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Hematological Alterations as a Response to Exposure to Selected Fungicides in Common Carp (*Cyprinus carpio* L.)*

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The aim of the present study was to assess the hematological response of common carp to fungicides and to determine recovery patterns in fungicide-free water. Fish were exposed to mancozeb, prochloraz or tebuconazole (at concentrations of 1.0, 1.0 and 2.5 mg l^{-1}, respectively) for 14 days followed by a 30-day recovery period. The following hematological parameters were examined after 1, 3 and 14 days of exposure as well as after recovery time: red blood cells (RBC), hematocrit (Hct), total hemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), total number of leukocytes (WBC) and leukograms. All analyzed parameters revealed alterations in relation to control samples. The pattern of these changes was irregular, showing either an increase or decrease at different time points of the experiment and not all observed differences were statistically significant. The most noticeable fungicide-specific changes were observed on the 1st and 14th days of chemical exposure. The majority of the parameters under investigation returned to the control levels after a detoxication period. However, some of the exerted effects were irreversible (Hb, MCH, MCHC and WBC for fish subjected to mancozeb; Hb, MCH, MCHC and monocyte count for fish subjected to prochloraz; Hct and monocyte number for fish subjected to tebuconazole). All of the observed hematological changes were not toxin-specific.

Key words: Mancozeb, prochloraz, tebuconazole, fish, blood parameters.

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Fungicides constitute a large group of pesticides which play an essential role in plant protection and production of large quantities of high quality crops that guarantee the economic viability of commercial agriculture (PROFFER *et al*. 2006). These compounds include substances of various chemical structure and diversified mechanisms of action. The most widely applied fungicides in Poland are derived from dithiocarbamate (mancozeb, metiram, thiuram, propineb), imidazole (imazalil,

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prochloraz) and triazole (cyproconazole, tebuconazole, propiconazole, epoxiconazole) (MINISTRY OF AGRICULTURE AND RURAL DEVELOPMENT OF THE REPUBLIC OF POLAND 2016) and constitute approximately 40% of all approved pesticides. Pesticides threaten the natural environment due to accidental penetration to surface water caused by spray drift, waste-water drainage and surface runoff (SADŁO & RUPAR 1991; SCHRIEVER & LIESS 2007). Fungicides are widely regarded as substances producing diverse effects in the aquatic environment. Dithiocarbamate derivatives are characterized by short-term residual effects as opposed to trialozes which are more resistant to oxidation and UV radiation (RÓŻAŃSKI 1992). Reference sources include scarce information regarding the occurrence of fungicide residues in surface waters. ANDREU-SÁNCHEZ *et al*. (2012) recorded the presence of tebuconazole in surface waters in Albufera Natural Park (Valencia, Spain) at a concentration of 5 μg l^{-1}. Research conducted on surface waters in Norway revealed propiconazole residues amounting to 0.1-10 μg l^{-1} (ALMLI *et al*. 2002). Finally, PADOVANI *et al*. (2006) detected tricyclazole (an equivalent of prochloraz) at a concentration of 10 μg l^{-1} in rice cultivation sites in Italy. Fungicides exhibit different levels of aquatic toxicity towards various organisms including fish. Due to quick fungicide penetration into the peripheral blood of fish (via gill epithelium) disruptions of hematological profiles are frequently utilized to study pathophysiological changes stimulated by these chemicals. The aim of the study was to determine effects produced by subtoxic concentrations of selected fungicides on various hematological parameters of common carp.

Material and Methods

Animals and experimental conditions

Animal research protocols were approved by the First Ethical Committee on Animal Experiments in Kraków, Poland (No. 124/2010). 150 individuals of common carp (*Cyprinus carpio* L.) with a mean weight of 60.00 ± 10 g were obtained from fish ponds at the Experimental Fisheries Station, Department of Ichthyology and Fisheries Sciences, University of Agriculture in Kraków. The fish were obtained during the summer fish harvest. Before commencement of the experiment fish underwent random clinical evaluation and verification for parasite occurrence. Then the fish were relocated to experimental tanks with water delivered at a continuous-flow and allowed to adapt to experimental conditions for a period of 1 month. During the experiment the fish were held in 300 liter aquariums in static conditions with the following water parameters: temperature 19.0 ± 2°C; pH 7-7,5; level of dissolved oxygen 7.20-9.11 mg l^{-1}; total hardness 16-18 °n; ammonium concentration 0.01-0.02 mg l^{-1}; nitrites concentration (NO_2^-) 0.5 mg l^{-1} and nitrates concentration (NO_3^-) 10-12 mg l^{-1}. During the course of the study animals were fed once a day with barley flakes and Chironomidae larvae.

