Vilniaus universitetas

Nature Research Centre

Milda
STANKEVIČIŪTĖ

Experimental studies of xenobiotics genotoxicity and cytotoxicity in fish erythrocytes

DOCTORAL DISSERTATION

Biomedical Sciences,
Ecology and Environmental Research (03B)

VILNIUS 2018

The work was carried out at Nature Research Centre during 2014–2018.

Research supervisor – **Dr habil. Janina Baršienė** (Nature Research Centre, Biomedical Sciences, Ecology and Environmental Research – 03B).

Defense of the dissertation is held at the public meeting of the Council:
Chairman – **Prof. Dr. Algimantas Paulauskas** (Vytautas Magnus University, Biomedical Sciencesi, Ecology and Environmental Research – 03B).
Members:
Dr Dalius Butkauskas (Nature Research Centre, Biomedical Sciences, Ecology and Environmental Research – 03B)
Dr Veronika Dedonytė (Vilnius University, Biomedical Sciences, Biology–01B)
Dr Gražina Stanevičiūtė (Nature Research Centre, Biomedical Sciences, Biology – 01B)
Dr Arvo Tuvikene (University of Tartu, Biomedical Sciences, Ecology and Environmental Research – 03B)

Defense of the dissertation will be held at the public meeting of the Council on 1st of October 2018 at 2.00 p.m. in the meeting Room (101 a) of Nature Research Centre.
Address: Akademijos 2, LT–08412, Vilnius, Lithuania, Tel. +370 5 2729257; E-mail: sekretoriatas@gamtostyrimai.lt

The summary of the doctoral dissertation was distributed on 1st September 2018.
The dissertation is available at the libraries of Vilnius University and Nature Research Centre and Vilnius University website: https://www.vu.lt/naujienos/ivykiu-kalendorius

Vilniaus universitetas

Gamtos tyrimų centras

Milda
STANKEVIČIŪTĖ

Eksperimentiniai ksenobiotikų genotoksiškumo ir citotoksiškumo tyrimai žuvų eritrocituose

DAKTARO DISERTACIJA

Mokslo sritis,
mokslo kryptis (03B)

VILNIUS 2018

Disertacija rengta 2014– 2018 metais Gamtos tyrimų centre
Mokslinius tyrimus rėmė Lietuvos mokslo taryba (projektas „ACTIS" S-MIP-17-10; parama už akademinius pasiekimus)

Mokslinis vadovas:
Habil. dr. Janina Baršienė (Gamtos tyrimų centras, biomedicinos mokslai, ekologija ir aplinkotyra – 03B)

Gynimo taryba:
Pirmininkas – **prof. dr. Algimantas Paulauskas** (Vytauto Didžiojo universitetas, biomedicinos mokslai, ekologija ir aplinkotyra – 03B)
Nariai:
dr. Dalius Butkauskas (Gamtos tyrimų centras, biomedicinos mokslai, ekologija ir aplinkotyra – 03B)
dr. Veronika Dedonyte (Vilniaus universitetas, biomedicinos mokslai, biologija – 01B)
dr. Gražina Stanevičiūtė (Gamtos tyrimų centras, biomedicinos mokslai, biologija – 1B)
dr. Arvo Tuvikene (Tartu universitetas, biomedicinos mokslai, ekologija ir aplinkotyra – 03B)

Disertacija ginama viešame Gynimo tarybos posėdyje 2018 m. spalio mėn. 1 d. 14 val. Gamtos tyrimų centro 101 auditorijoje. Adresas: Akademijos g. 2, LT-08412 Vilnius, Lietuva, tel. (8~5) 272 92 57; el. paštas sekretoriatas@gamtostyrimai.lt

Disertaciją galima peržiūrėti Vilniaus universiteto ir Gamtos tyrimų centro bibliotekose ir VU interneto svetainėje adresu: *https://www.vu.lt/naujienos/ivykiu-kalendorius*

Contents

LIST OF ORIGINAL PUBLICATIONS AND AUTHOR'S CONTRIBUTION 7
APROBATION OF THE RESULTS 9
ABBREVIATIONS 12
INTRODUCTION 13
1. Contaminants in the aquatic environment 14
2. Fish as bioindicators 16
3. Genotoxicity and cytotoxicity biomarkers in aquatic organisms 17
4. Scientific novelty of the thesis 18
5. Theoretical and practical significance 19
6. Aims of the thesis 20
7. Statements to defend 20
MATERIALS AND METHODS 21
1. Study design 21
MAIN RESULTS 24
Paper I: "Analysis of nuclear abnormalities in erythrocytes of rainbow trout (*Oncorhynchus mykiss*) treated with Cu and Zn and after 4-, 8-, and 12-day depuration (post-treatment recovery)" 24
Paper II: "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" 25
Paper IV: Responses of biomarkers in Atlantic salmon (Salmo salar) following exposure to environmentally relevant concentrations of complex metal mixture (Zn, Cu, Ni, Cr, Pb, Cd). Part II. 28
Paper XIII: Erythrocytic nuclear abnormalities, DNA damage, bioconcentration factor and haematological changes induced by metal mixture at environmentally relevant concentrations in *Rutilus rutilus* 31
Paper X: Experimental studies on the toxicity and geno-cytotoxicity effects of cadmium in embryos and larvae of rainbow trout, *Oncorhynchus mykiss* ... 33
Paper V: Toxicity, geno- and cytotoxicity of cadmium-based quantum dots and cadmium to rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) embryos 34

Paper III: Interaction of carboxylated CdSe/ZnS quantum dots with fish embryos: Towards understanding of nanoparticles toxicity......................... 36
Paper XIV: Geno-, cytotoxicity and toxicity induced by *Saprolegnia parasitica* and cadmium alone and in combination to *Oncorhynchus mykiss*………………………………………………………………………………….....36
DISCUSSION 39
CONCLUSIONS 47
SANTRAUKA 49
REFERENCES 72
ACKNOWLEDGEMENTS 80
COPIES OF PUBLICATIONS 81

LIST OF ORIGINAL PUBLICATIONS AND AUTHOR'S CONTRIBUTION

This thesis is based on the following co-authored original publications with an impact factor on the Clarivate Analytics Web of Science database (7 papers) and other publications with peer review process (7 papers). Publications are referred within the text using Roman numerals.

Publications with an impact factor on the Clarivate Analytics Web of Science database:

Experimental studies:

I. **Stankevičiūtė M**, Butrimavičienė L, Valskienė R, Greiciūnaitė J, Baršienė J, Vosylienė MZ, Svecevičius G (2016) Analysis of nuclear abnormalities in erythrocytes of rainbow trout (*Oncorhynchus mykiss*) treated with Cu and Zn and after 4-, 8-, and 12-day depuration (post-treatment recovery). *Mutation Research - Genetic Toxicology and Environmental Mutagenesis* 797: 26–35

II. **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

III. Rotomskis R, Jurgelėnė Ž, Stankevičius M, **Stankevičiūtė M**, Kazlauskienė N, Jokšas K, Montvydienė D, Kulvietis V, Karabanovas V (2018) Interaction of carboxylated CdSe/ZnS quantum dots with fish embryos: Towards understanding of nanoparticles toxicity. *Science of the Total Environment* 635: 1280–1291

IV. **Stankevičiūtė M**, Sauliutė G, Makaras T, Markuckas A, Virbickas T, Baršienė J. (2018) Responses of biomarkers in Atlantic salmon (*Salmo salar*) following exposure to environmentally relevant concentrations of complex metal mixture (Zn, Cu, Ni, Cr, Pb, Cd). Part II. *Ecotoxicology* https://doi.org/10.1007/s10646-018-1960-2

V. Jurgelėnė Ž, **Stankevičiūtė M**, Kazlauskienė N, Baršienė J, Montvydienė D, Rotomskis R (2018) Toxicity, geno- and cytotoxicity of cadmium-based quantum dots and cadmium to rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) embryos (manuscript)

In situ studies:

VI. Baršienė J, Butrimavičienė L, Grygiel W, Stunžėnas V, Valskienė R, Greiciūnaitė J, **Stankevičiūtė M** (2016) Environmental genotoxicity risk assessment along the transport routes of chemical munitions leading to the dumping areas in the Baltic Sea. *Marine Pollution Bulletin* 103(1–2): 45–53

VII. Valskienė R, Baršienė J, Butrimavičienė L, Grygiel W, Stunžėnas V, Jokšas K, **Stankevičiūtė M** (2018) Environmental genotoxicity and cytotoxicity levels in herring (*Clupea harengus*), flounder (*Platichthys flesus*) and cod (*Gadus morhua*) inhabiting the Gdansk Basin of the Baltic Sea. *Marine Pollution Bulletin* 133: 65–76

VIII. Butrimavičienė L, Baršienė J, Greiciūnaitė J, **Stankevičiūtė M**, Valskienė R (2018) Environmental genotoxicity and risk assessment in the Gulf of Riga (Baltic Sea) using fish, bivalves and crustaceans. *Environmental Science and Pollution Research*. https://doi.org/10.1007/s11356-018-2516-y

Further publications with peer review process:

IX. Valskienė R, **Stankevičiūtė M**, Butrimavičienė L, Greiciūnaitė J, Svecevičius G (2015) Induction of nuclear abnormalities in rainbow trout (*Oncorhynchus mykiss*) after exposure to model mixture of heavy metals (Zn, Cu, Ni, Cr, Cd, Pb) at maximum premissible concentration. *Proceedings of the 18th Conference for Junior Researchers "Science – Future of Lithuania"* ISSN 2029-5456. Vilnius, Technika. p. 100–105

X. Kazlauskienė N, Cibulskaitė Ž, **Stankevičiūtė M**, Baršienė J (2016) Experimental studies on the toxicity and geno-cytotoxicity effects of cadmium in embryos and larvae of rainbow trout, *Oncorhynchus mykiss. Proceedings of the 13th International Conference on Protection and Restoration of the Environment* ISBN 978-960-6865-94-7. Mykonos island, Greece. p. 449–459

XI. Cibulskaitė Ž, **Stankevičiūtė M**, Kazlauskienė N, Baršienė J, Kulvietis V, Rotomskis R (2016) Long-term toxicity and geno-cytotoxicity of quantum dots to rainbow trout *Oncorhynchus mykiss* embryos. *Proceedings of the 13th International Conference on Protection and Restoration of the Environment* ISBN: 978-960-6865-94-7. Mykonos island, Greece. p. 460–470

XII. 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*, eISBN 978-609-476-044-0 (doi: https://doi.org/10.3846/enviro.2017.043)

XIII. **Stankevičiūtė M**, Sauliutė G, Markuckas M, Virbickas T, Baršienė J (2018) Erythrocytic nuclear abnormalities, DNA damage, bioconcentration factor and haematological changes induced by metal mixture at environmentally relevant concentrations in *Rutilus rutilus*. *Proceedings of the 14th International Conference on Protection and Restoration of the Environment* ISBN: 978-960-99922-4-4. Thessaloniki, Greece. p. 785–794.

XIV. **Stankevičiūtė M**, Jurgelėnė Ž, Greiciūnaitė J, Markovskaja S, Kazlauskienė N, Baršienė J (2018) Geno-, cytotoxicity and toxicity induced by *Saprolegnia parasitica* and cadmium alone and in combination to *Oncorhynchus mykiss*. *Proceedings of the 14th International Conference on Protection and Restoration of the Environment* ISBN: 978-960-99922-4-4. Thessaloniki, Greece. p. 795–804.

XV. Jurgelėnė Ž, **Stankevičiūtė M**, Kazlauskienė N, Montvydienė D, Baršienė J, Jokšas K, Markuckas A (2018) Investigation of quantum dots toxicity, genotoxicity, cytotoxicity, and uptake in rainbow trout *Oncorhynchus mykiss* larvae. *Proceedings of the 14th International Conference on Protection and Restoration of the Environment* ISBN: 978-960-99922-4-4. Thessaloniki, Greece. p. 775–806.

[1]

APROBATION OF THE RESULTS

The key results of the thesis were presented for discussion and were approbated at international (10) and national (2) conferences.

International scientific conferences:

1. Cibulskaitė Ž, **Stankevičiūtė M**, Kazlauskienė N, Baršienė J. Toxicity and Geno-cytotoxicity of Cadmium to Rainbow Trout (*Oncorhynchus mykiss)* in early ontogenesis. „Vita Scientia 2016'' international Life Science Conference. 2016, January 4th Life Science Centre Saulėtekio Ave. 7.
2. Valskienė R, Butrimavičienė L, **Stankevičiūtė M**, Greiciūnaitė J, Dasevičiūtė L, Baršienė J. Environmental Genotoxicity Assessment in Chemical Munitions Dumping Zones in the Southern Baltic Sea. The Coins 2016 - International Conference of Natural and Life Sciences. 29th February - 3rd March 2016. Life Science Centre Saulėtekio Ave. 7.
3. Kazlauskienė N, Cibulskaitė Ž, Svecevičius G, Sauliutė G, Makaras T, Rotomskis R, Kulvietis V, Stankevičius M, Markuckas A, **Stankevičiūtė M**, Baršienė J. Nanoparticle And Heavy Metal

[1]Declaration of contribution
MS designed the study with JB. MS initiated and planned Papers I-II, XII, IV, XIII-XIV, performed sampling in the experiments, was responsible for the biomarkers analyses and statistical analysis. MS was responsible for all parts related to geno- and cytotoxicity assessment in Papers III, V, X-XI, XV. MS was responsible for the part of geno- and cytotoxicity analysis or preparation of certain parts in Papers VI-VIII and IX.

Toxicity Mechanisms In Fish During Ontogenesis: An Interdisciplinary Project. The Coins 2016 - International Conference of Natural and Life Sciences. 29th February - 3rd March 2016. Life Science Centre Saulėtekio Ave. 7.

4. Kazlauskienė N, Cibulskaitė Ž, **Stankevičiūtė M**, Baršienė J. Experimental studies on the toxicity and geno-cytotoxicity effects of cadmium in embryos and larvae of rainbow trout, *Oncorhynchus mykiss*. 13th International Conference on Protection and Restoration of the Environment, Mykonos island, Greece, | 3rd to 8th of July, 2016.
5. Cibulskaitė Ž, **Stankevičiūtė M**, Kazlauskienė N, Baršienė J, Kulvietis V, Rotomskis R. Long-term toxicity and geno-cytotoxicity of quantum dots to rainbow trout *Oncorhynchus mykiss* embryos. 13th International Conference on Protection and Restoration of the Environment, Mykonos island, Greece, | 3rd to 8th of July, 2016.
6. Ašmenaitė G, Petkutė G, **Stankevičiūtė M**, Butrimavičienė L. Genotoxicity assessment of heavy metal model mixture in swan mussel, european perch and common roach gills. 60th International Conference for Students of Physics and Natural Sciences „Open Readings 2017". March 14-17, 2017. Vilnius, Lithuania.
7. Sauliutė G, **Stankevičiūtė M**, Svecevičius G, Baršienė J, Valskienė R. Assessment of heavy metals bioconcentration factor (BCF) and genotoxicity response induced by metal mixture in *Salmo salar* tissues. 10th International Conference "Environmental Engineering" 27–28 April 2017, Vilnius, Lithuania.
8. **Stankevičiūtė M**, Sauliutė G, Markuckas M, Virbickas T, Baršienė J. Erythrocytic nuclear abnormalities, DNA damage, bioconcentration factor and haematological changes induced by metal mixture at environmentally relevant concentrations in *Rutilus rutilus*. Protection and Restoration of the Environment July 3-6, 2018, Thessaloniki, Greece.
9. **Stankevičiūtė M**, Jurgelėnė Ž, Greiciūnaitė J, Markovskaja S, Kazlauskienė N, Baršienė J (2018) Geno-, cytotoxicity and toxicity induced by *Saprolegnia parasitica* and cadmium alone and in combination to *Oncorhynchus mykiss*. Protection and Restoration of the Environment July 3-6, 2018, Thessaloniki, Greece.
10. Jurgelėnė Ž, **Stankevičiūtė M**, Kazlauskienė N, Montvydienė D, Baršienė J, Jokšas K, Markuckas A. Investigation of quantum dots toxicity, genotoxicity, cytotoxicity, and uptake in rainbow trout *Oncorhynchus mykiss* larvae. Protection and Restoration of the Environment July 3-6, 2018, Thessaloniki, Greece.

National scientific conferences:

1. Valskienė R, **Stankevičiūtė M**, Butrimavičienė L, Greiciūnaitė J, Svecevičius G. Induction of nuclear abnormalities in rainbow trout (*Oncorhynchus mykiss*) after exposure to an model mixture of heavy metals (Zn, Cu, Ni, Cr, Cd, Pb) at maximum permissible concentration. 18-osios Lietuvos jaunųjų mokslininkų konferencijos „Mokslas – Lietuvos ateitis“ antropogeninės taršos poveikis aplinkai sekcijoje. 2015 m. balandžio 9 d. Vilnius, žodinis pranešimas.

2. Baršienė J, Butrimavičienė L, Michailovas A, Rybakovas A, Valskienė R, **Stankevičiūtė M**, Eiva P, Greiciūnaitė J. Aplinkos genotksiškumo dėsningumai jūrinėse ekosistemose. Lietuvos mokslų akademijos konferencija ,, Šiuolaikiniai biologijos tyrimai Lietuvoje’’- jūros biologijai. Vilnius 2015-10-29.

ABBREVIATIONS

Ap – Apoptotic erythrocyte
BL – Blebbed nuclei erythrocyte
BNb – Binucleated erythrocyte with nucleoplasmic bridge
BN – Binucleated erythrocyte
CF – Condition factor
CAT – Catalase
cfu – colony-forming units
ENAs – Erythrocytic nuclear abnormalities
EN – Enucleus erythrocyte
EU – European Union
FA – Fragmented-apoptotic erythrocyte
Fr – Fragmented erythrocyte
GES – Good ecological status
Hb – Haemoglobin
Hct – Haematocrit
HIS – Hepatosomatic index
Kidney-shaped – Kidney-shaped erythrocyte
LSI – liver-somatic index
MN – Micronucleus
MPC – Maximum-Permissible- Concentrations
NB – Nuclear bud
NBf – Nuclear bud on filament
NPs – Nanoparticles
QDs – Quantum dots
RBC – Red Blood Cells
SCGE – Single cell gell electrophoresis
SOD – Superoxide dismutase
VacNuc – Vacuolated nuclei erythrocyte
WBC – White Blood Cells
8-shaped – 8-shaped nuclei erythrocyte

INTRODUCTION

The thesis pertains to ecological and toxicological relevance and addresses the problem of chemical mixtures, deploys an adequate animal models and seeks to contextualise the work in real EU context. One of the aims of the Water Framework Directive (WFD; European Union) is to attain the "Good Ecological Status" (GES) and "good chemical status" in all surface water bodies of the EU. These aims should be achieved by maintaining and restoring the health of aquatic ecosystems. The measurement of genotoxic effects in aquatic organisms under field provides early warning signals of adverse effects of chemical, physical or biological contaminants in aquatic environment, whereas assessment of genotoxic potential of single stressor or their combination have been disclosed under controlled laboratory conditions. Geno- and cytotoxicity evaluation of metal mixtures (Zn, Cu, Cr, Ni, Pb, Cd), quantum dots (CdSe/ZnS-COOH) and multiple stressors (chemical+biological (Cd+*Saprolegnia parasitica*)) in selected fish species under control laboratory conditions was performed. Environmental genotoxicity responses in various bioindicators from the Baltic Sea, reflects the complex of environmental conditions.

Anthropogenic activities have introduced complex mixtures of contaminants discharging with industrial, domestic and agricultural wastes into the environment that have increased concern about their adverse effects on freshwater and marine ecosystems. There is increasing attention in pollutants synergism, which has the most toxicological concern (Cedergreen 2014). Genotoxic agents are the ingredients of these mixtures that can provoke genetic damage and initiate toxic effects at various biological levels (Bolognesi and Hayashi 2011). In the environment aquatic organisms are exposed to a number of various pollutants covered by different EU regulations, while mixture effects are not currently regulated. According to SCHER, SCCS, SCENIHR, Opinion on the Toxicity and Assessment of Chemical Mixtures (2012) evaluation of risk assessment of chemical mixtures needs to take into consideration the potential mixture effects at realistic exposure levels in the environment, the possible impact of background exposure in the environment or diet, and health risks at low dose exposures to multiple chemicals. The focus of toxicology has shifted to investigating more subtle, chronic, low-dose effects, where cause and effect relationships may not be directly obvious and may greatly differ from the effects of the acute exposure (Klaassen et al., 2013). Moreover, cumulative effects of multiple stressors are becoming a major problem in ecotoxicology. Fish are considered as one of the most suitable bioindicators in ecotoxicological studies of various xenobiotics. Fish parasites can be used as indicators of water pollution and environmental quality. Parasites infections in the presence of pollution may further compromise the health of biondicators by reducing the immunocompetence of the host. Furthermore, exacerbation of contaminants

induced toxicity effects may be noted even parasites infestation occurs at low intensities (Marcogliese et al., 2005).

A growing interest in studies of environmental genotoxicity has led to the development of a variety of tests for the detection of genotoxic agents in aquatic media. Kroon with co-authors (2017) indicated the most suitable biomarkers of exposure. Micronuclei together with other nuclear abnormalities as irreversible genotoxic events and DNA damage assessment using the Comet assay were emphasized as suitable biomarkers of metal exposure (Kroon et al., 2017). Moreover, Comet assay and nuclear abnormalities test are considered as sensitive test systems for assessing geno- and cytotoxicity of pollutants.

Different fish species were used for the detection of genetically active compounds *in situ* and in laboratory exposures to various clastogenic and aneugenic compounds (Cavas and Ergene-Gozukara 2005; Cavas et al., 2005). Bioindicators used in an experimental and *in situ* studies of the thesis belong to different taxonomic groups and represent various functional positions, therefore may vary in responses to chemical pollution. Salmonid fish species such as *Salmo salar* and *Oncorhynchus mykiss* at different development stages are valuable bioindicators of ecological integrity of aquatic ecosystems (over their life cycle their integrate a wide range of riverine conditions, microhabitats) (Chovanec et al., 2003). While roach (*Rutilus rutilus*) is a suitable bioindicator for monitoring aquatic pollution in freshwater ecosystems (Łuczyńska et al., 2018). *Clupea harengus membras*, *Platichthys flesus*, *Gadus morhua*, *Zoarces viviparous* and two invertebrate species the Baltic clam *Macoma balthica* and the isopod *Saduria entomon* are sentinel organisms and typical components of the Baltic Sea communities and are used to monitor environmental pollution (Baršienė et al., 2014; 2015; Butrimavičienė et al., 2018; Góral et al., 2009). Comprehensive understanding of environmental conditions arises from evaluation and monitoring of hydroshepre pollution using various fish species.

1. Contaminants in the aquatic environment

Trace elements. As reported by European Environment Agency (EEA 2012) various chemicals, such as metals, pesticides and other pollutants are responsible for poor chemical status of surface water bodies across Europe. Metals are the main pollutants of any industrial or domestic discharges, and they are assumed as most hazardous in the toxicological studies (Javed and Usmani 2017). Heavy metals accounts for 20% of river water bodies in poor status; are the major pollutant (accounting for 60%) in lakes and accounts for 50% of coastal water bodies in poor status (EEA 2012). Wide distribution of metals in the aquatic environment is releated with their multiple industrial, domestic, agricultural and technological applications. Zn, Cu, Ni, Cr, Pb and Cd are the most common metal contaminants, whereas Pb and Cd are

indicated as priority metals due to the high degree of their toxicity. Toxicity of trace elements depends on their concentration, chemical species, exposure route, time and sensitivity of biological systems. In the aquatic environment metals exist in trace concentrations, therefore, evaluating mixture effects at realistic exposure levels a possible influence of background exposure should be taken into consideration. The necessity of studies related to interactions of low concentration toxic metals and essential metals mixtures was emphazised (Cobbina et al., 2015; Nys et al., 2017). As concluded by Nys with co-authors (2017), the understanding about metal mixture toxicity and their interactions is far from fully investigated, neither mechanism are fully understood nor performance of mixtures risk assessment. Future progress in mixture toxicology, must be able to provide experimental data for many more mixture elements, move in the direction of probabilistic exposure, ensure that data and methods are useful in the assessment of mixtures and provide a better perception of whether an assessment is too cautious or insufficiently protective (Evans et al., 2016). The experiments of the thesis were designed to evaluate metal mixture effects in fish by testing the mixture as a whole (whole mixture approach). According to Heys et al. (2016), whole mixture approach is the most logical because it reflects the simultaneous exposure that organisms in the environment face. Using this type of approach all interactions between the components in the mixture are accounted for.

Nanoparticles. Metal nanoparticles (NPs) are an emerging technology due to their optical, electronic properties and have wide application in industry and common use product. However, their also poses threats to aquatic environment due to release of metal ions and intrinsic charascteristics. Quantum dots (QDs) are one of emerging engineering nanomaterials. In the experiments of diseratation CdSe/ZnS negative-charged (covered with carboxyl groups), and coated with a polymer layer (PEG) QDs were used. They have advantageous properties in clinical imaging and diagnosis (Galeone et al., 2012). Moreover, nanoparticles can vary in size, shape or surface functionalizations leading to more difficult prediction of their toxicity. Nanopatricles has become the focus of ecotoxicology studies due to their unique properties compared to pure metals. In the thesis carboxylated CdSe/ZnS quantum dots were selected as the model multi-component, metal-based mixture. The potential for genotoxicity effects and mechanisms of DNA damage of QDs still remains unclear (Saez et al., 2015). Primary and secondary mechanisms of nanoparticles induced genotoxicity may exist. Primary genotoxicity is caused by direct interaction of nanoparticles or dissolved forms with the genetic material, proteins essential for DNA replication, transcription or repair (Mahaye et al., 2017). Inflamatory responses that lead to oxidative stress are thought to be the secondary mechanism of NPs genotoxicity. Studies with a greater focus on QDs-induced genotoxicity assessment using several DNA damage biomarkers to identify

the effects of physicochemical features and genotoxicity mechanisms are therefore suggested (Demir and Castranova 2017).

Biological stressors. The aquatic fungus-like heterotrophs or straminipilous fungi referred also as “water moulds” (traditionally oomycetes) of the order *Saprolegniales* is common and widespread in freshwater environment (Rietmüller 2000; Dick 2001). Moreover, *Saprolegnia parasitica* is thought to be the most frequent species of *Saprolegnia* genus infecting fish eggs (van West 2006; Shahbazian et al., 2010). Naturally, *Saprolegnia* species are found in all lotic and lentic freshwater basins (Rietmüller, 2000; Markovskaja, 2006). In aquaculture, *Saprolegnia* infection causes severe problem in incubating eggs and newly hatched fry (Hussein et al., 2001; Thoen et al., 2011; Van Den Berg et al., 2013). The lethal impact of saprolegniosis could cause major economic losses of the global fish industry production (Phillips et al., 2008). Since 2002, when the use of malachite green, an organic dye very efficient at killing the pathogen and previously widely used, was banned due to its toxicity, *Saprolegnia* infection has reemerged in aquaculture. In order to mitigate *Saprolegnia* infection in aquaculture, the development and testing of general or specific antifungal agents has increased (Ali et al., 2014). Therefore, it is crucial to determine *S. parastica* geno- and cytotoxicity potential alone and jointly with chemical stressors to predict toxicity outcomes.

2. Fish as bioindicators

Fish as bioindicators are suitable for the application of various methods which allow to assess toxic impacts from molecular to population levels (Chovanec et al., 2003). They are considered as the most useful bioindicators in marine and freshwater ecosystems for various chemical pollution. Moreover, fish have been successfully used in cytogenetic surveys. In the experiments of the thesis three fish species were used: Atlantic salmon (*Salmo salar* Linnaeus, 1758), rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) and roach (*Rutilus rutilus* Linnaeus, 1758) (accepted synonym for scientific name in FishBase (ver 02/2018, http://www.fishbase.org)).

Historically, *S. salar* is a keystone migratory species with a North Atlantic distribution, with presence in all countries whose rivers enter the North Atlantic (Hendry and Cragg-Hine 2003). The Atlantic salmon is included in annexes II and V of the European Union (EU) Habitats Directive as a species of European significance. Nevertheless, development of salmon populations since the mid-1990s has been encouraging (HELCOM 2011). The species is an economically (subsistence, recreational and commercial fisheries) and ecologically important fish species throughout the European region (Kulmala et al., 2012). Atlantic salmon in response to xenobiotics has been scantily investigated (Song et al., 2012).

Rainbow trout is one of widely studied fish species and is considered as a laboratory fish model in ecotoxicological research. Rainbow trout was used for genotoxicity assessment of various chemicals using Micronucleus and other erythrocytic nuclear abnormalities (ENAs) assay (Ayllón and Garcia-Vazquez 2001).

Roach is sedentary and abundant fish species, which is found in most European rivers, lakes and ponds (Persson 1983; Brabrand 1985). Fish occupy the lower trophic level of aquatic environment and are widely used to biologically monitor the level of chemical pollution of these ecosystems (Salem et al., 2014; Łuczyńska et al., 2018).

3. Genotoxicity and cytotoxicity biomarkers in aquatic organisms

Genotoxicity effects as biologically important endpoints are useful during pollution related toxicity assessment (Bolognesi and Cirillo 2014). A large number of biomarkers are used in the field of genetic ecotoxicology. Micronucleus and Comet assays or Single Cell Gel Electrophoresis (SCGE) are the most commonly used and validated methods in field and experimental studies (Bolognesi and Cirillo 2014). Other erythrocytic nuclear abnormalities also are considered as a proper markers of genetic instability (Gomes et al., 2015). Both Comet and nuclear abnormalities assays are highly sensitive, robust and relatively simple. These assays allows to detect damage at chromatic and chromosomal levels, respectively.

The micronucleus (MN) and other nuclear abnormalities assay is suitable to detect genotoxic and cytotoxic damage of wide range of chemical compounds and is widely applied in studies with aquatic organisms. Nuclear abnormalities such as nuclear buds (NB), nuclear buds on filament (NBf), bi-nucleated cells with nucleoplasmic bridge (BNb), blebbed (BL), vacuolated (VacNuc), 8-shaped, kidney-shaped nuclei, bi-nucleated (BN), fragmented-apoptotic (FA) and enucleus (EN) cells are considered to be indicators/markers of cytogenetic damage, and for this reason they complement micronucleus scoring in genotoxicity and cytotoxicity research (Gomes et al., 2015; Baršienė et al., 2014; Harabawy et al., 2014). Nuclear abnormalities such as MN, NB, NBf, BNb and BL are considered as genotoxicity endpoints, whereas 8-shaped, BN and FA are considered as cytotoxicity endpoints.

The Comet assay is a sensitive method that detects DNA strand breaks. This assay is widely applied in metal and nano-genotoxicology (Karlsson et al., 2015, Celá et al., 2014). The impact of genotoxins using Comet assay was evaluated in different marine and freshwater fish species (Khan et al., 2017; Vignardi et al., 2015; Munari et al., 2014).

The erythrocytes of fish have been indicated to be a suitable tool for Comet and nuclear abnormalities assays (Udroiu 2006). To bring better results, in the thesis geno- and cytotoxicity was evaluated in peripheral blood, liver, gills and

kidneys erythrocytes. In fish, the cephalic kidney is the major hematopoietic organ; the secondary hematopoietic organs (spleen, peri-portal areas of the liver, the intestinal submucosa and the thymus; occasional hematopoiesis occurrence reported in gills, brain and gonads) may have different sensitivities to xenobiotics (Agius and Roberts 2003; Macchi et al., 1992). Erythrocyte removal is related with liver and spleen (Soldatov 2005).

4. Scientific novelty of the thesis

This thesis contributes with the new scientific information on tissue-specific, time-related, concentration-dependent, multiple stressors influenced geno- and cytotoxicity responses. The influence of metal (Zn, Cu, Ni, Cr, Pb, Cd) mixtures and quantum dots (PEG coated CdSe/ZnS-COOH) as multicomponent chemical stressors and the effect of oomycetes *Saprolegnia parasitica* as a biological stressor on the induction of cytogenetic lesions have been analysed. Moreover, fluctuations of frequencies of genotoxic and cytotoxic lesions during fish recovery were described.

For the first time the reguliarities of geno- and cytotoxicity in fish were detected:

- The influence of complex metal mixture at Maximum-Permissible-Concentrations (MPC) on geno- and cytotoxicity endpoints was evaluated in different fish species. A likely influence of minor changes of even low metal exposure concentration (MPC) and markedly increased risk of toxicity was emphasized.
- The fluctuations of geno- and cytotoxicity endpoints frequencies in erythrocytes of different fish tissues was described in an experimental depuration process.
- Time-related, concentration-dependent and tissue-specific peculiarities of geno- and cytotoxicity responses were emphasized in different fish species.
- Geno- and cytotoxicity potencial of quantum dots (QDs) as one of emerging engineering nanomaterials was indicated in early life stages of fish.
- Genotoxic and cytotoxic effects of *Saprolegnia parasitica* pathogen as biological stressor was evaluated in *Oncorhynchus mykiss* larvae.
- Potential exacerbation of geno- and cytotoxicity endpoints after joint *S. parasitica* and Cd exposure was assessed in *Oncorhynchus mykiss* larvae.

5. Theoretical and practical significance

• Time-dependent and tissue-specific induction of erythrocytic nuclear abnormalities in *S. salar* was emphasized. Evaluation of *O. mykiss* potential recovery from cytogenetic damage after exposure to metal mixture provided information on tissue-specific, time-related, concentration-dependent fluctuations of geno- and cytotoxicity endpoints frequencies.

• Complex metal (Zn, Cu, Ni, Cr, Pb, Cd) mixture at Maximum-Permissible-Concentrations induce cytogenetic damage in erythrocytes of *S. salar* and *R. rutilus*. Reduction of concentration (MPC) of a single metal in a complex mixture can markedly increase levels of geno- and cytotoxic endpoints in different tissues.

• Genotoxic and cytotoxic effects of carboxylated CdSe/ZnS quantum dots was investigated in *O. mykiss* larvae and a hypothetical mechanism of adverse effects was suggested.

• Genotoxic and cytotoxic potential of *Saprolegnia parasitica* infection and exacerbation of these endpoints following joint parasitism and Cd exposure was evaluated in *O. mykiss* larvae.

• Experimental data on genotoxicity and cytotoxicity in fish tissues following exposure to complex metal mixtures at environmentaly relevant concentrations provide valuable information to develop a multimetal toxicity models that predict toxicity of mixtures to aquatic organisms.

• Detected regularities of fish recovery from cytogenetic damage can be applied for an aquatic ecosystem restoration planning.

• The results of the thesis could encourage revision of ecotoxicologically relevant water quality standards for metal mixtures.

• Characterization of genotoxic and cytotoxic lesions in hatchery-reared Atlantic salmon cells describes the cytogenetic status of the artificially breed fish and their suitability for reestablishment of salmon population in water bodies.

6. Aims of the thesis

The goal of the dissertation is an experimental evaluation of genotoxicity and cytotoxicity peculiarities of metal (Zn, Cu, Ni, Cr, Pb, Cd) mixtures and quantum dots (CdSe/ZnS-COOH) as multicomponent chemical stressors and oomycetes *Saprolegnia parasitica* as a biological stressor in fish erythrocytes.

The following objectives are investigated:

1. To assess time-dependent induction of nuclear abnormalities and time-related recovery in an experimental depuration process.
2. To assess geno- and cytotoxicity effects of complex metal mixtures at various concentrations in fish erythrocytes.
3. To evaluate geno- and cytotoxicity effects of complex metal mixtures in different tissues erythrocytes of bioindicators.
4. To assess geno- and cytotoxicity of quantum dots during early stages of fish development.
5. To identify genotoxicity and cytotoxicity impact of oomycetes *Saprolegnia parasitica* alone and jointly with Cd in fish erythrocytes.

7. Statements to defend

1. Exposure to complex metal mixture at MPC causes time-dependent and tissue-specific responses of geno- and cytotoxicity in *S. salar* erythrocytes.
2. Recovery of *O. mykiss* from cytogenetic damage is time-, tissue- and concentration-dependent; fluctuations of geno- and cytotoxicity endpoints, especially in *O. mykiss* liver and kidneys erythrocytes, is inherent to depuration period.
3. Reduction of MPC of a certain metals in a complex mixture markedly increases the levels of geno- and cytotoxicity in erythrocytes of *S. salar* and *R. rutilus*.
4. Peripheral blood erythrocytes indicate higher levels of geno- and cytotoxicity compared to liver, kidneys and gills erythrocytes. Peripheral blood erythrocytes show faster recovery rate after binary metal (Cu, Zn) mixture exposure compared to other tissues erythrocytes.
5. Carboxylated CdSe/ZnS quantum dots cause DNA strand breaks in *O. mykiss* embryos and larvae.
6. Exposure to *Saprolegnia parasitica* induces genotoxicity in *O. mykiss* larvae.

MATERIALS AND METHODS

1. Study design

Fish speciments from early life stages to juvenile ones, were investigated in the experiments of this thesis. The study design (including used fish species, biomarkers and chemical pollutants) is presented in Table 1. The method (erythrocytic nuclear abnormalities (ENAs) assay) for genotoxicity and cytotoxicity analysis in juvenile/adult fish is described in detail in Paper I-II, IV, VI-VIII, XII-XIII and IX, whereas the method for genotoxicity and cytotoxicity analysis in early stages of fish development is described in Paper V, X, XI, XIV and XV. This method was performed using criteria described by Heddle et al. (1991), Fenech et al. (2003) and Baršienė et al. (2006, 2014). The morphological features of ENAs are shown in Figs. 1 and 2. The method for DNA damage (Comet assay) analysis in juvenile fish is described in Paper XIII, whereas the DNA damage analysis in early stages of fish development is described in Paper III and V. The alkaline Comet assay was performed following the procedure of Singh et al. (1988) with some modifications (Fatima et al., 2014). Method for nuclear abnormalities analysis in invertebrates is described in paper VIII. Methods for analyses of haematological parameters followed by Svobodova et al. (1991) (erythrocytes, haemoglobin concentration, haematocrit level, leukocyte count) are described in Paper XIII. The methods for measurement of elements concentration in experimental and control water is described in the respective papers (I-V, IX-XV). The statistical methods applied in the papers are presented in detail in the respective papers (I-XV).

Table 1. The simplified scheme of experimental/study design for each of papers included in the thesis.

Fish species	Stage of development	Biomarkers	Test-chemicals or infection	Paper
Salmo salar	juvenile	ENAs	Metal mixture (Zn, Cu, Ni, Cr, Pb, Cd)	II, XII, IV
Rutilus rutilus		ENAs, Comet assay, haematological parameters		XIII
Oncorhynchus mykiss		ENAs		IX
			Cu/Zn mixture	I
	embryos	ENAs, Comet assay	carboxylated CdSe/ZnS QDs	XI, V
	embryos, larvae	ENAs	Cd	X
		Comet assay	carboxylated CdSe/ZnS QDs	III
	larvae	ENAs		XV
			Cd, *Saprolegnia parasitica*, Cd + *S. parasitica*	XIV
Clupea harengus membras	adult	ENAs	Environmental pollution (Baltic Sea)	VI
C. harengus membras, *Platichthys flesus*, *Gadus morhua*				VII
C. harengus membras, P. flesus, Zoarces viviparous, Macoma balthica, Saduria entomon				VIII

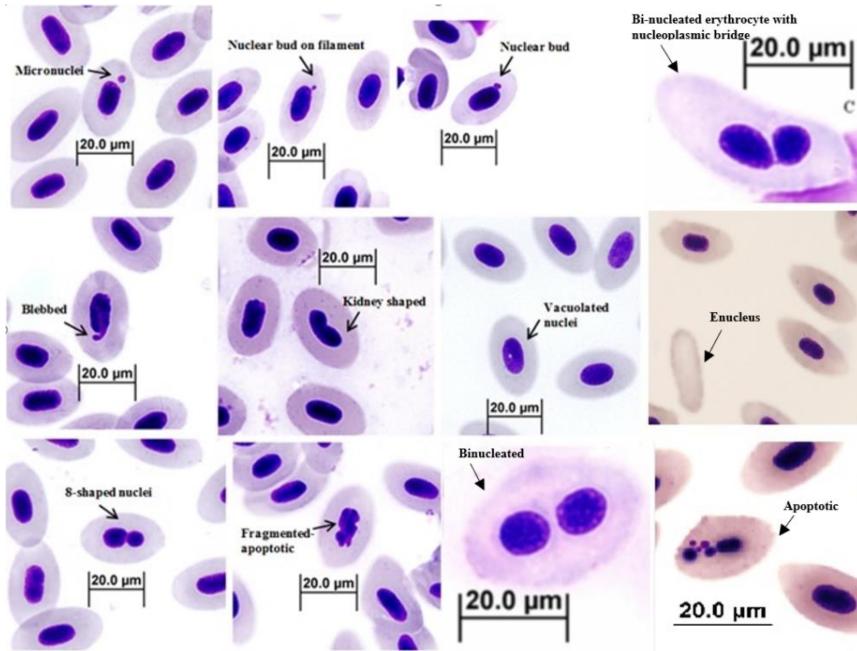


Fig. 1 Nuclear abnormalities in erythrocytes of *Salmo salar*, *Oncorhynchus mykiss* (bi-nucleated erythrocyte with nucleoplasmic bridge and binucleated erythrocyte) and *Rutilus rutilus* (enucleus and apoptotic erythrocyte (unpublished data)). From Paper I and II.

