Impacts of copper sulfate on hematological parameters of rainbow trout (Oncorhynchus mykiss, Walbaum, 1792)

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Abstract
Copper sulfate has been used for many years as an algaecide and parasite treatment. The usage of copper has certain issues as there is a thin line that separates effective treatment levels from overdoses. Copper sulfate can be extremely toxic to fish under certain conditions. This study is focused on the effects of copper sulfate on some blood indices in Oncorhynchus mykiss during a 24 hour period. Our research used one procedure 40 Oncorhynchus mykiss with the average weight of 220 ±10 g. The experimental group had 20 fish that were treated with the blue vitriol (dose of 0.012 g/40l), during a 24 hour period. The control group had 20 fish. The presence of copper in the water leads to the significant increase of erythrocyte, hemoglobin, MCHC and leukocyte levels while the MCV levels were noted to be considerably low. Monocytes, unsegmented and segmented granulocytes were significantly increased in the experimental group of fish. Lymphocyte count was considerably reduced in the same group of fish.

Keywords: Copper Sulphate, Rainbow trout, Toxic, Hematological values

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Introduction

Fish are one of the most widely distributed organisms in the aquatic environment and considered as one of the main protein sources for human (Rashed, 2001). Freshwater fish are similar to the sea water fish which plays an important role in determining resident’s diet (Ahmad et al., 2010). Fish has been found to be excellent indicator for the heavy metal contamination level in aquatic system because it occupies different food chain levels (Karadede-Akin and Unlu, 2007). Fish are often seen at the top of the aquatic food chain and may accumulate large amount of heavy metals from its environment (Mansour and Sidky, 2002). In addition, fish are one of the most indicative aspects in freshwater ambience, for the evaluation of heavy metals pollution and health risk potential of human consumption (Papagiannis et al., 2004). The fish respond and adapt differently to environmental changes (Kirin, 2002). Poor water quality may significantly affect health and haematological status of the fish. Water pollutants may affect the blood count and haematological status in fish (Durand et al., 2000; Guerriero, 2007). The early detection of water pollutants can be determined on a molecular level, tissue or organ abnormality and thus provide researchers timely actions that will prevent further degradation of the organism and the environmental habitat (Małgorzata, 2005). Christensen et al. (1977) have researched the impact of mercury and cadmium on brook trout (Salvelinus fontinalis) during the period of six to eight weeks. Significant increase of sodium and chlorine was detected in the blood plasma while the hemoglobin and glutamate were reduced. Cadmium caused an increase of chloride in the blood plasma while the mercury salts led to an increase of hemoglobin in the blood plasma. It has been determined that the pollutants that effect haematological status, may not necessarily effect the general health and survival. The answer is in the homeostatic mechanisms that enable organisms to maintain physical and physiological functions (Richards et al., 1996; Unver et al., 1999). The organisms maintain its functions when the pollutant level is small, thanks to the homeostatic mechanisms. The fish can manage elevated levels of poluted water without the significant metabolic damages. However, the higher levels of pollutants lead to an irreparable biochemical and physiological damages and sometimes death (Yilmaz et al., 2005).

Toxicological research often exploresthe effect of certain pollutants on the respiratory system of the fish. Due to the well developed techniques, it is possible to control the oxygen level, metabolite and pollutants in the blood system of fish (Kirin, 2000). Zink reduces transport of oxygen through the gill membranes, reducing its concentration in the blood, which leads to the hypoxia and death. However, if the zink is directly injected into the
blood stream of fish, death and hypoxia will not occur (Walter et al., 2005). The pollutants may also affect osmoregulation. Different amonia concentrations effect urine formation in the trout. Elevated amonia levels in the water lead to the elevated urine formation. Fish will die if amonia levels are 20 mg/L. Sublethal zink concentration leads to a decrease of protein, globulin and lysozyme in the carp. The haematological profile can reliably indicate presence of toxins in the water (Alakel and Shamsi, 1996). The phenol, pesticide and metal in the water decrease leukocyte levels. They also lead to changes in differential blood count, increase of granulocyte and a significant decrease of lymphocytes. Decrease of lymphocytes causes reduction of globulin transfer and respectively, a reduced antibody production (Dušek et al., 2005). There are matters in the water that originate from various human activities, different chemicals as well as heavy metals like cadmium and nickel. The concentration of these metals in the poluted water may reach critical levels and thus affect development of aquatic animals. These effects may lead to physiological and biochemical changes in organisms. The haematological monitoring provides early detection of these conditions (Svodoba et al., 2001; Seker et al., 2005). The haematological monitoring provides early detection of illness in fish. The haematological system is attacked fast, before the behavioral changes occur. The heavy metal toxicity attacks organisms very fast as the blood distributes metals to all parts of the body (Fijan, 2006). The haematological effects of cadmium and nickel on the carp, have been measured in the experiment.

