period proved to be insufficiently long for erythrocytes with nuclear abnormalities to recover to the unexposed level. In conclusion, changes in the MPCs of Ni, Pb and Cd in the test mixture produce biological effects similar to those previously determined in *S. salar*, *R. rutilus* and *P. fluviatilis* exposed to the mixture of the same metals but at lower MPCs of Ni and Pb and at higher MPC of Cd.

1. Introduction

Anthropogenic wastewater discharges into the environment rarely consist of single pollutants; therefore, the aquatic environment is rich in various chemical mixtures (Heys et al., 2016; Hinton and Aizawa, 2006). Wild fish populations are exposed to multiple chemical stressors, including mixtures of essential and non-essential metals. Hence, in the last decades, there arose the necessity for the improved assessment of chemical mixtures and their risks to the environment (Wang et al., 2020; Petitjean et al., 2019; Anyanwu et al., 2018). Good water quality is vital for human health and ecosystems, therefore, Good Ecological Status (GES) is the set objective of the European Water Framework Directive (WFD) for surface water bodies. Regrettably, recent reports indicate that many surface water bodies in Europe still have not attained GES (EEA, 2012, 2018; Schäfer et al., 2016). Therefore, Posthuma et al. (2019) have emphasised the need for more efficient mixture risk assessment methodologies, protective actions against chemical pollution, and restoration measures. However, environmental quality guidelines and standard toxicity testing methods are based on safe concentrations of individual pollutants in the environment rather than on those occurring in mixtures (e.g., Brack et al., 2017). Therefore, there is little information about interactions of chemicals (Hinton and Aizawa, 2006). The problem of the combined effects of multiple "below the threshold" toxic chemicals evoking adverse biological responses in the affected biota is

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well recognised (e.g., Nys et al., 2017; Brix et al., 2016). Assessment of the stress caused by chemical mixtures remains a challenge given that it cannot be predicted based on individual components. Therefore, to gain a better understanding of metal pollution in aquatic ecosystems, it is necessary to study effects of environmentally relevant metal mixtures in the aquatic environment (Castaldo et al., 2020). Such metals as copper (Cu), zinc (Zn), chromium (Cr) and nickel (Ni) are essential nutrients required for various biochemical and physiological functions (WHO/-FAO/IAEA, 1996). Other metals, such as cadmium (Cd) and lead (Pb), have no established biological functions and are considered to be non-essential metals (Kalay and Canli, 2000; Tchounwou et al., 2012). Zn, Cu, Ni, Cr, Pb and Cd are the most common metal contaminants released into European waters, whereas Pb, Ni and Cd are indicated as priority metals due to their high toxicity (EEA, 2018). According to EAA report (2018), Cd, Ni, Pb, Zn, Cu, Cr are the metal pollutants that most frequently exceed environmental quality standards (EQSs) in surface water bodies, and elevated concentrations of Cd, Pb and Ni are indicated as the most common reasons why surface water bodies fail to achieve good chemical status. Discharge of metals in EU waters varies depending on the type of industry and receiving water body, e.g. at present, Cr (VI) pollution in EU waters varies between 70 and 90 μg/L (Tumolo et al., 2020). As reported by EEA (2018), around 60% of surface water bodies are failing good ecological status.

Metal toxicity and carcinogenicity involve many mechanistic aspects, some of which are not clearly explained or understood. However, each metal is known to have unique properties and physicochemical properties that determine its specific toxicological action mechanisms. Our previous studies (Stankevičiūtė et al., 2017, 2018; Sauliutė et al., 2020) have shown that multicomponent mixtures of metals even at MPC (maximum permissible concentration) exert negative biological effects on geno-, cytotoxicity, biochemical and behavioural parameters in different fish species such as *Salmo salar*, *Rutilus rutilus* and *Perca fluviatilis*. These studies highlighted the significance of the impact that even small changes in metal concentrations (MPC) have on the geno-, cytotoxicity risks in erythrocytes of the exposed carnivorous and omnivorous fish species as well as on the accumulated metal amounts in them. The multimetal mixture containing Cr and Cu at 10 times reduced MPC was indicated to have the highest cytogenetic potential and, also, to affect fish behaviour and amounts of metal accumulation in different tissues. Moreover, results of our previous studies (Stankevičiūtė et al., 2018) suggested the occurrence of hormetic-like responses in the fish exposed to multimetal mixtures with reduced MPC of certain metals, indicating that some of the metals present in a mixture induce hormesis. Therefore, chemical risk assessment could be significantly improved if the hormetic dose was taken into consideration when planning ecotoxicological studies. However, the elucidation of the interaction and combined effects of trace metals and their concentrations in a complex mixture and their potential impact on fish behaviour throughout periods of exposure and recovery has received scant scholarly attention. The locomotor activity of fish is the most frequently assessed sublethal endpoint when determining behavioural changes in response to contaminants in toxicity tests and, therefore, it is considered to be a critical character when establishing fish survival in natural environment (Gui et al., 2014). Such endpoints as average velocity and angular velocity that are commonly used in behavioural studies provide useful information for the basic swimming performance assessment, and when combined, indicate behavioural complexity and occurrences of erratic movements (Melvin et al., 2017; Benhaim et al., 2012; Tierney, 2011). Since behaviour integrates biochemical, physiological, and ecological processes, it may be ideal for studying environmental pollution effects (Scott and Sloman, 2004).

To the best of our knowledge, the characteristics of metal depuration from tissues of multimetal mixtures-exposed fish have yet to be determined. Adverse effects of multimetal mixtures at MPC on various biomarkers in fish have been demonstrated already, but the potential of recovery has not been investigated in detail. As reported by Sharma et al. (2018), recovery indicates the compensatory potential of the fish immune system. The available limited data indicate fish recovery from the adverse biological effects produced by exposure to single metals (Yin et al., 2019; Lindh et al., 2019; Chen et al., 2018; Gomes et al., 2015; Palaniappan and Karthikeyan, 2009; Cerqueira and Fernandes, 2002), their mixtures (binary) (Stankevičiūtė et al., 2016; Arini et al., 2015; Palaniappan and Karthikeyan, 2009), other single substances (Sharma and Chadha, 2019; Guzmán-Guillén et al., 2016) or metal nanoparticles (Lindh et al., 2019). Most of these studies evaluated the depuration of metals from various fish tissues, and some of them also assessed such responses of other biomarkers as gene expression, oxidative stress (SOD, MT) (Arini et al., 2015), blood indices, and physiological parameters (restoration of gill structure) (Cerqueira and Fernandes, 2002). Less information is available on the recovery of genotoxicity, cytotoxicity and behavioural biomarkers (Sharma and Chadha, 2016, 2019; Sharma et al., 2018; Stankevičiūtė et al., 2016; Guilherme et al., 2014; Marques et al., 2014; Williams and Gallagher, 2013; Oss et al., 2013; Eissa et al., 2009; Bony et al., 2008). Moreover, in previous studies, recovery potential was evaluated during short periods of time (from 48 h up to 14 days), except for studies by Cerqueira and Fernandes (2002), Palaniappan and Karthikeyan (2009), Arini et al. (2015), Sharma and Chadha (2019), where fish recovery was investigated during a longer period (up to 75 days). As reported in these studies (except the study by Sharma and Chadha, 2019), the complete recovery of the biomarkers studied was not observed. As suggested in the above-mentioned studies, a longer depuration period may be needed for a full recovery from pollutants-induced adverse biological changes. Therefore, in the present study, the recovery period with frequent sampling was extended up to 28 days.

