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IMPACT OF COPPER AND ZINC MIXTURE ON BACTERIAL FLORA OF DIGESTIVE TRACT OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Abstract. The effects of 96-hour exposure and within 12 days after exposure to 0.25, 0.125, 0.06 portions of the 96-hour LC50 of Cu + Zn ions mixture on microbiological parameters of rainbow trout digestive tract were studied evaluating quantitative and functional characteristics of bacteria. The extinction of proteolytic bacteria was found in fish affected by 0.25 portion of the 96-hour LC50 of metal mixture. The recovery of proteolytic bacteria was determined in fish exposed to 0.125 and 0.06 portions of the 96-hour LC50 of Cu + Zn ions mixture after 8 days of keeping fish in clean water. The abundance of the investigated functional groups of bacteria was gradually recovering to the control level after 12 days of keeping fish in clean water.

Keywords: water pollution, environment monitoring, environmental sustainability.

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Introduction

Heavy metals and other man-made pollutants penetrating into the aquatic ecosystems from agriculture, urban and industrial sources, negatively affect the composition of bacterial communities of the digestive system of aquatic organisms, functional activity of intestinal bacteria and the whole physiological status of an organism. Moreover, due to pollution of aquatic ecosystems, separate species of autochthonous bacterial flora or even functional groups of bacteria in the digestive tract of aquatic species can be eliminated (Sugita *et al.* 2005; Bakke-McKellep *et al.* 2007; Navarrete *et al.* 2008).

Most of toxic contaminants enter fish via a food chain and from surrounding water. Whereas heavy metals are characterized by stability, they can persist in environment. Fish is at the top of the aquatic food chain and during its life can accumulate the large amount of metals (Couture, Pyle 2011). Eventually, through the food chains heavy metals can be transmitted to the humans (Saei-Dehkordi, Fallah 2011).

Low concentrations of copper (Cu) and zinc (Zn) ions exist in water bodies naturally. Fish use these metals as microelements (Watanabe *et al.* 1997) by consuming them together with food or directly from water (Handy 1996; Wood 2011). However, both Cu and Zn ions are determined to be toxic to aquatic fish when their levels increase above the background concentrations (Clearwater *et al.* 2002).

Pollution from industry and other anthropogenic activities has heightened the level of copper (Annual report of Water Quality of Rivers of Lithuania 2004). Copper sulphate ($CuSO_4$) is one of the most widely used algicides for phytoplankton control in fish ponds, reservoirs and lakes, due to leaching and runoff water from treated areas, it contaminates aquatic habitats (Gioda *et al.* 2007).

The toxic effects of waterborne and diet borne copper and zinc on fish are rather widely described in literature (Clearwater *et al.* 2002; Handy 2003; Bagdonas, Vosylienė 2006; Kazlauskienė, Vosylienė 2008). The acute, lethal and sublethal effects of Cu and Zn ions on physiological parameters of fish were assessed (De Boeck *et al.* 2004; Kazlauskienė, Vosylienė 2008; Eyckmans *et al.* 2011).

Genotoxic effects of different waterborne Cu and Zn ions mixture concentrations in the blood erythrocytes of *O. mykiss* were observed (Andreikėnaitė 2010).

Induction of toxicity of diet borne Cu and Zn ions have been described in rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*), common carp (*Cyprinus carpio*) (Lanno *et al.* 1985; Julshamn *et al.* 1988; Handy 1993). Reduced survival, growth and reproduction depending on diet borne metals in fish were determinated (Gundogdu *et al.* 2009). Interaction

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between diet borne metals and gastrointestinal tract of aquatic animals in ecosystems was noted (Liao *et al.* 2011).

Most of the metals ions in water bodies are found to be in the mixtures (or complexes ATSDR, Agency for Toxic Substance and Disease Registry, Draft 2004); therefore, it is important to evaluate effects of separate heavy metals and their mixtures (Kamunde, MacPhail 2011). The acute toxicity of Cu and Zn ions mixture and responses of different functional systems or changes in physiological parameters induced by these metals on *O. mykiss* at different life stages were studied by Kazlauskienė, Vosylienė (2008). According to these authors the effects of heavy metals ions singly could differ from the effects of Cu+Zn mixtures to fish.

