VILNIUS UNIVERSITY
NATURE RESEARCH CENTRE

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ACCUMULATION OF HEAVY METALS IN FISH, EXPOSED TO THE MULTI-METAL MIXTURES

Summary of doctoral dissertation

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VILNIAUS UNIVERSITETAS
GAMTOS TYRIMŲ CENTRAS

GINTARĖ SAULIUTĖ

SUNKIŲJŲ METALŲ KAUPIMASIS ŽUVYSE, VEIKIANT JAS DAUGIANARIAIS METALŲ MIŠINIAIS

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INTRODUCTION

Relevance of the study. Heavy metals (HM) are widely used in human activities, and, therefore they are classified as conventional pollutants of aquatic ecosystem. Metals such as zinc (Zn), copper (Cu), nickel (Ni), chromium (Cr), lead (Pb), cadmium (Cd), due to their toxicity, persistence, and bioaccumulation properties are attributed to priority hazardous substances in many countries (Directive 2008/105/EC; US EPA 2009).

Recently, more attention has been paid to the effects of various chemical mixtures (including mixtures of metals) on human health and the environment. The European Parliament is constantly indicating that the EU legislation on chemicals must take into account the additive effects of various chemicals (COM (2012) 252 final).

When metals accumulate in the body tissues, between them interactions occur that can be synergistic or antagonistic. The presence of some metals in the environment can quantitatively enhance or reduce the accumulation of other metals in the organisms (Cedergreen 2014). Metal interactions can substantially change important physiological processes in the organism: their uptake of chemical substances, their transport, metabolism, and elimination (Cedergreen 2014).

The effects of the metals mixtures in which the HM concentrations are environmentally relevant on fish are not fully investigated. Many studies analyze the accumulation of one or two metals and their toxicity to fish, but in the natural environment, multi-metal mixtures can be more toxic than the single metals (Jezierska and Witeska, 2001). Only some studies were carried out, in which the fish was exposed to multi-metal mixtures of the environmentally relevant HM concentrations (Pelgrom et al., 1995; Ribeyre et al., 1995; Komjarova and Blust, 2009a, 2009b). Such studies are relatively rare, furthermore, researchers use different experimental designs, different methods of testing the accumulation and toxicity to organisms. Therefore, the results are difficult to compare and often reflect the differences between the experimental systems used and not actual results. Most investigations are performed with a single species of fish, use high sublethal or lethal metal concentrations, and select experimentally unreasonable exposure duration (Duran et al., 2015; Driessnack et al., 2016, 2017). Therefore, it is unlikely that the results of such studies can be extrapolated to field conditions, except for accidental emergencies of pollution.

In the absence of data on the effects of chemical mixtures on organisms, the EU legislation so far has no systematic, comprehensive and integrated method that is provided for the assessment of the effects of mixtures that would take into account different uptake routes of the substances in organisms and the properties of the components of the mixture (COM (2012) 252 final). Therefore, in order to find out the patterns and mechanisms of the bioaccumulation of metals in the body tissues of the fish, it is necessary to carry out as many experimental studies as possible with multi-metal mixtures, in which there are environmentally relevant HM concentrations. Such studies would allow us to perform more accurately determination of the permissible limit values for the particular metals in water, taking into account the presence of other metals in the environment.

Scientific novelty of the study:

1. For the first time, differences in the accumulation of HM in the tissues of the fish were experimentally evaluated, exposing the fish to multi-metal mixture in the conditions of static water and rotating water-current.

2. For the first time, the optimal duration of exposure was experimentally determined, which is necessary to reach the steady-state of the HM concentration in the tissues, exposing the fish to multi-metal mixture at maximum permissible HM concentrations (MPC) in surface water (Directive 2000/60/EC).
3. It has been experimentally determined that exposing the fish to multi-metal mixtures with HM concentrations corresponding to MPC, the patterns of the HM accumulation in body tissues of the fish in most cases do not coincide with the patterns that are determined exposing the fish to the binary mixtures of HM.
4. For the first time, it has been determined that 10-fold reduced concentration of one of the metals in multi-metal mixture can significantly change the accumulation of some other HM in fish tissues.
5. It has been determined that even feeding with the same food, *Salmo salar* and *Perca fluviatilis*, which exclusively eat the food of animal origin in nature, accumulate more priority hazardous metals (Pb and Cd) in tissues than *Rutilus rutilus*, which is omnivorous in nature. However, the essential metals (Zn, Cu), by contraries accumulated more in the tissues of *R. rutilus* during the experiment. It shows that the differences in the accumulation of the above mentioned HM in fish tissues are not related to the diet and are caused by the other physiological processes.

Scientific and practical significance. The results of the study revealed that exposing the fish to multi-metal mixtures in the presence of environmentally relevant HM concentrations the interaction between HM specific for binary metal mixtures does not always reveal itself. When exposing to multi-metal mixtures, the *unilateral* change in the content of metals in fish tissues (decrease or increase) is more frequently observed. Therefore, the toxicity of the total mixture, which is directly related to the interaction of metals, cannot be predicted (tested) by the toxic effects of single or several metals. The components of the mixture interact by quantitatively increasing or reducing each other's accumulation in the organisms. For this reason, the toxicity of the metals mixture can change and can be stronger or weaker than predicted.

It has been determined that after 10-fold reduced concentration of a single metal in multi-metal mixture, the accumulation of some other metals in fish tissues can intensify. Correspondingly, decreasing in the concentration of a particular metal in the mixture does not necessarily result in a significantly lower accumulation of the metal in the fish tissues compared to the amount accumulated in the fish kept in the mixtures with a reduced concentration of any other HM. This shows that in the presence of other metals in the environment, the MPC that is determined for particular metal can be unsafe for the aquatic organisms.

The objective of the study is to investigate the patterns of the accumulation of six heavy metals (Zn, Cu, Ni, Cr, Pb, and Cd) in tissues (gills, liver, kidneys, and muscle) of different fish species exposed to the mixtures of metals in which the HM concentrations are not exceeding the maximum permissible concentrations.

The tasks of the study:

1. To determine the accumulation of metals (Zn, Cu, Ni, Cr, Pb, Cd) in the tissues of *S. salar*, *R. rutilus* and *P. fluviatilis* that are living in the natural environment.

2. To compare the patterns of the accumulation of heavy metals in *S. salar* tissues under the field conditions and experimental conditions (static water and rotating water-current condition).
3. To determine experimentally the optimal exposure duration, after reaching which the concentrations of HM in the tissues in *S. salar* do not increase significantly.
4. To determine experimentally the patterns of the accumulation of metals (Zn, Cu, Ni, Cr, Pb, and Cd) in body tissues of different fish species, using different combinations of multi-metal mixtures.
5. To determine the concentration of metallothioneins (MT) in the liver and kidneys of different fish species after exposure to multi-metal mixture.

Defensive statements:

1. Exposing the fish to multi-metal mixture under rotating water-current conditions, the patterns of the HM accumulation in the fish correspond to the patterns determined under field conditions. In the conditions of static water, the patterns of the HM accumulation change, therefore, such systems are unsuitable for the experimental study of processes that take place in nature.
2. The optimal time after the fish exposure to multi-metal mixtures is 14 days.
3. When exposing the fish to multi-metal mixtures with the reduced concentration of single metal, changes in the accumulation of HM in the fish tissues in most cases did not correspond to the patterns of the HM accumulation in the binary mixtures.
4. When exposing the fish to HM mixtures in which the MPC of one of the metals is reduced 10-fold, in certain cases, due to the interactions between HM the accumulation of metals can be higher than after the fish exposure to the mixture in which all HM concentrations correspond to MPC.
5. *Salmo salar* and *P. fluviatilis*, which exclusively eat the food of animal origin in nature, accumulate significantly more priority hazardous metals (Pb and Cd) in the tissues and less essential metals (Zn, Cu) than *R. rutilus*, which is omnivorous in nature. However, these differences in the accumulation of HM are not dependent on the fish diet, they should be caused by the differences in physiological processes in different diets of fish.

1. MATERIAL AND METHODS

The object of the study. The species of fish are classified using different taxonomic groups and according to specific needs for a habitation and food objects – Common roach (*Rutilus rutilus* Linnaeus, 1758), European perch (*Perca fluviatilis* Linnaeus, 1758) and Atlantic salmon (*Salmo salar* Linnaeus, 1758) have been selected for study.

The structure of the study. The research was carried out under field and experimental conditions.

Objective of the field study is to determine the main patterns of the HM accumulation in different body tissues of the fish and to select the appropriate experimental design based on these results. The study was conducted in 2012–2013. The bioaccumulation of metals was investigated in the body tissues of *P. fluviatilis* and *R. rutilus* from the aquatic ecosystem located near Kairiai landfill (Lithuania) and *S. salar*

from salmonid rivers Vilnia and Siesartis (Lithuania) that are hydrologically similar but their pollution level differs.

The experimental study was carried out in 2013–2016, in the Laboratory of Ecology and Physiology of Hydrobionts of the Nature Research Center. Different species of the fish (*S. salar, R. rutilus, P. fluviatilis*) were exposed to complex of different combinations in mixtures that consist of six metals (Zn, Cu, Ni, Cr, Pb, and Cd) under the same controlled test conditions. The accumulation of HM has been studied in the gills, liver, kidneys, and muscle. The experimental study was accomplished in four stages: during the first stage, the experimental design, which is the most appropriate for the field conditions was selected; during the second stage, the optimal exposure duration has been defined, after reaching it, the concentration of HM in body tissues of the fish does not increase significantly; in the third stage, it was carried out the study of the patterns on the accumulation of HM in the body tissues in different species of the fish, exposing the fish with seven different multi-metal mixtures, at the same exposure time; in the fourth stage, the concentration of metallothioneins (MT) in the liver and kidneys in different species of the fish after the exposure to multi-metal mixture was determined.

In both field and experimental studies, the contusion was performed on fish that was removed from the water before further investigation. Subsequently, fish were measured (total body length, mm) and weighed (total body weight, g). Later, they were used in the removal of needed tissues: muscle without skin, gills (whole organ), liver (whole organ) and kidneys (whole organ); organs were weighed to an accuracy of ±0.001 g. The concentration of HM in the samples was determined according to the ISO 15586:2003 standard.

1.1. The study of metal bioaccumulation in fish living in a natural environment

1.1.1 The study on the bioaccumulation of metals in the tissues of fish from the aquatic ecosystem located near Kairiai landfill

The Kairiai landfill site (55°55'42.7"23°23'42.81", WGS) was put into operation in 1960, closed in 2007. Large-scale household, municipal and industrial waste from various anthropogenic activities containing toxic substances has been deposited in it. The landfill is still continued to seep leachate, which is channeled into two isolated holding reservoirs, and maintained under open-air conditions. It is evident that landfill leachate is penetrating through permeable soils from holding reservoirs and pollute neighboring water bodies. The aquatic ecosystem incorporated in the landfill area consists of the nameless drainage channel surrounding the landfill which for the 1.5-km falls into the Ginkūnai Pond (of 1.1 km^2 area), and in turn the Švedė Creek flows out of the pond (Fig. 1).

For analysis of metals, *R. rutilus* and *P. fluviatilis* samples were collected (2012–2013) in six locations of the aquatic ecosystem (Fig. 1) at different distances from the landfill leachate reservoirs along the water flow direction [the number of individuals *P. fluviatilis* – 32, *R. rutilus* – 34; the number of samples: *N* (*P. fluviatilis*) = 11, *N* (*R. rutilus*) = 11]. For the analysis of metal accumulation, individuals of similar length and weight were selected. Concentration of metals were measured in fish gills, liver, and muscle and in water samples in the study sites.

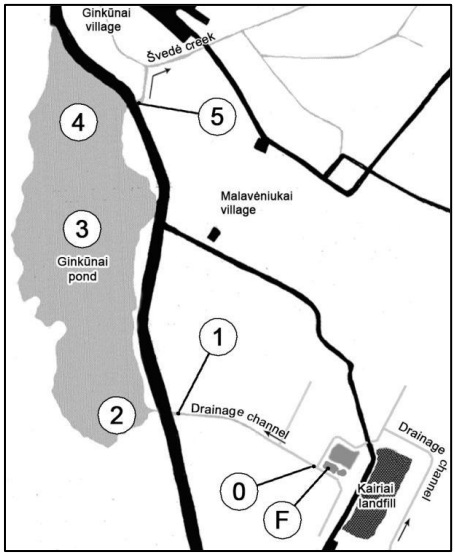


Fig. 1. The scheme of the study area and sampling stations: landfill leachate reservoir (F), drainage channel (station No. 0 and 1), Ginkūnai pond (station No. 2, 3, and 4) and Švedė creek flowing out of the pond (station No. 5).

1.1.2 The study on the bioaccumulation of metals in Atlantic salmon tissues in Vilnia and Siesartis rivers

Fish samples were collected in the Vilnia River below Nauja Vilnia town [54°41'25.67"25°21'33.31" (WGS)] and in the Siesartis River at the lower reaches (Ukmergė district) [55°17'24.67"24°51'54.67" (WGS)] in the middle of December 2013.

Vilnia flows through the urbanized area, in the past; it was heavily polluted with the domestic and industrial wastewaters and surface rainwaters (Gailiušis et al., 2001). There are no significant sources of pollution in the Siesartis river basin. Both rivers are typical salmonid water bodies, the monitoring of salmon fish is conducted in it (Praeivių žuvų būklės... 2009).