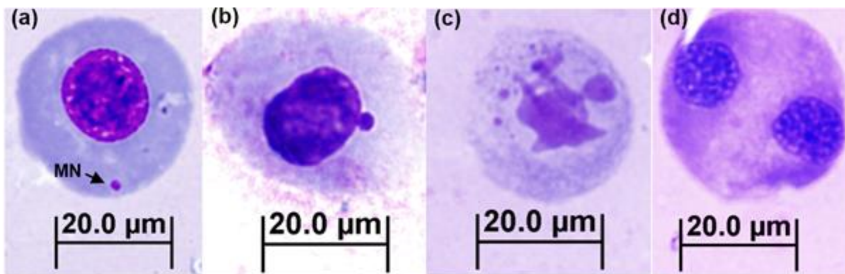


Fig. 2 *Oncorhynchus mykiss* embryo erythroblasts with **(a)** micronucleus (MN), **(b)** nuclear bud (NB), **(c)** fragmented-apoptotic (FA) and **(d)** bi-nucleated (BN) erythroblasts. From Paper V.

MAIN RESULTS

Paper I: "Analysis of nuclear abnormalities in erythrocytes of rainbow trout (*Oncorhynchus mykiss*) treated with Cu and Zn and after 4-, 8-, and 12-day depuration (post-treatment recovery)"

The induction of erythrocytic nuclear abnormalities was analysed in the peripheral blood, kidney and liver of rainbow trout *Oncorhynchus mykiss* after 4-day treatment with copper (Cu) and zinc (Zn) mixture solutions at 0.0625, 0.125 and 0.25% of 96-h LC50 (0.65 mg Cu/L and 3.79 mg Zn/L)) and in 4-, 8- and 12-day depuration process. Significantly increased MN levels were detected in peripheral blood of *O. mykiss* after all exposure concentrations, in liver erythrocytes at 0.125 and 0.25% and in kidney erythrocytes at 0.25% concentration (Fig. 3). Significant induction of NB and BL was detected in blood and kidney at 0.25%. Cytotoxicity analysis revealed significant induction of 8-shaped nuclei erythrocytes at all concentrations studied in kidney, at two (0.125 and 0.25%) concentrations — in liver and at the highest concentration in peripheral blood (Fig. 3).

During the depuration period, tissue-specific recovery trend and time-related fluctuations of geno- and cytotoxicity endpoints were detected in the fish. Micronuclei incidences were found to increase significantly after 4-day recovery (at 0.125%) in all tissues. Significant recovery was observed at 0.0625% concentration estimating the formation of MN in erythrocytes of peripheral blood (after 12-day recovery), of 8-shaped nuclei in liver (after 8- and 12-day) and kidneys erythrocytes (after 8-day recovery). Peripheral blood erythrocytes showed significant recovery of all analysed geno- and cytotoxicity endpoints after 8 and 12 days at 0.25%, however, recovery in this group was not observed after 12-day of depuration in liver and kidney erythrocytes (Fig. 3).

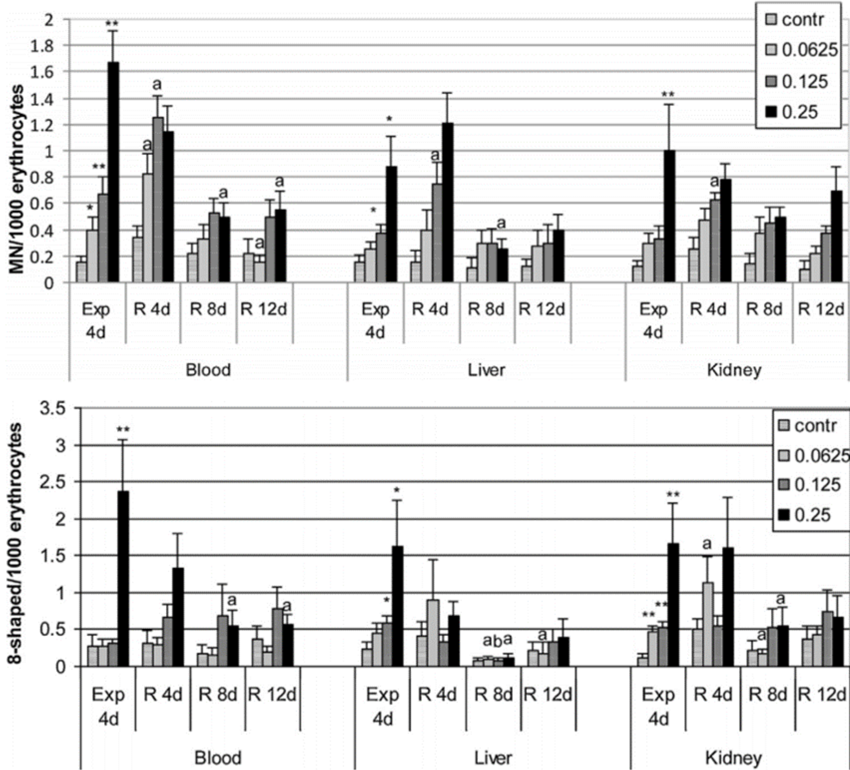


Fig. 3. Frequency of micronuclei (MN) and 8-shaped nuclei erythrocytes in blood, liver and kidney of rainbow trout exposed to different concentrations of copper–zinc mixture solution and in depuration process. Data are presented as means ± SE, differences between control and exposed fish groups shown: * $P < 0.05$, ** $P < 0.001$; differences between exposed and recovery fish groups shown: a $P < 0.05$, b $P < 0.001$, c $P < 0.0001$. Exp 4d –exposure 4 days; R – recovery 4, 8 and 12 days. From Paper I.

Paper II: "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"

Complex metal mixture at Maximum-Permissible-Concentrations (Zn–0.1, Cu–0.01, Ni–0.01, Cr–0.01, Pb–0.005 and Cd–0.005 mg/L) accepted for the inland waters in EU induced genotoxicity and cytotoxicity in different tissues erythrocytes of *S. salar* exposed for 28 days. The most obvious finding to emerge from the geno- and cytotoxicity analysis in tissues is that different non-linear response between exposure time versus endpoints levels was determined. Peripheral blood erythrocytes showed the highest levels of summed genotoxicity and cytotoxicity. Results for MN and other erythrocytic nuclei abnormalities assays in *S. salar* gills, liver, kidneys and peripheral

blood erythrocytes, are given in Fig. 4 A−D. Treatment with metal mixture significantly increased summed genotoxicity level at day 7 of exposure in liver and peripheral blood erythrocytes, and at day 14 of exposure in gills and kidneys erythrocytes. Significant elevation of cytotoxicity was found after 2 and 14 days of exposure in gills erythrocytes and after 28 days — in peripheral blood erythrocytes. Significant induction of cytotoxicity was not observed in liver and kidneys erythrocytes. During the exposure period, levels of summed genotoxicity (MN + NBf + NB) were higher than levels of summed cytotoxicity (FA + 8-shaped) in all analysed tissues (Fig. 4).

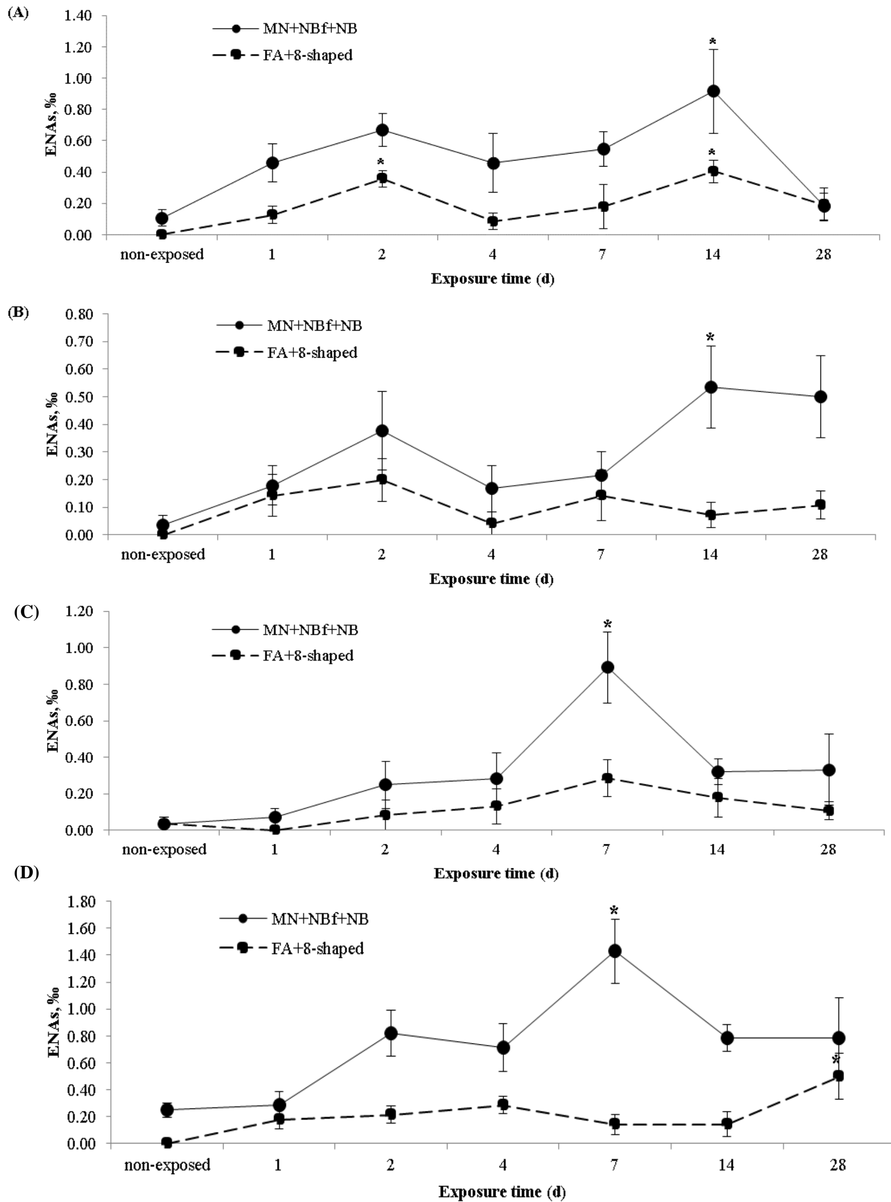


Fig. 4 The relationship between exposure time (d) and ENAs (summed genotoxicity (MN +NBf + NB) and cytotoxicity (FA + 8-shaped)) responses in Atlantic salmon: (A) gills, (B) kidneys, (C) liver and (D) peripheral blood erythrocytes (mean ± SEM, N = 7. Asterisks (*) denote significant differences from non-exposed group during exposure time ($p < 0.05$). From Paper II.

Paper IV: Responses of biomarkers in Atlantic salmon (Salmo salar) following exposure to environmentally relevant concentrations of complex metal mixture (Zn, Cu, Ni, Cr, Pb, Cd). Part II.

Possible influence and significance of 10-times reduced concentration of a single metal in a complex metal mixture (Zn – 0.1, Cu – 0.01, Ni – 0.01, Cr – 0.01, Pb – 0.005 and Cd – 0.005 mg/L) on geno- and cytotoxicity endpoints in *S. salar* was evaluated. Test fish were exposed for 14 days period to a six metal (Zn, Cu, Ni, Cr, Pb and Cd) mixture (herein after referred to as MIX) at a concentration corresponding to Maximum-Permissible-Concentrations (MPC) accepted for the inland waters in EU. Other treatments were performed by 10-times reducing MPC of single metal in the mixture (MIX) made of 6 metals, while other 5 metals concentrations remain constant (e.g. Zn↓ (10-times reduced Zn concentration in the mixture), while Cu, Ni, Cr, Pb, Cd concentrations remain constant (herein after referred to as Zn↓) and etc.).

The ENAs such as MN, BL and VacNuc exhibited the highest frequencies in most of the treatments. Micronuclei showed the highest frequencies in Cr↓ (10 times reduced Cr^{6+} concentration) or Cu↓ treatments, followed by MIX and Ni↓. The ENAs NBf, NB and VacNuc, showed the highest induction after treatment with MIX in gills and kidneys erythrocytes. The highest BL and Kidney-shaped frequencies were measured in Cd↓ treatment in all tissues, except gills erythrocytes for BL and kidneys – for Kidney-shaped frequencies. The highest induction of 8-shaped frequencies was measured in Cr↓, Cu↓ or Zn↓ treatments, depending on analysed tissue. FA and NBf exhibited the lowest frequencies in most of the treatments. The highest induction of total ENAs was detected in Cr↓ treatment, except in gills erythrocytes – after treatment with MIX. Significant differences of total ENAs induction was measured after Cr↓ treatment in all analysed tissues erythrocytes, after Cu↓ treatment in blood and after MIX, Pb↓ treatments in gills erythrocytes in comparison to control. Significant differences of separate ENAs induction, such as MN, Kidney-shaped and 8-shaped frequencies were measured in several treatments in blood; such as MN, NB, VacNuc and FA in several treatments in gills; such as MN in Cr↓ treatment in liver erythrocytes. No significant differences of separate ENAs induction were measured in kidneys erythrocytes in any treatment performed (Fig. 5).

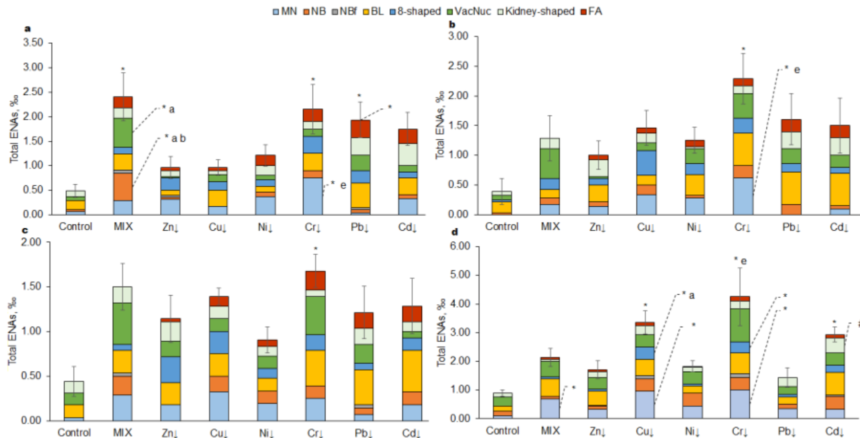


Fig. 5 Total number of ENAs in: (a) gills, (b) liver, (c) kidneys and (d) peripheral blood erythrocytes (mean ± SEM, n = 7). Asterisks (*) denote significant differences from control group, # – from MIX, a – from Zn↓; b – from Cu↓; c – from Ni↓; d – from Cr↓; e – from Pb↓; f – from Cd↓ ($p < 0.05$). From Paper IV.

Summed genotoxicity and cytotoxicity levels in erythrocytes of different tissues are shown in Fig. 6. The highest level of genotoxicity in peripheral blood and liver erythrocytes was detected after Cr↓ treatment, in gills – after MIX and Cr↓ treatments and in kidneys after treatment with MIX and Cu↓. The highest cytotoxicity level was detected after Cr↓ and Cu↓ treatment in peripheral blood, after Cr↓ treatment in kidneys; after Pb↓ treatment in gills and after Cu↓ treatment in liver erythrocytes. Significantly increased genotoxicity levels were measured in Cr↓ and Cu↓ treatments in peripheral blood erythrocytes, after Cr↓ treatment in gills and liver and after MIX and Cu↓ treatments in kidneys erythrocytes compared to control level. Significant cytotoxicity level was detected after Cr↓ and Cu↓ treatments in peripheral blood and after Pb↓ treatment in gills erythrocytes compared to control level.

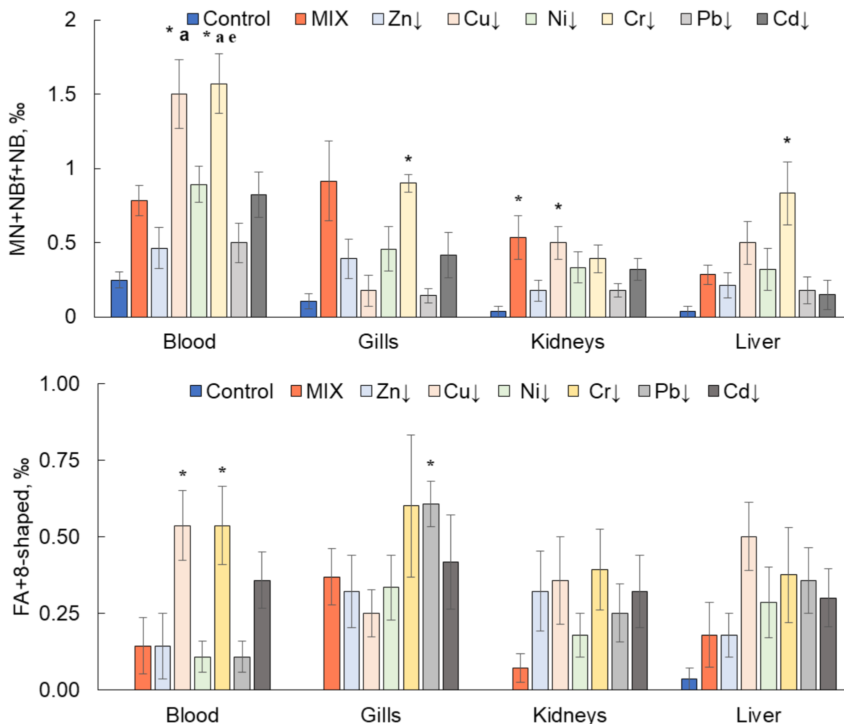


Fig. 6 Summed genotoxicity (MN+NBf+NB) and cytotoxicity (FA+8-shaped) responses in S. salar erythrocytes (mean ± SEM, n = 7). Asterisks (*) denote significant differences from control group, # – from MIX, a – from Zn↓; b – from Cu↓; c – from Ni↓; d – from Cr↓; e – from Pb↓; f – from Cd↓ ($p < 0.05$). From Paper IV.

A discriminant function analysis (DA) was performed to determine which ENAs coefficients distinguished treatments from each other and to identify any statistical similarity among data due to overlapping of statistical ellipses. Scatterplots of function 1 versus function 2 are presented in Fig. 7 a-d. Fig. 7 b-c shows that, data of Cd↓ treatment were entirely separated from other treatments data in liver and kidneys. Considering ENAs data, MN, NB and NBf contributed most to the discriminatory power of functions. The similarities were detected between data of MIX, Zn↓ and Pb↓ treatments in gills and liver tissues; between data of MIX and Pb↓ treatments in kidneys, they ellipses were overlapped (Fig. 7 a-c). Data of all treatments were separated from the control data in gills and liver tissues. Similar DA results were obtained using endpoints data in the same tissue, these results are presented in supplement 1 of Paper IV.

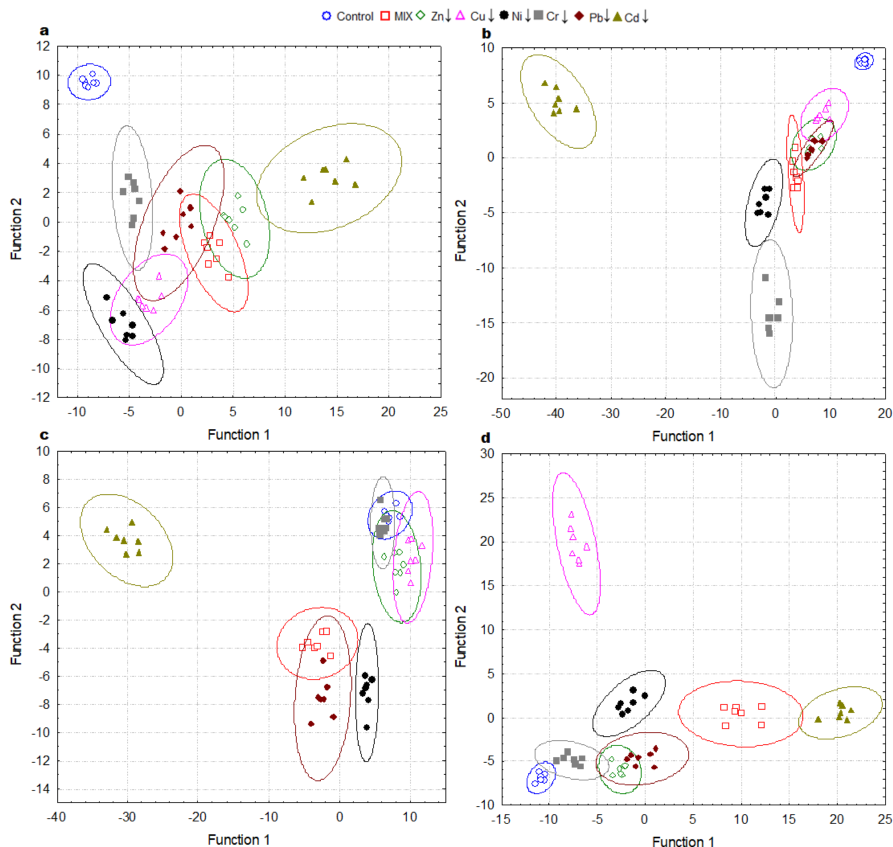


Fig. 7 Scatterplots of discriminant function 1 versus discriminant function 2 using metal concentration data in specific tissue ((a) gills, (b) liver, (c) kidneys, (d) muscle) and erythrocytic nuclear abnormalities in peripheral blood (ellipses show 95% confidence intervals). From Paper IV.

Paper XIII: Erythrocytic nuclear abnormalities, DNA damage, bioconcentration factor and haematological changes induced by metal mixture at environmentally relevant concentrations in *Rutilus rutilus*

Possible influence and significance of 10-times reduced concentration of a single metal in a complex mixture (Zn – 0.1, Cu – 0.01, Ni – 0.01, Cr – 0.01, Pb – 0.005 and Cd – 0.005 mg/L) on geno- and cytotoxicity endpoints in *Rutilus rutilus* was evaluated using the same experimental procedures as in *S. salar* experiment. 14 days treatment with metal mixtures significantly affected MN, enucleus (EN) and apoptotic (Ap) erythrocytes frequencies in *R. rutilus*. The frequencies of separate ENAs such as MN, EN were significantly elevated after Cr↓, Ni↓ treatments in peripheral blood erythrocytes, respectively; apoptotic erythrocytes – after MIX treatment in gills and enucleus after Ni↓

treatment in liver erythrocytes compared to control level. Significant elevations in total ENAs were measured after Cr↓, MIX or Ni↓ treatments in peripheral blood, gills or kidneys erythrocytes (Fig. 8).

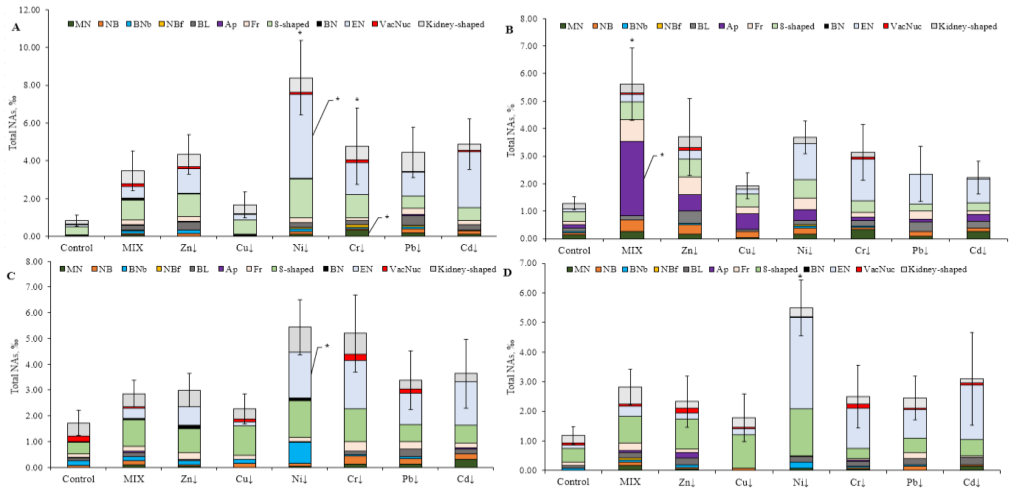


Fig. 8 Erythrocyte nuclear abnormalities (ENAs) in (A) peripheral blood, (B) gills, (C) liver and (D) kidneys erythrocytes in control fish and fish treated with metal mixtures (mean±SE, N=7). Asterisks (*) denote significant differences from control group ($p<0.05$). From Paper XIII.

The exposure of roach to Zn↓, Cr↓ and Pb↓ metal mixtures resulted in significant DNA strand breaks compared to those from the control group (Fig. 9). The highest percentage of DNA in the tail (22.59 %) was observed after Cr↓ treatment followed by Pb↓ (16.55 %) and Zn↓ (16.08 %) treatments.

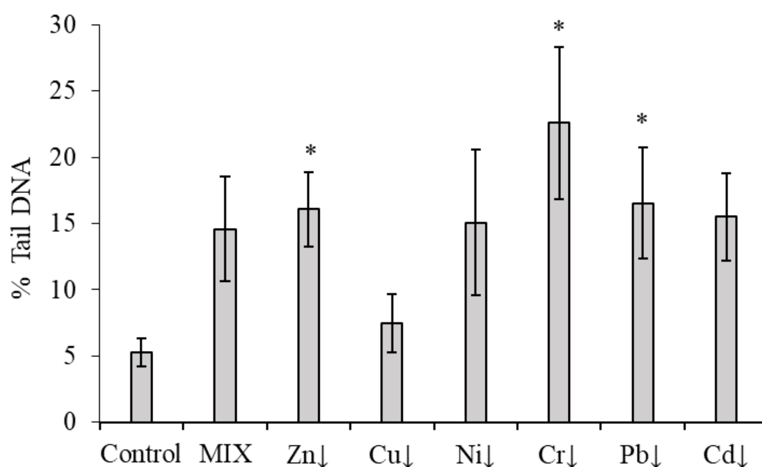


Fig. 9 DNA damage (percentage of DNA in the tail) in control fish and fish treated with metal mixtures (mean±SD, N = 7). Asterisks (*) denote significant differences from control group ($p<0.05$). From Paper XIII.

Decreased number of red blood cells, haematocrit level, haemoglobin concentration and increased number of white blood cells in peripheral blood was measured after MIX treatment (Table 2). However, only decrease in haemoglobin concentration was significant.

Table 2. Effects of metal mixture (MIX) on haematological parameters (mean±SD, N = 7) of *R. rutilus*. From Paper XIII.

Treatment	Hb, g/l	Hct, l/l	RBC count, $10^6 \times mm^{-3}$	WBC count, $10^3 \times mm^{-3}$
Control	84.83±11.07	0.328±0.07	1.33±0.12	23.42±9.05
MIX	64.33±14.88*	0.233±0.06	1.03±0.33	28.75±11.86

Asterisks (*) denote significant differences from control group ($p < 0.05$)

Paper X: Experimental studies on the toxicity and geno-cytotoxicity effects of cadmium in embryos and larvae of rainbow trout, *Oncorhynchus mykiss*

Cd (2 µg Cd L^{-1} as $CdCl_2 \cdot H_2O$) exposure caused both genotoxic and cytotoxic effects during developmental periods of rainbow trout (Fig. 10). After 96-hour exposure to Cd, significant elevation of MN and summed cytotoxicity (bi-nucleated (BN) + fragmented-apoptotic (FA) cells) were detected in the embryos erythroblasts. Micronuclei and NB frequencies increased approximately 4 times, pooled cytotoxicity increased 6 times compared to the control levels. The long-term exposure of larvae to Cd significantly induced the formation of MN, NB and summed cytotoxicity. Micronuclei, NB and

summed cytotoxicity levels increased approximately 8, 5, 11 times, respectively compared to control level.

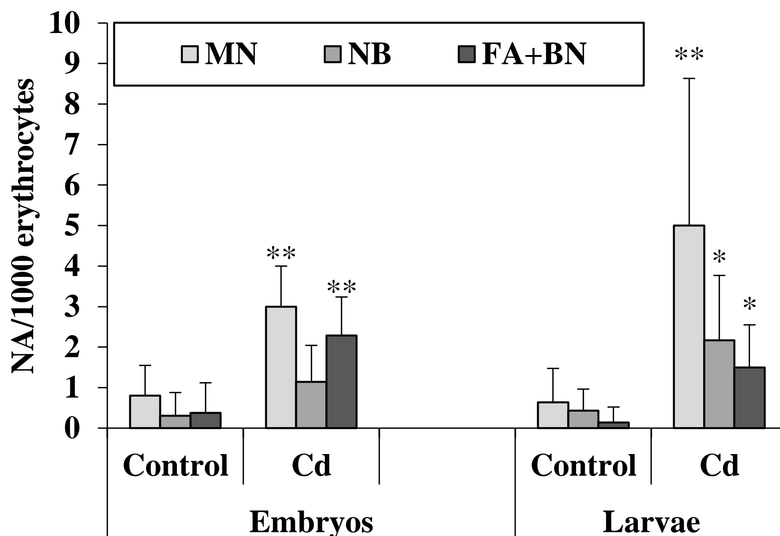


Fig. 10 The frequency of micronuclei (MN), nuclear buds (NB) and fragmented-apoptotic (FA) + bi-nucleated (BN) cells in erythroblasts of 4-day embryos and larvae. Differences between control and Cd exposed groups shown: $*p < 0.05$; $**p < 0.005$. From Paper X.

Paper V: Toxicity, geno- and cytotoxicity of cadmium-based quantum dots and cadmium to rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) embryos

Experiments of geno- and cytotoxicity potential of Cd and carboxylated CdSe/ZnS QDs in *O. mykiss* embryos erythroblasts was performed to compare toxicity outcomes. Four days exposure to Cd (2 µg Cd L-1 as $CdCl_2{\cdot}H_2O$) significantly increased the frequency of micronuclei (MN) in erythroblasts of embryos. Furthermore, a significant increase of total cytotoxicity level in erythroblasts of Cd-exposed embryos was detected. 4 days treatment with QDs (4×10^{-9} M) did not significantly affect the frequencies of nuclear abnormalities. However, all analysed genotoxicity and cytotoxicity endpoints in QD-exposed embryos were elevated approximately two times compared to the control levels (Fig. 11), whereas in Cd-exposed embryos, MN and NB frequencies increased approximately 4 times, and total cytotoxicity increased 6 times compared with the control levels.

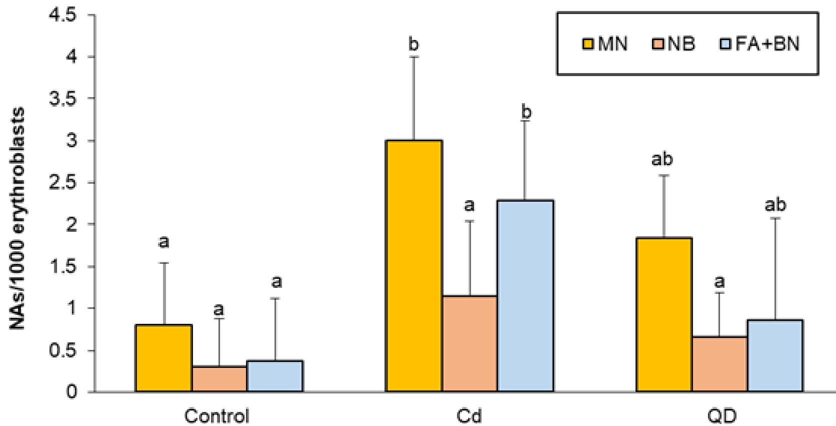


Fig. 11 Frequency of micronuclei (MN), nuclear buds (NB) and total cytotoxicity level (fragmented-apoptotic (FA) + bi-nucleated (BN) cells) in erythroblasts of embryos exposed to Cd and QDs for 4 days. Data are reported as mean ± SD, N = 7, Kruskal-Wallis test. Letters denote significant differences between groups ($p < 0.05$). From Paper V.

Figure 12 compares the DNA damage caused by Cd or QDs in embryos after 4 days treatment. A significant DNA damage (percentage of DNA in the tail) was measured in embryos as a result of Cd and QD exposures. However, DNA damage induced by Cd was 1.6-fold higher and statistically different compared to QDs-caused DNA damage.

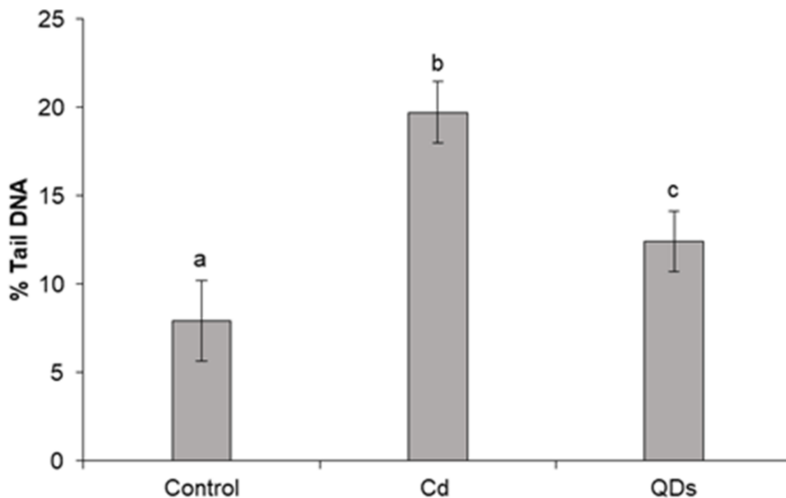


Fig. 12 The percentage (%) of DNA in the comet tail of rainbow trout embryos exposed to Cd or QDs for 4 days. Data are reported as (mean ± SD, N = 7, One-way ANOVA). Letters denote significant differences between groups. From Paper V.

Paper III: Interaction of carboxylated CdSe/ZnS quantum dots with fish embryos: Towards understanding of nanoparticles toxicity

Comet assay is considered to be more sensitive genotoxicity test compared to nuclear abnormalities assay. In order to detect a low level of genotoxic potential of QDs Comet assay was applied in erythroblasts of *O. mykiss* embryos exposed to QDs for 1 day. Moreover, to detect long-term genotoxicity and to test sensitivity of newly hatched larvae, Comet assay was applied in larvae after one-day post hatching. *O. mykiss* embryos were exposed to QDs (CdSe/ZnS-COOH) at concentration of 4 nM in embryos incubation water. Comet assay showed significantly increased DNA damage (percentage of DNA in the tail) in rainbow trout embryos after one day of exposure to QDs (27-day post fertilization) and in newly hatched larvae (one-day post hatching) after 11 days of exposure to QDs (Fig. 13).

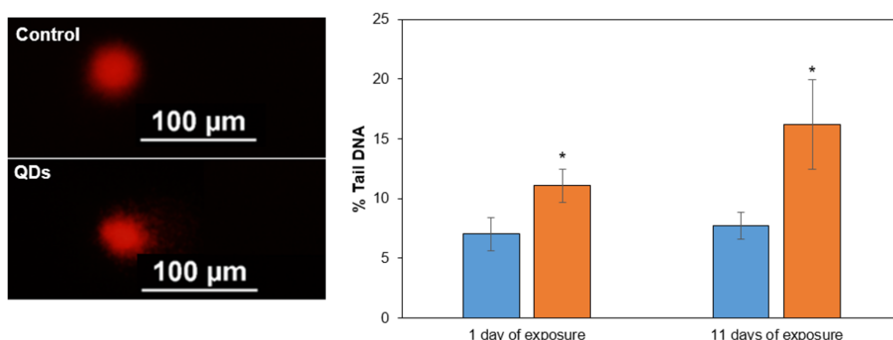


Fig. 13 The percentage of DNA in the comet tail (% Tail DNA, mean ± SD, N = 7) of embryos after one day of exposure (27-day post fertilization) and one-day old rainbow trout larvae (one day after hatching) at the end of the experiment (after 11 days of incubation to 4 nM QDs). From Paper III.

Paper XIV: Geno-, cytotoxicity and toxicity induced by *Saprolegnia parasitica* and cadmium alone and in combination to *Oncorhynchus mykiss*

Genotoxicity and cytotoxicity potential of *Saprolegnia parasitica* at concentrations 92000 (Sap1), 22400 (Sap2) and 5500 (Sap3) colony-forming units per milliliter (cfu/mL) and Cd (2 µg Cd/L as $CdCl_2{\cdot}H_2O$) alone and in combination with 5500 (Sap3) cfu/mL was assessed after 8-day treatment using *O. mykiss* larvae. Treatment with the lowest *S. parasitica* concentration (Sap3 – 5500 cfu/mL) significantly increased MN frequencies in erythroblasts of larvae. Cadmium alone did not induce significant MN formation (Fig. 14). However, Cd in combination with *S. parasitica* at concentration 5500 cfu/mL significantly increased MN frequencies. Notwithstanding, MN frequencies induced by Cd in combination with the lowest *S. parasitica* concentration

(Sap3) did not significantly differ from exposure to *S. parasitica* (Sap3) alone. Analysis of nuclear buds (NB) revealed a significant increase after treatment with the highest *S. parasitica* concentration (Sap1). Exposure to Cd, to other *S. parasitica* concentrations and co-exposure did not significantly affect NB responses in erythroblasts of *O. mykiss* larvae (Fig. 15).

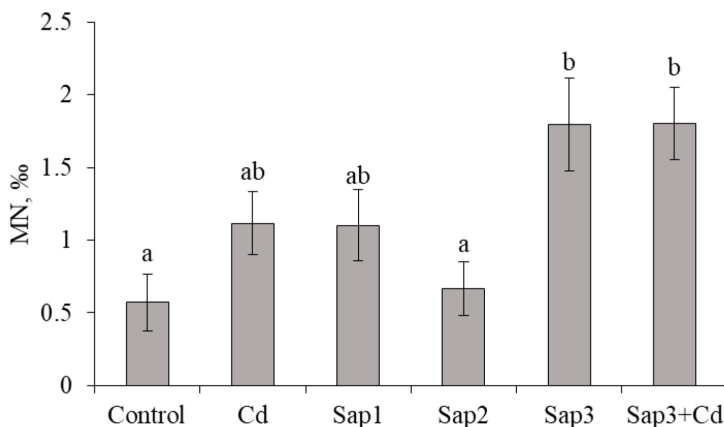


Fig. 14 Mean values (mean ± SEM, N = 10) of micronuclei (MN) frequencies in erythroblasts of *O. mykiss* larvae treated with cadmium (Cd, 2μg/L), three concentrations of *Saprolegnia parasitica* (Sap1 – 92000, Sap2 – 22400, Sap3 –5500 (cfu/mL)) and Cd in combination with *S. parasitica* (2 μg Cd/L + 5500 cfu/mL). Letters denote significant differences between groups. From Paper XIV.

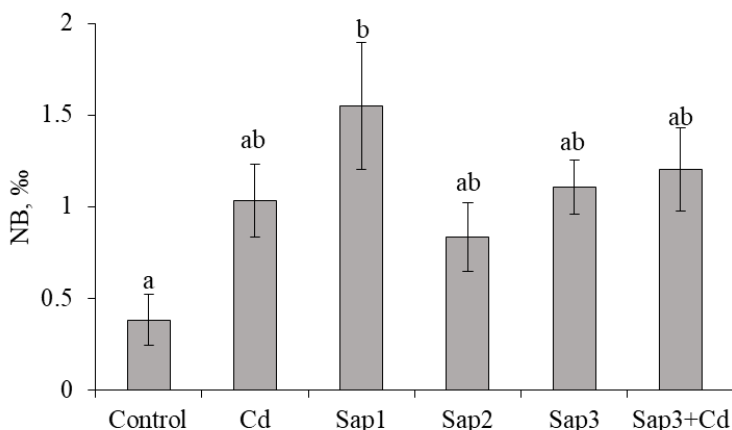


Fig. 15 Mean values (mean ± SEM, N = 10) of nuclear bud (NB) frequencies in erythroblasts of *O. mykiss* larvae treated with cadmium (Cd, 2μg/L), three concentrations of *Saprolegnia parasitica* (Sap1 – 92000, Sap2 – 22400, Sap3 – 5500 (cfu/mL)) and Cd in combination with *S. parasitica* (2 μg Cd/L + 5500 cfu/mL). Letters denote significant differences between groups. From Paper XIV.

Treatment with all *S. parasitica* concentrations and co-exposure treatment significantly increased total genotoxicity level in larvae erythroblasts, except for the 22400 cfu/mL (Sap2) concentration (Fig. 16). Significant changes in cytotoxicity endpoints were not detected after all treatments performed. Treatment with all *S. parasitica* concentrations and co-exposure treatment significantly increased total genotoxicity level in larvae erythroblasts, except for the 22400 cfu/mL (Sap2) concentration (Fig. 16). Significant changes in cytotoxicity endpoints were not detected after all treatments performed.