The intraperitoneal, sublethal dose of cadmium had been administered (10 μg Cd/kg) and nickel (60 μg Ni/kg).

We monitored haematological levels as well as detoxification potential after the transfer of fish to the safe zone. The individual entry of the sublethal dose has caused disintegration of erythrocytes in the carp, damages of the metabolism and hematopoiesis. The leukocytes and lymphocytes levels were on the wane in comparison to those of the control group (measured 6 hours after the ingestion). The nickel ingestion had caused reduction of erythrocytes in comparison to those of the control group and the group that has been treated by cadmium. The hemoglobin content was reduced after the cadmium exposure and decreased after the nickel exposure.

The aim of this study is to evaluate the role of the blue vitriol on some heamatological parameters in rainbow trout.

Materials and methods

Animals

This study includes 40 fish Oncorhynchus mykiss from river Rakitnica, which is the main tributary of the first section of the Neretva River. This research includes two groups of fish: control (n=20) and experimental group (n=20). Fish was placed in the aquariums of 40 litar volume. After the
adaptation, experimental group of fish was treated with the blue vitriol (CuSO₄·5H₂O) for 24 hours, (dose of 0.012 g/40l). No feeding was done for fish during the experiment. Handled animals were treated in accordance with the “Declaration on the Rights of Animals” (UNESCO, 1978), “Universal Declaration on Animal Welfare“ (WSPA, 2000) and Animal Protection and Welfare Law of Bosnia and Herzegovina (“Official Gazette” 25/09).

**Experimental Design**

Treated animals were with the weight of approximately 250 and 350 g. For bleeding, the caudal vein was punctured by using 2 mm heparinized the small gauge needle (21G; Semikem, Sarajevo, BiH). During the blood draw, the place of the puncture was disinfected and all other sterile rules were applied. The native blood, without the added antianticoagulants, was used for the further research.

**Heamatological Techniques**

The following heamatological parameters were analyzed: erythrocytes count, RBC (via Hayem solution, Semikem, Bosnia and Herzegovina), leukocyte count, WBC (via Türk reagens, Semikem, Bosnia and Herzegovina), hemoglobin concentration, HB by Drabkin’s hemoglobin cyanide method (Blaxhall and Daisley, 1973), hematocrit, HCT (centrifuging blood samples via micro hematocrit method, 5 min, 16 000 rpm by Hettich Haematokrit 24 zentri-fugen, Germany). Erythrocyte and leukocyte count was determined by hemocytometer chamber (Neuber’s chamber). Differential count of leukocytes was done by providing a blood smear and was examined with light microscope. The hematological indices: Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were determined from the following equations (Campbell 2004). Fresh smears were stained by May-Grünwald and Giemsa stains (Semikem, Sarajevo, BiH) for procentual analysis of leukocyte levels in the peripheral blood. Cell identification and procentual count of blood cells (were performed on Olympus BX41 light microscope).

**Morphometric Characteristics**

All fish were analized for body weight, total and standard body length and Fulton's Coefficient (Vuković, 1982). The total and standard length was measured by the Fishmeter while the body weight was measured with the precise scale.

**Statistical**

Results were evaluated by the IBM SPSS (Version 20.0, SPSS, Inc., Chicago, IL, USA). The collected data was analyzed through the methods of the descriptive statististics (mean, standard deviation and range). Variances between the groups were analized via One Way ANOVA method. P values lower than 0,05 were considered significant.
Table 1: Morphometric Characteristics of Controlled and Experimental fish.

| Parameters       | Control         | Experiment       | p-values |
|------------------|-----------------|------------------|----------|
| Total length (cm) | 27.45±2.04      | 27.05±1.74       | 0.286    |
| Standard length (cm) | 23.26±2.21     | 24.55±1.87       | 0.048    |
| Mass (g)         | 225.26±50.70    | 237.41±41.58     | 0.239    |

Results

Table 1 presents middle value, standard deviations, and p value (Student's t test) for morphometric parameters: total length, standard length and body mass. We were not able to determine significant differences in body length and body mass between the controlled and experimental fish. The experimental group has higher values of almost all parameters. The hematocrit and MCV count are the only parameters without the significant cross group differences (Table 2).

