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THE INFLUENCE OF CLOMAZONE-BASED HERBICIDE FORMULATION ON COMMON CARP (*CYPRINUS CARPIO*) – A LABORATORY STUDY INCLUDING PATHOPHYSIOLOGICAL AND HISTOPATHOLOGICAL ASSESSMENT*

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Abstract

Herbicides are widely applied in agriculture, which results in the pollution of ecosystems, including inland waters. Herbicides present in water may cause pathological changes in fish. The aim of this study was to evaluate the effects of a clomazone-based herbicide formulation, Command 480 EC, on the haematological and blood biochemical parameters of common carp as well as the microstructure of selected organs: gills, liver and trunk kidney. The fish were exposed to Command 480 EC at a concentration corresponding to 1 or 5 mg/l of clomazone (groups COM1 and COM2, respectively) for 10 days. The conducted analyses showed that the treatment caused numerous changes in red blood cell parameters, no significant changes in WBC count (in comparison to the control), and only a few transient changes in the leukogram. The only significant change in the biochemical parameters in comparison to the control was a decrease in the triglyceride concentration in fish observed after 3 days (group COM1) and 10 days (group COM2) of exposure to this herbicide. No considerable histopathological alterations were observed. The results of the present study suggest that exposure to the applied clomazone-based formulation can cause pathophysiological changes in common carp. It was also observed that blood parameters (especially haematological ones) are more sensitive markers of exposure to Command than the microstructure of the tested organs.

Key words: fish, pesticide, toxicity, welfare

It is known that pesticide contamination of water ecosystems has become an important ecological and epidemiological problem in many countries. The occurrence of these chemicals in water ecosystems has been confirmed in Portugal (Palma et al., 2014), USA (Stone et al., 2014), Greece (Papadakis et al., 2015), Spain (Aguilar et al., 2017), Netherlands (Sjerps et al., 2019), Italy (Montuori et al., 2020), Japan (Chidya et al., 2022), Poland (Kruć-Fijałkowska et al., 2022), India (Nag et al., 2023), etc. Pesticides can affect both vertebrates and invertebrates living in reservoirs and watercourses. Bunzel et al. (2015) demonstrated that pesticide run-off from sugar beet and rapeseed poses a serious ecological risk for aquatic invertebrates. The study conducted by Relyea (2005) showed that pesticide exposure led to changes in aquatic communities: species richness was reduced by 15% with Sevin (carbaryl), 22% with Roundup, and 30% with malathion. It should be added that by affecting invertebrates, pesticide contamination may indirectly affect fish populations. For example, it has been shown that pesticides can be toxic to primary producers and macroinvertebrates, thereby limiting recovery of populations of Pacific salmon (*Oncorhynchus* spp.) via adverse bottom-up impacts on aquatic trophic webs (Macneale et al., 2010). According to de Souza et al. (2020), pesticides that reach water bodies can impact the whole ecological food chain.

According to Ullah and Zorriehzahra (2015), exposure of fish to pesticides leads to changes in haematological parameters, plasma biochemical indices, microstructure of various organs, the antioxidant defence system, oxygen consumption, behaviour, nutrient profile, etc.; genotoxic effects have also been noted. According to Bojarski and Witeska (2020), the observed effects of pesticide exposure include anaemia or increase in red blood cell parameters (compensatory response), inflammation, immunosuppression, as well as increased activity of hepatic aminotransferases (hepatotoxicity) and increased cortisol concentration (exhibiting stress response), etc. Fish treated with pesticides may show various types of histopathological changes. Typical alterations that may

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be observed in gills of exposed individuals are telangiectasia, haemorrhages, clubbed lamellae, damaged rakers, and fused enlarged gill filaments (Rohani, 2023). Histopathological lesions are also often detected in the livers (e.g., vacuolization of hepatocytes) and kidneys (e.g., degeneration of glomeruli) of intoxicated fish (Rohani, 2023). Due to the fact that pesticides can lead to various pathophysiological as well as histopathological changes, both functional and structural aspects should be taken into account when assessing toxic effects of these chemical agents on fish.

Herbicides (weed control agents) are the most widely used type of pesticides; they represent about 50% of all crop-protection chemicals used throughout the world (Cooper and Dobson, 2007; De et al., 2014). One commonly used herbicide is clomazone, which inhibits the formation of photosynthetic pigments, electron carriers, tocopherol and gibberellins in vascular plants (Ferhatoglu and Barrett, 2006). Clomazone is used to control broad-leaved weeds and grasses in various types of crops. It is highly soluble in water and quite volatile. It is not persistent in the soil system but may be persistent in waterbodies (PPDB – Pesticide Properties DataBase, https://sitem.herts.ac.uk/aeru/ppdb/en/Reports/168.htm). The study conducted by Zanella et al. (2002) demonstrated that clomazone could be detected in agricultural water for at least 130 days after application, thus indicating the high stability of this chemical in aquatic environments. According to Van Scoy and Tjeerdema (2014), due to the high water solubility of clomazone, the potential impact of this herbicide on surface waters, groundwaters and aquatic organisms is of great concern. Stevanovic et al. (2017) revealed that technical (pure) clomazone as well as clomazone-based formulations can cause embryotoxic effects in zebrafish (*Danio rerio*). Interestingly, these authors demonstrated that emulsifiable concentrate formulation (Rampa® EC) showed higher toxicity than the pure active ingredient, while aqueous capsule suspension (GAT Cenit 36 CS) exhibited lower toxicity in comparison to technical clomazone. Thus, there is a need to investigate environmental impacts of various commonly used herbicide formulations, not only their active substances. To the best of our knowledge, very few scientific articles on the toxicity of clomazone-based herbicide formulations to fish have been published to date. Filling this gap, especially in the context of farmed fish such as common carp (*Cyprinus carpio*), seems to be an important task.

According to Balon (2004), common carp was the first domesticated fish species. Rahman (2015) explained that nowadays it is considered to be a very important aquaculture species in many Asian and some European countries. According to Shahi et al. (2022), common carp represents 3.4% of the global fish production and is cultured in more than 100 countries. Yancheva et al. (2022) emphasizes that this species is equally important for aquaculture and aquatic toxicology. These authors expressed the opinion that it is a sentinel species whose efficacy has been confirmed in various laboratory experiments, field studies and biomonitoring programs. According to Bojarski et al. (2022 a), the size of common carp allows for the collection of sufficient blood for haematological and biochemical analyses, which may not be possible in the case of significantly smaller fish. Moreover, our experience has shown that sampling of organs (e.g., gills, liver, kidneys) for histopathological examination does not pose difficulties in the case of both sexually mature and juvenile individuals.

One of the basic tools that is used in fish toxicology is haematological analysis (Witeska et al., 2023). Haematological changes in fish may be a result of exposure to various chemical agents, for example pesticides (e.g., Atamanalp and Yanik, 2003; Svobodova et al., 2003; Ramesh et al., 2009; Velisek et al., 2009 a; Lutnicka et al., 2019; Bojarski et al., 2022 a, 2023 a). Routine haematological analyses include evaluation of blood cell counts and other cell-related parameters. Standardly determined red blood cell parameters include basic erythrocyte indicators, i.e., erythrocyte (RBC) count, haemoglobin (Hb) concentration, haematocrit (Ht) value, as well as derived erythrocyte indices, i.e., mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC). White blood cell parameters include leukocyte (WBC) count and differential leukocyte count (leukogram), i.e., evaluation of the percentage or number of various types of white blood cells (Witeska et al., 2022). Sometimes, in addition to the above-mentioned red blood cell parameters, an erythrogram (i.e., characterization of erythrocyte morphology on the basis of analysis of a stained blood smear) is determined. According to Witeska et al. (2010, 2011), the morphology of fish's red blood cells is often more sensitive to various environmental agents than standard erythrocyte indices. Thus, cellular anomalies of fish's red blood cells can be observed even if the values of routinely determined erythrocyte parameters do not change significantly (Witeska et al., 2010, 2011). It has been shown that analysis of erythrocyte morphology can be used in the evaluation of the toxicity of pesticides (Ateeq et al., 2002; Babu et al., 2014; Bojarski et al., 2018 a; Fathy et al., 2019; Mahmood et al., 2022; Ritu et al., 2022). Blood biochemical analysis is often used in addition to haematological tests. Simultaneous analysis of haematological and blood biochemical parameters provides extensive information about the physiological state of fish, including level of stress, nutritional status, immune status, etc. (Witeska et al., 2022).