For the determination of the metal accumulation, in the rivers of Vilnia and Siesartis, 10 individuals of *S. salar* juvenile aged 1+ were caught (Sauliutė and Svecevičius, 2017). The concentration of metals was determined in *S. salar* in the gills, liver, kidneys, and muscle. At the same time, the water samples were taken at both litoral zones, and in the centre of riverbed of each river section analyzed ($N = 3$).

1.2. Experimental study on bioaccumulation of metals in fish tissues

The studies were conducted on hatchery-reared *S. salar* juveniles (age 1+ year) (Meškerinė fish hatchery) and 3-4 years old immature fish of *R. rutilus* and *P. fluviatilis* (UAB "Bartžuvė" fish hatchery).

The fish kept for acclimation in holding tanks (1000-L volume) supplied with flow-through aerated deep-well water at least two weeks prior to testing (minimum water flow rate1 L/g of their body mass per day). Fish were kept under a natural light cycle and fed commercial salmonids feed (ALLER PLATINUM) daily in the morning; the total amount was no less than 1% of their wet body mass per day. During the experiment, both water and diet was of the same type. Fish were accepted as acclimated to a new medium when they feed well.

During the experimental studies (see 1.2.1, 1.2.2, 1.2.3, 1.2.4), test fish were exposed to a six metal (Zn, Cu, Ni, Cr, Pb and Cd) mixture (MIX) prepared according to

Maximum-Permissible-Concentrations (MPC) accepted for the inland waters in EU (Directive 2000/60/EC) (Table 1).

Analytical grade metal salts (REACHIM, Russia) were used as the toxicants. Stock solution was prepared by dissolving necessary amount of the salt in distilled water, the final concentration being recalculated according to the amount of metal ion. Test solutions and clean water were renewed every day, and test fish were transferred into freshly prepared solutions after they were fed. Designed nominal metal concentrations in the test tanks (N = 3) were checked during blank tests (without fish) with an atomic absorption spectrophotometer (ISO 15586:2003). Each water sample was acidified with reagent-grade nitric acid (final concentration 0.5% v/v) and analyzed in triplicate. Mean measured concentrations were within 5% – 20% of the target (Table 1).

Table 1. Metals and their test waterborne concentrations (mg/L) in test media (Directive 2000/60/EC).

Metal	Source	Concentration (mg/L)	
		MPC nominal	Measured (mean ± SD)
Zn	$ZnSO_4 \cdot 7H_2O$	0.1	0.115 ± 0.014
Cu	$CuSO_4 \cdot 5H_2O$	0.01	0.009 ± 0.001
Ni	$NiSO_4 \cdot 7H_2O$	0.01	0.011 ± 0.002
Cr	$K_2Cr_2O_7$	0.01	0.012 ± 0.002
Pb	$Pb(NO_3)_2$	0.005	0.0045 ± 0.0004
Cd	$Cd(CH_3COO)_2 \cdot 2H_2O$	0.005	0.0052 ± 0.0003

Deep-well water was used as the dilution water. Its chemical and physical characteristics are presented in Table 2. The main physico-chemical parameters of the water (temperature, dissolved O_2, pH) were measured routinely with a hand-held multi-meter (WTW Multi 340i/SET, Germany).

Table 2. Chemical and physical characteristics of the dilution water.

Chemical and physical characteristics							
Metals (mg/L)		Cations (mg/L)		Anions (mg/L)		Other analytes	
Mn	0.068	Na^+	3.2	Cl^-	3.7	pH	7.9 – 8.1
Zn	0.0128	K^+	1.2	SO_4^{2-}	18.4	Temperature	12 – 13 °C
Cu	< 0.001	Ca^{2+}	70.1	HCO_3^-	258	Dissolved O_2	10 mg/L
Cr	< 0.001	Mg^{2+}	16.5	CO_3^-	0.18		
Ni	< 0.002	Fe^{2+}	0.1	NO_2^-	< 0.010		
Pb	< 0.001	Fe^{3+}	< 0.01	NO_3^-	< 0.050		
Cd	< 0.0003	Fe_{total}	0.1				
		NH_4^+	0.361				

1.2.1 The selection of the experimental system

This study was carried out in 2013–2014 with *S. salar* juvenile. Firstly, the experiment was conducted under semi-static *static water* conditions on five groups consisting of seven individuals (four treatments and one control) using glass tanks of 30-L total volume (20 x 30 x 50 cm) filled to a level of 2/3 with continuously aerated dilution water. Test fish were exposed for the 14-day period to multi-metal MIX mixture and singly to Ni, Cr and Pb (Svecevičius et al., 2014).

Subsequently, the study was conducted under semi-static *rotating water-current* conditions under analogous controlled test conditions. Aerators created the artificial flow of water in the test tanks. The system consists of polyethylene (PE) tanks (35 L); water

cooler, which was regularly supplied with flow-through aerated deep-well water (12–13 °C); air compressor; aerators.

Taking into account the quantitative accumulation results of HM in tissues of the fish, for further experimental studies, it was selected the experimental system that meets the field conditions better.

1.2.2 The determination of the optimal exposure duration

The objective of the study is to experimentally measure the time necessary to reach steady-state Zn, Cu, Ni, Cr, Pb, and Cd concentrations in analyzed fish tissues.

For the determination of the optimum exposure duration for HM, gills, liver, kidneys, and muscle of *S. salar* juvenile were used. The study was conducted in February-March 2015. Before the experiment, samples from non-exposed (fish after acclimation, 0 h of exposure, $N = 7$) fish were taken. The experiment was conducted under semi-static rotating water-current conditions on nine groups of fish (treatment and control, $N = 63$). Seven Atlantic salmons were put in each polyethylene (PE) tank of 35-L total volume filled to a level of 30 L with continuously aerate dilution water and with nine parallels, a total of 42 fish in treatment and 21 in control groups. In this experiment three controls for long-term exposure 7, 14 and 28 days were selected for making sure that animals used in minimum numbers (according to the "3Rs" – Reduce the number of animals used to a minimum, to obtain information from a smaller number of animals) (Directive 2010/63/EU). Test fish were exposed for the 1, 2, 4, 7, 14 and 28 days period to multi-metal MIX mixture.

1.2.3 The study of heavy metal accumulation patterns in the body tissues of different fish species

The patterns of the accumulation of metals (Zn, Cu, Ni, Cr, Pb, and Cd) were investigated in the gills, liver, kidneys, and muscle of the immature individuals of *S. salar, R. rutilus,* and *P. fluviatilis*. Studies of the metal accumulation with *S. salar* in the body tissues were carried out in April 2015, and *R. rutilus,* and *P. fluviatilis* in November-December 2016.

In this study, different species of the fish were exposed under the same controlled test conditions.

The experiment was conducted under semi-static rotating water-current conditions on 8 groups of fish (treatment and control, $N = 56$). Seven individuals were put in each polyethylene (PE) tank of 35-L total volume filled to a level of 30 L with continuously aerated dilution water and with 8 parallels, a total of 49 fish in treatment and 7 fish in control groups. The experiment was comprised of the seven treatments. Test fish were exposed for 14 days period to a six metal (Zn, Cu, Ni, Cr, Pb and Cd) mixture (MIX) at a concentration corresponding to Maximum-Permissible-Concentrations (MPC) (Table 1). Other treatments were performed by 10-fold reducing MPC of single metal in the mixture (MIX) made of 6 metals, while other 5 metals concentrations remain constant (e.g. Zn*0.1 (metal with 10-fold reduced concentration in MIX), while Cu, Ni, Cr, Pb, Cd concentrations remain constant (herein after referred to as Zn*0.1) and etc.).

The concentrations for waterborne metals (Cu, Zn, Ni, Cr, Pb and Cd) used in the current study are environmentally relevant, while 10-fold reduced concentration of a single metal in the mixture represent the possible influence of background exposure in the aquatic environment and to initiate the potential interactions of other HM that are

accumulating in the tissues of the fish contained in the mixture. Metals and their test concentrations in the mixtures of different metal combinations are presented in Tables 3 and 4.

Table 3. Mixtures of different combinations of metals and concentrations of HM (mg/L).

HM	Mixture / Source	MIX	Zn*0.1	Cu*0.1	Ni*0.1	Cr*0.1	Pb*0.1	Cd*0.1
		mg/L						
Zn	$ZnSO_4 \cdot 7H_2O$	0.1	0.01	0.1	0.1	0.1	0.1	0.1
Cu	$CuSO_4 \cdot 5H_2O$	0.01	0.01	0.001	0.01	0.01	0.01	0.01
Ni	$NiSO_4 \cdot 7H_2O$	0.01	0.01	0.01	0.001	0.01	0.01	0.01
Cr	$K_2Cr_2O_7$	0.01	0.01	0.01	0.01	0.001	0.01	0.01
Pb	$Pb(NO_3)_2$	0.005	0.005	0.005	0.005	0.005	0.0005	0.005
Cd	$Cd(CH_3COO)_2 \cdot 2H_2O$	0.005	0.005	0.005	0.005	0.005	0.005	0.0005

MIX – metal mixture corresponding to MPC (Directive 2000/60/EC)
HM*0.1 – mixtures of metals in which one of the metals MPC is reduced 10-fold
– metal MPC is reduced 10-fold

Table 4. Metals and their test concentrations in mixtures of different combinations of metals (mean ±SD, $N = 3$).

HM	Source	Concentration (mg/L)			
		MIX (MPC) nominal	MIX measured	HM*0.1 nominal	HM*0.1 measured
Zn	$ZnSO_4 \cdot 7H_2O$	0.1	0.115 ± 0.014	0.01	0.02 ± 0.001
Cu	$CuSO_4 \cdot 5H_2O$	0.01	0.009 ± 0.001	0.001	0.0018 ± 0.0003
Ni	$NiSO_4 \cdot 7H_2O$	0.01	0.011 ± 0.002	0.001	< 0.002
Cr	$K_2Cr_2O_7$	0.01	0.012 ± 0.002	0.001	0.0016 ± 0.0002
Pb	$Pb(NO_3)_2$	0.005	0.0045 ± 0.0004	0.0005	< 0.001
Cd	$Cd(CH_3COO)_2 \cdot 2H_2O$	0.005	0.0052 ± 0.0003	0.0005	0.00042 ± 0.00003

1.2.4. The study of metallothionein concentrations in tissues of different fish species

The concentration of metallothionein (MT) was investigated in 2015–2016, in the liver and kidneys of the *S. salar*, *R. rutilus*, and *P. fluviatilis* juveniles. The experiment was conducted under semi-static rotating water-current conditions on 2 groups of fish (treatment and control, $N = 14$). Seven individuals were put in each polyethylene (PE) tank of 35-L total volume filled to a level of 30 L with continuously aerated dilution water. Test fish were exposed for 14 days period to multi-metal MIX mixture.

For MT level assays, the liver and kidneys were removed, weighted and frozen (-80 °C). The concentration of MT in organs of the fish was determined at Vilnius University, the Center for Life Sciences, Laboratory of Biological Sciences (the methods for determining the concentration of HM and MT in fish tissues are described in detail in the dissertation). The concentration of MT and HM in the tissue was expressed in μg/g, mg/kg wet organ weight, respectively.

1.3. Statistical analysis

All of the study results obtained were statistically processed using STATISTICA 7.0 (StatSoft Inc., Tulsa, Oklahoma, USA). The normality of variable distributions was verified by the Kolmogorov-Smirnov and Shapiro-Wilk tests ($p > 0.05$).

The concentrations of heavy metals in the tissues of the fish in the Kairiai landfill site and salmon in Vilnia and Siesartis rivers were analyzed by two-way multidimensional dispersion analysis (*MANOVA*). Multiple comparisons of concentrations were performed using post-hoc multiple comparison Bonferroni criteria.

Data on the determination of the optimal exposure time (time necessary to stabilize concentrations of Zn, Cu, Ni, Cr, Pb, and Cd in body tissues) was analyzed by the one-way *ANOVA* (factor – exposure duration) and multiple comparisons post-hoc Bonferroni tests. The exposure time, from which the HM concentration did not significantly increase, was evaluated as optimal exposure duration.

The dependence of the metal amount in the tissues on the exposure time is also approximated by the logistic model of three parameters using the program CurveExpert Professional 2.6.3.

The patterns of metals Zn, Cu, Ni, Cr, Pb, and Cd accumulation in salmon, roach and perch body tissues exposing them to multi-metal mixtures were analyzed by two-way *ANOVA* and post-hoc Bonferroni criterion for multiple comparisons.

The differences in the concentrations of metallothioneins (MT) in the liver and kidneys of the control and test fish were evaluated using the one-way *ANOVA*. The relationship between MT and HM concentrations in body tissues of different fish species was determined using the Pearson correlation coefficient.

2. RESULTS AND DISCUSSION

2.1. The study of the metal accumulation in the fish living in a natural environment

During the field investigation, the accumulation of HM was investigated in tissues of *P. fluviatilis* and *R. rutilus* from Kairiai landfill aquatic ecosystem and *S. salar* from the rivers of Vilnia and Siesartis. Individuals of similar length and weight were selected for the determination of the concentrations of HM in the tissues (Table 5).

Table 5. Morphometric parameters of the *R. rutilus* and *P. fluviatilis* in the Kairiai landfill aquatic ecosystem and *S. salar* in Vilnia and Siesartis rivers (mean ± SD).

