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Effect of heavy metal model mixture on rainbow trout biological parameters

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Studies of the acute and long-term toxicity of a heavy metal model mixture (HMMM) (based on real metal concentrations and proportion in soil near the Vilnius–Kaunas–Klaipėda highway on rainbow trout were performed using a set of biological parameters. The concentration of HMMM solution considered to be equal to 100% was for Cu 0.874; Zn 0.93; Pb 4.7; Ni 0.66; Cr 0.33; Mn 18 mg/l. The calculated 48-hour LC_{50} value for rainbow trout juveniles comprised 129.7%, and the 96-hour LC_{50} value 108.97% of HMMM solution. No significant changes were found in the weight of HMMM-exposed fish, while the liver weight of fish exposed to 21.79% of HMMM solution and the hepato-somatic index of fish exposed to 21.79 and 10.89% of HMMM solution differed significantly from control. Changes in blood parameters (erythrocyte count, haematocrit level, leukocyte count) revealed a specific HMMM toxicity to fish. Erythrocyte count decreased in the blood of fish exposed to all HMMM concentrations studied. Our data suggest that even low amounts of traffic emission pollutants can negatively affect the fish organism, causing various disturbances in its health and wellbeing.

Key words: model heavy metal mixture, fish, toxicity

INTRODUCTION

First studies on traffic pollution in Lithuania were started in 1993–1995, and though available data from these studies are rather scarce (Adomaitis et al., 2001) they demonstrated the presence of a wide spectrum of heavy metals (Cu, Zn, Ni, Cr, Pb etc.) in soil near roadsides. Furthermore, heavy metals were found in the topsoil of dwelling districts (Taraškevičius, 1998). Later on, investigations of state road pollution were extended and the concentrations of traffic pollutants were analysed depending on road transport load, geographical situation, etc. (Baltrėnas, Vasarevičius, 2003; Балтренас, Янкайте, 2003; Baltrėnas et al., 2003). Comparison of data on soil pollution by heavy metals in some locations, e.g., near the Elektrėnai town in 1995 (Adomaitis et al., 2001), with our data (Балтренас, Янкайте, 2003; Lithuanian state roads, 2004) demonstrated that the concentrations of some metals increased almost 2, 4–5 times (chromium, copper) or by 16–18% (zinc and lead). These data may indicate a possible negative impact of traffic pollution on the environment, especially on soil ecosystems. However, the physico-chemical properties of soil, presence of other ions, clay minerals may reduce the toxicity of heavy metals, and vice versa, the toxicity may be exerted by many inorganic and organic metal complexes (Lugauskas et al., 2005). Due to rainfalls, pollutants may be washed away from the soil surface and carried into the surrounding streams, rivers and lakes. However, the most significant impact is induced by acid deposition (about half of the acidity in the atmosphere falls back to the earth through dry deposition as gases and dry particles). This acid deposition causes a direct and indirect damage of soil, not only dissolves and washes away nutrients but also enhances the washing out of toxic substances and may induce an indirect effect on aquatic organisms. The strength of the effects depends on many factors, such as water acidity, the chemistry and buffering capacity of the surrounding soils. The problem of acidification has been investigated in some countries taking into account even "episodic acidification", e.g., in spring when snow melts and pollutants that have accumulated over the winter period are suddenly released just at the time when many young fish and insects are most vulnerable (Heij, Erisman, 1995).

Heavy metals are diluted and affected by various surface water components (carbonate, sulphate, organic compounds – humic, fulvic, amino acids) after entering natural water bodies which induce formation of unsoluble salts or complexes. These salts and complexes are predicted to be not harmful to aquatic organisms (Eisler, 1998; Hogstrand, Wood, 1996). Part of them sink and are accumulated in bottom sediments. However, when water pH has declined (during acid rains or other acid episodes) heavy metals can be mobilised and released into the water column and become toxic to aquatic biota (Alabaster, Lloyd, 1986; Taylor et al., 1996). In addition, low concentrations of heavy metals can cause a chronic stress which may not kill individual fish, but lead to a lower body weight and smaller size and thus reduce their ability to compete for food and habitat.