The microstructure of various organs is another important marker of fish's exposure to toxic substances. According to Yancheva et al. (2016), histopathological alterations in organs such as gills, liver and kidneys have been successfully applied in ecotoxicological studies and risk assessment programs. According to Bojarski et al. (2022 a), the simultaneous use of haematological analysis, blood biochemical analysis and histopathological analysis allows for comprehensive evaluation of the influence of toxic agents on fish.

The aim of the current study was to assess the influence of clomazone-based herbicide formulation (Command 480 EC) on haematological parameters (including erythrogram), blood biochemical indices, and the microstructure of gills, liver and trunk kidney of common carp as an important experimental animal.

Material and methods

Experiment design

In the current study, 90 sexually immature common carp of the R3R8 line were used. The fish had always been kept under laboratory conditions. Before acclimation, they were subjected to veterinary examination and were considered healthy. The weight of the fish was 69.20±9.00 g (mean ± SD), and the length was 16.10±0.75 cm (mean ± SD). The animals were divided into 9 plastic tanks with a capacity of 300 litres each, filled with water to a volume of 200 litres (10 individuals per tank). The experiment began with acclimation, which lasted 14 days. Next, 3 equally sized groups (3 tanks with 10 fish per tank in each group) were established: control group (group C), group COM1 and group COM2. The fish in group C were kept in water without the addition of the tested herbicide formulation. The fish in group COM1 were exposed to the commercial herbicide Command 480 EC (FMC, USA) at a low concentration, corresponding to 1 mg/l of the active ingredient (clomazone), while the fish in group COM2 were exposed to the same herbicide formulation at a higher tested concentration, corresponding to 5 mg/l of the same active compound. The treatment lasted 1, 3 or 10 days. During exposure, the water (in the control group) or the water/herbicide mix (in the exposure groups) was changed every twelve hours; during acclimation, the water was changed at the same frequency, according to Bojarski et al. (2022 a, 2023 a). During acclimation, this procedure was applied in order to remove ammonia, nitrites and nitrates, and to accustom the fish to the changing of the water. During exposure, this was done to keep the concentration of the tested herbicide as constant as possible and to remove the above-mentioned nitrogenous metabolites. Water quality parameters (i.e., concentration of dissolved oxygen, ammonia, nitrites, nitrates, pH, general hardness, carbonate hardness, and temperature) were measured every two days. The temperature and oxygen concentration were determined using an Oxi 3310 oximeter (WTW, Poland), while pH was measured with a pH meter (Mettler Toledo, Switzerland). Colorimetric kits produced by Zoolek (Łódź, Poland) were used to determine ammonia (NH_3), nitrite (NO_2^-), and nitrate (NO_3^-) concentrations, as well as general hardness (GH) and carbonate hardness (KH). These kits were applied in our previous research (Bojarski et al., 2022 a, b, 2023 a, b). The results of the water quality analyses are presented in Table 1. The photoperiod was 14:10 (light : dark) during both acclimation and the treatment periods. The fish were fed with Aller Silver 3 mm (Aller Aqua), as described in our previous papers (Bojarski et al., 2022 a, 2023 a).

Table 1. Water parameters (mean ± SD) in the control and experimental groups

Parameter	Control group	Group COM1	Group COM2
Temp. (°C)	19.51±0.13	19.54±0.18	19.47±0.18
O_2 (mg/l)	6.52±0.40	6.48±0.41	6.38±0.41
pH	7.56±0.10	7.53±0.10	7.49±0.07
NH_3 (mg/l)	0.01±0.00	0.01±0.00	0.01±0.00
NO_2^- (mg/l)	0.02±0.03	0.02±0.03	0.02±0.02
NO_3^- (mg/l)	6.17±2.52	6.69±2.74	6.34±2.61
GH (°GH)	5.03±0.19	5.00±0.00	5.03±0.19
KH (°KH)	3.17±0.38	3.24±0.44	3.21±0.41

Biological material collection

After each exposure period (1, 3 and 10 days), 10 individuals from each group (C, COM1 and COM2) were used for the collection of biological material: blood was collected from 10 fish, while the organs (gills, liver and trunk kidney) were sampled from 6 randomly selected individuals. Blood was sampled from the caudal vein by an experienced fish haematologist with a needle and syringe into heparinized (Heparinum WZF, Polfa, Poland) tubes without the use of anaesthetic as it is well known that anaesthetics can affect haematological parameters (Bishkoul et al., 2015; Witeska et al., 2015, 2017; Rożyński et al., 2018) as well as plasma biochemical indicators (Velisek et al., 2009 b; Feng et al., 2011; Velisek et al., 2011; Lepic et al., 2014; Rożyński et al., 2018). Moreover, as we mentioned in our previous paper (Bojarski et al., 2022 b), the use of anaesthetics can be more stressful for common carp than a quick blood sampling without the application of pharmaceuticals. Some of the collected blood was subjected to haematological analysis immediately after sampling, while the rest of the blood was centrifuged (3000 g, 20 min) using a Mikro 200R centrifuge (Hettich, Germany) to obtain plasma. The plasma was frozen (–80°C) and was subjected to biochemical analysis after about two months of storage. After the blood sampling, the fish were euthanised by MS 222 (Merck, USA) applied at concentration of 500 mg/l, following Bojarski et al. (2022 a, 2023 a). The death of the fish was confirmed by decapitation. After this procedure, the organs were collected and preserved in 4% buffered formaldehyde.

Haematological analysis

To determine the red blood cell (RBC) count and the white blood cell (WBC) count, the blood was diluted 100 times with Hayem's solution (following Bojarski et al., 2022 b). The cells were then counted using a Bürker haemocytometer and a Delta Optical Evolution 300 microscope (100× magnification for erythrocytes and 200× for leukocytes). The erythrocytes were counted in an area of 0.2 mm^2, while the white blood cells were counted in an area of 4 mm^2 (following Bomski, 1983). Next, RBC

count and WBC count (per microlitre) were estimated using standard formulas (Bomski, 1983). For haematocrit (Ht) determination, glass capillary tubes were filled with blood to about 3/4 of their volume and then centrifuged (16 000 g, 5 min) using a Haematokrit 200 centrifuge (Hettich, Germany). Then, the percentage of the erythrocyte layer was measured with a standard reader. For determination of haemoglobin (Hb) concentration, the blood was mixed 1:250 with Drabkin's reagent (Chempur, Poland). The absorbance was read at 540 nm wavelength using a UV-1601PC UV-visible spectrophotometer (Shimadzu, Japan). The analysis of RBC count, WBC count, Hb concentration and Ht level was conducted within 8 hours of the end of blood collection. Derived erythrocyte parameters (i.e., MCV − mean corpuscular volume, MCH − mean corpuscular haemoglobin, and MCHC − mean corpuscular haemoglobin concentration) were calculated using standard formulas (Bomski, 1983). During the sampling, blood smears were made, which were dried for 3–4 days and then stained with Hem-Kolor kit (Stamar, Poland) according to the instructions provided by the manufacturer. Next, a leukogram (differential leukocyte count) and an erythrogram (i.e., characterization of erythrocyte morphology on the basis of analysis of a stained blood smear) were determined at 1000× magnification using a Nikon Eclipse Ci microscope. In each smear, 100 white blood cells and 300 red blood cells were inspected (according to Kondera et al., 2020, 2021). Analysis of the erythrogram included normal mature erythrocytes, normal immature erythrocytes (erythroblasts; shown in our previous article: Bojarski et al., 2023 b) and the following types of abnormal cells: erythrocytes showing changed cell shape (Figure 1), erythrocytes showing altered nucleus shape (Figure 2), erythrocytes exhibiting cytoplasm vacuolisation (Figure 3), and haemolysed erythrocytes (Figure 4). The digital images showing the abnormal red blood cells were acquired using a Nikon DS-Fi1c camera and NIS-Elements 4.12.01 software. Each haematological determination was conducted for n=10.