Food and feeding conditions significantly influence the composition of gastrointestinal microbiota of fish (Onarheim, Raa 1990; Ringø 1993; Ringø, Olsen 1999; Uchii *et al.* 2006; Korsnes *et al.* 2006; Martin-Antonio *et al.* 2007; Delzenne 2008). Many authors highlight on changes in fish bacterial flora depending on species (Bairagi *et al.* 2002), fish development study (Ringø, Birkbeck 1999; Fjellheim *et al.* 2007), feeding habitat and season (Šyvokienė 1989; Ringø *et al.* 2006, 2010; Šyvokienė *et al.* 2011).

The formation of microorganism populations in the digestive tract of fish was found to take place directly through the surrounding environment (Nayak 2010). Microorganisms compete for the feeding substrates, and with an occurrence of new substrate in the environment quantitative and qualitative changes take place in the endo-system of macroorganisms. Therefore, intensification of fermentation activity of bacteria of one or even several functional groups and at the same time inhibition or even elimination of other groups can take place. It causes changes in metabolites excluded by microorganisms, which affect the physiological status, productivity and immune system of an animal. The narrower the spectrum of qualitative composition and abundance of microorganisms the less are the possibilities to adapt to the changing feeding substrates (Šyvokienė 1989, 1990, 1991). However, the enzymes of digestive tract of fish perform the most important role in these processes (Kuzmina 2005).

In the bacterial communities of the digestive tract of fish dominating facultative bacteria are: *Vibrio*, *Aeromonas*, *Enterobacteriaceae* (Ringø, Birkbeck 1999; Al-Harbi, Uddin 2004).

Our previous studies of the impact of heavy metals ions on microorganism communities of fish digestive tract demonstrated the reliance of the changes on the composition of heavy metals or their mixtures, their concentration or exposure time (Šyvokienė, Mickėnienė 1999a, b; Mickėnienė, Šyvokienė 2001; Mickėnienė *et al.* 2007; Šyvokienė *et al.* 2003, 2006).

The purpose of this work was to investigate the effects of 96-hour exposure and within 12 days after the end of exposure to different concentrations of Cu and Zn ions mixture on the bacterial flora of the digestive tract of adult rainbow trout (*Oncorhynchus mykiss.*

1. Materials and methods

Microbiological investigations were carried out at the Laboratory of Genotoxicology of the Institute of Ecology of the Nature Research Centre. Rainbow trout adults were obtained from the Žeimena Fish Hatchery (Švenčionys district, Lithuania) and kept in the holding tanks of about 3000 l capacity supplied with flow-through artesian aerated water. For experiment, fish were transferred from the holding tanks to the aquaria of 40 l capacity and kept for 1 week in the new medium until acclimation, i.e. till they started to swim freely and feed well. The length of fish under study ranged from 15.5 ± 1.1 to 18.0 ± 1.2 cm, and the weight from 32.7 ± 1.0 to 43.1 ± 2.3 g, respectively. Artesian water of high quality was used for dilution. The average hardness of dilution water was approximately 284 mg/l ($CaCO_3$), alkalinity 244 mg/l (HCO_3^-), the mean pH was 8.0, temperature was 12 ± 0.5 °C, and the oxygen concentration ranged from 8 to 10 mg/l. Water in the aquarium was changed daily and fish were fed until satiety with the commercial DANA FEED fish food. Test fish were exposed to 0.25, 0.125 and 0.06 portion of the 96-hour LC50 of metal ions mixture. After the exposure part of fish was taken for microbiological analyses. Whereas other fish of each group were transferred to the new aquaria with constantly aerated metal-free water in which they were kept for 4, 8 and 12 days till analyses. Control fish were affected to the same manipulations (transferred to another aquaria) but were kept all the time in clean water.

The concentrations of Cu^{+2} and Zn^{+2} ions were chosen based on previous studies, which indicated that the 96-hour LC50 of copper was 0.65 mg/l and the 96-hour LC50 of zinc was 3.79 mg/l (Svecevičius, Vosylienė 1996; Svecevičius 1999; Kazlauskienė, Vosylienė 2008). The final concentrations were recalculated according to the amount of copper and zinc ions. Expressions of the metals concentrations studied are shown in Table 1 (Bagdonas, Vosylienė 2006).