Parameter	*P. fluviatilis*	*R. rutilus*	*S. salar* (Vilnia)	*S. salar* (Siesartis)
Total length (*L*), mm	257.8 ± 14.4	262.1 ± 13.4	177.5 ± 2.7	127.5 ± 8.1
Total weight (*Q*), g	326.8 ± 39.4	329.8 ± 31.4	50.1 ± 2.4	22.5 ± 3.7
Number of individuals (*N*)	32	34	10	10

Zinc, Cu, Ni, and Cr were detected in the gills, liver, and muscle of *R. rutilus* and *P. fluviatilis*. The content of Pb and Cd in the samples were below instrument detection limit (< 1.0; < 0.3 μg/L, respectively). Quantitatively, maximum levels in fish body tissues found were of Zn (4.67 – 76.7 mg/kg) while the minimum of Cr (0.196 – 0.370 mg/kg). The accumulation of HM in the fish varies according to the type of fish and body tissue. The majority of HM concentrations in the tissues of the omnivorous roach were higher than in tissues of carnivorous perch (eating the food of animal origin). In *R. rutilus* HM was more accumulated in the gills, *P. fluviatilis* – in the liver. The content of HM in the muscle of the fish was least. Roach 5.5-fold accumulated more Zn in gills and 3.0-fold more Cu in liver than perch. Both roach and perch Cr (all tissues), Zn (liver and muscle) and Ni (liver) accumulated similarly (post-hoc Bonferroni tests, $p > 0.05$).

According to hydrological parameters the rivers (Vilnia and Siesartis) were quite similar. No significant differences were found between site water physicochemical parameters as well. Meanwhile, heavy metal (Cu, Ni, Cr, and Pb) concentrations in the Vilnia River water in the most cases (4/6) were significantly higher (one-way *ANOVA*, a river effect, $p < 0.05$) than those in the Siesartis River water, although, did not exceed permissible values (for more detailed results see the dissertation, section 3.1.2).

In the most cases (10/15) the amount of HM in body tissues of fish from the Vilnia River was significantly higher as compared to those from the Siesartis River (two-way *MANOVA*, post-hoc Bonferroni tests, $p < 0.05$). The concentration of Ni in the gills of *S. salar* in Vilnia River was even 13.3-fold, and Cu concentration in the liver was 10.4-fold higher than in the tissues of *S. salar* in Siesartis. The salmon in Siesartis River accumulated significantly more Pb in kidneys and muscle, Ni and Cr in kidneys and Cd in gills compared to *S. salar* from Vilnia. In both rivers, the highest HM concentration was detected in gills and liver of fish, the least – in muscle. Fish tissues mostly accumulated Zn, the least – Pb, and Cd. However, the levels of HM in fish tissues did not exceed permissible values (EC 2014, 2015).

The tissues of different species (*S. salar, R. rutilus* and *P. fluviatilis*) mostly accumulated the essential metals (Zn, Cu), the least – toxic metals (Pb, Cd). The results of the study correspond to the conception of the distribution of metals into essential (Zn, Cu), non-essential non-toxic (Ni, Cr) and non-essential toxic (Pb, Cd) (Roy 2010). During the study, patterns of the same kind of HM accumulation in body tissues of different species of fish taken from different aquatic ecosystem were determined. Quantitative sequences of the bioaccumulation of metals in fish tissues are presented in Table 6.

Table 6. The quantitative sequences of the metal accumulation in tissues of different fish species.

Tissue	Rivers (*S. salar*)		Kairiai landfill aquatic ecosystem	
Gills	Vilnia	Zn > Cu > Ni > Cr > Pb > Cd	*R. rutilus*	Zn > Cu > Ni > Cr > Pb = Cd*
	Siesartis	Zn > Cu > Cr > Ni > Cd > Pb	*P. fluviatilis*	Zn > Cu > Ni > Cr > Pb = Cd*
Liver	Vilnia	Cu > Zn > Cr > Ni > Cd > Pb	*R. rutilus*	Zn > Cu > Ni > Cr > Pb = Cd*
	Siesartis	Zn > Cu > Ni > Cr > Cd > Pb	*P. fluviatilis*	Zn > Cu > Ni > Cr > Pb = Cd*
Muscle	Vilnia	Zn > Cu > Cr > Ni > Pb > Cd	*R. rutilus*	Zn > Cu > Ni > Cr > Pb = Cd*
	Siesartis	Zn > Cu > Cr > Ni > Pb > Cd	*P. fluviatilis*	Zn > Ni > Cu > Cr > Pb = Cd*
Kidneys	Vilnia	Zn > Cu > Cr > Ni > Cd > Pb	–	
	Siesartis	Zn > Cu > Ni > Cr > Cd > Pb		

Note: *The content of Pb and Cd in the samples was below the detection limit of the device (<1.0 and <0.3 μg/L, respectively).

According to the data of the field investigation, metals tend to accumulate in the gills (*S. salar, R. rutilus*) and in the liver (*P. fluviatilis*). These results are in agreement with those obtained by Kroglund et al. (2008), Heier et al. (2009) and Nunes et al. (2015). Some possible explanations for such metals distribution in tissues is that absorption of metal ions usually occurs through passive diffusion or carrier-mediated transport over the gills, due to different affinity of various metals to the tissues or rate of decontamination in specific tissue (Teien et al., 2006; Playle 2004). Liver is an important target organ involved in metabolic and detoxification mechanisms (Karadede et al., 2004). According to Allen-Gill and Martynov (1995), low levels of metals accumulated in muscle are due to slower synthesis of proteins in this tissue.

2.2. The accumulation of metals in fish tissues under experimental conditions

2.2.1 The selection of the experimental system

The results of the study on field and experimental HM bioaccumulation in the body tissues of *S. salar* are presented in Table 7.

Under the same controlled conditions, *S. salar* were exposed for 14 days in multi-metal MIX mixture under MPC. In both field and during the experiment in the rotating

water-current conditions, the HM was the least accumulated in the muscle (Table 7). Pb is the only exception which was found to accumulate at the highest level in *S. salar* muscle tissue in both field, static water and rotating water-current conditions. The most reasonable explanation for this result is tending to be a slower elimination of this metal from muscle (Yousafzai et al., 2010).

Table 7. The average contents of Zn, Cu, Ni, Cr, Pb, and Cd (mg/kg) in *S. salar* body tissues under field conditions and in the mixture of MIX (G – gills; L – liver; K – kidneys; M – muscle).

HM	**Field conditions,** *N* (number of fish) = 10	**Static water conditions,** *N* (number of fish) = 7	**Rotating water-current conditions,** *N* (number of fish) = 7
Zn	Vilnia: 60.4 > 23.9 > 19.2 > 2.24 G > K > L > **M** Siesartis: 36.8 > 18.3 > 14.8 > 6.37 G > K > L > **M**	28.3 > 4.89 > 3.42 > 1.89 **M** > G > K > L	36.0 > 31.3 > 29.4 > 2.69 G > L > K > **M**
Cu	Vilnia: 44.7 > 3.05 > 1.08 > 0.67 L > G > K > **M** Siesartis: 4.25 > 0.820 > 0.610 > 0.440 L > K > G > **M**	0.354 > 0.287 > 0.235 > 0.224 **M** > L > K > G	5.94 > 1.77 > 0.799 > 0.708 L > K > G > **M**
Ni	Vilnia: 1.06 > 0.170 > 0.100 > 0.037 G > L > K > **M** Siesartis: 0.164 > 0.110 > 0.080 > 0.030 K > L > G > **M**	0.751 > 0.562 > 0.143 > 0.081 **M** > G > L > K	0.089 > 0.085 > 0.049 > 0.038 G > K > L > **M**
Cr	Vilnia: 0.470 > 0.180 > 0.110 > 0.050 L > G > K > **M** Siesartis: 0.153 > 0.130 > 0.100 > 0.050 K > G > L > **M**	0.280 > 0.135 > 0.125 > 0.039 **M** > L > K > G	0.569 > 0.287 > 0.130 > 0.046 G > K > L > **M**
Pb	Vilnia: 0.030 > 0.010 = 0.010 = 0.010 G > L = K = **M** Siesartis: 0.020 = 0.020 > 0.017 > 0.010 G = **M** > K > L	0.413 > 0.255 > 0.237 > 0.189 **M** > L > G > K	0.054 > 0.044 > 0.028 > 0.015 **M** > G > K > L
Cd	Vilnia: 0.060 > 0.040 > 0.010 > 0.003 K > L > G > **M** Siesartis: 0.035 > 0.030 > 0.020 > 0.004 K > G > L > **M**	0.041 > 0.034 > 0.032 > 0.020 L > **M** > G > K	0.503 > 0.243 > 0.154 > 0.004 G > K > L > **M**

The results of the study showed that the patterns of the bioaccumulation of HM in fish tissues in rotating water-current conditions are relevant for the patterns determined during the field investigation. Similar results were obtained by Jezierska and Witeska (2001), Yancheva (2010) and Yousafzai et al. (2010). Meanwhile, the results of the experiment in static water conditions were opposite to the results of the field investigation. The fish accumulated the largest amounts of metal in the muscle (see Table 7, highlighted in red). Muscle is not an active tissue regarding the accumulation of metals due to the low content of metal-binding proteins (e.g. MT) (Yancheva et al., 2014; Jezierska and Witeska, 2001). Therefore, it is probable that experimental systems with the static water conditions are unsuitable for the accumulation of HM in the fish. We suppose that here could be a couple of reasons why the *S. salar* accumulated the highest amounts of metals in the muscle. It seems that the answer is in the test experimental design and fish behavior. When the *S. salar* were transferred into test tanks they distributed in the tank occupying corners and angles lying on the bottom of the tank. During all 2-week exposure period fish activity was low. Meanwhile, it is well known that Atlantic salmon is rheophilous species. In the field salmon are very active. They continuously demonstrate rheotaxis, actively search for food and catch it intensively,

perform distant and long-lasting anadromous and catadromous migrations. It seems that if the fish are active they rather release the metals from the muscle and other tissues. It has been confirmed by this field study and results of the experiment in the rotating water-current conditions. For this reason, for further experimental studies, the experimental system of the rotating water-current conditions was selected that correspond more the field conditions.

2.2.2 The determination of the optimal exposure time

The main objective of the study was to experimentally measure the time necessary to attain the steady-state Zn, Cu, Ni, Cr, Pb, Cd concentrations in analysed tissues of *S. salar*. The experiment was conducted under semi-static rotating water-current conditions. Test fish were exposed for the 1, 2, 4, 7, 14 and 28 days period to multi-metal MIX mixture.

The test was conducted on hatchery-reared *S. salar* juveniles, for control and test groups, individuals of similar length [*L* (control) = 171.7 ± 10.4 mm; *L* (test) = 170.6 ± 8.64 mm] and weight [*Q* (control) = 48.3 ± 9.69 g; *Q* (test) = 44.6 ± 6.70 g] (mean ± SD; *N* (control) = 28; *N* (test) = 42) were selected.

No significant differences among evaluated parameters were measured between long-term controls (7, 14 and 28 days) and non-exposed groups (0) (one-way *ANOVA*, post-hoc Bonferroni tests, $p > 0.05$). Statistical analysis between long-term controls and treatment groups (7, 14, 28 days), also between non-exposed and treatment groups were performed, the results showed same differences between values. Therefore, further statistical analyses are accomplished by comparing exposure groups with a non-exposed group (0) (Stankevičiūtė et al., 2017)

Statistically significant metal accumulation was observed in gills (of Ni and Cr), liver (of Cr), kidneys (of Ni) and muscle (of Zn and Cr) in comparison to non-exposed group even after 1 day of exposure (see the section 3.2.2 in the dissertation). Zinc and copper bioaccumulation in gills and liver did not differ significantly from non-exposed values during exposure period of 28 days. In contrast, accumulation of these metals in kidneys and muscle was significant. Quantitatively maximum levels were found for Zn and Cu, while the minimum levels for Ni and Pb. Overall, metals were accumulated mostly in the gills and kidneys, at least in the muscle. It was noted that the magnitude of Zn, Ni, Cr and Cd accumulation in the tissues showed the following sequence: gills > kidneys > liver > muscle; Cu – liver> kidneys > gills > muscle and Pb – muscle > gills > kidneys > liver.

Metals bioaccumulation analysis revealed that metal distribution was non-linear during exposure period and varied between body tissues. Therefore, the logistic model of the three parameters was used to approximate the increase in the relative amount of metals in the gills, liver, kidneys and muscle during the experiment (Fig. 2).

Logistic model was used to calculate the relative amount of metals in the tissues studied. It was expressed as the ratio between the HM concentrations measured after 1, 2, 4, 7 and 14 days and HM concentrations established in fish at the end of the experiment, i.e. after 28 exposition days. The results show that in case of all four tissues the dependence curves of the accumulation of HM exposure time similar in shape. The model chosen for approximation is appropriate, as shown by the high coefficient of determination of the model ($R^2 > 0.9$). Metals at the beginning of the exposure (on average up to 7 days) accumulate in fish tissues rather intensively. Later accumulation of

HM slowed down with increasing the exposure duration. After 7, 14 and 28 days the amount of HM in the gills, liver, kidneys and muscle increased slightly (Fig. 2).

