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# ALTERATIONS IN SELECTED HAEMATOLOGICAL PARAMETERS OF RAINBOW TROUT (*Oncorhynchus mykiss*) EXPOSED TO COMPLEX MIXTURES OF CONTAMINANTS

The aim of the study was to evaluate and compare changes in the selected haematological parameters of rainbow trout after long-term (14 days) exposure to sublethal concentrations of two kinds of contaminants: 1) highway traffic emission pollutants (model mixture of heavy metals) and 2) road maintenance salt. Four groups of fish exposed to sublethal concentrations of model mixture metal solutions demonstrated an increased frequency of abnormal erythrocytes and elevated percentage of neutrophiles. The percentage of juvenile and abnormal erythrocytes was significantly increased in the blood of fish exposed to sublethal concentrations of road maintenance salt solutions. Our data confirm that even low amounts of metals found in soil near roadsides and road maintenance salt can induce adverse effects on fish. Morphological analysis of erythrocytes seems to be a reliable tool for detection of toxic effects upon fish.

## 1. INTRODUCTION

Constantly expanding infrastructure of cities and roads has anthropogenic impact on nature [1]. Different flora and fauna communities thriving in natural ecosystems undergo a wide-scale human activity impact because of urban development. Increasing traffic flow induces the elevation of contaminants in soil near roads and the amount of heavy metals such as Cu, Pb, Ni, Cr, Zn, which constitute the large majority of chemicals found in soil [2], [3]. Heavy metals can be accumulated by soil particles and living organisms, they may migrate in soil and destroy natural processes, and can enter surface and underground waters [1].

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Intense traffic flows urge that the traffic safety measures be implemented, one of them being road maintenance salt (RMS) widely used in cold seasons, which consequently causes the negative impact on the components of road environment. RMS concentrations were evaluated in snow cover, soil [4], underground water [5] in countries using salts for road maintenance. As the findings show, high concentrations of RMS (up to 3200 mg/l) accumulated in snow together with snow melt water enter the soil and negatively affect roadside vegetation [6]. The evaluation of the negative impact of RMS on aquatic ecosystems according to diatom flora biodiversity demonstrated that this salt could influence the spreading of diatom flora which is specific to saline waters [7].

Due to snow melting and rainfalls, traffic pollutants may be washed off and away from the soil surface and carried into the surrounding streams, rivers and lakes. The studies concerning the negative impact of traffic pollutants (and their mixtures) on aquatic biota are very scarce. However, it is thought that the adverse impact of traffic pollutants on aquatic ecosystems can differ, depending on the level of contaminants, dynamics of exposure and on the characteristics of a water receiver. On the other hand, pollutants released from soil into aquatic ecosystems can affect freshwater chemistry [5] and pose threat to biota, especially in spring when many aquatic animals are most vulnerable. This negative impact can be short-term or intermittent; however, low concentrations of contaminants can cause a chronic stress in fish organisms, which in turn can reduce their resistance to various environmental stressors. A better understanding of the impact of traffic emission pollutants requires integrated studies on pollutant paths in the soil-water cycle and their impact on aquatic biota.

Haematological parameters are important in diagnosing the structural and functional status of fish exposed to toxicants, they are considered to be a reliable approach in the assessment of toxicity of different single chemicals [8] and their mixtures to fish [9]. Changes in haematological parameters depend on the magnitude of the impact of contaminant (concentration), the duration of exposure and, on the other hand, on fish species, their age and health status [8]. Disrupted haematological patterns appear very quickly and precede changes in fish behaviour and visible lesions [10]. Alterations in white blood cell numbers might be regarded as a prognostic tool or an early-warning signal of the disturbance in homeostatic defence abilities of fish [11].

In our previous studies [12], [13], the main toxic characteristics of two kinds of pollutants (model mixture of Cu, Pb, Ni, Cr, Zn, Mn (MMM) and road maintenance salt (RMS)) were determined: the 96-hour LC50 of MMM and RMS in acute toxicity tests were estimated, and the long-term effect of these contaminants on certain biological parameters of fish was determined. The present study is the continuation of these investigations aimed at evaluating and comparing the haemotoxic effects caused by long-term (14 days) exposure of rainbow trout (*Oncorhynchus mykiss*) to two different kinds of complex mixtures of contaminants (model mixture of metals Cu, Zn, Pb, Ni, Cr, Mn and road maintenance salt).