Table 1. Concentrations of Cu and Zn in mixture studied

Metals	Concentrations Portion of LC50	mg/l
Copper	0.25	0.16
Zinc	0.25	0.948
Mixture of Cu and Zn	0.25+0.25	0.16+0.948
Copper	0.125	0.08
Zinc	0.125	0.474
Mixture of Cu and Zn	0.125+0.125	0.08+0.474
Copper	0.0625	0.625
Zinc	0.0625	0.238
Mixture of Cu and Zn	0.0625+0.0625	0.04+0.238

By (Bagdonas, Vosylienė 2006)

Copper and zinc sulphate ($CuSO_4 \cdot 5H_2O$), $ZnSO_4 \cdot 7H_2O$, reagent grade 99%) (<REAKHIM> Company, Russia) were used as the toxicants, and stock solutions were prepared by dissolving a necessary amount of salts in distilled water (Svecevičius 2012).

For each microbiological analysis 8–10 fish specimens were used. Populations of aerobic and facultative anaerobic heterotrophic bacteria occurring in the digestive system of fish were counted using the dilution plate technique (Hansen, Olafsen 1999). The surface of rainbow trout specimens was sterilised with 95% ethanol, and then fish were dissected to remove their digestive tract. The content of the digestive tract was removed to sterile Petri dishes. Each set of experiment involved ten specimens of fish. All digestive tract samples were weighed and placed into a test tube, and then nine volumes of diluents were added. The tenfold dilution was further done serially. The volume of 0.1 ml of least dilutions (expected to give 30 to 300 colony-forming units, CFUs), was plated in triplicate on solid media. Incubation was carried out aerobically at 20 °C for seven days, except for Mac Conkey agar plates, which were incubated at 37 °C for 7 days. The number of CFU in the digestive tract of *O. mykiss* was established on four media: tryptone soya agar (OXOID) was chosen for isolation of total heterotrophic bacteria (THB), milk agar for proteolytic bacteria (PB) as a separate group of total heterotrophic bacteria, PB were identified according to the zones of protein (casein) hydrolysis on milk agar, MacConkey agar (OXOID) was used for total coliform bacteria (TCB). Bacterial colonies appearing on each plate were counted, and a CFU per 1 g (wet weight) of the digestive tract content was obtained (Kuznetsov, Dubinina 1989).

All data were analysed using standard statistical methods. Mean values, standard deviations and 95% confidence intervals were assessed.

The average values and mean square deviations were calculated (Sakalauskas 2003).

2. Results and discussion

Normal intestinal microflora of aquatic organisms is a natural biosorbent of toxic substances penetrating from the environment and produced in the host organism. Therefore, it responds to pollutants most sensitively (Šyvokienė, Mickėnienė 1999a). The dominating heterotrophic bacteria were observed among bacterial communities of the digestive tract of control fish (Figs 1–4) in the experiment of 96-hour exposure of rainbow trout to different parts of LC50 of Cu + Zn ions mixture.

The abundance of heterotrophic bacteria constituted 5.2×10^4 (4.72 log CFU g) CFU g of cells per gram of the content of the fish digestive tract. After 96-hour exposure to 0.06 portion of the LC50 of Cu + Zn ions mixture (concentrations of metal ions in this portion of mixture were ~16 times lower than 96-hour LC50 of Cu and 96-hour LC50 of Zn, respectively) the abundance of total heterotrophic bacteria decreased 6 times; after the same duration exposure to 0.125 portion of the LC50 of Cu + Zn ions mixture (concentrations of metal ions in this portion of mixture ~8 times were lower than 96-hour LC50 of Cu and 96-hour LC50 of Zn, respectively) – 26 times; and after exposure to 0.25 portion of the LC50 of Cu + Zn ions mixture (concentrations of metal ions in this portion of mixture were lower ~4 times than 96-hour LC50 of Cu and 96-hour LC50 of Zn, respectively) – 18 times as compared with this Parameter of control fish.

The exposure to the least concentration – 0.06 portion of LC50 Cu + Zn mixture induced two-fold elevation of the abundance of *O. mykiss* proteolytic bacteria, whereas the density of PB in digestive tract of fish lowered more than 11.5 times after exposure to medium – 0.125 portion of the LC50 Cu + Zn mixture.

Exposure of fish to the highest (0.25 portion of the 96-hour of the LC50 Cu + Zn mixture) concentration induced the extinction of proteolytic bacteria from bacterial communities of the digestive tract of rainbow trouts. In contrast, the abundance of coliform bacteria increased 140 times, as compared to the controls (Fig. 1).