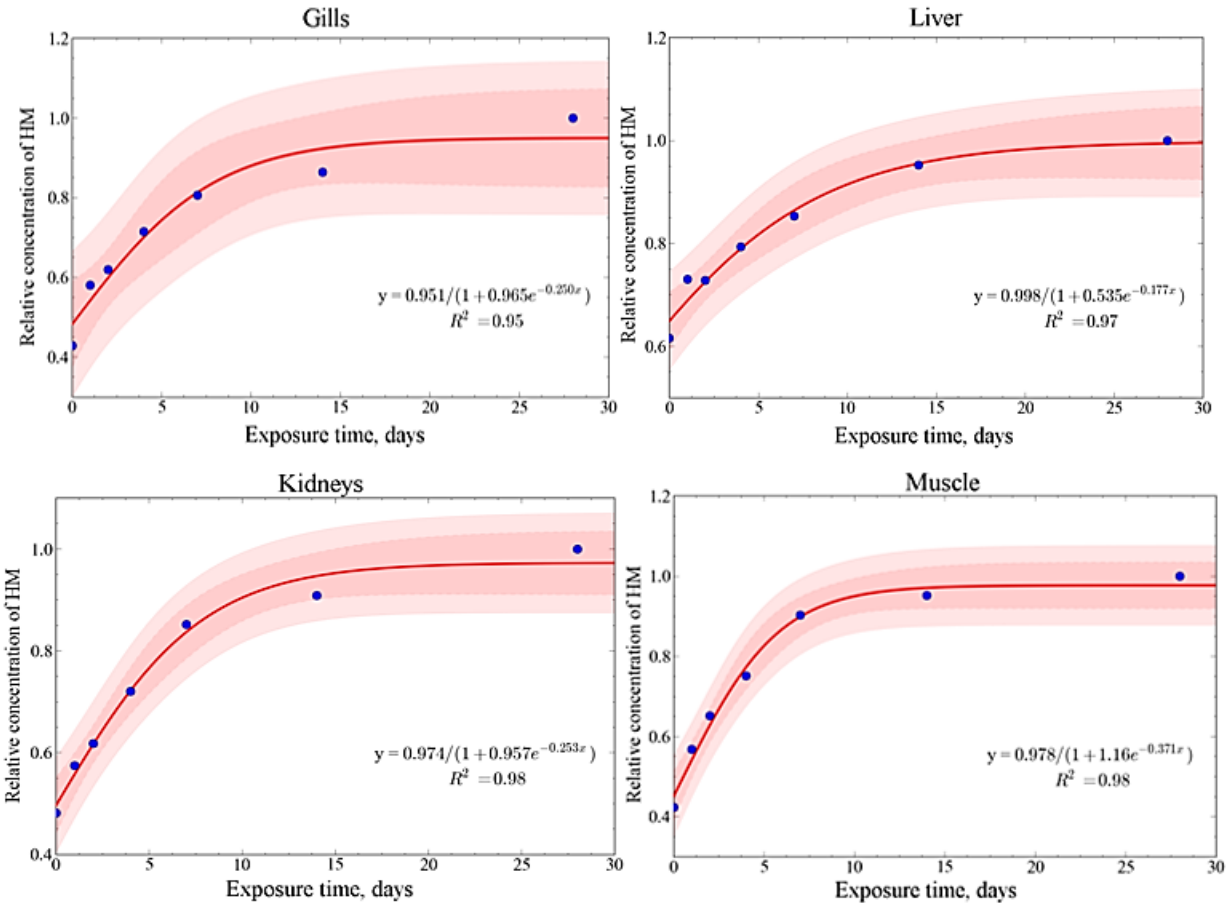


Fig. 2. The logistic model of the dependence of relative HM content in the body tissues of *S. salar* on the duration of the exposure (the means of the relative HM contents, the prediction and its 95% confidence interval are depicted).

The time necessary to reach the steady-state Zn, Cu, Ni, Cr, Pb, Cd concentrations in analysed tissues of *S. salar* exposed to multi-metal MIX mixture is shown in Table 8.

The time necessary to reach steady-state was metal specific and varied between organs. Zinc and Cu, which are essential metals, did not reach steady-state in gills and liver, because no significant differences ($p > 0.05$) were measured between exposed and non-exposed groups. While, steady-state Zn and Cu concentrations in kidneys and muscle were reached within 4, 14 and 14, 7 days, respectively. Nickel has not reached steady-state only in gills tissue, whereas liver, kidney s and muscle Ni concentrations appeared to reach steady-state at different exposure period. Steady-state Cr and Pb concentrations were reached in all analysed tissues. It should be noted, that Cd accumulation increased with increasing exposure periods at all tissues. Cadmium, which has no known biological function in fish, has not reached steady-state in all tissues except the muscle tissue. It should be mentioned, that all metals have reached steady-state in muscle of *S. salar*, but at different exposure period (Table 8). Several studies have shown, that accumulation tendency of metals by the muscle tissue is comparatively low, while decontamination rate in muscle tissue is very fast (Perera et al., 2015; Yeşilbudak and Erdem, 2014).

To summarize, steady-state for metals were reached within 14 days in most of the tested tissues. This is confirmed by the relative changes of HM content shown in Figure 2 – after seven days, the concentrations of HM significantly decrease. According to this, the duration of the 14-day exposure was chosen for further experimental studies.

Table 8. The time necessary to attain the steady-state Zn, Cu, Ni, Cr, Pb and Cd concentrations in analysed tissues of *S. salar* exposed to multi-metal mixture (MIX)

Metal	Tissue	Exposure time, days					
		1	2	4	7	14	28
Zn	Gills						
	Liver						
	Kidneys						
	Muscle						
Cu	Gills						
	Liver						
	Kidneys						
	Muscle						
Ni	Gills						
	Liver						
	Kidneys						
	Muscle						
Cr	Gills						
	Liver						
	Kidneys						
	Muscle						
Pb	Gills						
	Liver						
	Kidneys						
	Muscle						
Cd	Gills						
	Liver						
	Kidneys						
	Muscle						

Steady-state not reached

Steady-state

This research sheds new light on tissue-specific and time related necessary to reach the steady-state of HM concentrations during exposure to an entire metal mixture. At the beginning of the experiment, the accumulation of HM in the tissues was more intensive and when increasing the exposure time, the accumulation of HM was slowed down, i.e. the concentration did not increase significantly. Statistically significant bioaccumulation of several metals (Ni and Cr) was observed even after 1 day of exposure. In general, metals were bioaccumulated mainly in the gills and kidneys, at lesser extent in the muscle. Some possible explanations for such metals distribution in tissues is that absorption of metal ions usually occurs through passive diffusion or carrier-mediated transport over the gills, due to different affinity of various metals to the tissues or rate of decontamination in specific tissue (Jezierska and Witeska, 2006). In addition, the bioaccumulation of metals is a dynamic process in which the uptake and elimination of HM from the body tissues occurs simultaneously (Streit 1998). It should be noted that Cd and Ni have not reached steady-state in gills, liver, kidneys and gills, respectively, i.e. it was not enough within 28 days to reach a steady state. Metal elimination routes are slower than uptake routes and depend on the biological half-life of the metal. As reported by Larson et al. (1985), in rainbow trout biological half-life for liver and kidney Cd was

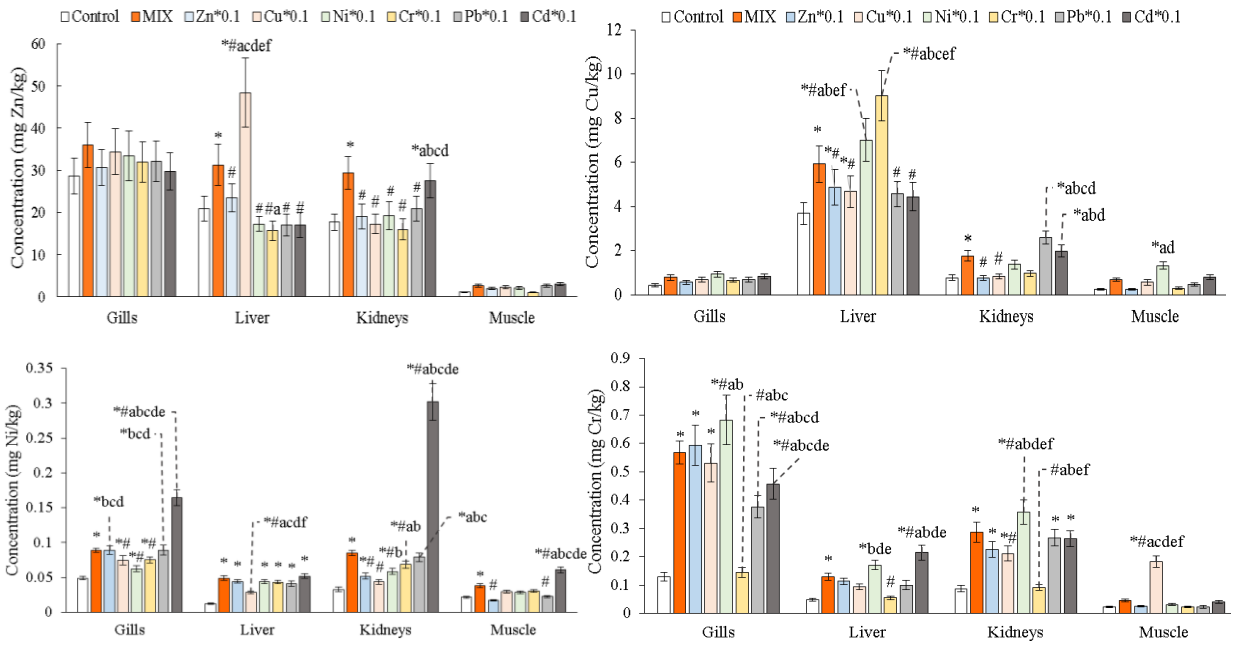
more than a year. Otherwise, especially a long uptake phase to reach Cd steady-state in all tissues may be required. Essential metals such as Zn and Cu are quickly eliminated, levels of these metals are homeostatically controlled (Bury et al., 2003). Whereas Cd and Ni are released much more slowly from the tissues (Kargin and Çogun, 1999).

This is a first attempt to evaluate the time needed to attain steady-state internal body concentrations in Atlantic salmon tissues for metals after exposure to multi-metal mixture at MPC. While, many researchers prefer unreasoned exposure time ranging from several hours to 270 days (Komjarova and Blust, 2009a; Calamari et al., 1982). It is necessary to determine exposure duration experimentally (under the same controlled conditions), due to further investigations of interactions in metal mixture. The time required to achieve a steady-state level in fish exposed to an entire mixture is likely different from that necessary in single-metal exposures. Whereas, in the primary period of exposure metal is absorbed and accumulated at a high rate, level stabilizes when an equilibrium of metal uptake and excretion rates is achieved (Jezierska and Witeska, 2006). The results of this study will allow us to continue to carry out and compare the results of the experimental study, which will analyze the patterns of accumulation of the same HM in the body tissues of different fish species.

2.2.3 The study of heavy metal accumulation patterns in the body tissues of different fish species

The objective of this study was to determine the patterns in the accumulation of metals (Zn, Cu, Ni, Cr, Pb, and Cd) in *S. salar*, *R. rutilus*, and *P. fluviatilis* body tissues (gills, liver, kidneys, and muscle), exposing to different combinations of multi-metal mixtures. For the experiment (control and treatment group) individuals of approximately equal length and weight was selected (see the dissertation). The treatment group was exposed to seven different combinations of multi-metal mixtures (MIX, Zn*0.1, Cu*0.1, Ni*0.1, Cr*0.1, Pb*0.1, and Cd*0.1) for 14 days.

The contents (mg/kg of w/w) of metals accumulated in the body tissues of *S. salar*, *R. rutilus*, and *P. fluviatilis* in the control and treatment groups are shown in figure 3, 4 and 5.



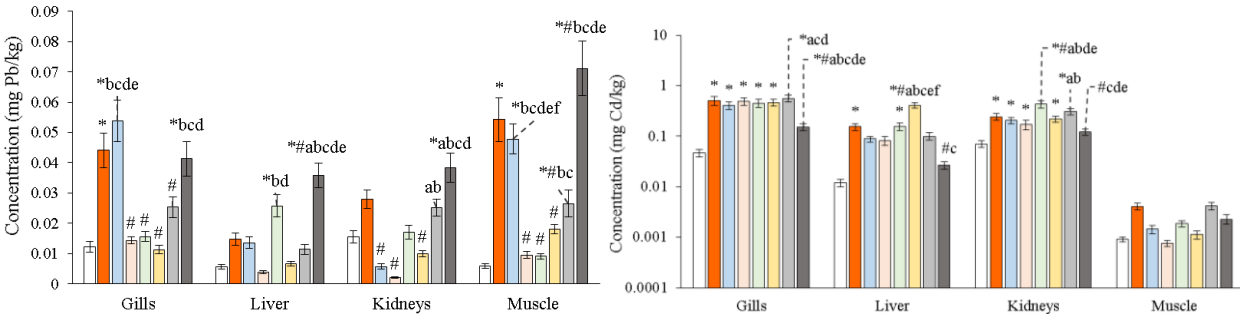


Fig. 3. The content of metals in *S. salar* of the body tissues, exposing to multi-metal mixtures (mean ± SD, *N* = 7). The asterisk (*) marks significant differences in values from control; **#** – from MIX; **a** – from Zn*0.1; **b** – from Cu*0.1; **c** – from Ni*0.1; **d** – Cr*0.1; **e** – Pb*0.1; **f** – Cd*0.1) (two-way *ANOVA*, post-hoc Bonferroni tests, $p < 0.05$). In the Y-axis, the concentration of Cd is given on a logarithmic scale.