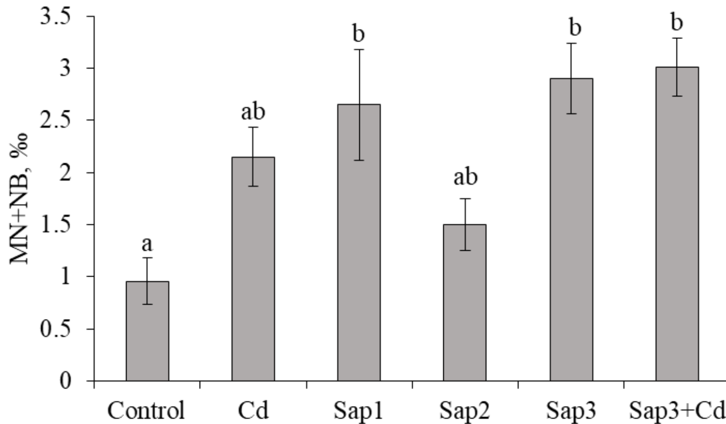


Fig. 16 Total genotoxicity (MN+NB) level (mean ± SEM, N = 10) in erythroblasts of *O. mykiss* larvae treated with cadmium (Cd, 2µg/L), three concentrations of *Saprolegnia parasitica* (Sap1 – 92000, Sap2 – 22400, Sap3 – 5 500 (cfu/mL)) and Cd in combination with *S. parasitica* (2 µg Cd/L + 5500 cfu/mL). Letters denote significant differences between groups. From Paper XIV.

DISCUSSION

Cumulative effects of chemical mixtures and multiple stressors as realistic exposure scenario are becoming a major problem in ecotoxicology. However, geno- and cytotoxicity of multiple stressors, quantum dots (metal-based nanoparticles) as an emerging engineering nanomaterials and complex metal mixtures at environmentally realistic concentrations are not well explored. This thesis sheds new light on tissue-specific, time-related, concentration-dependent, multiple stressors influenced geno- and cytotoxicity responses; fluctuations of geno- and cytotoxicity endpoints frequencies during fish exposure to complex, binary metal mixture, metal-based nanoparticles (QDs) or biological stressor (pathogen). Furthermore, the potential to exacerbate toxicity endpoints after fish exposure to multiple environmental stressors (pathogen infection and pollution) is emphasized.

High concentrations of individual metals have a potential genotoxicity and cytotoxicity effects. However, geno- and cytotoxicity responses to metal mixtures at environmentally relevant concentrations are scarcely investigated. Particularly, changes in geno- and cytotoxicity responses and their toxicological significance after reduction of concentration of single metal in the mixture have not been investigated. The experiments of the thesis indicated that complex metal mixture at MPC accepted for the inland water in EU induce genotoxicity and cytotoxicity in different tissues erythrocytes of *S. salar*, *R. rutilus* and *O. mykiss*. Moreover, for the first time, the reference level of geno- and cytotoxicity in hatchery-reared *S. salar* was evaluated. The results showed very low cytotoxicity (0.00–0.07‰) and low genotoxicity (0.04–0.50‰) reference levels in erythrocytes of reared salmon. The data suggest a good health status of salmon used for re-establishing or supplementing the wild population in rivers flowing to the Baltic Sea.

All the concentrations for waterborne metals (Cu, Zn, Ni, Cr, Pb and Cd) assessed in the experiments are environmentally relevant, while 10-times reduced concentration of a single metal in the mixture represent the possible influence of background exposure in the aquatic environment. Considering time-dependent induction of nuclear abnormalities, the most obvious finding to emerge from the geno- and cytotoxicity analysis in tissues of *S. salar* exposed to metal mixture for 28 days is that different non-linear response between exposure time versus analysed endpoints levels was determined. Genotoxicity levels peaked on day 7 in liver and peripheral blood erythrocytes of *S. salar*, while in gills and kidney—on day 14, and these levels were statistically higher than those of a non-exposed group. Statistically elevated cytotoxicity levels were detected on day 2 and day 14 in gills erythrocytes and on day 28 in *S. salar* peripheral blood. Previously genotoxicity or cytotoxicity induction of low metal mixtures concentration was shown in carp (*Cyprinus carpio*) exposed to binary–ternary metals mixtures Cd + Cr, Cd + Cu, Cd + Cr + Cu and synergistic toxic effects of multiple metals were emphasized (Zhu

et al. 2004). Complex effect of metal mixture causing DNA damage, also was measured using higher concentrations (0.5 mg/L of each) of Cd + Pb, Cd + Zn, Pb + Zn, Cd + Pb + Zn mixtures in *Misgurnus anguillicaudatus* (Zhang et al., 2008). Cu/Cd-co-exposed fish (0.1 ppm of each) showed slightly increased total nuclear abnormalities, while micronuclei frequencies were not significantly affected (Güner and Muranlı 2011). Experimental data on the relationship between exposure time and ENAs frequencies after fish exposure to metal mixture is scarce. Zhu et al. (2004) showed that micronuclei frequencies increased with increasing exposure time after carp exposure to low concentrations of single Cr and Cd. Micronuclei frequencies after exposure to single Cr (0.001 mg/L), Cd + Cr (0.001 + 0.001 mg/L) and single Cd (0.001 mg/L), Cd + Cr + Cu (0.001 + 0.001 + 0.01 mg/L) peaked on day 4 and day 9, respectively, and then smoothly changed (Zhu et al., 2004). The fluctuations of DNA damage during exposure time were indicated by Zhang et al. (2008). DNA damage caused by mixed ions in *M. anguillicaudatus* increased significantly during the first seven days, and then rose moderately during 7–14 days, then greatly decreased during 14–21 days and followed by a steady decrease after 21-day treatment.

The influence on geno- and cytotoxicity outcome after 10-times reduction of even low concentration (MPC) of single metal in a complex mixture was assessed using *S. salar* and *R. rutilus*. The results of an experimental research demonstrated that reduction of even low (MPC) metal concentration in the mixture significantly affected the frequencies of genotoxicity and cytotoxicity endpoints in Atlantic salmon and roach erythrocytes. Exposure to toxic and essential metals mixture with slight change of a single metal concentration resulted in different risk of geno- and cytotoxicity, which can apparently be influenced by metal-metal interactions in both outside and inside the organism. The most severe genotoxicity or cytotoxicity responses in Atlantic salmon were detected after treatments with Cr↓ (10 times reduced Cr^{6+} concentration) followed by MIX, Cu↓ or Pb↓ mixtures. While the most severe cytogenetic damage in roach was detected after Cr↓, Ni↓ and MIX, whereas Comet assay indicated Cr↓ followed by Pb↓ and Zn↓ treatments as having a significant effect on DNA strand breaks. These results may suggest occurrence of hermetic-like responses, that some of the metals in a mixture induce hormesis. Exposure to low concentrations of Cr may produce a beneficial or stimulatory effect. Perez-Benito (2006) emphasized hormetic effect of low Cr (VI) concentrations such as an increased lifespan of exposed fish. Antioxidant effect of Cr, Pb and Cd mixture at environmentally relevant concentrations due to significantly altered levels of SOD and CAT in different tissues of *Cyprinus carpio* was reported by Rajeshkumar with co-authors (2017). As concluded by Calabrese and Mattson (2011) low concentration hormetic stimulation indicates an adaptive response that represent an environmentally-induced altered phenotype and allows a quantitative estimate of biological plasticity. In the present study, changes of the geno- and

cytotoxic endpoints frequencies following treatments with different concentration of a single metal in the mixtures may be related to variations of antioxidant defense enzyme activities leading to bioprotective functions. This presumption may be considered as a possible mechanism for the hormetic effects. However, more research must be conducted to predict hormesis in chemical mixtures. Moreover, the results on geno- and cytotoxicity of metal mixtures in Atlantic salmon and roach suggested that interactions at low metal exposure concentrations are likely to occur and elicit toxicologically significant effects. Therefore, environmental risk assessments addressing individual substances are insufficiently protective for such complex mixtures. Each metal in the mixture may provoke specific toxicity endpoints due to their involvement in a spectrum of metabolic pathways in the biological systems (Wah Chu and Chow 2002). Previously an experimental study showed, that metals with higher covalent index such as Pb and Cu, elicit synergistic effect, while metals with low covalent index (Cd, Ni and Zn) have a volatile impact (Wah Chu and Chow 2002). Also, metal with low covalent index, such as Zn, have neutralizing effect on other metals with lower covalent index, e.g. Cd, Ni toxicity (Wah Chu and Chow 2002). Cobbina with co-authors (2015) showed that exposure to metal mixtures at low dose influences homeostatic regulation of toxic and essential metals. A literature review survey on single metal genotoxicity at low concentrations showed that some of metals comprising mixture in the current research singly were genotoxic to certain fish species even at MPC. According to the research data 0.01 mg/L of Cu causes different responses depending on fish species and tissues. Zhu with co-authors (2004) study shows, that individually Cu at 0.01, 0.1 and 1mg/L did not induce significant MN formation in carp blood. Copper (0.01 mg/L) did not cause increase in micronucleus and binuclei in *Carassius gibelio* blood, gills and liver cells, but induce micronuclei formation in *C. carpio* gills (Cavas et al., 2005). Cadmium at 0.01 mg/L is known to induce MN formation in carp blood, while exposure to Cd at 0.001mg/L concentrations did not significantly induce MN frequencies (Zhu et al., 2004). However, cadmium (0.005 mg/L) treatment caused binuclei formation in liver of *C. carpio* and micronuclei and binuclei formation in gills and liver of *Corydoras paleatus*, respectively. Blood, liver and gills of *C. gibelio* was not sensitive to Cd exposure at 0.005 mg/L considering micronuclei and binuclei formation (Cavas et al., 2005). While, all tested Cr concentrations (0.001, 0.01, 0.1 mg/L) induced significant MN frequencies in carp erythrocytes in comparison to control level (Zhu et al., 2004). DNA damage increased in both blood cells and gills of *Prochilodus lineatus* exposed to Ni concentrations (25, 250 and 2500 µg L(-1)) (Palermo et al. 2015). Cadmium, Cr and Cu are able to induce genotoxic effects at environmentally relevant concentration in Argentinean silverside (*Odontesthes bonariensis*) gills and liver cells (Gasulla et al., 2016).

Time-related recovery from geno- and cytotoxicity endpoints was assessed in *O. mykiss* after co-exposure to different concentrations of Zn-Cu mixture in

an experimental depuration process. Co-exposure to mixture of essential metals (Zn and Cu) at various concentrations resulted in an increase of geno- and cytotoxicity endpoints in rainbow trout erythrocytes. Exposure to the highest Cu-Zn concentration (0.25%) resulted in significant increase of MN, 8-shaped frequencies and total genotoxicity level in all analysed tissues. Significant induction of analysed endpoints in lower test concentrations (0.0625 and 0.125%) varied depending on tissue. The main concern is that geno- and cytotoxic endpoints provoked by joint copper–zinc mixture may be influenced by concentration of each metal in the mixture. The ratios of metals in the mixtures can lead to different coergisms types as have been shown by Obiakor and Ezeonyejiaku (2015). Synergistic coergisms were determined in most of the explored ratio mixtures of Cu and Zn, except in the ratio 1:1—antagonistic effect. Bagdonas and Vosylienė (2006) as well as Obiakor et al. (2010) studies revealed that Cu and Zn jointly (in all of three concentrations tested) exert a larger genotoxic effect when compared to the single action of each metal. In comparison to results obtained in the current thesis, they also detected the highest levels of MN in fish blood erythrocytes co-exposed to 0.25% concentration of copper–zinc mixture, but detected frequencies of MN were higher. Obiakor and Ezeonyejiaku (2015) concluded that copper and zinc jointly provoke higher or cumulative toxicity. Probably mechanisms causing metal/metal synergies are interactions on metal availability and uptake (Cedergreen 2014). Genotoxic properties of metals are related evidently to the accumulation of DNA damaging free radicals, clastogenic process or simultaneously to clastogenic and aneugenic action in aquatic organisms (Nepomuceno et al., 1997).

During the depuration period, MN were found to increase after 4-day recovery and decrease after 8- and 12-day recovery period in all analysed tissues of rainbow trout. This may be due to several factors: the cell cycle kinetics and the mechanism of MN induction. MN arises in cell divisions, and their expression can appear at different times after the DNA damage (Bolognesi and Hayashi 2011). Grisolia and Cordeiro (2000) evaluated the time-dependent response of MN formation during hematopoiesis in the kidney and MN peak in circulating blood erythrocytes after fish exposure to cytochalasin B. An experiment showed that micronuclei formed in the young kidney erythrocyte cells were detected in peripheral blood 2–4 days later, and this time lag depends on species and clastogens. In the current experiment, recovery of analysed tissues from some genotoxic or cytotoxic endpoints was observed after 8 or 12 days at 0.0625 and 0.125% concentrations. Blood erythrocytes showed significant recovery after 8 and 12 days in 0.25% concentration. Significant recovery was not observed after 12-day recovery in liver and kidney erythrocytes after co-exposure to the highest concentration. This study shows that 12 days term was not enough for recovery of juvenile rainbow trout from genotoxicity and cytotoxicity after treatment with copper–zinc mixture. Moreover, the present experiment revealed significant effects of

copper–zinc mixture on gross morphometric indices. During the depuration period, the LSI increased, but CF decreased. The current results are in agreement with those of Omar et al. (2012) who reported that decrease in CF values was observed in fish Nile tilapia (*Oreochromis niloticus*) and mullet (*Mugil cephalus*) collected from highly degraded aquatic habitats polluted with metals (Cu, Zn, Pb, Fe and Mn) as compared with those from the reference site. Liu et al. (2010) found that growth declined, but hepatosomatic index (HSI) increased in fish *Synechogobius hasta* with increasing waterborne copper levels. Zheng et al. (2001) showed that waterborne zinc exposure significantly reduced HSI in *S. hasta*, but did not significantly influenced CF. The mean CF and HSI in three-spined stickleback (*Gasterosteus aculeatus*) exposed to copper showed no significant differences between treatments (Santos et al., 2010). Bonga and Lock (1992) reported that a reduced growth is a result of significantly increased energy requirements for maintenance of water and ion homeostasis, because waterborne toxicants increase the permeability to water and ions of the gill epithelium and inhibit the ion-exchange activity of the chloride cells.

Considering tissue-specific geno- and cytotoxicity responses, peripheral blood erythrocytes mostly exhibited the highest levels of summed genotoxicity and cytotoxicity during all experiments. Moreover, during recovery assessment peripheral blood erythrocytes showed faster recovery rate and less fluctuating frequencies of analysed endpoints compared with liver and kidneys erythrocytes. The highest ENAs frequencies in peripheral blood could be explained by the fact that peripheral blood receives erythrocytes and erythrocytes with nuclear abnormalities from several hematopoietic organs. Sites of erythropoiesis in teleost fish are concentrated predominantly in pro- and mesonephros (Soldatov 2005). The secondary hematopoietic organs such as periportal areas of the liver and gills may have different sensitivities to xenobiotics (Agius and Roberts 2003; Macchi et al., 1992). Cavas et al. (2005) suggest that use of different tissues other than peripheral blood in micronucleus assays brings better results. The thesis indicated that peripheral blood was a more sensitive tissue and better indicator of the geno- and cytotoxicity of metal mixtures compared to gills, kidney or liver erythrocytes. Moreover, the higher recovery rate was observed in peripheral blood. The tissue specificity depends on the bioaccumulation of toxicants, different sensitivity and defensive mechanism of the certain organ (Ahmad et al., 2006; Velma and Tchounwou 2010). In teleost fish, the main erythropoietic tissue is a cephalic kidney; cell division may be more active in this tissue (Baršienė et al., 2006; Velma and Tchounwou 2010). In fish, liver and kidney play a major role in the detoxification and excretion of toxins via the induction of metal-binding proteins such as metallothioneins (Roesijadi and Robinson 1994). In general, elimination routes of metals from fish are through bile, urine, gills and mucus (Varanasi and Markey 1978; Kim et al., 2004). Kidneys are more susceptible to oxidative stress than liver, which is

the primary site for the biotransformation of xenobiotic compounds (Velma and Tchounwou 2010).

Geno- and cytotoxicity potential of metal-based nanoparticles (CdSe/ZnS-COOH QDs) was assessed using early life stages of rainbow trout. Geno- and cytotoxic effect induced by single Cd exposure was used for comparison with QDs provoked effects taking into account possible Cd ions leakage from QDs. Considering Cd and QDs geno- and cytotoxicity in rainbow trout erythroblasts, different frequencies of analysed endpoints were noted. In the present experiments, exposure to Cd induced significant cytogenetic damage and DNA strand breaks in erythroblasts of embryos and significant cytogenetic damage in erythroblasts of rainbow trout larvae. The findings of this study are in agreement with those obtained by other researchers evaluating Cd-induced genotoxicity and cytotoxicity in various fish species. Increased frequencies of micronucleated and binucleated cells in different tissues of fish exposed to Cd were recorded previously (Cavas et al., 2005; Jindal and Verma 2015). Chronic exposure to CdC_{l2} (0.37 and 0.62 mg/L) induced elevated formation of micronuclei and other nuclear abnormalities (nuclear bud, binucleates, lobed, notched and vacuolated nuclei) in peripheral blood erythrocytes of fish *Labeo rohita* (Jindal and Verma 2015). Cd-induced cell death by apoptosis was determined in rainbow trout hepatocytes (Risso-de Faverneyet al. 2001). According to Hsu et al. (2013), Cd at sublethal levels induced oxidative stress in zebrafish embryos. Consequently, oxidative stress and DNA repair inhibition are major mechanisms triggering Cd genotoxicity (Hsu et al., 2013).

Performed experiments demonstrate that the Comet assay successfully detected DNA damage in erythroblasts of rainbow trout embryos and larvae after exposure to QDs, while the nuclear abnormalities assay did not allways detect significant elevation in genotoxicity and cytotoxicity endpoints. Carboxylated CdSe/ZnS at 4nM concentration significantly increased DNA damage after 1, 4 days of exposure in embryos and in newly hatched larvae after 11 days of exposure. The clastogenic/aneugenic properties of CdSe/ZnS QDs have been observed in some studies (Aye et al., 2013; Galeone et al., 2012). For this reason, Galeone et al. (2012) suggested classifying CdSe/ZnS QDs as significantly toxic in long-term *in vivo* treatments. The reason of QD-induced DNA damage is likely to be related to ROS generation. As concluded by Singh et al. (2009), nanoparticle-induced oxidative stress is thought to be a key mechanism responsible for genotoxicity effects. Several studies have pointed to PEG-coating that can protect from Cd^{2+} leak (Galeone et al., 2012; Zhang et al., 2006). It was concluded that coating with PEG decreases toxicity but does not eliminate it (Ju et al., 2013; Galeone et al., 2012). Saez et al. (2015) showed that genotoxicity potential and reactivity of Cd with intracellular targets are influenced by its nano or ionic form. Nano-Cd showed a stronger genotoxic activity compared with ionic-Cd in *Hediste diversicolor*. However, the results obtained by Aye et al. (2013) showed that even though

QDs induced ROS, the mutagenic/clastogenic properties of QDs were not completely accounted for. The inherent physico-chemical properties of QDs may generate various reactions, which result in genotoxicity/mutagenicity effects in cells or organisms. Other possible mechanisms underlying genotoxicity of QDs are inflammation, aberrant signalling responses and direct interaction with DNA and nuclear proteins (Brunetti et al., 2013). Moreover, disturbed gas exchange due to detected QDs aggregations on the surface of embryos would be expected to produce genotoxicity endpoints in embryos (Rotomskis et al., 2018). Hypoxia induced reactive oxygen species (ROS) may be a physiological response to oxygen deficiency. However, hypoxia-induced ROS production remains unclear. Several studies reported hypoxia-induced genetic instability in organisms and cell cultures (Snyder and Diehl 2005; Kim et al., 2007), and concluded that hypoxia alone may act as genotoxic agent. Several reports have shown that hypoxia is a mutagen, teratogen, because it affects fish embryonic development (Shang and Wu 2004; Shang et al., 2006). Hypoxia is known to induce mortality, malformation, delay fish (*Danio rerio*) embryonic development, hatching, disrupt the apoptotic pattern, balance of sex hormones, affect sex differentiation and sex ratio (Shang and Wu, 2004; Shang et al., 2006). However, hypoxia inducing development impairments in fish, remains unclear (Wu 2009). In many studies, oxidative stress and inflammatory response is proposed as key mechanisms for understanding nanoparticles-induced toxicity and genotoxicity (Magdolenova et al., 2014; Xiao et al., 2016). Nevertheless, such findings are hypothesis generating and require further detailed study. Thus, potential for genotoxicity effects and mechanisms of DNA damage of QDs still remains unclear (Saez et al., 2015). Further studies with a greater focus on QD-induced genotoxicity assessment using several DNA damage biomarkers to identify the effects of physicochemical features and genotoxicity mechanisms are therefore suggested (Demir and Castranova 2017).

Genotoxic and cytotoxic potential of infections (*Saprolegnia parasitica*) and exacerbation of these effects after multiple stressors (*S. parasitica* and Cd) exposure was evaluated using rainbow trout larvae. Genotoxic potential of *S. parasitica* infection in rainbow trout larvae was identified. The findings indicated a significant increase of separate genotoxicity endpoints and total genotoxicity depending on exposure concentration of *S. parasitica*. However, genotoxicity endpoints did not show a clear tendency to increase with increasing *S. parasitica* exposure concentration. Belmonte and co-authors (2014) detected the immune suppression in Atlantic salmon before the pathogen infection (establishment) or after early stages of interaction. Moreover, 12 days exposure of fish to *S. parasitica* (104 zoospores/cysts liter^{-1}) did not cause evidence of infection and no suppression of the antigen, and no induction of proinflammatory genes were detected. These responses might indicate a protection against the oomycetes. In this study, exposure to

the highest concentrations of *S. parasitica* did not induce the highest frequencies of all analysed geno- and cytotoxicity endpoints. These results might indicate the threshold for inhibition of certain geno- and cytotoxicity responses. Scientific literature data related to direct or indirect genotoxic effects induced by *Saprolegnia* do not exist. The genotoxic potential of *S. parasitica* in fish has not been investigated at all. This study provides first toxicity data that show significantly increased genotoxic activity in rainbow trout after *S. parasitica* exposure. Azimzadeh and Amniattalab (2017) indicated oxidative stress, haematological and histopathological changes in rainbow trout infected with *S. parasitica*. Oomycetes are known to secrete toxins, proteinaceous substances or hydrolytic enzymes (Soanes et al., 2007). Torto-Alalibo et al. (2005) have exuded and isolated several proteins of *S. parasitica* (CBD proteins, CBEL-like proteins, glycosyl hydrolases, proteases, protease inhibitors) and emphasized that these proteins can have a range of impacts on health. Moreover, *Saprolegnia* infection induce a strong inflammatory response in fish. As concluded by Belmonte et al. (2014) *S. parasitica* produces the metabolite prostaglanding E2 (PGE2), which increases the inflammatory response in fish leukocytes. However, one of the limitations of these findings is that it does not explain which mechanisms (direct or indirect) are responsible for such genotoxicity outcome. Marcogliese et al. (2005) concluded that parasitism in the presence of pollution may further compromise the health by reducing the immunocompetence of the host. Furthermore, exacerbation of toxicity effects may be noted even parasites infestation occurs at low intensities. In agreement with that, the findings of this study, showed the highest total genotoxicity level after joint treatment with the lowest *S. parasitica* concentration and Cd in comparison to other treatments. In the present experiment, significant cytotoxicity was not induced by any *S. parasitica* concentration tested, as well as after co-exposure treatment. As emphasized by Schaumburg et al. (2006), parasites can induce anti-apoptotic activities in the host.

CONCLUSIONS

1.The highest genotoxicity induction in liver and peripheral blood erythrocytes occurred at day 7 of exposure to complex metal mixture (MIX) at MPC, and at day 14 – in gills and kidneys erythrocytes of *Salmo salar*. Significant cytotoxicity levels were found after 2 and 14 days of exposure in gills erythrocytes and after 28 days — in peripheral blood erythrocytes. Significant induction of cytotoxicity was not observed in liver and kidneys erythrocytes.

2.Depending on the test concentrations co-exposure to Cu-Zn induced significant formation of micronucleus, nuclear buds, blebbed and 8-shaped nuclei erythrocytes in different tissues of *O. mykiss*. Peripheral blood erythrocytes showed significant recovery from genotoxicity and cytotoxicity after 8 and 12 days at the highest (0.25%) Cu-Zn co-exposure concentration.

3.During the recovery, liver and kidney erythrocytes exhibited more significant fluctuations in the frequency of cytogenetic lesions compared to peripheral blood erythrocytes. A significant increase in the frequency of cytogenetic lesions was determined on the 4th day of recovery, depending on the concentration of the binary metal (Cu-Zn) mixture and tissue. On the 8th day of recovery, depending on the exposure concentration, a significant decrease in the frequency of cytogenetic lesions in liver and kidney erythrocytes was observed, there was no significant decrease on the 12th day of recovery (except for 8-shaped at 0.0625% concentration in liver).

4.Exposure to metal mixtures at MPC elicit significant genotoxic and cytotoxic effects in different fish species. Minor changes of even low concentration (MPC) of single metal in a complex mixture significantly influence the outcome of geno- and cytotoxicity. Significant induction of cytogenetic lesions in erythrocytes of *S. sala*r was detected after treatments with Cr↓ (10 times reduced Cr^{6+} concentration), MIX, Cu↓ and Pb↓ mixtures. Significant cytogenetic damage in *R. rutilus* was detected after Ni↓, MIX and Cr↓, whereas Comet assay indicated Cr↓ followed by Pb↓ and Zn↓ treatments as inducing a significant DNA strand breaks.

5.Fish peripheral blood erythrocytes indicated higher levels of cytogenetic damage during metal mixture treatments, whereas faster recovery rate was observed in peripheral blood compared to other tissues erythrocytes.

6.Exposure to 4nM carboxylated CdSe/ZnS quantum dots resulted in significant DNA strand breaks in embryos (after 1 and 4 days) and in newly hatched larvae of *O. mykiss* after 11 days of exposure. Exposure to 2 µg Cd/L induced significant formation of MN, total cytotoxicity (FA+BN) and DNA strand breaks in embryos, whereas significantly elevated frequencies of MN, NB and total cytotoxicity were measured in larvae of *O. mykiss* after 4 days treatment.

7. Significant induction of MN and NB frequency in erythroblasts of *O. mykiss* larvae was detected after treatment with *Saprolegnia parasitica*

concentration 5500 and 92000 cfu/mL, respectively. Total level of genotoxicity endpoints showed significant elevation after the highest and the lowest *S. parasitica* concentrations.

8.*S. parasitica* (5500 cfu/mL) and Cd co-exposure resulted in significantly elevated MN frequencies and total genotoxicity level, however, no significant synergistic interaction was found comparing with the individual effects of the two stressors (*S. parasitica* and Cd).

SANTRAUKA

ĮVADAS

Šioje disertacijoje pateikti eksperimentiniai cheminių medžiagų bei mišinių tyrimai itin svarbūs ekotoksikologiniu aspektu, kadangi leidžia išsamiau pažvelgti į vandens aplinkoje vyraujančią taršą cheminiais mišiniais. Pasirinkti bioindikatoriai bei atlikti moksliniai tyrimai suteikė naujos informacijos, kuri gali būti naudinga ES vandens taršos cheminių medžiagų mišiniais reguliavimo kontekste. Vienas iš Vandens pagrindų direktyvos (Vandens pagrindų direktyva, Europos Sąjunga) tikslų yra pasiekti ES paviršinių vandens telkinių "gerą ekologinę būklę" (angl. GES) ir "gerą cheminę būklę". Šis tikslas turėtų būti pasiektas išlaikant ir atkuriant gerą vandens ekosistemų būklę. Aplinkos genotoksiškumo įvertinimas bioindikatoriniuose organizmuose, suteikia pirminius signalus apie neigiamą cheminės, fizikinės ar biologinės taršos poveikį vandens ekosistemoms, tuo tarpu genotoksinio potencialo vertinimas, kontroliuojamomis eksperimentinėmis sąlygomis, leidžia atskleisti atskiro stresoriaus ar jų komplekso poveikį pasirinktiems bioindikatoriams. Disertacijoje pateikti eksperimentiniai tyrimai apie daugianarių metalų mišinių (Zn, Cu, Cr, Ni, Pb, Cd), esant didžiausioms leistinoms koncentracijoms (DLK), daugiakomponentinių nanodalelių (karboksilintų CdSe/ZnS kvantinių taškų), *Saprolegnia parasitica*, kaip biologinio stresoriaus indukuotą genotoksinį ir citotoksinį poveikį įvairiuose bioindikatoriniuose organizmuose. Galiausiai, po metalų mišinio poveikio žuvyse įvykę citogenetiniai pokyčiai toliau tirti organizmų atsisatymo procesuose. Atlikti Baltijos jūros aplinkos genotoksiškumo ir citotoksiškumo tyrimai leido palyginti pasirinktų geno- ir citotoksiškumo biožymenų atsakus vyraujant aplinkoje kompleksui veiksnių (biotiniai, abiotiniai).

Antropogeninės veiklos metu su pramoninėmis, buitinėmis ir žemės ūkio nuotekomis į aplinką patenka cheminių medžiagų mišiniai, kurie kelia rūpestį dėl jų neigiamo poveikio gėlavandenėms ir jūrinėms ekosistemoms. Genotoksinai yra šių mišinių sudedamosios dalys, kurie gali sukelti genetinius pažeidimus ir inicijuoti toksinį poveikį įvairiuose biologinių sistemų lygmenyse (Bolonese, Hayashi 2011). Vandens organizmai yra veikiami įvairių teršalų, kuriems taikomi skirtingi Europos Sąjungos reglamentai, tačiau cheminių mišinių poveikis šiuo metu nėra reglamentuojamas. Remiantis SCHER, SCCS, SCENIHR (2012) nuomone dėl cheminių mišinių rizikos vertinimo, cheminių mišinių rizikos vertinimas turi atsižvelgti į galimus mišinių efektus aplinkoje realiai egzistuojančiomis koncentracijomis bei į galimą foninį šių cheminių medžiagų poveikį aplinkoje. Suminis kelių stresorių poveikis tampa pagrindine problema ekotoksikologijoje. Kadangi, esant cheminei taršai, kitų stresorių būvimas, pvz., parazitų infekcijos, gali dar labiau pakenkti organizmų būklei, sumažindamos jų atsparumą. Toksinio

poveikio sustiprėjimas gali būti stebimas net esant mažam parazitų intensyvumui (Marcogliese et al., 2005). *Saprolegnia parasitica* yra oomicetas natūraliai paplitęs gėlavandenėse ekosistemose, kuris sukelia ligą saprolegniozę. Ši grybelinė liga yra dažna žuvininkystės ūkiuose ir sukelia didelius ekonominius nuostolius. Malachito žaliojo, kaip prevencinės priemonės nuo *S. parasitica* infekcijos, naudojimas buvo uždraustas dėl toksinio jo poveikio. Efektyvesnių ir saugesnių apsaugos priemonių nuo šio žuvų ligos sukėlėjo nerasta. Svarbu nustatyti *S. parasitica* inicijuotą geno- ir citotoksinį potencialą, tam kad būtų galima atskirti patogeno sukeliamą toksiškumą nuo cheminių medžiagų, naudojamų kovoti su saprolegnioze, indukuojamo toksiškumo. Vienalaikio *S. parasitica* kartu su cheminiu stresoriumi (Cd) poveikio tyrimai atskleistų stresorių potencialą sukelti geno ir citotoksinių efektų paūmėjimą.

Kroon su bendraautoriais (2017) įvardijo tinkamiausius cheminių medžiagų sukeliamo genotoksinio ir citotoksinio poveikio biožymenis. Mikrobranduolių kartu su kitomis branduolio pažaidomis ir DNR pažaidų vertinimas, taikant Kometų metodą (ang. Comet assay), buvo pasiūlyti kaip tinkamiausi metalų geno- ir citotoksinio poveikio žymenys (Kroon et al., 2017). Pateikti metodai plačiai taikomi ir nano-genotoksikologiniuose tyrimuose (Karlsson et al., 2015, Celá et al., 2014). Kometų metodas leidžia įvertinti DNR grandinės trūkius atskirose ląstelėse. Branduolio pažaidų analizė leidžia identifikuoti įvairių stresorių sukeliamus citogenetinius efektus, išskiriant genotoksines ir citotoksines pažaidas organizmų ląstelėse. Mikrobranduoliai, branduolio pumpurai, branduolio pumpurai su nukleoplazmine jungtimi, dvibranduoliai tiltai ir branduolio ataugos yra priskiriamos genotoksinėms pažaidoms, 8-formos branduolį turinčios, dvibranduolės ir fragmentuotos-apoptotinės ląstelės yra priskiriamos citotoksinėms pažaidoms. Kitos pažaidos, kaip vakuolės branduolyje, bebranduolės ar pupelės formos branduolį turinčios ląstelės taip pat laikomos citogenetinio poveikio biožymenimis, kurie papildo branduolio pažaidų analizę genotoksiškumo ir citotoksiškumo tyrimuose (Gomes et al., 2015; Harabawy et al., 2014).

Įvairios žuvų rūšys, plačiai taikomos aplinkos geno-citotoksinių junginių poveikio tyrimams *in situ* bei klastogeninių ir aneugeninių junginių poveikio tyrimuose laboratorinėmis sąlygomis (Cavas, Ergene-Gozukara 2005; Cavas et al., 2005). Šio darbo eksperimentiniuose ir *in situ* tyrimuose naudoti įvairūs bioindikatoriniai organizmai, kurie priklauso skirtingoms taksonominėms grupėms ir atspindi įvairias ekologines grupes, kadangi jų geno-citotoksinis atsakas į cheminę taršą gali varijuoti. Lašišinės žuvys, pvz., Atlantinė lašiša *Salmo salar* ir vaivorykštinis upėtakis *Oncorhynchus mykiss*, skirtingose vystymosi stadijose yra vertingi vandens ekosistemų ekologinio integralumo biologiniai indikatoriai (Chovanec et al., 2003). Paprastoji kuoja (*Rutilus rutilus*) yra tinkamas bioindikatorius vandens užterštumo stebėjimui įvairiose gėlavandenėse ekosistemose (Łuczyńska et al., 2018). *Clupea harengus*

membras, *Platichthys flesus*, *Gadus morhua*, *Zoarces viviparous* ir dvi bestuburių rūšys *Macoma balthica* bei *Saduria entomon* yra būdingi Baltijos jūros bendrijų komponentai, naudojami aplinkos taršai stebėti (Baršienė et al., 2008; 2014; 2015; Góral et al., 2009).

DARBO NAUJUMAS

Šio darbo rezultatai suteikia naujos mokslinės informacijos apie genotoksiškumo ir citotoksiškumo parametrų atsakus įvairiose žuvų rūšyse bei skirtinguose jų audiniuose. Išanalizuota metalų (Zn, Cu, Ni, Cr, Pb ir Cd) mišinių ir kvantinių taškų (CdSe/ZnS-COOH), kaip daugianarių cheminių stresorių, bei oomicetų *Saprolegnia parasitica*, kaip biologinio stresoriaus, įtaka citogenetinių pažaidų indukcijai. Apibūdinti genotoksinių ir citotoksinių pažaidų dažnių svyravimai žuvų atsistatymo po poveikio metu. Pirmą kartą žuvyse nustatyti šie genotoksiškumo ir citotoksiškumo ypatumai:

- įvertintas didžiausių leistinų metalų koncentracijų (DLK) geno- ir citotoksinis poveikis daugianariame mišinyje skirtingoms žuvų rūšims; sumažinus daugianariame mišinyje atskiro metalo koncentraciją 10 kartų žuvų audiniuose nustatyti reikšmingi toksiškumo pokyčiai;
- pašalinus cheminių stresorių poveikį, apibūdinti geno- ir citotoksinių pažaidų dažnių svyravimai žuvų skirtingų audinių eritrocituose;
- skirtingose žuvų rūšyse nustatyti nuo poveikio laiko, cheminių medžiagų koncentracijų priklausomi ir audiniui specifiški geno- ir citotoksiškumo atsakai;
- ankstyvose žuvų vystymosi stadijose nustatytas kvantinių taškų, kaip naujų inžinerinių nanomedžiagų, geno- ir citotoksinis poveikis;
- įvertintas biologinio stresoriaus - patogeno *Saprolegnia parasitica* geno- ir citotoksinis poveikis *Oncorhynchus mykiss* lervose;
- išnagrinėtas genotoksiškumo ir citotoksiškumo atsakas *O. mykiss* lervose, po poveikio dviem stresoriais t.y. biologiniu (*S. parasitica*) ir cheminiu (Cd).

MOKSLINĖ IR PRAKTINĖ DARBO REIKŠMĖ

• Nustatyta *Salmo salar* eritrocitų branduolio pažaidų indukcijos priklausomybė nuo poveikio metalų mišiniais trukmės bei apibrėžtas geno- ir citotoksiškumo atsakų specifiškumas žuvų audiniuose. *O. mykiss* atsistatymo potencialo po citogenetinio metalų mišinio poveikio vertinimas atskleidė audiniui specifinius, nuo metalo mišinio koncentracijų bei atsistatymo trukmės priklausomus citogenetinių pažaidų dažnio svyravimus.

• Metalų (Zn, Cu, Ni, Cr, Pb ir Cd) mišinys, esant DLK, sukelia citogenetinius pažeidimus *S. salar* ir *R. rutilus* eritrocituose. Nustatyta, kad atskiro metalo koncentracijos šiame mišinyje sumažinimas gali reikšmingai padidinti geno- ir citotoksinių atsakų lygį skirtinguose žuvų audiniuose.

• Pirmą kartą įvertinti karboksilintų CdSe/ZnS kvantinių taškų genotoksiniai ir citotoksiniai efektai ankstyvose *O. mykiss* vystymosi stadijose bei aprašytas hipotetinis nustatytų efektų mechanizmas.

• Įvertintas geno- ir citotoksinis potencialas *Saprolegnia parasitica* užkrėstose *O. mykiss* lervose bei šių atsakų paūmėjimas veikiant *S. parasitica* kartu su metalu (Cd).

• Daugianarių metalų mišinių (esant DLK) eksperimentiniai genotoksiškumo ir citotoksiškumo tyrimų žuvyse rezultatai gali suteikti naujos ir svarbios informacijos apie mišinių toksiškumą vandens organizmams bei gali būti taikomi mišinių toksiškumo vertinimo modelių kūrimui.

• Tyrimuose nustatyti žuvų atsistatymo po citogenetinio metalų mišinio poveikio dėsningumai gali būti taikomi sudarant ar koreguojant ekologiškai pažeistų vandens ekosistemų atkūrimo planus.

• Nustatytas daugianarių metalų mišinių geno- ir citotoksinis poveikis galėtų paskatinti ekotoksikologiškai svarbių vandens kokybės standartų persvarstymą įtraukiant į juos ir mišinių poveikį.

• Apibrėžtas geno- ir citotoksinių pažaidų lygis dirbtinai veisiamose Atlantinėse lašišose, leidžia spręsti apie veisyklose vyraujančią šių žuvų citogenetinę būklę ir tinkamumą populiacijų skaitlingumo atsatymui Lietuvos vandens telkiniuose.

DARBO TIKSLAS

Eksperimentiniai metalų (Zn, Cu, Ni, Cr, Pb, Cd) mišinių ir kvantinių taškų (CdSe/ZnS-COOH), kaip daugianarių cheminių stresorių, bei oomicetų *Saprolegnia parasitica*, kaip biologinio stresoriaus, geno- ir citotoksiškumo dėsningumų tyrimai žuvų eritrocituose.

Darbo uždaviniai:

1.Įvertinti branduolio pažaidų indukciją laike ir žuvų atsistatymo metu po poveikio metalų mišiniais.

2.Įvertinti skirtingų koncentracijų daugianarių metalų mišinių genotoksinį ir citotoksinį poveikį žuvų eritrocituose.

3.Įvertinti daugianarių metalų mišinių geno- ir citotoksinį poveikį skirtinguose indikatorinių organizmų audinių eritrocituose.

4.Įvertinti kvantinių taškų geno- ir citotoksinį poveikį žuvims ankstyvojo vystymosi stadijose.

5.Įvertinti oomicetų *Saprolegnia parasitica* bei šio patogeno kartu su Cd geno- ir citotoksinį potencialą žuvų eritrocituose.

GINAMIEJI TEIGINIAI

1.Didžiausios leistinos metalų koncentracijos daugianariame mišinyje sukelia nuo poveikio laiko priklausomus ir audiniui specifinius geno- ir citotoksiškumo atsakų dažnių pokyčius *S. salar* eritrocituose.