Our previous studies of rainbow trout exposure to a range of concentrations from 0.16 to 0.67 parts of the 96-hour LC50 of equitoxic copper-zinc mixture showed that the mixture at concentration 0.16 part of the 96-hour LC50 induced significant changes in microbiological parameters. The abundance of heterotrophic bacteria decreased more than 2 times as compared to the controls ($p < 0.05$) and that of total coliform bacteria lowered, but the difference was not significant as compared to the controls. The densities of cultivable total coliform bacteria actually increased following the addition of the toxicant, possibly as a result of the release of organic carbon from poisoned, copper-zinc sensitive organism (Šyvokienė *et al.* 2006).

During experiment the recovery of the balance of bacterial flora of the digestive tract of rainbow trout after 4, 8 and 12 days of keeping fish in clean water was observed. The recovery of proteolytic bacteria in the digestive tract of fish groups affected to 0.125 and 0.25 portions of 96-hour of the LC50 Cu + Zn after 4 days keeping fish in clean water was not determined (Fig. 2). However, the recovery of the abundance of bacteria of all functional groups in bacterial communities of the digestive tract of *O. mykiss* was detected after 8 days of keeping fish in clean water. While, the restoration of proteolytic bacteria in bacterial communities of the digestive tract was observed almost entire only after 12 days of keeping rainbow trouts in clean water (Fig. 3).

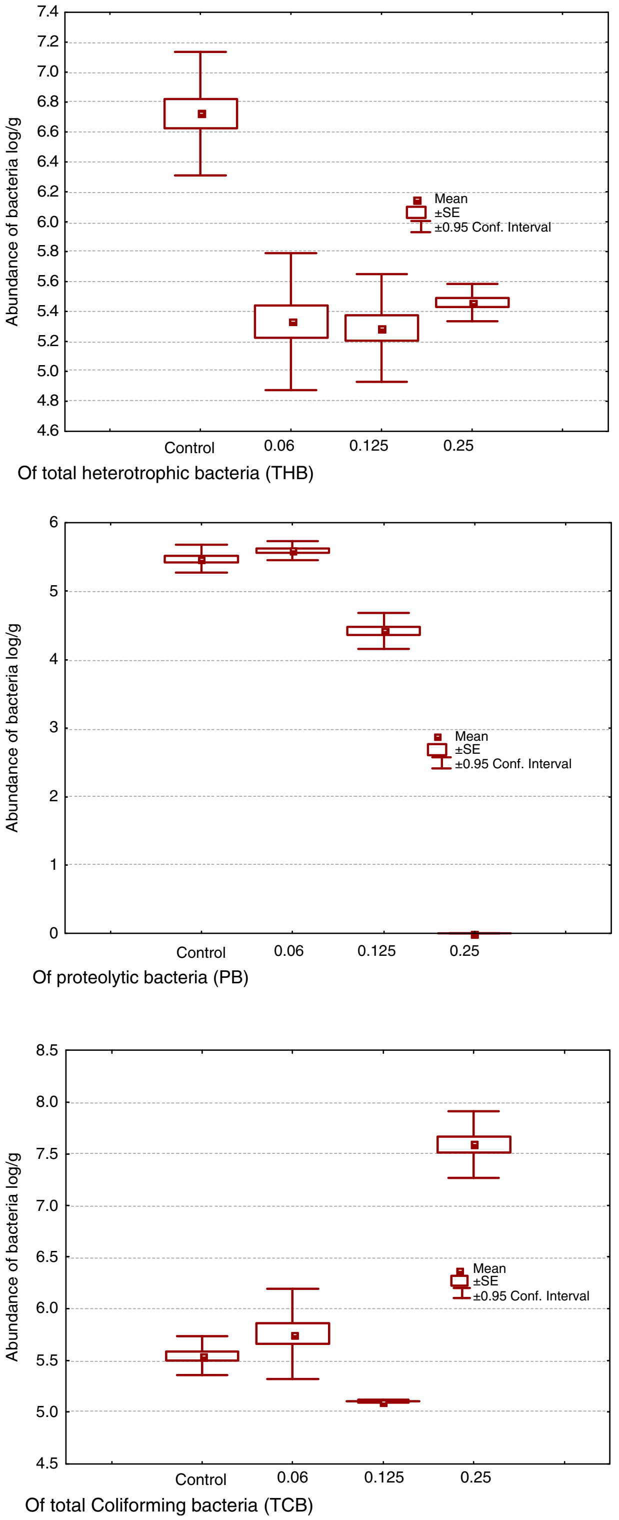

Fig. 1. The impact of the 96-hour exposure to the LC50 Cu+Zn ions mixture on the abundance of bacteria in digestive tract of rainbow trout (Oncorhynchus mykiss) Concentration in portion of equitoxic 96-hour LC50 mixture