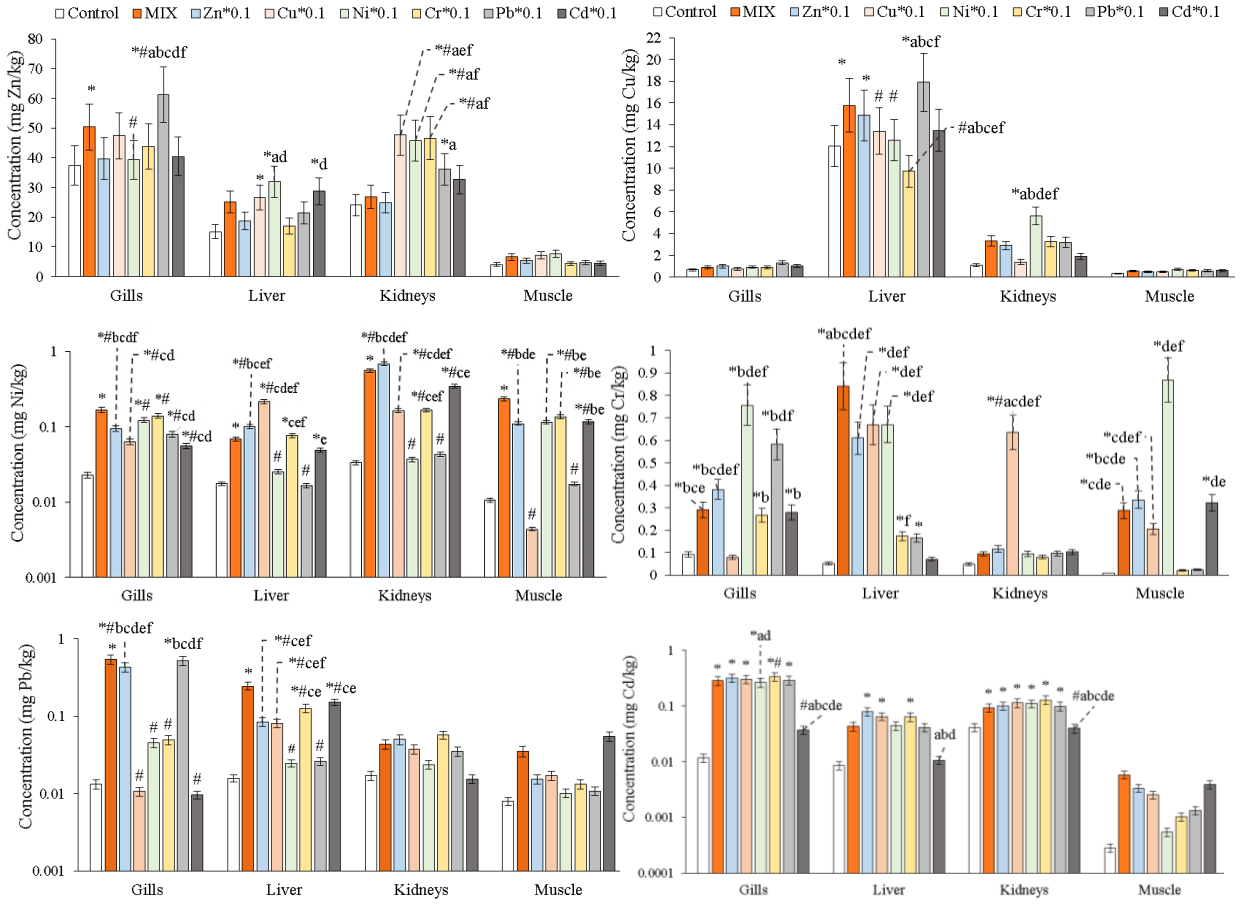


Fig. 4. The content of metals in *R. rutilus* of the body tissues, exposing to multi-metal mixtures (mean ± SD, *N*= 7). The asterisk (*) marks significant differences in values from control; **#** – from MIX; **a** – from Zn*0.1; **b** – from Cu*0.1; **c** – from Ni*0.1; **d** – Cr*0.1; **e** – Pb*0.1; **f** – Cd*0.1) (two-way *ANOVA*, post-hoc Bonferroni tests, $p < 0.05$). In the Y-axis, the concentration of Ni, Pb, and Cd is given on a logarithmic scale.

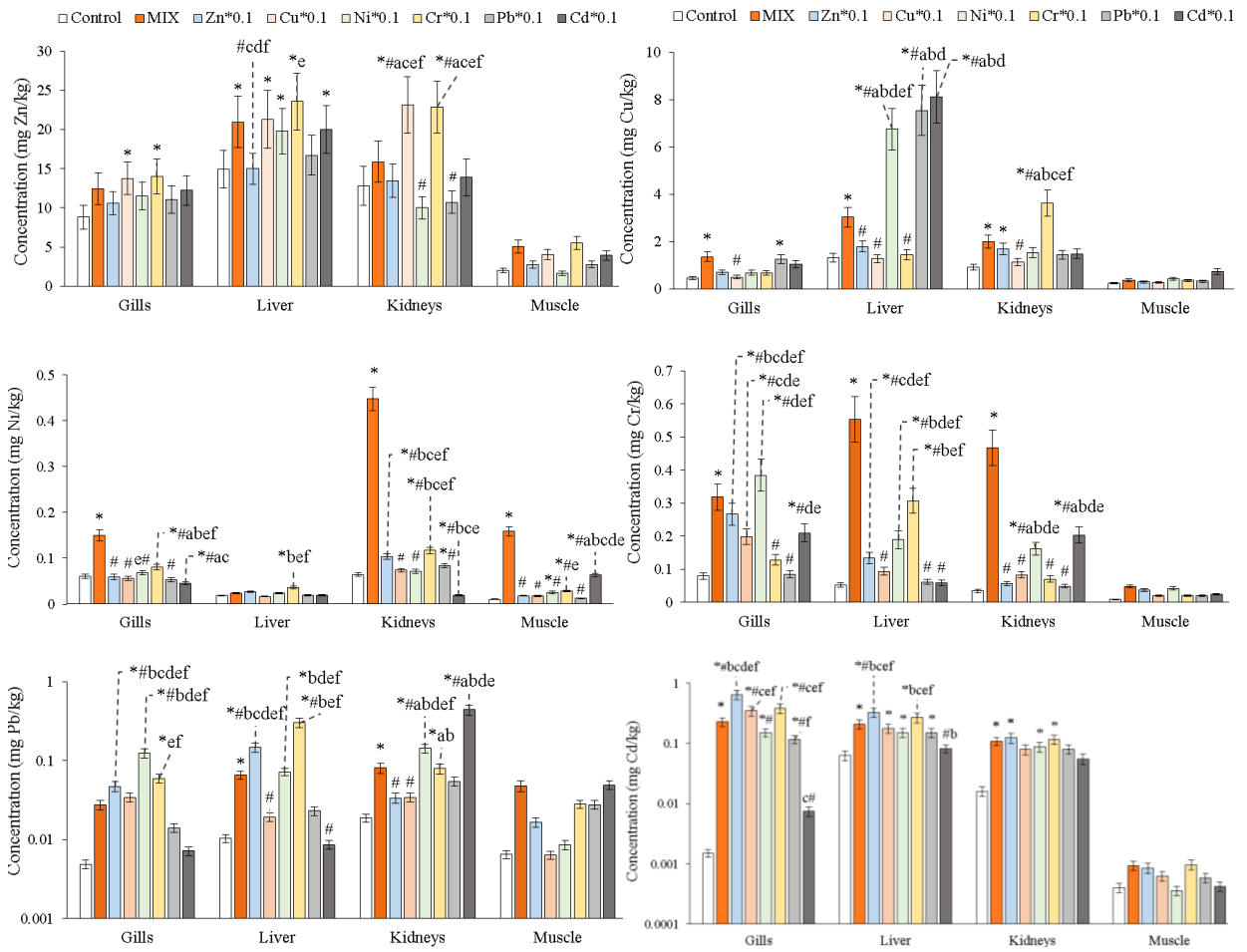


Fig. 5. The content of metals in *P. fluviatilis* of the body tissues, exposing to multi-metal mixtures (mean ± SD, *N*= 7). The asterisk (*) marks significant differences in values from control; **#** – from MIX; **a** – from Zn*0.1; **b** – from Cu*0.1; **c** – from Ni*0.1; **d** – Cr*0.1; **e** – Pb*0.1; **f** – Cd*0.1) (two-way *ANOVA*, post-hoc Bonferroni tests, $p < 0.05$). In the Y-axis, the concentration of Pb and Cd is given on a logarithmic scale.

Different fish species exposed to HM*0.1 mixtures were observed differences in a specific tissue of the same HM content change (decrease or increase) compared to the MIX mixture (Table 9).

After comparison of the MIX mixture, in all three fish species in a particular tissue, in most cases, *unilateral change* was observed, only one of the metal content change (decreasing or increasing), when the MPC of another metal is reduced 10-fold (i.e. the reduction of the second HM concentration in the mixture does not cause the changes in the accumulation of the first HM in tissue). Less ordinarily, there was a change in the content of the same two HM in the *same direction* (when reducing the concentration of one of two specific metals in the MPC mixture 10-fold, the accumulation of another metal in the fish tissues also decreases) or the change in the content of the same amount HM but the *opposite direction* (when the concentration of the first of two specific metals in the MPC mixture is reduced 10-fold, the accumulation of the second HM in fish tissues decreases, but after the reduction of the second HM in the MPC, the accumulation of the first HM increases). The most common patterns of the accumulation of HM were found in the muscle (Table 9).

Table 9. The recurrent change of the content of HM in the gills, liver, kidneys, and muscle of three different fish species (*S. salar, R. rutilus, P. fluviatilis*), exposing to multi-metal HM*0.1 mixtures in which the MPC of a single metal was reduced 10-fold (↓ – *unilateral* decrease of metal content; ↑ – *unilateral* increase of metal content; ↓↓ – change in contents of HM in the *same direction*; ↓↑ – change in contents of HM in the *opposite direction*).

Compered to MIX					
GILLS			**LIVER**		
HM mixture	HM	Change content of HM	HM mixture	HM	Change content of HM
Cu*0.1	Ni	↓	Zn*0.1	Cu	↓↑
Cu*0.1	Cr	↓↓	Zn*0.1	Cr	↓
Ni*0.1	Zn	↓	Cu*0.1	Cr	↓
Ni*0.1	Cd	↓	Cu*0.1	Pb	↓
Cr*0.1	Ni	↓↑	Cr*0.1	Cd	↑
Cd*0.1	Zn	↓	Pb*0.1	Zn	↓
Cd*0.1	Cr	↓	Pb*0.1	Ni	↓
Cd*0.1	Pb	↓	Pb*0.1	Cr	↓
			Pb*0.1	Cd	↓
MUSCLE			**KIDNEYS**		
Zn*0.1	Cu	↓	Zn*0.1	Cu	↓
Zn*0.1	Ni	↓	Cu*0.1	Ni	↓
Zn*0.1	Pb	↓	Cu*0.1	Pb	↓
Zn*0.1	Cd	↓	Cr*0.1	Ni	↓
Cu*0.1	Ni	↓↑	Pb*0.1	Ni	↓
Cu*0.1	Pb	↓			
Cu*0.1	Cd	↓↑			
Ni*0.1	Pb	↓↓			
Ni*0.1	Cd	↓			
Cr*0.1	Ni	↓			
Cr*0.1	Pb	↓↓			
Cd*0.1	Pb	↑			

In the gills of different fish species, it was observed the decrease of Cr and Cu accumulation exposing to Cu*0.1 and Cr*0.1 mixtures, respectively (*a change in the same direction*). In gills, an increase in Cr accumulation was observed exposing to Ni*0.1 mixture, but exposing to Cr*0.1 mixture the Ni content significantly decreased (*a change in the opposite direction*). In the liver, the opposite direction change of the metals revealed itself, during which the Cu content decreased exposing fish to Zn*0.1 mixture, but exposing to Cu*0.1, the content of Zn increased. In kidneys, only the unilateral change (decrease) in metal content was detected exposing the fish to a specific HM*0.1 mixture. The changes in the levels of Pb in the same direction were determined in the muscle exposing the fish to Ni*0.1 and Cr*0.1 mixtures, and the opposite direction change was determined in Ni and Cd, exposing different species of fish to the Cu*0.1 mixture (Table 9). It is also regular that significant decrease in accumulated Ni amounts in all tissues (except the liver) of all three fish species after treatment with Cu*0.1 and Cr*0.1 mixtures. Similarly, significantly lower of Ni concentration in gills and muscle of *R. rutilus* and *P. fluviatilis* all treatments groups (HM*0.1) were measured compared to MIX mixture.

The fact that the ratio of concentrations of different metals in the mixture significantly influences the accumulation of HM in the body tissues of fish is illustrated in Table 10 by the sequences of HM content after treatment with different multi-metal

mixtures. In the position of the first sequence indicates the HM mixture in which the fish of the particular species was kept and in the tissue of that fish the highest content of specific metal accumulated, and in the last one the mixture in which the fish was kept and the content of the same metal was the lowest.