Chemicals tested

The experiment included the following fungicide active ingredients: mancozeb, prochloraz and tebuconazole. The selection of fungicides was based on their potential to control a broad spectrum of fungal diseases and common application in agriculture. Mancozeb is widely used to protect many fruit, vegetable, nut and field crops and it proved effective against a wide array of fungal diseases including potato blight, leaf spot, scab, and rust (KAMRIN 1997). Prochloraz is used against a wide range of diseases affecting field crops, fruit, turf and vegetables (BOZDOGAN 2014). Tebuconazole is a synthetic fungicide effective against various smut and bunt diseases in cereals and other field crops and is very commonly used on peppers (NAVARRO *et al.* 2011).

Our study covered exposure to active ingredients (analytical standards) of tested fungicides. Mancozeb (reagent grade 96.4%; Sigma-Aldrich) was tested at a nominal concentration of 1.0 mg l^{-1}. This fungicide, due to its low durability in the aquatic environment (BISSON & HONTELA 2002), was applied to water 3 times at regular intervals at the same concentration amounting to 1.0 mg l^{-1}. Before the 2nd and 3th applications, the aquarium water was gradually exchanged. Experimental animals were exposed to a single dose of prochloraz (grade 99.3 ± 0.4%; Institute of Industrial Organic Chemistry, Warsaw) at a nominal concentration of 1.0 mg l^{-1} due to its long-term residual effects in the aquatic environment (ANKLEY *et al.* 2005; KINNBERG *et al.* 2007). Tebuconazole is characterized by long-term residual effects in the aquatic environment (PESTICIDE PROPERTIES DATABASE 2016) and therefore a single dose (grade 99.9 ± 0.1%; Institute of Industrial Organic Chemistry, Warsaw) at a nominal concentration of 2.5 mg l^{-1} was applied in our experiment.

The tested concentrations of the fungicides were selected on the basis of available reference literature regarding the LC_{50} value (HEJDUK & SVOBODOVÁ 1980; KAMRIN 1997; KREUTZ *et al*. 2008; SANCHO *et al*. 2010) and were lower than 25% of that value. The available literature on tested fungicide concentrations detected in surface waters is insufficient.

Experimental design

The fish were divided into three experimental groups (exposed to mancozeb, prochloraz or tebuconazole) and three respective control groups (C_M, C_P, C_T). Each experimental group was exposed to single fungicide for 14 days. After exposure the fish underwent a 30-day recovery in clean water to assess whether the effects of fungicides on fish are temporary or permanent.

Hematological analysis

The following hematological parameters were studied to assess the effects of exposure to selected fungicides: total number of erythrocytes (RBC), hematocrit value (Hct), total hemoglobin concentration (Hb), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), total number of leukocytes (WBC) and leukograms (BOMSKI 1995).

To assess hematological parameters peripheral blood was obtained by cardiac puncture with the use of glass Pasteur pipettes washed with heparin sodium (Heparinum, Polfa). Blood samples were taken from 10 individuals from each experimental group. Hematological analyses were repeated after 1, 3 and 14 days of exposure to a given fungicide and at the end of the 30-day recovery period. Hematological parameters in respective control groups, including 10 fish per each control group, were evaluated at the beginning of the experiment. The fish were decapitated after taking blood samples.

An aliquot of the blood was transferred to microcapillary tubes which were centrifuged (2000 g, 10 min) using an MPW-351R centrifuge (MPW Med. Instruments). RBC was obtained manually using a Bürker hemocytometer according to the method of NATT and HERRICK (1952). Hct value was calculated as the percentage of red blood cell pellet in the total blood column. Hemoglobin content was determined in total blood using the cyanmethemoglobin method. The amount of cyanmethemoglobin was measured spectrophotometrically at 540 nm using BioTek Eon spectrophotometer (BioTek® Instruments), as described in the Biochemtest® kit instruction. WBC count was obtained manually using a Bürker hemocytometer according to a method provided by NATT and HERRICK (1952). Different kinds of leukocytes (lymphocytes, monocytes, mature and immature neutrophiles) were quantified by blood smear analysis.

Statistical analysis

Normality of distribution was tested by the Shapiro-Wilk's test, homogeneity of variance was determined utilizing Levene's test. Then, data were analyzed by a one-way ANOVA, followed by Tukey's post-hoc test. The level of significance was set at $\alpha = 0.05$. Data were presented as means ± SD. Results were analyzed using STATISTICA 10 software.

Results

Alterations in red blood cell parameters: RBC, Hct, Hb, MCV, MCH, MCHC

Disruptions in red blood cell parameters during exposure to mancozeb, prochloraz and tebuconazole as well as after the recovery period are summarized in Tables 1-3.

