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Effects of MCPA Herbicide on Hematological Parameters and Ultrastructure of Hematopoietic Tissues of Common Carp (*Cyprinus carpio* L.)

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Original article

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Common carp juveniles were subjected to 14 day exposure to 100 µg/l of MCPA (phenoxy acid herbicide) and 30 day depuration. Peripheral blood parameters were analyzed after 1, 3, 7 and 14 days of exposure at 7, 14 and 30 days of depuration. Ultrastructure of hematopoietic tissues of head and trunk kidney and spleen were analyzed after the end of exposure and purification. Results showed that MCPA exposure induced only minor and transient alterations in red blood parameters but more pronounced changes in leukocyte differential count: a significant and persistent depletion of mature neutrophils during both exposure and depuration, and monocytosis during exposure. These changes indicate a possible inflammatory process and immunosuppression caused by this herbicide. Analysis of hematopoietic tissue revealed no major pathologic lesions but some minor ultrastructural anomalies. Hematopoietic precursor cells with blurred ultrastructure, some vacuoles in cytoplasm with different electron density and size, melanomacrophage and myelin-like structures were observed during exposure and particularly during depuration. These changes indicate a weak cytotoxic effect of MCPA on carp hematopoietic system.

Key words: MCPA, herbicide, toxicity, hematology, hematopoietic tissues, common carp.

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Phenoxy acids, as 2,4-D, MCPA and MCPP, are herbicides widely used in agriculture, forestry and horticulture (KUDSK & STREIBIG 2003). The presence of these substances in the aquatic environment has been reported in monitoring studies. Many scientific studies confirmed that shellfish and fish are good models to evaluate the toxicity in aquatic system due to their ability to metabolize xenobiotics, their sensitivity to pollutants (BARTOSKOVA *et al*. 2013; ALIKO *et al*. 2015; CHROMCOVA *et al*. 2015; BURGOS-ACEVES *et al*. 2016; FAGGIO *et al*. 2016; MATOZZO *et al*. 2016; SAVORELLI *et al*. 2017; SEHONOVA *et al*. 2017a, b; BURGOS-ACEVES *et al*. 2018) and the position into the aquatic food chain (TORRE *et al*. 2013; FAGGIO *et al*. 2016; PAGANO *et al*. 2017; BURGOS-ACEVES & FAGGIO 2017). SADOWSKI *et al*. (2014) detected MCPA (2.20 µg/l) and 2,4-D (1.36 µg/l) in surface water in agricultural areas of Lower Silesia, Poland. MCPA (up to 60 µg/l) was observed in stream water in southern Sweden (KREUGER 1998). The same herbicide, at concentration of 0.27 µg/l, was found in urban water in Melbourne, Australia (ALLINSON *et al*. 2017). GAILLARD *et al*. (2016) revealed that MCPA (26 µg/l) was present in water of fishponds located

in Lorraine Region, France. The presence of herbicides in surface waters may adversely affect fish (GLUSCZAK *et al*. 2007; CATTANEO *et al*. 2008; MORAES *et al*. 2009; BOTELHO *et al*. 2012; GOSIEWSKI *et al*. 2012). Hematological studies are important for environmental monitoring of fish and their health condition during culture because fish are generally so intimately associated with the aquatic environment (FAGGIO *et al*. 2014a; FAZIO *et al*. 2015; NATH *et al*. 2018). These parameters are closely related to the response of the animal to the environment, an indication that the environment where fish lives could exert some influence on the blood characteristics (FAGGIO *et al*. 2014 b, c). Various laboratory techniques are used to evaluate herbicide toxicity (VAN DER OOST *et al*. 2003; CHROMCOVA *et al*. 2015; PLHALOVA *et al*. 2017), including hematological tests, that appear to be a reliable and sensitive indicator of herbicide toxicity to fish (BOJARSKI *et al*. 2015). Anemic response including decrease in the values of red blood parameters such as hematocrit (Ht), hemoglobin concentration (Hb) and erythrocyte count (RBCc) often accompanied by the alterations in the mean cell volume (MCV) and mean corpuscular hemoglobin indices (MCH and MCHC) was observed after exposure to various herbicides: molinate (SANCHO *et al*. 2000), metribuzin (VELISEK *et al*. 2009b), glyphosate (GHOLAMI-SEYEDKOLAEI *et al.* 2013; FIORINO *et al.* 2018), and clomazone (PEREIRAB *et al*. 2013). Very scarce available data indicate that herbicides may also cause leukopenia in fish. Leukocyte count (WBCc) reduction after molinate exposure was reported by SANCHO *et al.* (2000) and after glyphosate exposure by GHOLAMI-SEYEDKOLAEI *et al.* (2013), while decreased leukocrit value after metribuzin treatment was observed by VELISEK *et al*. (2009b). However, the data concerning histopathological lesions caused by herbicides in hematopoietic tissue that may accompany changes in peripheral blood are very scarce (TEH *et al*. 1997; GÓMEZ *et al.* 1998; SANCHO *et al*. 2000; CAPKIN *et al.* 2010).

The presence of the phenoxy acid herbicides may pose a threat to fish living in contaminated environment. The effects of these herbicides on fish organism have not been extensively explored so far. The aim of the study was to determine the influence of phenoxy acid herbicide MCPA on blood parameters and ultrastructure of hematopoietic tissue of common carp (*Cyprinus carpio* Linnaeus, 1758).

Materials and Methods

Animals and experimental conditions

The study was approved by the Ist Local Ethical Committee on Animal Testing in Kraków (permission No. 124/2010). The study was done on common carp juveniles of body mass 60±10 g obtained from the Department of Ichthyobiology and Fishery Management of Polish Academy of Science in Gołysz. The fish were harvested from the rearing pond in spring. Before the experiment they were subjected to clinical and parasitological examination and acclimated for 2 weeks to the laboratory conditions. During the experiment the fish were kept in 14 aquaria (300 l each), 10 fish in each aquarium. Water was constantly aerated using LP-60 aerator (Resun, China) and filtered using external filters Unimax (Aquael, Poland). Water quality parameters were measured every 3 days (temperature, pH, dissolved oxygen level, total hardness, concentrations of ammonia, nitrite and nitrate) using reagent kits and multiparameter photometer HI83200 (Hanna Instruments, Olsztyn, Poland). The average values of these parameters during the experiment were: temperature 17-18°C, pH 7.2-8.0, O_2 8.26-9.15 mg/l, hardness 16-18 n, NH_3 0.02-0.07 mg/l, NO_2^- 1-2 mg/l and NO_3^- 18-24 mg/l. Water was renewed every 3-4 days during exposure to maintain the nominal concentration of the tested herbicide and prevent the accumulation of fish nitrogen metabolites. Similarly, water was exchanged every 3-4 days during the purification period. Fish were fed daily *ad libitum* with barley flakes and frozen chironomid larvae.