Table 10. The accumulations of metals (Zn, Cu, Ni, Cr, Pb, Cd; in the first column of table are specified bold and underlined) in body tissues of fish species, exposing to different types of multi-metal mixtures (HM*0.1 mixtures in the table are indicated in Italic, without "*0.1" symbol). In the first position of the sequence, the mixture of HM is indicated, in which the fish of the particular species accumulated heavy metals in tissues at the highest concentrations.

The black asterisk (*) indicates the HM*0.1 mixture in which the significantly higher content amount of particular metal was accumulated compared with the content that accumulated in the tissues of the fish that was kept in MIX (two-way *ANOVA*, post-hoc Bonferroni tests, $p < 0.05$);
The red asterisk (*) indicates the HM*0.1 mixture, in which the fish was kept and accumulated significantly higher content of the same metal (MPC was reduced 10-fold in the mixture, indicated in red) compared to the content accumulated in the fish tissue kept in the mixture indicated in the last place of the sequence (two-way *ANOVA*, post-hoc Bonferroni tests, $p < 0.05$).

	HM, mg/kg	*S. salar*	*R. rutilus*	*P. fluviatilis*
		Metal mixture		
GILLS	**Zn**	**MIX**>*Cu->Ni->Pb->Cr->Zn->Cd-*	*Pb->***MIX**>*Cu->Cr->Cd->Zn->Ni-*	*Cr->Cu->***MIX**>*Cd->Ni->Pb->Zn-*
	Cu	*Ni->Cd->***MIX**>*Cu->Pb->Cr->Zn-*	*Pb->Cd->Zn->Ni->***MIX**>*Cr->Cu-*	**MIX**>*Pb->Cd->Zn->Ni->Cr->Cu-*
	Ni	*Cd->*Zn->Pb->***MIX**>*Cr->Cu->Ni-*	***MIX**>*Cr->Ni->*Zn->Pb->Cu->Cd-*	***MIX**>*Cr->Ni->*Zn->Cu->Pb->Cd-*
	Cr	*Ni->*Zn->***MIX**>*Cu->Cd->Pb->Cr->*	*Ni->*Pb->*Zn->***MIX**>*Cd->Cr->*Cu-*	*Ni->***MIX**>*Zn->Cd->Cu->Cr->-Pb-*
	Pb	*Zn->***MIX**>*Cd->Pb->Ni->Cu->Cr-*	**MIX**>*Pb->*Zn->Cr->Ni->Cu->Cd-*	*Ni->Cr->*Zn->*Cu->***MIX**>*Pb->Cd-*
	Cd	*Pb->***MIX**>*Cu->Cr->Ni->Zn->Cd->*	*Cr->*Zn->Cu->Pb->***MIX**>*Ni->Cd->*	*Zn->*Cr->*Cu->***MIX**>*Ni->Pb->Cd->*
LIVER	**Zn**	*Cu->***MIX**>*Zn->*Ni->Cd->Pb->Cr-*	*Ni->Cd->Cu->***MIX**>*Pb->Zn->Cr-*	*Cr->Cu->***MIX**>*Cd->Ni->Pb->Zn-*
	Cu	*Cr->Ni->***MIX**>*Zn->Cu->Pb->Cd-*	*Pb->***MIX**>*Zn->Cd->Cu->*Ni->Cr-*	*Cd->Pb->Ni->***MIX**>*Zn->Cr->Cu-*
	Ni	*Cd->***MIX**>*Zn->Ni->*Cr->Pb->Cu-*	*Cu->*Zn->*Cr->***MIX**>*Cd->Ni->Pb-*	*Cr->Zn->***MIX**>*Ni->Pb->Cd->Cu-*
	Cr	*Cd->*Ni->***MIX**>*Zn->Pb->Cu->Cr-*	***MIX**>*Ni->Cu->Zn->Cr->*Pb->Cd-*	***MIX**>*Cr->*Ni->Zn->Cu->Pb->Cd-*
	Pb	*Cd->*Ni->***MIX**>*Zn->Pb->Cr->Cu-*	***MIX**>*Cd->Cr->Zn->Cu->Pb->Ni-*	*Cr->*Zn->*Ni->***MIX**>*Pb->Cu->Cd-*
	Cd	*Cr->*Ni->***MIX**>*Pb->Zn->Cu->Cd-*	*Zn->Cu->Cr->Ni->***MIX**>*Pb->Cd-*	*Zn->*Cr->***MIX**>*Cu->Ni->Pb->Cd-*
KIDNEYS	**Zn**	**MIX**>*Cd->Pb->Ni->Zn->Cu->Cr-*	*Cu->*Cr->*Ni->*Pb->Cd->***MIX**>*Zn-*	*Cu->*Cr->***MIX**>*Cd->Zn->Pb->Ni-*
	Cu	*Pb->Cd->***MIX**>*Ni->Cr->Cu->Zn-*	*Ni->***MIX**>*Cr->Pb->Zn->Cd->Cu-*	*Cr->*Zn->***MIX**>*Zn->Ni->Cd->Pb->Cu-*
	Ni	*Cd->*Zn->***MIX**>*Pb->Cr->Ni->*Zn->Cu-*	*Zn->*Zn->***MIX**>*Cd->Cu->Cr->Pb->Ni-*	***MIX**>*Cr->Zn->Pb->Cu->Ni->*Cd-*
	Cr	*Ni->*Zn->***MIX**>*Pb->Cd->Zn->Cu->Cr->*	*Cu->*Zn->Cd->Pb->***MIX**>*Ni->Cr-*	***MIX**>*Cd->Ni->Cu->Cr->Zn->Pb-*
	Pb	*Cd->***MIX**>*Pb->*Ni->Cr->Zn->Cu-*	*Cr->Zn->***MIX**>*Cu->Pb->Ni->Cd-*	*Cd->*Ni->*Ni->***MIX**>*Cr->Pb->Cu->Zn-*
	Cd	*Ni->*Pb->***MIX**>*Cr->Zn->Cu->Cd-*	*Cr->Cu->Ni->Zn->Pb->***MIX**>*Cd->*	*Zn->Cr->***MIX**>*Ni->Pb->Cu->Cd-*
MUSCLE	**Zn**	*Cd->Pb->***MIX**>*Cu->Ni->Zn->Cr-*	*Ni->Cu->***MIX**>*Zn->Pb->Cd->Cr-*	*Cr->***MIX**>*Cu->Cd->Pb->Zn->Ni-*
	Cu	*Ni->Cd->***MIX**>*Cu->Pb->Cr->Zn-*	*Ni->Cr->Cd->Pb->***MIX**>*Cu->Zn-*	*Cd->Ni->***MIX**>*Cr->Pb->Zn->Cu-*
	Ni	*Cd->*Zn->***MIX**>*Cr->Cu->Ni->Pb->Zn-*	***MIX**>*Cr->Cd->Ni->*Zn->Pb->Cu-*	***MIX**>*Cd->Cr->Ni->Zn->Cu->Pb-*
	Cr	*Cu->*Zn->***MIX**>*Cd->Ni->Zn->Cr->Pb-*	*Ni->*Zn->Cd->***MIX**>*Cu->Pb->Cr-*	**MIX**>*Ni->Zn->Cd->Pb->Cu->Cr-*
	Pb	*Cd->*Zn->***MIX**>*Zn->Pb->*Cr->Cu->Ni-*	*Cd->***MIX**>*Cu->Zn->Cr->Pb->Ni-*	*Cd->***MIX**>*Cr->Pb->Zn->Ni->Cu-*
	Cd	*Pb->***MIX**>*Cd->Ni->Zn->Cr->Cu-*	**MIX**>*Cd->Zn->Cu->Pb->Cr->Ni-*	*Cr->***MIX**>*Zn->Cu->Pb->Cd->Ni-*

As it was mentioned before, when analyzing the accumulation of HM in the tissues of different fish species, the highest concentrations of metals do not necessarily accumulate in fish tissues that were kept in MIX mixture. In most cases, in HM mixture sequences, MIX doesn't occupy the first position. This approves the fact that in mixtures (HM*0.1) in which the MPC of one metal was reduced 10-fold, the accumulation of any metals in fish body tissues can be induced.

Similarly, reducing the MPC of the specific metal in a mixture 10-fold, a lower accumulation of the same metal in the tissue was not always detected in comparison to the contents in the same tissue of fish exposed to other HM*0.1 mixture (see Table 10, highlighted in red). This shows that fish body tissues, in the presence of other metals in the mixture, tend to accumulate a specific metal even if the metal concentration in the mixture is relatively low. For example, the content of Cd accumulated in the muscle of the fish after treatment with Cd*0.1 mixture (i.e. in a mixture with a 10-fold reduced Cd concentration) was not significantly lower than in the fish after treatment with MIX mixture. However, in other tissues, by contrast, the lowest Cd concentrations were accumulated after exposure to the Cd*0.1 mixture.

There is also a regularity that the contents of Cr in the gills and Pb in the muscle were significantly higher in the fish after treatment with Ni*0.1 and Cd*0.1 mixtures, respectively. It is quite high probability that interactions between Cr-Ni and Pb-Cd may occur, which increase the accumulation of one metal in the fish tissue, reducing the concentration of the other metal in the mixture.

Exposing different fish species to multi-metal mixtures, metals accumulated in the fish body tissues quite differently (Table 11).

Table 11. The comparison of metal accumulation in the body tissues of different fish species (roach (R), perch (P), salmon (S)) exposing the fish to multi-metal mixtures.

Mixture	Tissue	Zn	Cu	Ni	Cr	Pb	Cd
Control	Gills	R	R	P	S	R	S
	Liver	S	R	P	P	R	P
	Kidneys	R	R	P	S	P	S
	Muscle	R	R	S	S	R	S
MIX	Gills	R	P	R	S	R	S
	Liver	S	R	R	R	R	P
	Kidneys	S	R	R	P	P	S
	Muscle	R	S	R	R	S	R
Zn*0.1	Gills	R	R	R	S	R	P
	Liver	S	R	R	R	P	P
	Kidneys	R	R	R	S	R	S
	Muscle	R	R	R	R	S	R
Cu*0.1	Gills	R	R	S	S	P	S
	Liver	S	R	R	R	R	P
	Kidneys	R	R	R	R	R	S
	Muscle	R	S	S	R	R	R
Ni*0.1	Gills	R	S	R	R	P	S
	Liver	R	R	S	R	P	S
	Kidneys	R	R	P	S	P	S
	Muscle	R	S	R	R	R	S
Cr*0.1	Gills	R	R	R	R	P	S
	Liver	P	R	R	P	P	S
	Kidneys	R	P	R	S	P	S
	Muscle	P	R	R	S	P	S
Pb*0.1	Gills	R	R	S	R	R	S
	Liver	R	R	S	R	R	P
	Kidneys	R	R	P	S	P	S
	Muscle	R	R	S	R	P	S
Cd*0.1	Gills	R	P	S	S	S	S
	Liver	R	R	S	S	R	P
	Kidneys	R	S	R	S	P	S
	Muscle	R	S	R	R	S	R

Note: the different colors (pink – roach; yellow – salmon; green – perch) indicate a significant (two-way ANOVA, post-hoc Bonferroni tests, $p < 0.05$) higher content of HM in fish tissues of the particular species compared to other fish species.

R. rutilus tended to accumulate significantly more HM than *S. salar* and *P. fluviatilis*. The table below shows that in the tissues of omnivorous *R. rutilus* higher content of Zn, Cu, Ni accumulated, and Pb, Cd in the carnivorous fish (*S. salar* and *P. fluviatilis*). Chromium in tissues approximately equally accumulated in both omnivorous and carnivorous fish. Although *R. rutilus* tissues accumulated less Cd than *S. salar* and *P. fluviatilis*, however, under MIX; Zn*0.1; Cu*0.1 and Cd*0.1 mixtures, the content of Cd accumulated in the muscle of *R. rutilus* are significantly higher than in the muscle of other fish species. The information mentioned above confirms that the accumulation of HM in the tissues depends on the type of fish and body tissue, and of metal and metal concentration in multi-metal mixture where fish are exposed.

The current study is the first to examine the effects of exposure to multi-metal HM mixture low waterborne concentration and importance of 10-fold reduction of single mixture component on tissue-specific metal accumulation in fish. The results of the research showed that during the exposure of fish to multi-metal HM*0.1 mixtures, the interaction was possible between the HM and resulted in higher or lower content of metals in fish tissues. After comparison to the MIX mixture, in all three fish species in a particular tissue, in most cases, *unilateral change* was observed, only one of the metal content alteration (decreasing or increasing), when the MPC of another metal is reduced 10-fold (i.e. the reduction of the second HM concentration in the mixture does not cause the changes in the accumulation of the first HM in tissue). Less ordinarily, there was a change in the content of the same two HM in the *same direction* (when reducing the concentration of one of two specific metals in the MPC mixture 10-fold, the accumulation of another metal in the fish tissues also decreases) or the change in the content of the same amount HM but the *opposite direction* (when the concentration of the first of two specific metals in the MPC mixture is reduced 10-fold, the accumulation of the second HM in fish tissues decreases, but after the reduction of the second HM in the MPC, the accumulation of the first HM increases). When analyzing the studies of other authors, it can be observed that the interaction of HM is often investigated between two or three metals, exposing the fish to single metals and their mixture (Driessnack et al., 2016, 2017; Duran et al., 2015; Winter et al., 2012, McGeer et al., 2007; Eroglu et al., 2005; Kargin and Çogun, 1999; Pelgrom et al., 1995). The test waterborne concentrations are chosen to be the same for exposing the fish to both single metals and the mixture, and the content of metals in fish tissues is compared. Most of the time the interaction between metals is determined during the study. However, there are several researches, in which only the unilateral change in one of the metals was determined. Driessnack et al. (2017) analyzed the interaction between Cd and Zn in a mixture. Zinc significantly inhibited the accumulation of Cd in the gills and liver. However the content of Zn in the tissues did not decrease in the presence of Cd (unilateral change). McGeer et al. (2007) investigated the absorption of metals in the gills, exposing the fish to Cu + Cd mixture. It has been shown that Cu inhibited the accumulation of Cd in the gills, but the Cu absorption in the gills does not change in the presence of Cd in the mixture. Pelgrom et al. (1995) also investigated the interaction between Cd and Cu by exposing the fish to single metals and their mixture. They determined unilateral interaction between these metals. When exposing to the mixture, the content of Cd in the tissues decreased, unlike exposing to Cd separately. However, the author did not determine the impact of Cd on the accumulation of Cu in fish under the Cd + Cu mixture. Similarly, in this study, there

were several changes in the content of HM in the fish tissues of the same or opposite direction. The following changes in HM content are discussed below.