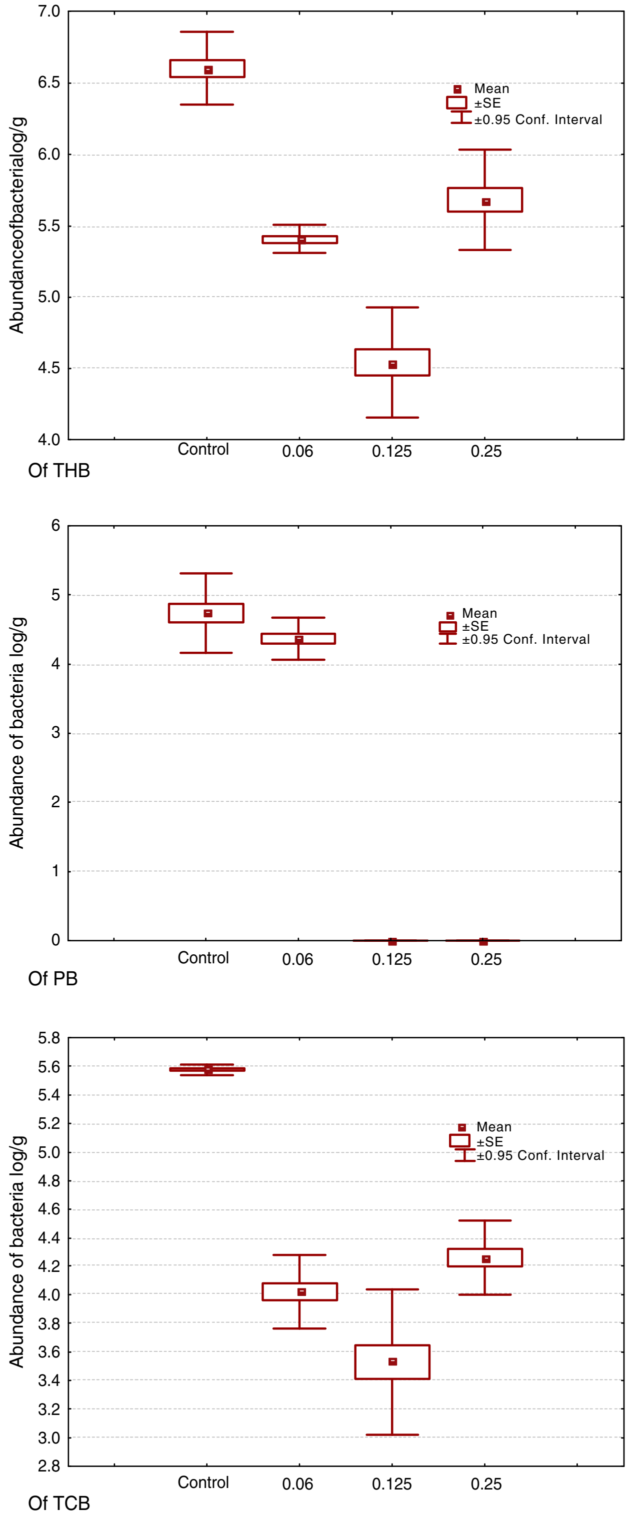

Fig. 2. The impact of the 96-hour exposure to the LC50 Cu+Zn ions mixture on abundance of bacteria in digestive tract of trouts after 4 days keeping fish in clean water Concentration in parts of equitoxic 96-hour LC50 mixture

The constantly changing environment is the main factor controlling the growth and development of microorganisms. Experimental studies on heavy metals toxicity demonstrate the negative effects of these contaminants on the structures of bacterial communities of the digestive tract and bacterial digestion of aquatic organisms as well as existence of imbalance of bacterial communities. Contamination of environment