Experimental design

The fish (140 individuals) were equally divided into 2 groups: control and MCPA-exposed. The fish were exposed to tested herbicide at sublethal concentration of 100 µg/l for 14 days and then subjected to depuration in clean water for another 30 days. Blood was sampled from 10 fish of each group after 1, 3, 7 and 14 days of exposure and after 7, 14 and 30 days of depuration. Blood was sampled by heart puncture using glass heparinized Pasteur pipettes (sodium heparin 5000 IU/ml, Polfa, Poland) to heparinized plastic Eppendorf tubes. Blood from each fish was taken only once (after anesthesia with MS-222) and subjected to standard hematological analysis. RBCs and WBCs were counted in blood diluted 1:200 with Natt-Herrick solution using Bürker hemocytometer. Hematocrit value (Ht) was measured using microhematocrit method. Hemoglobin concentration (Hb) was measured spectrophotometrically at 540 nm wave length after conversion of hemoglobin to cyanmethemoglobin with Drabkin solution. Next, mean cell volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated. Blood smears were also made and stained using Hemacolor® staining kit (Merck, Germany) to evaluate differential leukocyte count. Various types of leukocytes were identified per 100 cells at ×600 magnification. The

following types of cells were identified: lymphocytes, monocytes, juvenile neutrophils, mature neutrophils and eosinophils. Ultrastructural analysis of hematopoietic organs was also performed. Head and trunk kidney and spleen tissues were sampled from 5 fish of the control and MCPA-exposed groups twice: immediately after the end of exposure (14 days) and after the end of purification (44 days of the experiment). The sections were treated using standard method (KARNOVSKY 1965) and embedded in epoxy resin (Epoxy Embedding Medium, Sigma Aldrich). The preparations were subjected to transmission electron microscope analysis (TEM) using JOEL JEM-100 SX and Tecna G2 Spirit (FEI Company).

Chemical tested

MCPA (2-methyl-4-chlorophenoxyacetic acid $C_9H_9ClO_3$) is a systemic postemergence herbicide used to control annual and perennial dicotyledon weeds in cereal, linen, rice, peas and potato crops. It is also used in grasslands and forestry. MCPA is a component of 33 commercial formulas available in Poland, in 11 being a unique active substance, and in remaining 22 – one of at least 2 different active substances (THE REGISTER OF PLANT PROTECTION PRODUCTS, Polish Ministry of Agriculture and Rural Development, https://bip.minrol.gov.pl/Informacje-Branzowe/Produkcja-Roslinna/Ochrona-Roslin/Rejestr-Srodkow-Ochrony-Roslin, February 2017). MCPA is quite persistent in aquatic environment with half-life of 13.5 days (PESTICIDE PROPERTIES DATABASE – PPDB, University of Hertfordshire, https://sitem.herts.ac.uk/aeru/ppdb, February 2017). Analytical standard of purity 99.7 ± 0.1% obtained from the Institute of Organic Industry in Warsaw (Branch in Pszczyna) was used.

Statistical analysis of data

Normality of distribution was tested by the Shapiro-Wilk's test, and homogeneity of variance using Levene's test. The data were analyzed by ANOVA, followed by Tukey's post-hoc test. For the data that did not meet the assumptions of ANOVA (differential leukocyte count), a non-parametric U Mann–Whitney test was performed. The level of significance was set at $\alpha = 0.05$. Data were presented as means ± SD. Results were analyzed using STATISTICA 10 program.

Results

Hematological parameters

During the experiment neither mortality nor distress symptoms were observed in the exposed fish. The values of red blood parameters are shown in Table 1. RBCc in the control group ranged from 0.70 ± 0.11 to 1.12 ± 0.18 $\times 10^6$/µl. After 7 days of MCPA exposure RBCc value significantly increased and after 14 days of purification decreased compared to the control. Ht in the control fish ranged from 27.0 ± 4.5 to 34.7 ± 5.1% and no sta-

Table 1

Values of red blood cell parameters in common carp during exposure to MCPA (100 µg/l) and purification (asterisks indicate the values significantly different from the control at the same sampling time, Tukey's test, $P<0.05$)

Time of blood collecting/parameter tested		Exposure to MCPA herbicide (100 µg/l)				Purification		
		1 day	3 days	7 days	14 days	7 days	14 days	30 days
RBCc ($\times 10^6$/µl)	Control	0.81 ± 0.16	1.06 ± 0.10	0.88 ± 0.20	1.00 ± 0.08	0.70 ± 0.11	1.12 ± 0.18	1.08 ± 0.07
	MCPA	0.85 ± 0.30	1.06 ± 0.38	1.20* ± 0.25	0.80 ± 0.19	0.73 ± 0.15	0.82* ± 0.23	0.93 ± 0.15
Ht (%)	Control	27.00 ± 4.52	31.00 ± 2.78	32.90 ± 5.90	28.70 ± 3.62	27.40 ± 2.72	34.70 ± 5.10	34.00 ± 2.58
	MCPA	30.20 ± 4.02	32.90 ± 4.86	35.40 ± 4.25	29.30 ± 3.89	28.50 ± 2.68	31.00 ± 4.67	30.60 ± 4.90
Hb (g/dl)	Control	6.08 ± 1.42	8.33 ± 1.37	5.88 ± 0.86	7.93 ± 1.37	6.85 ± 1.77	7.79 ± 0.94	4.80 ± 0.57
	MCPA	9.98* ± 1.13	8.62 ± 1.14	4.53 ± 0.80	6.19* ± 0.76	6.89 ± 0.78	6.27 ± 0.86	5.65 ± 0.76
MCV (fl)	Control	342.07 ± 75.93	302.57 ± 48.30	394.50 ± 117.69	290.29 ± 49.31	399.44 ± 75.65	319.65 ± 82.80	319.23 ± 38.67
	MCPA	393.58 ± 134.22	350.13 ± 137.56	311.13 ± 63.48	389.45 ± 141.11	411.92 ± 111.34	399.04 ± 99.00	334.14 ± 55.61
MCH (pg)	Control	78.10 ± 26.06	78.93 ± 14.58	69.14 ± 13.79	79.70 ± 13.00	97.48 ± 21.65	71.02 ± 12.51	44.51 ± 5.05
	MCPA	129.05* ± 40.22	91.18 ± 34.23	40.12 ± 10.55	80.55 ± 20.73	100.70 ± 35.76	80.32 ± 18.49	61.98 ± 10.90
MCHC (g/dl)	Control	22.53 ± 4.62	26.20 ± 3.77	18.21 ± 3.38	28.00 ± 5.80	25.08 ± 6.70	22.66 ± 2.63	14.04 ± 1.63
	MCPA	33.37* ± 4.27	26.71 ± 5.38	13.06 ± 3.36	21.37* ± 3.25	24.44 ± 4.15	20.37 ± 2.11	19.16 ± 5.71