In the present study, the rainbow trout, *Oncorhynchus mykiss*, was chosen as a laboratory fish model because of its economic importance worldwide and its use as a bioindicator species in ecotoxicological studies due to its susceptibility to various environmental pollutants (Makaras et al., 2020a, 2020b; Saibu et al., 2018; Stankevičiūtė et al., 2016; Niyogi et al., 2015; Heydarnejad et al., 2013). *Oncorhynchus mykiss* as a fish model has been widely used in ecotoxicological studies of environmental pollutants (Stankevičiūtė et al., 2016) for the following advantages: low rearing costs, suitability for sensitive early life-stage bioassay tests, sensitivity to many classes of pollutants, and importance for commercial farming in cold waters (Elmore and Boorman, 2013; Ahmed and Ahmad, 2020). Moreover, fish models are becoming increasingly accepted in toxicological studies of environmental chemicals, given the extensive homology between fish and human genomes (Elmore and Boorman, 2013). To represent environmentally relevant multiple chemical stressors, the mixture of Zn, Cu, Ni, Cr, Pb and Cd at the concentration corresponding to Maximum-Permissible-Concentrations (MPC) acceptable for the EU inland waters (EC, 2000, 2008; EU, 2013) was used. The MPCs for Cr (0.01 mg/L), Cu (0.01 mg/L) and Zn (0.1 mg/L) accepted for the inland waters remain unchanged. However, the MPCs for other metals such as Ni (from 0.01 (EC, 2000) to 0.034 mg/L (EU, 2013)), Pb (from 0.005 (EC, 2000) to 0.014 mg/L (EU, 2013)) and Cd (from 0.005 (EC, 2000) to 0.0015 mg/L (EC, 2008; EU, 2013) (category 5 of the hardness class of the)) have been changed. In the present paper, we sought to find out whether the above-mentioned changes in the MPCs of metals (Ni, Pb, Cd) in the test mixture could bring about marked changes in biomarker responses in salmonids and produce biological effects different from those previously determined in *S. salar*, *R. rutilus* and *P. fluviatilis* exposed to the mixture of the same metals but at lower MPCs of Ni and Pb. In addition, this study investigated whether the toxicity tendency (higher toxicity of the mixture containing Cr and Cu at 10 times reduced MPCs reported in previous studies (Sauliutė et al., 2020; Stankevičiūtė et al., 2018)) can be maintained by changing the concentration of other metals in the mixture. In addition, the depuration potential and dynamics in *O. mykiss* under environmentally realistic exposure scenario was assessed. Therefore, the main aim of the present study was to determine the biological consequences of multimetal mixture exposure as a multiple chemical stressor

on fish at molecular, cellular, physiological and whole-organism levels and alterations in biomarker responses during the depuration period.

2. Material and methods

2.1. Fish maintenance

All applicable international, national, and institutional guidelines for the care and use of animals were followed (EU, 2010; Permission No: LT 61–13–005). Rainbow trout (*Oncorhynchus mykiss* Walbaum 1792) at the embryonic stage were purchased from the Simnas hatchery (Lithuania). The test fish were selected from a population of a single stock, from the same spawning. After transportation to the laboratory, the eggs were placed into incubators (43 × 51 × 15 cm) made of perforated (2 mm in diameter) stainless steel. The present study was performed on *O. mykiss* juveniles (age 0+, initial mean total weight and standard deviation (SD) 11.69 ± 3.67 g, initial mean standard length and SD 100.2 ± 9.8 mm; $N = 360$). Before experimentation, the fish were kept under the same water quality and illumination conditions as those in the test. The fish were kept in flow-through 1 m^3 capacity tanks filled with aerated deep-well water (minimum flow rate 1 L/1 g of their wet body mass per day) at densities of 35 kg m^{-3} and were fed on commercial trout feed every morning (Aller Platinum), the total amount of the daily feed intake being not <1% of their wet body mass. Fish-holding conditions represented their optimal environment: ~12 °C, pH ~8.1, natural light-dark cycle, and the concentration of dissolved oxygen ~9.6 mg O_2/L (OECD, 2019).

2.2. Materials

The certified standard single-element metal (Pb^{2+}, Cu^{2+}, Cd^{2+} and Ni^{2+} 1000 mg·L^{-1} in HNO_3 0.5 mol·L^{-1} (Certipur®)) solutions were purchased from Merck (Germany), zinc (Zn^{2+} 10,000 mg·L^{-1} in 5% HNO_3 (Roti®Star)) solution from Roth (Germany) and potassium dichromate ($K_2Cr_2O_7$, CAS No. 7778–50–9) from Sigma-Aldrich (Germany). All chemical reagents were of analytical grade unless otherwise mentioned.

2.3. Preparation of metal mixture solutions

The certified standard single-element metal solutions (Pb^{2+}, Cu^{2+}, Cd^{2+} and Ni^{2+} 1000 mg/L in HNO_3 0.5 mol/L (Certipur®), and Zn^{2+} 10,000 mg/L in 5% HNO_3 (Roti® Star), respectively) were used for the metal mixture solution preparation. For Cr, stock solutions of 1000 mg/L were prepared by dissolving analytical grade $K_2Cr_2O_7$ (Sigma-Aldrich) in distilled water. A certain volume of each individual metal (Zn, Cu, Ni, Pb, Cd and Cr, respectively) from the stock solution was diluted in 1000 mL of distilled water using a volumetric flask with 0.1 M nitric acid. We tested the influence of water hardness on real metal ions concentrations experimentally, and, taking into account water hardness, prepared the metal mixture solution in which the concentration of each metal was 1.5–3 times higher than its nominal value. The stock solution was prepared in a HDPE bottle a day prior to test initiation to allow for chemical equilibration.

2.4. Deep-well water

The main physico-chemical parameters of the deep-well water, metals and their test waterborne concentrations (mg/L) used in the experiments are presented in Table 1. The data indicate that concentrations of metals (Cr, Ni, Pb and Cd) in the deep-well water used for dilution were below the device detection limit. Water temperature, dissolved O_2, pH and conductivity were measured routinely with a hand-held multi-metre (WTW Multi 340i/SET, Germany). Deep-well water was analysed for a number of standard physical and chemical properties according to standard analytical methods (ISO, 1988, 1990, 1994, 1998, 2003, 2007, 2008). Measurements of the concentration of metal ions were performed using an atomic absorption spectrophotometer Varian's SpectrAA 55, USA.

2.5. Experimental protocols

The experiments were conducted under semi-static rotating water-current conditions in polyethylene (PE) 80-L total volume tanks filled with 70 L of continuously aerated deep-well water. The test solutions (treatment group) and clean water (control group) were refreshed every day. Before transfer, the fish were fed. The test fish were transferred into other tanks with the same amount of freshly-produced solutions. The empty tanks were washed.

In the course of this study, metal mixture exposure and post-treatment recovery experiments were conducted. During the metal exposure experiment, *O. mykiss* individuals were exposed to the mixture (further referred to as MIX) of six metals (Zn – 0.1, Cu – 0.01, Ni – 0.034, Cr – 0.01, Pb – 0.014 and Cd – 0.0015 mg/L) at concentrations corresponding to MPC for inland waters in EU (Directive 2000/60/EB, 2000; EC, 2008; Directive 2013/39/EB, 2000). In the other two tests, the fish were exposed to the multimetal mixture, in which the concentration of Cr or that of Cu was 10-fold lower than its respective MPC, the concentrations of the other 5 metals remaining unchanged (e.g. Cr↓ (the metal whose concentration in MIX was reduced)), and those of Cd, Zn, Cu, Ni and Pb remaining constant (further referred to as Cr↓ or Cu↓). The treated fish were sampled after 1, 4, 7 and 14 days. During the recovery experiment, which was conducted after 14 days of exposure to metal mixtures (MIX, Cr↓ and Cu↓), *O. mykiss* specimens were transferred to

Table 1
The physico-chemical parameters of the deep-well water used in the experiments, metals and their waterborne concentrations (mg/L) in test media.

Chemical and physical characteristics of deep-well water							
Metal (μg/L)		Cations (mg/L)		Anions (mg/L)		Other analytes	
Mn	6	Na^+	5.2	Cl^-	4.4	pH	8.1 ± 0.15
Zn	12.8	K^+	3.8	SO_4^{2-}	23.1	Temperature	12 ± 0.5 °C
Cu	4	Ca^{2+}	68.0	HCO_3^-	203	Dissolved O_2	9.5 ± 0.25 mg/L
Cr	< 1	Mg^{2+}	15.0	CO_3^-	1.03		
Ni	< 2	Fe^{2+}	0.1	NO_2^-	< 0.05		
Pb	< 1	Fe^{3+}	< 0.01	NO_3^-	< 0.05		
Cd	< 0.3	Fe_{total}	0.1				
		NH_4^+	0.13				
Metals and their test waterborne concentrations (mg/L) in test media							
Metal	MIX (MPC)	MIX Measured		Cr↓ Measured		Cu↓ Measured	
Zn	0.1	0.115		0.091		0.110	
Cu	0.01	0.0087		0.0087		0.0025	
Ni	0.034	0.029		0.029		0.029	
Cr^{total}	0.01	0.008		0.001		0.008	
Pb	0.014	0.0091		0.0091		0.011	
Cd	0.0015	0.0013		0.0013		0.0013	

aquariums with clean (deep-well) water. Fish were sampled after 1, 4, 7, 14 and 28 days of recovery.