2.Citogenetinių pažaidų dažnių pokyčiai *O. mykiss* eritrocituose vykstant atsistatymui priklauso nuo atsistatymo trukmės, metalų mišinio koncentracijos bei yra specifiniai tiriamam audiniui. Atsistatymo periodui būdingi pažaidų dažnio svyravimai, aukščiausi svyravimai stebimi *O. mykiss* kepenų ir inkstų eritrocituose, lyginat su kraujo.

3.Daugianariame metalų mišinyje didžiausios leistinos kai kurių metalų koncentracijos sumažinimas padidina geno- ir citotoksiškumo atsakų lygį *S. salar* ir *R. rutilus* eritrocituose.

4.Žuvų periferinio kraujo eritrocituose aptinkamas didesnis geno- ir citotoksinių pažaidų dažnis, nei kepenų, inkstų ar žiaunų eritrocituose. Po poveikio binariniu metalų (Zn ir Cu) mišiniu greitesniu atsistatymo laiku pasižymi periferinio kraujo eritrocitai, nei kitų audinių eritrocitai.

5.Karboksilinti CdSe/ZnS kvantiniai taškai sukelia DNR grandinės trūkius *O. mykiss* embrionų ir lervų eritroblastuose.

6.*Saprolegnia parasitica* infekcija sukelia genotoksines pažaidas *O. mykiss* lervose.

TYRIMŲ MEDŽIAGA IR METODIKA

1. Tyrimų schema

Eksperimentiniai tyrimai vykdyti kaip bioindikatorius naudojant skirtingo vystymosi stadijų žuvis. Tyrimų schema, nurodant tyrimuose naudotas žuvų rūšis, biožymenis ir chemines medžiagas, pateikta 1 lentelėje.

Genotoksiškumo ir citotoksiškumo analizė, atlikta suaugusių žuvų ir jauniklių eritrocituose, taikant mikrobranduolių ir kitų branduolio pažaidų metodą, išsamesnė informacija apie šį metodą pateikta I-II, IV, VI-VIII, XII-XIII ir IX publikacijose; genotoksiškumo ir citotoksiškumo analizė ankstyvojo vystymosi stadijų žuvų eritroblastuose aprašyta V, X, XI, XIV ir XV publikacijose. Branduolio pažaidų analizė buvo atlikta remiantis Heddle ir kiti (1991), Fenech ir kiti (2003) bei Baršienės ir kiti (2006, 2014) publikacijose aprašytais kriterijais. Eritrocitų branduolio pažaidų morfologiniai bruožai pateikti I, II ir V publikacijose. DNR grandinės trūkiams žuvų jaunikliuose nustatyti naudotas Kometų metodas aprašytas XIII publikacijoje, tuo tarpu Kometų metodas taikytas žuvų ankstyvose vystymosi stadijose aprašytas III ir V publikacijose. Kometų metodas atliktas pagal Singh ir kitų (1988) pateiktas procedūras su tam tikrais Fatima ir bendraautorių (2014) aprašytais pakeitimais. Branduolio pažaidų analizės metodai bestuburiuose aprašyti VIII publikacijoje. Tyrimuose naudotų cheminių elementų koncentracijų ir fiziko-cheminių parametrų vandenyje matavimo metodai aprašyti atitinkamose publikacijose (I-V, IX-XV). Tyrimų metu taikyti statistiniai metodai yra išsamiai aprašyti kiekvienoje iš pateiktų publikacijų (I-XV).

1 lentelė. Atliktų tyrimų schema (informacija pateikta pagal parengtas publikacijas).

Žuvų rūšis	Vytymosi stadija	Biožymenys	Cheminės medžiagos ar infekcija	Publikacija
Salmo salar	jaunikliai	Branduolio pažaidos	Metalų mišiniai (Zn, Cu, Ni, Cr, Pb, Cd)	II, XII, IV
Rutilus rutilus		Branduolio pažaidos, Kometų metodas, hematologiniai parametrai		XIII
Oncorhynchus mykiss		Branduolio pažaidos		IX
			Cu/Zn mišinys	I
	embrionai	Branduolio pažaidos, Kometų metodas	karboksilinti CdSe/ZnS kvantiniai taškai	XI, V
	embrionai, lervos	Branduolio pažaidos	Cd	X
		Kometų metodas	karboksilinti CdSe/ZnS kvantiniai taškai	III
	lervos	Branduolio pažaidos		XV
			Cd, *Saprolegnia parasitica*, Cd + *S. parasitica*	XIV
Clupea harengus membras	suaugėliai	Branduolio pažaidos	Aplinkos tarša (Baltijos jūra)	VI
C. harengus membras, *Platichthys flesus*, *Gadus morhua*				VII
C. harengus membras, P. flesus, Zoarces viviparous, Macoma balthica, Saduria entomon				VIII

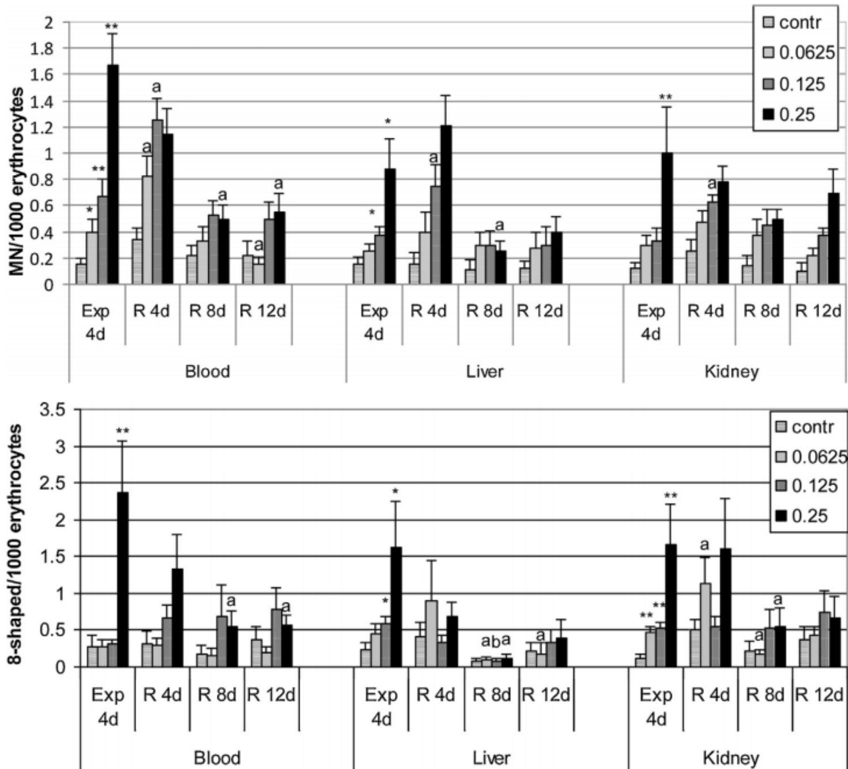
REZULTATAI IR JŲ APTARIMAS

Eritrocitų branduolio pažaidų indukcija organizmų atsistatymo metu ir skirtinguose jų audiniuose

Eksperimentiniai žuvų citogenetinio atsistatymo, po Zn ir Cu mišiniu, esant skirtingoms koncentracijoms (t.y. 0,0625; 0,125 ir 0,25% 96 val. LC50) poveikio tyrimai buvo atlikti *O. mykiss* periferinio kraujo, kepenų ir inkstų eritrocituose. Skirtingos Zn ir Cu mišinio koncentracijos po 4 parų poveikio indukavo geno- ir citotoksines pažaidas *O. mykiss* eritrocituose. Reikšminga mikrobranduolių, branduolio pumpurų, ataugų, bendro genotoksiškumo ir 8-formos eritrocitų branduolio indukcija nustatyta visuose 3 audiniuose po poveikio didžiausia (0,25%) dvinario metalų mišinio koncentracija. Po poveikio mažesnėmis šio mišinio koncentracijomis (0,0625 ir 0,125%) reikšminga pažaidų indukcija nustatyta priklausomai nuo audinio (4 pav.). Ankstesnių tyrimų metu nustatyta, kad toksinis Zn ir Cu mišinio poveikis gali priklausyti nuo kiekvieno iš mišinyje esančio metalo koncentracijos. Obiakor ir Ezeonyejiaku (2015) nustatė, kad metalų koncentracijų santykis mišiniuose sąlygoja skirtingus koergizmo tipus. Sinergetinė sąveika nustatyta daugelyje skirtingų Cu ir Zn koncentracijų santykio mišiniuose, išskyrus santykį 1:1, čia stebima antagonistinė sąveika. Bagdono ir Vosylienės (2006), Obiakor ir kitų autorių (2010) atlikti tyrimai atskleidė, kad Zn ir Cu mišinys (analogiškomis šiam tyrimui koncentracijomis) sukelia žymesnius genotoksinius efektus lyginant su atskiru šių metalų poveikiu. Šių autorių gauti rezultatai sutampa su šiame darbe gautais rezultatais, kad didžiausi mikrobranduolių dažniai aptikti žuvų eritrocituose po poveikio didžiausia Zn ir Cu mišinio koncentracija. Obiakor ir Ezeonyejiaku (2015) tyrimas atskleidė, kad bendras Cu ir Zn poveikis pasižymi suminiu arba didesniu toksiškumu. Pagrindinis mechanizmas lemiantis metalų sinergetinį poveikį yra sąveikos lemiančios metalų prieinamumą ir jų pasisavinimą (Cedergreen 2014). Metalų genotoksinės sąvybės yra susijusios su oksidacinėmis DNR pažaidomis, klastogeniniu ir aneugeniniu jų poveikiu vandens organizmuose (Nepomuceno et al., 1997).

Atsistatymo po citogenetinių pažeidimų laikotarpyje (12 parų), nustatytas mikrobranduolių dažnio padidėjimas 4-ą atsistatymo parą, tuo tarpu 8-ą ir 12-ą atsistatymo parą jų dažnis sumažėjo visuose tirtuose *O. mykiss* audiniuose (4 pav.). Šiuos rezultatus galėjo lemti ląstelės ciklo kinetika ir mikrobranduolių indukcijos mechanizmai. Mikrobranduoliai susiformuoja ląstelės dalijimosi metu, ir jų ekspresija laike gali varijuoti (Bolognesi, Hayashi 2011). Grisolia ir Cordeiro (2000) analizavo mikrobranduolių indukciją laike. Eksperimentų metu nustatyta, kad mikrobranduoliai susiformavę jaunuose inkstų eritrocituose po 2-4 parų aptinkami periferinio kraujo eritrocituose. Šis laikas priklauso nuo žuvų rūšies ir cheminės medžiagos. Atsistatymo laikotarpio 8-tą ir 12-tą parą nustatytas kai kurių geno- ir citotoksinių pažaidų reikšmingas dažnio sumažėjimas po poveikio

0,0625 ir 0,125% koncentracija (4 pav.). Periferinio kraujo eritrocituose reikšmingas citogenetinių pažaidų dažnio sumažėjimas po poveikio didžiausia (0,25%) Zn ir Cu mišinio koncentracija nustatytas 8-tą ir 12-tą atsistatymo parą. Tačiau 12-tą atsistatymo parą reikšmingo pažaidų dažnio sumažėjimo kepenų ir inkstų eritrocituose nenustatyta.

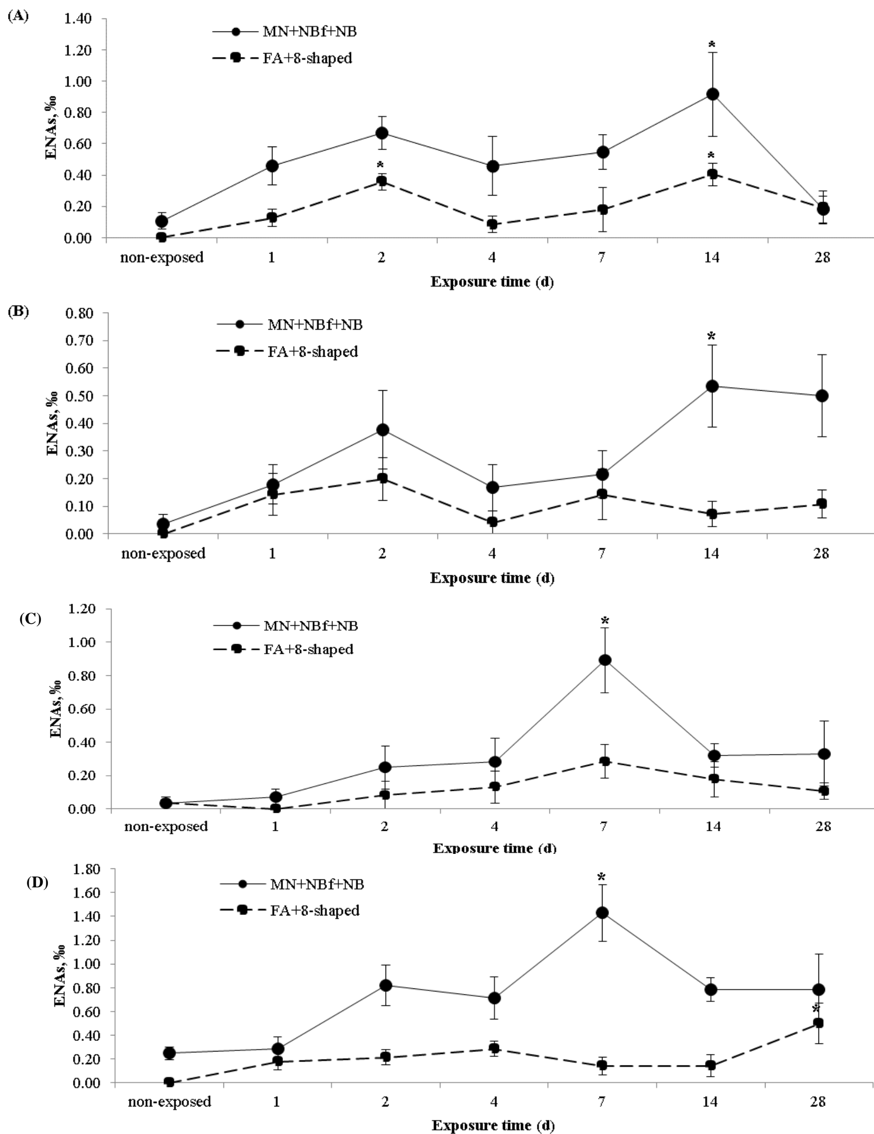


4 pav. Mikrobranduolių (MN) ir 8-formos (8-shaped) branduolių dažniai *O. mykiss* periferinio kraujo, kepenų ir inkstų eritrocituose po poveikio skirtingomis Zn ir Cu mišinio koncentracijomis bei atsistatymo procese. Vidurkis ± SE; skirtumai nuo kontrolės: * $p < 0.05$, ** $p < 0.001$; skirtumai nuo poveikio grupės ir atsistatymo grupės: a $p < 0.05$, b $p < 0.001$, c $p < 0.0001$. Exp 4d – 4 parų poveikis; R – 4, 8 ir 12 parų atsistatymo laikas; Contr – kontrolė. Publikacija I.

Eritrocitų branduolio pažaidų indukcija laike, įvairiuose audiniuose bei skirtingų koncentracijų poveikyje

Šio darbo eksperimentiniai tyrimai atskleidė, kad didžiausios leistinos metalų koncentracijos (Zn – 0,1; Cu – 0,01; Ni – 0,01; Cr – 0,01; Pb – 0,005 ir Cd – 0,005 mg/L) daugianariuose mišiniuose sukelia genotoksiškumą ir citotoksiškumą *Salmo salar*, *Rutilus rutilus* ir *O. mykiss* skirtingų audinių eritrocituose. Eksperimentiniais tyrimais buvo siekiama nustatyti nuo poveikio trukmės priklausomą branduolio pažaidų indukciją eritrocituose 28 paras paveikus *S.salar* daugianariu metalų mišiniu esant DLK. Tyrimo

rezultatai atskleidė netiesinį geno- ir citotoksiškumo atsakų nuo poveikio trukmės ryšį. Didžiausias reikšmingas genotoksiškumo lygis periferinio kraujo ir kepenų eritrocituose nustatytas 7-tą poveikio parą, tuo tarpu žiaunų ir inkstų eritrocituose – 14-tą poveikio parą (5 pav.). Reikšminga citotoksiškumo indukcija nustatyta 2-ą ir 14-tą poveikio parą žiaunų eritrocituose ir 28-tą poveikio parą *S. salar* periferinio kraujo eritrocituose (5 pav.). Svarbu pažymėti, kad šio tyrimo metu buvo nustatyti geno- ir citotoksinių pažaidų dažniai *S. salar* ląstelėse, kurie leidžia apibūdinti dirbtinai veisiamų žuvų citogenetinę būklę ir tinkamumą populiacijų skaitlingumo atstatymui vandens telkiniuose. Tyrimo rezultatai parodė labai žemus citotoksiškumo (0,00–0,07‰) ir genotoksiškumo (0,04–0,50‰) lygius kontrolinės grupės lašišų eritrocituose (5 pav.).

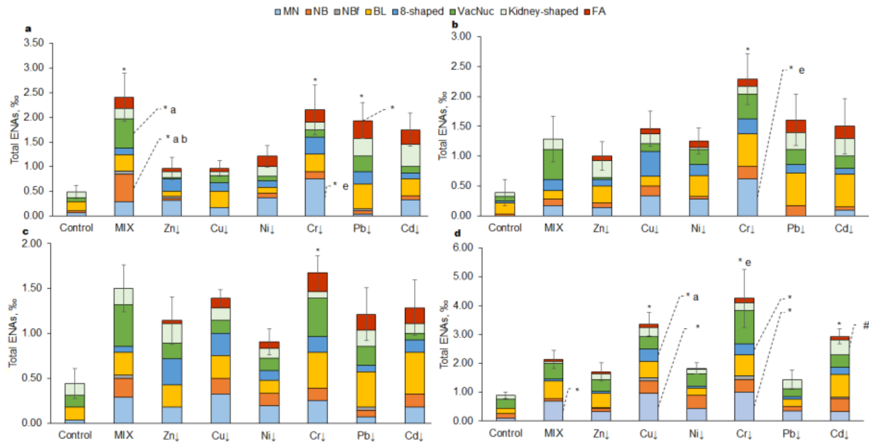


5 pav. *S. salar* eritrocitų branduolio pažaidų dažnių (bendras genotoksiškumas (MN +NBf + NB) ir citotoksiškumas (FA + 8-shaped)) kitimas laike po poveikio daugianariu metalų (Zn – 0,1; Cu – 0,01; Ni – 0,01; Cr – 0,01; Pb – 0,005 ir Cd – 0,005 mg/L mg/L) mišiniu: (A) žiaunų, (B) inkstų, (C) kepenų ir (D) periferinio kraujo eritrocituose (mean ± SE, n = 7). * žymi reikšmingus skirtumus nuo kontrolės (non-exposed) ($p < 0.05$). Publikacija II.

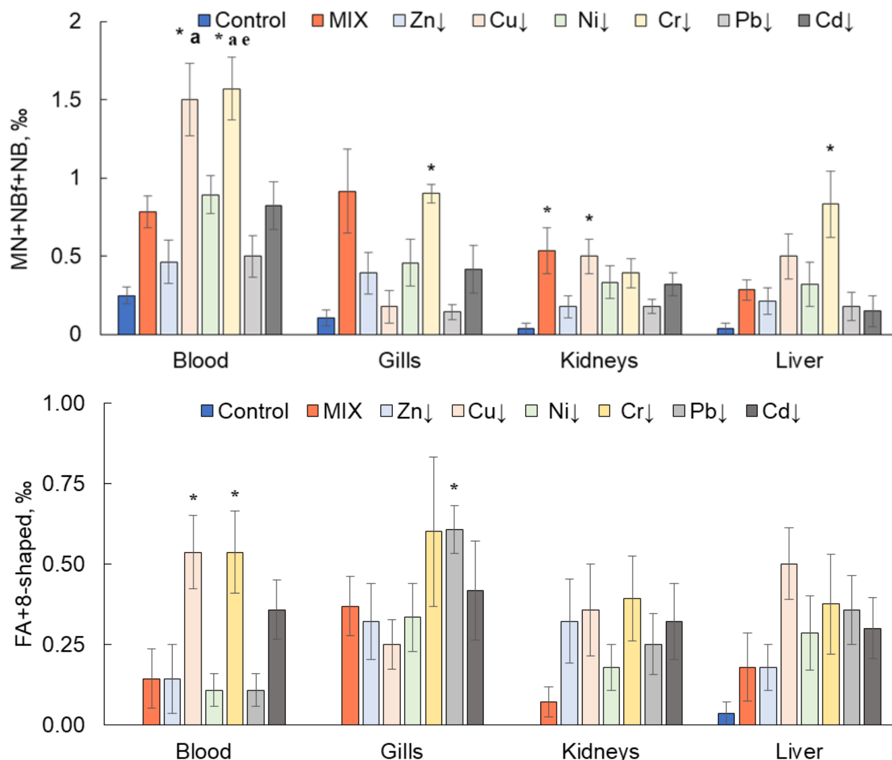
Remiantis mokslinės literatūros analize, pavienės daugianariame metalų mišinyje naudotos metalų koncentracijos taip pat gali sukelti geno- ir citotoksines pažaidas skirtingų žuvų rūšių audiniuose (Zhu et al., 2004; Cavas

et al., 2005; Palermo et al., 2015; Gasulla et al., 2016). Genotoksiniai ar citotoksiniai efektai po poveikio dvinariais ar trinariais metalų mišiniais, esant mažoms koncentracijoms, buvo nustatyti įvairiose žuvų rūšyse Zhu et al., 2004; Zhang et al., 2008; Güner, Muranlı 2011 atliktuose tyrimuose. Sinergetinis metalų (Cd + Cr, Cd +Cu ir Cd + Cr + Cu) mišinių mažų koncentracijų poveikis geno- ir citotoksiškumo indukcijai nustatytas karpio (*Cyprinus carpio*) eritrocituose (Zhu et al., 2004). DNR pažaidas *Misgurnus anguillicaudatus* žuvyse po poveikio Cd + Pb, Cd + Zn, Pb + Zn ir Cd + Pb + Zn mišiniais (taikyta 0,5 mg/L kiekvieno iš metalų koncentracija) nustatė Zhang su kitais bendraautoriais (2008). Bendras branduolio pažaidų dažnio padidėjimas nustatytas žuvyse paveiktose 0,1 ppm Cu + Cd mišiniu (Güner, Muranlı 2011). Apžvelgus esamus genotoksinių ir citotoksinių pažaidų dažnių žuvų audiniuose kitimo poveikio laike tyrimus, kaip ir šiame darbe nustatyti pažaidų dažnio svyravimo laike ypatumui. Didžiausias mikrobranduolių dažnis karpio eritrocituose, po poveikio Cr (0,001 mg/L), Cd + Cr (0,001 + 0,001 mg/L) ir Cd (0,001 mg/L), Cd + Cr + Cu (0,001 + 0,001 + 0,01 mg/L), nustatytas 4-tą ir 9-tą poveikio parą, atitinkamai (Zhu et al., 2004). Zhang su bendraautoriais (2008) nustatė DNR pažaidų variaciją laike *M. anguillicaudatus* žuvyse metalo mišinių poveikyje. DNR pažaidos reikšmingai didėjo iki 7-tos poveikio paros, 7–14-tą paromis pažaidų dažnis didėjo vidutiniškai, o žymus jų sumažėjimas nustatytas 14–21-ą poveikio parą.

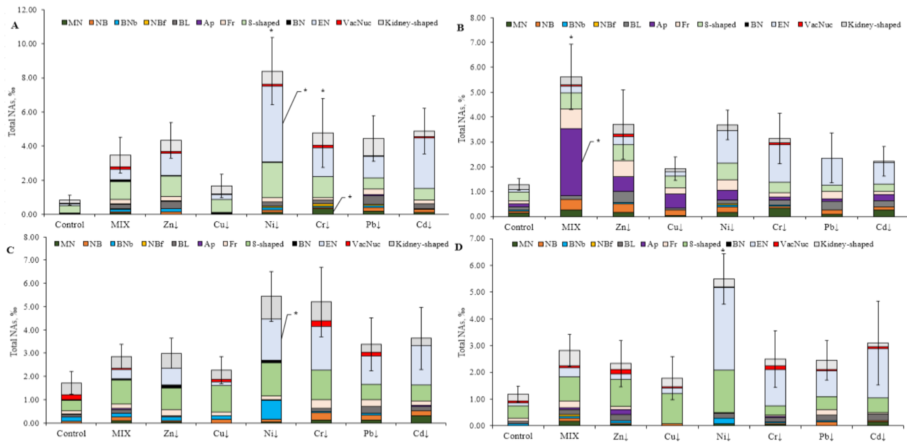
Vieno iš metalų daugianariame mišinyje koncentracijos sumažinimo įtaka genotoksinių ir citotoksinių efektų pokyčiams ir jų toksikologiniam reikšmingumui nėra tirta. Disertacijos eksperimentiniuose tyrimuose taikytas vieno iš metalo koncentracijos mišinyje sumažinimas 10 kartų atspindi galimą foninį/aplinkos lygį vandens ekosistemose. Šio darbo eksperimentinių tyrimų metu, buvo tirta 10 kartų vieno iš DLK mišinyje esančių metalų sumažinimo įtaka geno- ir citotoksiškumo atsakams *S. salar* ir *R. rutilus* skirtingų audinių eritrocituose 14-os parų poveikyje. Eksperimentinių tyrimų rezultatai parodė, kad net mažos (DLK) metalo koncentracijos mišinyje sumažinimas reikšmingai paveikė genotoksinių ir citotoksinių pažaidų dažnius *S. salar* ir *R. rutilus* eritrocituose (6–9 pav.). Nebūtinųjų ir būtinųjų metalų mišiniai, mažinant vieno iš metalo koncentraciją 10 kartų, lėmė skirtingą geno- ir citotoksiškumo laipsnį, kuris galimai yra sąlygotas metalų sąveikos tiek už organizmo ribų, tiek pačiame organizme. Reikšmingi geno- ar citotoksiškumo atsakai *S. salar* eritrocituose buvo nustatyti po poveikio Cr↓ (10 kartų sumažinta Cr^{6+} DLK daugianariame mišinyje), MIX, Cu↓ ar Pb↓ mišiniais (6–7 pav.). Tuo tarpu reikšmingi citogenetiniai pažeidimai *R. rutilus* eritrocituose aptikti po poveikio Cr↓, Ni↓ ir MIX mišiniais (8 pav.). Kometų analizė parodė, kad Cr↓, Pb↓ ir Zn↓ mišinių poveikis sukėlė reikšmingus DNR grandinės trūkius *R. rutilus* eritrocituose (9 pav.).



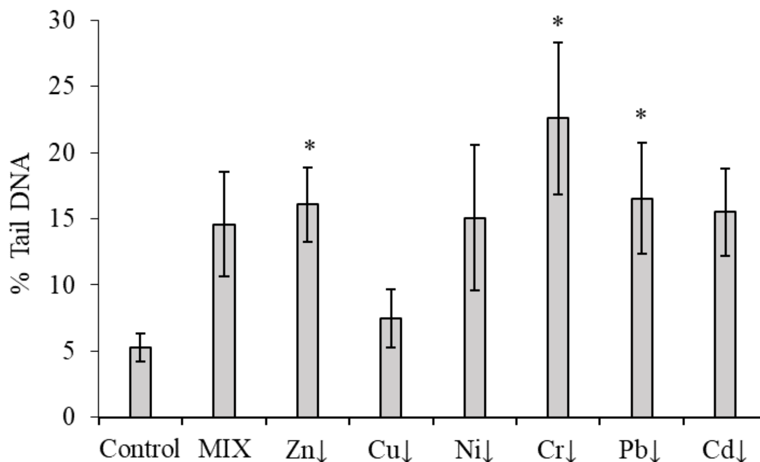
6 pav. Bendras eritrocitų branduolio pažaidų lygis *S. salar*: (a) žiaunų, (b) kepenų, (c) inkstų ir (d) periferinio kraujo eritrocituose (vidurkis ± SE, n = 7). * žymi reikšmingus skirtumus nuo kontrolinės grupės, # – nuo MIX, a – nuo Zn↓; b – nuo Cu↓; c – nuo Ni↓; d – nuo Cr↓; e – nuo Pb↓; f – nuo Cd↓ ($p < 0,05$). Publikacija IV.



7 pav. Bendras genotoksiškumo (MN+NBf+NB) ir citotoksiškumo (FA+8-shaped) lygiai *S. salar* eritrocituose (vidurkis ± SE, n = 7). * žymi reikšmingus skirtumus nuo kontrolinės grupės, # – nuo MIX, a – nuo Zn↓; b – nuo Cu↓; c – nuo Ni↓; d – nuo Cr↓; e – nuo Pb↓; f – nuo Cd↓ ($p < 0.05$). Publikacija IV.



8 pav. Eritrocitų branduolio pažaidų lygis *R. rutilus* (A) periferinio kraujo, (B) žiaunų, (C) kepenų ir (D) inkstų eritrocituose (kontrolinėje ir daugianariais metalų mišiniais paveiktose grupėse, vidurkis±SE, n = 7). * žymi reikšmingus skirtumus nuo kontrolės ($p < 0,05$). Publikacija XIII.



9 pav. DNR pažeidimai (procentas DNR kometos uodegoje) *R. rutilus* eritrocituose, kontrolinėje ir daugianariais metalų mišiniais paveiktose grupėse (vidurkis ± SD, n = 7). * žymi reikšmingus skirtumus nuo kontrolinės grupės ($p < 0,05$). Publikacija XIII.

Nustatyti daugianarių metalų mišinių geno- ir citotoksinio poveikio skirtumai, gali būti iš dalies aiškinami hormezės efekto egzistavimu. Mažos Cr koncentracijos gali sąlygoti naudingą organizmui stimuliuojantį efektą. Perez-Benito (2006) nustatė hormetinį mažos Cr (VI) koncentracijos efektą, kuris pasireiškia pailgėjusia žuvų gyvenimo trukme. Antioksidantiniai metalų (Cr, Pb ir Cd) mišinio, aplinkoje egzistuojančiomis koncentracijomis, efektai nustatyti *Cyprinus carpio* audiniuose, dėl reikšmingai pakitusių antioksidantinių fermentų (SOD ir CAT) lygio (Rajeshkumar et al., 2017). Geno- ir citotoksinių pažaidų variacija po poveikio daugianariais metalų

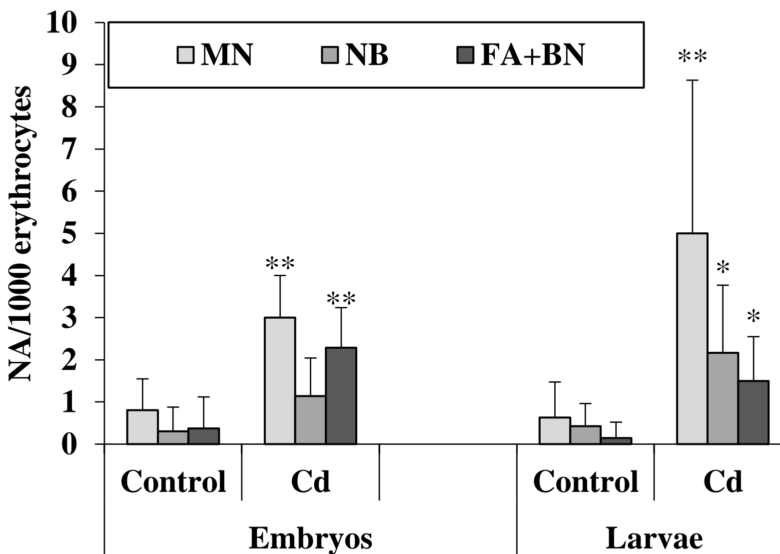
mišiniais su 10 kartų sumažinta vieno iš metalų koncentracija gali būti aiškinama fermentinės antioksidantinės sistemos suaktyvėjimu, o tai lemia apsaugą nuo oksidacinių pažeidimų. Tačiau, kad patvirtinti hormezės reiškinį cheminių medžiagų mišiniuose, reikalingi išsamesni tyrimai. Gauti rezultatai gali būti aiškinami ir tuo, kad kiekvienas metalas dalyvaudamas metabolizmo procesuose gali sukelti specifinius pažeidimus biologinėse sistemose (Wah Chu, Chow 2002). Kitų autorių tyrimai atskleidė, kad metalai su didesniu kovaleniniu indeksu, kaip Pb ir Cu, sukelia sinergetinius efektus, tuo tarpu metalai (Cd, Ni ir Zn), kuriems būdingas žemesnis kovalentinis indeksas, pasižymi kintamu poveikiu (Wah Chu, Chow 2002). Taip pat metalai su mažu kovalentiniu indeksu, kaip Zn, pasižymi neutralizuojančiu efektu kitų metalų sukeliamam toksiškumui, pvz., Cd ir Ni (Wah Chu, Chow 2002). Cobbina su bendraautoriais (2015) nustatytė, kad metalo mišiniai, esant mažoms koncentracijoms, paveikia homeostatinį toksinių ir biologiškai svarbių metalų reguliaciją.

Įvertinus metalų mišinių geno- ir citotoksinį poveikį skirtinguose žuvų audiniuose, nustatyta, kad dažniausiai periferinio kraujo eritrocituose buvo aptinkami didžiausi geno- ir citotoksiškumo lygiai. Atsistatymo periodo metu, *O. mykiss* periferinio kraujo eritrocitai pasižymėjo greitesniu išsivalymu bei mažesne citogenetinių pažaidų dažnio variacija, lyginant su kepenų ir inkstų eritrocitais. Aukšti citogenetinių pažaidų dažniai periferinio kraujo eritrocituose gali būti paaiškinami tuo, kad į periferinį kraują patenka eritrocitai iš kelių hematopoetinių organų. Kaulinėse žuvyse pagrindinę eritropoezės funkciją atlieka priekinė inkstų dalis (Soldatov 2005). Tuo tarpu antriniai hematopoetiniai organai, kaip kepenys ar žiaunos gali pasižymėti skirtingu ksenobiotikams jautrumu (Agius, Roberts 2003; Macchi et al., 1992). Cavas su bedraautoriais (2005) rekomendavo, branduolio pažaidų analizę atlikti skirtinguose žuvų audiniuose. Specifinį audinių atsaką gali sąlygoti cheminių medžiagų polinkis akumuliuotis tam tikruose audiniuose, skirtingas audinių jautrumas ir juose esantys apsauginiai mechanizmai (Ahmad et al., 2006; Velma, Tchounwou 2010). Kadangi kaulinėse žuvyse inkstai yra pagrindinis eritropoezės organas, ląstelių dalijimasis šiame audinyje yra intensyviausias (Baršienė et al., 2006; Velma, Tchounwou 2010). Žuvų inkstai ir kepenys atlieka detoksikacijos funkciją toksinų ekskrecijai indukuodami metalotioneinų sintezę (Roesijadi, Robinson 1994). Metalų šalinimas žuvyse gali vykti per blužnį, šlapimą, žiaunas ir gleives (Varanasi, Markey 1978; Kim et al., 2004).

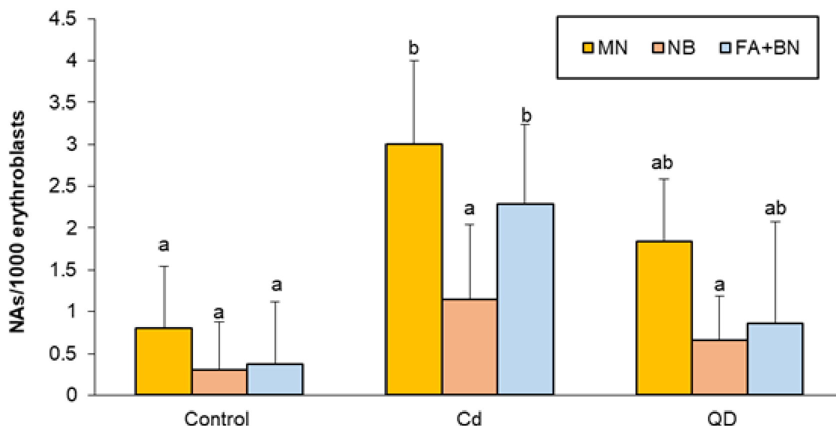
Kvantinių taškų geno- ir citotoksinis poveikis ankstyvojo žuvų vystymosi stadijose

Disertacijos tyrimų metu, nustatytas 4 nM koncentracijos polietilenglikolio dangalą turinčių karboksilintų CdSe/ZnS kvantinių taškų (KT) geno- ir citotoksinis poveikis *O. mykiss* embrionuose ir lervose. Eksperimentiniai Cd (2μg/L) citogenetinio poveikio *O. mykiss* akstyvojo vystimosi stadijose

tyrimai buvo atlikti siekiant palyginti efektus su KT sukeliamais geno- ir citotoksiniais efektais, galimai atsirandančiais dėl Cd jonų atsipalaidavimo subyrėjus KT struktūrai. Nustatytas skirtingas Cd ir KT geno- ir citotoksinis poveikis *O. mykiss* eritroblastuose. Cd poveikis sukėlė reikšmingus geno-, citotoksinius ar DNR grandinės trūkius (Kometų metodas) *O. mykiss* embrionų bei lervų eritroblastuose (10–12 pav.). Šio tyrimo rezultatus patvirtina ankstesni tyrimai, kurių metu buvo nustatytas Cd geno- ir citotoksinis potencialas įvairiose žuvų rūšyse. Po poveikio Cd žuvų audiniuose buvo aptikta mikrobranduolių ir dvibranduolių ląstelių indukcija (Cavas et al., 2005; Jindal, Verma 2015). $CdCl_2$ (0,37 ir 0,62 mg/L) indukuotas mikrobranduolių ir kitų branduolio pažaidų formavimasis nustatytas *Labeo rohita* periferinio kraujo eritrocituose (Jindal, Verma 2015). Cd sukelta apoptozė nustatyta *O. mykiss* hepatocituose (Risso-de Faverneyet et al., 2001). Hsu su bedraautoriais (2013) nustatė, kad subletalios Cd koncentracijos sukelia oksidacinį stresą zebražuvės (*Danio rerio*) embrionuose. Oksidacinis stresas ir DNR reparacijos inhibicija yra pagrindiniai mechanizmai sąlygojantys Cd genotoksiškumą (Hsu et al., 2013).

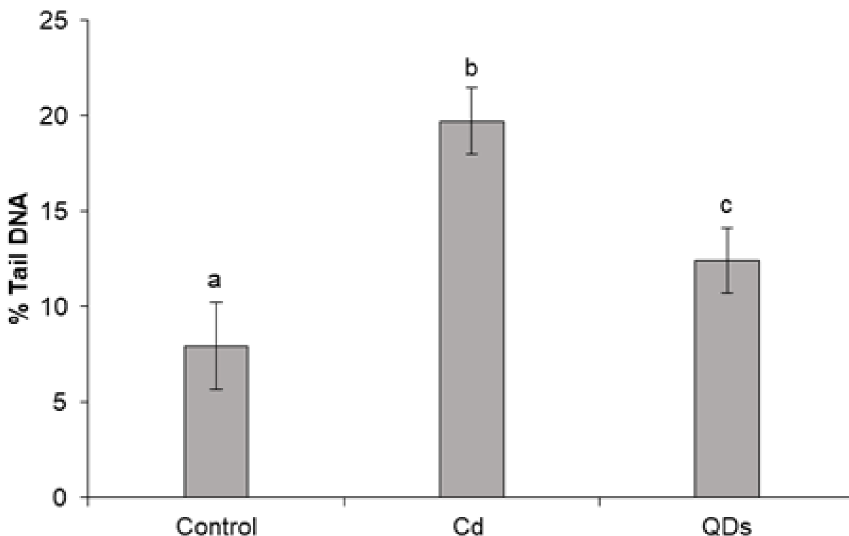


10 pav. Mikrobranduolių (MN), branduolio pumpurų (NB) ir fragmentuotų-apoptozinių (FA) + dvibranduolių (BN) eritroblastų dažnis *O. mykiss* embrionuose ir lervose po 4 parų poveikio 2µg/L Cd koncentracija. Vidurkis± SE, reikšmingi skirtumai nuo kontrolės: *$p < 0,05$; **$p < 0,005$. Publikacija X.

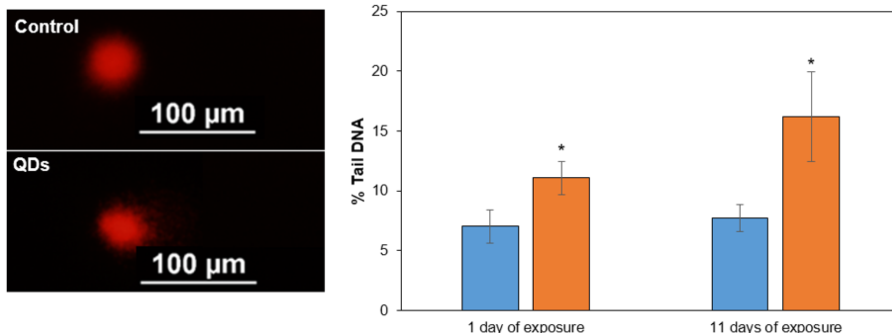


11 pav. Mikrobranduolių (MN), branduolio pumpurų (NB) ir bendras citotoksiškumo lygis (FA + BN) *O. mykiss* embrionų eritroblastuose po 4 parų poveikio 2µg/L Cd ir 4 nM KT koncentracija. Vidurkis ± SD, n = 7. Raidės žymi reikšmingus skirtumus tarp grupių ($p < 0.05$). Publikacija V.