## 2. MATERIALS AND METHODS

Toxicity tests were conducted in the Laboratory of Ecology and Physiology of Hydrobionts (Institute of Ecology of Vilnius University). Rainbow trout (*Oncorhynchus mykiss*) juveniles obtained from the Žeimena hatchery were maintained in holding tanks of about 3 000 l capacity supplied with flow-through artesian aerated water. The weight of fish under study ranged from 12.9 to 14.4 g. Long-term toxicity tests were performed under semi-static conditions. Control water and solutions of contaminants were renewed daily. During studies, fish were fed until satiety using the commercial DANA FEED fish food. Artesian water of high quality was used for dilution.

A model metal mixture, consisting of six heavy metals, was formed based on available analytical data of average amounts of representative heavy metals in soil near the main highway of Lithuania (No. A1 Vilnius–Kaunas–Klaipėda). The determined concentrations of metals consisted of: Cu – 40–60 mg/kg; Zn – 20–50 mg/kg; Pb – 160–200 mg/kg; Ni – 20–30 mg/kg; Mn – 600–800 mg/kg; Cr –15–20 mg/kg. The soil samples for pollution assessment were collected at a distance of 1–150 m from both roadsides and from 0–10 cm deep soil layer.

In the present study, MMM concentrations were artificially decreased by ~ 50 times. The stock solution of MMM was prepared in distilled water using the following chemically pure substances: $Cu(NO_3)_2 \cdot 2.5H_2O$; $Zn(NO_3)_2 \cdot 6H_2O$; $Ni(NO_3)_2 \cdot 6H_2O$; $Cr(NO_3)_3 \cdot 9H_2O$; $Pb(NO_3)_2$; $Mn(NO_3)_3 \cdot 4H_2O$, the final concentration being recalculated according to the number of heavy metal ion. The concentration of MMM solution considered to be equal to 100% was Cu – 0.874; Zn – 0.93; Pb – 4.7; Ni – 0.66; Cr – 0.33; Mn – 18 mg/l, respectively.

The investigation covered the effect of road maintenance salt (RMS), which is applied in Vilnius city during the autumn and winter seasons. The chemical analysis of salt solution was performed by ICP-MS (inductively-coupled plasma mass spectrometry) and analytical data revealed the metal ion composition of the salt under study (Cl, Na, Ca, Mg, K (g/l) and V, Sr, Br, As, P, Si, Mn, Zn (mg/l)) [13].

The concentrations of salt in long-term studies constituted 4.56; 2.28; 1.14; 0.1825 g/l, respectively.

After the evaluation of the main toxic characteristics of pollutant mixtures (96-hour LC50) in acute toxicity tests [12], [13] the studies were continued to investigate the sublethal effects of highway emission pollutants: 1) model metal mixture (MMM) and 2) road maintenance salt (RMS). Sublethal effects of MMM were studied starting with the 0.2 portion of 96-hour LC50 (108.97% of MMM), i.e. 21.79%, and then the concentrations were reduced accordingly to 10.89%, 5.45% and 1.1% of MMM. Sublethal effects of RMS were evaluated starting with the 4 times diluted 96-hour LC50 of RMS (18.25 g/l) and the examined concentrations constituted 4.56; 2.28; 1.14; 0.1825 g/l of RMS solutions, respectively. Four groups of fish, each consisting of ten individuals, were exposed to MMM solutions and four groups of fish –

to RMS solutions for 14 days. The control group of rainbow trout was kept in an aquarium with artesian aerated water.

After the treatment, blood samples were collected from 40 rainbow trout specimens exposed to MMM and from 40 specimens treated with RMS solutions using routine methods [14]. Blood smears were stained by May-Grünwald-Giemsa and Romanovsky (Merck) stains. In each smear, 300 erythrocytes and 200 leukocytes were classified according to their differential count, and erythrograms (%) and leukograms (%) were assessed with regard to each fish.

The significance of all results was verified by Student's *t*-test at $p \leq 0.05$.