2.6. Analysis of metal bioaccumulation in gills

All the chemicals used were of analytical reagent grade, solutions were prepared in the deionized water. Calibration standards of each metal were prepared by appropriately diluting stock solutions of 1000 ppm standards (*Merck*). Special care was taken to prevent contamination of the samples with metal: all laboratory glassware was soaked in 2 M HNO_3 for 48 h and rinsed several times with deionized water prior to use. For metal analysis, an adapted procedure based on Malik et al. (2009), Sekhar et al. (2004) and Aucoin et al. (1999) was applied. The frozen fish specimens were dissected using stainless steel instruments. 0.2–1 g of gill samples were used for the analysis. The samples were digested with 10 mL ultra pure nitric acid (*Sigma Aldrich*) at 100 °C until solution became clear (around 90 min). Subsequently, 3 mL of 30% H_2O_2 (*Sigma Aldrich*) was added and the solution was kept at 100 °C for 120 min. Afterwards, the volume of the solution was reduced to approx. 2 mL by evaporating the acid at 80 °C. The cooled digested solutions were filtered through a Whatman filter into 15 mL volumetric flasks and made up to volume using deionized water. Blank samples were prepared following the same procedure. The as-prepared samples were analysed for metals using ICP-OES Perkin Elmer Optima 7000 DV. In order to maintain the quality assurance, the quality control sample was run every ten samples, and necessary corrections and re-sloping were made in the standard curve using freshly run standards. Reagent blanks were analysed to provide a baseline correction. No metals were measured in any of the blank samples analysed (1 blank for each batch of 10 samples). For metal bioaccumulation, *O. mykiss* gills were sampled after 14 days of treatment with MIX and after 7, 14 and 28 days of recovery. Metal concentrations in gill tissues were expressed in micrograms per gram of wet weight (ww).

2.7. Comet assay

The alkaline comet assay was performed in erythrocytes of *O. mykiss* peripheral blood following the procedure of Singh et al. (1988) with some modifications (Fatima et al., 2014). A suspension of peripheral blood cells from fish individuals was prepared in Dulbecco's Phosphate Buffered Saline. Cell viability was measured by the method of trypan blue exclusion (Anderson and Wild, 1994). Only cell suspensions with viability >80% were used. The slides were stained with ethidium bromide, placed under a glass cover and analysed by fluorescence microscopy (Olympus BX51 microscope with an Olympus U-RFL-T fluorescent burner, Tokyo, Japan). The photos were taken with an Olympus U-CMAD3 (Tokyo, Japan) camera. Fifty (50) nuclei of each individual were scored randomly and captured at 40× magnification. Images were analysed and the percentage of DNA in the tail (% Tail DNA) were assessed using the Comet assay IV image analysis software (version 4.2).

2.8. Analysis of nuclear abnormalities (NAs) in an in vivo assay

Blood samples were obtained by puncturing the caudal vein with a 25 G needle attached to a 1 mL sterile syringe (B. Braun, Germany) using 3.8% sodium citrate as an anticoagulant. A drop of blood was directly smeared on microscopic slides and air-dried. The stained slides were analysed under bright-field Olympus BX51 microscopes (Tokyo, Japan) using an immersion objective (1000×). 4000 erythrocytes with intact cellular and nuclear membranes per fish were evaluated by blind scoring. The final results were expressed as the mean value (‰) of the sums of the analysed individual lesions scored in 1000 cells per organism sampled from every study group. The formation of micronuclei (MN), nuclear buds (NB), nuclear buds on filament (NBf) were assessed as genotoxicity endpoints, and 8-shaped nuclei, fragmented apoptotic (FA) and binucleated (BN) cells as cytotoxicity endpoints. Total genotoxicity (ΣGentox) levels were assessed as the sum of the frequencies of the detected genotoxicity (MN + NB + NBf) endpoints. Nuclear abnormalities were identified using the criteria described by Heddle et al. (1991), Fenech et al. (2003), and Baršienė et al. (2014).

2.9. Haematological analyses

Blood samples from fish were taken from the caudal vein with a disposable syringe (1 mL volume) by drawing approximately 0.1 mL of blood and then transferred to blood collection tubes (washed with 3.8% sodium citrate). Concentrations of glucose in fish blood were determined using the automatic Glucose Analyser (EKSAN-Gm, Analita, Joint-Stock Company Ltd, Lithuania). The minimum detection limit of the blood glucose method is from 2 to 30 mmol/L and the error for repeated measurements (precision) is ≤5%. Minimal blood sample volume per measurement is 50 μL. Haematocrit level (Hct, l/l) was determined using a routine method (Svobodova et al., 1991). The blood-filled heparinized capillary tubes were centrifuged for 5 min at 100,000 rpm (Microhematocrit Centrifuge), after which, haematocrit was determined. In the present study, glucose and haematocrit levels were measured during all periods of blood sampling from fish of both exposure and recovery groups.

2.10. Behavioural assay

The behavioural test was performed in order to collect data on the locomotor activity of the test fish species during 14 exposure days and 14 days of recovery after exposure. On the day of the experiment, fish individuals ($N = 15$) were randomly taken from the holding 80-L tanks and placed into experimental 210 × 310 × 130 mm-sized, approximately 5 L, 5 mm thick glass aquariums. Sections were filled with 3 L of continuously aerated deep-well water containing the necessary concentration of metals in tested mixtures (see Table 1), with an air stone separated from the main testing area by a baffle (1.5 mm mesh size). This experiment was performed in static water conditions. Each aquarium section was covered with a translucent polymeric glass to prevent fish from jumping out of the aquarium. Water temperature and aeration were constant (Table 1). Fish were allowed to acclimate to the new experimental tanks for 2 h. After acclimation, fish locomotor activity endpoints were recorded for 1 min at selected time intervals (every 10 min) for a 1-h period using a digital video camera (PANASONIC HC-V770 Wi-Fi, 12.76 MP). The video camera was positioned 3 m above the test aquarium. The researcher was absent from the experimental space during filming to avoid disturbing the fish. The tracked video data were processed using the ETHOVISION XT 12 (Noldus Technologies, Inc., The Netherlands) software.

2.11. Measurement of supporting metrics for fish: CF, GSI, LIS and KSI

Condition factor (CF), liver-somatic (LSI), gills-somatic (GSI) and kidneys-somatic (KSI) indexes were computed using the following formulas (ICES, 2011): CF=[[body mass(g) / (total length)^3] *100]; LSI = [LW / (BW − LW) *100]; GSI = [GW / (BW − GW) * 100]; KSI = [KW / (BW − KW) * 100]; where LW − liver mass (g), GW − gills mass (g), KW − kidneys mass (g), and BW − body weight (g).

2.12. Data analysis and statistics

All the data obtained were tested for normality using Kolmogorov-Smirnov's and Shapiro-Wilk's tests and homogeneity of variance was tested applying Levene's and Bartlett's tests. When the data met ANOVA assumptions, multiple-comparison tests (two-way ANOVA, Tukey post hoc test) were conducted to reveal differences among the experimental groups. In the case of deviation from normality, the data were transformed (square root) and re-analysed. A two-way ANOVA test was used to analyse the locomotor activity data and to determine the interaction

between treatment and exposure duration. A one-way ANOVA was applied to test statistically significant differences between the control and treatment groups at the same time points. All ANOVAs were followed by Tukey's HSD post-hoc tests, which were conducted to identify significant differences among the groups. The significance threshold of $p < 0.05$ was specified for all statistical comparisons a priori. All the data obtained were analysed using the STATISTICA 10.0 Software, Inc. PA, USA. Statistical analysis graphs were drawn using the GraphPad Prism version 8.0.0 Software, San Diego, California, USA.

3. Results

3.1. General observations

To measure changes over time, CF and body somatic indices (LSI, KSI and GSI) of all fish specimens were recorded before the trial, during each treatment and recovery period. The comparison of CF and body somatic indices within and among treatments over time revealed slightly increased CF, LSI and GSI in all treatments, except for the treatment with Cr↓, in the case of which, these indices remained decreased both after exposure and recovery periods. KSI showed a slight increase after exposure and recovery periods in all treatments. CF and all somatic indices did not show significant ($p > 0.05$) variation among and within treatments over time. No mortality was recorded during the experimental period.

3.2. Bioaccumulation of metals

The concentrations of metals in fish gills after the 14-day exposure to MIX are presented in Fig. 1. The obtained results show that concentrations of all the six metals contained in the mixture (MIX) increased in the MIX-treated fish compared to those of the control group. Statistically significant differences were observed in the accumulated level of Cr ($F_{1.18} = 13.014$, $p = 0.002$), Ni ($F_{1.16} = 8.920$, $p = 0.009$) and Cd ($F_{1.18} = 92.917$, $p < 0.001$).