Fish exposed to mancozeb showed changes in most examined red blood cell parameters (Table 1).

RBC measured in control fish (C_M) directly after commencement of exposition reached a value of 1.6 ± 0.3 mln μl^{-1}. Exposure to mancozeb followed by detoxication period had a statistically significant influence on the red blood cell count

Table 1

Alterations of red blood cell parameters during exposure to mancozeb and after the recovery period (h – hour; C_M (0 h) – control group; detox – after the recovery period; n = 10).

Time period	RBC (mln μl^{-1})	Ht (%)	Hb (g dl^{-1})	MCV (μm^3)	MCH (pg)	MCHC (g dl^{-1})
C_M (0 h)	1.57±0.31	28.14±1.57	10.18±1.14	179.73±38.78	68.75±7.70	39.44±6.47
24 h	2.14±0.34*	34.89±2.52***	12.12±1.36*	165.15±29.05	54.75±11.32	37.06±5.17
3 days	1.37±0.29	22.11±3.22***	8.76±1.57	153.91±25.51	63.90±12.18	40.71±5.58
14 days	0.95±0.19*	20.88±2.80***	6.62±0.88***	217.70±54.42	66.09±14.41	30.40±6.11*
detox	1.57±0.45	25.10±1.10	6.76±0.97***	171.69±55.46	47.66±13.54*	27.12±3.67***

*differences statistically significant in comparison to the control group at P<0.05

*** differences statistically significant in comparison with to control group at P<0.001.

(F = 13.28; P<0.00001). RBC increased significantly to the amount of 2.1 ± 0.3 mln μl^{-1} after 1 day of mancozeb application. Further exposure to this fungicide until the end of the experimental period caused a decrease of RBC and the value reached a statistically significant level after 14 days (0.9 ± 0.2 mln μl^{-1}). After the recovery period, the total number of erythrocytes was comparable to the control value.

Ht value in control carps (C_M) amounted to 28 ± 2 %. Exposure to mancozeb followed by detoxification also had a statistically significant influence on the hematocrit value in tested fish (F = 41.20; P<0.00001). Ht evaluated after 1 day of fungicide administration showed a significant increase up to 35 ± 2 % followed by a significant decline recorded after the 3rd and 14th day (22 ± 3 % and 21 ± 3 %, respectively). After the recovery period the Ht value was almost similar to the control value.

Hb concentration in the peripheral blood of the control fish (C_M) evaluated directly after the commencement of the study equaled 10 ± 1 g dl^{-1}. We observed a statistically significant influence of the exposure to mancozeb and detoxication on hemoglobin content in the blood of tested fish (F = 33.86; P<0.00001). Hb concentration increased after 1 day of exposure and reached statistically significant level of 12 ± 1 g dl^{-1}. Measurements taken afterward and the end of exposure time revealed gradual decline of Hb concentration which finally reached the level of 7 ± 1 g dl^{-1}, which was statistically lower than the control value. After the recovery period Hb concentration slightly increased but still was significantly lower in comparison with the control value.

Other experimental red blood cells parameters in control fish (C_M) reached the following values: MCV = 180 ± 39 μm^3, MCH = 69 ± 8 pg and MCHC = 40 ± 7 g dl^{-1}. The lowest value of MCV was detected after 3 days (154 ± 25 μm^3) and the highest after 14 days of exposure (218 ± 54 μm^3). However, both these changes were not statistically significant (F = 2.49; p = 0.06). MCH (F = 4.30; P = 0.006) and MCHC (F = 10.10; P<0.00001) was statistically lower as compared to the control value either after 14 days of fungicide treatment (MCHC) or after the recovery period (MCH). The lowest values of MCH and MCHC were observed after the recovery period (48 ± 14 pg and 27 ± 4 g dl^{-1}, respectively). The highest value of MCH was detected after 14 days and MCHC after 3 days of fungicide exposure.

Exposure of fish to prochloraz resulted in the disruption of most examined red blood cell parameters (Table 2).

RBC, Ht and Hb measured in the control group (C_P) at the beginning of the experimental period amounted to the following values: 1.6 ± 0.3 mln μl^{-1}, 27 ± 2 % and 10 ± 2 g dl^{-1}. The fish exposed to prochloraz, as compared to the control values, showed a decrease in these parameters during the whole experimental period: RBC (F = 8.64; p = 0.00005), Ht (F = 19.65; P<0.00001) and Hb (F = 34.01; P<0.00001). The lowest statistically significant values were recorded after 1 day of exposure and the respective values were as follows: 0.8 ± 0.3 mln μl^{-1}, 14 ± 4 % and 3 ± 1 g dl^{-1}. Then, after 3 days of fungicide exposure, these parameters increased and reached a statistically significant level only for Hb (6 ± 2 g dl^{-1}). After the 14th day of the experiment, fish subjected to prochloraz showed a statistically significant decrease of RBC (1.1 ± 0.3 mln μl^{-1}) and Ht (21 ± 3 %). Hb measured after 14 days of exposure revealed a statistically significant increase (7 ± 1 g dl^{-1}) as compared to the control value. After the recovery period RBC and Ht exhibited an insignificant increase while Hb was characterized by a statistically significant decrease (6 ± 2 g dl^{-1}), as compared to the control values.