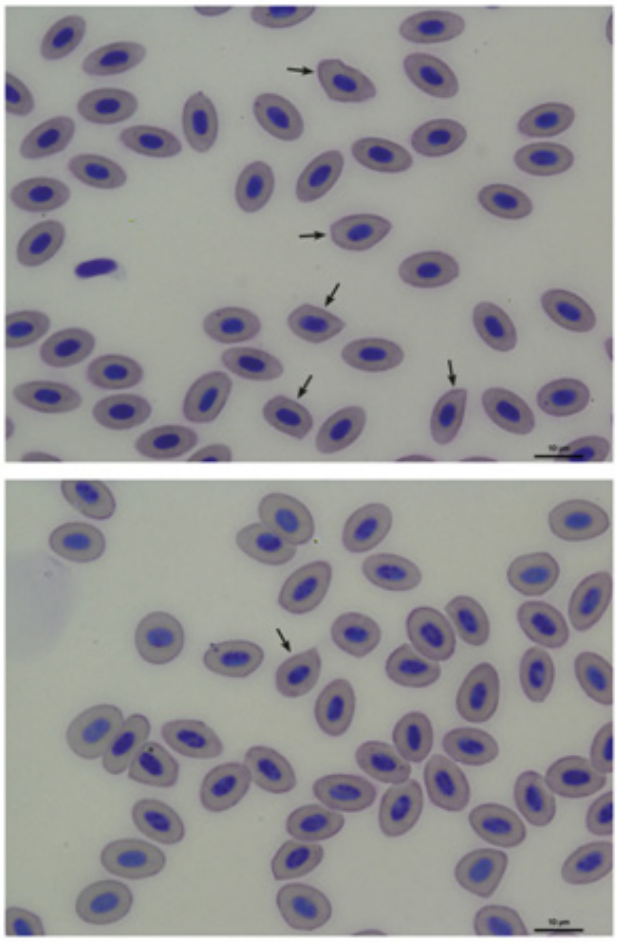


Figure 1. Abnormal cell shape of common carp red blood cells (erythrocytes with abnormal cell shape are indicated with arrows)

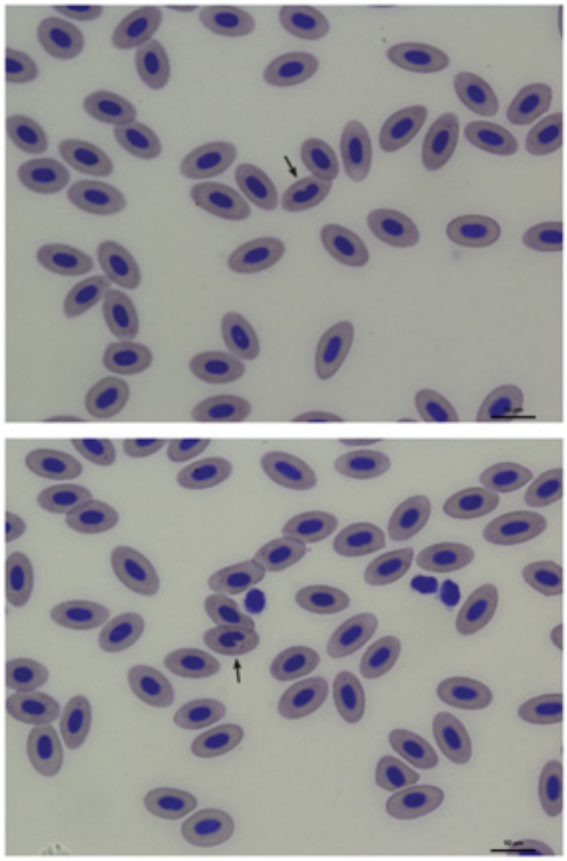


Figure 2. Abnormal nucleus shape of common carp red blood cells (erythrocytes with abnormal nucleus shape are indicated with arrows)

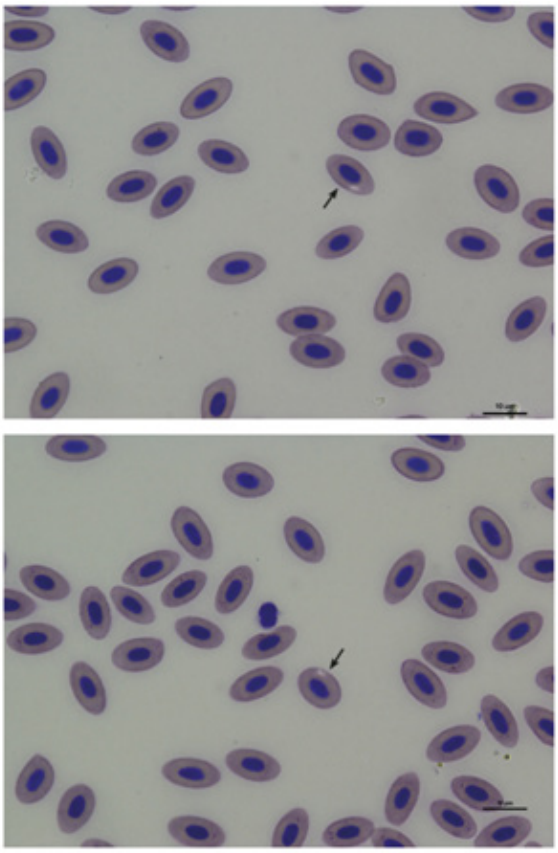


Figure 3. Vacuolization of common carp red blood cells (vacuolated erythrocytes are indicated with arrows)

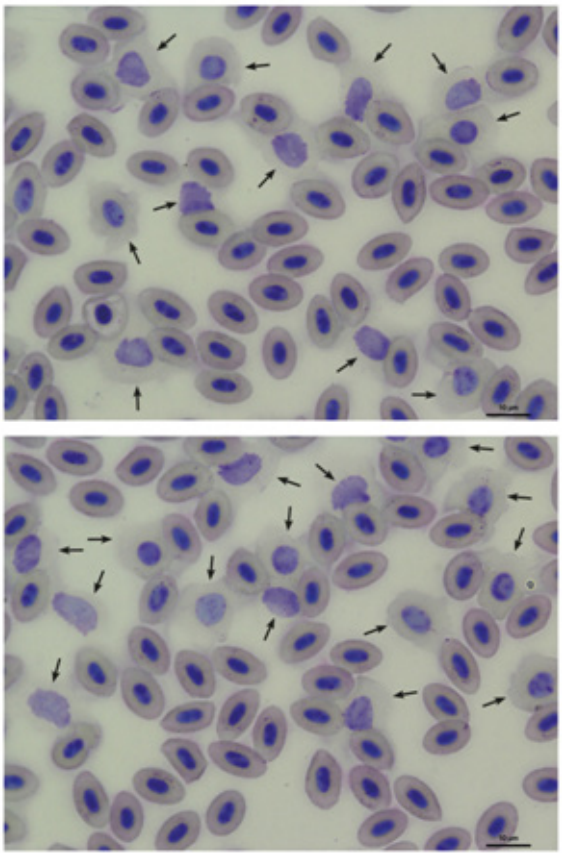


Figure 4. Haemolysis of common carp red blood cells (haemolysed erythrocytes are indicated with arrows)

Biochemical analysis

The blood plasma was used to determine the following biochemical parameters: glucose (Glu), total protein (TP), triglyceride (Tg) and total cholesterol (TC) concentration, as well as alanine aminotransferase (ALT) activity. All the biochemical indices tested in the current study were determined using BioSystems kits (Barcelona, Spain) according to the instructions provided by the manufacturer; the only modification of the procedure was that ALT activity calculations were performed on the basis of two absorbance measurement points instead of four points, following Bojarski et al. (2022 a). Determination of non-enzymatic biochemical parameters was conducted with an EPOCH microplate reader (BioTek Instruments, USA). For measurement of ALT activity, a V-730 UV-visible spectrophotometer (Jasco, Japan) was used. The principle of the Glu, TP and TC concentration determination method has been described previously (Bojarski et al., 2021). The determination of ALT activity was based on two chemical reactions and the decrease in the amount of NADH accompanying the second of them. In the first reaction, ALT catalyses the transfer of the amino group from alanine to 2-oxoglutarate, forming pyruvate and glutamate; in the next reaction, lactate dehydrogenase (LDH) converts pyruvate to lactate (Schumann et al., 2002). The NADH decrease was measured at a wavelength of 340 nm. Triglyceride concentration was determined based on previously described chemical reactions (Fossati and Prencipe, 1982). Triglycerides were enzymatically hydrolysed to glycerol and fatty acids by lipase. The concentration of the released glycerol was then assayed in reactions catalysed by glycerol kinase and glycerol-phosphate oxidase in a system that generates hydrogen peroxide; the final product was coloured quinoneimine, which is generated from hydrogen peroxide and other reagents under catalytic action of peroxidase. Absorbance of quinoneimine was determined at 500 nm. Each biochemical determination was conducted for n=10.