3.3. Depuration of metals

Percentage changes in the accumulated amounts of Zn, Cr, Cu, Ni, Cd and Pb in the gills over recovery periods relative to control group values and relative to those of the MIX treatment group are presented in Fig. 2. The mean values of Zn, Cr, Ni and Pb measured after 7, 14 and 28 days of recovery did not differ significantly from the control ones. However, during all recovery periods, the accumulated Cd amount remained significantly higher ($H = 23.36$, d.f. 3, $p < 0.001$) than the control one. The level of Cu in the gills was not significantly affected as a result of 14-day MIX treatment, however, on the 28th day of recovery, it was found significantly ($F_{3.36} = 4.028$, $p = 0.014$) decreased (the mean percentage change approximated 20%) in comparison to the control one. Cr and Ni amounts were found significantly elevated after 14 days of MIX treatment. However, after the 14- and 28-day recovery periods, their levels almost reached control values. Such results suggest a faster elimination of these metals from *O. mykiss* gills. In summary, percentage changes in metal concentrations demonstrate an insignificant increase in the accumulated Zn, Ni, Cr, Cu and Pb amounts during the 7- and/or 14-day recovery periods and a decrease at the end of recovery (28 day).

During recovery periods, the concentrations of accumulated metals showed a tendency to decrease with increasing recovery period relative to the 14-day MIX treatment. The levels of Cd, Ni and Cr, which were significantly affected by MIX treatment, showed decreasing concentrations in the gills during recovery periods. On the 28th day of the recovery period, Cd concentration decreased by approximately 40%, but this change was not statistically significant ($H = 6.440$, d.f. 3, all $p > 0.092$). Meanwhile, significant decreases were detected in Cr ($H = 23.93$, d.f. 3, $p < 0.001$) and Ni ($H = 9.728$, d.f. 3, $p = 0.021$) levels, which dropped approximately by 70% compared to the levels measured after MIX treatment. As well as in relation to control values, the percentage change of Cu in the gills was not significantly affected by the 14-day MIX treatment. However, on the 28th day of recovery, the amount of Cu decreased approximately by 40% and this change was significant ($F_{3.36} = 4.028$, $p = 0.014$).

In summary, significantly elevated concentrations of Cr and Ni in MIX-treated *O. mykiss* gills reached control values after 14 and 28 days of recovery, respectively, indicating that the selected depuration period was sufficient for the elimination of these metals from the gills. Although Cd concentration in the gills declined during the 28 day-long depuration period, a significant recovery was not achieved. Moreover, the depuration period resulted in a significantly reduced concentration of Cu

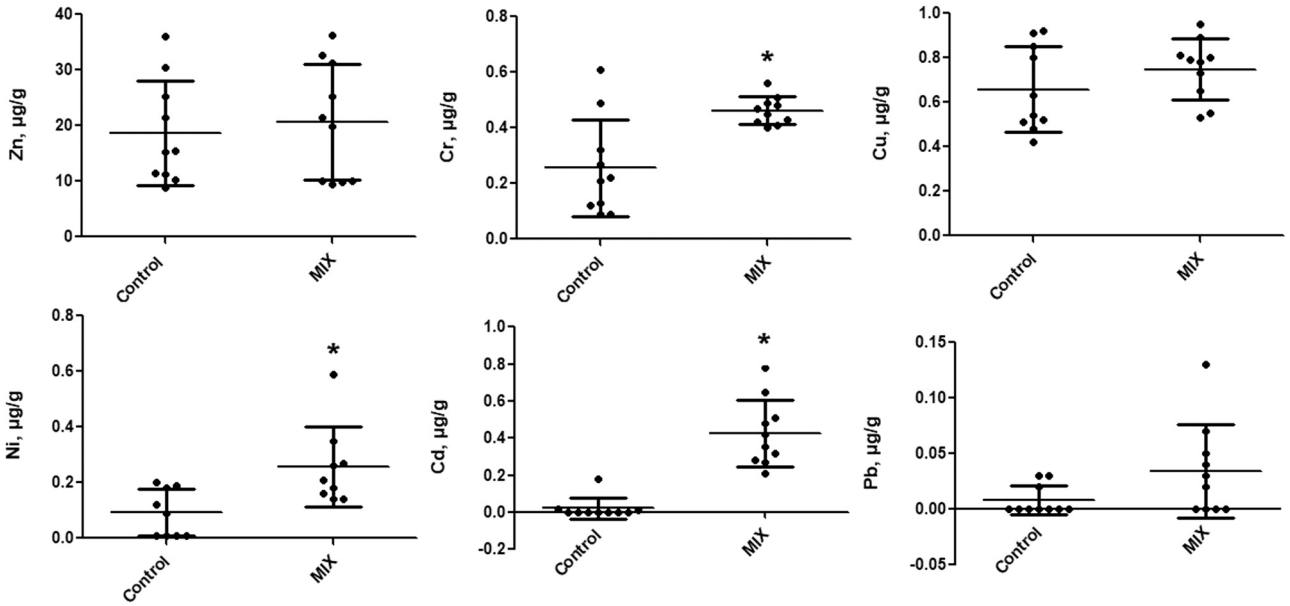

Fig. 1. The mean concentration (µg/g) of metals in the gills (mean ± SD, $N = 9$–10) of the control group fish and in those exposed to metal mixture (MIX) for 14-days. MIX solution was prepared in accordance with the maximum-permissible-concentrations (MPC) of Pb, Zn, Cu, Cd, Ni and Cr set for the inland waters in EU (EC, 2008). Asterisks (*) denote significant differences from the control group ($p < 0.05$).

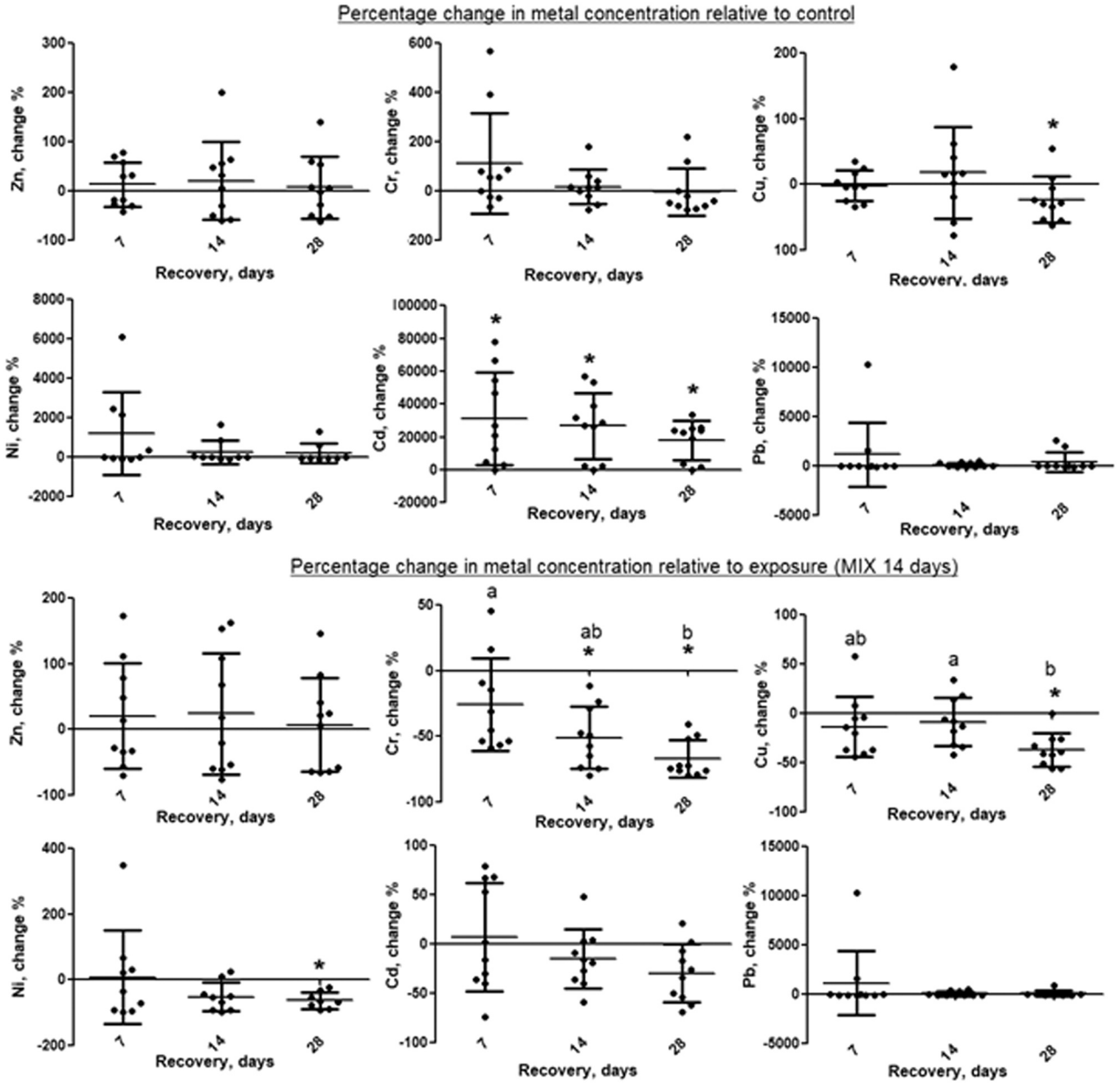

Fig. 2. The relative percentage change in metal concentrations in the gills of *O. mykiss* (mean ± SD, $N = 8–10$) after 7-, 14- and 28-days of recovery in clean water. Asterisks (*) denote significant differences from control or MIX groups ($p < 0.05$). Letters denote significant differences among recovery groups ($p < 0.05$). MIX solution was prepared in accordance with the maximum-permissible-concentrations (MPC) of Pb, Zn, Cu, Cd, Ni and Cr set for the inland waters in EU (EC, 2008).

compared to the control value.