## 3. RESULTS AND DISCUSSION

A variety of contaminants that are accumulated on a road and soil surface could be transported with storm water runoff or snow-melt water and enter surrounding water bodies [15]. According to US EPA, organisms in a water body typically are not experiencing a constant, steady exposure but rather fluctuating exposures, including periods of high concentrations, which may have adverse effects [16]. The main aim of the present study was to evaluate the haemotoxic effects of some contaminant mixtures that can enter aquatic environment via traffic emission.

### 3.1. HAEMATOLOGICAL PARAMETERS OF RAINBOW TROUT EXPOSURE TO MMM SOLUTION

Our previous studies demonstrated the obvious toxic effects of MMM on morphological parameters and some haematological parameters of rainbow trout. Erythrocyte count in blood of MMM-exposed fish was significantly lower ($0.60 \pm 0.04 – 0.74 \pm 0.04 \cdot 10^6 \times mm^{-3}$) as compared to the control ($0.84 \pm 0.04 \cdot 10^6 \times mm^{-3}$) even in fish exposed to the lowest concentration of MMM (1.1%) studied [12]. Cu, Zn, Cr and Ni ion amounts in MMM solution of this concentration were lower than maximum permissible concentrations of these metals accepted for Lithuanian natural water-receivers, whereas Pb and Mn concentrations were 7 times and 15 times higher. The present study showed that juvenile erythrocyte values did not significantly differ among the treated groups and the control ones (table 1). Juvenile erythrocytes encompassed basophilic, polychromatic and orthochromic erythrocytes. The share of old, removal forms of erythrocytes in blood of treated fish tended to be higher, however, the statistically significant elevation was detected only in the group of fish exposed to 5.45% of MMM. The erythrocyte aging process involved some stages: gradual change of nucleus (rounding) and cell (irregular) shapes, enlargement of cell, and changing of nucleus colour (figure 1 A, D). The percentage of abnormal erythrocytes was significantly elevated in all groups of fish treated with MMM (table 1). The abnormalities included irregular cell shape and vacuolisation of cytoplasm (figure 1 B).

Table 1

The alterations in erythrograms and leukograms of blood of rainbow trout treated with MMM solutions

| Parameter | MMM concentration, % | | | | |
|---|---|---|---|---|---|
| | Control | 21.79% | 10.89% | 5.45% | 1.10% |
| | Erythrogram, % | | | | |
| Juvenile erythrocytes (%) | 5.62±0.50 | 5.58±0.38 | 5.74±0.56 | 5.38±0.84 | 4.33±0.40 |
| Old forms of erythrocytes (%) | 2.44±0.51 | 3.65±0.80 | 3.26±0.39 | 4.62±0.42* | 2.46±0.12 |
| Abnormal erythrocytes (%) | 3.50±0.60 | 5.21±0.5* | 7.8±0.6* | 7.74±0.7* | 7.67±0.8* |
| | Leukogram, % | | | | |
| Small and medium lymphocytes (%) | 92.9±0.87 | 91.1±1.05 | 90.1±1.64 | 89.0±1.5 | 92.1±1.55 |
| Juvenile lymphocytes (%) | 2.75±0.36 | 2.45±0.25 | 3.75±0.50 | 2.90±0.54 | 2.25±0.32 |
| Neutrophiles (%) | 4.40±0.80 | 7.00±1.03* | 6.33±1.1* | 8.10±1.50* | 6.25±1.0* |
| Myelocytes (%) | 1.22±0.21 | 2.10±0.36* | 1.83±0.37* | 2.50±0.44* | 2.0±0.38* |
| Metamyelocytes (%) | 1.48±0.48 | 1.40±0.30 | 1.42±0.25 | 1.78±0.55 | 2.0±0.0.5 |
| Neutrophiles with elongated + lobed nucleus (%) | 1.70±0.41 | 3.5±0.53* | 3.08±0.7* | 3.82±0.5* | 2.25±0.5 |
| Thrombocytes/200 white blood cells | 81.7±9.05 | 107.3±14.1 | 89.4±3.52 | 91.5±9.43 | 80.3±4.8 |

* Different from control at $p < 0.05$.