tistically significant differences between the control and herbicide-exposed fish were observed. Hb in blood of the control fish ranged from 4.80 ± 0.57 to 7.93 ± 1.37 g/dl. Hb significantly increased in MCPA-exposed group already after 1 day of exposure, while after 14 days significantly decreased compared to the control. MCV in the control fish was between 290.29 ± 49.31 fl and 399.44 ± 75.65 fl. In MCPA-exposed fish MCV values were usually slightly higher but no significant differences were observed compared to the control. MCH of the control fish was from 44.51 ± 5.05 to 97.48 ± 21.65 pg, while in the MCPA-exposed group it was more variable and after 1 day of exposure to the herbicide MCH value was significantly higher compared to the control. MCHC in the control showed the values between 14.04 ± 1.63 and 28.0 ± 5.80 g/dl. In MCPA-exposed group MCHC significantly increased after 1 day of exposure and decreased at the end of exposure compared to the control.

The results of leukocyte analysis are presented in Table 2. WBCc in the control ranged from 23.4 ± 4.7 to 45.8 ± 9.4 $\times 10^3/\mu l$. A significant increase in WBCc was observed in MCPA group after 7 days of exposure compared to the control. In the control group lymphocytes comprised from 85.0 ± 6.4 to 96.8 ± 1.6%. After 3 days of exposure to the herbicide a significant decrease in lymphocyte percentage was observed, followed by an increase after 30 days of purification. Percentage of immature neutrophils in the control fish was between 1.9 ± 1.1 and 8.0 ± 4.3% and no significant differences were observed between the groups. Percentage of mature neutrophils in the control ranged from 1.5 ± 0.9 to 7.7 ± 3.0%. After 1 day of exposure to MCPA a significant decrease occurred followed by an increase in 7 days compared to the control. Percentage of mature neutrophils decreased again in fish subjected to the herbicide treatment after 7, 14 and 30 days of purification compared to the control. Percentage of monocytes in the control was low and ranged from 0.3 ± 0.4 to 1.4 ± 1.2%. After 3 and 7 days of MCPA exposure contribution of monocytes significantly increased compared to the control.