3.4. Comet assay

Results of the comet assay are shown in Fig. 3A. The DNA damage caused by metal mixtures (MIX, Cr↓ and Cu↓) was assessed based on the DNA percentage in the tail. Mixtures with reduced Cr and Cu concentrations were found to induce the heaviest DNA damage during the exposure period. The percentage of DNA in the tail increased with increasing duration of MIX and Cr↓ exposure reaching the highest values (15.08 ± 3.56 and 18.61 ± 4.18, respectively) after 14 days of treatment, while in the case of Cu↓ treatment, the maximum mean value (16.89 ± 3.80) of tail DNA was recorded after 7 days of exposure. There were significant differences found among and within treatments over time ($F_{9,14} = 17.855$, $p < 0.001$). A significantly heavier DNA damage was detected after 4 days of treatment with Cr↓ and Cu↓ ($p = 0.037$ and $p < 0.001$, respectively), after 7 days of treatment with Cu↓, while after 14 days, all treatments were found to induce a significant (all $p < 0.001$) DNA damage compared to the control value. In addition, it was found that different treatments induced a different extent of damage, e.g. after 4- and 7-days of exposure, the DNA damage induced by exposure to Cu↓ was significantly heavier than that induced by other treatments and significantly lower than that caused by Cr↓ at the end of the exposure period.

Changes in DNA damage during the recovery period are presented in Fig. 3A. The first significant depuration did not occur until day 14. During the recovery period, significant differences among treatments became apparent over time ($F_{15,21} = 9.799$, $p < 0.001$). The DNA damage inflicted by MIX treatment was found to decrease relatively steadily with increasing recovery time. However, a significant decrease in the tail DNA percentage compared to that in control and 14 day-exposure groups was observed in all exposure groups only at the end

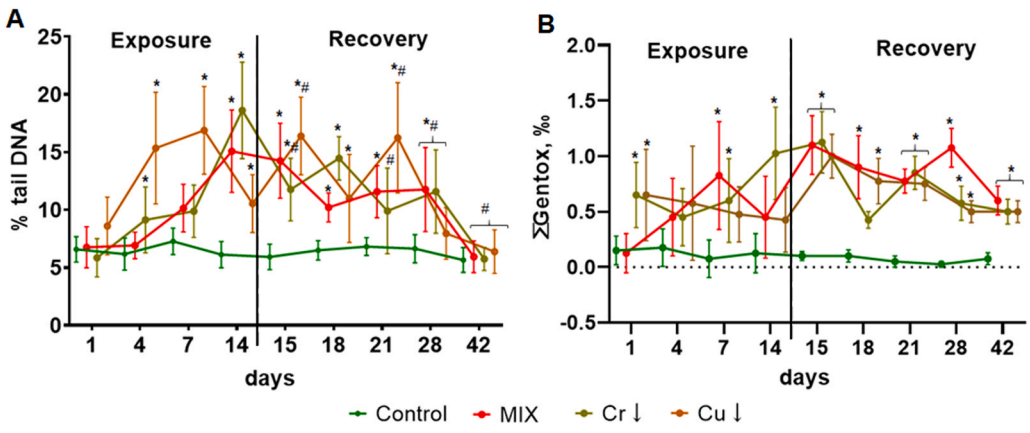

Fig. 3. The extent of the DNA damage (A) determined by the Comet assay (percentage of tail DNA, mean ± SD, $N = 10$) and the level of total genotoxicity (B) (∑Gentox, mean ± SEM, $N = 10$) in peripheral blood erythrocytes of the fish after 1-, 4-, 7- and 14-days of exposure to metal mixtures and after 1-, 4-, 7-, 14- and 28-days of recovery in clean water. MIX solution was prepared in accordance with the maximum-permissible-concentrations (MPC) of Pb, Zn, Cu, Cd, Ni and Cr set for the inland waters in EU (EC, 2008). Arrows (↓) indicate a 10-fold decrease in the nominal concentration of Cr^{6+} and Cu^{2+} in the metal mixture compared to MIX. Asterisk (*) indicates values significantly different ($p < 0.05$) from the control ones, while "#" indicates values significantly different ($p < 0.05$) from those recorded on the14th day of exposure.

of the recovery period. The process of recovery after Cr↓ and Cu↓ treatments was less consistent than that after MIX treatment, i.e. during the 28-day recovery period, there were both significant increases and decreases in DNA damage observed. In the case of Cu↓ treatment, DNA damage recovery over time proved to be the most uneven. However, in this group, recovery was faster, with a significant reduction in DNA damage observed as early as the 14th day of the recovery period. Complete recovery of DNA damage was observed on the 28th day, when the percentage of tail DNA did not differ significantly among all treatments and the control group. At the end of the recovery period, the percentage of tail DNA in the MIX-treated *O. mykiss* specimens decreased approximately by 60%, in the Cr↓-treated fish by 70%, and in the specimens treated with Cu↓ by 40% compared to the 14 day-treatment values.

3.5. Analysis of nuclear abnormalities (NAs) in an in vivo assay

Metal mixture-induced increases in total genotoxicity (ΣGentox) levels in *O. mykiss* peripheral blood erythrocytes are presented in Fig. 3B. No clear tendency on the part of the genotoxic effect was revealed. A significant ($F_{9.14} = 4.472$, $p < 0.001$) genotoxic effect was recorded on the first day (after Cr↓ and Cu↓ treatments, both $p = 0.046$), the 7th day (after MIX ($p < 0.001$) and Cr↓ ($p = 0.026$) treatments), and after 14 days (after Cr↓ ($p < 0.001$) treatment) of exposure compared to the control. Cytotoxicity endpoints were not significantly affected by metal mixture treatments.

In the course of the recovery period, total genotoxicity in all treatment groups was observed to slightly increase or decrease compared to the 14-day exposure level (Fig. 3B). There were no significant ($F_{15.22} = 1.693$, $p = 0.054$) differences observed in total genotoxicity levels within and among treatments over time during the recovery period. At the end of the recovery period, the MIX-, Cr↓- and Cu↓-induced total genotoxicity level was found to be significantly (all $p < 0.049$) higher than the control one.

3.6. Haematological analyses

Metal mixtures were not found to induce a significant ($F_{9.14} = 1.789$, $p = 0.079$) decrease in *O. mykiss* blood glucose level during 1-, 4-, 7- and 14-day exposure, except for the 14-day exposure to Cr↓ ($p = 0.014$) (Fig. 4A). During the 28-day-recovery period, significant changes in glucose level were not observed either ($F_{15.17} = 2.925$, $p < 0.001$), except for a significant ($p = 0.002$) increase in blood glucose in the MIX-treated fish on the 1st day of the recovery period.

The level of haematocrit in control fish did not ($F_{9.14} = 1.92$, $p = 0.056$) differ significantly from that in the metal-treated fish throughout the exposure period, yet there was a significantly higher haematocrit level recorded in the Cr↓ mixture-treated fish on the 1st ($p = 0.035$) and on the 4th days ($p = 0.003$) compared to the control (Fig. 4B). During the recovery period, significant ($F_{15.17} = 1.77$, $p = 0.043$) changes in haematocrit level in the metal mixture-treated fish were not observed either, except for a significant ($p = 0.006$) decrease in the Cu↓-treated fish on the 4th day and a significant increase in the MIX-treated *O. mykiss* specimens on the first day.