Studies of metal toxicity to fish demonstrated that metals exert their principal toxic effects on the haemopoietic system by altering blood cell formation in the kidney or by direct effects on the formed elements, particularly red blood cells, following their entrance into the circulation [8]. We consider that in this study MMM caused the specific haemotoxic effect on directly circulating erythrocytes as the share of old forms of erythrocytes in blood tended to be higher, and the percentage of abnormal erythrocytes was significantly elevated in all groups of fish treated with MMM (table 1). These adverse effects could be partially explained by the data obtained by the authors who studied the toxicity of single metals. Heavy metals such as zinc, copper and lead might alter the properties of erythrocyte membranes rendering them more fragile and permeable, which probably resulted in cell swelling, deformation (erythrocyte elongation, roundness) and damage [8]. Studies in vitro demonstrated that the elevated concentrations of Zn ions caused marked changes in the composition and structure of membrane lipids in carp erythrocytes and also caused alterations in the erythrocyte plasma membrane fluidity [17]. Swollen erythrocytes may degenerate, and it might have been a cause of a decrease in their number especially pronounced after Pb treatment [8]. However, it should be noted that the effect of the mixture of metals on fish organism can differ from the effect of single metals, and sometimes interactions of metals in a mixture may be either more harmful or beneficial to the organism [9].

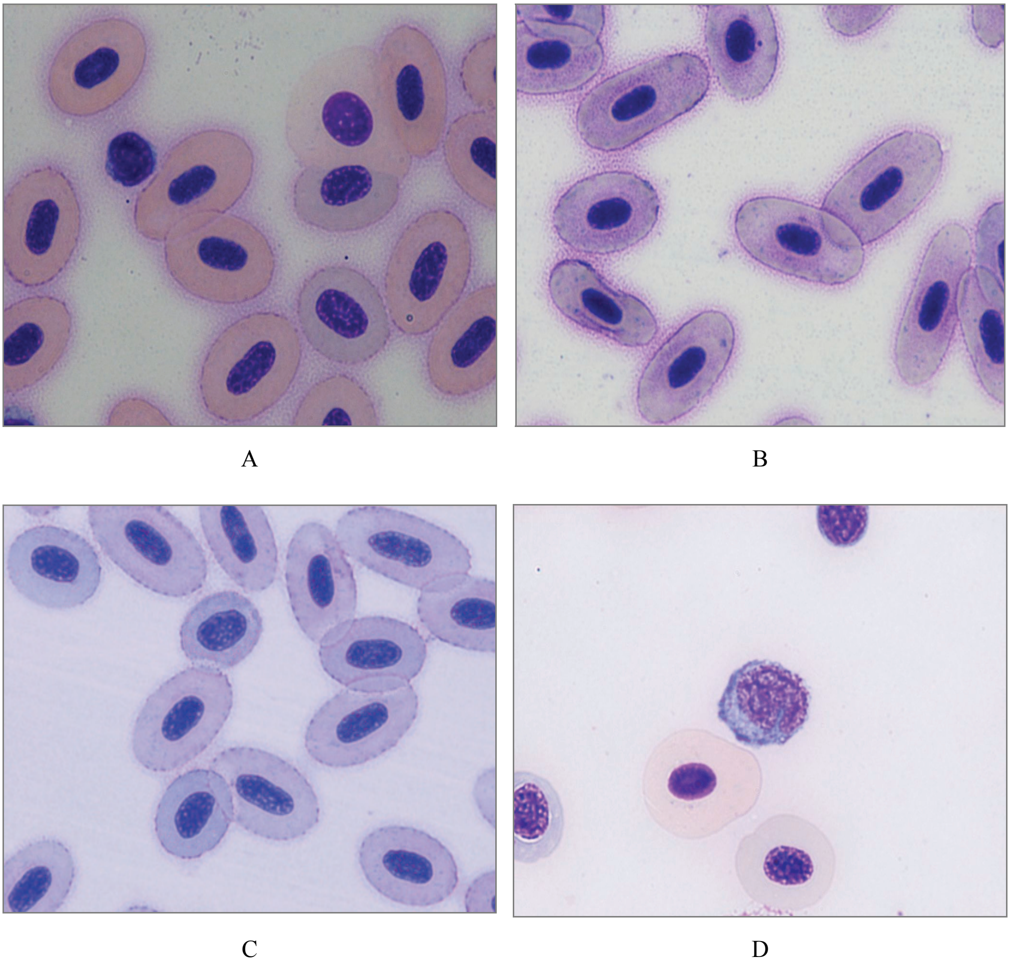


Fig. 1. Rainbow trout erythrocytes under light microscope:
A – old, removal erythrocyte and lymphocyte; B – changed (elongated, sagged) forms of erythrocytes;
C – three juvenile erythrocytes (polychromatic erythroblasts); D – juvenile lymphocyte and two old, removal erythrocytes (magnification of 1000×)