Table 2

Alterations of red blood cell parameters during exposure to prochloraz and after the recovery period (h – hour; C_P (0 h) – control group; detox – after the recovery period; n = 10).

Time period	RBC (mln μl^{-1})	Ht (%)	Hb (g dl^{-1})	MCV (μm^3)	MCH (pg)	MCHC (g dl^{-1})
C_P (0 h)	1.57±0.31	27.00±2.33	9.58±2.40	179.73±38.78	70.66±7.92	40.54±6.65
24 h	0.80±0.28***	14.22±4.41***	3.15±1.11***	175.30±28.42	48.82±23.92*	23.85±11.42*
3 days	1.23±0.29	24.44±5.34	5.89±1.57***	184.91±18.17	46.63±9.05**	25.22±7.35*
14 days	1.11±0.16*	20.56±2.55**	6.66±1.04***	200.72±31.83	59.45±9.13	32.15±7.13
Detox	1.26±0.31	22.70±3.95	5.97±1.69***	207.81±30.07	48.74±9.95*	28.88±9.10*

* differences statistically significant in comparison to the control group at P<0.05
** differences statistically significant in comparison to the control group at P<0.01
*** differences statistically significant in comparison to the control group at P<0.001.

Other red blood cell parameters (MCV, MCH and MCHC) in control fish (C_P) amounted to: 80 ± 39 μm^3, 71 ± 8 pg i 40 ± 7 g dl^{-1}, respectively. A statistically significant decline of MCH (F= 4.73; P= 0.003) and MCHC (F= 5.81; P= 0.0003) in prochloraz administered fish was observed during the whole exposition period and after the recovery. Post-hoc analysis revealed a statistically significant differences after 1 day and 3 days of exposure (47 ± 9 pg and 25 ± 7 g dl^{-1}, respectively) as well as after the recovery (49 ± 10 pg and 29 ± 9 g dl^{-1}, respectively). No statistically significant differences were detected for MCV during the whole experimental period (F = 0.76; P = 0.56). The lowest values for RBC, Ht, Hb, MCV and MCHC were observed after 1 day of exposure, while the lowest measure for MCH was recorded 3 days after prochloraz application.

Some red blood cell parameters of fish exposed to tebuconazole were statistically different as compared to the control group (Table 3).

RBC, Ht and Hb evaluated in control fish (C_T) upon the start of the experiment reached the following values: 1.1 ± 0.4 mln μl^{-1}, 18 ± 3 % and 6 ± 2 g dl^{-1}. RBC in fungicide exposed fish decreased slightly in the course of the experiment, especially 3 days after tebuconazole application (0.8 ± 0.3 mln μl^{-1}). After the recovery period RBC returned to the control value. The differences observed were not statistically significant (F = 4.05; P = 0.40). However, statistically significant fluctuations regarded Ht (F = 20.79; P = 0.0009) and Hb (F = 29.25; P<0.0001) levels. Hb and Ht increased statistically after 1 day of exposure (24 ± 3 % and 9 ± 2 g dl^{-1}, respectively). Hb and Ht measured at other time points (3 and 14 days after the exposure to the fungicide) declined in comparison with the control values but this decrease was not statistically significant. After the recovery period, both Ht and Hb increased again but statistical significance was achieved only for Ht (23 ± 2 %). Control values (C_T) for other red blood cell parameters, namely MCV, MCH, MCHC, were as follows: 202 ± 50 μm^3, 70 ± 11 pg and 31 ± 5 g dl^{-1}. These parameters did not show any statistically significant differences during the whole experimental period. The lowest values for Hb, Ht, MCV, MCH and MCHC were observed after the exposure period (14 days). Detoxication in clean water caused a minor increase in the above-mentioned parameters.

White blood cell parameters: WBC and leukograms

Results of the study of white blood cell parameters during exposition of common carp to mancozeb, prochloraz and tebuconazole as well as after the recovery period are shown in Tables 4-6.

Exposition to mancozeb influenced most of examined white blood cell parameters (Table 4).