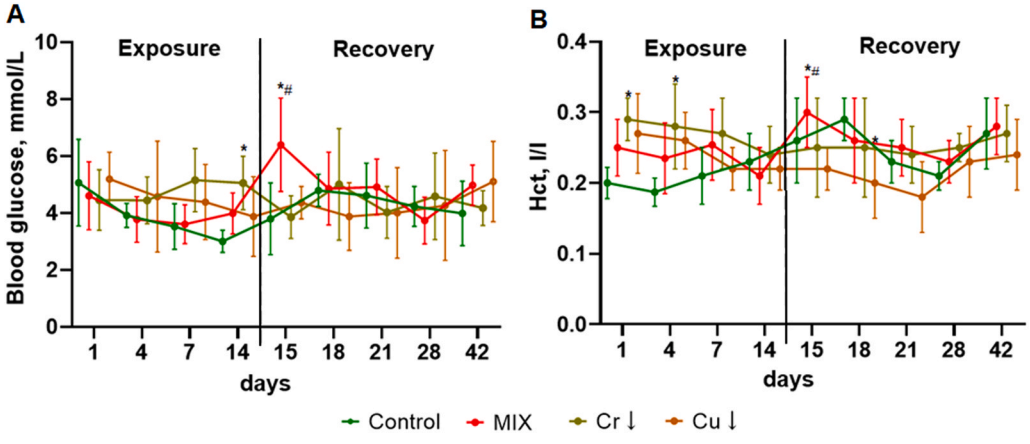

Fig. 4. Blood glucose concentration (A) and hematocrit (B) level in fish blood (mean ± SD, $N = 10$) after 1-, 4-, 7- and 14-day exposure to metal mixtures and after 1-, 4-, 7-, 14- and 28-day recovery in clean water. MIX solution was prepared in accordance with the maximum-permissible-concentrations (MPC) of Pb, Zn, Cu, Cd, Ni and Cr set for the inland waters in EU (EC, 2008). Arrows (↓) indicate a 10-fold decrease in the nominal concentration of Cr^{6+} and Cu^{2+} in the metal mixture compared to MIX. Asterisk (*) indicates values significantly different ($p < 0.05$) from the control ones, while "#" indicates values significantly different ($p < 0.05$) from those recorded on the14th day of exposure.

3.7. Behavioural responses

During the 14-day period of exposure to different concentrations of metal mixtures (MIX, Cr↓ and Cu↓), the locomotor activity of fish juveniles showed clear differences from that of the control group (Fig. 5), i.e. the average velocity was decreased (Fig. 5A), while the angular velocity, on the contrary, was increased (Fig. 5B). Throughout the recovery period, fish locomotor activity used to slightly increase with increasing exposure duration. The average and angular velocity data were found to differ significantly among different treatments (Fig. 6). The average velocity of the fish exposed to MIX ($p < 0.001$), Cr↓ ($p = 0.008$) and Cu↓ ($p = 0.015$) was significantly lower than that of the control group, while no significant differences were found in the average velocity of the fish exposed to different metal mixtures (Fig. 6A). As for the angular velocity, only that of the MIX-exposed fish was found to be significantly different from that of the control ($p < 0.001$) and from that of the Cu↓-exposed fish ($p = 0.022$) (Fig. 6B). However, the performed data analysis of the main effect produced by the interaction between treatment and exposure duration revealed no statistically significant differences for these endpoints (two-way ANOVA, $p > 0.05$) (see Table 2).

In comparison with the control period at the same time points, exposure to MIX ($p = 0.013$), Cu↓ ($p = 0.028$) and Cr↓ ($p = 0.031$) mixtures was found to induce a significant decrease in the average velocity of the fish after 7 days of exposure, the most dramatic decrease being established after 14 days of exposure to MIX ($p = 0.034$) and Cr↓ ($p = 0.015$) (one-way ANOVA, $p < 0.05$) (Fig. 6A). Meanwhile, only one significant difference ($p = 0.045$) was observed between the angular velocity of the MIX-exposed fish and the control level after 21 days of the recovery period (Fig. 5B).

4. Discussion

To our knowledge, the present study is one of the first to thoroughly investigate the accumulation and depuration of Zn, Cu, Cr, Ni, Cd and Pb from the gills tissues of *O. mykiss* treated with the multimetal mixture at environmentally relevant concentrations (MPC), and to evaluate a wide variety of the biomarker responses elicited during exposure and depuration periods. In the present study, *O. mykiss* specimens were exposed to a mixture of Zn, Cu, Ni, Cr, Cd and Pb at MPC accepted for the EU inland water, which resulted in a significant accumulation of Cd, Cr and Ni in the gill tissue. Fish gills are recommended as indicators of environmental pollution more often than other fish organs (Rajeshkumar and Li, 2018), which is because of their heightened sensitivity to contaminants resulting from their direct exposure to the external environment (Arini et al., 2011). Similar results were obtained in our previous studies (Sauliutė et al., 2020; Stankevičiūtė et al., 2017, 2018), where significantly increased amounts of Cd, Cr and Ni were detected in the gills of *S. salar*, *R. rutilus* and *P. fluviatilis* exposed to the mixture of the same metals, but at different MPCs of Cd, Ni and Pb. Our results are in agreement with those of our previous studies, which have shown that in the gills of the MIX-treated fish, Cd concentration increased the most followed by those of Cr or Ni. In our previous studies, significantly increased concentrations of metals were found in the gills of the following fishes: Cu in *P. fluviatilis*, Zn, Pb in *R. rutilus,* and Pb in *S. salar*. However, significant increases in concentrations of these metals were not observed in the gills of *O. mykiss*. In the present study, metal accumulation in the gills of the fish treated with Cr↓ and Cu↓ mixtures was not measured. However, in the previous studies, exposure to these mixtures was found to result in significantly elevated amounts of Cd in the gills of the mixture-exposed fish. The amount of Cd detected in the gills of *P. fluviatilis* and *R. rutilus* treated with Cr↓ mixture was significantly higher than that in the fish treated with MIX mixture (Sauliutė et al., 2020). A significantly higher amount of Cd was recorded in the gills of *P. fluviatilis* after the treatment with Cu↓ mixture as well. Exposure to Cu↓and Cr↓ mixtures was found to induce a lower accumulation of Cr, Cu and Ni in the gills of all the tested fish species (*S. salar, P. fluviatilis, R. rutilus*) than exposure to MIX. After treatment with Cu↓ and Cr↓ mixtures, Ni accumulation in the gills and kidneys of all the tested fish species significantly decreased. Moreover, treatments with Cu↓ and Cr↓ mixtures induced a significantly lower accumulation of Ni in the muscle tissue of *R. rutilus* and *P. fluviatilis* than that induced by the treatment with MIX (Sauliutė et al., 2020).

The present study has revealed that the duration and patterns of Cd, Ni and Cr depuration from the gills of *O. mykiss* differ. The post-exposure 28-day recovery resulted in a significant depuration of accumulated Cr and Ni amounts from the gills of the exposed fish. The results of the study by Palermo et al. (2015) showed that Ni interferes in gill antioxidant defense, indicating protection of the gills tissue and elimination of toxic compounds. In addition, their study revealed that the maximum permissible Ni concentration (25 μg/L) in Brazilian natural fresh water is unsafe for the neotropical fish *Prochilodus lineatus*. Chen et al. (2018) evaluated patterns of hexavalent Cr depuration from the *Oryzias latipes* juveniles treated with 0.5 and 8 mg/L. Initially, the process of Cr accumulation was rapid, but then slowed down and continued at a slower rate until the end of the exposure period showing no signs of the reached steady-state. The initially rapid Cr accumulation was accompanied by increased lipid peroxidation and elevated activities of antioxidants. During the 14-day recovery period, depuration of both exposure concentrations of Cr reached 50%. The initial Cr loss in *O. latipes* juveniles was similar to the 40% loss from liver, gill and intestine after 4 days of depuration in the *O. latipes* adults exposed to 4 mg/L Cr (VI) (Chen et al., 2016). This result is in agreement with that obtained in our study, where Cr concentration decreased by

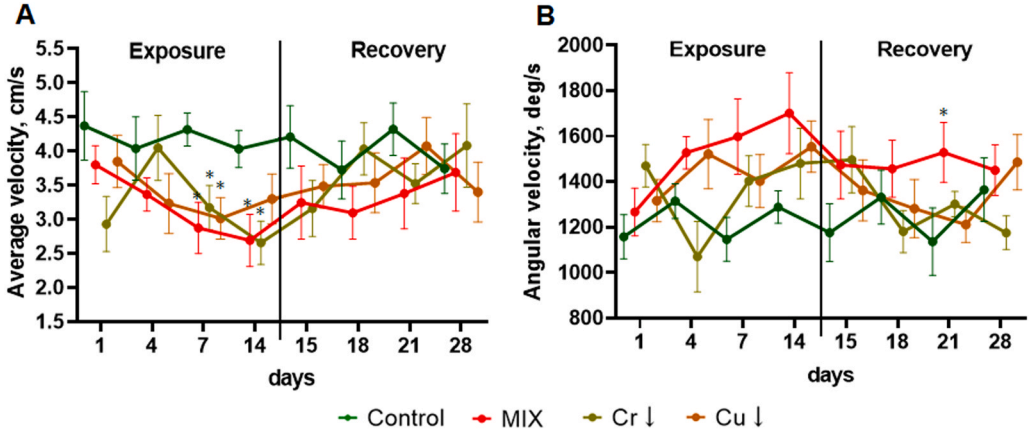

Fig. 5. Average velocity (cm/s) (A) and angular velocity (deg/s) (B) (mean ± SEM, $N = 15$) of rainbow trout juveniles (*Oncorhynchus mykiss*) after 1, 4, 7 and 14 days of exposure to metal mixtures and after 1, 4, 7, 14 days of recovery in clean water. MIX solution was prepared in accordance with the maximum-permissible-concentrations (MPC) of Pb, Zn, Cu, Cd, Ni and Cr set for the inland waters in EU (EC, 2008). Arrows (↓) indicate a 10-fold decrease in the nominal concentration of Cr^{6+} and Cu^{2+} in the metal mixture compared to MIX. Asterisks (*) denote significant differences between locomotor activity parameters of the control and treatment groups recorded at the same time points (one-way ANOVA, $p < 0.05$).