Histopathological analysis

Standard light microscopy procedures were used for histopathological examination. Samples of gills, liver and trunk kidney tissues were embedded in Paraplast Regular (Sigma, St. Louis, MO, USA). Transverse sections of 6 µm were made on Zeiss Hyrax M55 microtome and affixed to the glass slides. The slides were then treated with haematoxylin solution according to Delafield (Chempur, Poland; time of staining: 5 minutes) and, after washing in tap water, with eosin solution (time of staining: 5 minutes) (H+E) for general cytology. The slides were subjected to blind analysis using a light microscope (Nikon Eclipse E600), and the digital images were acquired and processed using a Nikon DS-Fi1c camera and

NIS-Elements F software. The analysis focused on the overall histological structure of the organs and detection of potential abnormalities. The methodological details of the histological procedures are provided in our previous article (Bojarski et al., 2022 a).

Statistical analysis

For statistical analysis of the experimental data, one-way ANOVA was used, followed (if significant) by the Tukey HSD test; alternatively, we used the Kruskal-Wallis test, then (if significant) the Dunn test with Bonferroni correction. The compliance of the data with the assumptions of the one-way ANOVA model was verified using the Shapiro-Wilk test of normality and the Levene homogeneity of variance test. On the basis of the verification of the assumptions mentioned above in the case of the haematological parameters (including neutrophil to lymphocyte ratio, except the leukogram and the erythrogram) and plasma biochemical indicators, it was concluded that the data met the assumptions of the one-way ANOVA model, therefore this model was applied. In the case of the erythrogram and the leukogram, it was concluded that the data did not meet the assumptions of the one-way ANOVA model, therefore the Kruskal-Wallis and Dunn tests were used as mentioned above. The statistical analysis was carried out using R software (R Foundation for Statistical Computing, version 4.1.3). The results of the statistical analysis are presented in detail in the Results section, while Tables 2–7 present only statistically significant differences found between each experimental group and the control group.

Table 2. Haematological changes in common carp (*Cyprinus carpio*) after 1 day of exposure to Command 480 EC (mean ± SD; significant differences compared to the control values are marked with asterisks; * 0.01 ≤ P.adj < 0.05; ** 0.001 ≤ P.adj < 0.01; *** 0 ≤ P.adj < 0.001)

Parameter	Control group	Group COM1	Group COM2
RBC (10^6/µl)	1.54±0.27	1.62±0.23	2.05±0.21 ***
Ht (%)	29.05±2.08	27.10±2.35	26.23±2.02 *
Hb (g/dl)	7.53±0.89	7.34±0.77	7.75±0.69
MCV (fl)	194.70±43.05	170.87±29.27	128.83±13.65 ***
MCH (pg)	50.26±10.96	46.33±8.76	38.10±4.43 **
MCHC (g/dl)	25.94±2.61	27.07±1.47	29.55±0.85 ***
MNE (%)	97.47±0.95	97.97±0.88	97.30±1.30
EAS (%)	1.73±0.91	1.17±0.69	1.87±1.23
EAN (%)	0.23±0.16	0.20±0.23	0.10±0.16
EVC (%)	0.13±0.17	0.37±0.43	0.10±0.16
HE (%)	0.43±0.61	0.27±0.49	0.63±0.85
EB (%)	0.00±0.00	0.03±0.11	0.00±0.00
WBC (10^3/µl)	26.25±5.60	26.70±8.94	32.43±6.09
Lym (%)	93.40±4.12	93.90±4.07	91.50±2.95
Seg (%)	2.80±2.10	2.80±2.49	3.30±1.34
ImNeu (%)	3.70±2.50	3.10±1.73	5.10±2.92
Mono (%)	0.10±0.32	0.20±0.42	0.10±0.32
NLR	0.07±0.05	0.06±0.04	0.09±0.03

RBC – red blood cell count; Ht – haematocrit value; Hb – haemoglobin concentration; MCV – mean corpuscular volume; MCH – mean corpuscular haemoglobin; MCHC – mean corpuscular haemoglobin concentration.

MNE – mature normal erythrocytes; EAS – erythrocytes of altered cell shape; EAN – erythrocytes of altered nucleus shape; EVC – erythrocytes of vacuolated cytoplasm; HE – haemolysed erythrocytes; EB – erythroblasts.

WBC – white blood cell count; Lym – lymphocytes; Seg – segmented neutrophils; ImNeu – immature neutrophils; Mono – monocytes; NLR – neutrophil to lymphocyte ratio.

Table 3. Haematological changes in common carp (*Cyprinus carpio*) after 3 days of exposure to Command 480 EC (mean ± SD; significant differences compared to the control values are marked with asterisks; * 0.01 ≤ P.adj < 0.05; ** 0.001 ≤ P.adj < 0.01; *** 0 ≤ P.adj < 0.001)

Parameter	Control group	Group COM1	Group COM2
1	2	3	4
RBC (10^6/µl)	1.48±0.20	1.64±0.27	1.81±0.25 *
Ht (%)	30.18±2.37	27.55±2.28 *	26.60±1.31 **
Hb (g/dl)	7.68±0.61	7.56±0.45	7.69±0.49
MCV (fl)	207.38±33.17	170.62±22.59 *	149.89±22.04 ***
MCH (pg)	52.86±8.75	46.94±6.80	43.45±7.48 *
MCHC (g/dl)	25.48±1.21	27.51±1.47 **	28.91±1.49 ***

Table 3 – contd.

1	2	3	4
MNE (%)	97.33±0.70	97.00±0.86	92.57±1.56 ***
EAS (%)	1.93±0.75	2.50±0.77	6.70±2.08 ***
EAN (%)	0.17±0.18	0.27±0.41	0.23±0.22
EVC (%)	0.10±0.16	0.10±0.16	0.13±0.28
HE (%)	0.40±0.54	0.10±0.16	0.37±0.95
EB (%)	0.07±0.14	0.03±0.11	0.00±0.00
WBC (10^3/µl)	23.13±5.48	19.98±3.57	20.15±2.92
Lym (%)	97.40±2.50	93.40±2.80 *	92.90±6.26
Seg (%)	1.00±1.25	3.60±2.46 *	3.60±3.86
ImNeu (%)	1.40±1.78	2.90±2.08	3.20±2.94
Mono (%)	0.20±0.42	0.10±0.32	0.30±0.48
NLR	0.03±0.03	0.07±0.03	0.08±0.08

RBC – red blood cell count; Ht – haematocrit value; Hb – haemoglobin concentration; MCV – mean corpuscular volume; MCH – mean corpuscular haemoglobin; MCHC – mean corpuscular haemoglobin concentration.

MNE – mature normal erythrocytes; EAS – erythrocytes of altered cell shape; EAN – erythrocytes of altered nucleus shape; EVC – erythrocytes of vacuolated cytoplasm; HE – haemolysed erythrocytes; EB – erythroblasts.

WBC – white blood cell count; Lym – lymphocytes; Seg – segmented neutrophils; ImNeu – immature neutrophils; Mono – monocytes; NLR – neutrophil to lymphocyte ratio.