Mancozeb produced statistically significant variances in WBC during the whole course of the experiment (F = 20.69; P<0.00001). WBC recorded for control fish (C_M) upon the commencement of the procedure amounted to 97 ± 11 x 10^3 μl^{-3}. After 3-day exposition to mancozeb WBC dropped to 71 ± 13 x 10^3 μl^{-3} and subsequently increased to a statistically significant level of 85 ± 49 x 10^3 μl^{-3}. The value of WBC measured after the recovery period was comparable to the control value. Percentages of different leukocytes for control fish were as follows: lymphocytes – 87.7 ± 7.8 %; neutrophils – 7.2 ± 2.5 %, monocytes – 2.5 ± 0.9 %. In common carp subjected to mancozeb lymphocytes accounted for 76-95 %, neutrophils for 1.7-23 % and monocytes for 2 % of all leukocytes. After 14 days of fungicide exposure, we observed a statistically significant decrease of percentage values for lymphocytes (F = 15.40; P<0.00001) and statistically significant increase of percentage value for neutrophils (F = 45.25; P<0.00001), as compared to the control values. During the entire

Table 3

Alterations of red blood cell parameters during exposure to tebuconazole and after the recovery period (h – hour; C_T (0 h) – control group; detox – after the recovery period; n = 10).

Time period	RBC (mln μl^{-1})	Ht (%)	Hb (g dl^{-1})	MCV (μm^3)	MCH (pg)	MCHC (g dl^{-1})
C_T (0 h)	1.06±0.36	18.22±2.99	6.10±1.89	202.00±49.96	59.65±10.78	31.09±5.51
24h	0.95±0.18	24.22±2.64*	9.47±1.89**	268.54±53.71	83.13±28.95	37.46±3.53
3 days	0.77±0.11	20.22±4.89	7.11±1.65	225.52±42.14	83.88±35.24	32.95±3.75
14 days	0.92±0.25	18.78±2.82	5.13±0.56	204.68±37.51	54.03±11.83	28.18±5.22
detox	1.02±0.24	23.13±2.42*	5.75±1.02	221.09±31.62	66.16±20.22	30.20±7.96

* differences statistically significant in comparison to the control group at P<0.05

** differences statistically significant in comparison to the control group at P<0.01.

Table 4

Leukograms of fish during exposure to mancozeb and after the recovery period (h – hour; C_M (0 h) – control group; detox – after the recovery period; n = 10).

	C_M (0 h)	24 h	3 days	14 days	detox
WBC $\times 10^3$ μl^{-1}	96.94±11.4	109.65±16.10	71.44±13.00	185.08±48.90***	105.05±26.60
Lymphocytes %	87.70±7.80	94.98±3.40	91.95±2.10	76.41±8.30**	92.10±9.50
Neutrophils %	7.23±2.50	2.17±0.90	6.00±1.90	23.31±6.10***	1.73±0.70
– mature %	12.19±6.90	37.51±8.70***	22.30±8.00	15.18±3.80	27.90±9.00*
– immature %	87.81±6.90	62.49±8.70***	77.70±8.00	84.82±3.80	72.10±9.00*
Monocytes %	2.54±0.90	2.06±1.40	1.86±0.70	1.88±1.30	0.78±0.50

* differences statistically significant in comparison to the control group at P<0.05
** differences statistically significant in comparison to the control group at P<0.01
*** differences statistically significant in comparison to the control group at P<0.001.

course of the experiment (fungicide exposure and the recovery period) the number of immature neutrophils exceeded the number of mature forms. Percentage value for immature neutrophils was variable during the exposure period and reached the highest statistically significant value after 1 day of exposure (37.5 ± 8.7%). After termination of the fungicide stimulus it dropped to a value of 15.2 ± 3.8%. The percentage of mature neutrophils after the recovery period increased to the value of 27.9 ± 9.0% and was statistically significant (F = 8.87; P<0.001). The percentage of monocytes in control fish amounted to 2.5 ± 0.9%. The number of monocytes in fungicide treated fish dropped gradually throughout the experimental period and reached the lowest value after the recovery – 0.8 ± 0.5% (Table 4).

Exposure to prochloraz caused alterations in most studied white blood cell parameters (Table 5).