Toxic effects of heavy metals on soil microorganisms *in situ* (near the roadside of the Vilnius–Kaunas–Klaipėda highway) were investigated by Lugauskas et al. (2005) and a negative influence of the test metals on actinomycetes, mineral nitrogen assimilating and oligonitrophilic bacteria was found. Comprehensive studies performed by Marčiulionienė and colleagues (Marčiulionienė et al., 2002; Montvydienė, Marčiulionienė, 2004; Marčiulionienė et al., 2005) evaluating different kinds of pollutant impacts demonstrated the genotoxic effect as well as adverse effects of chemicals on the growth and development of soil and aquatic plants. The risk of traffic pollutants to spread into the environment may impede the assessment of their negative impact on aquatic animals. This negative impact can be short-term or intermittent, however, the possibility of such effect could be hardly denied, especially when considering ponds, small lakes, or rivers situated near the Vilnius–Kaunas–Klaipėda highway (Lake Didžiulis, Kaunas reservoir, the Nevėžis, Dubysa, Šaltuona, Kalnupis, Ančia, Aitra, Jūra Rivers). A better understanding of the impact of traffic emission pollutants requires integrated studies on the fate of pollutants in the soil–water cycle and their impact on aquatic biota.

In the majority of ecotoxicological studies, effects of single metals on fish have been evaluated, while studies of biological responses of fish to the impact of a mixture of heavy metals (more than three components) are scarce (Larsson et al., 1987; Kloepper-Sams et al., 1994; Kazlauskienė et al., 1996; Kazlauskienė, Burba, 1997). However, these studies have demonstrated that the effects of metal mixtures can differ in their toxicity on living organisms from the effects of single components. The toxicity of heavy metal mixtures depends on their concentrations, specific composition, and duration of fish exposure (Kazlauskienė et al., 1996; Kazlauskienė, Burba, 1997; Vosylienė, et al., 2003). The present study is a first step in evaluating the possible toxic impacts of some traffic emission pollutants on aquatic animals.

The aim of the current study was to evaluate the acute and long-term toxicity of a model mixture of heavy metals on rainbow trout by the use of a set of biological parameters based on real metal concentrations and proportion in soil near the Vilnius–Kaunas–Klaipėda highway.

MATERIALS AND METHODS

The toxicity tests were conducted at the Laboratory of Ecology and Physiology of Hydrobionts (Institute of Ecology of Vilnius University). Juvenile rainbow trout (*Oncorhynchus mykiss*) specimens were obtained from the Žeimena hatchery and kept in holding tanks of about 3000 l capacity supplied with flow-through artesian aerated water. The weight of fish in the study ranged from 12.9 to 14.4 g. During long-term studies fish were fed the commercial DANA FEED fish food until satiety.

Artesian water of high quality was used for dilution. The average hardness of dilution water was approximately 284 mg/l as $CaCO_3$, alkalinity was 244 mg/l as HCO_3^-, the mean pH was 8.0, temperature ranged from 12° C to 13.5° C and oxygen concentration ranged from 8 to 10 mg/l.

The effect of a model mixture consisting of six heavy metals was investigated. The formation of the model mixture was based on available analytical data on the amounts of representative heavy metals in soil near the main highway of Lithuania (Vilnius–Kaunas–Klaipėda). The determined concentrations of metals were as follows: 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 a soil layer 0–10 cm deep (Lithuanian state roads, 2004). The stock solution of HMMM was prepared in distilled water using the following chemically pure substances: $Cu(NO_3)_2 \times 2.5H_2O$, $Zn(NO_3)_2 \times 6H_2O$, $Ni(NO_3)_2 \times 6H_2O$, $Cr(NO_3)_3 \times 9H_2O$, $Pb(NO_3)_2$, $Mn(NO_3)_3 \times 4H_2O$, the final concentration being recalculated according to the amount of heavy metal ions. According to scientific data, nitrates are not toxic to aquatic organisms (Russo, 1985). In our previous studies, the 96-hour LC_{50} of copper for adult rainbow trout was determined to be 0.65 mg/l (Vosylienė, Svecevičius, 1996). Therefore, in the present study HMMM concentrations were artificially decreased ~50 times with the aim to be closer to LC_{50} levels of copper to rainbow trout. The HMMM solution considered to be equal to 100% contained Cu 0.874, Zn 0.93, Pb 4.7, Ni 0.66, Cr 0.33, Mn 18 mg/l. The determination of LC_{50} of HMMM started from a 180% concentration of the mixture. The different HMMM concentrations applied in the study are presented in Table 1.