Table 4. Haematological changes in common carp (*Cyprinus carpio*) after 10 days of exposure to Command 480 EC (mean ± SD; significant differences compared to the control values are marked with asterisks; * $0.01 \leq P.adj < 0.05$; ** $0.001 \leq P.adj < 0.01$)

Parameter	Control group	Group COM1	Group COM2
RBC (10^6/µl)	1.83±0.31	1.68±0.21	1.43±0.24 **
Ht (%)	30.25±1.81	27.90±1.63 *	26.85±2.60 **
Hb (g/dl)	7.56±0.42	7.14±0.44	7.12±0.63
MCV (fl)	169.56±30.56	168.51±20.17	194.89±47.01
MCH (pg)	42.29±6.96	43.18±5.62	51.79±13.41
MCHC (g/dl)	25.02±1.22	25.60±0.92	26.60±1.66 *
MNE (%)	95.77±1.20	96.27±1.65	96.77±1.43
EAS (%)	3.73±1.19	2.77±1.66	2.50±1.01
EAN (%)	0.20±0.17	0.23±0.27	0.17±0.18
EVC (%)	0.03±0.11	0.20±0.23	0.07±0.14
HE (%)	0.07±0.14	0.20±0.42	0.50±0.92
EB (%)	0.20±0.32	0.33±0.63	0.00±0.00
WBC (10^3/µl)	32.23±4.60	33.60±6.36	27.55±4.80
Lym (%)	94.40±2.22	95.10±2.77	93.70±1.95
Seg (%)	2.90±1.29	2.90±1.79	2.90±1.29
ImNeu (%)	2.50±1.35	1.90±1.29	3.00±1.70
Mono (%)	0.20±0.42	0.10±0.32	0.40±0.52
NLR	0.06±0.03	0.05±0.03	0.06±0.02

RBC – red blood cell count; Ht – haematocrit value; Hb – haemoglobin concentration; MCV – mean corpuscular volume; MCH – mean corpuscular haemoglobin; MCHC – mean corpuscular haemoglobin concentration.

MNE – mature normal erythrocytes; EAS – erythrocytes of altered cell shape; EAN – erythrocytes of altered nucleus shape; EVC – erythrocytes of vacuolated cytoplasm; HE – haemolysed erythrocytes; EB – erythroblasts.

WBC – white blood cell count; Lym – lymphocytes; Seg – segmented neutrophils; ImNeu – immature neutrophils; Mono – monocytes; NLR – neutrophil to lymphocyte ratio.

Table 5. Biochemical changes in common carp (*Cyprinus carpio*) after 1 day of exposure to Command 480 EC (mean ± SD; no significant differences in comparison to the control values were detected)

Parameter	Control group	Group COM1	Group COM2
TP (g/l)	30.96±3.09	31.17±3.49	31.38±2.49
Glu (mg/dl)	157.74±37.99	146.64±37.57	181.76±29.61
Tg (mg/dl)	164.37±52.04	150.70±20.92	137.22±23.07
TC (mg/dl)	143.80±25.04	146.18±21.54	146.77±28.35
ALT (U/l)	32.15±11.28	31.86±11.59	30.77±11.53

TP – total protein; Glu – glucose; Tg – triglycerides; TC – total cholesterol; ALT – alanine aminotransferase.

Table 6. Biochemical changes in common carp (*Cyprinus carpio*) after 3 days of exposure to Command 480 EC (mean ± SD; significant differences compared to the control values are marked with asterisks; * 0.01 ≤ P.adj < 0.05)

Parameter	Control group	Group COM1	Group COM2
TP (g/l)	31.71±7.94	30.09±3.41	28.51±2.97
Glu (mg/dl)	168.15±45.79	176.04±52.05	152.92±33.14
Tg (mg/dl)	204.18±33.67	166.52±25.10 *	183.42±37.43
TC (mg/dl)	161.51±23.80	147.64±30.41	149.24±24.50
ALT (U/l)	39.27±6.96	40.33±19.40	32.10±9.28

TP – total protein; Glu – glucose; Tg – triglycerides; TC – total cholesterol; ALT – alanine aminotransferase.

Table 7. Biochemical changes in common carp (*Cyprinus carpio*) after 10 days of exposure to Command 480 EC (mean ± SD; significant differences compared to the control values are marked with asterisks; ** 0.001 ≤ P.adj < 0.01)

Parameter	Control group	Group COM1	Group COM2
TP (g/l)	27.67±2.99	27.19±2.84	26.41±5.14
Glu (mg/dl)	158.51±51.33	168.36±44.43	112.47±25.31
Tg (mg/dl)	205.38±54.90	171.27±13.25	143.92±34.86 **
TC (mg/dl)	129.43±19.50	133.23±14.95	121.74±22.60
ALT (U/l)	33.61±4.60	30.32±6.56	31.98±6.26

TP – total protein; Glu – glucose; Tg – triglycerides; TC – total cholesterol; ALT – alanine aminotransferase.

Results

No mortalities were recorded during the current experiment. Moreover, no signs of disease or distress were observed.

Haematological parameters

Tables 2–4 present statistically significant differences in haematological results between each experimental group and the control group. The following description also shows differences between groups COM1 and COM2; it also contains the P-values obtained in the statistical analysis.

The fish exposed to Command at the higher concentration showed higher RBC count after 1 and 3 days of treatment in comparison to the fish from the control group. The differences were statistically significant (P.adj=0.000168 and P.adj=0.0146). Moreover, RBC count after 1 day of exposure, determined in the case of individuals in group COM2, was higher as compared to group COM1. This difference was statistically significant (P.adj=0.00102). However, longer exposure (10 days) resulted in an RBC count decrease in group COM2 in comparison to the control; the difference was significant at P.adj=0.00402.

After 1 day of exposure, the fish exposed to Command at the higher tested concentration exhibited lower Ht level in comparison to control individuals. This difference was statistically significant at P.adj=0.0181. Exposure lasting 3 days led to a decrease in Ht value in both experimental groups, i.e., COM 1 and COM2, compared to the control group; the differences were significant (P.adj=0.0207 and P.adj=0.00157, respectively). Similarly, after 10 days of treatment the experimental fish (group COM1 and group COM2) showed lower Ht values in comparison to the control animals; the differences were significant at P.adj=0.0421 and P.adj=0.0027, respectively.

In the case of Hb concentration, no significant differences were found between the individual groups, regardless of the exposure time (P=0.506 for 1 day, P=0.822 for 3 days, P=0.108 for 10 days).

The fish exposed to the herbicide at the higher concentration showed a decrease in MCV, as compared to the control value after 1 day of exposure. This difference was statistically significant (P.adj=0.000176). Moreover, the animals from group COM2 showed lower MCV than the fish from group COM1; this difference was significant at P.adj=0.0145. After 3 days of treatment, the fish in the experimental groups (COM1 and COM2) showed lower MCV than the value detected in the case of the control individuals (P.adj=0.0118 and P.adj=0.000127, respectively). No significant changes in MCV were found after 10 days of exposure (P=0.171).

Short-term (1 day) exposure caused a decrease in MCH in the fish exposed to Command at the higher tested concentration in comparison to the control individuals. This difference was statistically significant (P.adj=0.00943). Similarly, after 3 days of treatment, the fish in group COM2 exhibited decreased MCH in comparison to the control individuals; this difference was significant at P.adj=0.029. After 10 days of exposure, no significant changes in MCH were found (P=0.058).

After 1 day of exposure, an increase in MCHC in the fish treated with the tested herbicide at the higher con-

centration (group COM2) in comparison to the control fish was detected. Moreover, the fish from group COM2 showed higher MCHC as compared to those from group COM1. These differences were statistically significant (P.adj=0.000342 and P.adj=0.0127, respectively). After 3 days of treatment, the fish from both experimental groups showed higher MCHC than the control specimens. The difference between group COM1 and the control group was significant at P.adj=0.0086, while the difference between group COM2 and the control was significant at P.adj=0.0000234. After 10 days of treatment, the fish from group COM2 still had higher MCHC in comparison to the control individuals; this difference was significant at P.adj=0.0309.

No significant changes in WBC count were detected after 1 day and 3 days of treatment (P=0.11 and P=0.179). However, after 10 days of exposure, this haematological parameter in the fish treated with Command at the lower concentration was higher in comparison to the individuals from group COM2; this difference was statistically significant at P.adj=0.0431.

The leukogram changed statistically significantly only after 3 days of exposure. It was detected that the fish from group COM1 had a lower percentage of lymphocytes and a higher percentage of segmented neutrophils in comparison to the control individuals. These differences were significant at P.adj=0.0210 and P.adj=0.0219, respectively. No significant changes in the percentage of lymphocytes or segmented neutrophils were found after 1 day (P=0.246, P=0.695) and 10 days (P=0.339, P=0.991) of exposure.