Histology of hematopoietic organs

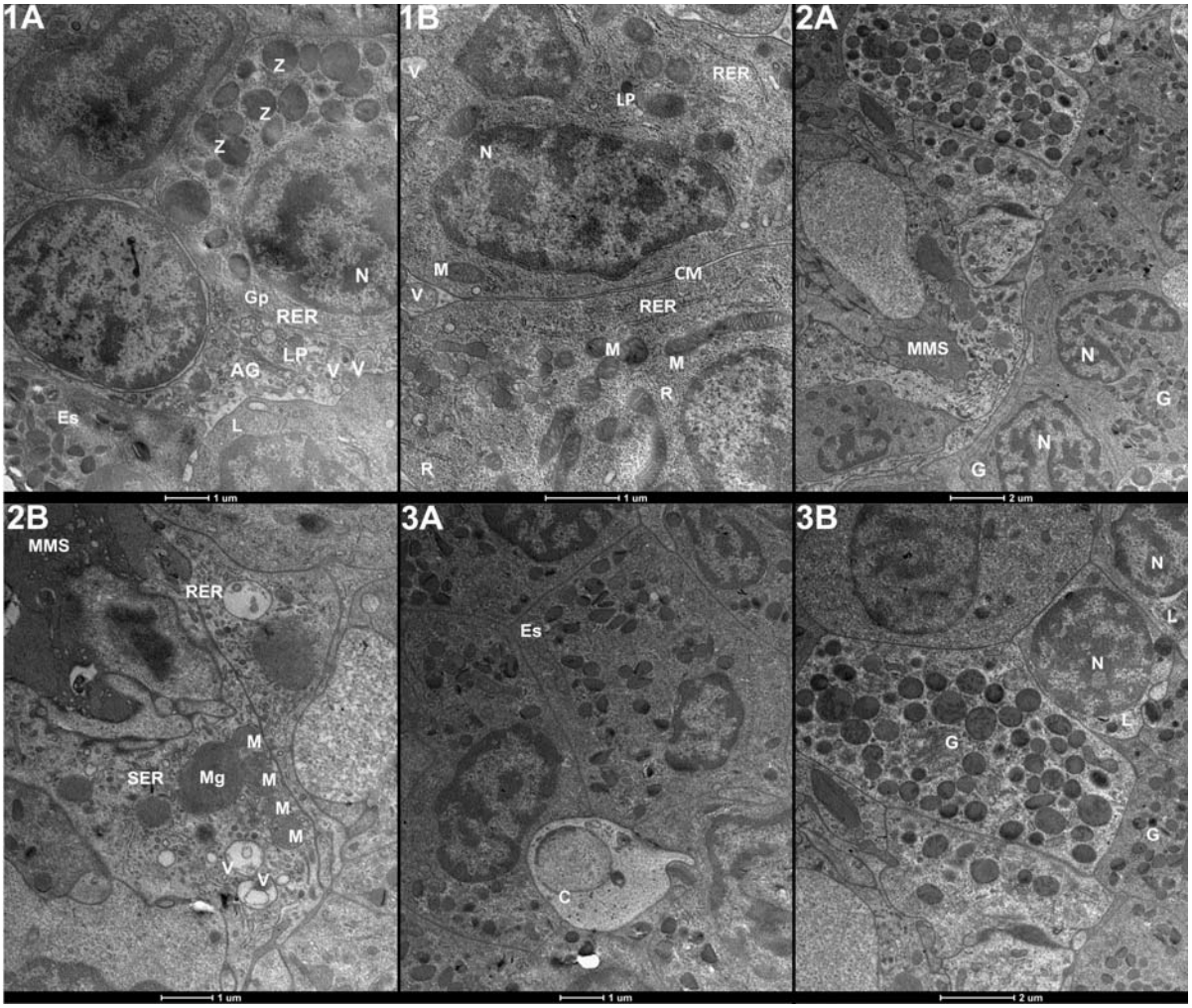
Head kidney

In fish from the control group head kidney hematopoietic tissue ultrastructure showed no pathological lesions and the results obtained after 14 and 44 days of experiment were similar (Fig. 1A and B). Head kidney of the control fish showed firm structure. Juvenile blood cells: promyelocytes, metamyelocytes, eosinophils, lymphocytes and scarce erythrocytes were present in the stroma, usually tightly packed. In the cells following organelles were observed: mitochondria, short RER sections, free ribosomes, single Golgi apparatuses, single vacuoles containing various materials, and granulocytes showed also numerous granules (Fig. 1A and B). After 14 days of MCPA exposure the head kidney structure was still firm and blood precursor cells were tightly packed (Fig. 2A and B). However, focally the cells were slightly deformed (Fig. 2B). Numerous neutrophil precursors, eosinophils and lymphocytes were observed (Fig. 2A). Single cells with electron-light nuclei or cytoplasm were observed among the normal cells (Fig. 2A and B). These cells showed also melanomacrophage struc-

Table 2

Values of white blood cell parameters in common carp during exposure to MCPA (100 μg/l) and purification (asterisks indicate the values significantly different from the control at the same time, Tukey's test for WBCc and U-Mann Whitney test for differential leukocyte count, $P<0.05$)

Time of blood collecting / parameter tested		Exposure to MCPA herbicide (100 μg/l)				Purification		
		1 day	3 days	7 days	14 days	7 days	14 days	30 days
WBCc ($\times 10^3/\mu l$)	Control	35.00 ± 5.90	42.60 ± 5.41	34.20 ± 5.29	33.80 ± 9.82	23.40 ± 4.72	24.20 ± 3.33	45.80 ± 9.36
	MCPA	36.80 ± 16.06	65.20 ± 25.98	77.10* ± 20.29	47.90 ± 11.70	30.20 ± 9.31	66.20* ± 15.16	52.00 ± 17.00
Lymphocytes (%)	Control	90.50 ± 2.58	93.60 ± 1.74	96.80 ± 1.60	95.60 ± 1.91	92.10 ± 4.06	88.70 ± 2.03	85.00 ± 6.36
	MCPA	94.08 ± 2.34	86.78* ± 3.42	89.10 ± 3.75	94.35 ± 4.06	96.15 ± 2.25	93.00 ± 3.55	92.85* ± 2.68
Juvenile neutrophils (%)	Control	3.85 ± 2.04	1.65 ± 0.85	1.90 ± 1.17	1.90 ± 1.11	2.90 ± 1.98	4.65 ± 2.71	8.00 ± 4.33
	MCPA	2.23 ± 1.51	5.47 ± 2.73	1.80 ± 1.49	1.75 ± 1.80	1.55 ± 0.42	4.00 ± 2.98	3.90 ± 1.78
Mature neutrophils (%)	Control	4.65 ± 2.20	3.80 ± 1.92	1.50 ± 0.85	1.50 ± 0.85	3.90 ± 1.82	5.70 ± 2.54	7.72 ± 3.00
	MCPA	2.03* ± 1.47	2.99 ± 1.50	2.85* ± 1.20	2.20 ± 1.32	1.65* ± 0.97	2.20* ± 1.99	3.20* ± 1.77
Monocytes (%)	Control	1.60 ± 1.07	1.40 ± 1.18	0.95 ± 0.55	0.75 ± 0.68	1.40 ± 1.22	0.50 ± 0.53	0.60 ± 0.22
	MCPA	1.53 ± 0.78	4.71* ± 1.83	3.75* ± 1.67	1.40 ± 1.02	0.95 ± 0.93	0.40 ± 0.61	0.05 ± 0.16