Šio darbo metu nustatyta, kad Kometų metodas sėkmingai aptinka DNR pažeidimus *O. mykiss* emrionų ir lervų eritroblastuose po poveikio KT (12 ir 13 pav.). Tuo tarpu, taikant branduolio pažaidų analizę reikšmingų geno- ir citotoksinių pažaidų indukcijos KT poveikyje nenustatyta (11 pav.).



12 pav. DNR procentas (%) kometos uodegoje po 4 parų ekspozicijos 2µg/L Cd ir 4 nM KT koncentracijoje *O. mykiss* embrionų eritroblastuose. Vidurkis ± SD, n = 7, raidės žymi reikšmingus skirtumus tarp grupių ($p < 0.05$). Publikacija V.



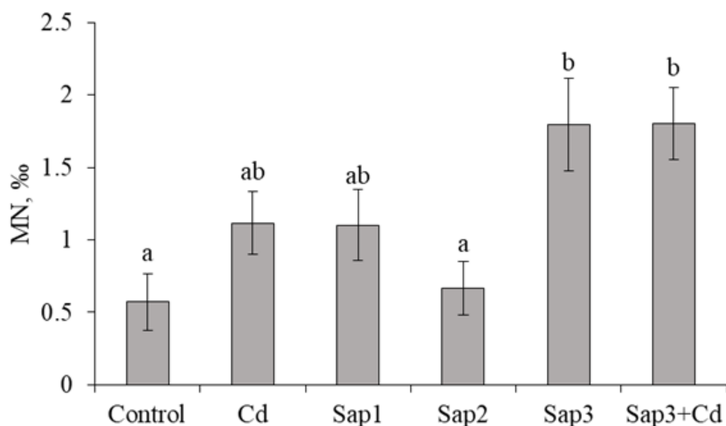
13 pav. Procentas DNR kometos uodegoje (% Tail DNA, vidurkis ± SD, n = 7) po 1 paros poveikio *O. mykiss* embrionuose ir vienadienėse lervose eksperimento pabaigoje (po 11 parų inkubacijos 4 nM KT). Publikacija III.

Klastogeninės/aneugeninės KT sąvybės buvo pabrėžtos ankstesniuose tyrimuose (Aye et al., 2013; Galeone et al., 2012). Galeone su bendraautoriais (2012) pasiūlė CdSe/ZnS KT klasifikuoti kaip reikšmingai toksiškus *in vivo*. KT sukelti DNR pažeidimai yra susiję su reaktyvių deguonies formų generavimu. Singh ir kiti (2009) nustatė, kad nanodalelių indukuotas oksidacinis stresas yra laikomas pagrindiniu mechanizmu lemiančiu jų genotoksiškumą. Ankstesnių tyrimų metu nustatyta, kad polietilenglikolio (PEG) dangalas gali apsaugoti nuo Cd^{2+} jonų išsiskyrimo iš KT (Galeone et al., 2012; Zhang et al., 2006). Galeone su bendraautoriais (2012) bei Ju su bendraautoriais (2013) nustatė, kad PEG dangalas sumažina, bet nepašalina KT toksiškumo. Saez ir kiti (2015) tyrimuose atskleidė, kad genotoksiškumo potencialas ir Cd reaktyvumas su viduląsteliniais komponentais yra sąlygojamas jo nano arba joninės formos. Daugiašerėse žieduotosiose kirmėlėse *Hediste diversicolor* Cd nano formoje pasižymi stipresniu genotoksiniu aktyvumu nei joninė jo forma. Nors Aye su bendraautoriais (2013) gauti rezultatai parodė, kad KT indukuoja reaktyvias deguonies formas, mutageninės/klastogeninės KT sąvybės nėra pilnai paaiškinamos. KT būdingos fiziko-cheminės sąvybės gali sąlygoti įvairias reakcijas, kurios iššaukia įvairius genotoksiškumo/mutageniškumo efektus organizmų ląstelėse. Kiti galimi KT genotoksiškumo mechanizmai: uždegiminės reakcijos, tiesioginės sąveikos su DNR ar branduolio baltymais (Brunetti et al., 2013). Šio tyrimo metu, straipsnio bendraautoriai nustatė KT agregaciją ant *O. mykiss* ikrų paviršiaus (Rotomskis et al., 2018). KT agregacija gali sutrikdyti dujų apykaitą embriono viduje ir lemti oksidacinio streso sukeltus genotoksinius pažeidimus. Hipoksijos indukuota reaktyvios deguonies formų generacija gali būti fiziologinis organizmo atsakas į deguonies nepakankamumą. Tačiau, hipoksijos sąlygota reaktyvių deguonies formų indukcija yra mažai tirta. Keletas tyrimų atskleidė hipoksijos nulemtą genetinį nestabilumą įvairiuose organizmuose ir ląstelių kultūrose (Snyder, Diehl 2005; Kim et al., 2007). Buvo nustatyta, kad hipoksija gali veikti kaip genotoksinis agentas. Taip pat tyrimais yra įrodyta, kad hipoksija gali būti

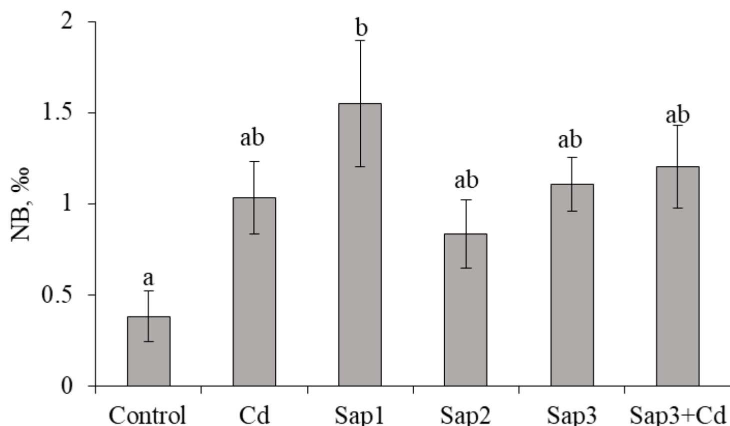
mutagenas, teratogenas, nes sutrikdo žuvų embrionų vystymąsi (Shang, Wu 2004; Shang et al., 2006). Hipoksija sukelia mirtingumą, išsigimimus, embrioninį žuvų (*D. rerio*) vystymosi, ritimosi sutrikimą, trikdo apoptotinius procesus, sukelia lytinių hormonų disbalansą ir daro įtaką lyčių santykiui (Shang, Wu, 2004; Shang et al., 2006). Oksidacinis stresas ir uždegiminiai procesai yra nurodomi, kaip pagrindiniai nanodalelių toksiškumą ir genotoksiškumą lemiantys mechanizmai (Magdolenova et al., 2014; Xiao et al., 2016).

S. parasitica bei šio patogeno kartu su Cd geno- ir citotoksinis poveikis žuvų eritrocituose

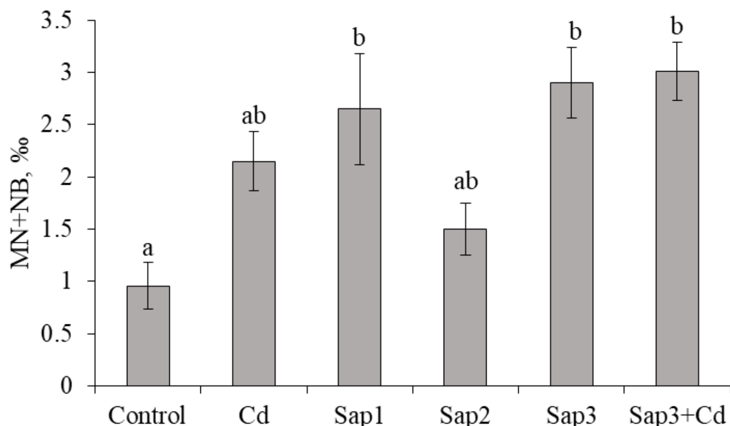
Genotoksinis ir citotoksinis oomicetų *Saprolegnia parasitica* infekcijos įvairiomis koncentracijomis (92000, 22400 ir 5500 colony-forming units per milliliter (cfu/mL)) potencialas ir poveikio paūmėjimas, esant vienalaikiam dviejų stresorių (*S. parasitica* (5500 cfu/mL) ir Cd (2µg/L)) poveikiui, buvo tiriamas *O. mykiss* lervose po 8-ių parų poveikio. Eksperimentinių tyrimų metu buvo nustatytas *S. parasitica* infekcijos genotoksinis poveikis *O. mykiss* lervų eritroblastuose. Tyrimų metu buvo nustatyta reikšminga mikrobranduolių (MN), branduolio pumpurų (BP) ir bendro genotoksiškumo (MN+NB) indukcija priklausomai nuo taikytos *S. parasitica* koncentracijos (14–16 pav.). Genotoksinių pažaidų dažnio didėjimas, didėjant *S. parasitica* koncentracijai, nenustatytas. Gauti rezultatai gali rodyti citogenetinių pažaidų slopinimą priklausomai nuo poveikio koncentracijos.



14 pav. Mikrobranduolių (MN) dažnis (vidurkis ± SE, n = 10) O. mykiss lervų eritroblastuose po poveikio 2µg/L Cd, *Saprolegnia parasitica* (Sap1 – 92000, Sap2 – 22400, Sap3 –5500 (cfu/mL)) ir veikiant kartu Cd ir *S. parasitica* (2 µg Cd/L + 5500 cfu/mL). Raidės žymi reikšmingus skirtumus tarp grupių ($p < 0.05$). Publikacija XIV.



15 pav. Branduolio pumpurų (NB) dažnis (vidurkis ± SE, n = 10) *O. mykiss* lervų eritroblastuose 2µg/L Cd, *Saprolegnia parasitica* (Sap1 – 92000, Sap2 – 22400, Sap3 –5500 (cfu/mL)) ir veikiant kartu Cd ir *S. parasitica* (2 µg Cd/L + 5500 cfu/mL). Raidės žymi reikšmingus skirtumus tarp grupių ($p < 0.05$). Publikacija XIV.



16 pav. Bendras genotoksiškumo (MN+NB) lygis (vidurkis ± SE, n = 10) *O. mykiss* lervų eritroblastuose 2µg/L Cd, *Saprolegnia parasitica* (Sap1 – 92000, Sap2 – 22400, Sap3 –5500 (cfu/mL)) ir veikiant kartu Cd ir *S. parasitica* (2 µg Cd/L + 5500 cfu/mL). Raidės žymi reikšmingus skirtumus tarp grupių ($p < 0.05$). Publikacija XIV.

Iki šiol nebuvo atlikta jokių *S. parasitica* genotoksiškumo ir citotoksiškumo indukcijos tyrimų. Azimzadeh ir Amniattalab (2017) nustatė oksidacinį stresą, hematologinius ir histopatologinius pakitimus O. mykiss individuose užkrėstuose *S. parasitica*. Nustatyta, kad tiriami oomicetai išskiria įvairias toksines medžiagas ir hidrolizinius fermentus (Soanes et al., 2007). Torto-Alalibo su bendraautoriais (2005) išskyrė keletą *S. parasitica* baltymų (CBD baltymai, CBEL tipo baltymai, glikozilo hidrolazės, proteazės, proteazių inhibitoriai) ir pabrėžė, kad šie baltymai gali turėti įvairų poveikį organizmų sveikatai. Nustatyta, kad *S. parasitica* infekcija gali sukelti stiprius

uždegiminius procesus žuvyse. Belmonte su bendraautoriais (2014) pateiktame tyrime nurodyta, kad *S. parasitica* produkuoja metabolitą prostaglandiną E2 (PGE2), kuris skatina uždegiminius procesus žuvyse. Marcogliese su bendraautoriais (2005) nustatė, kad parazitizmas kartu su chemine tarša gali dar labiau pabloginti oganizmo būklę, kadangi sutrikdomas šeimininko imuninės sistemos atsparumas. Taip pat kelių stresorių vienalaikis poveikis gali paskatinti toksiškumo efektų padidėjimą, net ir esant mažam parazitų intensyvumo laipsniui. Šio tyrimo rezultatai neatskleidė sinergetinės sąveikos. Didžiausias bendras genotoksiškumo lygis buvo nustatytas po poveikio *S. parasitica* kartu su Cd (16 pav.), tačiau šis padidėjimas nebuvo statistiškai reikšmingas. Tyrimų metu citotoksinio *S. parasitica* poveikio *O. mykiss* lervų eritroblastuose nenustatyta. Schaumburg su bedraautoriais (2006) nustatė, kad parazitai gali indukuoti anti-apoptotinį aktyvumą šeimininko ląstelėse.

Apibendrinant, šiame darbe pateikta nauja ir moksliškai svarbi informacija apie genotoksinių ir citotoksinių efektų tyrimų reikšmingumą bei sukeltus daugianarių metalų mišinių aplinkoje galimai egzistuojančiomis koncentracijomis, daugiakomponentinių nanodalelių (kvantinių taškų) bei biologinių stresorių (patogenų) genotoksinius ir citotoksinius efektus. Eksperimentinis žuvų atsistatymo, po metalų mišinio sukelto citogenetinio poveikio, vertinimas suteikė naujų žinių apie susiformavusių branduolio pažaidų dažnių svyravymus vykstančius organizmo atsistatymo metu bei audinių specifiškumą. Darbe taip pat pabrėžiamas galimas geno- ir citotoksiškumo efektų sustiprinimas esant vienalaikiam kelių stresorių poveikiui (patogenų infekcija ir cheminė tarša).

IŠVADOS

1. Daugianaris metalų mišinys, esant DLK, 7-tą poveikio parą sukelia didžiausią genotoksiškumo lygį *S. salar* periferinio kraujo ir kepenų eritrocituose, 14-tą parą – žiaunų ir inkstų eritrocituose. Didžiausias citotoksiškumo lygis nustatytas 2-ą ir 14-tą poveikio parą žiaunų eritrocituose, 28-tą parą – periferinio kraujo eritrocituose. Citotoksinio poveikio kepenų ir inkstų eritrocituose nenustatyta.

2. Cu ir Zn mišinys, priklausomai nuo taikytų koncentracijų, indukavo mikrobranduolių, branduolio pumpurų, ataugų ir 8-formos branduolio eritrocitų formavimąsi skirtinguose *O. mykiss* audiniuose. Reikšmingas periferinio kraujo eritrocitų, po didžiausios Cu-Zn (0,25%) mišinio koncentracijos sukelto geno- ir citotoksinio poveikio, atsistatymas nustatytas 8-tą ir 12-tą parą.

3. Atsistatymo metu kepenų ir inkstų eritrocitams ypač būdingi citogenetinių pažaidų dažnio svyravimai. Ketvirtą atsistatymo parą nustatytas reikšmingas citogenetinių pažaidų dažnio padidėjimas priklausomai nuo dvinario metalų (Cu-Zn) mišinio koncentracijos ir audinio. Aštuntą atsistatymo parą, priklausomai nuo poveikio koncentracijų, nustatytas reikšmingas citogenetinių pažaidų dažnio sumažėjimas kepenų ir inkstų eritrocituose, 12-tą atsistatymo parą reikšmingo (išskyrus 8-formos branduolių dažnį kepenyse 0,0625% koncentracijoje) sumažėjimo nestebima.

4. Daugianariai metalų mišiniai, esant DLK, sukelia genotoksinius ir citotoksinius efektus skirtingose žuvų rūšyse. Atskiro metalo DLK sumažinimas daugianariame mišinyje turi įtakos citogenetinių pažaidų lygiui skirtinguose žuvų audiniuose. Reikšminga citogenetinių pažaidų indukcija *S. salar* eritrocituose nustatyta po poveikio Cr↓ (10 kartų sumažinta Cr6+ koncentracija daugianariame mišinyje), MIX, Cu↓ ir Pb↓ mišiniais. Reikšminga citogenetinių pažaidų indukcija *R. rutilus* eritrocituose nustatyta po poveikio Ni↓, MIX ir Cr↓ mišiniais; reikšmingus DNR trūkius sukelia Cr↓, Pb↓ ir Zn↓ mišiniai.

5. Žuvų periferinio kraujo eritrocituose nustatomi didžiausi citogenetinių pažaidų lygiai po poveikio metalų mišiniais, taip pat periferinio kraujo eritrocitams būdingas greitesnis citogenetinių pažaidų dažnio sumažėjimas, lyginant su kitais audiniais.

6. Karboksilinti CdSe/ZnS kvantiniai taškai, esant 4nM koncentracijai, sukelia DNR grandinės trūkius *O. mykiss* embrionuose (po 1 ir 4 parų) ir vienadienėse lervose po 11 parų poveikio. Kadmio 2 μg/L koncentracija po 4 parų poveikio indukuoja reikšmingą mikrobranduolių dažnio, citotoksiškumo ir DNR trūkių padidėjimą *O. mykiss* embrionuose bei reikšmingą mikrobranduolių, branduolio pumpurų ir citotoksiškumo lygio padidėjimą lervose.

7. *Saprolegnia parasitica* infekcija (esant 5500 cfu/mL ir 92000 cfu/mL, atitinkamai) sukelia mikrobranduolių ir branduolio pumpurų *formavimąsi O.*

mykiss lervų eritroblastuose. Reikšmingą genotoksiškumo lygio padidėjimą žuvų ląstelėse sukėlė mažiausia (5500 cfu/mL) ir didžiausia (92000 cfu/mL) *S. parasitica* koncentracija.

8. Bendras *S. parasitica* (5500 cfu/mL) ir Cd poveikis sukėlė reikšmingą mikrobranduolių dažnio bei bendro genotoksiškumo lygio padidėjimą *O. mykiss* lervų eritroblastuose, tačiau reikšminga sinergetinė sąveika, lyginat su atskiru dviejų stresorių (*S. parasitica* ir Cd) poveikiu, nenustatyta.

REFERENCES

Agius C, Roberts RJ (2003) Melano-macrophage centres and their role in fish pathology. Journal of Fish Diseases 26: 499–509

Ahmad I, Maria VL, Oliveira M, Pacheco M, Santos MA (2006) Oxidative stressand genotoxic effects in gill and kidney of *Anguilla anguilla* L. exposed to chromium with or without pre-exposure to beta-naphthoflavone. Mutation Research 608(1): 16–28

Ali SE, Thoen E, Evensen Ø, Skaar I (2014) Boric acid inhibits germination and colonization of *Saprolegnia* spores in vitro and in vivo. PLoS One 9: e91878.

Aye M, Di Giorgio C, Berque-Bestel I, Aime A, Pichon BP, Jammes Y, Barthélémy P, De Méo M (2013) Genotoxic and mutagenic effects of lipid-coated CdSe/ZnS quantum dots. Mutation Research 750 (1–2): 129–138

Ayllón F, Garcia-Vazquez E (2001) Micronuclei and other nuclear lesions as genotoxicity indicators in rainbow trout *Oncorhynchus mykiss*. Ecotoxicology and Environmental Safety 49(3): 221–225

Azimzadeh K, Amniattalab A (2017) Total Sialic Acid, Oxidative Stress and Histopathological Changes in Rainbow Trout Saprolegniasis (*Oncorhynchus mykiss*). Kafkas Universitesi Veteriner Fakultesi Dergisi 23(1): 55–62

Bagdonas E, Vosylienė MZ (2006) A study of toxicity and genotoxicity of copper, zinc and their mixture to rainbow trout (*Oncorhynchus mykiss*). Biologija 1: 8–13

Baršienė J, Dedonytė V, Rybakovas A, Andreikenaitė L, Andersen OK (2006) Investigation of micronuclei and other nuclear abnormalities in peripheral blood and kidney of marine fish treated with crude oil. Aquatic Toxicology 78S: S99–S104

Baršienė J, Andreikėnaitė L, Garnaga G, Rybakovas A (2008) Genotoxic and cytotoxic effects in the bivalve mollusks *Macoma balthica* and *Mytilus edulis* from the Baltic Sea. Ekologija 54(1): 44–50

Baršienė J, Butrimavičienė L, Grygiel W, Lang T, Michailovas A, Jackūnas T (2014) Environmental genotoxicity and cytotoxicity in flounder (*Platichthys flesus*), herring (*Clupea harengus*) and Atlantic cod (*Gadus morhua*) from chemical munitions dumping zones in the southern Baltic Sea. Marine Environmental Research 96: 56–67

Baršienė J, Butrimavičienė L, Michailovas A, Grygiel W (2015) Assessing the environmental genotoxicity risk in the Baltic Sea: frequencies of nuclear buds in blood erythrocytes of three native fish species. Environmental Monitoring and Assessment 187(1): 4078

Belmonte R, Wang T, Duncan GJ, Mélida I, Bulone H, Van West P, Secombes CJ (2014) Role of Pathogen-Derived Cell Wall Carbohydrates and Prostaglandin E2 in Immune Response and Suppression of Fish Immunity by

the Oomycete *Saprolegnia parasitica*. Infection and Immunity 8 2(11): 4518–4529

Bolognesi C, Cirillo S (2014) Genotoxicity biomarkers in aquatic bioindicators. Current Zoology 60(2): 273–284

Bolognesi C, Hayashi M (2011) Micronucleus assay in aquatic animals. Mutagenesis 26(1): 205–213

Bonga W, Lock RAC (1992) Toxicants and osmoregulation in fish. Netherlands Journal of Zoology 42(2–3): 478–493

Brabrand A (1985) Food of Roach (*Rutilus-rutilus*) and Ide (*Leusiscus-idus*) –significance. Oecologia 66: 461–467

Brunetti V, Chibli H, Fiammengo R, Galeone A, Malvindi MA, Vecchio G, Cingolani R, Nadeau JL, Pompa PP (2013) InP/ZnS as a safer alternative to CdSe/ZnS core/shell quantum dots: in vitro and in vivo toxicity assessment. Nanoscale 5(1): 307–331

Calabrese EJ, Mattson MP (2011) Hormesis provides a generalized quantitative estimate of biological plasticity. Journal of Cell Communication and Signaling 5(1): 25–38

Cavas T, Ergene-Gozukara S (2005) Induction of micronuclei and nuclearabnormalities in Oreochromis niloticus following exposure to petroleumrefinery and chromium processing plant effluents. Aquatic Toxicology 74: 264–271

Cavas T, N.N. Garanko, V.V. Arkhipchuk (2005) Induction of micronuclei and binuclei in blood, gill and liver cells of fishes subchronically exposed to cadmium chloride and copper sulphate. Food and Chemical Toxicology 43: 569–574

Cedergreen N (2014) Quantifying synergy: a systematic review of mixture toxicitystudies within environmental toxicology. PLoS One 9(5): e96580

Celá P, Veselá B, Matalová E, Večeřa Z, Buchtová M (2014) Embryonic toxicity of nanoparticles. Cells Tissues Organs 199(1): 1–23

Chovanec A, Hofer R, Schiemer F (2003) Fish as bioindicators. In: Markert BA, Breure AM, Zechmeister HG (eds) Bioindicators and biomonitors: principles, concepts and applications. Elsevier, Amsterdam, 6: 639–675

Cobbina SJ, Chen Y, Zhou Z, Wu X, Feng W, Wang W, Mao G, Xu H, Zhang Z, Wu X, Yang L (2015) Low concentration toxic metal mixture interactions: Effects on essential and non-essential metals in brain, liver, and kidneys of mice on sub-chronic exposure. Chemosphere 132: 79–86

Dick MW (2001) Straminipilous Fungi: systematics of the peronosporomycetes, including accounts of the marine straminipilous protists, the plasmodiophorids, and similar organisms. Kluwer Academic Publishers, Dordrecht.

Demir E, Castranova V (2017) Evaluation of the Potential Genotoxicity of Quantum Dots. A Review. Aspects Nanotechnol 1(1): 1–19

EEA (2012) European waters — assessment of status and pressures. Report No 8/2012, ISSN 1725-9177

Evans RM, Martin OV, Faust M, Kortenkamp A (2016) Should the scope of human mixture risk assessment span legislative/regulatory silos for chemicals? Science of the Total Environment 543: 757–764

Fatima M, Usmani N, Hossain MM, Siddiqui MF, Zafeer MF, Firdaus F, Ahmad S (2014) Assessment of genotoxic induction and deterioration of fish quality in commercial species due to heavy-metal exposure in an urban reservoir. Archives of Environmental Contamination and Toxicology 67(2): 203–2013

Fenech M, Chang WP, Kirsch-Volders M, Holland N, Bonassi S, Zeiger E (2003) HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutation Research 534: 65–75

Galeone A, Vecchio G, Malvindi MA, Brunetti V, Cingolani R, Pompa PP (2012) In vivo assessment of CdSe-ZnS quantum dots: coating dependent bioaccumulation and genotoxicity. Nanoscale 4: 6401–6407

Gasulla J, Picco SJ, Carriquiriborde P, Dulout FN, Ronco AE, de Luca JC (2016) Genotoxic Effects Induced by Cd($^{+2}$), Cr($^{+6}$), Cu($^{+2}$) in the Gill and Liver of *Odontesthes bonariensis* (Piscies, Atherinopsidae). Bulletin of Environmental Contamination and Toxicology 96(5): 591–595

Gomes JMM, Ribeiro HJ, Procópio MS, Alvarenga BM, Castro ACS, Dutra WO, et al. (2015) What the erythrocytic nuclear alteration frequencies could tell us about genotoxicity and macrophage iron storage? PLoS One 10(11): e0143029

Góral M, Szefer P, Ciesielski T, Warzocha J (2009) Distribution and relationships of trace metals in the isopod *Saduria entomon* and adjacent bottom sediments in the southern Baltic. Journal of Environmental Monitoring 11(10): 1875–82

Grisolia CK, Cordeiro CMT (2000) Variability in micronucleus induction with different mutagens applied to several species of fish. Genetics and Molecular Biology 23 (1): 235–239

Güner U, Muranlı FDG (2011) Micronucleus test, nuclear abnormalities and accumulation of Cu and Cd on *Gambusia affinis* (Baird & Girard, 1853). Turkish Journal of Fisheries and Aquatic Sciences 11: 615–622

Harabawy ASA, Mosleh YYI (2014) The role of vitamins A, C E and selenium asantioxidants against genotoxicity and cytotoxicity of cadmium, copper, leadand zinc on erythrocytes of Nile tilapia, *Oreochromis niloticus*. Ecotoxicology and Environmental Safety 104 (2014) 28–35

Heddle JA, Cimino MC, Hayashi M, Romagna F, Shelby MD, Tucker JD, Vanparys P, MacGregor JT (1991) Micronuclei as an index of cytogenetic damage: past, present, and future. Environmental and Molecular Mutagenesis 18(4): 277–291

HELCOM (2011) Salmon and Sea Trout Populations and Rivers in the Baltic Sea – HELCOM assessment of salmon (*Salmo salar*) and sea trout (Salmo trutta) populations and habitats in rivers flowing to the Baltic Sea. Baltic Sea Environment Proceedings No. 126A

Hendry K, Cragg-Hine D (2003) Ecology of the Atlantic Salmon. Conserving Natura 2000 Rivers Ecology Series No. 7. English Nature, Peterborough

Heys KA, Shore RF, Pereira MG, Jones KC, Martin FL (2016) Risk assessment of environmental mixture effects. RSC Advances 6: 47844–47857

Hsu T, Huang KM, Tsai HT, Sung ST, Ho TN (2013) Cadmium (Cd)-induced oxidative stress down-regulates the gene expression of DNA mismatch recognition proteins MutS homolog 2 (MSH2) and MSH6 in zebrafish (*Danio rerio*) embryos. Aquatic Toxicology 126: 9–16

Hussein MA, Hatai K, Nomura T (2001) Saprolegniasis in salmonids and their eggs in Japan. Journal of Wildlife Diseases 37(1): 204–207.

Javed M, Usmani N (2017) An overview of the adverse effects of heavy metal contamination on fish health. The National Academy of Sciences Proc. doi: 10.1007/s40011-017-0875-7

Jindal R, Verma S (2015) In vivo genotoxicity and cytotoxicity assessment of cadmium chloride in peripheral erythrocytes of *Labeo rohita* (Hamilton). Ecotoxicology and Environmental Safety 118: 1–10

Ju L, Zhang G, Zhang C, Sun L, Jiang Y, Yan C, Duerksen-Hughes PJ, Zhang X, Zhu X, Chen FF, Jun Y (2013) Quantum dot-related genotoxicity perturbation can be attenuated by PEG encapsulation. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 753(1): 54–64

Karlsson HL, Di Bucchianico S, Collins AR, Dusinska M (2015) Can the comet assay be used reliably to detect nanoparticle-induced genotoxicity? Environmental and Molecular Mutagenesis 56: 82–96

Khan MS, Qureshi NA, Jabeen F (2017) Assessment of toxicity in fresh water fish *Labeo rohita* treated with silver nanoparticles. Applied Nanoscience 7:167–179

Khan I, Saeed K, Khan I (2017) Nanoparticles: Properties, applications and toxicities. Arabian Journal of Chemistry (In Press)

Kim BM, Choi JY, Kim YJ, Woo HD, Chung HW (2007) Reoxygenation following hypoxia activates DNA-damage checkpoint signaling pathways that suppress cellcycle progression in cultured human lymphocytes. FEBS Letters 581(16): 3005–3012

Kim SG, Jee JH, Kang JC (2004) Cadmium accumulation and elimination in tissues of juvenile olive flounder, *Paralichthys olivaceus* after sub-chronic cadmium exposure. Environmental Pollution 127: 117–123

Klaassen C, Casarett LJ, Doull J (2013) Chapter 23: Toxic Effects of Metals. In: Klaassen CD Casarett and Doull’s toxicology: the basic science of poisons 8th ed. McGraw-Hill Education, New York, NY

Kroon F, Streten C, Harries S (2017) A protocol for identifying suitable biomarkers to assess fish health: A systematic review. PLoS One 12(4): e0174762.

Kulmala S, Haapasaari P, Karjalainen TP, Kuikka S, Pakarinenm T, Parkkila K, Romakkaniemi A et al. (2012) Ecosystem services provided by the Baltic salmon – a regional perspective to the socio-economic benefits associated with a keystone species, In Socio-economic Importance of Ecosystem Services in the Nordic Countries – Synthesis in the Context of The Economics of Ecosystems and Biodiversity (TEEB), Copenhagen Nordic Council of Ministers 266–276

Liu XJ, Luo Z, Xiong BX, Liu X, Zhao YH, Hu GF, Lv GJ (2010) Effect of waterborne copper exposure on growth, hepatic enzymatic activities and histology in *Synechogobius hasta*. Ecotoxicology and Environmental Safety 73: 1286–1291

Łuczyńska J, Paszczyk B, Łuczyński MJ (2018) Fish as a bioindicator of heavy metals pollution in aquatic ecosystem of Pluszne Lake, Poland, and risk assessment for consumer's health. Ecotoxicology and Environmental Safety 153: 60–67

Macchi GJ, Romanol A, Christiansen HE (1992) Melano-macrophage centres in white mouth croaker *Micropogonias furneri*, as biological indicators of environmental changes. Journal of Fish Biology 40: 971–973

Magdolenova Z, Collins A, Kumar A, Dhawan A, Stone V, Dusinska M (2014) Mechanisms of genotoxicity. A review of in vitro and in vivo studies with engineered nanoparticles. Nanotoxicology 8(3): 233–278

Mahaye N, Thwala M, Cowan DA, Musee N (2017) Genotoxicity of metal based engineered nanoparticles in aquatic organisms: A review. Mutation Research 773: 134–160

Marcogliese DJ, Brambilla LG, Gagné F, Gendron AD (2005) Joint effects of parasitism and pollution on oxidative stress biomarkers in yellow perch *Perca flavescens*. Diseases of Aquatic Organisms 63(1): 77–84

Markovskaja S (2006) *Saprolegniaceae* (*Peronosporomycetes*) in Lithuania. II. The genus Saprolegnia. Botanica Lithuanica 12(2): 97–112.

Munari M, Sturve J, Frenzilli G, Sanders MB, Brunelli A, Marcomini A, Nigro M, Lyons BP (2014) Genotoxic effects of CdS quantum dots and Ag2S nanoparticles in fish cell lines (RTG-2). Mutation Research/Genetic Toxicology and Environmental Mutagenesis 16(775–776): 89–93

Nepomuceno JC, Ferrari I, Spano MA, Centeno AC (1997) Detection of micronuclei in peripheral erythrocytes of *Cyprinus carpio* exposed to metallicmercury. Environmental and Molecular Mutagenesis 30: 293–297

Nys C, Versieren L, Cordery KI, Blust R, Smolders E, De Schamphelaere KAC (2017) Systematic evaluation of chronic metal-mixture toxicity to three species and implications for risk assessment. Environmental Science & Technology 51(8): 4615–4623

Obiakor MO, Ezeonyejiaku CD (2015) Copper–zinc coergisms and metal toxicity atpredefined ratio concentrations: predictions based on synergistic ratio model. Ecotoxicology and Environmental Safety 117: 149–154

Obiakor MO, Okonkwo JC, Ezeonyejiaku CD, Ezenwelu CO (2010) Genotoxicology: single and joint action of copper and zinc to *Synodontis clarias* and *Tilapia nilotica*. Journal of Applied Sciences and Environmental Management 14(3): 59–64

Omar WA, Zaghloul KH, Abdel-Khalek AA, Abo-Hega S (2012) Genotoxic effectsof metal pollution in two fish species *Oreochromis niloticus* and *Mugil cephalus*, from highly degraded aquatic habitats. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 746(1): 7–14

Palermo FF, Risso WE, Simonato JD, Martinez CBR (2015) Bioaccumulation of nickel and its biochemical and genotoxic effects on juveniles of the neotropical fish Prochilodus lineatus. Ecotoxicology and Environmental Safety 116:19–28

Perez-Benito JF (2006) Effects of chromium (VI) and vanadium (V) on the lifespan of fish. Journal of Trace Elements in Medicine and Biology 20(3): 161–170

Persson L (1983) Food-consumption and the significance of detritus and algae to intraspecific competition in Roach *Rutilus rutilus* in a Shallow Eutrophic Lake. Oikos 41: 118–125

Phillips AJ, Anderson VL, Robertson EJ, Secombes CJ van West P (2008) New insights into animal pathogenic oomycetes. Trends in Microbiology 16(1): 13–19.

Rietmüller A (2000) Morphologie, Ökologie und Phylogenie aquatischer Oomyceten. Bibliotheca Mycologica185: 1–344.

Risso-de Faverney C, Devaux A, Lafaurie M, Girard JP, Bailly B, Rahmani R (2001) Cadmium induces apoptosis and genotoxicity in rainbow trout hepatocytes through generation of reactive oxygene species. Aquatic Toxicology 53(1): 65–76

Roesijadi G, Robinson WE (1994) Metal Regulation in Aquatic Animals: mechanisms of uptake, accumulation and release. Aquatic Toxicology, Molecular, Biochemical and Cellular Perspectives, in: D.C. Malins, G.K.Ostrander (Eds.), CRC Press, Boca Raton, FL, US, pp. 387–420

Saez G, Aye M, De Meo M, Aimé A, Bestel I, Barthélémy P, Di Giorgio C (2015) Genotoxic and oxidative responses in coelomocytes of *Eisenia fetida* and *Hediste diversicolor* exposed to lipid-coated CdSe/ZnS quantum dots and CdCl2. Environmental Toxicology 30(8): 918–926

Salem ZB, Capelli N, Laffray X, Elise G, Ayadi H, Aleya L (2015) Seasonal variation of heavy metals in water, sediment and roach tissues in a landfill draining system pond (Etueffont, France). Ecological Engineering 69: 25–37

Santos EM, Ball JS, Williams TD, Wu H, Ortega F, van Aerle R, Katsiadaki I, Falciani F, Viant MR, Chipman JK, Tyler CR (2010) Indentifying health impacts ofexposure to copper using transcriptomics and metabolomics in a fish model. Environmental Science and Technology 44(2): 820–826

Schaumburg F, Hippe D, Vutova P, Lüder CG (2006) Pro- and anti-apoptotic activities of protozoan parasites. Parasitology 132(S1): S69–S85

Shahbazian N, Ebrahimzadeh Mousavi HA, Soltani M, Khosravi AR, Mirzargar S, Sharifpour I (2010) Fungal contamination in rainbow trout eggs in Kermanshah province propagations with emphasis on *Saprolegniaceae*. Iranian Journal of Fisheries Sciences 9(1): 151–160.

Shang EH, Wu RS (2004) Aquatic hypoxia is a teratogen and affects fish embryonic development. Environmental Science and Technology 38(18): 4763–4767

Shang EH, Yu RM, Wu RS (2006) Hypoxia affects sex differentiation and development, leading to a male-dominated population in zebrafish (*Danio rerio*). Environmental Science and Technology 40(9): 3118–3122

SCHER, SCCS, SCENIHR (2012) Opinion on the toxicity and assessment of chemical mixtures

Singh N, Manshian B, Jenkins GJ, Griffiths SM, Williams PM, Maffeis TG, Wright CJ, Doak SH (2009) NanoGenotoxicology: the DNA damaging potential of engineered nanomaterials. Biomaterials 30(23–24): 3891–3914

Snyder RD, Diehl MS (2005) Hypoxia-induced micronucleus formation in mice. Drug and Chemical Toxicology 28: 373–37

Svobodova Z, Vykusova B (Eds.) (1991) Diagnostics, Prevention and Therapy of Fish Diseases and Intoxications. Vodnany: Czechoslovakia

Soanes DM, Richards TA, Talbot NJ (2007) Insights from sequencing fungal and oomycete genomes: what can we learn about plant disease and the evolution of pathogenicity? Plant Cell 19(11): 3318–3326

Soldatov AA (2005) Peculiarities of organization and functioning of the fish red blood system. Journal of Evolutionary Biochemistry and Physiology 41: 272–281

Song Y, Salbu B, Heier LS, Teien HC, Lind OC, Oughton D, Petersen K, Rosseland BO, Skipperud L, Tollefsen KE (2012) Early stress responses in Atlantic salmon (*Salmo salar*) exposed to environmentally relevant concentrations of uranium. Aquatic Toxicology 112–113: 62–71

Thoen E, Evensen O, Skaar I (2011) Pathogenicity of *Saprolegnia* spp. to Atlantic salmon, *Salmo salar* L., eggs. Journal of Fish Diseases 34(8): 601–608.

Torto-Alalibo T, Tian M, Gajendran K, Waugh ME, Van West P, Kamoun S (2005) Expressed sequence tags from the oomycete fish pathogen *Saprolegnia parasitica* reveal putative virulence factors. BMC Microbiology 5: 46

Udroiu I (2006) The micronucleus test in piscine erythrocytes. Aquatic Toxicology 79: 201–204

Van Den Berg AH, McLaggan D, Dieguez-Uribeondo J, van West P (2013) The impact of the water moulds *Saprolegnia diclina* and *Saprolegnia parasitica* on natural ecosystems and the aquaculture industry. Fungal Biology Reviews 27(9): 33–42.

Van West P (2006) *Saprolegnia parasitica*, an oomycete pathogen with a fishy appetite: New challenges for an old problem. Mycologist 20(3): 99–104.

Varanasi U, Markey D (1978) Uptake and release of lead and cadmium in skin andmucus of coho salmon (Oncorhynchus kisutch). Comparative Biochemistry and Physiology – Part C: 60: 187–191

Velma V, Tchounwou PB (2010) Chromium induced biochemical, genotoxic and histopathologic effects in liver and kidney of Goldfish, *Carassius auratus*. Mutation Research 698(1–2): 43–51

Vignardi CP, Hasue FM, Sartório PV, Cardoso CM, Machado AS, Passos MJ, Santos TC, Nucci JM, Hewer TL, Watanabe IS, Gomes V, Phan NV (2015) Genotoxicity, potential cytotoxicity and cell uptake of titanium dioxide nanoparticles in the marine fish *Trachinotus carolinus* (Linnaeus, 1766). Aquatic Toxicology 158: 218–229

Wah Chu K, Chow KL (2002) Synergistic toxicity of multiple heavy metals is revealed by a biological assay using a nematode and its transgenic derivative. Aquatic Toxicololy 61: 53–64

Wu T, Tang M (2014) Toxicity of quantum dots on respiratory system. Inhalation Toxicology 26: 128–139

Xiao YY, Liu L, Chen Y, Zeng YL, Liu MZ, Jin L (2016) Developmental Toxicity of Carbon Quantum Dots to the Embryos/Larvae of Rare Minnow (*Gobiocypris rarus*). BioMed Research International Volume Article ID 4016402: 11 p.