White blood cell count was significantly elevated or tended to be higher in all groups of MMM-exposed fish ($25.7\pm3.3$–$36.0\pm0.4\cdot10^{3} \times mm^{-3}$) as compared to the control ($22.1\pm1.8\cdot10^{3} \times mm^{-3}$) [12]. The percentage of juvenile forms, small and medium lymphocytes in the blood of treated fish did not differ significantly from the control (table 1). The data obtained by various authors show that the intoxication of fish with heavy metals may cause adverse alterations in the level of white blood cells depending on the concentrations of metals and duration of exposure [18]. The alterations in leukocyte count of rainbow trout exposed to heavy metal

(Cu, Zn, Cr, Ni, Pb, Cd, Mn) mixture depended on the mixture concentrations, and differences in white blood cell count, as compared to the control fish, significantly diminished along with the lowering of metal mixture concentration [12]. Juvenile lymphocytes with an irregularly shaped nucleus and the deep basophilic colour cytoplasm, which is characteristic of the blast cells of high protein synthesis activity, were noticed (figure 1 D) in the blood smears of fish exposed to 10.89–21.79% concentrations of MMM. Studies of SNYDER and VALEE [18] demonstrated that lymphocytes were activated by a heavy metal, thereby altering their structure and subsequently influencing latent functions.

Neutrophile percentage was significantly increased in the blood of MMM-treated fish. Among the neutrophiles, both myelocytes and forms with elongated or lobed nucleus significantly predominated in the fish exposed to 5.45–21.79% concentrations of MMM. Thrombocyte count was marginally significantly elevated in the blood of fish exposed to the highest (21.79%) studied concentration of MMM. The increase in neutrophile count of both young forms and mature cells detected in the fish affected by MMM in the present study might be a response of the organism to a chronic toxicant effect reflecting a modulation of the immune defence system. Neutrophile maturation is usually accelerated by pathological processes in the organism and deviation in the share of neutrophiles to mature forms was found to be accompanied by the changes in erythrocyte forms [19]. Modulator effects of Mn on the immune response of fish were found to be dependent on the metal concentrations [20]. Ni at sublethal concentrations (11.7 μg/l) also altered the immunoregulatory mechanisms of rainbow trout [21]. Although thrombocyte count was found to increase compared with the control in *Clarias gariepinus* exposed to lead [22], no significant alterations in the number of these cells were observed in MMM-exposed fish in the present study.

### 3.2. HAEMATOLOGICAL PARAMETERS OF RAIBOW TROUT AFTER EXPOSURE TO RMS SOLUTION

Erythrocyte count was significantly decreased as compared to the control even in fish treated with the lowest studied concentration (0.18 g/l) of RMS [12]. In the control group of rainbow trout, the percentage of juvenile erythrocytes was 5.62±0.50%, and a significantly decreased amount of juvenile erythrocytes was found in all groups of fish treated with RMS solutions ($p < 0.05$) (table 2). In contrast to the data obtained with MMM, the percentage of juvenile erythrocytes was significantly reduced, which could be related to the adverse impact of RMS on erythropoiesis of rainbow trout. No significant alterations were found in the percentage of old, removal forms of erythrocytes in the blood of RMS-exposed fish (table 2), whereas a 2-, 3-fold elevation of abnormal erythrocyte level was noted after the exposure of fish to 0.18–4.56 g/l RMS which was consistent with the data obtained with MMM.