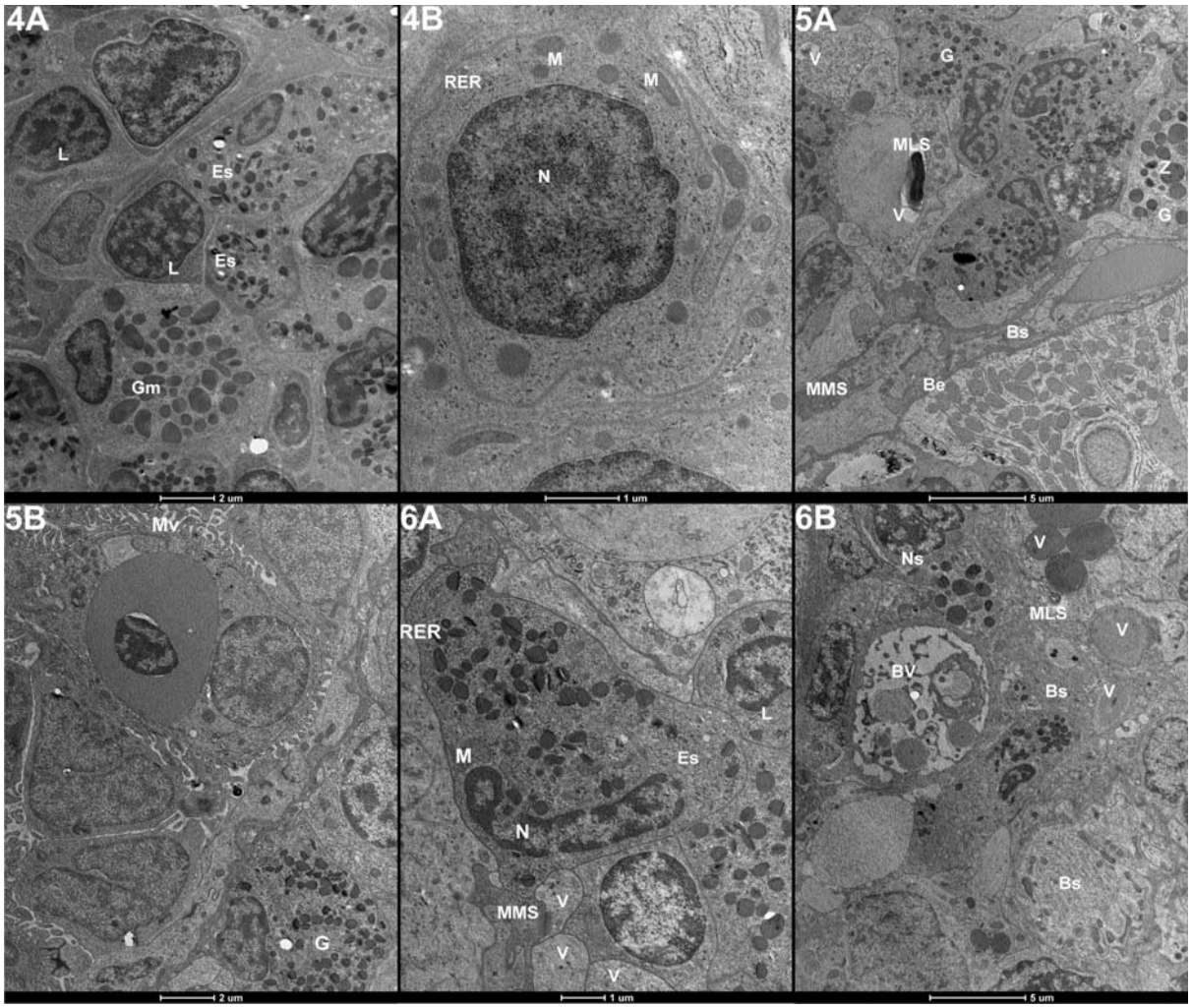


Figs 1-3.
Fig. 1A. Electron micrograph of common carp head kidney, control. Hematopoietic precursors: promyelocyte (Gp), eosinophil (Es) and lymphocyte (L). Numerous granules (Z), vacuoles (V), Golgi apparatus (AG) and primary lysosome (LP) in granulocytes. 9900 x. Fig. 1B. Electron micrograph of common carp head kidney, control. Organelles in hematopoietic precursor cells: rough endoplasmic reticulum (RER), free ribosomes (R), mitochondria (M), single vacuoles (V), primary lysosome (LP), cell nuclei (N) and cellular membrane (CM) 16500 x.
Fig. 2A. Electron micrograph of common carp head kidney after 14 days of exposure to 100 µg/l of MCPA. Numerous usually correct granulocytes (G) with nucleus (N) and damaged cell with melanomacrophage structure (MMS). 6000 x. Fig. 2B. Electron micrograph of common carp head kidney after 14 days of exposure to 100 µg/l of MCPA. Large melanomacrophage structure (MMS), numerous vacuoles (V) of different size and electron density, rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER), mitochondria (M) and megamitochondria (Mg). 11500 x.
Fig. 3A. Electron micrograph of common carp head kidney after 30 days of depuration post MCPA exposure. In firm tissue structure eosinophils (Es) and electron-light cells (C). 9900 x. Fig. 3B. Electron micrograph of common carp head kidney after 30 days of depuration post MCPA exposure. Normal structure of granulocytes (G) and lymphocytes (L) with correct nucleus (N). 6000 x.

tures of various size. In granulocytes numerous granules were visible (Fig. 2A). After 30 days of depuration of MCPA-exposed fish their head kidneys usually had firm structure (Fig. 3A and B). Among the hematopoietic precursors granulocytes predominated. Cell nuclei showed chromatin diversification: both euchromatin and heterochromatin were observed. Nuclei of some cells were slightly deformed. Electron-light cells or cells with electron-light cytoplasm also occurred. Nuclei of lymphocytes were often cleft.

Trunk kidney – hematopoietic tissue

Analyses of trunk kidney hematopoietic tissue ultrastructure revealed no pathological alterations in fish from the control group and no differences between the samples taken after 14 and 44 days of experiment (Fig. 4A and B). The organ had firm structure and tightly packed cells. Mitochondria showed distinct cristae. RER and single Golgi apparatuses were also observed. Granulocytes showed numerous granules. In nuclei heterochromatin and euchromatin zones were visible. Hematopoietic tissue of trunk kidney consisted mainly of granulocyte and lymphocyte precursors. After 14 days of MCPA exposure hematopoietic and excretory parts of trunk kidney were connected and the cells were usually tightly packed (Fig. 5A) but some of them were slightly separated (Fig. 5B). Various types of granulocytes accompanied by