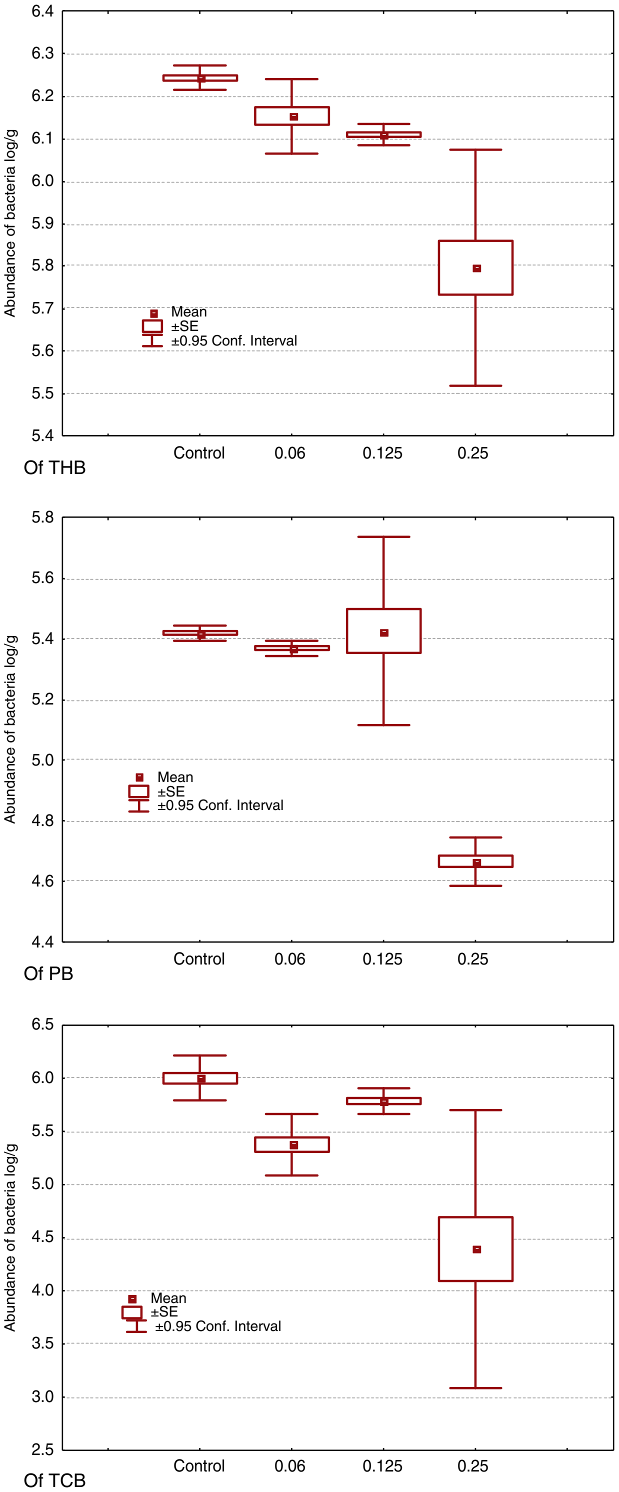

Fig. 3. The impact of the 96-hour exposure to the LC50 of Cu + Zn ions mixture on abundance of bacteria in digestive tract of trouts after 8 days keeping fish in clean water
Concentration in parts of equitoxic 96-hour LC50 mixture