Zhang T, Stilwell JL, Gerion D, Ding L, Elboudwarej O, Cooke PA, Gray JW, Alivisatos AP, Chen FF (2006) Cellular effect of high doses of silica-coated quantum dot profiled with high throughput gene expression analysis and high content cellomics measurements. Nano Letters 6(4): 800–808

Zhang Y, Wang YJ, Yu RL, Zhang S, Wu ZB (2008) Effects of heavy metals Cd^{2+}, Pb^{2+} and Zn^{2+} on DNA damage of loach *Misgurnus anguillicaudatus*. Frontiers in Biology 3(1): 50–54

Zheng JL, Luo Z, Chen QL, Liu X, Liu CX, Zhao YH, Gong Y (2001) Effect of waterborne zinc exposure on metal accumulation, enzymatic activities and histology of *Synechogobius hasta*, Ecotoxicology and Environmental Safety 74: 1864–1873

Zhu Y, Wang J, Bai Y, Zhang R (2004) Cadmium, chromium, and copper induce polychromatocyte micronuclei in carp (*Cyprinus carpio* L.). Bulletin of Environmental Contamination and Toxicology 72(1): 78–86

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor Dr habil. Janina Baršienė for being the greatest mentor and guiding me on the right path, expertise, giving me so many valuable advices, constant support and continuous motivation throughout the study. She always supports me with trust and inspired me to be positive and confident; I am especially grateful for placing her trust and confidence in my abilities to do this research, for seeing the potential in me and for the freedom she gave me to conclude this project. I am so thankful for everything that I have learned from her.

I wish to thank my co-authors, Dr Laura Butrimavičienė, Dr Gintaras Svecevičius, Dr Wlodzimierz Grygiel, Dr Nijolė Kazlauskienė, Dr Zita Milda Vosylienė, Dr Gintarė Sauliutė, Živilė Jurgelėnė, Roberta Valskienė, Janina Greiciūnaitė, Tomas Makaras, Dr Svetlana Markovskaja, Dr Kęstutis Jokšas, Dr Tomas Virbickas, Dr Danguolė Montvydienė and others, for collaborations and expertise.

I express my gratitude to Dr Laura Butrimavičienė and Dr Aleksandras Rybakovas for their advices and support. I am grateful to Dr Svetlana Markovskaja for her guidance and expertise during S. parastica infection experiments. I express my gratitude to Dr Zita Milda Vosylienė who taught me haematological analysis; to Dr Vaidotas Morkūnas and Gintarė Urbonaitė of Vilnius University who taught me SSGE for their time and interest. I sincerely thank to Dr Kęstutis Arbačiauskas and Dr Eglė Šidagytė for the suggestions in data analysis and to Dalia Baršytė Lovejoy (Toronto University) and Irena Žalakevičienė for the language check. I thank Dr Laura Butrimavičienė or her valuable suggestions and encouragement.

To my colleagues and friends Dr Gintarė Sauliutė, Živilė Jurgelėnė, Janina Greiciūnaitė, Roberta Valskienė and Tomas Makaras, I thank them for all the crazy hours we have spent together in the lab and for sharing all these happy and sad moments with me. I sincerely thank all of the personnel of the Laboratory of Genotoxicology and the Laboratory of Ecology and Physiology of Hydrobionts for co-operation and assistance.

I would like to thank to my family and my closer friends for unconditional support and patience throughout these years.

This work was supported in part by the Research Council of Lithuania through the project ACTIS S-MIP-17-10.

COPIES OF PUBLICATIONS

I

Analysis of nuclear abnormalities in erythrocytes of rainbow trout (Oncorhynchus mykiss) treated with Cu and Zn and after 4-, 8-, and 12-day depuration (post-treatment recovery)

Stankevičiūtė M, Butrimavičienė L, Valskienė R, Greiciūnaitė J, Baršienė J, Vosylienė MZ, Svecevičius G

Mutation Research - Genetic Toxicology and Environmental Mutagenesis
2016, 797:26–35

Reprinted with permission from Elsevier Ltd.

Mutation Research 797 (2016) 26–35

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Analysis of nuclear abnormalities in erythrocytes of rainbow trout (*Oncorhynchus mykiss*) treated with Cu and Zn and after 4-, 8-, and 12-day depuration (post-treatment recovery)

Milda Stankevičiūtė*, Laura Butrimavičienė, Roberta Valskienė, Janina Greiciūnaitė, Janina Baršienė, Milda Zita Vosylienė, Gintaras Svecevičius

Nature Research Centre, Akademijos 2, 08412 Vilnius, Lithuania

ARTICLE INFO

Article history:
Received 22 July 2015
Received in revised form 12 January 2016
Accepted 15 January 2016
Available online 19 January 2016

Keywords:
Micronuclei
Nuclear abnormalities
Rainbow trout
Copper–zinc mixture
Genotoxicity

ABSTRACT

The induction of micronuclei (MN), nuclear buds (NB), bi-nucleated erythrocytes with nucleoplasmic bridge (BNb), vacuolated (VacNuc), blebbed (BL), 8-shaped nuclei, bi-nucleated (BN) and fragmented-apoptotic (FA) erythrocytes was analysed in the peripheral blood, cephalic kidney and liver of rainbow trout *Oncorhynchus mykiss* after 4-day treatment with copper (Cu) and zinc (Zn) mixture solutions and in 4-, 8- and 12-day depuration process. Fish (three treatment and one control group, $N = 40$) were exposed to 0.0625, 0.125 and 0.25 fractions of 96-h LC50, respectively under semi-static conditions. Exposure of *O. mykiss* to Cu and Zn induced significant increase of MN (in blood in all test groups; in liver 0.125, 0.25 and in kidney 0.25 groups, respectively), NB and BL (in blood and kidney 0.25 group), 8-shaped (in blood 0.25; in liver 0.125, 0.25 and in kidney all test groups, respectively) and VacNuc (in liver and kidney 0.0625 and 0.125 groups). After 4-day recovery, significantly elevated levels of MN (in blood 0.0625, 0.125; in liver and kidney 0.125 group, respectively) and 8-shaped (in kidney—0.0625 group) were observed in fish. Significant recovery was observed in 0.0625 group after 12-day depuration, estimating the formation of MN in erythrocytes of blood, of 8-shaped nuclei erythrocytes in liver and kidney (after 8-, 12-day and 8-day recovery, respectively). Significant decrease of MN in blood (after 8- and 12-day recovery), in liver (after 8-day recovery), of NB in blood and kidney (after 8-day recovery) and of 8-shaped nuclei erythrocytes in blood (after 8 and 12-day recovery), kidney and liver (after 8-day recovery) was determined in 0.25 group. Changes in gross morphometric indices and biological parameters were observed. The binary metal mixture did not induce FA erythrocytes in any tissue at any test concentration.
© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Anthropogenic activities have introduced complex mixtures of contaminants discharging with industrial, domestic and agricultural wastes into the environment that have increased concern about their adverse effects on freshwater and marine ecosystems. There is increasing attention in pollutants synergism which has the most toxicological concern [1]. Genotoxic agents are the ingredients of these mixtures that can provoke genetic damage and initiate effects at various biological levels [2]. Therefore, a growing interest in studies of environmental genotoxicity has led to the development of a variety of tests for the detection of genotoxic agents in aquatic media. Different fish species were used for the detection of genetically active compounds *in situ* and in laboratory exposures to various clastogenic and aneugenic compounds [3,4].

At low levels, copper and zinc are essential for many biological processes. It is known that zinc plays a crucial role in different cellular mechanisms, including defence against free radicals and maintaining cell viability and genomic stability. Zinc is a vital micronutrient for fish normal growth and development. However, at high concentrations the element acts as a toxic agent [5]. Nevertheless, fish evolved mechanisms regulating uptake, excretion and binding of Zn into metallothioneins. In rainbow trout, the carcass is the main depot (84–90%) for Zn, while in the gills accumulation of waterborne zinc predominantly consists of metabolically active fraction [6]. The highest levels of accumulated Cu and Zn have been found in the liver, than in the muscle or gill tissues of gilthead seabream (*Sparus aurata*) and common sole (*Solea senegalensis*) [7].

Copper is an essential nutrient for organisms, but at high cellular concentrations can act as a toxic agent [8]. This metal is a widely

* Corresponding author.
E-mail address: milda.stan@gmail.com (M. Stankevičiūtė).

http://dx.doi.org/10.1016/j.mrgentox.2016.01.003
1383-5718/© 2016 Elsevier B.V. All rights reserved.

distributed contaminant in aquatic ecosystems. It can cause harmful effects due to bioavailability in tissues and capability to induce oxidative stress and affect DNA integrity [9]. Oxidative stress initiated by Cu in rainbow trout may trigger off significant induction of DNA strand breaks and apoptosis in damaged cells [10].

Since many compounds in the environment are able to induce genotoxic effects with or without directly damaging DNA, the necessity to measure both types of alterations is evident. In order to assess both clastogenic and aneugenic effects of contaminants in organisms, many studies have applied the micronucleus test, which has served as an index of cytogenetic damage for many years [11]. Micronuclei (MN) are produced from chromosome fragments or whole chromosomes that lag at cell division due to lack of centromere, damage in centromere or a defect in cytokinesis [12]. The investigation of other nuclear abnormalities–nuclear buds, bi-nucleated cells with nucleoplasmic bridge, blebbed, 8-shaped, vacuolated nuclei, fragmented-apoptotic and bi-nucleated cells, has also been considered as a reliable approach in assessment of genotoxicity and cytotoxicity of contaminants in aquatic organisms [13–15]. Morphological nuclear alterations, firstly, have been described in fish peripheral blood erythrocytes by Carrasco et al. [16] and were suggested as indicators of genotoxic damage in genotoxicity surveys. The formation of nuclear buds (NB) may reflect the clastogenic action of the agent and unequal capacity of organisms to expel damaged, amplified, failed DNA replication or improperly condensed chromatin, chromosome fragments without telomeres and centromeres from the nucleus [17]. MN, NB and blebbed nuclei (BL) cells suggest a similar origin and all can be applied as genotoxicity analogues [17–19]. Vacuolated nuclei are probably related to aneuploidy leading to micronuclei formation [20]. Induction of bi-nucleated cells with nucleoplasmic bridges is an indicator of dicentric chromosomes [21]. Bi-nucleated erythrocytes are formed in abnormal cell division due to blocking of cytokinesis [4]. A formation of 8-shaped erythrocytes also reflects a failure of the erythropoiesis, and they are formed from a part of the mitotic spindle [22]. Elimination of cytogenetic damage by the apoptosis and necrosis is a key process, which occurs at different rates in various organisms [23].

Micronuclei have been induced in fish cells exposed to various heavy metals under both laboratory and field conditions [13,14,24]. The piscine micronucleus test has been successfully used to evaluate the genotoxic or cytotoxic damage in several fish tissues, such as fin cells [25,26], gill epithelium [4,25], kidney [27], liver [4] and peripheral blood [27]. In fish, the cephalic kidney is the major hematopoietic organ; the secondary hematopoietic organs (spleen, peri-portal areas of the liver, the intestinal submucosa and the thymus; occasional hematopoiesis occurrence reported in gills, brain and gonads) may have different sensitivities [28,29]. Peripheral blood receives erythrocytes and erythrocytes with nuclear abnormalities from several hematopoietic organs [30]. The tissue-specific Cu and Zn geno-cytotoxicity data in fish is scarce. The tissue specificity depends on the bioaccumulation of toxicants, different sensitivity and defensive mechanism of the certain organ [31,32]. Evaluation of genotoxicity and cytotoxicity in blood and cephalic kidney erythrocytes of turbot (Scophthalmus maximus) and Atlantic cod (Gadus morhua) treated with nonylphenol, crude oil and mixture of oil spiked with alkylphenols and PAHs showed inter-tissue differences in their responses [27].

Most of the studies focus on the individual DNA damaging potential of single metal in fishes [4,24,25,33]. Only several studies have been addressed to the joint action genotoxicity of copper–zinc mixtures. The joint toxic action of binary copper–zinc mixtures differs from single action of these essential trace metals in fish [34]. The induction of MN by copper, zinc and their binary mixture was previously demonstrated in rainbow trout (*Oncorhynchus mykiss*) [35], planet catfish (*Synodontis clarias*) and Nile tilapia (*Tilapia nilotica*) [36] blood erythrocytes. Significant increase in micronuclei was observed in rainbow trout after 14-day exposure to the heavy metal mixture solution at 21.79% and 10.89% concentration. The absolute concentration (100%) of the heavy metal mixture solution was accepted as Cu—0.874; Zn—0.93; Pb —4.7; Ni—0.66; Cr—0.33 and Mn—18 mg/L, respectively [37]. European eel (*Anguilla anguilla*) treated with 0.2 μM Cu/L ($CuCl_2$) after 7 days has demonstrated the highest concentrations of copper in the liver, and, in parallel, there was evolved sub-cellular protection via Cu-binding into metallothioneins and significant elevation of cytogenetic damage in blood erythrocytes [38].

However, there is a lack of information about genotoxicity and cytotoxicity of copper and zinc mixtures at different concentrations, exposure time and variations in recovery responses and duration in different fish tissues. In the present study, we focused on the assessment of the induction of micronuclei (MN), nuclear buds (NB), bi-nucleated erythrocytes with nucleoplasmic bridge (BNb), vacuolated (VacNuc), blebbed (BL) and 8-shaped nuclei, bi-nucleated (BN), and fragmented-apoptotic (FA) erythrocytes in rainbow trout blood, kidney and liver after the exposure to different concentrations of copper and zinc mixture solution and to describe time-related recovery in the experimental depuration process. The concentrations of Cu and Zn were chosen considering 96-h LC50 values—for copper 0.65 mg/L and for zinc—3.79 mg/L [39,40].

2. Material and methods

2.1. Experimental set-up

The experimental treatment was conducted on hatchery-reared one-year-old rainbow trout (*O. mykiss* Walbaum 1792) (average total weight 39.7 g and average total length 173 mm). The fish was obtained from Meškerinė fish hatchery (Švenčionys District, Lithuania) and kept for acclimation in holding tanks (3000-L volume) supplied with flow-through aerated deep-well water at least one week prior to testing. Fish were fed commercial trout feed (DANA FEED) daily in the morning; the total amount was no less than 1% of their wet body mass per day. During the experiment, the fish were fed in the same manner. Trout were accepted as acclimated to a new medium when their behavior became normal and they feed well.

Deep-well water was used as the dilution water. Average water hardness and alkalinity were 284 (271–296) and 200 (190–210) mg/L as $CaCO_3$, respectively, mean pH was 8.0, temperature was maintained at 10–11 °C, dissolved O_2 concentration was no less than 10 mg/L, and dissolved organic carbon (DOC) was below the instrumental detection limit (<0.3 mg/L) [41].

After an acclimation period, trout were transferred from the holding tanks into four glass tanks of 100-L volume with continuously aerated water in groups of 40 specimens (one control and three treatments). Test fish were exposed for 4 days to equitoxic copper–zinc mixture solutions at a concentration corresponding to the sum of 0.25, 0.125 and 0.0625 96-h LC50 fraction under semi-static conditions. Test concentrations of copper and zinc were chosen based on 96-h LC50 values of 0.65 mg Cu/L and 3.79 mg Zn/L derived from acute toxicity tests in previous studies under the same laboratory conditions [39,40]. These concentrations are 65-fold for copper and 37.9-fold for zinc higher than those Maximum-Allowable-Concentrations (MAC) of 0.01 and 0.1 mg/L, respectively accepted for receiving inland water-bodies of Lithuania [42]. Reagent-grade metal sulphates ($CuSO_4 \cdot 5H_2O$), ($ZnSO_4 \cdot 7H_2O$, 99% purity, «REACHIM» Company, Russia) were used as the toxicants. Mixture stock solution was prepared by dissolving a necessary amount of metal salt in distilled water, final concentration being recalculated according to the metal ion amount. Clean water and mixture solutions were renewed every 24 h and test fish were transferred either into freshly prepared solution or into clean water after they were fed.

After the 4-day mixture exposure period a part of fish (10 specimens from each exposed and control group) was taken for genotoxicity and cytotoxicity analysis. Whereas other fish from each group were transferred into clean, metal-free water and were kept for 4, 8 and 12 days for recovery until genotoxicity and cytotoxicity analysis using 10 specimens for each period of the depuration.

2.2. Analytical procedures

The main physico-chemical parameters of the water (temperature, dissolved O_2, pH and conductivity) were measured routinely with a hand-held multi-meter (WTW Multi 340i/SET, Germany). Designed nominal metal concentrations in the tanks were checked during blank tests (without fish) ($N = 4$) with an atomic absorption spectrophotometer (SHIMADZU AA-6800, Japan) by graphite furnace technique using proprietary software. Each water sample was acidified with reagent-grade

Table 1
Heavy metal concentration (μg/L) in binary mixture used in the laboratory exposure.

Heavy metal	Concentration	
	Nominal (fraction of 96-h LC50)	Measured (mean ± standard deviation)
Copper	0 (Control)	2.5 ± 0.6
	160 (0.25)	172 ± 13.4
	809 (0.125)	86 ± 8.8
	40 (0.0625)	44 ± 3.9
Zinc	0 (Control)	10.8 ± 0.5
	948 (0.25)	965 ± 80
	474 (0.125)	507 ± 65
	328 (0.0625)	358 ± 30

nitric acid (final concentration 0.5% v/v) and analysed in triplicate. Mean measured concentrations were within 10% of the target (Table 1).

2.3. Sampling, biological parameters and gross morphometric indices

Peripheral blood samples from the caudal vein and erythrocytes from kidney and liver tissues were obtained from 160 rainbow trout specimens. Biological parameters (total fish body weight (Q) and total length (L), body weight without stomach weight (q), liver weight (Qlw)) were measured for all specimens in control, exposed and on 4-, 8- and 12-day in recovery groups. Gross morphometric indices were evaluated according to the following formulas [43]: condition factor (CF) = total body weight (g)/(total length)^3; liver-somatic index (LSI) = liver weight × 100/(total body weight$^\#$ − liver weight) $^\#$subtract stomach content.

2.4. Sample preparation and analysis

A drop of blood was directly smeared on microscopic slides and air-dried. After the sacrifice, small pieces of cephalic kidney and liver were dissected, softly dragged along clean slide and allowed to dry for 1–2 h [27]. Dried smears were fixed in methanol for 10 min and were stained with 10% Giemsa solution for 8 min [44]. The stained slides were analysed under bright-field microscopes Olympus BX51, or Nikon eclipse 50*i* at final magnification of 1000×. Blind scoring of nuclear abnormalities was performed on coded slides. The formation of micronuclei (MN), nuclear buds (NB), bi-nucleated erythrocytes with nucleoplasmic bridge (BNb), blebbed (BL), vacuolated (VacNuc), 8-shaped nuclei, bi-nucleated (BN) and fragmented-apoptotic (FA) erythrocytes were identified using criteria described by Fenech et al. [11] and Baršienė et al. [22]. For each studied specimen of rainbow trout, 4000 erythrocytes with intact cellular and nuclear membrane were examined. Final results were expressed as the mean value (‰) of sums of analysed individual lesions scored in 1000 erythrocytes per fish sampled from every study group.

Induction of micronuclei and nuclear buds, bi-nucleated erythrocytes with nucleoplasmic bridge and blebbed nuclei in fish blood, kidney and liver erythrocytes was used as genotoxicity endpoints, and induction of vacuolated and 8-shaped nuclei as well as bi-nucleated and fragmented-apoptotic erythrocytes was assessed as cytotoxicity endpoints. The morphological features of studied nuclear abnormalities are shown in Fig. 1.

2.5. Data analysis and statistics

The means of studied nuclear abnormalities, standard errors and *P* values were calculated for each experimental and control group using PRISM statistical package. Differences were accepted as significant at the 95% level of confidence ($P < 0.05$). Non-parametric Mann–Whitney *U*-test was used to compare frequencies of MN and other nuclear abnormalities between control and exposed fish groups, between exposed and recovery groups. Principal component analysis (PCA) was accomplished to obtain an integrated view of the relationships between the investigated variables. The extraction of principal components (PCs) was based on eigenvalues greater than 1. Variables with loading factor >0.50 and 0.40–0.49 were considered as significant and moderately significant, respectively.

3. Results

3.1. Genotoxicity of Cu and Zn mixture (induction of micronuclei, nuclear buds, bi-nucleated erythrocytes with nucleoplasmic bridge, blebbed erythrocytes)

Significantly increased MN levels were detected in peripheral blood of all exposed groups, in liver erythrocytes of 0.125 and 0.25 groups and in kidney erythrocytes of 0.25 group (Fig. 2).

Four-day depuration revealed significant elevation of MN in blood, liver and kidney erythrocytes of fish, previously exposed to copper–zinc mixture consisting of 0.125 fraction of LC50, and blood cells exposed to 0.0625 fraction of LC50. Micronuclei level in control group of rainbow trout remained almost at the same level as in 4-day treatment. Further post-treatment depuration during 8 days revealed comparatively lower levels of genotoxicity in all tested tissues. However, after 12 days of depuration, statistically lower frequencies of MN were found only in blood erythrocytes of fish in 0.0625 and 0.25 groups in comparison to exposure levels. The values of nuclear buds were lower than the frequency of micronuclei in studied tissues of all exposed fish groups. The highest induction (0.67 NB/1000 cells) was observed in blood in 0.25 group. It should be pointed out that during the depuration process significant decrease in nuclear buds was found only after 4 days of recovery in blood erythrocytes of 0.125 group, also after 8-day recovery in blood and kidney erythrocytes scored in 0.25 group. Significant frequencies of nuclear abnormalities with similar type of origin—nuclear buds and blebbed nuclei (NB + BL) were found in fish (in all tissues) treated with the highest concentration of copper–zinc mixture. Significant recovery was observed in blood and kidney erythrocytes after 8, only in blood after 12 days of exposure. No recovery was observed in liver erythrocytes.

Analysis of summed genotoxicity (MN + NB + BNb) in all analysed tissues showed significant differences in 0.25 exposure group in comparison to control group. After 4-day recovery, significantly elevated levels of summed genotoxicity in kidney erythrocytes were established in 0.125 group. After the 8-day period of depuration, statistically decreased genotoxicity levels were observed in blood and kidney erythrocytes in 0.25 group. Twelve days of depuration caused statistically significant decrement of genotoxicity only in blood erythrocytes (Fig. 3).

3.2. Cytotoxicity of Cu and Zn mixture (induction of erythrocytes with 8-shaped and vacuolated nuclei)

Cytotoxicity analysis revealed significant induction of 8-shaped nuclei erythrocytes in all concentrations studied in kidney, in two (0.125 and 0.25)—in liver and in the highest concentration in blood. The frequency of 8-shaped nuclei in kidney erythrocytes of 0.0625 group was found increased after 4-day post-treatment recovery. Recovery was observed in all tissues after 8-day of depuration. Twelve days of depuration did not cause significant reduction in the 8-shaped nuclei in liver and kidney erythrocytes, except in the liver of 0.0625 group (Fig. 4).

Statistically enlarged frequencies of vacuolated nuclei (VacNuc) erythrocytes were detected only in liver and kidney of rainbow trout from 0.0625 and 0.125 groups in comparison to control groups. After 4 days of depuration, significantly lowered values of VacNuc were registered in blood and liver erythrocytes of the fish from 0.125 group. Eight- and 12-day depuration provoked decreased VacNuc levels only in blood erythrocytes of fish from 0.0625 and 0.25 groups (Fig. 5).

3.3. Biological parameters and gross morphometric indices

The rainbow trout length, weight without stomach weight, liver weight, condition factor (CF) and liver-somatic index (LSI) of the fish are presented in Table 2. Fish body weight without stomach weight was found to decrease in all treatments and remained decreased after 12-day recovery as compared to control. During the exposure and depuration periods, changes in fish length showed similar trends as body weight without stomach weight. Liver weight did not show significant differences between the treatments and recovery groups, except 0.125 8-day recovery group in comparison to exposure groups. Statistically decreased liver weight

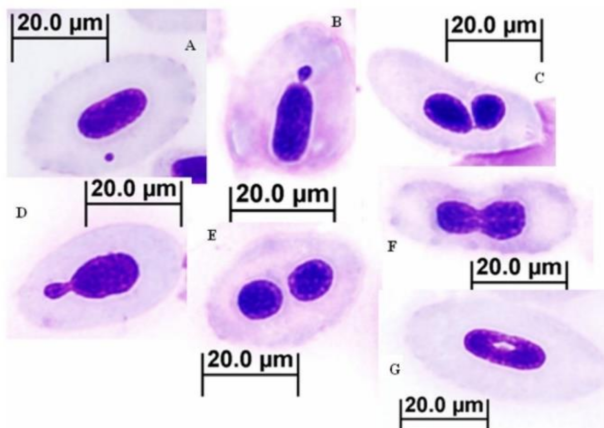


Fig. 1. Rainbow trout mature erythrocytes with micronucleus (A), nuclear bud (B), bi-nucleated erythrocyte with nucleoplasmic bridge (C), blebbed (D), bi-nucleated (E), 8-shaped (F) and vacuolated nuclei in fish erythrocytes (G).

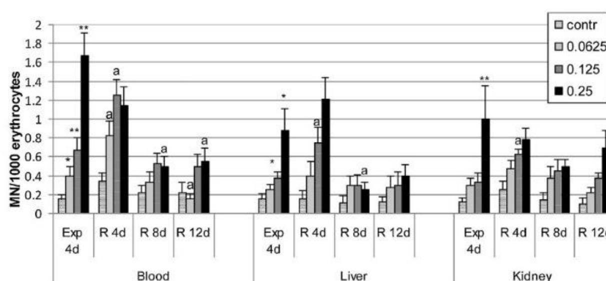


Fig. 2. Frequency of micronuclei (MN) in blood, liver and kidney erythrocytes of rainbow trout exposed to different concentrations of copper–zinc mixture solution and in depuration process. Data in this and all other figures are represented as means ± SE, differences between control and exposed fish groups shown: one asterisk at level $P < 0.05$, two asterisks $P < 0.001$; differences between exposed and recovery fish groups shown: a $P < 0.05$, b $P < 0.001$, c $P < 0.0001$.

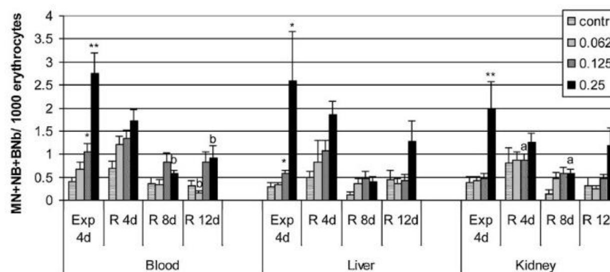


Fig. 3. Frequency of summed genotoxicity (MN + NB + BNb) in blood, liver and kidney erythrocytes of rainbow trout exposed to different concentrations of copper–zinc mixture solution.

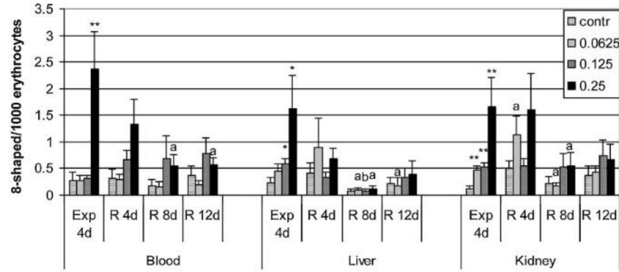


Fig. 4. Frequency of 8-shaped nuclei in blood, liver and kidney erythrocytes of rainbow trout exposed to different concentrations of copper–zinc mixture solution.

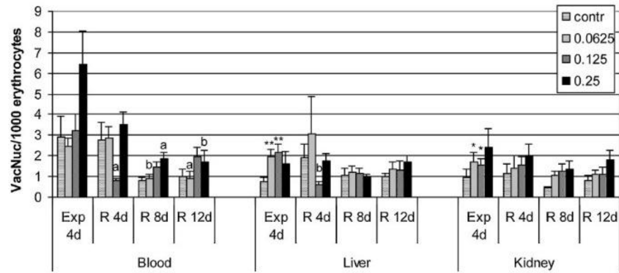


Fig. 5. Frequency of VacNuc in blood, liver and kidney erythrocytes of rainbow trout exposed to different concentrations of copper–zinc mixture solution.

Table 2
Gross morphometric indices and biological parameters of *O. mykiss* after treatment with copper–zinc mixture solution and during depuration process. Data are represented as means ± SE. Differences between control and exposed fish groups shown: *at level $P < 0.05$, **$P < 0.001$, ***$P < 0.0001$. Differences between exposed and recovery fish groups shown: a $P < 0.05$, b $P < 0.001$, c $P < 0.0001$.

	Control	0.0625	0.125	0.25
Length (L), cm				
Exposure	17.95 ± 0.41	18.05 ± 0.22	16.81 ± 0.17**	15.58 ± 0.08**
Recovery 4 day	17.50 ± 0.28	17.50 ± 0.33	16.25 ± 0.59	16.00 ± 0.45*
Recovery 8 day	17.14 ± 0.39	16.55 ± 0.2b	15.10 ± 0.29**c	15.85 ± 0.30*
Recovery 12 day	18.13 ± 0.26	17.85 ± 0.24	15.92 ± 0.66*	15.95 ± 0.41**
Weight without stomach weight (q), g				
Exposure	39.43 ± 2.37	39.39 ± 1.68	32.1 ± 1.03*	33.34 ± 1.08*
Recovery 4 day	38.26 ± 2.02	35.52 ± 2.13	28.15 ± 2.75**	26.53 ± 2.36**
Recovery 8 day	34.23 ± 2.2	32.79 ± 1.16b	22.77 ± 1.13***c	26.76 ± 1.38**b
Recovery 12 day	43.82 ± 1.93	39.78 ± 1.35	27.92 ± 3.6**	27.61 ± 1.86***a
Liver weigh (Qlw), g				
Exposure	0.39 ± 0.03	0.45 ± 0.03	0.44 ± 0.02	0.35 ± 0.01
Recovery 4 day	0.41 ± 0.02	0.39 ± 0.02	0.34 ± 0.04	0.33 ± 0.03
Recovery 8 day	0.41 ± 0.04	0.43 ± 0.02	0.37 ± 0.01a	0.41 ± 0.03
Recovery 12 day	0.57 ± 0.05	0.48 ± 0.02	0.47 ± 0.06	0.41 ± 0.03*
CF				
Exposure	0.68 ± 0.01	0.67 ± 0.02	0.67 ± 0.02	0.88 ± 0.02**
Recovery 4 day	0.71 ± 0.01	0.66 ± 0.01**	0.65 ± 0.02*	0.64 ± 0.02*b
Recovery 8 day	0.67 ± 0.01	0.68 ± 0.01	0.66 ± 0.02	0.67 ± 0.01b
Recovery 12 day	0.73 ± 0.01	0.70 ± 0.02	0.68 ± 0.04	0.67 ± 0.02*b
LSI				
Exposure	0.99 ± 0.05	1.16 ± 0.08	1.39 ± 0.07**	1.05 ± 0.03
Recovery 4 day	1.07 ± 0.02	1.14 ± 0.06	1.20 ± 0.09	1.28 ± 0.06**b
Recovery 8 day	1.24 ± 0.1	1.33 ± 0.06a	1.66 ± 0.08**a	1.54 ± 0.05*b
Recovery 12 day	1.32 ± 0.07	1.23 ± 0.04	1.73 ± 0.11*a	1.50 ± 0.05b

was found only in 0.25 group after 12-day depuration in comparison to control.

There were significant differences between CF in fish from 0.25 treatment control and recovery groups. During the 4-day recovery, CF significantly decreased in all groups as compared to control. Changes in LSI were also measured. The LSI showed a tendency to increase after treatment with binary metal mixture and during the depuration period.

3.4. Relationships between biological parameters, gross morphometric indices and nuclear abnormalities—PCA

The highest induction of MN and other nuclear abnormalities (NAs) was found in all tissues exposed to the highest concentration of binary metal mixture. Principal components analysis (PCA) was applied to obtain an integrated view of the relationships between the investigated variables in all tissues in 0.25 exposure group (Fig. 6).

Principal component statistical analysis in the blood showed 3PCs, in the liver and kidney—4PCs with eigenvalues > 1, which explained over 90% of the total variance. The first component (PC1, 45.8% of the total variance) in blood grouped significant loadings of all biological parameters, gross morphometric indices and three NAs (MN, NB and 8-shaped). The PC1 loadings of all biological parameters (*Q* (−0.957), *q* (−0.964), *L* (−0.837), Qlw (−0.610)) and CF (−0.796) indicated that these parameters were positively intercorrelated to one another and negatively with LSI (0.597). MN (−0.690) and 8-shaped (−0.698) were positively intercorrelated to one another, CF and with all biological parameters, negatively with NB (0.410) and LSI. BL (−0.489) was extracted with PC2 (26.88% of the total variance) along with VacNuc (0.851), whereas NB (−0.692), BL (−0.811), 8-shaped (−0.698) and VacNuc (−0.443) were significant or moderately significant variables extracted with PC3 (19.09% of the total variance).

The first component (PC1, 38.47% of the total variance) in liver grouped significant loadings of all biological parameters and gross morphometric indices. The PC1 loadings of all biological parameters (Q (−0.998), *q* (−0.997), *L* (−0.778), Qlw (−0.415) and CF (−0.895) indicated that these parameters were positively intercorrelated to one another and negatively with LSI (0.784). NAs such as MN (0.703), NB (0.756), BL (0.689), 8-shaped (0.715), VacNuc (0.998) had significant loadings on PC2 (32.67% of the total variance). NB (−0.602), BL (0.624), LSI (−0.443), *L* (−0.505) and Qlw (−0.633) were extracted with PC3 (17.00% of the total variance), whereas MN (0.687) and 8-shaped (−0.574) were significant variables extracted with PC4 (10.85% of the total variance).

The first component (PC1, 41.45% of the total variance) in kidney grouped significant loadings of all gross morphometric indices, Q, *q*, *L*, BL and 8-shaped. The PC1 loadings of 8-shaped (−0.416) and BL (−0.495) indicated that these parameters were positively intercorrelated to one another, biological parameters (*Q* (−0.958), *q* (−0.959), *L* (−0.765)), CF (−0.848)) and was negatively correlated with LSI (0.876). Other NAs (MN (0.874), NB (0.886) and VacNuc (0.601)), LSI (0.434) and Qlw (0.940) had significant or moderately significant loadings on PC2 (28.66% of the total variance). Apart from the moderate loadings on PC1, BL (0.830) and 8-shaped (0.840) had significant loadings on PC3 (15.77% of the total variance). VacNuc (0.617) and *L* (0.581) had significant loadings on PC4 (11.88% of the total variance).

4. Discussion

The exposure of *O. mykiss* to copper–zinc mixture solutions induced statistically significant increase of MN (in the blood in all groups, in the liver in 0.125 and 0.25, in the kidney in 0.25 groups, respectively), NB (in the blood and kidney only in 0.25 groups), BL (in the blood and kidney in 0.25 groups), 8-shaped (in the blood in 0.25, in the liver in 0.125, 0.25, in the kidney in all groups, respectively) and VacNuc (in the liver and kidney in 0.0625 and 0.125 groups). The highest induction of MN was found in all tissues exposed to the highest concentration of binary metal mixture. Summed genotoxicity (MN + NB + BNb) rates followed a trend similar to that shown by the MN. The frequencies of nuclear buds were low in all tissues studied. Significant induction of NB was observed in the blood and kidney of fish after exposure to 0.25 fraction of LC50. Significantly increased levels of NB + BL were observed in all tissues of rainbow trout only after treatment with the highest concentration of binary metal mixture. A significant increase of VacNuc occurred only in the liver and kidney erythrocytes in 0.0625 and 0.125 groups. Significant enumeration of 8-shaped erythrocytes was observed in kidney in all exposure groups, the liver in 0.25, 0.125 and in the blood in 0.25 groups. The applied mixture of heavy metals did not significantly increase the incidences of BN and BNb cells. Copper–zinc mixture did not induce any fragmented-apoptotic cells. In study of Andreikėnaitė et al. [37] the used heavy metals mixture solution (Cu, Zn, Pb, Ni, Cr, Mn) did not induce significant changes in BN and FA cell frequencies between treatments and control groups. Failure in cytokinesis can result in an increase of BN and 8-shaped nuclei cells. Genotoxic agents' induced genomic instability may lead to formation of MN, NB and BL nuclei cells. MN, NB and BL nuclei cells suggest a similar origin as genotoxic analogist [17–19].

Many studies describing the biological effects of heavy metals under laboratory and field conditions demonstrate that at the certain concentrations they are genotoxic to fish [25,45,46,47–49]. The main concern is that geno-cytotoxic endpoints provoked by joint copper–zinc mixture may be influenced by concentration of each metal in the mixture. The ratios of metals in the mixtures can lead to different coergisms types as have been shown by Obiakor and Ezeonyejiaku [34]. Synergistic coergisms were determined in most of the explored ratio mixtures of Cu and Zn, except in the ratio 1:1—antagonistic effect. Bagdonas and Vosylienė [35] as well as Obiakor et al. [36] studies revealed that Cu and Zn jointly (in all of three concentrations tested) exert a larger genotoxic effect when compared to the single action of each metal. In comparison to our study, they also detected the highest levels of MN in fish blood erythrocytes exposed to 0.25 concentration of copper–zinc mixture, but the frequencies of MN were higher. Obiakor and Ezeonyejiaku [34] concluded that copper and zinc jointly provoke higher or cumulative toxicity. Probably mechanisms causing metal/metal synergies are interactions on metal availability and uptake [1]. The combined (Cd, Cu, Pb and Zn (1.25 mg/L of each)) heavy metal mixture induced the formation of MN, BN, vacuolated, blebbed nuclei and other NAs in Nile tilapia (*Oreochromis niloticus*) blood erythrocytes. After 5 days of exposure, inductions of vacuolated nuclei were approximately two, blebbed nuclei erythrocytes six and MN more than eighty times higher in comparison with the control group [14]. Frequencies of vacuolated nuclei and nuclear buds in peripheral erythrocytes of fish Roho labeo (*Labeo rohita*) exposed to $CdCl_2$ (0.37 and 0.62 mg/L) for 100 days increased with the exposure period up to the 10th day, after 15 days of the exposure the rate of nuclear abnormalities began to decline [50].

Genotoxic properties of heavy metals are related evidently to the accumulation of DNA damaging free radicals, clastogenic process or simultaneously to clastogenic and aneugenic action in aquatic organisms [51]. Bopp et al. [10] using the rainbow trout gill cell line RTgill-W1 showed that Cu treatment resulted in a dose-dependent elevation in cytotoxicity and formation of cellular ROS. A significant induction of DNA strand breaks was observed after 2 h exposure of RTgill-W1 cells to 1 and 2.5 μM CuSO4 at pH 7. According to Santos et al. [52] exposure to Cu resulted in DNA

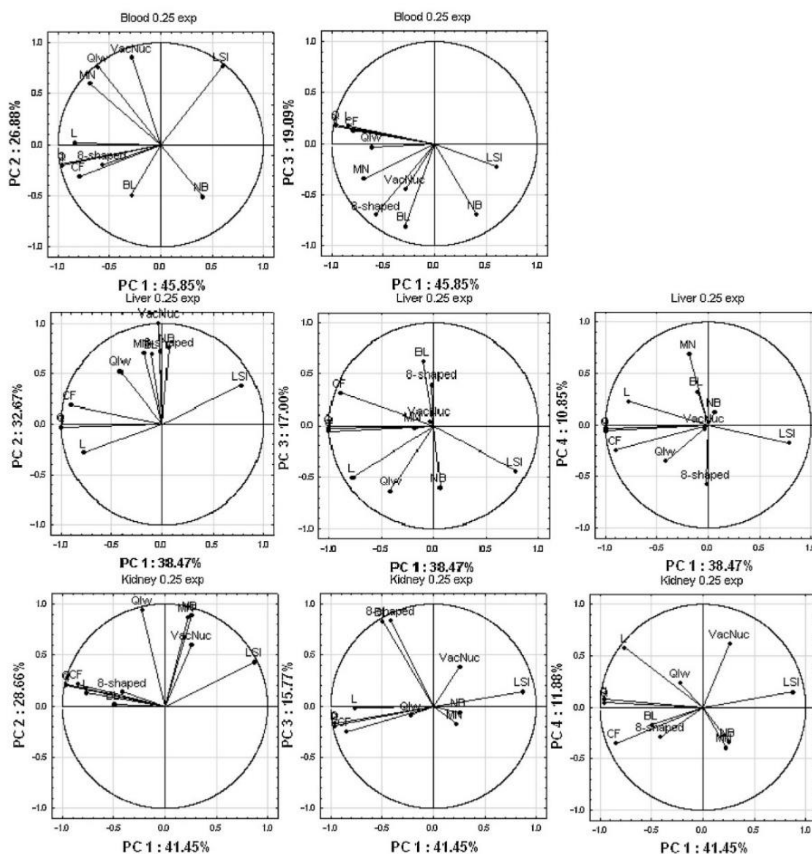


Fig. 6. PCA results: loadings integrating biological parameters, gross morphometric indices and nuclear abnormalities. Abbreviations: *L*—length, *Q*—body weight, *q*—body weight without stomach weight, Qlw— liver weight.

strand breaks induction in blood cells of three-spined stickleback (*Gasterosteus aculeatus*) at all used concentrations (3.2, 10, 32, 64, and 128 μg/L). The percentage tail DNA ranged from 17.38 ± 1.23% for the control fish to 37.23 ± 1.78% for fish exposed to the highest 128 μg Cu/L concentration. Exposure to copper also caused alterations in hepatic gene expression and metabolite concentrations in a concentration-dependent manner (from 10 μg Cu/L) [52].