Acute (96-hour) toxicity studies were performed under semi-static conditions according to the LST ISO 7346-2:1999. The system consisted of six 40 l aquaria. For the experiments, seven fish were transferred from holding tanks to each aquarium and kept in the new medium until acclimation, i. e. till they started to swim freely and feed well. Different amounts of stock solution were added to five aquaria and nothing was added to one of them (control). Each test was replicated twice. Every day the water was changed and the number of dead fish was recorded. Dead fish were weighed individually (after being slightly blotted dry). No mortality was observed among control fish. The concentrations of metal ions in stock solution were established by ICP-MS (inductively-coupled plasma mass spectrometry),

Table 1. **Concentrations of metals in HMMM tested and their MPC accepted for Lithuanian inland waters (Valstybės žinios, 2002, No. 62–2533)**

HMMM concentration (%)	**Heavy metal concentration (mg/l)**					
	Cu	Zn	Pb	Ni	Cr	Mn
180	1.57	1.26	6.48	0.85	0.66	25.20
162	1.41	0.88	5.76	0.76	0.59	22.68
108.97	0.95	0.76	3.92	0.51	0.398	15.18
100	**0.87**	**0. 7**	**3.6**	**0.47**	**0.367**	**14.0**
21.79	1.827	1.47	0.756	0.0987	0.077	2.94
5.45	0.044	0.004	0.189	0.24	0.019	0.71
1.1	0.0095	0.008	0.037	0.005	0.004	0.15
MPC	**0.01**	**0.1**	**0.005**	**0.01**	**Total 0.01**	**0.01**

and the analytical data confirmed that the metal concentrations met the estimated ones quite satisfactorily.

Long-term (14 days) toxicity tests were also performed under semi-static conditions. The fish were transferred from holding tanks into five aquaria of 100 l capacity. Control water and HMMM solutions were renewed daily. During the tests fish were fed every day.

The body weight of fish was measured at the start and at the end of exposure. After 14 days of exposure, fish under study and control ones were taken for the measurement of biological parameters. The fish were caught gently in a small net, avoiding stress as much as possible. The morphological parameters included total weight and liver weight. The hepato-somatic index was calculated according to Vosylienė, Svecevičius (1997). Gill ventilation frequency (counts/min) and "coughing" rate (counts/min) were measured during 3-minute periods for each fish individually and the mean value for 10 fish was calculated. Blood samples were collected by punction of the caudal blood vessels. Heparin sodium salt was used for stabilization of the fish blood. Blood was sampled from 10 fish of each exposure group. Erythrocytes (RBC, $10^6 \times mm^{-3}$), haemoglobin concentration (Hb, g/l), haematocrit level (Hct, l/l), leukocyte count (WBC, $10^3 \times mm^{-3}$) were determined using routine methods (Diagnostics, prevention, and therapy of fish diseases and intoxications, 1991).

The median acutely lethal concentration (LC_{50}) values and their 95% confidence intervals were estimated by the trimmed Spearman–Karber method (Hamilton et al., 1977).

The statistical analysis of the data was performed with SPSS 10.0 software, and the significance of the data was determined by the one-way ANOVA test.

RESULTS AND DISCUSSION

Acute toxicity studies were performed in order to determine the basic toxic characteristics of HMMM. After introducing different amounts of stock HMMM solution into the aquaria, the majority of fish died within 48 hours: a total mortality (100%) was registered at 162% and 180% concentrations of HMMM. The calculated 48-hour LC_{50} value for rainbow trout was 129.7% and the 96-hour LC_{50} value 108.97% of HMMM (Table 2). The method applied did not allow to calculate the 24-hour LC_{50} value.

The duration of exposure was found to influence the LC_{50} value, as the 48-hour LC_{50} of HMMM was by 19% higher as compared with the 96-hour LC_{50} value (Table 2), and a sharp boundary between the lethal and sublethal zones specific to the HMMM lethal effects (Vosylienė et al., 2003) was observed. The 96-hour LC_0 according to data on fish mortality covered very narrow ranges ($83.2\% < LC_0 < 104\%$), and similar data were observed for the 96-hour LC_{100} ($104\% < LC_{100} < 129.6\%$).

Table 2. **The calculated LC_{50} of HMMM (percent solution) for rainbow trout**

Exposure duration (hours)	**LC_{50} (%)**	**95% confidence intervals**
48	129.7	122.24 ÷ 137.61
96	108.97	103.30 ÷ 114.96

The fish that survived recovered very soon and within 24 hours after exposure appeared to be in satisfactory condition. With regard to behaviour, the locomotor activity of fish significantly increased after introducing the HMMM solution at lethal concentrations; random swimming, body tremors and respiratory disorders were observed.