Table 2

The alterations in erythrograms and leukograms of the blood of rainbow trout treated with RMS solutions

| Parameter | RMS concentration, % | | | | |
|---|---|---|---|---|---|
| | Control | 4.56 g/l | 2.28 g/l | 1.14 g/l | 0.18 g/l |
| | Erythrogram, % | | | | |
| Juvenile erythrocytes (%) | 5.62±0.50 | 4.0±0.44* | 3.67±0.43* | 3.99±0.57* | 2.88±0.79* |
| Old forms of erythrocytes (%) | 2.44±0.51 | 3.06±0.34 | 2.64±0.0.24 | 3.1±0.39 | 2.63±0.33 |
| Abnormal erythrocytes (%) | 3.50±0.60 | 8.98±0.91* | 11.18±0.81* | 10.50±1.3* | 5.68±0.64* |
| | Leukogram, % | | | | |
| Small and medium lymphocytes (%) | 93.9±0.87 | 95.4±0.96 | 94.3±0.77 | 94.0±0.46 | 93.7±1.0 |
| Juvenile lymphocytes (%) | 2.75±0.36 | 2.0±0.47 | 2.35±0.50 | 2.6±0.29 | 2.95±0.59 |
| Neutrophiles (%) | 4.40±0.80 | 2.60±0.64* | 3.34±0.63 | 3.4±0.57 | 3.35±0.53 |
| Myelocytes (%) | 1.22±0.21 | 0.40±0.09* | 1.14±0.20 | 0.81±0.16 | 1.40±0.17 |
| Metamyelocytes (%) | 1.48±0.48 | 1.0±0.72 | 1.00±0.25 | 0.94±0.22 | 0.50±0.15* |
| Neutrophiles with elongated + lobed nucleus (%) | 1.7±0.41 | 1.20±0.47 | 1.20±0.35 | 1.65±0.42 | 1.45±0.33 |
| Thrombocytes/200 white blood cells | 81.7±9.1 | 61.3±6.9* | 53.23±5.9* | 47.7±6.5* | 45.8±6.6* |

* Different from control at $p < 0.05$.

Common freshwater fish (roach (*Rutilus rutilus*), perch (*Perca fluviatilis*) and bream (*Abramis brama*)) are very sensitive to changes in water salinity and many studies reported the influence of water salinity on fish development and growth [23]. The sensitivity of rainbow trout to changes in water salinity was confirmed in the present study also, as higher concentrations of RMS caused significant changes in some haematological parameters. Although concentrations of RMS constituents (Ca, Mg, V, Sr, Br, As, P) were low [13], salinity could enhance their toxic effects as alterations in the bioavailability and toxicity of chemicals to aquatic organisms depend on water salinity [8].

White blood cells count decreased significantly in the fish exposed to two highest (2.28 and 4.56 g/l) concentrations of RMS [13]. No significant alterations in the percentage of juvenile lymphocytes were observed in the fish treated with RMS solutions; however juvenile lymphocytes with an irregularly shaped nucleus and the deep basophilic colour cytoplasm were detected in the blood of fish exposed to 2.28–4.56% concentrations of RMS. A statistically significant decrease in total neutrophile and myelocyte percentages was observed only in the group of rainbow trout exposed to 4.56 g/l of RMS solution ( $p = 0.05$). In contrast to the data obtained with the effect of MMM, thrombocyte count was significantly lowered in all groups of RMS-treated fish. These data confirm the negative effect of RMS on the defence properties of fish. Changes in lymphocyte, neutrophile, and thrombocyte count demonstrated disturbances in both specific and non-specific immune mechanisms in fish. Such disturbances may result in impaired resistance of fish to the adverse affect of environmental factors.

## 4. CONCLUSIONS

The results of the present study demonstrated that experimental treatment of rainbow trout with different concentrations of the metal model mixture and road maintenance salt caused considerable changes in the blood cell morphology. Cytotoxic damage to blood erythrocytes of fish was detected. Morphological analysis of erythrocytes seems to be a reliable tool for the detection of toxic effects on fish.

The effect of MMM and RMS on lymphocytes, neutrophiles and platelets of the treated fish differed and depended on the specific effect of pollutants and their concentrations.

Our data suggest that even low concentrations of traffic pollutants can cause adverse effects on the fish haematological parameters which in turn might affect the health status of fish. Their impact would depend on concentrations of pollutants in water, water characteristics, and specific sensitivity of organisms.

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