The percentage of immature neutrophils as well as the percentage of monocytes did not differ significantly, regardless of the exposure time (P=0.278 and P=0.757 for 1 day; P=0.115 and P=0.547 for 3 days; P=0.262 and P=0.283 for 10 days).

Similarly, in the case of NLR (neutrophil to lymphocyte ratio), no significant differences were found between the analysed groups, regardless of the sampling time (P=0.302 for 1 day, P=0.081 for 3 days, P=0.611 for 10 days).

As in the case of the leukogram, statistically significant differences in the erythrogram were observed only after 3 days of treatment. The fish from group COM2 showed a lower percentage of normal mature red blood cells in comparison to the fish in the control group. Moreover, the individuals from group COM2 had less normal mature erythrocytes than the animals from group COM1. These differences were significant at P.adj=0.0000855 and P.adj=0.00143, respectively. No significant changes in this haematological parameter were found after 1 and 10 days of exposure (P=0.389 and P=0.242, respectively).

It was also found that the fish exposed to Command (3 days) at the higher tested concentration exhibited a higher percentage of erythrocytes with altered cell shape in comparison to the control individuals. Moreover, the individuals in group COM2 showed a higher percentage of this type of erythrocyte in comparison to the fish from group COM1. These differences were significant at P.adj=0.000194 and P.adj=0.0229, respectively. No significant changes in this parameter were found after 1 and 10 days of exposure (P=0.328 and P=0.113, respectively).

The percentage of erythrocytes showing altered nucleus shape, the percentage of red blood cells exhibiting vacuolated cytoplasm, and the percentage of haemolysed erythrocytes did not differ significantly, regardless of the exposure time (P=0.239, P=0.195 and P=0.569 for 1 day; P=0.827, P=0.974 and P=0.217 for 3 days; P=0.884, P=0.102 and P=0.451 for 10 days).

Similarly, no statistically significant changes in the percentage of erythroblasts were detected, regardless of the sampling point (P=0.368 for 1 day, P=0.342 for 3 days and P=0.11 for 10 days).

Biochemical parameters

Tables 5–7 present statistically significant differences in plasma biochemical results between each experimental group and the control group. The following description also shows differences between groups COM1 and COM2 and contains P-values obtained in the statistical analysis.

The concentration of TP in the analysed fish did not differ among the groups, regardless of the exposure time (P=0.954, P=0.411 and P=0.758 respectively for 1, 3 and 10 days of treatment).

The concentration of Glu in the tested fish did not differ among the groups after 1 and 3 days of exposure (P=0.093 and P=0.504, respectively). However, after 10 days of treatment the concentration determined in the animals from group COM1 was higher as compared to the individuals in group COM2; this difference was statistically significant at P.adj=0.0158.

The concentration of Tg detected after 1 day of exposure did not differ significantly among the analysed groups (P=0.24). After 3 days of exposure, the concentration of Tg recorded in the fish from group COM1 was lower than the value found in the case of the control animals, while longer (10 days) treatment resulted in a decrease in this biochemical parameter in the fish in group COM2 (in comparison to the specimens from the control group). These differences were significant at P.adj=0.0391 and P.adj=0.00362, respectively.

The concentration of TC in the analysed fish did not differ among the groups, regardless of the sampling time (P=0.962, P=0.449 and P=0.41 respectively for 1, 3 and 10 days of exposure).

Similarly, the activity of ALT did not differ among the groups (P=0.96 for 1 day, P=0.324 for 3 days and P=0.466 for 10 days of exposure).

Histopathology

No visible macroscopic signs of intoxication were observed in the current study. The results of the histopathological analysis are presented in Figures 5–7.

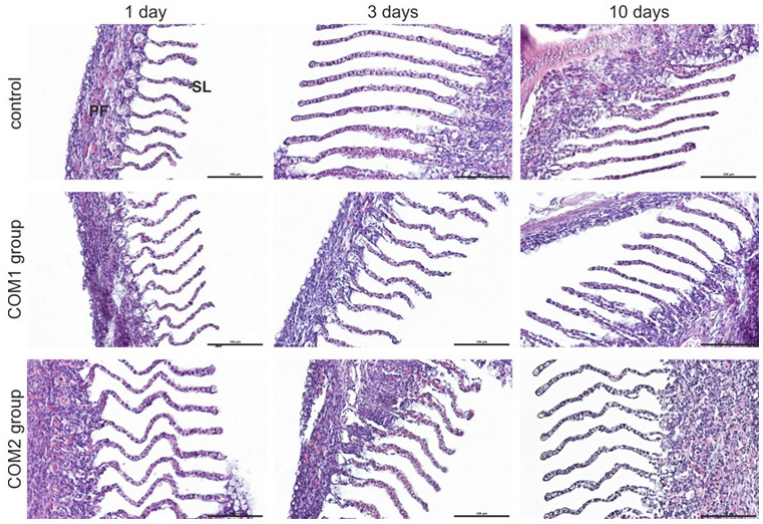


Figure 5. Photomicrographs of the gills of the common carp in the control and experimental groups. PF – primary filament; SL – secondary lamellae. Haematoxylin-eosin staining. Scale bars: 100 µm. No histopathological changes were found

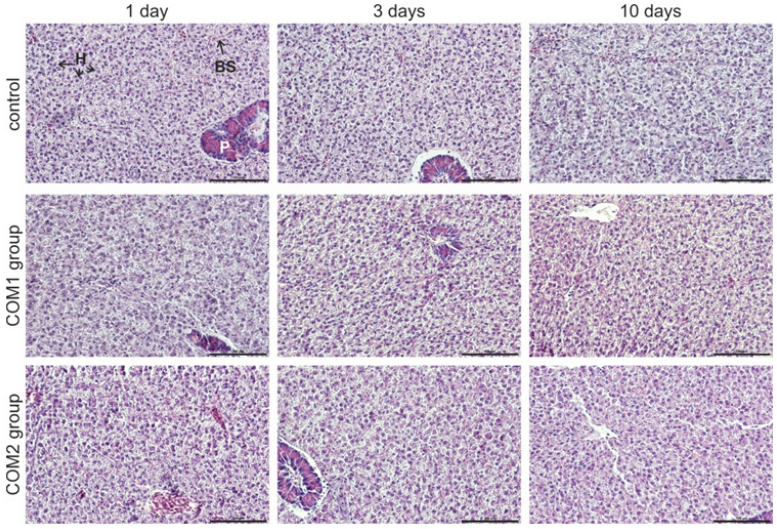


Figure 6. Photomicrographs of the liver of the common carp in the control and experimental groups. H – hepatocytes; BS – blood sinusoid; P – pancreas. Haematoxylin-eosin staining. Scale bars: 100 µm. No histopathological changes were found

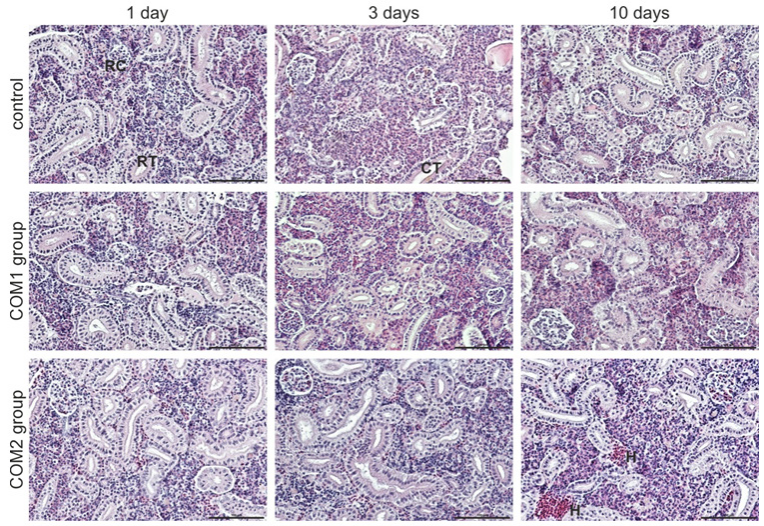


Figure 7. Photomicrographs of the trunk kidney of the common carp in the control and experimental groups. RC – renal corpuscle; RT – renal tubule; CT – collecting tubule; H – haemorrhage. Haematoxylin-eosin staining. Scale bars: 100 µm

The histological image of the gills was the same in all specimens, regardless of the analysed group of fish and time of exposure. The gills presented typical structure, with primary and secondary lamellae lined with epithelium (Figure 5). None of pathological changes typical of herbicide exposure (i.e., hypertrophy and hyperplasia of the epithelium, lamellar lifting and fusion, necrosis of epithelial cells) were detected.