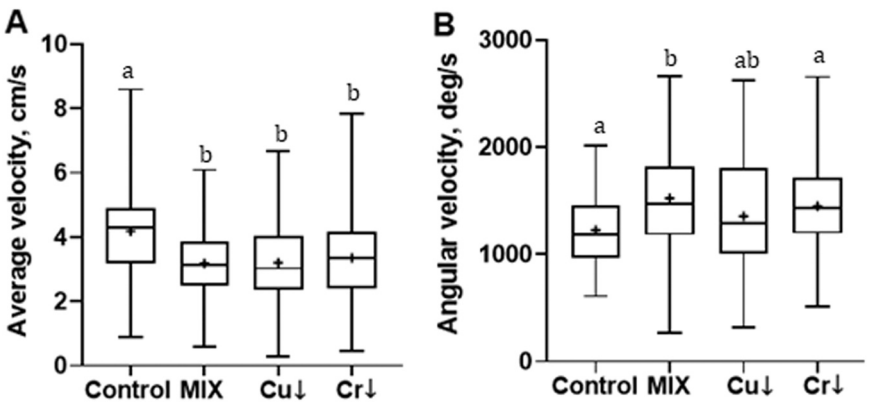

Fig. 6. Comparison of the average velocity (cm/s) (A) and angular velocity (deg/s) (B) (minimum, 25th percentile, median, 75th percentile and maximum values) of rainbow trout juveniles (*Oncorhynchus mykiss*) of different exposure groups (MIX, Cr↓ and Cu↓) after the overall 28-day exposure and recovery period. MIX solution was prepared in accordance with the maximum-permissible-concentrations (MPC) of Pb, Zn, Cu, Cd, Ni and Cr set for the inland waters in EU (EC, 2008). Arrows (↓) indicate a 10-fold decrease in the nominal concentration of Cr^{6+} and Cu^{2+} in the metal mixture compared to MIX. The means are indicated by plus signs. Letters denote significantly different groups (two-way ANOVA, $p < 0.05$).

Table 2
Analysis of the effects that test variables have on locomotor activity endpoints of *O. mykiss* juveniles during 14-d exposure to metal mixtures and after 14 d recovery in clean water using the data collected within 1-h observation time frames (two-way ANOVA).

Effect	Sum-of-squares	Degrees of freedom	Mean squares	F	P value
Average velocity, cm/s					
Intercept	6137.667	1	6137.667	2466.641	<0.001
Treatment	46.394	3	15.465	6.215	<0.001
Time (days)	20.518	7	2.931	1.178	0.314
Treatment × time (days)	45.826	21	2.182	0.877	0.622
Error	1114.745	448	2.488		
Angular velocity, deg/s[a]					
Intercept	634,047.5	1	634,047.5	15,622.02	<0.001
Treatment	791.1	3	263.7	6.50	<0.001
Time (days)	311.4	7	44.5	1.10	0.364
Treatment × time (days)	1022.4	21	48.7	1.20	0.246
Error	18,182.9	448	40.6		

[a] Square-root transformation was used for parametric statistical calculation.

approximately 50% on the 14th day of depuration compared to the level of Cr accumulated after MIX treatment. These results suggest that Cr depuration may be not significantly influenced by the presence of other metals in the gills tissue and that Cr elimination rate is not affected by single- or multiple-metal exposures. However, to confirm this hypothesis, further studies should be performed. As for Cd, during recovery, the accumulated amount of this metal decreased (by approximately 40%), but not significantly, indicating a slower Cd removal from the gill tissue.

Arini et al. (2015) revealed the recovery potential of the *Danio rerio* gill tissue after the 15-day treatment with Cd and Zn, singly or in combination. The 75-day depuration period proved to be insufficiently long for complete elimination of Cd (93.4% and 82.2% of the accumulated Cd amount were eliminated respectively from the gills of the fish exposed to Cd and Cd/Zn). Meanwhile, Zn was poorly accumulated, but quickly depurated. Cd depuration from the gills is likely to be associated with the transfer of this metal to the liver. Moreover, in the case of Cd contamination, genetic responses in the test fish were more pronounced, i.e. differentially expressed genes in the gills and liver were more strongly affected than in the case of fish exposure to zinc.

Cu accumulation in the MIX-exposed fish was not significantly affected; however, at the end of the recovery period, a significant decrease in Cu concentration was detected. The gills are known to play a key role in homeostasis, and branchial Cu excretion has been reported in previous studies (Kamunde et al., 2002; Grosell et al., 2001). The study conducted by Mansouri et al. (2016) was designed to assess the uptake (20 days) and depuration (10 days) of Cu after exposure to CuO nanoparticles (NPs) in the presence of TiO_2 NPs in the liver, intestine, muscle, and gills of *Cyprinus carpio*. During the depuration period, Cu concentrations decreased in all the examined tissues, which, in descending order of this metal concentration, are listed as follows: gill>intestine>muscle>liver. According to the authors, after the recovery period, there was no significant difference recorded between residual concentrations of Cu in most tissues and that of the control group. Thus, the concentration of Cu in *C. carpio* tissues returned to the near-baseline level in clean water, suggesting that longer time was required for complete elimination. In another study, Gündogdu et al. (2011) investigated Cu elimination from the tissues and organs of *O. mykiss* after dietary Cu exposure. The highest and the lowest Cu elimination from *O. mykiss* tissues was from the intestine and liver, respectively. The authors also reported that the elimination of Cu from gills and muscle tissues in fish of different concentration-exposure groups was higher than that from the liver tissue.

Metal treatment was not found to affect growth and somatic indices of *O. mykiss* juveniles; no significant changes were detected during the 28-day recovery period, either. No evidence of pathology or mortality was found in the fish groups exposed to metal mixtures. In previous studies, impaired growth of fish was not associated with exposure to low metal concentrations and resultant accumulation of metals (Kamunde and MacPhail, 2008; Farag et al., 2006). As reported by Stankevičiūtė et al. (2016), after a 12-day depuration period, there was a decrease determined in CF (condition factor) of *O. mykiss* exposed to the binary mixture of Cu and Zn at sublethal concentrations. In addition, such haematological parameters as haematocrit level and blood glucose concentration did not show any significant tendencies during the period of exposure, except for the exposure to Cr↓ mixture, where significantly increased Hct and blood glucose levels were observed after 1- and 14-day exposure, respectively. The assessed haematological parameters did not indicate any trend changes during the recovery of MIX-, Cr↓- and Cu↓- mixture-treated fish. Blood glucose as an indicator of stress was evaluated as inaccurate in studies by Makaras et al. (2020a, 2020b), Iversen et al. (2003), Pérez-Casanova et al. (2008) and Fast et al. (2008). Moreover, as reported in the study by Makaras et al. (2020a), blood

glucose as a nonspecific physiological-biochemical stress response to chemical stimuli was found to be insensitive, and was assumed to be species- or stressor-specific. Hence, it was concluded that to complement toxicity data, blood glucose should be used only in combination with other endpoints.