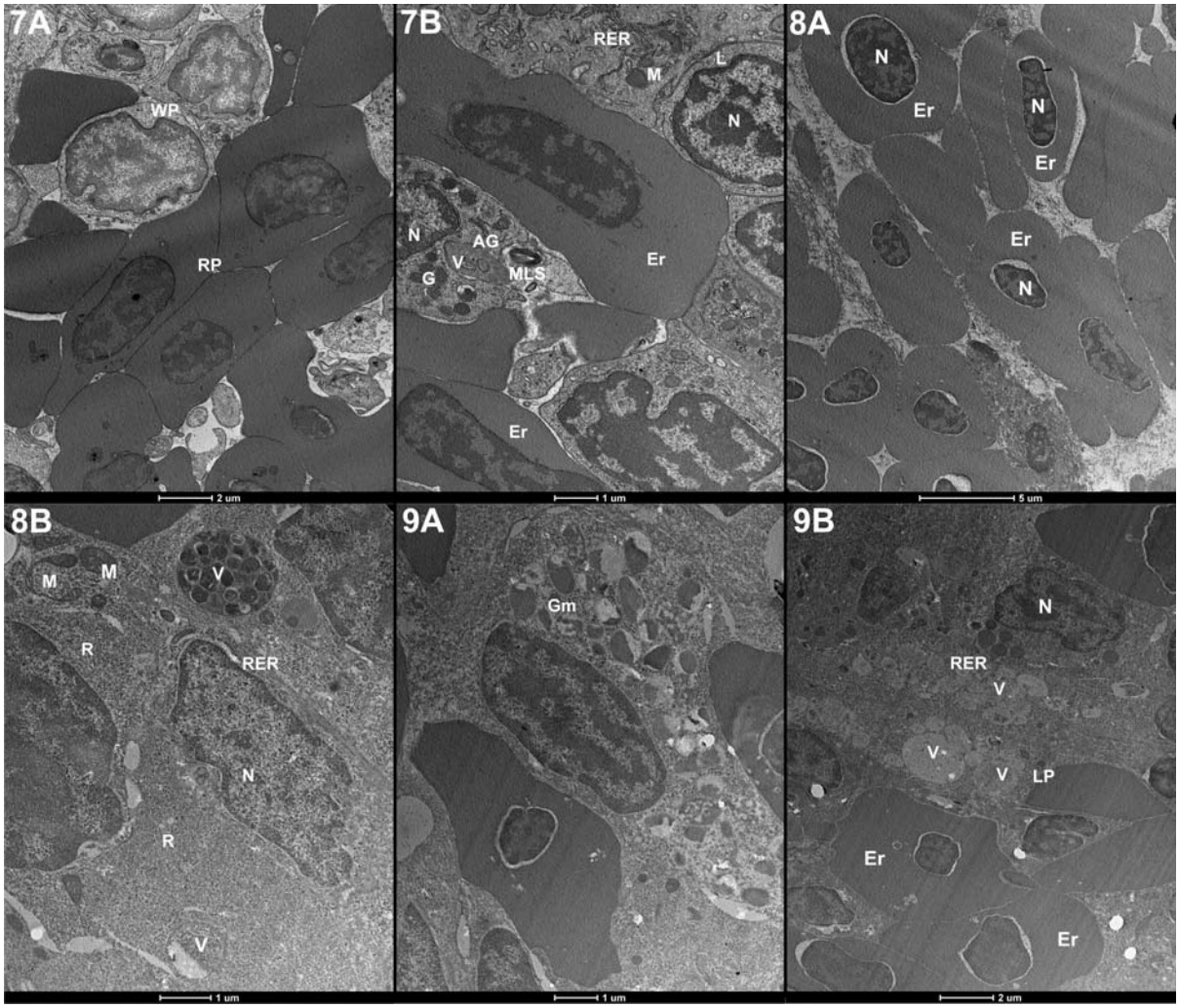


Figs 4-6.
Fig. 4A. Electron micrograph of common carp trunk kidney hematopoietic tissue, control. Firm tissue structure, numerous neutrophil precursors (Gm), eosinophils (Es) and lymphocytes (L). 6000 x. Fig. 4B. Electron micrograph of common carp trunk kidney hematopoietic tissue, control. Lymphocyte precursor with visible mitochondria (M), rough endoplasmic reticulum (RER) and nucleus (N). 16500 x.
Fig. 5A. Electron micrograph of common carp trunk kidney hematopoietic tissue near the boundary (Be) of excretory part after 14 days of exposure to 100 µg/l of MCPA. Firm tissue structure with some hematopoietic precursor cells of blurred ultrastructure (Bs). Granulocytes (G) containing numerous granules (Z) and damaged cells with single vacuoles (V) of various size and content. Single melanomacrophage (MMS) and myelin-like (MLS) structures. 4200 x. Fig. 5B. Electron micrograph of common carp trunk kidney hematopoietic tissue after 14 days of exposure to 100 µg/l of MCPA. Slight tissue structure loosening. Single granulocyte (G) and numerous microvilli (Mv) 6000 x.
Fig. 6A. Electron micrograph of common carp trunk kidney hematopoietic tissue after 30 days of depuration post MCPA exposure. Lymphocyte (L) and correct eosinophil (Es) with mitochondria (M), rough endoplasmic reticulum (RER) and nucleus (N). Hematopoietic precursor cells showing blurred ultrastructure, some vacuoles (V), and melanomacrophage structure (MMS). 9900 x. Fig. 6B. Electron micrograph of common carp trunk kidney hematopoietic tissue after 30 days of depuration post MCPA exposure. Hematopoietic precursor cells of normal (Ns) and blurred (Bs) ultrastructure near blood vessel (BV). Vacuoles (V) and myelin-like structures (MLS) in the cells. 4200 x.

lymphocytes were observed (Fig. 5A and B). They usually showed correct ultrastructure but in some cells it was slightly blurred (Fig 5A). Granulocytes (particularly eosinophils) showed numerous granules (Fig. 5A and B). In some cells single vacuoles, melanomacrophage structures and myelin-like bodies occurred (Fig. 5A). After 30 days of depuration hematopoietic and excretory parts of trunk kidney were connected or partly separated. Hematopoietic tissue showed firm structure (Fig. 6A and B) but cell ultrastructure was often blurred, particularly near excretory zone and blood vessels (Fig. 6B). Hematopoietic cells contained melanomacrophage and myelin-like structures accompanied by vacuoles (Fig 6A and B).

Spleen

Ultrastructure of splenic hematopoietic cells of the control fish revealed no pathological lesions and histological picture after 14 and 44 days of experiment did not differ (Fig. 7A and B). The spleen tissue showed numerous hematopoietic precursors and erythrocytes. Erythrocytes at various stages of



Figs 7-9.
Fig. 7A. Electron micrograph of common carp spleen, control. Firm tissue structure, numerous red pulp (RP) and white pulp (WP) cells. 6000 x.Fig. 7B. Electron micrograph of common carp spleen, control. Lymphocytes (L) and granulocytes (G) with ultrastructure: mitochondria (M), rough endoplasmic reticulum (RER), single mielin-like structure (MLS), Golgi apparatus (AG), vacuoles (V) and nuclei (N); single erythrocytes (Er) also visible. 9900 x.
Fig. 8A. Electron micrograph of common carp spleen after 14 days of exposure to 100 µg/l of MCPA. Group of erythrocytes (Er) with nuclei (N) at various stages of physiological degradation and electron-light stroma. 4200 x. Fig. 8B. Electron micrograph of common carp spleen after 14 days of exposure to 100 µg/l of MCPA. White pulp cell ultrastructure showing mitochondra (M) of different size and single in dividing process, short rough endoplasmic reticulum sections (RER), free ribosomes (R), vacuoles (V) and nuclei with euchromatin and heterochromatin. 11500 x.
Fig. 9A. Electron micrograph of common carp spleen after 30 days of depuration post MCPA exposure. Damaged metamyelocyte (Gm). 9900 x. Fig. 9B. Electron micrograph of common carp spleen after 30 days of depuration post MCPA exposure. Numerous vacuoles (V), primary lysosome (LP), rough endoplasmic reticulum (RER) in blurred ultrastructure of white pulp cells. Cell with nucleus (N) and some erythrocytes (Er) also visible. 6000 x.