After the evaluation of the main toxic characteristics of the mixture, the sublethal effects of HMMM on fish were investigated. Sublethal effects of HMMM on rainbow trout juveniles were studied starting with 0.2 part of 96-hour LC_{50}, i.e. a 21% concentration of HMMM.

HMMM exposure did not induce significant changes in the weight of rainbow trout juveniles. The weight of fish at the initiation of exposure ranged from 13.2 ± 0.4 to 13.8 ± 0.5 g, and no significant differences were determined in the weight of control fish and those exposed to HMMM at the end of exposure. The mean weight of control fish was 22.3 ± 0.7 g, while the weight of exposed fish ranged from 22.0 ± 0.9 to 23.2 ± 0.8 g. A significant difference was found in the liver weight of fish exposed to 21% of HMMM solution and in the hepato-somatic index of fish exposed to 21.79 and 10.89% of HMMM solution (Table 3). The fact that no significant differences in the weight of exposed and control fish were found suggests a little effect of HMMM on the development and growth of fish. However, the decrease in liver weight and hepato-somatic index indicates some liver function disturbances induced by exposure to HMMM. Many heavy metals such as copper, zinc, lead, mercury or cadmium may be deposited in this organ, and this deposition is directly related to liver detoxifying ability (Jezierska, Witeska, 2001). Authors who studied metal-induced lesions in fish liver often observed changes in liver size and the value of hepato-somatic index which depended on the concentration of metals and the duration of exposure (Singh, Reddy, 1990; Baker et al., 1997; Kazlauskienė, Vosylienė, 1999). The decline of glycogen level, inhibition of enzymatic activity are the most common symptoms of metal-induced changes in fish liver (Jezierska, Witeska, 2001).

Furthermore, no significant changes were found in the respiratory parameters of fish exposed to HMMM. The gill ventilation frequency of fish exposed to the highest studied concentration of HMMM (21.79%) was 89.0 ± 0.6 counts/min, while of the control fish 86.6 ± 1.3 counts/min; the coughing rate of control and exposed fish was registered as 1.5 ± 0.5 and 2.3 ± 0.6 counts/min, respectively.

Changes in blood parameters (erythrocyte count, haematocrit level, leukocyte count) revealed HMMM toxicity to fish. Erythrocyte count decreased significantly ($p < 0.005$) in the blood of fish exposed to almost all HMMM concentrations studied, while alterations of haematocrit level depended on HMMM concentration. The highest concentration of HMMM (21.79%) induced a drop in haematocrit level, meanwhile the level of haematocrit in blood of fish exposed to all lower HMMM concentrations was higher as compared to control (Table 4). White blood cell count was significantly elevated in the group of fish exposed to 21.79% and 5.45% solution of HMMM as compared to the control.

The present study demonstrated obvious toxic effects of HMMM on the red blood cell count of rainbow trout. Erythrocyte count in the blood of HMMM-exposed fish was significantly lower as compared to control, even in fish exposed to the lowest concentration of HMMM (1.1%). Though Cu, Zn, Cr and Ni ion amounts in HMMM solution of this concentration were lower than MPC of these metals, Pb and Mn concentrations were 7 and 15 times higher (Table 1). Probably the HMMM induced a specific haemotoxic effect on blood erythrocytes. 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 (indicated by an increase in haematocrit level), deformation and damage (Witeska, Kosciuk, 2003; Kazlauskienė, Vosylienė, 1999). This suggestion was confirmed in our studies by alterations in haematocrit level which decreased only in fish exposed to a 21% solution of HMMM. The exposure of fish to all lower concentrations of HMMM induced an increase in this parameter. Red blood cell count decrease in the blood of rainbow trout was previously determined when studying the long-term effect of the Švedė pond water on fish. It was supposed that various heavy metals that had entered the pond water exerted a specific haemotoxic effect on fish blood (Vosylienė, Kazlauskienė, 2004).