During the depuration period, MN were found to increase after 4-day recovery and decrease after 8- and 12-day recovery period in all tissues. Decreased MN incidences in blood erythrocytes of rainbow trout and Nile tilapia (*T. nilotica*) after 96-h recovery in clean water were described by Bagdonas and Vosylienė [35] as well as in Obiakor et al. [36] investigation. After 96-h recovery of *S. clarias* exposed to copper–zinc mixture, the frequency of micronucleated erythrocytes showed no significant decrease at all concentrations studied [36]. This may be due to several factors: the cell cycle kinetics and the mechanism of MN induction. MN arises in cell divisions, and their expression can appear at different times after the DNA damage [2]. Grisolia and Cordeiro [53] evaluated the time-dependent response of MN formation during hematopoiesis in the kidney and the micronucleus peak in circulating blood erythrocytes after fish exposure to cytochalasin B. The experiment showed that micronuclei formed in the young kidney erythrocyte cells were detected in peripheral blood 2–4 days later, and this time lag depends on species and clastogens.

Recovery from some genotoxic or cytotoxic endpoints was observed after 8 or 12 days in 0.0625 and 0.125 groups of explored tissues. Blood erythrocytes showed significant recovery after 8 and

12 days in 0.25 group. Statistically significant recovery was not observed after 12-day recovery in liver and kidney erythrocytes after exposure to the highest heavy metals concentration. Our study shows that 12 days term was not enough for recovery of juvenile rainbow trout from genotoxicity and cytotoxicity after treatment with copper–zinc mixture. Juveniles of fat snook (*Centropomus parallelus*) were exposed to 25 or 50 μg Cu/L for 30 days and recovery was observed at 0, 4, 10, and 30 days after exposure. No recovery from genotoxicity was observed during 30-day post-exposure [24].

Our study revealed significant effects of copper–zinc mixture on gross morphometric indices. During the depuration period, the LSI increased, but CF decreased. The current results are in agreement with those of Omar et al. [54]. who reported that decrease in CF values was observed in fish Nile tilapia (*O. niloticus*) and mullet (*Mugil cephalus*) collected from highly degraded aquatic habitats polluted with metals (Cu, Zn, Pb, Fe and Mn) as compared with those from the reference site. Liu et al. [55] found that growth (weight gain, specific growth rate) declined, but hepatosomatic index (HSI) increased in fish *Synechogobius hasta* with increasing waterborne copper levels. Zheng et al. [56] showed that waterborne zinc exposure significantly reduced HSI in *S. hasta*, but did not significantly influenced CF. The mean CF and HSI in three-spined stickleback (*G. aculeatus*) exposed to copper showed no statistically significant differences between treatments [52]. Bonga and Lock [57] reported that a reduced growth is a result of significantly increased energy requirements for maintenance of water and ion homeostasis, because waterborne toxicants increase the permeability to water and ions of the gill epithelium and inhibit the ion-exchange activity of the chloride cells.

Other authors showed histopathological changes caused by copper and zinc in fish liver and kidney. Ciji and Nandan [58] study revealed that *Cyprinidae* fish species *Puntius parrah* exposed to a sublethal concentration of 0.05 mg Cu/L and 0.9 mg Zn/L showed a higher accumulation of these metals in the liver than in the kidney, gills, and muscle. Furthermore, there were found the significant decreases in erythrocytes after exposure to sublethal concentrations and histopathological alterations in liver and kidney tissues. Histopathological changes (cellular and nuclear degenerations) were found in the liver of fish exposed to 0.03, 0.05 and 0.1 mg Cu/L and to 0.6, 0.9 and 1.8 mg Zn/L, for 3, 7, 14 and 28 days, respectively. Histopathological changes such as degenerations of cell nuclei in kidney tissue also were detected after 28 days of exposure to sublethal concentrations of copper sulphate. Higher copper toxicity to *P. parrah* in comparison to zinc was marked [58]. Some findings show Cu and Zn effect on enzyme activities in fish. Hepatic enzymatic activities (SOD, CAT, SDH, PK, LDH, LPL and HL) were changed after copper exposure, hepatic lipid peroxidation level increased in *S. hasta* [55]. Waterborne Zn exposure induced the hepatic SOD activity, hepatic CAT and GST activities in *S. hasta* [56]. The Zn exposure also resulted in reduced hepatic lipid content and vacuoles in hepatocytes of *S. hasta*.

Principal component analysis (PCA) was applied to obtain an integrated view of the relationships between the investigated variables. The first component (PC1) in all tissues has grouped significant loadings of almost all biological parameters and gross morphometric indices. PCA results showed that all biological parameters intercorrelated to one another and negatively with LSI in all explored tissues. PCA revealed connection between MN, NB, 8-shaped, biological parameters and gross morphometric indices in blood. In liver significant loadings of biological parameters, gross morphometric indices (PC1) and NAs (PC2) on different PCs suggested that these parameters were influenced by other factors. PC1 revealed relation between BL, 8-shaped, gross morphometric indices and biological parameters, except Qlw, whereas relation between MN, NB, VacNuc, LSI and Qlw was revealed on PC2 in kidney. A correlation between MN frequency and NB, deformed and vacuolated nucleus was found in irradiated fish Catla (*Catla catla*) in Anbumani and Mohankumar [59] study.

Cavas et al. [4] suggest that use of different tissues other than peripheral blood in micronucleus assays brings better results. The results of our study showed that copper–zinc mixture was genotoxic for all the three tissues analyzed after exposure for 4 days. Our data demonstrates that rainbow trout peripheral blood was a more sensitive tissue and better indicator of the genotoxicity of Cu and Zn than either kidney or liver erythrocytes. The higher recovery rate was observed in blood tissue. The tissue-specific Cu and Zn geno-cytotoxicity data in fish is scarce. The tissue specificity depends on the bioaccumulation of toxicants, different sensitivity and defensive mechanism of the certain organ [31,32]. In teleost fish, the main erythropoietic tissue is a cephalic kidney; cell division may be more active in this tissue [27,32]. In fish, liver and kidney play a major role in the detoxification and excretion of toxins via the induction of metal-binding proteins such as metallothioneins [60]. In general, elimination routes of metals from fish are through bile, urine, gills and mucus [61,62]. Kidneys are more susceptible to oxidative stress than liver, which is the primary site for the biotransformation of xenobiotic compounds [32]. Cavas et al. [4] observed that in fish species (*Cyprinus carpio*, *Carassius gibelio*, *Corydoras paleatus*) their various tissues showed differential sensitivity to cadmium (0.005–0.1 mg/L) and copper (0.01–0.25 mg/L) treatment for 21 days. Gill and liver cells were more sensitive than peripheral blood to micronucleus and binuclear inducing agents. Dubey and Tripathi [63] found that the highest MN frequencies were observed in gills followed by blood and least in kidney cells after spotted snakehead (*Channa punctatus*) exposure to cadmium chloride.

5. Conclusions

The present study revealed, that binary-metal mixture of copper and zinc causes geno-cytotoxicity effects in blood, liver and kidney erythrocytes, changes in gross morphometric indices (LSI, CF) and biological parameters of rainbow trout. During the depuration period, tissue-specific recovery trend and time-related fluctuations of geno-cytotoxicity levels were detected in the fish. Micronuclei incidences were found to increase significantly after 4-day recovery (in 0.125 group) in all tissues. Significant recovery was observed in 0.0625 group estimating the formation of MN in erythrocytes of blood (after 12-day recovery), of 8-shaped nuclei in liver (after 8- and 12-day) and kidney erythrocytes (after 8-day recovery). Blood erythrocytes showed significant recovery of all analysed geno-cytotoxicity endpoints after 8 and 12 days in 0.25 group, however, recovery in this group was not observed after 12-day of depuration in liver and kidney erythrocytes. Further investigations related to geno-cytotoxicity of heavy metals mixtures in different fish tissues will add new information on impact mechanisms, and recovery peculiarities during the depuration process.

Conflict of interest

None

Acknowledgments

We are thankful to Dalia Baršytė Lovejoy (Toronto University) and Irena Žalakevičienė for the language check.

References

[1] N. Cedergreen, Quantifying synergy: a systematic review of mixture toxicity studies within environmental toxicology, PLoS One 9 (5) (2014) e96580.

[2] C. Bolognesi, M. Hayashi, Micronucleus assay in aquatic animals, Mutagenesis 26 (1) (2011) 205–213.

[3] T. Cavas, S. Ergene-Gozukara, Induction of micronuclei and nuclear abnormalities in *Oreochromis niloticus* following exposure to petroleum refinery and chromium processing plant effluents, Aquat. Toxicol. 74 (2005) 264–271.

[4] T. Cavas, N.N. Garanko, V.V. Arkhipchuk, Induction of micronuclei and binuclei in blood, gill and liver cells of fishes subchronically exposed to cadmium chloride and copper sulphate, Food Chem. Toxicol. 43 (2005) 569–574.

[5] C.M. Wood, Toxic responses of the gill, in: D. Schlenck, W.H. Benson (Eds.), Target Organ Toxicity in Marine and Freshwater Teleosts, vol. 1, Organs. Taylor and Francis, London, New York, 2001, pp. 1–89.

[6] R. Sappal, J. Burka, S. Dawson, C. Kamunde, Bioaccumulation and sub-cellular partitioning of zinc in rainbow trout (*Oncorhynchus mykiss*): cross-talk between waterborne and dietary uptake, Aquat. Toxicol. 91 (2009) 281–290.

[7] J.J. Vicente-Martorell, M.D. Galindo-Riano, M. Garcia-Vargas, Bioavailability of heavy metals monitoring water, sediments and fish species from a polluted estuary, J. Hazard. Mater. 162 (2009) 823–836.

[8] N.R. Bury, P.A. Walker, C.N. Glover, Nutritive metal uptake in teleost fish, J. Exp. Biol. 206 (2003) 11–23.

[9] I. Ahmad, M. Oliveira, M. Pacheco, M.A. Santos, *Anguilla anguilla* L. oxidative stress biomarkers responses to copper exposure with or without β-naphtoflavone pre-exposure, Chemosphere 61 (2005) 267–275.

[10] S.K. Bopp, H.K. Abicht, K. Knauer, Copper-induced oxidative stress in rainbow trout gill cells, Aquat. Toxicol. 86 (2008) 197–204.

[11] M. Fenech, W.P. Chang, M. Kirsch-Volders, N. Holland, S. Bonassi, E. Zeiger, HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures, Mutat. Res. 534 (2003) 65–75.

[12] J.A. Heddle, M.C. Cimino, M. Hayashi, F. Romagna, M.D. Shelby, J.D. Tucker, P. Vanparys, J.T. MacGregor, Micronuclei as an index of cytogenetic damage: past, present, and future, Environ. Mol. Mutagen. 18 (4) (1991) 277–291.

[13] M. Fatima, N. Usmani, M. Mobarak Hossain, M.F. Siddiqui, M.F. Zafeer, F. Firdaus, S. Ahmad, Assessment of genotoxic induction and deterioration of fish quality in commercial species due to heavy-metal exposure in an urban reservoir, Arch. Environ. Contam. Toxicol. 67 (2014) 203–213.

[14] A.S.A. Harabawy, Y.Y.I. Mosleh, The role of vitamins A, C E and selenium as antioxidants against genotoxicity and cytotoxicity of cadmium, copper, lead and zinc on erythrocytes of *Nile tilapia*, *Oreochromis niloticus*, Ecotoxicol. Environ. Saf. 104 (2014) 28–35.

[15] J. Baršienė, L. Butrimavičienė, A. Michailovas, W. Grygiel, Assessing the environmental genotoxicity risk in the Baltic Sea: frequencies of nuclear buds in blood erythrocytes of three native fish species, Environ. Monit. Assess. 187 (1) (2015) 4078.

[16] K.R. Carrasco, K.L. Tilbury, M.S. Myers, Assessment of the piscine micronucleus test as an *in situ* biological indicator of chemical contaminant effects, Can. J. Fish. Aquat. Sci. 47 (1990) 2123–2136.

[17] H.K. Lindberg, X. Wang, H. Järventaus, G.C.M. Falck, H. Norppa, M. Fenech, Origin of nuclear buds and micronuclei in normal and folate-deprived human lymphocytes, Mutat. Res. 617 (2007) 33–45.

[18] L. Serrano-Garcia, R. Montero-Montoya, Micronuclei and chromatid buds are the result of related genotoxic events, Environ. Mol. Mutagen. 38 (1) (2001) 38–45.

[19] J. Crott, M. Fenech, Preliminary study of the genotoxic potential of homocysteine in human lymphocytes in vitro, Mutagenesis 16 (2001) 213–217.

[20] F.N. Ghadially, Ultrastructural Pathology of the Cell and Matrix, Butterworths, London, 1982.

[21] M. Fenech, Cytokinesis-block micronucleus cytome assay, Nat. Protoc. 2 (5) (2007) 1084–1104.

[22] J. Baršienė, L. Butrimavičienė, W. Grygiel, T. Lang, A. Michailovas, T. Jackūnas, Environmental genotoxicity and cytotoxicity in flounder (*Platichthys flesus*), herring (*Clupea harengus*) and Atlantic cod (*Gadus morhua*) from chemical munitions dumping zones in the southern Baltic Sea, Mar. Environ. Res. 96 (2014) 56–67.

[23] M. Mičić, N. Bihari, Z. Jaksic, E.G. Muller, R. Batel, DNA damage and apoptosis in the mussel *Mytilus galloprovincialis*, Mar. Environ. Res. 53 (2002) 243–262.

[24] R.N. Oss, V.D. Baroni, R.M. Duarte, A.L. Val, V.M. Fonseca Almeida Val, L.C. Gomes, Recovery of fat snook, *Centropomus parallelus* (*Teleostei: Perciformes*) after subchronic exposure to copper, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 157 (2013) 306–309.

[25] V.V. Arkhipchuk, N.N. Garanko, Using the nucleolar biomarker and the micronucleus test on in vivo fish fin cells, Ecotoxicol. Environ. Saf. 62 (2005) 42–52.

[26] W. Jiraungkoorskul, S. Sahaphong, P. Kosai, M.H. Kim, Micronucleus test: the effect of ascorbic acid on cadmium exposure in fish (*Puntius altus*), Res. J. Environ. Toxicol. 1 (1) (2007) 27–36.

[27] J. Baršienė, V. Dedonytė, A. Rybakovas, L. Andreikenaitė, O.K. Andersen, Investigation of micronuclei and other nuclear abnormalities in peripheral blood and kidney of marine fish treated with crude oil, Aquat. Toxicol. 78S (2006) S99–S104.

[28] C. Agius, R.J. Roberts, Melano-macrophage centres and their role in fish pathology, J. Fish Dis. 26 (2003) 499–509.

[29] G.J. Macchi, A. Romanol, H.E. Christiansen, Melanomacrophage centres in white mouth croaker *Micropogonias* furneri, as biological indicators of environmental changes, J. Fish Biol. 40 (1992) 971–973.

[30] I. Udroiu, The micronucleus test in piscine erythrocytes, Aquat. Toxicol. 79 (2006) 201–204.

[31] I. Ahmad, V.L. Maria, M. Oliveira, M. Pacheco, M.A. Santos, Oxidative stress and genotoxic effects in gill and kidney of *Anguilla anguilla* L. exposed to chromium with or without pre-exposure to beta-naphthoflavone, Mutat. Res. 608 (1) (2006) 16–28.

[32] V. Velma, P.B. Tchounwou, Chromium induced biochemical, genotoxic and histopathologic effects in liver and kidney of Goldfish, *Carassius auratus*, Mutat. Res. 698 (1–2) (2010) 43–51.

[33] K.K. Yadav, S.P. Trivedi, Sublethal exposure of heavy metals induces micronuclei in fish, *Channa punctata*, Chemosphere 77 (2009) 1495–1500.

[34] M.O. Obiakor, C.D. Ezeonyejiaku, Copper–zinc coergisms and metal toxicity at predefined ratio concentrations: predictions based on synergistic ratio model, Ecotoxicol. Environ. Saf. 117 (2015) 149–154.

[35] E. Bagdonas, M.Z. Vosylienė, A study of toxicity and genotoxicity of copper, zinc and their mixture to rainbow trout (*Oncorhynchus mykiss*), Biologija 1 (2006) 8–13.

[36] M.O. Obiakor, J.C. Okonkwo, C.D. Ezeonyejiaku, C.O. Ezenwelu, Genotoxicology: single and joint action of copper and zinc to *Synodontis clarias* and *Tilapia nilotica*, J. Appl. Sci. Environ. Manage. 14 (3) (2010) 59–64.

[37] L. Andreikėnaitė, J. Baršienė, M.Z. Vosylienė, Studies of micronuclei and other nuclear abnormalities in blood of rainbow trout (*Oncorhyncus mykiss*) treated with heavy metal mixture and road maintenance salts, Acta Zool. Lituanica 17 (3) (2007) 213–219.

[38] M. Oliveira, A. Serafim, M.J. Bebianno, M. Pacheco, M.A. Santos, European ell (*Anguilla anguilla* L.) metallothionein endocrine, metabolic and genotoxic responses to copper exposure, Ecotoxicol. Environ. Saf. 70 (2008) 20–26.

[39] G. Svecevičius, M.Z. Vosylienė, Acute toxicity of copper to common freshwater fishes of Lithuania, Ekologija 2 (1996) 17–21.

[40] G. Svecevičius, Acute toxicity of zinc to common freshwater fishes of Lithuania, Acta Zool. Lituanica Hydrobiol. 9 (2) (1999) 114–118.

[41] G. Svecevičius, G. Sauliutė, R.L. Idzelis, J.G. Grigelevičiūtė, Accumulation of heavy metals in different body tissues of Atlantic salmon, *Salmo salar* L., exposed to a model mixture (Cu, Zn, Ni, Cr, Pb, Cd) and singly to nickel, chromium, and lead, Bull. Environ. Contam. Toxicol. 92 (4) (2014) 440–445.

[42] Directive 2008/105/EC of the European Parliament and of the Council of 496 16 December 2008 on environmental quality standards in the field of water 497 policy, amending and subsequently repealing Council Directives 82/176/EEC, 498 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 499 2000/60/EC of the European Parliament and of the Council. Official Journal of the European Communities L 500 348, 24/12/2008 p. 0084–0097.

[43] ICES Report of Study Group on Integrated Monitoring of Contaminants and Biological Effects (SCIMC), 14–18 March 2011, Copenhagen, Denmark; (2011), p.57. [ICES CM 2011/ACOM].

[44] J. Baršienė, J. Lazutka, J. Šyvokienė, V. Dedonytė, A. Rybakovas, A. Bjornstad, O.K. Andersen, Analysis of micronuclei in blue mussels and fish from the Baltic and the North Seas, Environ. Toxicol. 19 (2004) 365–371.

[45] C.T. de Lemos, P.M. Rodel, N.R. Terra, B. Erdtmann, Evaluation of basal micronucleus frequency and hexavalent chromium effects in fish erythrocytes, Environ. Toxicol. Chem. 20 (2001) 1320–1324.

[46] S. Sanchez-Galan, A.R. Linde, F. Ayllon, E. Garcia-Vazquez, Induction of micronuclei in eel (*Anguilla anguilla* L.) by heavy metals, Ecotoxicol. Environ. Saf. 49 (2001) 139–143.

[47] M.V.M. Ferraro, A.S. Fenocchio, M.S. Mantovani, C. de Oliveira Ribeiro, M.M. Cestari, Mutagenic effects of tributyltin and inorganic lead (P BII) on the fish *H. malabaricus* as evaluated using the comet assay and the piscine micronucleus and chromosome aberration tests, Genet. Mol. Biol. 27 (2004) 103–107.

[48] J.I.R. Porto, C.S.O. Araujo, E. Feldberg, Mutagenic effects of mercury pollution as revealed by micronucleus test on three Amazonian fish species, Environ. Res. 97 (2005) 287–292.

[49] S. Ergene, T. Cavas, A. Celik, N. Koleli, F. Kaya, A. Karahan, Monitoring of nuclear abnormalities in peripheral erythrocytes of three fish species from the Goksu Delta (Turkey): genotoxic damage in relation to water pollution, Ecotoxicology 16 (2007) 385–391.

[50] R. Jindal, S. Verma, In vivo genotoxicity and cytotoxicity assessment of cadmium chloride in peripheral erythrocytes of *Labeo rohita* (Hamilton), Ecotoxicol. Environ. Saf. 118 (2015) 1–10.

[51] J.C. Nepomuceno, I. Ferrari, M.A. Spano, A.C. Centeno, Detection of micronuclei in peripheral erythrocytes of *Cyprinus carpio* exposed to metallic mercury, Environ. Mol. Mutagen. 30 (1997) 293–297.

[52] E.M. Santos, J.S. Ball, T.D. Williams, H. Wu, F. Ortega, R. van Aerle, I. Katsiadaki, F. Falciani, M.R. Viant, J.K. Chipman, C.R. Tyler, Indentifying health impacts of exposure to copper using transcriptomics and metabolomics in a fish model, Environ. Sci. Technol. 44 (2) (2010) 820–826.

[53] C.K. Grisolia, C.M.T. Cordeiro, Variability in micronucleus induction with different mutagens applied to several species of fish, Genet. Mol. Biol. 23 (1) (2000) 235–239.

[54] W.A. Omar, K.H. Zaghloul, A.A. Abdel-Khalek, S. Abo-Hega, Genotoxic effects of metal pollution in two fish species *Oreochromis niloticus* and *Mugil cephalus*, from highly degraded aquatic habitats, Mutat. Res. 746 (2012) 7–14.

[55] X.J. Liu, Z. Luo, B.X. Xiong, X. Liu, Y.H. Zhao, G.F. Hu, G.J. Lv, Effect of waterborne copper exposure on growth, hepatic enzymatic activities and histology in *Synechogobius hasta*, Ecotoxicol. Environ. Saf. 73 (2010) 1286–1291.

[56] J.L. Zheng, Z. Luo, Q.L. Chen, X. Liu, C.X. Liu, Y.H. Zhao, Y. Gong, Effect of waterborne zinc exposure on metal accumulation, enzymatic activities and

histology of *Synechogobius hasta*, Ecotoxicol. Environ. Saf. 74 (2001) 1864–1873.

[57] W. Bonga, R.A.C. Lock, Toxicants and osmoregulation in fish, Neth. J. Zool. 42 (2–3) (1992) 478–493.

[58] P.P. Ciji, S.B. Nandan, Toxicity of copper and zinc to *Puntius parrah* (Day, 1865), Mar. Environ. Res. 93 (2014) 8–46.

[59] S. Anbumani, M.N. Mohankumar, Gamma radiation induced micronuclei and erythrocyte cellular abnormalities in the fish *Catla catla*, Aquat. Toxicol. 122–123 (2012) 125–132.

[60] G. Roesijadi, W.E. Robinson, Metal Regulation in Aquatic Animals: Mechanisms of Uptake, Accumulation and Release, Aquatic Toxicology, Molecular, Biochemical and Cellular Perspectives, in: D.C. Malins, G.K. Ostrander (Eds.), CRC Press, Boca Raton, FL, US, 1994, pp. 387–420.

[61] U. Varanasi, D. Markey, Uptake and release of lead and cadmium in skin and mucus of coho salmon (*Oncorhynchus kisutch*), Comp. Biochem. Physiol. C 60 (1978) 187–191.

[62] S.G. Kim, J.H. Jee, J.C. Kang, Cadmium accumulation and elimination in tissues of juvenile olive flounder, *Paralichthys olivaceus* after sub-chronic cadmium exposure, Environ. Pollut. 127 (2004) 117–123.

[63] A. Dubey, N.K. Tripathi, Evaluation of time, dose and tissue dependent genotoxic effect of cadmium chloride in a fresh water fish, *channa punctatus* (*pisces*: family–*channidae*), Int. J. Recent. Sci. Res. 5 (5) (2014) 934–939.

II

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

Stankevičiūtė M, Sauliutė G, Svecevičius G, Kazlauskienė N, Baršienė J

Ecotoxicology 2017, 26(8): 1051–1064

Reprinted with permission from Springer Nature

Ecotoxicology
DOI 10.1007/s10646-017-1833-0

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

Milda Stankevičiūtė[1] · Gintarė Sauliutė[1] · Gintaras Svecevičius[1] · Nijolė Kazlauskienė[1] · Janina Baršienė[1]

Accepted: 21 June 2017
© Springer Science+Business Media, LLC 2017

Abstract Health impact of metal mixture at environment realistic concentrations are difficult to predict especially for long-term effects where cause-and-effect relationships may not be directly obvious. This study was aimed to evaluate metal mixture (Zn—0.1, Cu—0.01, Ni—0.01, Cr—0.01, Pb—0.005 and Cd—0.005 mg/L, respectively for 1, 2, 4, 7, 14 and 28 days at concentrations accepted for the inland waters in EU) genotoxicity (micronuclei, nuclear buds, nuclear buds on filament), cytotoxicity (8-shaped nuclei, fragmented-apoptotic erythrocytes), bioaccumulation, steady-state and the reference level of geno-cytotoxicity in hatchery-reared Atlantic salmon tissues. Metals accumulated mostly in gills and kidneys, to the lesser extent in the muscle. Uptake of metals from an entire mixture in the fish for 14 days is sufficient to reach steady-state Cr, Pb concentrations in all tissues; Zn, Cu—in kidneys and muscle, Ni—in liver, kidneys, muscle and Cd—in muscle. Treatment with metal mixture significantly increased summed genotoxicity levels at 7 days of exposure in peripheral blood and liver erythrocytes, at 14 days of exposure in gills and kidney erythrocytes. Significant elevation of cytotoxicity was detected after 2 and 14 days of exposure in gills erythrocytes and after 28 days—in peripheral blood erythrocytes. The amount of Cu, Cr, Pb and Cd accumulated in tissues was dependent upon duration of exposure; nuclear buds, 8-shaped nuclei frequencies also were dependent upon duration of exposure. This study indicates that metals at low levels when existing in mixture causes significant geno-cytotoxicity responses and metals bioaccumulation in salmon.

Keywords Genotoxicity · Cytotoxicity · *Salmo salar* · Bioaccumulation · Metal mixture · Steady-state

✉ Milda Stankevičiūtė
milda.stan@gmail.com

[1] Nature Research Centre, Akademijos 2, 08412 Vilnius, Lithuania

Introduction

The focus of metal toxicology has shifted to investigating more subtle, chronic, low-dose effects, where cause-and-effect relationships may not be directly obvious and may greatly differ from the effects of the acute exposure (Klaassen et al. 2013). Metals (Zn, Cu, Ni, Cr, Pb, Cd) are categorized as priority hazardous substances (pollutants) in many countries all over the world due to their toxicity, persistence, and propensity for bioaccumulation. Many of them are perfect toxic indicators of general ambient water quality (US EPA 2009; Scorecard 2011). Genotoxicity, cytotoxicity of single metals such as Zn, Cu, Ni, Cr, Cd, Pb to different fish species was emphasized by many authors (Ahmed et al. 2013; Arunachalam et al. 2013; Arkhipchuk and Garanko 2008; Cavas et al. 2005; Jindal and Verma 2015; Jiraungkoorskul et al. 2007; Kousar and Jeved 2015; Palermo et al. 2015). The potential of metal induced damage to the genetic material was evaluated using high concentrations (critically exceeding the permitted standards in the natural water environment).

Studies associated with complex metal mixture geno-cytotoxicity and bioaccumulation are scarce, especially considering environment realistic (environmentally-relevant) metal mixture concentrations. In view of recent publications dealing with metal mixture toxicity, only several studies use complex metal mixtures of more than two

Published online: 01 July 2017

metals (Zhang et al. 2008; Zhu et al. 2004). According to SCHER, SCCS, SCENIHR, Opinion on the Toxicity and Assessment of Chemical Mixtures (2012) evaluation of risk assessment of chemical mixtures needs to take into consideration the potential mixture effects at realistic exposure levels in the environment, the possible impact of background exposure in the environment or diet, and health risks at low dose exposures to multiple chemicals. Most studies on the effects of metals on fish examine one particular metal. Several studies showed that low concentrations of some single metals do not induce genotoxicity in fish (Zhu et al. 2004). However, low concentrations of metals in a complex mixture may act synergistically, thus resulting in drastically different levels of toxicity (Zhu et al. 2004). It seems that interactions between different metals are related to their competitive uptake from the environment and different distribution in fish tissues, which results in some metals influencing the accumulation of other metals in fish (Jezierska and Witeska 2001; Svecevičius et al. 2014). As concluded by SCHER, SCCS, SCENIHR, Opinion on the Toxicity and Assessment of Chemical Mixtures (2012) interactions of chemicals in mixtures are difficult to predict especially for long-term effects. However, research studies focusing on long-term geno-cytotoxicity evaluation of realistic metal concentrations in different fish species are rare (Zhu et al. 2004).

Geno-cytotoxicity (genotoxicity endpoints—micronuclei (MN), nuclear buds (NB), nuclear buds on filament (NBf) and cytotoxicity endpoints—8-shaped nuclei and fragmented-apoptotic (FA) erythrocytes) evaluation of Atlantic salmon (*Salmo salar*), an ecologically and economically important fish species in the Europe, in response to xenobiotics has been scantily investigated. Micronucleus assay in Atlantic salmon peripheral blood erythrocytes was previously used to evaluate genotoxicity of uranium (Song et al. 2012). To the best of our knowledge, this is the first research study evaluating geno- and cytotoxicity of complex metal ions mixture to Atlantic salmon. 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 metal mixture. While, many researchers prefer unreasoned exposure time ranging from several hours to 270 days (Komjarova and Blust 2009; 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 present study evaluated metal mixture geno-cytotoxicity in peripheral blood, gills, kidneys and liver erythrocytes, and accumulation in gills, liver, kidneys and muscle tissues of Atlantic salmon. The potential of combination of the most common metal contaminants (Zn, Cu, Ni, Cr, Pb and Cd) to induce tissue-specific and time-related geno-cytotoxicity and metal bioaccumulation in Atlantic salmon was assessed. This study was designed to evaluate metal mixture genotoxicity, cytotoxicity, bioaccumulation and steady-state in Atlantic salmon tissues by testing the mixture as a whole (whole mixture approach). According to Heys et al. (2016), on initial examination, whole mixture approach is the most logical because it reflects the simultaneous exposure that organisms in the environment face. Using this type of approach all interactions between the components in the mixture are accounted for.

The main objectives of the present study were: (1) to assess bioaccumulation of metals in different tissues (gills, liver, kidneys and muscle) of Atlantic salmon after exposure to complex metal mixture at a concentration corresponding to Maximum-Permissible-Concentrations (MPC) accepted for the inland waters, (2) to experimentally measure the time necessary to attain the steady-state Zn, Cu, Ni, Cr, Pb, Cd concentrations in analysed tissues, (3) to assess geno- and cytotoxicity in erythrocytes from different tissues and fluctuations of such responses during the exposure period.

Material and methods

Experimental set-up

The test was conducted on hatchery-reared 1-year-old Atlantic salmon (*Salmo salar* Linnaeus, 1758) smolts, average total weight 46.1 ± 8.2 g and average total length 171.0 ± 9.3 mm (mean ± SD, $N = 70$, respectively). The fish was obtained from Meškerinė fish hatchery (Švenčionys District, Lithuania) and 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 their behavior became normal and they feed well. Deep-well water was used as the dilution water. Its chemical characteristics are given in Table 1 (ISO 15586 2003; ISO 6332 1988; ISO 5814 1990; ISO 10523 2008; ISO 14911 1998; ISO 10304-1 2007; ISO 9963-1 1994).

Reagent grade metal salts («REACHIM» Company, Russia) were used as the toxicants. Stock solution was

Table 1 Chemical and physical characteristics of the dilution water (all values are in mg/L, unless otherwise noted)

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				

prepared by dissolving necessary amount of the salt in distilled water, the final concentration being recalculated according to the amount of metal ion.

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 aerated 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 "3 Rs" – Reduce the number of animals used to a minimum, to obtain information from a smaller number of animals) (Directive 2010/63/EU). Metal bioaccumulation in tissues, genotoxicity and cytotoxicity levels in fish peripheral blood, gills, kidneys and liver erythrocytes from non-exposed, 7, 14 and 28 days controls are represented in Table 2. No significant ($p > 0.05$) differences among evaluated parameters were measured between controls and non-exposed groups. Statistical analysis between 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.

Test fish were exposed for the 1, 2, 4, 7, 14 and 28 days period to a six metal (Zn, Cu, Ni, Cr, Pb and Cd) mixture at a concentration corresponding to maximum-permissible-concentrations (MPC) accepted for the inland waters in EU (Directive 2008/105/EC) (Table 3). Test solutions and clean water were renewed every day, and test fish were transferred into freshly prepared solutions after they were fed.

Analytical procedures

The main physico-chemical parameters of the water (temperature, dissolved O_2, pH and conductivity) were measured routinely with a hand-held multi-meter (WTW Multi 340i/SET, Germany). Designed nominal metal concentrations in the tanks were checked during blank tests (without fish) ($N = 4$) with an atomic absorption spectrophotometer (SHIMADZU AA-6800, Japan) by graphite furnace technique using proprietary software. Each water sample was acidified with reagent-grade nitric acid (final concentration 0.5% v/v) and analysed in triplicate. Mean measured concentrations were within 5–20% of the target (Table 3).

Metal bioaccumulation analysis

After the testing was completed, fish (of control and metal-exposed groups) were sacrificed. Fish were measured (total body length, mm) and weighed (total body weight, gram). Later, they were used in the removal of needed tissues: muscle without skin (~3 g), gills (whole organ), liver (whole organ) and kidneys (whole organ); organs were weighed to an accuracy of ± 0.001 g. Fish samples were hot air oven-dried at 85 °C for 24 h until reached constant weight, pre-digested tightly in a concentrated ultrapure HNO_3 (60%) and H_2O_2 (30%) (Lach-Ner, Chempur, respectively) at a ratio of 5:1 v/v for 8 h at a room temperature and then microwave-digested quickly (Jia et al. 2005). After cooling solutions were filtered through a 0.45 µm glass filter and diluted with deionized water. Metal concentrations were measured by atomic absorption spectrophotometry on Varian Spectr AA 55 (USA) with a graphite furnace technique in accordance with standardized procedure (ISO 15586 2003) final concentration being expressed as mg/kg of wet weight. Accuracy of analytical procedure was checked using certified reference material fish homogenate (IAEA–407). Recoveries were in acceptable range (within 10%) of the certified values.

Micronucleus (MN) and erythrocyte nuclear abnormalities (ENAs) analysis in *in vivo* assay

Micronucleus (MN) and erythrocyte nuclear abnormalities (ENAs) analysis was performed in peripheral blood, gills, kidneys and liver erythrocytes. Blood was immediately

Table 2 Metal bioaccumulation in tissues and genotoxicity–cytotoxicity levels in erythrocytes of non-exposed and control fish

Metal bioaccumulation, mg/kg of w/w (±SD); genotoxicity, cytotoxicity, ‰ (±SE)					
Tissue		Non-exposed	Control 7 days	Control 14 days	Control 28 days
Gills	Zn	30.2 ± 4.54	30.1 ± 4.21	28.6 ± 4.28	29.2 ± 3.42
	Cu	0.645 ± 0.091	0.697 ± 0.060	0.696 ± 0.099	0.759 ± 0.078
	Ni	0.060 ± 0.004	0.060 ± 0.006	0.053 ± 0.007	0.052 ± 0.005
	Cr	0.075 ± 0.008	0.075 ± 0.006	0.076 ± 0.030	0.077 ± 0.029
	Pb	0.010 ± 0.001	0.011 ± 0.002	0.012 ± 0.002	0.013 ± 0.003
	Cd	0.049 ± 0.010	0.049 ± 0.005	0.046 ± 0.008	0.048 ± 0.006
	MN + NBf + NB	0.11 ± 0.05	0.43 ± 0.14	0.11 ± 0.05	0.21 ± 0.12
	FA + 8-shaped	0 ± 0	0.04 ± 0.04	0 ± 0	0.04 ± 0.04
Liver	Zn	20.2 ± 3.79	20.3 ± 3.33	21.0 ± 2.93	21.4 ± 1.17
	Cu	4.83 ± 0.704	4.81 ± 0.379	4.91 ± 0.826	4.82 ± 0.775
	Ni	0.032 ± 0.003	0.033 ± 0.004	0.028 ± 0.013	0.032 ± 0.010
	Cr	0.055 ± 0.006	0.056 ± 0.006	0.049 ± 0.005	0.050 ± 0.004
	Pb	0.007 ± 0.001	0.007 ± 0.001	0.006 ± 0.001	0.006 ± 0.001
	Cd	0.020 ± 0.003	0.018 ± 0.002	0.016 ± 0.004	0.019 ± 0.004
	MN + NBf + NB	0.04 ± 0.04	0.26 ± 0.11	0.11 ± 0.05	0.25 ± 0.09
	FA + 8-shaped	0.04 ± 0.04	0.07 ± 0.05	0.07 ± 0.05	0.07 ± 0.05
Kidneys	Zn	16.6 ± 2.24	16.9 ± 1.53	17.7 ± 1.95	17.9 ± 1.52
	Cu	0.793 ± 0.094	0.807 ± 0.126	0.783 ± 0.128	0.793 ± 0.131
	Ni	0.056 ± 0.004	0.057 ± 0.004	0.051 ± 0.012	0.054 ± 0.012
	Cr	0.116 ± 0.013	0.119 ± 0.012	0.118 ± 0.035	0.112 ± 0.024
	Pb	0.020 ± 0.003	0.021 ± 0.005	0.018 ± 0.002	0.019 ± 0.002
	Cd	0.058 ± 0.010	0.056 ± 0.007	0.062 ± 0.010	0.063 ± 0.010
	MN + NBf + NB	0.04 ± 0.04	0.07 ± 0.05	0.07 ± 0.05	0.04 ± 0.04
	FA + 8-shaped	0 ± 0	0.04 ± 0.04	0.04 ± 0.04	0 ± 0
Muscle	Zn	0.653 ± 0.099	0.666 ± 0.093	0.956 ± 0.412	0.892 ± 0.367
	Cu	0.513 ± 0.052	0.507 ± 0.052	0.429 ± 0.092	0.418 ± 0.143
	Ni	0.022 ± 0.004	0.020 ± 0.005	0.021 ± 0.001	0.019 ± 0.007
	Cr	0.022 ± 0.003	0.022 ± 0.003	0.023 ± 0.003	0.020 ± 0.004
	Pb	0.011 ± 0.001	0.010 ± 0.002	0.009 ± 0.005	0.010 ± 0.005
	Cd	0.002 ± 0.0003	0.001 ± 0.0002	0.001 ± 0.0004	0.001 ± 0.0004
Blood	MN + NBf + NB	0.25 ± 0.05	0.5 ± 0.17	0.43 ± 0.11	0.25 ± 0.08
	FA + 8-shaped	0 ± 0	0.07 ± 0.07	0.07 ± 0.05	0 ± 0

Table 3 Metals and their test waterborne concentrations (mg/L) in test media

Metal	Source	Concentration (mg/L)	
		Maximum-permissible-Concentration (MPC)	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

taken from the caudal vein. A drop of blood was directly smeared on microscopic slides and air-dried. After the sacrifice, small pieces of cephalic kidneys, liver and gills were dissected, softly dragged along clean slide and allowed to dry for 1–2 h (Baršienė et al. 2006). Dried smears were fixed in methanol for 10 min and were stained with 10% Giemsa solution in phosphate buffer pH = 6.8 for 8 min (Baršienė et al. 2004). The stained slides were analysed under bright-field microscopes Olympus BX51 (Tokyo, Japan) using an immersion objective (1000×) and the photos were taken with an Olympus U-CMAD3 (Tokyo, Japan) camera. Four thousands erythrocytes with intact cellular and nuclear membrane per fish were evaluated using blind scoring by a single observer. Total 28,000 erythrocytes were analysed in each treatment group.