Analysis of variances revealed significant differences of WBC measured in various experimental groups and at different time points (F = 43.34; P<0.00001). WBC for control fish assessed at the beginning of the experiment amounted to 97 ± 11 x 10^3 μl^{-3}. The initial insignificant decrease of WBC in response to prochloraz (after 1 day) was followed by an increase. The lowest (statistically significant) increase of WBC was recorded after 14 days of fungicide exposure (257 ± 55 x 10^3 μl^{-3}). After the recovery period WBC was almost similar to the control value. The percentage distribution of individual types of leukocytes in control fish was as follows: lymphocytes – 87.7 ± 7.8 %; neutrophils – 7.2 ± 2.5 %, monocytes – 2.5 ± 0.9 %. Leukogram analysis revealed that the percentage of leukocytes in fish subjected to prochloraz decreased significantly (71 ± 9%) after 1 day of the experiment. Further fungicide exposition and recovery caused steady increases in the percentage of leukocytes (91.3 ± 3.5%) – F = 7.61; P<0.001. Fluctuations in the percentage of leukocytes were accompanied by a statistically significant drop in the number of neutrophils: from 26.5 % after 1 day of exposure to 5 and 7 % after 14 days and the recovery period, respectively – F = 34.74; P<0.000001. In fish exposed to prochloraz immature forms accounted for

Table 5

Leukograms of fish during exposure to prochloraz and after the recovery period (h – hour; C_P (0 h) – control group; detox – after the recovery period; n = 10).

	0 hours	24 hours	3 days	14 days	detox
WBC $\times 10^3$ μl^{-1}	96.94±11.4	73.88±25.90	127.50±38.10	256.90±55.00***	103.94±18.00
Lymphocytes %	87.70±7.80	71.00±9.90*	86.75±2.50	90.73±6.40	91.33±3.50
Neutrophils %	7.23±2.50	26.50±5.90***	11.19±2.50	5.31±1.50	7.25±1.30
– mature %	12.19±6.90	19.04±2.90	16.73±6.70	16.33±5.10	17.80±4.30
– immature %	87.81±6.90	80.96±2.90	83.27±6.70	83.67±5.10	82.20±4.30
Monocytes %	2.54±0.90	1.00±0.50	2.06±0.70	1.26±0.60*	1.22±0.80*

* differences statistically significant in comparison to the control group at P<0.05
*** differences statistically significant in comparison to the control group at P<0.001.

80 % of all neutrophils. The number of monocytes remained at the level 1-2 % until reaching the 14th day of the experiment. Measurements taken after the 14th day and the recovery period showed a statistically significant decline of monocytes (Table 5) – F = 4.78; P<0.01.

Exposure to tebuconazole caused alterations in most examined white blood cell parameters Table 6.

WBC measured in control fish (C_T) directly at the beginning of the experiment reached the value of 116 ± 29 x 10^3 μl^{-3}. Exposition to tebuconazole had a significant influence on WBC (F = 24.06; p = 0.0001). Increase in WBC reached a statistically significant level after 3 days of exposure (169 ± 55 x 10^3 μl^{-3}). During further exposition and after the recovery period WBC records were lower in comparison to the control fish and reached the lowest value after the recovery (102 ± 35 x 10^3 μl^{-3}). However, these alterations were not significant statistically. Other white blood cell parameters, that is percentage of lymphocytes (F = 12.30; P<0.00001), neutrophils (F = 21.19; P<0.000001) and monocytes (F = 8.04; P<0.001), exhibited statistically significant variations throughout the experiment. The percentage distribution of leukocytes in control animals showed the following pattern: lymphocytes – 94.3 ± 1.8 %; neutrophils – 2.8 ± 1.5 %, monocytes – 2.3 ± 1.4 %. Lymphocytes accounted for 73-93 %, neutrophils for 6-18 % and monocytes for 4-7 % of all leukocytes. The percentage distribution of lymphocytes underwent a statistically significant decline at the beginning of the exposure (after 1 day) and amounted to 73 ± 10 %. The percentage distribution of neutrophils and monocytes at the same time exhibited a statistically significant increase up to 18 ± 5 % and 7 ± 3 %, respectively. Continuation of fungicide exposure resulted in a steady increase of the number of lymphocytes while the percentage of neutrophils and monocytes was in decline. However, recovery in clean water again caused a statistically significant increase of the percentage of neutrophils. During fungicide application neutrophils were mostly represented by immature forms. The largest percentage share of immature neutrophils was recorded after 1 day of exposure (83 ± 6 %). Neutrophil counts obtained later revealed a gradual decrease of the percentage of immature forms. However, the recorded decline had no influence on statistical significance as the percentage of immature neutrophils was still statistically higher as compared to the control value. After the recovery period the number of immature neutrophils was similar to the control value.