Table 3 presents Spearman's Test of Correlation between erythrocytes and HCT, Hb, MCV, MCH and MCHC. R value shows positive or a negative correlation, p values and the level of significance.

There is a negative correlation between erythrocytes and other hematological parameters in the blood of controlled fish. There is a positive correlation between erythrocytes and hemoglobin concentration in the blood of experimental fish, as well as the negative correlation for HCT and MCHC, and MCV and MCH. Both groups have significantly different erythrocyte, MCV and MCH values. The experimental group has significantly increased levels of monocytes, unsegmented and segmented granulocytes as well as considerably lower lymphocyte count (Table 4).

Table 2: Haematological Parameters Values and Between Differences.

| Haematological Parameters | Control group | Experimental group | Sig. |
|---------------------------|---------------|--------------------|------|
|                           | Mean          | Range              | Mean  | Range   |
| RBC (109/L)               | 1.21±0.21     | 1.02-1.74          | 1.55±0.12 | 1.34-1.75 | 0.00*  |
| HCT (g/L)                 | 0.41±0.09     | 0.237-0.51         | 0.392±0.06 | 0.29-0.48 | 0.267  |
| HB (g/L)                  | 56.29±20.85   | 31.95-98.55        | 76.22±4.67 | 66.66-82.45 | 0.00*  |
| MCV                       | 367.72±119.27 | 208.04-591.91     | 281.67±49.23 | 153.57-338.99 | 0.02*  |
| MCH                       | 47.63±18.34   | 24.13-72.03        | 49.16±3.96 | 43.55-58.04 | 0.394  |
| MCHC (g/L)                | 152.19±36.89  | 105.79-235.20      | 198.51±30.84 | 159.68-260.99 | 0.00*  |
| WBC (109/L)               | 3.34±1.04     | 1.75-5.11          | 4.14±0.28  | 3.55-4.56  | 0.01*  |

* Statistical significant values at 0.05.
Table 3: Spearman’s Test of Correlation

| Parameters | Erythrocytes |          |          |          |          |
|------------|--------------|----------|----------|----------|----------|
|            | Experiment   | r        | p        | Control  | r        | p        |
| HCT        | -0.213       | 0.446    | -0.364   | 0.182    |
| Hb         | +0.127       | 0.652    | -0.405   | 0.135    |
| MCV        | -0.947       | 0.000**  | -0.848   | 0.000**  |
| MCH        | -0.892       | 0.000**  | -0.800   | 0.000**  |
| MCHC       | -0.236       | 0.397    | -0.023   | 0.934    |

Table 4: Blood leukocytes values and cross group differences

| Blood leukocytes | Control group | Experimental group | Sig. |
|-----------------|---------------|--------------------|------|
|                 | Mean          | Range              | Mean  | Range |      |
| Limfociti       | 81.27±3.22    | 77-87              | 71.85±3.03 | 66-76 | 0.00* |
| Monociti        | 1.77±0.83     | 1-3                | 2.50±0.76 | 1-4   | 0.02* |
| Eozinofili      | 1.23±0.42     | 1-2                | 1.33±0.51 | 1-2   | 0.08  |
| Nesegmentirani  | 10.±3.03      | 6-15               | 15.35±3.24 | 11-22 | 0.00* |
| Segmentirani    | 6.72±1.79     | 3-9                | 9.71±1.73 | 7-13  | 0.00* |

Discussion

Fish is one of the most expensive sources of food in the water and are sensitive to the environmental pollution. It is possible to detect toxic symptom of herbicides by studying cytological and serological indices in fish. Fish are directly exposed to the environmental changes. Sensitivity to different toxic materials varies among fish. This is the reason why we used different types of fish in our study (Naji et al., 2019).

Toxic metals in fish may have negative impact on the health of humans (kidney failure, liver damage) (Al-Busaidi, 2011). Toxic materials may be absorbed through the gills or through the digestive system. The absorbed toxic metals effect health of fish. Testing of hematological parameters enables early detection of health in fish. Early detection of water pollutants can be determined on a molecular level, through the tissue or organ abnormality.