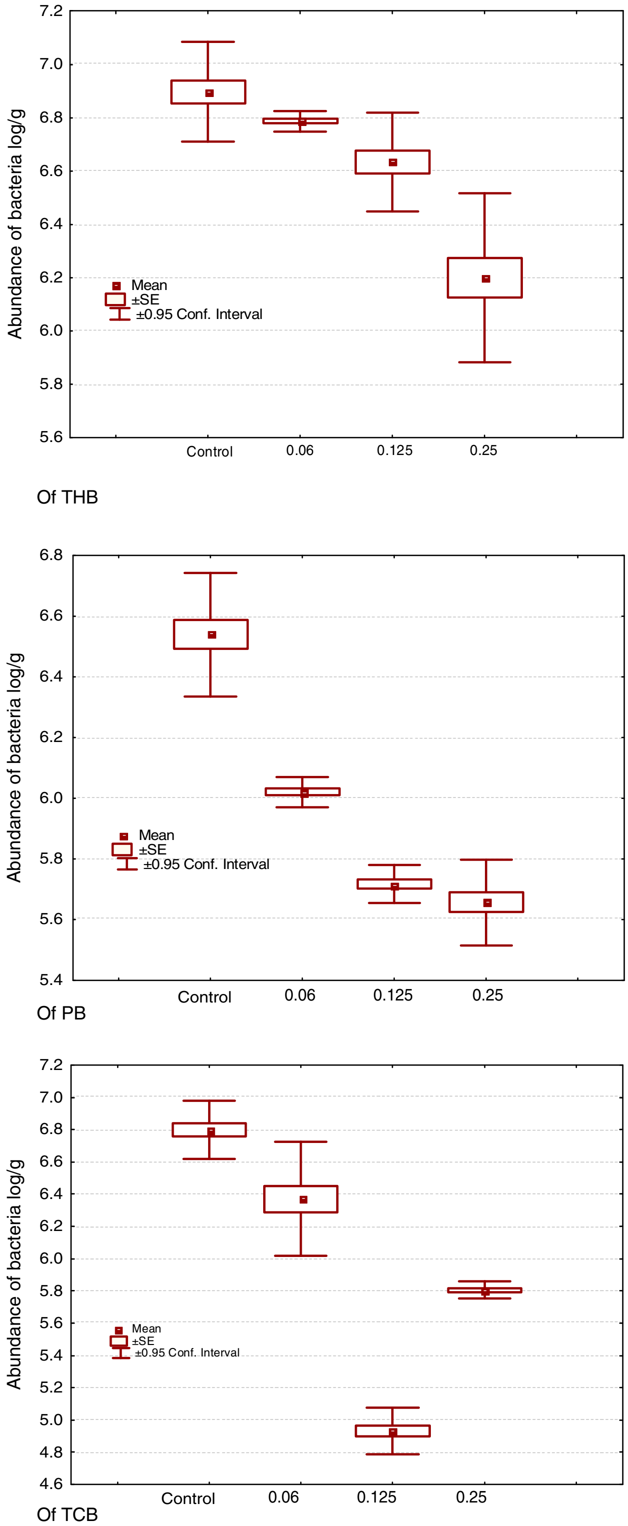

Fig. 4. The impact of the 96-hour exposure to the LC50 of Cu + Zn ions mixture on abundance of bacteria in digestive tract of trouts after 12 days keeping fish in clean water
Concentration in parts of equitoxic 96-hour LC50 mixture

was found to reveal a tendency of extinction of some proteolytic bacteria beneficial for the digestion of an organism (Šyvokienė 1989, 1990, 1991; Nayak 2010). Summarising obtained data, we can consider that proteolytic bacteria of the digestive tract of rainbow trout were the most sensitive to (Cu + Zn ions) mixture exposure. Proteolytic bacteria determine the regularities of the formation and functioning of autochthonous bacterial flora in the early ontogenesis of fish (Šyvokienė *et al.* 1995, 1999b).

After exposure to higher (0.125 portion of the LC50) concentration of mixture of studied metals the disturbances in the bacterial flora of the digestive tract of rainbow trout were observed. The decrease in the abundance of vitally important functional groups of bacteria was detected (Figs 2–4), a tendency of extinction of proteolytic bacteria was observed (Figs 1, 2). The recovery of PB was observed after 8 days of keeping fish in clean water (Figs 2, 3). According to Witeska and Kosciuk (2003) waterborne heavy metals, initially bound to gills and subsequently deposited in other tissues, might affect the fish, even if toxic agents were removed from the water.

Conclusions

1. The results of this study indicate that exposure of rainbow trout to 0.06 portion of the 96-hour LC50 of Cu+Zn ions mixture induced a six-fold decrease in the abundance of total heterotrophic bacteria in digestive tract of rainbow trout. Exposure to Cu+Zn mixture at concentration 0.125 portion of the LC50 decreased the abundance of heterotrophic bacteria 26 times and to Cu+Zn mixture at concentration 0.25 portion of the 96-hour LC50 –18 times.

2. The imbalance of trout's bacterial flora and the extinction of proteolytic bacteria from bacterial communities of the digestive tract of exposed fish were determined after treatment to the highest concentration (0.25 portion of the LC50 of Cu+Zn mixture). The abundance of total coliform bacteria group after exposure with the same metal mixture concentration increased 140 times.

3. The recovery of investigated proteolytic bacteria in bacterial cenoses of the digestive tract of *O. mykiss* was detected only after 8 days of transfering fish to clean water and the restoration of proteolytic bacteria in bacterial communities of the digestive tract was observed almost entire only after 12 days of transfering fish to clean water.

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