Comparison of the influence of tested chemicals

Mancozeb, tebuconazole and prochloraz had different influences on RBC. Exposure to mancozeb led to an increase of RBC whereas exposure to tebuconasole and prochloraz resulted in decreased RBC. This effect occurred after 24 hours (F=25.66; P<0.00001). Similarl significantly different influence of the tested fungicides on Ht occurred after 24 hours (F=74.75; P<0.00001), when fish exposed to mancozeb had a much higher level of Ht than fish exposed to other fungicides. During the whole experiment fish exposed to mancozeb also had a significantly higher Hb level than specimens exposed to tebuconazole and prochloraz (24 hours – F=40.96; P<0.00001; 3 days – F=4.96; P0.05; 14 days – F=282.41; P<0.0001). On the other hand after detoxication all fish had similar Hb concentrations (F=0.63; P=0.53). After 24 hours (F=3.61; P<0.05) and 3 days (F=5.16; P<0.05) fish exposed to tebuconazole had higher MCV values than fish exposed to other chemicals. After 14 days the highest MCV value was recorded in fish treated with mancozeb (F=3.58; P<0.05). During detoxication MCV was nearly the same in fish

Table 6

Leukograms of fish during exposure to tebuconazole and after the recovery period (h – hour; C_T (0 h) – control group; detox – after the recovery period; n = 10)

	0 hours	24 hours	3 days	14 days	detox
WBC $\times 10^3 \cdot \mu l^{-1}$	115.75±28.60	153.13±53.90	169.44±47.10*	102.17±34.80	92.50±11.00
Lymphocytes %	94.33±1.80	72.84±9.90***	87.17±6.80	91.65±2.10	86.56±4.50
Neutrophils %	2.80±1.50	18.28±4.60***	5.78±1.90	5.44±2.10	12.46±4.50**
– mature %	41.84±5.20	16.98±6.00***	20.10±5.60***	29.27±6.60*	42.91±9.10
– immature %	58.16±5.20	83.02±6.00***	79.90±5.60***	70.73±6.60*	57.09±9.10
Monocytes %	2.33±1.40	7.13±3.00**	5.92±3.10	3.92±1.40	1.06±0.90

* differences statistically significant in comparison to the control group at P<0.05
** differences statistically significant in comparison to the control group at P<0.01
*** differences statistically significant in comparison to the control group at P<0.001.

from all studied groups. Similarly MCH was the highest in fish exposed to tebuconazole for 24 hours (F=4.43; P<0.05) and 3 days (F=4.12; P<0.05), whereas after 14 days the highest MCV value was recorded in fish exposed to macozeb (F=61.17; P<0.00001). During detoxication, MCV reached similar levels in all the fish. Taking into consideration MCHC, significant differences between fish exposed to different fungicides occurred on day 14 when specimens exposed to prochloraz had the highest MCHC (F=30.34; P<0.00001).

During the first 3 days of the experiment, the highest WBC value was recorded in fish exposed to tebuconazole and the lowest in fish exposed to mancozeb for three days. The differences concerning WBC between fish exposed to the tested chemicals were statistically significant on the 1st (F=7.68; P<0.01), 3rd (F=15.08; P<0.0001) and 14th (F=18.51; P<0.0001) day of exposure. There were no differences after detoxication. The percentage of lymphocytes during the first three days was the highest in the group of fish exposed to mancozeb, but on the 14th day the highest percentage of lymphocytes was found in fish exposed to tebuconazole. The differences in lymphocyte percentage were statistically significant on the 1st (F=19.71; P<0.0001) and 14th day (F=13.04; P<0.001). Moreover significant differences were found after the detoxication period with the highest percentage of lymphocytes in fish exposed to mancozeb and the lowest in fish exposed to tebuconazole (F=13.28; P<0.001).

A decreasing tendency of neutrophil percentage was found in fish exposed to prochloraz and tebuconazole and an increasing tendency was recorded in fish exposed to mancozeb. The highest percentage of neutrophils was found in fish exposed to prochloraz for 24 h and 3 days. The lowest percentage occurred in specimens exposed to mancozeb for 24 hours. On the other hand, after 14 days of exposure the highest concentration of neutrophils was recorded in fish treated with mancozeb. The differences between fish exposed to the tested fungicides were statistically significant on the 1st (F=47.59; P<0.00001), 3rd (F=13.37; P<0.001) and 14th (F=33.84; P<0.00001) day of exposure. Moreover significant differences occurred also after detoxication, when fish exposed to mancozeb had the lowest percentage of neutrophils and fish exposed to tebuconazole had the highest percentage of neutrophils (F=22.91; P<0.0001). The percentage of mature neutrophils showed a decreasing tendency in fish exposed to macozeb and prochloraz. In fish poisoned with tebuconazole, an increasing tendency in mature neutrophil percentage was recorded. After 24 hours the highest percentage of mature neutrophils was found in fish poisoned with mancozeb. After 14 days and after detoxication the highest percentage of mature neutrophils was found in specimens treated with tebuconazole. During the whole experiment differences in percentage of mature neutrophils that occurred between fish exposed to different chemicals were statistically significant – day 1 F=265.03, P<0.00001; day 3 F=212.56, P<0.00001; day 14 F=108.14, P<0.00001; detoxication F=32.20, P<0.0001).