The microstructure of the liver was typical: polygonal hepatocytes with homogenous cytoplasm and centrally located nucleus were recorded (Figure 6). Blood sinusoids that contained erythrocytes and formed communication channels between the hepatocytes were observed. The experimental fish did not differ from the control ones and did not show any symptoms of intoxication in liver tissue (e.g., vacuolization of hepatocyte cytoplasm, nuclear alterations in hepatocytes, haemorrhage, necrosis of the liver tissue).

The histological image of the trunk kidney was also typical: nephrons with a renal corpuscle (containing glomerulus), renal tubule, collecting tubule and interstitial tissue were observed. Generally, no pathological changes typical of pesticide exposure, such as glomerular degeneration, atrophy of the tubules or tubular necrosis, were detected in the exposed fish. However, extensive haemorrhaging was noted in the case of one individual out of six specimens from group COM2 that were analysed after 10 days of treatment (Figure 7).

Discussion

As was mentioned in the Introduction of this article, haematological analysis is one of the basic tools applied in evaluation of the toxicity of chemicals. Toxicants present in the water environment can affect haematological parameters in fish, including the number of red blood cells. As previously shown by Bojarski and Witeska (2020), exposure of fish to herbicides may result in both an increase in RBC count (which indicates a compensatory response) and a decrease in the number of erythrocytes in the blood (suggesting an anaemic response). In the present study, short-term exposure (1 and 3 days) led to an increase in the number of red blood cells in fish from group COM2, which could be a result of the release of red blood cells from the spleen (erythrocyte storage organ) or/and the head kidney (hematopoietic organ). Longer treatment (10 days) resulted in a reduction in RBC count observed in fish in the same group. This indicates a compensatory mechanism followed by an anaemic response. Regardless of the concentration of the tested herbicide and the exposure time, a decrease in haematocrit was observed (except for fish from group COM1 after 1 day of treatment), which seems to confirm the assumption regarding an anaemic response. Similarly, *Rhamdia quelen* exposed to another commercial clomazone formulation (Gamit, FMC, USA) showed lower haematocrit in comparison to the value detected in the control fish (Crestani et al., 2006). Also, other herbicides, such as atrazine (Blahova et al., 2014; Popoola, 2018), 2,4-D (Soni et al., 2018), oxadiazon-based formulation Ronstar® (Oluah et al., 2020), and some glyphosate-based herbicides, such as Sunsate® (Okomoda et al., 2013) and Roundup (Salbego et al., 2010), caused a decrease in haematocrit in fish. The literature analysis we conducted indicates that an increase in haematocrit in fish exposed to herbicides has been observed less frequently than a decrease (Husak et al., 2014; Kondera et al., 2018). In order to determine whether a given herbicide caused an anaemic response or a compensatory response, other red blood cell parameters, especially Hb concentration, should also be considered. According to Witeska (2015), the mechanisms of anaemia are different, but they all produce similar effects, i.e., a decrease in Hb concentration and RBC count, which results in reduced oxygen supply to the tissues and thus deterioration of fish health. Witeska added that MCV, MCH and MCHC in anaemic fish also sometimes change, which indicates alterations in the size and staining properties of red blood cells. In the present study, fish exposed to Command showed a reduction in MCV (except the last sampling), indicating microcytosis. On the other hand, *Prochilodus lineatus* exposed to a clomazone formulation (Gamit® 500) showed no changes in MCV (Pereira et al., 2013), while Roundup-treated common carp exhibited an increase in this parameter (Bojarski et al., 2022 a). In the present study, microcytosis was accompanied by an increase in the mean corpuscular haemoglobin concentration (MCHC) in exposed fish, which indicates a reaction to prevent hypoxia; however, a decrease in erythrocyte volume was also associated with poikilocytosis (group COM2 after 3 days of treatment). Little data about the effects of herbicides on the morphology of fish red blood cells has been published so far. The study conducted by Bojarski et al. (2018 a) showed that exposure of common carp to pendimethalin resulted in increased percentage of erythrocytes showing altered cell shape, while simultaneous exposure to pendimethalin and ethofumesate led to a higher percentage of nucleus malformations. Ateeq et al. (2002) demonstrated that exposure of *Clarias batrachus* to 2,4-D or butachlor caused an increase in numbers of altered erythrocytes. These authors stated that echinocytes accompanied by altered nuclei and vacuoles were typical of 2,4-D, while anisochromasia and anisocytosis were characteristic of butachlor. Moreover, Fathy et al. (2019) observed poikilocytosis in *Oreochromis niloticus* treated with various herbicide formulations, i.e., Harness 84% EC, Nomine 2% SL, Basagran 48% AS, Inpul 75% WG, Reto 60% WG, and Queen 75% WG. To the best of our knowledge, the mechanisms of changes in the shape of fish erythrocytes are not yet fully understood. According to Witeska (2013), deformation of fish erythrocytes are an effect of cell membrane deterioration. We failed to find scientific data on the relationship between fish erythrocyte deformations and the oxygen-carrying capacity of these cells, but it can be assumed that significantly

altered erythrocytes transport oxygen less efficiently in comparison to normal ones.

As shown above, common carp exposed to Command showed lots of changes in red blood cell parameters. It is worth noting that the commonly determined basic and derived erythrocyte indices turned out to be more sensitive to the tested herbicide than the erythrogram, i.e., morphology of erythrocytes determined on the basis of blood smear analysis. Similarly to the erythrogram, white blood cell parameters exhibited high stability. The total number of white blood cells in experimental fish did not differ significantly from the leukocyte count in control fish, regardless of the concentration of the tested herbicide and the exposure time. However, neutrocytosis and lymphopenia were detected in fish exposed to Command at the lower concentration after 3 days of treatment, which may indicate a stress reaction or inflammation. However, the presence of inflammation may be questioned due to the lack of increase in WBC count in fish from this group in comparison to the number of leukocytes in control individuals. Brum et al. (2014) revealed that *Rhamdia quelen* obtained from a clomazone-contaminated site of the Madre River (Southern Brazil) exhibited an increased number of monocytes, which in the authors' opinion could suggest a possible defence response as a result of chronic exposure to this herbicide. No significant difference was observed in the number of neutrophils, therefore it can be assumed that changes in the leukogram of fish exposed to clomazone (or clomazone-based formulations) depend to a large extent on the fish species. On the other hand, it has been documented that *Oncorhynchus mykiss* subjected to butachlor exposure exhibited increased percentage of neutrophils and decreased lymphocyte percentage in comparison to the control (Ahmadivand et al., 2014), which is consistent with our own results and may suggest a reaction that is non-species specific and independent of the herbicide type.