physiological degradation were organized in lines or groups with lymphoid tissue among them or they surrounded lymphoid tissue forming sinus-like figures. The cells were usually tightly packed but sometimes empty intercellular spaces were observed. White pulp contained granulocytes: eosinophils and neutrophils at various development stages, and lymphocytes (Fig. 7B). After 14 days of exposure to MCPA spleen structure was generally compact (Fig. 8B). Red pulp consisted of lesser or larger groups of erythrocytes at various stages of more pronounced destruction compared to the control. (Fig. 8A). White pulp consisted of correct and abnormal cells – showing blurred ultrastructure (Fig. 8B). The cells showed mitochondria, RER, free ribosomes and vacuoles of different electron density, and the nuclei with euchromatin and heterochromatin zones (Fig. 8B). After 30 days of depuration white pulp had firm structure with tightly packed hematopoietic cells (Fig. 9A and B). Most of the cells showed correct ultrastructure but some cells with blurred ultrastructure and distinct anomalies were observed. These anomalies included numerous vacuoles of uniform or diverse electron density (Fig. 9B). The red pulp contained numerous erythrocytes undergoing physiological destruction.

Discussion

Exposure of fish to MCPA resulted in a significant but transient alterations in red blood parameters: increase in Hb, MCH and MCHC on the 1 day of exposure that might have resulted from stress followed by a decrease in Hb and MCHC after 14 days. After 14 days of depuration erythropenia was also observed. These results indicate that MCPA may induce in fish a transient anemic response. According to VOSYLIENE (1999), the quantitative red blood parameters in fish are rather stable and little sensitive to environmental factors, due to considerable compensatory abilities of organism.

It is known that different pesticides present in the aquatic environment can cause chemical stress in fish, which is expressed by changes in hematological parameters (SVOBODOVÁ *et al*. 2003; LI *et al*. 2011; LUTNICKA *et al*. 2016). However, little works concerning the effects of herbicides on the hematological profile of fish has been published. Alterations in the values red blood cell parameters of fish exposed to herbicides were observed by various authors but most data concern acute exposures. Anemia was reported by VELISEK *et al.* (2009b) who revealed that short-term (96 h) metribuzin exposure of *Cyprinus carpio* (175.1 mg/l) caused a significant decrease in Ht, Hb, MCV, and RBCc values. Similarly, short-time (96 h) Roundup exposure (3-20 mg/l) of *Leporinus obtusidens* reduced RBCc, Ht and Hb levels. RAMESH *et al*. (2009) showed that acute atrazine treatment (24 h, 18.5 mg/l) caused significant reduction of RBCc and Hb content in *C. carpio*. HUSSEIN *et al*. (1996) revealed that the exposure of *Oreochromis niloticus* and *Chrysichthyes auratus* to 3 and 6 mg/l of atrazine resulted in significant decrease in RBCc, Hb and Ht compared the control group in both species. DOBŠÍKOVÁ *et al*. (2011) observed a decrease of Ht in *C. carpio* exposed for 96 h to 13 mg/l of Gardoprim Plus Gold 500 SC (corresponding to 2.25 mg/l and 3.75 mg/l of terbuthylazine and S-metolachlor, respectively). KREUTZ *et al*. (2011) revealed a significant decrease in RBCc of *Rhamdia quelen* exposed to glyphosate (96 h, 0.73 mg/l). CRESTANI *et al*. (2006) observed that clomazone caused a significant decrease of Ht values in *Rhamdia quelen* after 96 h of exposure at a concentration of 0.05 mg/l and after 192 h at concentrations of 0.05 mg/l and 1.0 mg/l. After purification (192 h), the Ht levels in treated fish (0.5 and 1.0 mg/l) were similar to control values indicating a recovery. Butachlor administration for 48-72 h at concentrations 0.5-1.0 mg/l led to hematological alterations in *Labeo rohita* including time- and concentration-related decrease in RBCc and Ht values (GHAFFAR *et al*. 2015). Sublethal exposure of *Anguilla anguilla* to molinate (96 h, 11.15 mg/l) induced a significant decrease in Ht, Hb and RBCc (SANCHO *et al*. 2000). Some authors, however, reported that herbicide exposures of fish caused increase in the values of their red blood parameters. The results obtained by MODESTO and MARTINEZ (2010) demonstrated a significant increase of Ht and RBCc in *Prochilodus lineatus* exposed to Roundup Transorb® (24 and 96 h at 5 mg/l). Sub-chronic exposure of *C. carpio* to terbutryn (2-40 mg/l) led to a significant increase in RBCc (VELISEK *et al.* 2010). The results obtained by BOJARSKI *et al*. (2015) revealed that exposure of *C. carpio* to pendimethalin was time- and concentration-related in a non-linear way: at 2.5 μg/l a significant increase of Hb and MCHC were observed after 7 days of treatment. At 25 μg/l Hb decreased after 1 day and Ht after 3 days, while RBCc, Ht and Hb increased after 7 days of herbicide exposure. Ethofumesate (7 days, 0.11 μg/l) caused increase of RBCc, Ht and Hb, while at 1.1 μg/l Hb and MCHC values increased after 3 days of exposure. In fish exposed to mixture of both herbicides (2.5 μg/l of pendimethalin + 0.11 μg/l of ethofumesate or 25 μg/l and 1.1 μg/l, respectively) RBCc and Ht increased after 1 and 3 days of exposure, while longer exposures (7 days) resulted in reduction of RBCc and Ht values and increase of MCV and MCHC.

More pronounced alterations occurred in white blood cell system: despite transient leukocytosis after 7 days of exposure and 14 days post exposure, a persistent depletion of mature neutrophils, both during and after the end of exposure was observed, accompanied by significant monocytosis in 7 and 14 days of exposure. These changes indicate a possible inflammatory and immunosuppressive response in fish exposed to MCPA and increased migration of granulocytes to the affected tissues.