The evaluation of geno- and cytotoxicity endpoints using a nuclear abnormalities assay revealed a significant elevation in the total genotoxicity level in peripheral blood erythrocytes of *O. mykiss* exposed to metal mixtures. The levels of total genotoxicity varied depending on exposure duration and metal mixture. Our findings are consistent with those reported by Stankevičiūtė et al. (2016), who observed exposure duration-related fluctuations in genotoxicity levels in Cu+Zn mixture-treated *O. mykiss*. The highest level of total genotoxicity was recorded after treatment with Cr↓ mixture on the 14th day of exposure. During the recovery period, total genotoxicity levels slightly decreased, but remained higher and significantly different from the control even on the 28th day of the recovery period. Similar results were observed in Cu-exposed *Centropomus parallelus*, where recovery from genotoxicity was not observed 30 days after exposure (Oss et al., 2013). The DNA damage results obtained from the comet assay revealed negative effects of metal mixtures on DNA integrity. The 14-day exposure to the metal mixture containing a reduced concentration of Cr caused the most increased percentage of tail DNA in peripheral blood erythrocytes. DNA damage was found to decrease with increasing depuration period. The fastest recovery of DNA damage was observed in the *O. mykiss* specimens previously treated with Cu↓ mixture, where DNA damage in the exposed fish was repaired to the control level on the 14th day. The control-level DNA integrity in the specimens treated with Cr↓ and MIX mixtures was regained at the end of the recovery period (on the 28th day). Previous studies have shown that a 30-day recovery period is sufficiently long for the DNA damage in blood cells of 4-nonylphenol-treated *Channa punctatus* (Sharma and Chadha, 2016, 2019; Sharma et al., 2018) and in those of vineyard pesticide-treated *Salmo trutta* (Bony et al., 2008) to be fully repaired. A shorter recovery period was observed in *Anguilla anguilla* (Guilherme et al., 2014; Marques et al., 2014) treated with glyphosate-based herbicide Round-up® (recovered at day 14 and day 1, respectively). A significant recovery capacity (observed after 8 and 12 days) was pointed out for the peripheral blood erythrocytes of the Cu and Zn mixture-treated *O. mykiss* specimens in the study by Stankevičiūtė et al. (2016). However, such duration of the recovery period was insufficient for the significant recovery of liver and kidney erythrocytes. The fluctuations in total genotoxicity levels observed during the 14-day exposure period and the 28-day period of recovery may be linked to the release of erythrocytes with nuclear abnormalities from kidneys and liver into blood circulation. A significant difference between recovery responses of erythrocytic cellular abnormalities (ECA), recorded on day 7 and day15, was revealed by Khan et al. (2018) in the study investigating the recovery potential of *Barbonymus gonionotus* after 30-day exposure to profenofos (pesticides). In addition, this study determined significant variations in recovery responses relative to those of control groups, and among recovery days, which is in agreement with our results. The 7-day recovery period showed the highest recovery response and the greatest decrease in formation of micronuclei, which may suggest the repair of damaged DNA, the high-degree loss of defective cells, or both (Grover et al., 2001). The highest recovery based on ENA frequency was observed on the 15th day, while stability of abnormalities on the 30th day of the recovery period. Bonomo et al. (2020) studied *D. rerio* recovery potential after 24 h exposure to magnesium-hesperidin complex (MgHP) and determined that the exposure-induced nuclear abnormalities in zebrafish disappeared after 96 h, evidencing the possible stimulation of DNA repair mechanisms. The findings of Islam et al. (2019) showed that the recuperation of erythrocytes with nuclear aberrations and those with cellular abnormalities depends on recovery duration and sampling frequency, as the investigated parameters were found to steadily normalise after exposure to pesticide-free water. The most noteworthy recuperation reaction was ascertained within a span of 15 days before moderating down (Islam et al., 2019).

In summary, after the 28-day depuration period, the adverse genotoxic effects (erythrocytic nuclear abnormalities and DNA damage) recorded in the specimens of *O. mykiss* exposed to the mixture of metals at MPCs did not reach the control level (in the case of erythrocytic nuclear abnormalities). The observed cytogenetic effect may be related to the significant amount of Cd in the gills, which remained significantly higher on the 28th day of recovery. In addition, there was no significant cytotoxic activity observed, which might eliminate resultant genotoxic lesions. Cd is a non-redox metal that may directly induce oxidative stress via bonding to the sulfhydryl, disulfide, and amine groups of cell compounds, including nucleus, and, thus, significantly disrupt their homeostasis (Drąg-Kozak et al., 2019; Ghiasi et al., 2017; Abalaka, 2015). The study conducted by Williams and Gallagher (2013) revealed persistent behavioural deficits, histological injury and the altered expression of olfactory biomarkers in Cd-exposed *Oncorhynchus kisutch* after the16-day depuration in clean water.

The results of our study on fish behaviour can be summarised as follows: the locomotor activity of *O. mykiss* juveniles exposed to different metal mixtures was found to significantly vary depending on the endpoints analysed. The locomotor activity of the exposed fish decreased with increasing exposure duration (number of exposure days) reaching a peak after 7 and 14 days of exposure to the tested metal mixtures. In general, we noted that exposure to metal mixtures had a lethargic effect on fish behaviour. The observed hypoactivity in fish could be explained by the metal-induced metabolic system impairment. Some authors demonstrated that metals have a negative impact on the metabolic enzyme activity associated with glycolysis (e.g., LDH), TCA cycle (e.g., citrate synthase), and the electron transport chain (e.g., cytochrome C oxidase) (Couture et al., 2008; Couture and Kumar, 2003). Therefore, the impaired ATP production could lead to the observed hypoactivity in test species. Mishra and Mohanty (2009) revealed that metals also adversely affect various organs/systems in fish, which ultimately may lead to the overall systemic toxicity and the resultant reduced swimming activity. In contrast, Eissa et al. (2009) showed that the locomotor activity of different fish species (*C. carpio, Australoheros facetum*, and *Astyanax fasciatus*) significantly increased after 4–7 days of exposure to sublethal Cd^{2+} concentrations, while *A. facetum* exhibited a clear-cut tendency to return to baseline behaviour after 7 days of recovery, and the locomotor activity of *C. carpio* remained high until the end of the experiment. These authors hypothesised that such changes could be associated with internal mobilisation of metal-binding proteins, whose synthesis is known to be induced by metals (Van der Oost et al., 2003). According to some authors, the impaired behavioural responses and morphological deformities observed even during recovery periods may be associated with the inhibition of brain and muscular Acetylcholinesterase (AChE) activity induced by specific chemicals, and their bioaccumulation in tissues (Ramesh and David, 2009). In contrast with other studies, Harit and Srivastava (2018) showed that under exposure to sublethal concentrations (0.01 ppm and 0.02 ppm) of endosulfan for 5 days, the locomotor activity of *Channa punctatus,* significantly decreased, while the frequency of fish surfacing, the rate of opercular movements and mucus secretion increased. However, after 5 days, the locomotor activity of the exposed fish reverted to normal behavioural patterns, which were observed until the end of the recovery period. In summary, the impact of metal mixtures on fish behaviour is associated with their impact on physiological systems and could be species-specific because of differences in sensitivities of physiological compensatory–adaptive responses to contaminant exposure among different fishes (Beyers et al., 1999). Hence, the impacts of various metal mixtures on all of these factors need to be carefully considered.

5. Conclusion

The present study showed that exposure to mixtures of metals at

MPCs causes genotoxic effects in blood erythrocytes of *O. mykiss*, and a significantly higher accumulation of Cd, Cr and Ni in the gills tissue of the exposed fish. In addition, multimetal mixtures were found to produce a lethargic effect on fish behaviour, the locomotor activity of the exposed fish decreasing with increasing exposure duration. According to the results obtained in this study, *O. mykiss* successfully depurated Cr and Ni in less than 28 days, whereas the level of Cd decreased by only approximately 40% over the same period. The performed comet assay revealed a significant capacity of *O. mykiss* to restore its DNA integrity after exposure to the mixture of metals at MPC. However, the 28-day recovery period proved to be insufficiently long for the erythrocytes with nuclear abnormalities to recover to the control level. The accumulation of Cd in the gills and its slow elimination therefrom may account for the persistent cytogenetic damage, which was revealed by the performed nuclear abnormalities assay. In conclusion, changes in the MPCs of metals (Ni, Pb, Cd) in the test mixture did not induce marked changes in *O. mykiss* biomarker responses and the induced biological effects were similar to those previously determined in *S. salar*, *R. rutilus* and *P. fluviatilis* after exposure to the mixture of the same metals but at lower MPCs of Ni and Pb and at a higher MPC of Cd. Our study, which was performed applying the whole-mixture approach, has extended the knowledge essential for assessing metal mixture exposure risks in the aquatic ecosystems failing to meet good chemical status requirements, as well as the understanding of the recovery potential of organisms therein, thus providing a more sound basis for the development of ecosystem restoration measures. Further research is needed to test the depuration potential and dynamics of the aquatic organisms exposed to chemical mixtures under the environmentally realistic scenario.

CRediT authorship contribution statement

Milda Stankevičiūtė: Conceptualization, Methodology, Investigation, Formal analysis, Supervision, Writing - original draft. **Tomas Makaras:** Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **Janina Pažusienė**: Investigation, Formal analysis, Writing - original draft. **Brigita Čapukoitienė:** Investigation. **Gintarė Sauliutė:** Methodology, Investigation, Formal analysis, Writing - original draft. **Živilė Jurgelėnė:** Investigation. **Eva Raudonytė-Svirbutavičienė:** Investigation, Writing - original draft. **Kęstutis Jokšas:** Investigation, Writing - original draft.

Declaration of Competing Interest

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

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