During the period of exposure to fungicides, an increasing tendency of percentage of young neutrophils was indicated in fish exposed to mancozeb and a decreasing tendency was found in fish exposed to tebuconazole. Thus, on the 1st day the highest percentage of young neutrophils was found in fish exposed tebuconazole and on the 14th day the highest percentage of young neutrophils was found in fish exposed to mancozeb. Significant differences were detected on the 1st (F=14.16; P<0.001), and 14th day (F=11.90; P<0.01). After detoxication the highest percentage of young neutrophils was recorded in fish poisoned with prochloraz and the lowest in fish poisoned with tebuconazole (F=13.12; P<0.001).

The percentage of monocytes was the highest in fish exposed to tebuconazole during the whole period of poisoning comparing to fish exposed to other chemicals. On the other hand, the highest percentage of monocytes after detoxication had fish previously exposed to prochloraz. Significant differences in monocyte percentage between fish exposed to different chemicals were detected on the 1st (F=14.71; P<0.001), 3rd (F=10.32; P<0.01), 14th days (F=10.90; P<0.001). There was no statistically significant differences in monocyte percentage after detoxication.

Discussion

Hematological parameters are sensitive indicators of the physiological condition of fish (ROCHE & BOGÉ 1996; SOPIŃSKA 1984). The presence of toxic substances in the aquatic environment induces quick hematological alterations (ATAMANALP & YANIK 2003; DRASTICHOVÁ *et al.* 2004; SVOBODOVÁ *et al.* 2003; WITESKA *et al.* 2010; WOJTASZEK *et al.* 1992). Stress stimuli generate the following disruptions in the peripheral blood of common carp: increased RBC and Ht as well as elevated hemoglobin concentration. Such changes support the transport of oxygen in fish blood (ANTYCHOWICZ 2007). Results obtained in our laboratory (Table 1-3) indicate that selected fungicides cause alterations of red blood cell parameters.

ATAMANALP and YANIK (2003) after 3-week (24-h intervals) exposure of rainbow trout to mancozeb at a concentration amounting to ½ LC_{50} recorded a slight increase of RBC and decrease of all

other red blood cell parameters. Statistically significant alterations were detected only for Hb and MCV. Such a significant drop in Hb concentration may have detrimental influence on transport of oxygen to various tissues and in result it may decease metabolic rate. Hb shortage could also be a consequence of its increased degradation or reduced synthesis. Mechanisms of mancozeb-induced alterations in Hb concentration in fish have not been studied yet. Exposure of goldfish to mancozeb produced a completely different response. Ht and Hb did not change after fungicide exposure (KUBRAK *et al.* 2012). Similarly, 3-week chronic exposure to sub-lethal concentrations of benomyl (benzimidazole) did not affect Hb concentration in blood and other parameters such as MCV, MCH and MCHC. A slight insignificant reduction regarded only RBC and Ht (MIN & KANG 2008). Chronic exposure to propiconazole (triazole) caused disruptions of hematological parameters, namely decreased RBC, Ht and Hb as well as elevated MCH and MCHC (LI *et al.* 2011). Analysis of the influence of selected fungicides on a given red blood cell parameter indicate that fungicides may produce diversified effects which are difficult to interpret.

Leukocytes of fish exposed to selected fungicides show either decline or enhancement of white blood cell parameters and alterations observed depend on the time of exposure and fungicide applied (Tables 4-6). ATAMANALP and YANIK (2003) reported a decline in the number of leukocytes in rainbow trout subjected to 3-week mancozeb application at a concentration of ½ LC_{50}. Similar declining changes were recorded for WBC measured after chronic exposure of fish to propiconazole (LI *et al.* 2011). Studies conducted on goldfish revealed lymphopenia and increase of the percentage of mature neutrophils after short-term exposure to mancozeb (KUBRAK *et al.* 2012). In summary, our investigations as well as results obtained by other authors suggest that fungicides polluting the environment exert an influence on white blood cell parameters of fish. However, leukocyte response to fungicide application does not seem to be specific.

Alterations of hematological parameters as a result of chemical stress seem to depend on various factors such as the type of fungicide applied, its concentration, duration of the residual effect, physiochemical parameters of water, fish species as well as fish body weight (MURTY 1986). The results of our research indicate that fungicides modify hematological parameters in common carp but the nature of these disruptions remains unclear and effects produced are mostly temporary. Fungicide exposure also activates the body's defense mechanisms. Detected increases or decreases of hematological parameters are not uniform. Despite not showing any regular response to a given toxic substance, the observed alterations may be regarded as sensitive markers of the actual physiological condition of fish.

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