Little literature concerning the effects of herbicides on white blood parameters in fish is available and reported results are divergent. According to VELISEK *et al. (*2009b), 96 h exposure of *C. carpio* to 175.1 mg/l of metribuzin caused a significant WBCc decrease. Similar reaction was reported in *C. carpio* by RAMESH *et al*. (2009) after acute atrazine treatment (18.5 mg/l, 24 h). DOBŠÍKOVÁ *et al*. (2011) observed a decrease of WBCc and lymphopenia in *C. carpio* exposed for 96 h to 13 mg/l of Gardoprim Plus Gold 500 SC. According to KREUTZ *et al*. (2011), WBCc significantly decreased in the blood of glyphosate-exposed *Rhamdia quelen* (96 h, 0.73 mg/l). Also in molinate-exposed (96 h, 11.15 mg/l) *Anguilla anguilla* WBCc significantly decreased (SANCHO *et al*. 2000). On the other hand, increase in WBCc accompanied by lymphocytosis and neutropenia were reported by MODESTO and MARTINEZ (2010) in *Prochilodus lineatus* exposed to Roundup Transorb® (24 and 96 h, 5 mg/l). Butachlor exposure (48-72 h, 0.5-1.0 mg/l) re-

sulted in WBCc increase in *Labeo rohita* (GHAFFAR *et al*. 2015). BOJARSKI *et al*. (2015) found that exposure of *C. carpio* to 0.11 µg/l of ethofumesate caused increase of WBCc after 3 days of exposure. Hematological alterations induced in fish by various contaminants may be different and probably depend on various factors: type of toxic compound and its concentration, time of exposure, fish species, water quality parameters and other factors.

The observed hematological disturbances might have been related to herbicide-induced pathological lesions in hematopoietic organs: head and trunk kidneys and spleen. Our electron microscope observations of the hematopoietic organs suggest that MCPA caused minor ultrastructural alterations: deformed and blurred blood precursor cells, numerous vacuoles with auto- and heterophagic content, myelin-like and melanomacrophage structures were observed. In the spleen, ultrastructure of white and red pulp cells was usually correct but some cells were destroyed. The presence of abundant vacuoles, myelin-like and melanomacrophage structures in the cells indicates cell recovery and elimination of degenerated debris after herbicide-induced damage. The decrease in frequency of mature neutrophils during depuration period after fish exposure to MCPA might have been related to their destruction or migration to damaged tissues and involvement in the inflammatory process. Very few literature data are available concerning histopathological lesions in hematopoietic organs of fish exposed to aquatic contaminants, and their correlation with coexisting hematological changes. GÓMEZ *et al*. (1998) studied the lesions in hematopoietic part of posterior (trunk) kidney tissue in tench (*Tinca tinca*) caused by a continuous exposure to 400 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D). Using light microscope they found marked alterations characterized by progressive swelling and cell necrosis, and degeneration in the intertubular space in 48 h after intoxication. Intracytoplasmic vacuoles of different size were also visible. The lesions increased with time. Ultrastructural analysis revealed that at the beginning of exposure necrosis and phagocyte activation occurred. After 5 days the cells showed increased necrotic degeneration, numerous myelin figures and auto- and heterophagic vacuoles. Maximum necrosis level was observed after 12 days when mass disruption of cell-specific granules was observed, particularly in heterophils. Occasionally, electron-dense hyaline droplets were observed in venous sinus myoepithelial cells in the hematopoietic portion, arranged along the major axis of the cell. Hematopoietic tissue lesions were accompanied by changes in peripheral blood: Ht and Hb values decreased progressively. According to the authors, these hematological alternations indicated changes in cell membrane permeability, complementing the findings in hematopoietic tissue. CAPKIN *et al*. (2010) studied the effects of sublethal concentrations of carbofuran (25, 50 and 200 µg/l), propineb (3, 6 and 24 mg/l) and benomyl (2, 5 and 20 mg/l) on trunk kidney and spleen of juvenile rainbow trout (*Oncorhynchus mykiss*). The fish were exposed to the pesticides for 14 days. The most important lesions were observed at the highest concentrations of pesticides. Trunk kidney and spleen showed necrosis and abundant melanomacrophage centers. In the spleen lipid infiltration and increase of sinusoidal space were observed. TEH *et al.* (1997) observed histopathologic alterations of spleen of feral fish (sunfish, *Lepomis auritus* and bass, *Micropterus salmoides*) from three freshwater ecosystems polluted with discharges from a nuclear weapons facility, bleached craft mill or showing high levels of PCBs. Lymphopenia vascular congestion and reticuloendothelial cell necrosis were found in fish from first and third ecosystem. Melanomacrophage aggregations were also abundant in the spleen of sunfish from first ecosystem. VELISEK *et al*. (2009a) found no histological effects in the spleen of common carp (*C. carpio*) exposed for 96 h at 57.5 µg/l of bifenthrin (Talstar EC 10) HAAPARANTA *et al.* (1996) observed melanomacrophage centers in spleen, liver and hematopoietic part of the trunk kidney of two species of fish: perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*). Similarly as in the present study, the results obtained by other authors (TEH *et al*. 1997; CAPKIN *et al*. 2010) showed that abundance and the surface of melanomacrophage centers and structures in the internal organs usually increased as a result of water contamination by different toxic substances. These results indicate high intensity of elimination of cellular debris after damage caused by herbicide intoxication in hematopoietic cells, particularly during depuration period.

Changes in the values of hematological parameters accompanied by the ultrastructural lesions in the hematopoietic organs caused by sublethal exposure to 100 µg/l of MCPA may be interpreted as a result of chemical stress. Most of hematological alterations were transient but ultrastructural lesions in hematopoietic organs seemed more persistent which was probably related to cellular regeneration processes.

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Research concept and design: H.L., B.B.; Collection and/or assembly of data: H.L., B.B., M.Ch.-G., W.T., E.T., A.K.-B.; Data analysis and interpretation: H.L., B.B., M.W., W.T., E.T., A.K.-B., M.L.; Writing the article: H.L., B.B., M.W., W.T., E.T., A.K.-B.; Critical revision of the article: H.L., B.B., M.W., M.Ch.-G., M.L.; Final approval of article: H.L., B.B., M.W., M.L.

Conflict of Interest

The authors declare no conflict of interest.

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