Biochemical parameters determined in plasma or serum are one of basic markers in laboratory diagnostics used in assessment of the health and welfare of fish. The directions of changes in selected blood biochemical parameters (e.g., glucose, cholesterol, triglyceride or lactate concentration) in fish subjected to stress conditions have been listed in a review by Seibel et al. (2021). Biochemical parameters tested in the current study were much less sensitive to the effect of the tested herbicide than haematological indicators. The only significant change in the biochemical parameters in comparison to the control was reduction of triglyceride concentration in fish after 3 days (group COM1) and 10 days (group COM2) of exposure to the herbicide. We failed to find scientific articles showing that herbicides cause reduction in triglyceride concentration in plasma or serum obtained from fish blood. However, Kondera et al. (2018) demonstrated that common carp treated with Roundup exhibited increased blood triglyceride concentration and cholesterol level. It is well known that the liver is involved in various biochemical processes, including the metabolism of fats. The results of the biochemical analysis presented in the current study may suggest lipid metabolism disorders. Georgieva et al. (2023) demonstrated that exposure of common carp to pesticides of various types led to fatty degeneration in the liver. It cannot be ruled out that these phenomena are connected, but the type of histological procedure we used in our experiment does not allow this presumption to be verified. In our opinion, application of fat-specific histological techniques could be an interesting continuation of the research that we have conducted. To the best of our knowledge, there is very little scientific data on the effect of clomazone-based herbicides on the biochemical parameters of fish blood. Cattaneo et al. (2012) revealed that exposure to Gamit 500 CE (commercial clomazone formulation) led to increased glucose concentration and decreased total protein level; it also resulted in changes in lactate concentration in blood plasma of common carp. These authors noted that the changes in glucose and protein concentrations may indicate a long-term response against the applied herbicide. Crestani et al. (2006) demonstrated that exposure of *Rhamdia quelen* to Gamit resulted in increased concentration of plasma glucose, lactate, as well as plasma ALT and AST activity. Interestingly, the total protein level decreased or increased, depending on the exposure duration: 24 hours of treatment led to a decrease, while longer exposure (48 and 96 hours) caused an increase in this biochemical parameter. It should be mentioned that other herbicides can also cause changes in blood biochemical indicators in fish. For example, our previous study demonstrated that common carp treated with Chwastox Extra® 300 SL (MCPA-based formulation) exhibited transient hypoproteinemia and a temporary decrease in ALT activity (Bojarski et al., 2023 a). Moreover, common carp treated with Roundup showed minor and temporary changes in blood biochemical parameters: increased glucose concentration and cholesterol level (Bojarski et al., 2022 a). Thus, it was concluded that the biochemical parameters tested turned out to be less-sensitive markers of exposure to the tested herbicide in comparison to the analysed haematological indices (Bojarski et al., 2022 a). As previously mentioned, the same results were found in the case of the results of the current study. According to Bojarski and Witeska (2020), haematological and blood biochemical parameters are useful biomarkers in the evaluation of the physiological state of fish exposed to pesticides, but they are not specific markers of intoxication. These authors added that pathophysiological changes observed in pesticide-exposed fish depend on many factors, such as the type of active ingredient and its concentration, exposure duration, fish species, environmental conditions, etc.

In the current study, no histopathological lesions were detected in the studied organs of Command-treated common carp, with the exception of extensive haemorrhaging detected in one individual. Due to the fact that it was observed only in one specimen, it cannot be ruled out that this change occurred independently of exposure to the applied herbicide. The results of the current study

may indicate that blood parameters (especially haematological ones) are more-sensitive markers of exposure to Command than the microstructure of the analysed organs. In our previous experiments, fish of the same species treated with other herbicide formulations (Roundup® 360 Plus or Chwastox Extra® 300 SL) also showed no alterations in microstructure of the gills, liver and trunk kidney (Bojarski et al., 2022 a, 2023 a). Similarly, histopathological analysis of the liver microstructure of hybrid fish *Leiarius marmoratus* × *Pseudoplatystoma reticulatum* treated with Roundup Original® did not reveal pathological changes (de Moura et al., 2017). However, the vast majority of articles published on the influence of weed control agents on the microscopic structure of individual organs of fish show that herbicide exposure can lead to various types of histopathological lesions. For example, common carp treated with atrazine showed severe multifocal lamellar teleangiectasia and pooling of the blood, with formation of thrombi in the gills as well as hydropic-to-vacuolar degeneration of hepatocytes, dilatation of capillaries, and hyperaemia in the liver (Blahova et al., 2014). It has also been demonstrated that atrazine-treated *Ctenopharyngodon idella* exhibited lesions in the gills: epithelial hyperplasia, epithelial lifting, aneurism, desquamation, necrosis, etc. The hepatic injuries were described as swelling of hepatocytes, followed by karyolysis, karyohexis and dilation of sinusoids (Ahmad et al., 2021). Similarly, exposure of common carp to simazine has been shown to lead to necrotic changes in the liver and kidney. The necrosis in the liver was characterized by the presence of cellular debris associated with melanomacrophage centres and infiltration of inflammatory cells; steatosis was also detected. According to these authors, the most remarkable finding in the renal microstructure in simazine-treated individuals was globular eosinophilic foci (Oropesa et al., 2009). Moreover, *Carassius auratus* treated with metribuzin-based formulation (Sencor 70 WG) showed changes in microscopic kidney images, such as glomerular shrinkage, haemorrhages, as well as degradation and necrosis of the tubular epithelium (Husak et al., 2014). These authors concluded that induction of oxidative stress may be a possible mechanism of the toxic action of the herbicide formulation tested in their study. It has also been demonstrated that *Channa punctata* exposed to pendimethalin exhibits cellular hypertrophy, fusion of secondary lamellae, cellular degeneration and necrosis of epithelium in the gills. Hepatocellular damage, inflammatory cell infiltration, as well as dilation of sinusoids with blood congestion were detected in the liver of the exposed fish. Moreover, pendimethalin exposure resulted in lesions in the kidney, such as cellular hypertrophy or hydropic swelling of tubules and tubular necrosis with interstitial edema (Tabassum et al., 2016). The liver of *Clarias gariepinus* exposed to thiobencarb-based herbicide formulation (Citron® 50 EC) showed vacuolar degeneration and necrobiotic changes as well as karyopyknosis of hepatocytes. Infiltration of melanomacrophages and mononuclear cells in portal areas of the liver was also detected (Elias et al., 2020). *Channa striatus* treated with 2,4-D (2,4-dichlorophenoxyacetic acid) exhibited lesions in gills and liver. The gills of treated animals had lamellar degeneration, epithelial lifting and necrotic changes of epithelium. The alterations detected in the liver of exposed fish included loss of cellular architecture, necrosis, and accumulation of fat in parenchymal cells (Anusuya and Hemalatha, 2015). Ornamental fish *Trichogaster trichopterus* exposed to paraquat showed hyperplasia, fusion, and necrosis of gill lamellae. The liver of the treated specimens was enlarged. Moreover, vacuolar degeneration, hypertrophy, necrosis and bleeding were noted (Banaee et al., 2013). Furthermore, *Channa punctatus* exposed to Ozone (paraquat dichloride 24% SL) also showed pathological changes in the vital organs. The changes in the gills included curling of secondary lamellae, aneurysm, gill bridging, and enlargement of the cartilaginous core. The liver tissue exhibited pyknotic nuclei, large sinusoidal congestion, cell fusion, necrosis, etc. The changes in microscopic kidney structure included, for example, an increase in Bowman's space and necrosis of glomeruli (Badroo et al., 2020).

The results of research into the impact of herbicides on the microstructure of fish organs differ but some of the changes listed above are similar, regardless of the type of weed control agent or fish species. According to Bojarski et al. (2018 b), fish's reactions to intoxication with herbicides are nonspecific and toxic effects of herbicides depend on various factors, such as type and concentration of herbicide, duration of exposure, and fish species.

The mechanisms of toxic action of clomazone and clomazone-based formulations in the context of fish have not yet been explained. Although some data suggest that clomazone toxicity may be related to oxidative stress and decreased acetylcholinesterase activity (Cattaneo et al., 2012), the evidence in this regard is very limited.

Conclusion

Exposure to Command resulted in many changes in haematological parameters, but especially in erythrocyte indices. In the leukogram, only minor and transient changes suggesting stress reaction or inflammatory response were detected. The analysis of the blood biochemical parameters tested in the current research may suggest disorders in lipid metabolism. No considerable histopathological lesions were observed, which suggests that blood parameters are more sensitive markers of exposure to Command than the microstructure of the gills, liver and kidney. However, to confirm this conclusion it is necessary to carry out similar toxicological tests involving other species of fish. Further research, including more-detailed histological techniques, analysis of the activity of hematopoietic tissue, and analysis of a broad spectrum of biochemical parameters not tested in the current experiment, are necessary to explain the mechanisms of the observed changes. Due to the fact that scientific data on the toxicity of clomazone-based herbicide

formulations to fish are very limited, therefore we highly recommend conducting further research in this area.

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Ethics approval

The experiment was approved by the Local Ethics Committee for Animal Testing at the Medical University of Silesia in Katowice (Resolution No. 28/2021 of 19.05.2021).

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