The researchers can thus implement timely measures that will prevent further degradation of habitat (Malgorzata, 2005). Hematological profile is a reliable indicator of toxic pollutants in the water (Alakel and Shamsi, 1996). Physiological diagnostics of fish understands that both erythrocytes and leukocytes have a high diagnostic value (Bond, 1979; Kekić et al., 1985).

Copper is the main component of multicellular organisms. Hemocyanins are copper containing respiratory pigments. Copper deficiency lowers erythrocyte count. Daily copper needs for an adult humans are 1 to 1,5 mg (Mercer, 1998). Copper plays an important role in the overall blood count: iron absorption, hemoglobin synthesis and erythrocyte production, colagen and elastin synthesis, bone formation. Copper is also an integral part of many enzymes. Copper deficiency has an impact on enzyme processes. The copper
absorption is influenced by the vitamin C, phytates, sulfides etc. Copper binds with albumin and is thus transported into the liver. Metallothionen is crucial for the safe copper storage (Cvjetko et al., 2010). Concentration of copper in the water significantly increases erythrocyte and hematocrit count while it reduces leukocyte and hemaglobin count and hematological parameters. There are various reasons that lead to these changes. Intraparitoneal injection of cadmium and nickel causes destruction and lowered erythrocyte count, particularly the lymphocyte count in carp (Demirak et al., 2006). Lowered leukocyte count was recorded by Dušek et al. (2005). Concentration of nickel in the water leads to hypoxia and death (Walter et al., 2005). Our research also records lowered leukocyte count. Increased erythrocyte count prevents hematopoiesis of blood cells in both directions (erythropoiesis and leukopoiesis), due to the short term acute effects of copper. On the other hand, considerably high erythrocyte count may lead to hypoxia. Copper protein is proved to be non toxic and thus enables temporary storage of copper in the liver or it can act as a donor for ceruloplasmin synthesis. Copper protein is transported from the liver into the blood stream and it enters erythrocytes in the form of erythrocuorein. Ceruloplasmin is a safe, non toxic component in mammals that enters the blood from the digestive system. This process is different for fish. Therefore the copper is directly available to erythrocytes. Excessive amounts of copper are excreted mostly through the bile (Stalović et al., 2013). This process is different in fish as the copper is directly available to erythrocytes. CuSO₄ is a copper sulphate that effects osmoregulation and pH of blood in fish. Copper sulphate effects ion state of protein, respectively hemoglobin in erythrocytes, which prevents oxygenation. This mechanism causes hypoxia that stimulates erythropoiesis and increased erythrocyte count while the volume (MCV) is low. Hemoglobin, that is non functional due to the copper binding, does not perform its transporting role. That leads to a lowered hemoglobin concentration and other hematological parameters. Heavy metals are distributed differently in fish. The highest concentration of Cu, Zn and Fe is recorded in the liver, Pb and Mn in the gills, while the muscles have the lowest concentration of metals. The concentration of metal is influenced by the diet, for example Herbivor S. Gibbus has the lowest Zn concentration in all of its organs (El-Moselhy et al., 2014). Gills are the main absorbants of metal but its large surface rapidly difuses toxic metals (Dhaneesh et al., 2012). This facilitates blood diffusion. Depending on the type of metal and its concentration, toxic effects are directly manifested in erythrocytes. Various studies have recorded heavy metal concentration in the gills (Quadir and Malik, 2011; Shriadah, 2004). Concentration of metal varies in different organs, fish species, dietary processes and genetic properties (El-Moselhy, 2014).
transport of these metals can cause major changes in the blood count, plasma protein functions, changes in pH and ultimately severe respiratory functions.

**Conclusion**
The effects of toxic metals depend on the type of tissue affected and transporting routes. The absorption of copper through the gills directly impacts pH, ion state of plasma and the process of oxygenation in erythrocytes. An acute and short term accumulation of erythrocuprein in erythrocytes changes ion shape of hemoglobin. This process causes hypoxia, considerable increase in the red blood cells and reduced hemoglobin concentration. On the other hand, leukopoiesis is decreased which weakens the immunity.

The presence of copper in the water leads to the significant increase of erythrocyte, hemoglobin, MCHC and leukocyte levels while the MCV levels were noted to be considerably low. Monocytes, unsegmented and segmented granulocytes were significantly increased in the experimental group of fish. Lymphocyte count was considerably reduced in the same group of fish.

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