Table 3. **Effects of HMMM on rainbow trout morphological parameters**

Concentration, %	Length, cm	Weight, g	Liver weight, g	HSI
21.79	11.9 ± 0.16	22.7 ± 0.7	0.32 ± 0.01*	1.60 ± 0.09*
10.89	11.6 ± 0.17	23.2 ± 0.8	0.34 ± 0,02	1.65 ± 0.06*
5.45	11.8 ± 0.13	22.0 ± 0.9	0.40 ± 0.01	2.05 ± 0.07
1.1	11.9 ± 0.18	23.0 ± 0.03	0.36 ± 0.03	1.78 ± 0.12
Control	11.8 ± 0.19	22.3 ± 0.7	0.38 ± 0.02	1.92 ± 0.08

* Values significantly different from control ($p < 0.01$).

Table 4. **Effects of HMMM on rainbow trout haematological parameters**

Concentration %	RBC count, $10^6 \times mm^{-3}$	Haematocrit level, l/l	WBC count, $10^3 \times mm^{-3}$
21.79	0.63± 0.02*	0.38 ± 0.01*	32.9 ± 2.7*
10.89	0.74 ± 0.04*	0.47 ± 0.02*	25.7 ± 3.3
5.45	0.81 ± 0.05	0.46 ± 0.02	36.0 ± 0.4*
1.1	0.60 ± 0.04*	0.44 ± 0.02	26.6 ± 3.0
Control	0.84 ± 0.04	0.42 ± 0.01	22.1 ± 1.8

* Values significantly different from control ($p < 0.05$).

The elevated leukocyte count determined in HMMM-exposed fish, as compared to the control, may indicate that HMMM induced a stress response in fish.

CONCLUSIONS

The present study evaluated the short- and long-term effects of a heavy metal model mixture on rainbow trout by using a set of biological parameters. The composition of metals was based on their real concentrations and proportion in soil near the Vilnius–Kaunas–Klaipėda highway.

The calculated value of the 96-hour LC_{50} of HMMM solution was 108.97%.

No significant changes were found in the weight of HMMM-exposed fish, while the liver weight of fish exposed to 21.79% of HMMM solution and the hepatosomatic index of fish exposed to 21.79 and 10.89% of HMMM solution differed significantly from controls.

Changes in blood parameters (erythrocyte count, haematocrit level, leukocyte count) revealed a specific toxicity of the HMMM to fish. Erythrocyte count decreased in the blood of fish exposed to most of the HMMM concentrations studied.

Our data suggest that even low amounts of traffic emission pollutants can negatively affect fish, causing various disturbances in its health and well-being.

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SUNKIŲJŲ METALŲ MODELINIO MIŠINIO POVEIKIS VAIVORYKŠTINIO UPĖTAKIO BIOLOGINIAMS RODIKLIAMS

Santrauka

Sunkiųjų metalų modelio mišinio (SMMM) ūminio ir ilgalaikio toksiškumo vaivorykštiniam upėtakiui tyrimai buvo atlikti naudojant kompleksą biologinių parametrų. Mišinys buvo suformuotas, remiantis realiomis metalų koncentracijomis ir proporcija dirvoje prie greitkelio Vilnius–Kaunas–Klaipėda. SMMM tirpalo koncentracija, prilyginta 100%, sudarė atitinkamai: Cu – 0,874; Zn – 0,93; Pb – 4,7; Ni – 0,66; Cr – 0,33; Mn – 18 mg/l. Apskaičiuota 48-val. mišinio LC50 vertė vaivorykštinio upėtakio jaunikliams sudarė 129,7% ir 96-val. LC50 vertė – 108,97% SMMM tirpalo. Ilgalaikiais tyrimais nenustatyta patikimų SMMM paveiktų žuvų masės skirtumų, lyginant su kontrole. Tuo tarpu žuvų, paveiktų 21,79% SMMM, kepenų masė bei paveiktųjų 21,79 ir 10,89% SMMM tirpalu kepenų somatinis indeksas patikimai skyrėsi nuo kontrolės. Kraujo rodiklių (eritrocitų skaičiaus, hematokrito lygio ir leukocitų skaičiaus) pokyčiai atskleidė specifinį SMMM toksiškumą žuvims. Žuvų, paveiktų visų tirtų koncentracijų SMMM, kraujyje eritrocitų skaičius sumažėjo. Mūsų gautieji duomenys leidžia manyti, kad net maži transporto teršalų kiekiai gali neigiamai veikti žuvų organizmą ir sukelti įvairių sveikatos sutrikimų.

Raktažodžiai: modelinis sunkiųjų metalų mišinys, žuvys, toksiškumas