Exposing three different species of fish to Cu*0.1 mixture, the Cr concentration accumulated in the gills of the fish investigated was lower (significantly lower in the gills of *R. rutilus* and *P. fluviatilis*) compared to the content accumulated in the gills of the MIX-affected fish group. Moreover, treatment with Cr*0.1 mixture, the content of Cu accumulated in the gills was also lower than in the fish affected by MIX (*change in same directions of HM content*) (see Table 9). This process was observed in the gills of all three fish species, but differences in Cu and Cr content were not statistically significant in comparison to content in gills of the MIX-affected fish group. On the other hand, the tendency of a change in the accumulation of Cu and Cr in gills of all three fish species, exposing to the mixtures of Cr*0.1 and Cu*0.1, respectively, presumes that synergistic interactions can exist between these metals. According to the scientific literature, the interaction between Cu-Cr exposing the fish to multi-metal mixture has not been studied until now.

Compared to MIX mixture, the Cr concentration in the fish gills was significantly higher in the fish kept in the Ni*0.1 mixture, but the Ni content was significantly lower after treatment with Cr*0.1 mixture (*change in opposite directions of HM content*) (see Table 9). This indicates that there is an interaction between these metals, but only in opposite directions (the content of HM decreased/increased). This result is different comparing with results of other authors (Palaniappan and Karthikeyan, 2009; Van Hoof and Nauwelaers, 1984), which determined the strong synergistic interactions between these metals. However, it should be noted that the fish (*C. mrigala, R. rutilus*) in the above-mentioned studies were exposed to binary mixtures (Ni + Cr).

Compared to MIX mixture, Ni accumulation was significantly lower in the gills, kidneys, and muscle of three different fish species after treatment with Cu*0.1 and Cr*0.1 mixtures, however, the content of Cr and Cu in the fish gills and muscle were tended to increase after the exposure to Ni*0.1 mixture. This shows that there is only one direction synergy between Ni-Cu and Ni-Cr. In the kidney of different fish species, unilateral change of Ni was detected – less accumulation after treatment with Cu*0.1 and Cr*0.1 compared to the MIX mixture. According to our data, this process has not been described so far.

The change in the content of Zn-Cu in opposite directions has been determined in the liver of three different fish species. When fish were exposed to Zn*0.1 mixture, a lower concentration of Cu was accumulated, but under the Cu*0.1 mixture, the content of Zn accumulated in the liver, on the contrary, was higher. As is known, Cu and Zn are essential elements that are involved in metabolic activities (Bury et al., 2003). The antagonistic interaction between these metals occurs due to the chemical similarity, the same valency, equal binding sites in the gill membrane, and equal transport proteins (Pelgrom et al., 1995; Schjolden et al., 2007). According to the authors (Nadella et al., 2007; Ojo et al., 2009; Qiu and Hogstrand, 2005) Zn and Cu tend to inhibit each other's uptake to the rainbow trout organism. The results of my study only partly coincide with the results of these authors' research (as mentioned above, when the Cu concentration is reduced in multi-metal mixture, a higher content of Zn is accumulated in the fish liver, but a reduction in the content of Zn in the mixture results in the opposite change in Cu content (Cu accumulation decreases)).

When the fish were exposed to a specific HM*0.1 mixture, in the kidney of S. *salar, R. rutilus* and *P. fluviatilis* it was observed *unilateral change* in the content of metal compared to the MIX mixture. In addition, in the kidneys, the least number of equal patterns of HM accumulation was determined, which were typical for three different fish species (see Table 9). This may be due to kidney function in the organism. Kidneys are responsible for urine production and fluid excretion from the organism, which acts as a mechanism for the elimination of toxicants (Ojeda et al., 2003).

According to the data of field and experimental studies, fish muscle, as compared to other body tissues, accumulates the least amount of HM (Jezierska and Witeska, 2006; Guérin et al., 2011; Mercari et al., 2014). However, in this study, in the muscle, the changes in HM content were mostly specific, found in all three different fish species. The changes in the contents of Cd-Cu and Ni-Cu of the opposite direction are found in fish muscle. The change in Cu and Cd content, exposing to Cd*0.1 and Cu*0.1 mixtures, respectively is consistent with the results of McGeer et al. (2007) and Pelgrom et al. (1995) study, in which the interaction between these metals was not determined. Similarly, when the concentration of Cu in the mixture was reduced, the content of Ni in the muscle of the fish decreased (compared to the fish in the MIX mixture) and this coincides with the results of other authors' researches (Brix et al., 2016; Komjarova and Blust, 2009a). However, by reducing the concentration of Ni in the mixture, the Cu content in the muscle was tended to increase (a change in the opposite direction of HM content). The same interaction was determined by Komjarova and Blust (2009a), but only in the gills of the *Danio rerio.*

In addition, the change (decrease) in the same directions of Ni-Pb and Cr-Pb was determined in the muscle. Komjarova and Blust (2009a) investigated interactions between five metals (Pb, Cd, Cu, Ni, and Zn) *Danio rerio* were exposed to multi-metal mixture. The results showed that accumulation of Pb in the whole body directly increased, increasing the concentrations of other metals in the mixture. There is few studies on the interaction between Cr and Pb. Ghosh et al. (2007) *L. rohita* were exposed to the binary Cr + Pb mixture and Cr, Pb separately. However, the interaction of opposite directions has been determined. The content of Pb in the tissues decreased in the presence of Cr in the mixture, but the Cr content increased in the presence of Pb in the mixture.

The results of the study are quite difficult to compare to the results of other authors' studies. Many of the researches have been carried out on the selection of single metals, binary, and ternary metal mixtures, and using high concentrations that cannot reflect the real processes occurring in nature. Contrary to most of the research done, in this study, the patterns of the accumulation of HM in fish body tissues were investigated exposing to multi-metal mixtures and reducing the concentration of HM in mixture 10-fold, rather than increasing. Meanwhile, in most studies, the interaction of HM in the mixture exposing the fish has been analyzed without changing HM concentrations (Duran et al. 2015) or by increasing the concentrations of HM in the mixture (Brix et al., 2016; Kargin and Çogun, 1999; Pelgrom et al., 1995). The results of this study showed that when reducing the concentrations of a specific HM up to the environmentally relevant HM concentrations, and if the HM was removed from the mixture, fish tissues accumulated significantly higher contents of some other HM than after the MIX mixture exposure (see Table 10). Exposing the fish to HM*0.1 mixtures significantly increased the content of HM in *S. salar* tissues accumulated for 14 cases out of 168; *R. rutilus* – 12 cases out

of 168; *P. fluviatilis* – 18 cases out of 168 compared to the MIX mixture. This indicates that a significant reduction in the concentration of one HM in the mixture leads to more intense accumulation of some other metals in the fish tissues.

For the same reason, the reduction of a specific metal concentration in the mixture does not necessarily lead to significantly lower accumulation of the same metal in the tissues as compared to the content accumulated in fish in the exposed mixtures with a reduced any one metal concentration (see Table 10 highlighted in red). This was especially characteristic for the accumulation of Ni, Pb, and Cd. When fish were exposed to Ni*0.1, Pb*0.1, and Cd*0.1 mixtures, respectively, Ni, Pb (in most fish body tissues) and Cd (in muscle) were accumulated no less than exposing to other HM*0.1 mixtures (see Table 10). And contrary: the reduction in the concentration of one of the metals in the mixture in some cases reduces the accumulation of another metal in fish tissues, so that the content of the latter even becomes significantly lower than the content in fish tissues exposed to mixtures with reduced concentration of this specific metal (*S. salar* – 7 cases out of 168, *R. rutilus* – 13 cases out of 168 and *P. fluviatilis* – 6 cases out of 168) (e.g. the amount of Ni in the gills of the perch exposed to the mixture of Pb*0.1 is significantly lower than in the gills of the perch exposed to Ni*0.1 (see Figure 5).

At the moment, a considerable amount of data on the bioaccumulation of HM in fish tissues from natural water bodies has been accumulated, but interspecies differences in fish that may affect the accumulation of metals in tissues are still insufficiently investigated. The results of the study show similar, but not identical trends (see Table 11). The highest content of metals in the tissues was accumulated by *R. rutilus*, less – *S. salar*, the least – *P. fluviatilis*. In the tissues of roach, it was more tend to accumulate Zn, Cu, Ni, and salmon and perch that are eating only the food of animal origin – Pb and Cd. Yousafzai et al. (2010) investigated the accumulation of Zn, Ni, Cr, Cu, Cd, and Pb in the tissues of the freshwater predators fish (*Wallago attu*) and omnivorous fish (*Labeo dyocheilus*), depending on their different eating habits. The results of the study showed that omnivorous fish in the body tissues investigated – skin, gills, intestines, liver and muscle, accumulated more metals (except for Pb) (43%, 36%, 63%, 105%, 86%, respectively) than predatory fish. According to authors further studies are needed in order to find out the mechanisms of the uptake and excretion of Pb in the body tissues. However, it is assumed that the excretion of Pb from the predatory fish organism is slower than that of the omnivorous fish. Jia et al. (2017) also found that the omnivorous fish (*C. auratus, S. curriculus*) tended to accumulate essential metals (Cu, Fe, Mn, and Zn) and predatory fish (*P. fulvidraco*) – toxic metals (As, Cd, and Pb). Hashim et al. (2014) studied the accumulation of Ni, Cd, and Pb in the muscle of benthophagous, omnivorous, and predatory fish caught in the Kelantan River, Malaysia. The authors determined that because of the greater variety of food in the aquatic ecosystem, omnivorous fish accumulates more Cd and Ni in the muscle than benthophagous or predatory fish. Lead was mostly accumulated in predatory fish body tissues, and the least – in the benthophagous fish tissues.

According to the results of this study, it can be seen that the patterns of the accumulation of HM in the body tissues of the exposing the fish to multi-metal mixtures did not coincide with the patterns that occur when fish are exposed to binary HM mixtures. Furthermore, new changes in the accumulation of some HM pairs in fish tissues under mixtures with reduced concentrations of one or another HM have not been described so far. Therefore, the toxicity of the whole of the HM mixture cannot be

predicted solely by the correspondence of metal concentrations present in the mixture with the MPC, which are, in most cases, determined by the toxicity of one metal. The accumulation of metals and their toxic effects depend on the presence of other HM in the mixture. In this case, it is necessary to gather as much information as possible about the mutual role on accumulation in living organisms and, in turn, determining one or another toxic effect of heavy metals that are widespread in the environment and more frequently with household and industrial wastewater getting in the surface water. Such information would produce preconditions for creating more precision in predictive models.

2.2.4. The concentration of metallothioneins in the fish body tissues after treatment with multi-metal mixture

The concentration of metallothioneins (MT) was measured in the liver and kidney of different fish species. Individuals of similar length and weight were selected for this study (see dissertation). Fish were exposed for 14 days to multi-metal MIX mixture. The concentration of MT in liver and kidney of different fish species are presented on Figure 6.

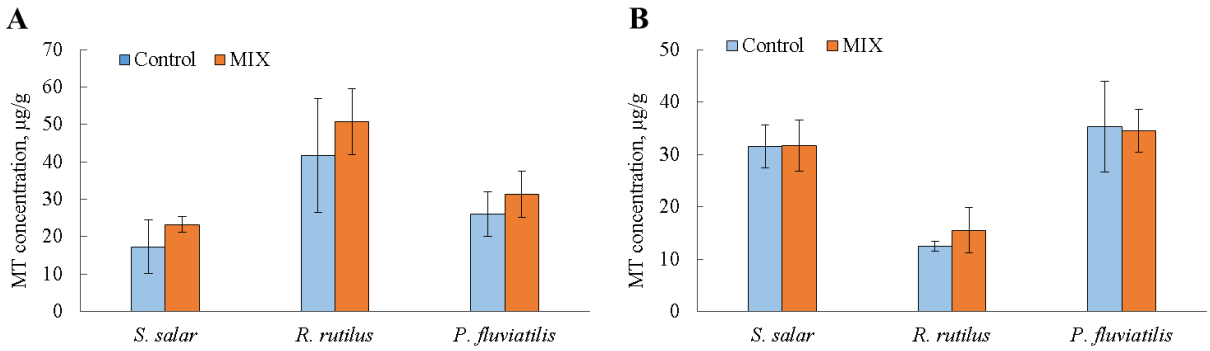


Fig. 6. MT concentration (µg/g wet weight) in liver (**A**) and kidneys (**B**) in different species of fish (mean ± SD, $N = 7$) after exposure to the MIX mixture.

This study showed, that MIX mixture did not induce significant increase of MT in the treatment group (MIX) compared to control group (one-way *ANOVA*, $p > 0.05$). It is probable that the absence of the difference in MT concentration is determined by a small sample of data. In this study, carnivorous fish that is eating food of animal origin (*S. salar* and *P. fluviatilis*) accumulated more the priority toxic metals – Cd (in the gills, liver, kidneys) and Pb (in the kidneys, muscle) than omnivorous *R. rutilus*, which accumulated more Zn, Cu and Ni (see Table 11). Taking into account the identical character of Cd and Pb accumulation in fish that is eating the food of animal origin, during the further analysis, the *S. salar* and *P. fluviatilis* could be combined into one group, thus increasing the data sample. However, before the data combining, it was verified if in the liver of the different fish species MT concentrations did not differ. It was determined that the concentration of MT in the liver of salmon and perch was not different both in control and test groups. However, MT concentration in the liver of roach was significantly higher than in the liver of other fish species (one-way ANOVA, $p < 0.05$). This confirms that the roach cannot be grouped together with salmon or perch. After the combination of fish that eats the food of animal origin, a significant difference in MT concentrations ($p = 0.047$) in the liver of fish tested (MIX) compared to control. This shows that multi-metal MIX mixture, which consists of six metals MPC, is inducing MT synthesis in the liver of *S. salar* and *P. fluviatilis* that more accumulates Cd and Pb.

Pearson correlations analyze was conducted between MT and HM content in fish tissue. It was found that the content of MT in the liver of *R. rutilus* significantly correlates with the content of Zn ($r = 0.83$; $p = 0.022$). This relationship could occur due to the fact, that *R. rutilus*, in comparison to *S. salar* and *P. fluviatilis*, tended to accumulate significantly more Zn, Cu, and Ni in body tissues.

The increase in MT concentration in fish tissues is induced by Zn, Cu, and Cd (Hylland et al., 1992; Knapen et al., 2007; Min et al., 2016). The main role of MT in the organisms is to maintain the homeostasis of essential metals (Zn and Cu) and protect the organisms from toxic metals (Pb and Cd) (Vasak 2005; Coyle et al., 2002). The results of our study revealed an unequal reaction of different fish species to the HM. Fish that is eating the food of animal origin and accumulating Cd and Pb has a significant increase in MT concentration in the liver. Meanwhile, in the liver of omnivorous roach, which accumulated more essential metals, a significant correlation was found between MT and Zn. However, no significant induction of MT in the *R. rutilus* liver was induced. This may be related to the fact that Zn is a necessary metal for the organisms, and that the higher Zn content is necessary for the induction of MT.

The results of this study showed that multi-metal MIX mixture (consist of HM environmentally relevant concentrations) did not result in significant induction of MT in the liver and kidney of different fish species, but combining groups of carnivorous fish (*S. salar* and *P. fluviatilis*) a significant increase in MT concentration in the fish liver was observed compared to control group. This shows that, treatment with multi-metal mixture even relatively safe concentrations (maximum permissible) cause the protective physiological reactions of the organism.

CONCLUSIONS

1. When the fish was exposed to multi-metal mixture in the rotating water-current conditions, the patterns of the accumulation of heavy metals (HM) in the fish correspond to the patterns determined in the field conditions – the HM is the least accumulated in fish muscle. Under the static water conditions, HM more accumulate in the muscle, contrary to rotating water-current conditions or field conditions. As the patterns of the HM accumulation in static water conditions change, such systems are unsuitable for the experimental study of the processes that take place in nature.
2. It has been determined that the optimal time of the fish exposure to multi-metal mixture, after which the concentrations of the majority of HM in the *S. salar* tissues do not increase significantly, is 14 days.
3. Exposing the different species of fish (*S. salar, R. rutilus, P. fluviatilis*) to multi-metal mixtures, the following common patterns have been determined: Ni content decreased in gills and kidneys, exposing fish to mixtures in which the maximum permissible concentrations (MPC) of Cu and Cr was reduced 10-fold and in muscle exposing fish to mixtures in which the MPC of Zn and Pb was reduced 10 fold; Cr content increased in gills exposing fish to mixture in which the MPC of Ni was reduced 10-fold.
4. It has been determined that when exposing the different species of the fish to multi-metal mixtures in which the MPC of one of the metals was reduced 10-fold, some other metals in tissues accumulated more than in the mixture, in which all the MPC concentrations corresponded the MPC (the reduction of the concentration of one HM significantly increased the accumulation of another HM: *S. salar* – 14 cases out of 168, *R. rutilus* – 12 cases out of 168, and *P. fluviatilis* – 18 cases out of 168).
5. It has been determined that when exposing the different species of the fish to multi-metal mixtures, where the MPC of one of the metals was reduced 10-fold (HM*0.1), the accumulation of some specific metals in tissues was less than that in the HM*0.1 mixture with reduced concentration of precisely this a particular metal (the reduction of one HM concentration significantly inhibited the accumulation of another HM: *S. salar* – 7 cases out of 168, *R. rutilus* – 13 cases out of 168 and *P. fluviatilis* – 6 cases out of 168).
6. In the exposure of multi-metal mixtures, omnivorous *R. rutilus* accumulates more Zn, Cu, and Ni, while eating the food of animal origin *S. salar* and *P. fluviatilis* – Pb and Cd.
7. It was determined that multi-metal MIX mixture at MPC caused the induction of metallothioneins (MT) in the liver of fish tested (*S. salar* and *P. fluviatilis*) that is eating the food of animal origin compared to control group.

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2. **Sauliutė G**, Svecevičius G (**2014**) Sunkiųjų metalų biokoncentracijos potencialo atlantinės lašišos (*Salmo salar* L.) kūno audiniuose įvertinimas veikiant modeliniu mišiniu ir atskirais metalais. *17-osios Lietuvos jaunųjų mokslininkų konferencijos „Mokslas – Lietuvos ateitis“ straipsnių rinkinys* Vilnius, Technika. 228 p. 142–148.
3. Svecevičius G, Kazlauskienė N, Kesminas V, Staponkus R, Taujanskis E, **Sauliutė G** (**2014**) Heavy Metal Accumulation in Fishes of Different Ecological Groups from Kairiai Landfill Regional Aquatic Ecosystem. *9th International Conference on Environmental Engineering* (doi: 10.3846/enviro.2014.060).
4. Svecevičius G, Kazlauskienė N, Kesminas V, Staponkus R, **Sauliutė G**, Taujanskis E, Slučkaitė A, Makaras T (**2014**) Complex Study into Toxic Effects of Heavy Metals Discharged from Closed Landfill on Neighbouring Aquatic Ecosystem. *Journal of International Environmental Application and Science* 9(5): 619–628.
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6. **Sauliutė G**, Svecevičius G (**2015**) Siesarties ir Vilnios upių ekotoksikologinės būklės įvertinimas pagal atlantinės lašišos (*Salmo salar* L.) jauniklių morfologinius rodiklius. *„Mokslas – Lietuvos ateitis/Science – Future of Lithuania“, Environmental Protection Engineering* 7(4): 424–429.
7. **Sauliutė G**, Svecevičius G (**2016**) Assessment of Landfill Pollution Load on Hydroecosystem by Use of Heavy Metal Bioaccumulation Data in Fish. *World Academy of Science, International Journal of Environmental, Chemical, Ecological, Geological and Geophysical Engineering* 10(1): 44–51.
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9. **Sauliutė G**, Svecevičius G (**2017**) Heavy Metals (Zn, Cu, Ni, Cr, Pb, Cd) in Water and Body Tissues of Young Atlantic Salmon *Salmo salar* in Two Rivers of Different Pollution Level: A Comparison with Fish Condition Parameters. *Fresenius Environmental Bulletin* 26(1a): 666–673.
10. Stankevičiūtė M, **Sauliutė G**, Svecevičius G, Kazlauskienė N, Baršienė J (**2017**) Genotoxicity and cytotoxicity response to environmentally relevant complex metal mixture (Zn, Cu, Ni, Cr, Pb, Cd) accumulated in Atlantic salmon (*Salmo salar*). Part I: importance of exposure time and tissue dependence, *Ecotoxicology* 26(8): 1051–1064.

11. **Sauliutė G**, Stankevičiūtė M, Svecevičius G, Baršienė J, Valskienė R (**2017**). Assessment of heavy metals bioconcentration factor (BCF) and genotoxicity response induced by metal mixture in *Salmo salar* tissues. *10th International Conference on Environmental Engineering* (doi: https://doi.org/10.3846/enviro.2017.043).
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3. **Sauliutė G,** Svecevičius G (**2015**) Siesarties ir Vilnios upių ekotoksikoginės būklės įvertinimas pagal atlantinės lašišos (*Salmo salar* L.) jauniklių morfologinius rodiklius. 18-osios Lietuvos jaunųjų mokslininkų konferencijos „Mokslas – Lietuvos ateitis“ antropogeninės taršos poveikis aplinkai sekcijoje. 2015 m. balandžio 9 d. Vilnius. Oral presentation.
4. **Sauliutė G**, Svecevičius G (**2016**) Assessment of Landfill Pollution Load on Hydroecosystem by Use of Heavy Metal Bioaccumulation Data in Fish. ICEEB 2016: 18th International Conference on Ecology and Environmental Biology. January, 18-19, 2016, London, United Kingdom. Oral presentation.
5. **Sauliutė G**, Svecevičius G (**2016**) Heavy Metal Accumulation Patterns In The Body Tissues Of Ecologically Different Fish Species. The Coins'16 – 11th international conference of natural and life sciences, 29 February – 3 March 2016, Vilnius, Lithuania. Poster presentation.
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7. **Sauliutė G,** Svecevičius G (**2016**) Atlantinių lašišų (*Salmo salar* L.) jauniklių morfologinių rodiklių pokyčiai veikiant sunkiųjų metalų (Zn, Cu, Ni, Cr, Pb, Cd) mišiniu: eksperimentinis tyrimas. 19-oji Lietuvos jaunųjų mokslininkų

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9. **Sauliutė G** (**2017**) Sunkiųjų metalų kaupimosi žuvų kūno audiniuose dėsningumai, veikiant daugianariais metalų mišiniais. *Jaunųjų mokslininkų konferencija BIOATEITIS: gamtos ir gyvybės mokslų perspektyvos.* 2017 m. gruodžio 7 d. Vilnius. Oral presentation.

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SANTRAUKA

Disertacinis darbas apėmė iki šiol itin mažai tyrinėtą sritį: metalų sąveikos nulemtos sunkiųjų metalų (SM) akumuliacijos žuvų kūno audiniuose dėsningumus, veikiant skirtingas žuvų rūšis (*S. salar*, *R. rutilus* ir *P. fluviatilis*) daugianariais SM mišiniais, esant didžiausioms leistinoms SM koncentracijoms (DLK), kurios reglamentuojamos ES vidaus vandenims. Iki šiol absoliuti dauguma tyrimų buvo vykdomi su binariniais, trinariais metalų mišiniais, test-organizmus veikiant letaliomis ar subletaliomis koncentracijomis, t. y., sukuriant dirbtines, gamtinėje aplinkoje realiai neegzistuojančias sąlygas. Šio tyrimų rezultatai parodė, kad veikiant žuvis daugianariais mišiniais esant SM didžiausioms leistinoms koncentracijoms (DLK), metalų kaupimosi kūno audiniuose dėsningumai pakinta, t. y., tie patys SM daugianariuose mišiniuose elgiasi kitaip nei binariniuose mišiniuose. Be to, eksponuojant skirtingas žuvų rūšis mišiniais, kuriuose vieno konkretaus metalo DLK buvo sumažinta 10 kartų nustatyta, kad suintensyvėja kai kurių kitų metalų kaupimasis žuvų audiniuose. Atitinkamai, konkretaus metalo koncentracijos sumažinimas mišinyje nebūtinai nulemia to metalo reikšmingai mažesnį sukaupimą audiniuose, lyginant su sukauptu kiekiu žuvyse, laikytose mišiniuose su sumažinta kurio nors kito SM koncentracija.

Taip pat skirtingai nei buvo iki šiol manoma, šio tyrimo rezultatai įrodo, kad net ir maitinant vienodu pašaru, *S. salar* ir *P. fluviatilis*, gamtoje mintantys tik gyvūninės kilmės maistu, audiniuose sukaupia daugiau prioritetinių pavojingų metalų (Pb ir Cd) nei *R. rutilus*, kuri gamtoje yra visaėdė. Tačiau būtinieji metalai (Zn, Cu), atvirkščiai, eksperimento metu labiau kaupėsi *R. rutilus* audiniuose. Tai rodo, kad minėtų SM kaupimosi žuvų audiniuose skirtumai nesusiję su mityba ir yra sąlygojami kitų fiziologinių procesų. Be to daugianaris SM mišinys, esant realioms, gamtinei aplinkai artimoms koncentracijoms, sukėlė reikšmingą MT koncentracijos padidėjimą gyvūninės kilmės maistu mintančių bandymo žuvų (*S. salar* ir *P. fluviatilis*) kepenyse, lyginant su kontrole. Tai rodo, kad net ir sąlyginai saugiomis laikomos (didžiausios leistinos) koncentracijos jau sukelia apsaugines fiziologines organizmo reakcijas.