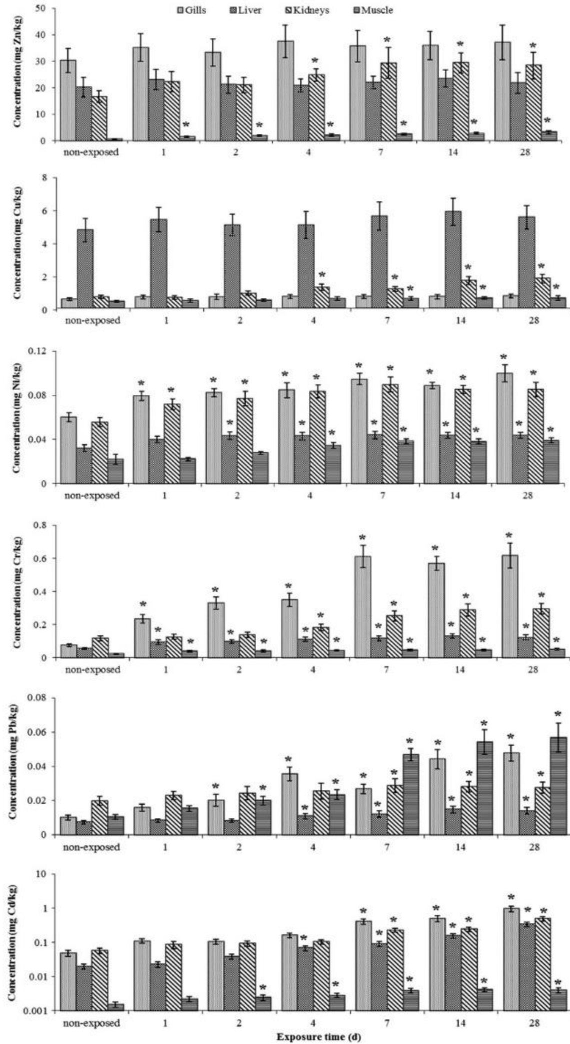


Fig. 1 Metal (Zn, Cu, Ni, Cr, Pb and Cd) concentration (mg/kg of w/w) in body tissue (gills, liver, kidneys and muscle) of Atlantic salmon (mean ± SD, $N = 7$). *Asterisks* (*) denote values significantly different from non-exposed group during exposure time ($p < 0.05$)

Final results were expressed as the mean value (‰) of sums of analysed individual lesions scored in 1000 erythrocytes per fish sampled from every study group. The formation of micronuclei (MN), nuclear buds (NB), nuclear buds on filament (NBf) (as genotoxicity endpoints), 8-shaped nuclei and fragmented-apoptotic (FA) (as cytotoxicity endpoints),

kidney-shaped, blebbed (BL), vacuolated (VacNuc) erythrocytes were identified using criteria described by Fenech et al. (2003) and Baršienė et al. (2014). The genotoxicity levels were assessed as the sum of the frequencies of micronuclei, nuclear buds and nuclear buds on filament (MN + NB + NBf). Cytotoxicity levels were assessed as the sum of the frequencies of fragmented-apoptotic and 8-shaped erythrocytes (FA + 8-shaped).

Data analysis and statistics

Significance of differences between the non-exposed, control and treated groups were tested using one-way analysis of variance ANOVA followed by Bonferroni post hoc test through STATISTICA 7.0 (StatSoft Inc., Tulsa, Oklahoma, USA) software. The results were expressed as mean ± standard error or standard deviation. Multiple linear regressions were performed to assess if micronucleus and other ENAs frequencies and metals bioaccumulation were dependent upon duration of exposure. Spearman correlation coefficients (using GraphPad Prism® 5.01 (GraphPad Software Inc., San Diego, CA, USA)) were calculated in order to elucidate correlations between metal bioaccumulation and ENAs frequencies. The level of significance was established at $p < 0.05$.

To determine steady-state, a quantitative approach was used in the data analysis. The measurement of time necessary to attain the steady-state Zn, Cu, Ni, Cr, Pb and Cd concentrations was based on the mean metal level accumulated in specific tissue and their statistical (one-way analysis of variance ANOVA followed by Bonferroni post hoc test) differences during the exposure period.

Results

Analysis of metals in fish

The levels of accumulation for Zn, Cu, Ni, Cr, Pb and Cd (means ± SD (mg/kg of w/w)) in gills, liver, kidneys and muscle of Atlantic salmon are presented on Fig. 1. Statistically significant metal accumulation was observed in gills

Table 4 The time necessary to attain the steady-state Zn, Cu, Ni, Cr, Pb and Cd concentrations in analysed tissues of Atlantic salmon exposed to an entire mixture

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

(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. 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.

Data obtained showed that metal accumulation in Atlantic salmon was time-related and tissue specific, i.e. different tissues showed a different capacity for accumulating metals. 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.

The time necessary to reach the steady-state Zn, Cu, Ni, Cr, Pb, Cd concentrations in analysed tissues of Atlantic salmon exposed to an entire mixture is shown in Table 4. The time necessary to reach steady-state was metal specific and varied between organs. Zinc and Cu, which are

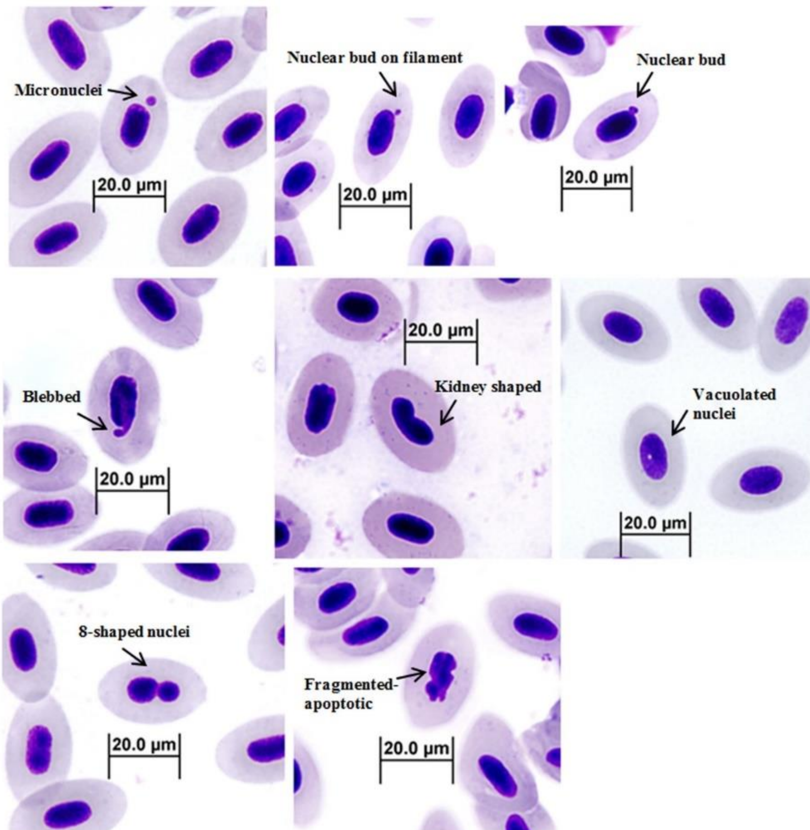


Fig. 2 Nuclear abnormalities in erythrocytes of Atlantic salmon

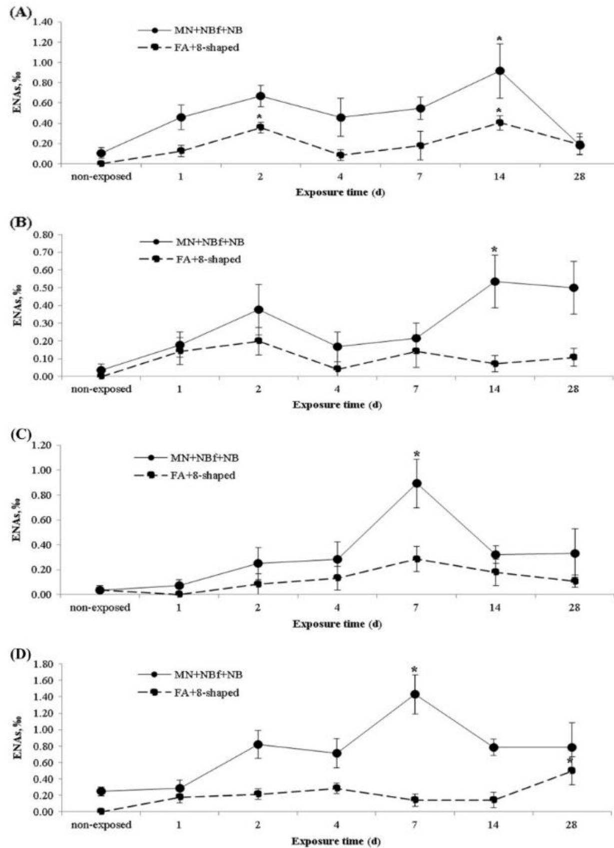


Fig. 3 The relationship between exposure time (**d**) and ENAs (summed genotoxicity (MN + NBf + NB) and cytotoxicity (FA + 8-shaped)) responses in Atlantic salmon: **a** gills, **b** liver, **c** kidneys and **d** peripheral blood erythrocytes (mean ± SEM, $N = 7$. *Asterisks* (*) denote significant differences from non-exposed group during exposure time ($p < 0.05$)

essential metals, did not reach steady-state in gills and liver, because no significant differences 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, kidneys 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 tissue, but at different exposure period. To summarize, steady-state for metals were reached within 14 days in most of the explored tissues.

Genotoxicity and cytotoxicity analysis

The erythrocytic nuclei abnormalities (ENAs) determined in Atlantic salmon blood were micronuclei (MN), nuclear bud on filament (NBf), nuclear bud (NB), blebbed (BL), kidney shaped, vacuolated (VacNuc), 8-shaped nuclei and

Table 5 Summary of the results obtained in the multiple linear regression analysis

$N = 196$	β	Standard error of β	B	Standard error of B	p-level	R^2
Exposure time						0.71
Intercept			−99.11	40.39	0.015	
Zn	−0.104	0.092	−1.817	1.614	0.262	
Cu	0.351	0.054	38.67	5.910	0.001	
Ni	−0.006	0.095	−58.79	881.40	0.947	
Cr	−0.361	0.093	−467.25	119.77	0.001	
Pb	0.558	0.052	8636.26	809.17	0.001	
Cd	0.821	0.070	842.11	72.14	0.001	
Exposure time						0.08
Intercept			136.29	26.21	0.001	
MN	−0.064	0.080	−41.89	52.25	0.424	
NB	0.159	0.073	143.13	65.62	0.030	
NBf	0.028	0.076	78.07	211.51	0.712	
BL	0.068	0.083	49.55	60.26	0.412	
8-shaped	0.157	0.075	208.58	100.30	0.039	
VacNuc	0.073	0.080	50.42	55.05	0.361	
Kidney shaped	−0.058	0.075	−63.26	81.90	0.441	
FA	0.015	0.076	27.81	139.12	0.842	

Relationship between duration of exposure (28 days) and metal concentration in Atlantic salmon body tissues (mg/kg of w/w) and erythrocytic nuclei abnormalities

β standardized coefficient, p level of statistical significance

fragmented-apoptotic (FA) erythrocytes. The morphological features of nuclear abnormalities are shown in Fig. 2.

Results for MN and other erythrocytic nuclei abnormalities assays in Atlantic salmon gills, liver, kidneys and peripheral blood erythrocytes, are given in Fig. 3a–d. Treatment with metal mixture significantly increased summed genotoxicity level at day 7 of exposure in liver and peripheral blood erythrocytes, and at day 14 of exposure in gills and kidneys erythrocytes. Statistically significant elevation of cytotoxicity was found after 2 and 14 days of exposure in gills erythrocytes and after 28 days —in peripheral blood erythrocytes. Significant induction of cytotoxicity was not observed in liver and kidneys erythrocytes. During the exposure period, levels of summed genotoxicity (MN + NBf + NB) were higher than summed cytotoxicity (FA + 8-shaped) levels in all analysed tissues. Fluctuations of geno- and cytotoxicity responses in different tissue erythrocytes were noted. The induction of genotoxicity and cytotoxicity declined at 4 days of exposure in peripheral blood, gills and kidneys erythrocytes, except for the cytotoxicity level in peripheral blood. In general, during all 28 days of exposure time, genotoxicity and cytotoxicity increased in exposed fish. Significant induction of other nuclear abnormalities such as BL and VacNuc was observed only in liver erythrocytes at day 7 while in gills erythrocytes at day 7 and day 14 of exposure, respectively. No significant elevation of kidney shaped nuclei erythrocytes was observed with the increasing exposure time.

Using multiple regression analysis, statistically significant relationship was found between the Cu, Cr, Pb and Cd concentrations in body tissues and duration of exposure also between the NB, 8-shaped nuclei frequency and duration of exposure (Table 5).

Relationship between geno-cytotoxicity responses and metals bioaccumulation in tissues

In the present study, a significant relationship between the bioaccumulation of several metals and the genetic damage was observed in different tissues. Interestingly in a few metal specific cases the correlations observed were tissue specific. Correlations were detected between Ni bioaccumulation in kidneys and summed cytotoxicity responses in kidneys erythrocytes (7 days, $r = 0.791$, $p = 0.048$) and Pb bioaccumulation in gills and summed cytotoxicity in gills erythrocytes (4 days, $r = 0.860$, $p = 0.024$) as well as Cu, Cd and Ni bioaccumulation in gills and summed cytotoxicity in blood (14 days, $r = 0.791$, $p = 0.048$; 28 days, 0.855, $p = 0.024$, respectively).

The summed genotoxicity and bioaccumulated metals correlated in: (a) gills: with Ni (7 days, $r = 0.882$, $p = 0.012$; 28 days, $r = 0.800$, $p = 0.034$); (b) liver: with Cu (4 days, $r = 0.767$, $p = 0.048$); (c) kidneys: with Zn and Pb

(2 days, $r = 0.764$, $p = 0.048$ and 7 days, $r = 0.771$, $p = 0.048$, respectively); (d) muscle: correlation with Cd bioaccumulation (28 days, $r = 0.811$, $p = 0.034$). No correlations were observed with Cr bioaccumulation and geno-cytotoxicity response in analysed tissues.

Discussion

This research sheds new light on tissue-specific and time-related geno-cytotoxicity responses and time necessary to reach the steady-state concentrations during exposure to an entire metal mixture. Mixture toxicity has received increasing attention, because it reveals realistic exposure scenario. However, geno-cytotoxicity of metal mixtures at environmentally realistic concentrations is not well explored. This study shows that the used complex metal mixture at MPC induced genotoxicity and cytotoxicity in different tissues erythrocytes of Atlantic salmon. The most obvious finding to emerge from the geno-cytotoxicity analysis in tissues is that different non-linear response between exposure time versus endpoints levels was determined. Peripheral blood erythrocytes showed the highest levels of summed genotoxicity and cytotoxicity. These results are in agreement with those obtained by Valskienė et al. (2015) and Stankevičiūtė et al. (2016). The highest ENAs frequencies in peripheral blood could be explained by the fact that peripheral blood receives erythrocytes and erythrocytes with nuclear abnormalities from several hematopoietic organs. Sites of erythropoiesis in teleost fish are concentrated predominantly in pro- and mesonephros (Saldatov 2005). The secondary hematopoietic organs such as periportal areas of the liver and gills may have different sensitivities to xenobiotics (Agius and Roberts 2003; Macchi et al. 1992). Several studies have shown genotoxicity or cytotoxicity induction in rainbow trout (*Oncorhynchus mykiss*) after exposure to the same metal mixture for 14 days (Valskienė et al. 2015), in carp (*Cyprinus carpio*) exposed to binary–ternary metals mixtures Cd + Cr, Cd + Cu, Cd + Cr + Cu and synergistic toxic effects of multiple metals were emphasized (Zhu et al. 2004). Complex effect of metal mixture causing DNA damage, also was measured using higher concentrations (0.5 mg/L of each) of Cd + Pb, Cd + Zn, Pb + Zn, Cd + Pb + Zn mixtures in *Misgurnus anguillicaudatus* (Zhang et al. 2008). Cu/Cd-co-exposed fish (0.1 ppm of each) showed slightly increased total nuclear abnormalities, while micronuclei frequencies were not significantly affected (Güner and Muranlı 2011). Erythrocytic nuclear abnormalities are genetic biomarkers of organism's exposure to genotoxic agents. Due to their ecological implications, they are considered excellent markers of exposure for assessing aquatic environments. It is well established that DNA damage is the most important cause of cancer, teratogenesis, embryotoxicity and genotoxic disease syndrome, like reduced productive and reproductive capacity (Ramsdorf et al. 2012). Therefore, disturbances in population and community-level dynamics may occur as an ecological impact due to these adverse effects (Nacci et al. 1996).

In the present research, for the first time, the reference level of geno-cytotoxicity in hatchery-reared salmon was evaluated. These hatchery-reared salmons for many years are extensively used for the re-establishment of salmon populations in rivers of Lithuania, including the HELCOM SALAR project (HELCOM 2011). This study showed very low cytotoxicity (0.00–0.07‰) and low genotoxicity (0.04–0.50‰) reference levels in erythrocytes of reared (captive-bred population from a particular wild population) salmon. The data suggest a good health status of salmon used for re-establishing or supplementing the wild population in rivers flowing to the Baltic Sea.

Metals bioaccumulation analysis revealed that metal distribution was non-linear during exposure period and varied between body tissues. Statistically significant bioaccumulation of several metals 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). However, the findings of the current study do not support the previous research. Prior study, that has evaluated metals bioaccumulation in gills, liver, kidneys and muscle of Atlantic salmon exposed for 14 days to an entire mixture (Zn, Cu, Ni, Cr, Pb, Cd) and to Ni, Cr, and Pb individually at the same concentrations, have demonstrated different results of metals accumulation in tissues (Svecevičius et al. 2014). Overall, metals were accumulated mostly in the muscle, gills, liver and kidneys, which is contrary to our results. While, accumulation of Pb was partly similar to our results; the highest accumulation was detected in muscle tissue, at the least—in kidneys. This inconsistency may be due to different experimental design in a current study (rotating water-current conditions), which changed fish behavior (situated by the water flow versus being at the bottom). Such experiment design has been chosen as it depicts a more realistic situation (Atlantic salmon is rheophilic species, prefer flowing water) as compared to experimental design—without water flow.

In the present study, significant relationship was found between the Cu, Cr, Pb and Cd concentrations in tissues and duration of exposure also between the NB, 8-shaped nuclei frequency and duration of exposure. As reported by Stankevičiūtė et al. (2016) and Gomes et al. (2015), 8-shaped

nuclei erythrocytes and nuclear buds can be considered as markers of depuration. Tissue concentration reaches steady-state when metal uptake from water is balanced by metal excretion and growth dilution. In the present study, aqueous exposure test (metal uptake phase) was run for 28 days. However, current study has demonstrated that steady-state was reached earlier. In most of the explored tissues, steady-state metals concentrations were reached within 14 days. It should be noted that Cd and Ni have not reached steady-state in gills, liver, kidneys and gills, respectively. Metal elimination routes are more numerous 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 cadmium was more than a year. Otherwise, particularly 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. Whereas Cd and Ni are released much more slowly from the tissues (Kargin and Çogun 1999). Notwithstanding, all metals have reached steady-state in muscle tissue, but at the different exposure period. 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). In contrast, the highest concentration of Pb was detected in the muscle of Atlantic salmon. It has been suggested that carnivorous fish species show different pattern of Pb accumulation in muscle as compared to omnivorous (Yousafzai et al. 2010). Notwithstanding, Cd and Pb levels were low in Atlantic salmon in the present study (Cd: 0.002–0.004 mg/kg and Pb: 0.015–0.057 mg/kg), and below the EU maximum level for these metals in fish (0.05 mg Cd/kg and 0.3 mg Pb/kg muscle tissue (EC 2014, 2015, respectively)).

The highest levels of accumulated metals were measured during 7–28 days exposure period. Genotoxicity levels peaked on day 7 in liver and blood erythrocytes, while in gills and kidney—on day 14, and these levels were statistically higher than those of a non-exposed group. Statistically elevated cytotoxicity levels were detected on day 2 and day 14 in gills erythrocytes and on day 28 in peripheral blood. Experimental data on the relationship between exposure time and ENAs frequencies after fish exposure to metal mixture is scarce. Zhu et al. (2004) showed that micronuclei frequencies increased with the exposure day after carp exposure to low concentrations of single Cr and Cd. Micronuclei frequencies after exposure to single Cr (0.001 mg/L), Cd + Cr (0.001 + 0.001 mg/L) and single Cd (0.001 mg/L), Cd + Cr + Cu (0.001 + 0.001 + 0.01 mg/L) peaked on day 4 and day 9, respectively, and then smoothly changed (Zhu et al. 2004). The DNA damage level fluctuations were showed by Zhang et al. (2008). DNA damage caused by mixed ions in *M. anguillicaudatus* increased significantly during the first seven days, and then rose moderately during 7–14 days, then greatly decreased during 14–21 days and followed by a steady decrease after 21-day treatment.

However, there are some limitations of an entire mixture evaluation, because it is impossible to identify the metal responsible for geno- and cytotoxic effects and potential interactions. Therefore, in the present case, correlations between metals accumulation and geno-cytotoxic endpoints in tissues were estimated. Correlation between geno-cytotoxicity responses in erythrocytes from different tissues and bioaccumulation of metals, except Cr, was found. Cytotoxicity responses positively correlated with Pb, Cu, Cd and Ni bioaccumulation, while summed genotoxicity responses correlated with these four metals and Zn. At the end of exposure period (28 days), correlation was found between Ni accumulation in gills and summed geno-, cytotoxicity in peripheral blood. This finding may be explained by the fact that Ni accumulation appears to occur rapidly within gills tissue, followed by either negligible stabilization or a mild continued increase over time. For this reason, Ni did not reach steady-state in gills. Interestingly, in the liver correlations were found only between Cu accumulation and summed genotoxicity responses. Controversially, Cu and Zn accumulation in liver, gills were not significant in comparison to non-exposed level. Copper and Zn did not reach steady-state in gills and liver. A possible explanation for these results may be homeostasis of these metals in organism (Bury et al. 2003). Relationship between metal tissue concentration and genotoxic response does not always exist. The relationship between Cd, Cr residues in kidneys and micronuclei frequencies had been described by Zhu et al. (2004), while this relationship was not detected in muscle tissue. In contrast, in this study, relationship between Cd accumulation in muscle and genotoxicity was detected. Gasulla et al. (2016) concluded that genotoxic damage may occur in the fish even before metal accumulation in tissues is detectable.

As concluded by Jirtle and Skinner (2007), epigenetic alterations are involved in MN formation; alterations in gene expression may provide a potentiality for the acclimation to a changing environment. The aspects of acclimation to elevated waterborne metal concentrations are not currently known (Wood et al. 2011). It is known that fish liver, kidneys, gills and gonads are target tissues for metal accumulation (Driessnack et al. 2016). For this reason, gene expression analysis in target tissues after exposure to metal mixtures may provide information on possible acclimation to elevated waterborne metal concentrations. No sign of acclimation in the field to the contamination by mixture of Cu (38 μg/L) and Zn (480 μg/L) was noticed in Atlantic salmon. Such contamination eliminated the upstream

spawning migration of Atlantic salmon (Saunders and Sprague 1967). Concentrations of avoidance responses of Atlantic salmon to Cu and Zn mixture were lower than when tested individually, significant avoidance detected at 0.4 µg/L of copper and 6.1 µg/L of zinc mixture (Sprague 1964).

Conclusions

In summary, this is the first report showing induction of geno-cytotoxicity in response to metal mixture exposure at MPC and establishing the time necessary to attain the steady-state concentrations of metals in tissues of Atlantic salmon. The time needed to attain steady-state concentrations of metals in most of the tissues is 14 days. All metals have reached steady-state in muscle, which is the important part of fish due to its consumption by human beings. However, genotoxicity levels peaked on day 7 in liver and blood erythrocytes, while in gills and kidneys—on day 14 of exposure, and these levels were statistically higher than those of a non-exposed group. This study indicated that metals at low levels (at MPC) when existing in mixture causes significant geno-cytotoxicity responses and metals bioaccumulation in Atlantic salmon. This study could encourage revising of ecotoxicologically relevant water quality standards for metal mixtures covering both geno-cytotoxicological and bioaccumulation processes in aquatic organisms. The present study provides the first insights into the reference levels of genotoxicity and cytotoxicity in hatchery-reared European Atlantic salmon (used for re-establishment of salmon in potential rivers of Lithuania). To ensure a good status of Atlantic salmon populations, further studies should be conducted to establish the reference levels of geno-cytotoxicity in wild Atlantic salmon.

Specific toxic endpoint such as genotoxicity and cytotoxicity was identified and measured; however, it is impossible to identify the toxicant responsible for this outcome. Further experimental research will be conducted to evaluate effects, which arises due to interactions between metals in the mixture at steady-state (Part II). Furthermore, future research should be undertaken to investigate the potential of metal mixture at MPC to cause long-term or permanent gonadal toxicity in *Salmo salar*. Moreover, can viability of early life stages be affected, if such gonadal damage occurred. Analysis of altered gene expression in target tissue may provide a potentiality for the acclimation to a changing environment. Additionally, research conducted at the organism level, such as behavioral responses to contaminants at low exposure levels can supplement the results obtained in the investigations at a cellular/molecular level.

Acknowledgements This research was supported by the research infrastructure Open Access Centre for Nature Research at the initiative of the Open R&D Lithuania network. This work is funded by the Research Council of Lithuania, Project No MIP-108/2015. Geno-cytotoxicity studies were performed under Nature Research Centre program 3. We are thankful to Dalia Baršytė Lovejoy (Toronto University) for the language check.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

References

Agius C, Roberts RJ (2003) Melano-macrophage centres and their role in fishpathology. J Fish Dis 26(9):499–509

Ahmed M, Kundu G, Al-Mamun M, Sarkar S, Akter M, Khan M (2013) Chromium (VI) induced acute toxicity and genotoxicity in freshwater stinging catfish, Heteropneustes fossilis. Ecotoxicol Environ Saf 92:64–70

Arkhipchuk VV, Garanko NN (2008) Using the nucleolar biomarker and the micronucleus test on in vivo fish fin cells. Ecotoxicol Environ Saf 62(1):42–52

Arunachalam KD, Subhashini S, Annamalai SK (2013) In-vivo evaluation of hexavalent Chromium induced DNA damage by alkaline comet assay and oxidative stress in *Catla catla*. Am J Environ Sci 9(6):470–482

Baršienė J, Lazutka J, Syvokienė J, Dedonytė V, Rybakovas A, Bagdonas E, Bjornstad A, Andersen OK (2004) Analysis of micronuclei in blue mussels and fish from the Baltic and the North Seas. Environ Toxicol 19(4):365–371

Baršienė J, Dedonytė V, Rybakovas A, Andreikenaitė L, Andersen OK (2006) Investigation of micronuclei and other nuclear abnormalities in peripheral blood and kidney of marine fish treated with crude oil. Aquat Toxicol 78S:S99–S104

Baršienė J, Butrimavičienė L, Grygiel W, Lang T, Michailovas A, Jackūnas T (2014) Environmental genotoxicity and cytotoxicity in flounder (*Platichthys flesus*), herring (*Clupea harengus*) and Atlantic cod (*Gadus morhua*) from chemical munitions dumping zones in the southern Baltic Sea. Mar Environ Res 96:56–67

Bury NR, Walker PA, Glover CN (2003) Nutritive metal uptake in teleost fish. J Exp Biol 206:11–23

Calamari D, Gaggino GF, Pachetti G (1982) Toxicokinetics of low levels of Cd, Cr, Ni, and their mixture in long-term treatment on *Salmo gairdneri* Rich. Chemosphere 11:59–70

Cavas T, Garanko NN, Arkhipchuk VV (2005) Induction of micronuclei and binuclei in blood, gill and liver cells of fishes subchronically exposed to cadmium chloride and copper sulphate. Food Chem Toxicol 43(4):569–574

Directive 2008/105/EC of the European Parliament and of the Council of 496 16 December 2008 on environmental quality standards in the field of water 497 policy, amending and subsequently repealing Council Directives 82/176/EEC, 498 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 499 2000/60/EC of the European Parliament and of the Council. Official Journal of the European Communities L 500 348, 24/12/2008, p. 0084–0097

Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes
Driessnack MK, Matthews AL, Raine JC, Niyogi S (2016) Interactive effects of chronic waterborne copper and cadmium exposure on tissue-specific metal accumulation and reproduction in fathead minnow (*Pimephales promelas*). Comp Biochem Physiol Part C 179:165–173
EC (2014) (EU) No 488/2014 of 12 May amending Regulation (EC) No 1881/ 2006 as regards maximum levels of cadmium in foodstuffs. Official Journal of the European Union 2014, L138/75
EC (2015) (EU) 2015/1005 of 25 June amending Regulation (EC) No 1881/2006 as regards maximum levels of lead in certain foodstuffs. Official Journal of the European Union 2015, L161/9
Fenech M, Chang WP, Kirsch-Volders M, Holland N, Bonassi S, Zeiger E (2003) HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutat Res 534:65–75
Gasulla J, Picco SJ, Carriquiriborde P, Dulout FN, Ronco AE, de Luca JC (2016) Genotoxic Effects Induced by Cd^{+2}, Cr^{+6}, Cu^{+2} in the Gill and Liver of *Odontesthes bonariensis* (Piscies, Atherinopsidae). Bull Environ Contam Toxicol 96:591–595
Gomes JMM, Ribeiro HJ, Procópio MS, Alvarenga BM, Castro ACS, Dutra WO, da Silva JBB, Junior JDC (2015) What the erythrocytic nuclear alteration frequencies could tell us about genotoxicity and macrophage iron storage? PLoS One 10(11): e0143029
Güner U, Muranlı FDG (2011) Micronucleus test, nuclear abnormalities and accumulation of Cu and Cd on *Gambusia affinis* (Baird & Girard, 1853). Turk J Fish Aquat Sci 11:615–622
Heys KA, Shore RF, Pereira MG, Jones KC, Martin FL (2016) Risk assessment of environmental mixture effects. RSC Adv 6:47844–47857
HELCOM (2011) Salmon and Sea Trout Populations and Rivers in the Baltic Sea—HELCOM assessment of salmon (*Salmo salar*) and sea trout (*Salmo trutta*) populations and habitats in rivers fl owing to the Baltic Sea. Balt. Sea Environ. Proc. No. 126A
ISO 6332 (1988) Water quality. Determination of iron—spectrometric method using 1.10-phenanthrolinxe
ISO 5814 (1990) Water quality. Determination of dissolved oxygen—electrotechnical probe method
ISO 9963-1 (1994) Water quality. Determination of alkalinity—Part 1: determination of total and composite alkalinity
ISO 14911 (1998) Water quality. Determination of dissolved Li^{+}, Na^{+}, NH_4^{+}, K^{+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, Sr^{2+} and Ba^{2+} using ion chromatography—method for water and waste water
ISO 15586 (2003) Water quality. Determination of trace elements using atomic absorption spectrometry with graphite furnace. International Organization for Standardization, Geneva
ISO 10304-1 (2007) Water quality. Determination of dissolved anions by liquid chromatography of ions. Determination of bromide, chloride, fluoride, nitrate, nitrite, phosphate and sulfate
ISO 10523 (2008) Water quality. Determination of pH
Jezierska B, Witeska M (2001) Metal toxicity to fish. University of Podlasie, Siedlce
Jezierska B, Witeska M (2006) The metal uptake and accumulation in fish living in polluted waters. In: Twardowska I, Allen HE, Häggblom MM, Stefaniak S (eds) Soil and water pollution monitoring, protection and remediation. NATO Science Series, vol 69. Springer, Dordrecht
Jia H, Ren H, Satoh S, Endo H, Hayashi T (2005) Comparison of pretreatment condition of cadmium in fish sample and diet by microwave digestion method for ICP-AES. J Tokyo Univ Mar Sci Technol 1:41–46
Jindal R, Verma S (2015) In vivo genotoxicity and cytotoxicity assessment of cadmium chloride in peripheral erythrocytes of Labeo rohita (Hamilton). Ecotoxicol Environ Saf 118:1–10
Jiraungkoorskul W, Kosai P, Sahaphong S, Kirtputra P, Chawlab J, Charucharoen S (2007) Evaluation of micronucleus test's sensitivity in freshwater fish species. Res J Env Sci 1(2):56–63
Jirtle RL, Skinner RL (2007) Environmental epigenomics and disease susceptibility. Nat Rev Genet 8:253–262
Kargin F, Cogun Hy (1999) Metal interactions during accumulation and elimination of zinc and cadmium in tissues of the freshwater fish tilapia nilotica. Bull Environ Contam Toxicol 63(4):511–519
Klaassen C, Casarett LJ, Doull J (2013) Chapter 23: Toxic Effects of Metals. In: Klaassen CD Casarett and Doull's toxicology: the basic science of poisons 8th ed. McGraw-Hill Education, New York, NY. 1922
Komjarova I, Blust R (2009) Multimetal interactions between Cd, Cu, Ni, Pb, and Zn uptake from water in the zebrafish (*Danio rerio*). Environ Sci Technol 43(19):7225–7229
Kousar S, Javed M (2015) Diagnosis of metals induced DNA damage in fish using comet assay. Pak Vet J 35(2):168–172
Larson A, Haux C, Sjöbeck M (1985) Fish physiology and metal pollution: results and experiences from laboratory and field studies. Ecotoxicol Environ Saf 9:250–281
Macchi GJ, Romanol A, Christiansen HE (1992) Melano-macrophage centres in white mouth croaker Micropogonias furneri, as biological indicators of environmental changes. J Fish Biol 40:971–973
Nacci DE, Cayula S, Jackim E (1996) Detection of DNA damage in individual cells from marine organisms using the single cell gel assay. Aquat Toxicol 35:197–210
Palermo FF, Risso WE, Simonato JD, Martinez CBR (2015) Bioaccumulation of nickel and its biochemical and genotoxic effects on juveniles of the neotropical fish Prochilodus lineatus. Ecotoxicol Environ Saf 116:19–28
Perera PACT, Kodithuwakku SP, Sundarabarathy TV, Edirisinghe U (2015) Bioaccumulation of Cadmium in freshwater fish: an environmental perspective. Insight Ecol 4(1):1–12
Ramsdorf WA, Vicari T, De Almeida MIM, Ferreira Artoni R, Cestari MM (2012) Handling of Astyanax sp. for biomonitoring in Cangüiri Farm within a fountainhead (Iraí River environment preservation area) through the use of genetic biomarkers. Environ Monit Assess 184:5841–5849
Saldatov AA (2005) Peculiarities of organization and functioning of the fish red blood system. J Evolut Biochem Physiol 41 (3):272–281
Saunders RL, Sprague JB (1967) Effects of copper–zinc mining pollution on a spawning migration of Atlantic salmon. Water Res 1 (6):419–432
SCHER, SCCS, SCENIHR (2012) Opinion on the toxicity and assessment of chemical mixtures
Scorecard (2011) Water quality indicators. Ambient water quality: four toxic pollutants. http://scorecard.goodguide.com/env-releases/def/iwi_wqi.html. Accessed 07 Jul 2016
Song Y, Salbu B, Heier LS, Teien HC, Lind OC, Oughton D, Petersen K, Rosseland BO, Skipperud L, Tollefsen KE (2012) Early stress responses in Atlantic salmon (*Salmo salar*) exposed to environmentally relevant concentrations of uranium. Aquat Toxicol 112–113:62–71
Sprague JB (1964) Avoidance of copper zinc solutions by young salmon in the laboratory. J Water Pollut Control Fed 36:990–1104
Stankevičiūtė M, Butrimavičienė L, Valskienė R, Greiciūnaitė J, Baršienė J, Vosylienė MZ, Svecevičius G (2016) Analysis of nuclear abnormalities in erythrocytes of rainbow trout (*Oncorhynchus mykiss*) treated with Cu and Zn and after 4-, 8-, and 12-

day depuration (post-treatment recovery). Mutat Res/Genet Toxicol Environ Mutagen 797:26–35

Svecevičius G, Sauliutė G, Idzelis RL, Grigelevičiūtė J (2014) Accumulation of heavy metals in different body tissues of Atlantic Salmon *Salmo salar* L., exposed to a model mixture (Cu, Zn, Ni, Cr, Pb, Cd) and singly to nickel, chromium, and lead. Bull Environ Contam Toxicol 92:440–445

US EPA (2009) National recommended water quality criteria. Office of water, office of science and technology (4304T). http://water.epa.gov/scitech/swguidance/standards/criteria/current/. Accessed 13 Jul 2016

Valskienė R, Stankevičiūtė M, Butrimavičienė L, Greiciūnaitė J, Svecevičius G (2015) Induction of nuclear abnormalities in rainbow trout (*Oncorhynchus mykiss*) after exposure to a model mixture of heavy metals (Zn, Cu, Ni, Cr, Cd, Pb) at maximum permissible concentration. In: Proceedings of the 18th conference for junior researchers "Science – Future of Lithuania", Aplinka 15:100–105

Wood CM, Farrell AP, Brauner CJ (eds) (2011) Fish physiology: homeostasis and toxicology of essential metals, 1st Edition. Academic Press, Cambridge, p 528

Yeşilbudak B, Erdem C (2014) Cadmium accumulation in gill, liver, kidney and muscle tissues of common carp, cyprinus carpio, and nile tilapia, oreochromis niloticus. Bull Environ Contam Toxicol 92(5):546–550. doi:10.1007/s00128-014-1228-3

Yousafzai AM, Chivers DP, Khan AR, Ahmad I, Siraj M (2010) Comparison of heavy metals burden in two freshwater fishes Wallago attu and Labeo dyocheilus with regard to their feeding habits in natural ecosystem. Pak J Zool 42(5):537–544

Zhang Y, Wang YJ, Yu RL, Zhang S, Wu ZB (2008) Effects of heavy metals Cd^{2+}, Pb^{2+} and Zn^{2+} on DNA damage of loach misgurnus anguillicaudatus. Front Biol China 3(1):50–54

Zhu Y, Wang J, Bai Y, Zhang R (2004) Cadmium, chromium, and copper induce polychromatocyte micronuclei in carp (Cyprinus carpio L.). Bull Environ Contam Toxicol 72(1):78–86

III

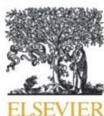
Interaction of carboxylated CdSe/ZnS quantum dots with fish embryos: Towards understanding of nanoparticles toxicity

Rotomskis R, Jurgelėnė Ž, Stankevičius M, Stankevičiūtė M, Kazlauskienė N, Jokšas K, Montvydienė D, Kulvietis V, Karabanovas V

Science of the Total Environment 2018, 635: 1280–1291

Reprinted with permission from Elsevier Ltd.

Science of the Total Environment 635 (2018) 1280–1291



Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Interaction of carboxylated CdSe/ZnS quantum dots with fish embryos: Towards understanding of nanoparticles toxicity

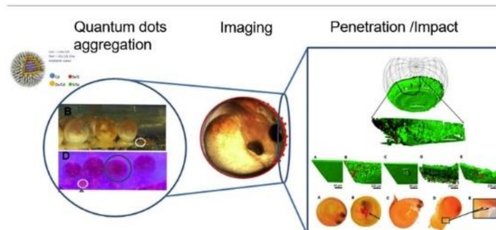
Ričardas Rotomskis [a,b,*], Živilė Jurgelėnė [c,**], Mantas Stankevičius [a,b], Milda Stankevičiūtė [d], Nijolė Kazlauskienė [c], Kęstutis Jokšas [e,f], Danguolė Montvydienė [c], Vytautas Kulvietis [a], Vitalijus Karabanovas [a,g]

[a] Laboratory of Biomedical Physics, National Cancer Institute, Baublio st. 3b, LT-08660 Vilnius, Lithuania
[b] Biophotonics Group of Laser Research Center, Vilnius University, Sauletekio ave. 9, LT-10222 Vilnius, Lithuania
[c] Laboratory of Ecology and Physiology of Hydrobionts, Nature Research Centre, Akademijos st. 2, LT-08412 Vilnius, Lithuania
[d] Laboratory of Genotoxicology, Nature Research Centre, Akademijos st. 2, LT-08412 Vilnius, Lithuania
[e] Geology and Geography Institute of Nature Research Centre, Akademijos st. 2, LT-08412 Vilnius, Lithuania
[f] Vilnius University, Faculty of Chemistry and Geosciences, Naugarduko st. 24, LT-03225 Vilnius, Lithuania
[g] Department of Chemistry and Bioengineering, Vilnius Gediminas Technical University, Sauletekio ave. 11, LT-10223 Vilnius, Lithuania

HIGHLIGHTS

- Quantum dots (QDs) are aggregating and forming the agglomerates on the surface of fish embryos.
- The aggregation of the chemically stable QDs caused the partial disintegration of the fish embryos chorion.
- Confocal fluorescence microscopy is a valuable tool for detection and visualization of QDs migration in the fish embryos.
- QDs aggregates didn't penetrate the embryos; they caused the chorion damage which could affect the development of fish.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 1 February 2018
Received in revised form 15 April 2018
Accepted 16 April 2018
Available online xxxx

Editor: Henner Hollert

Keywords:
Nanoparticles
Quantum dots aggregation and agglomeration on fish embryos
Chorion
Confocal fluorescence microscopy
3D reconstruction, nanotoxicity

ABSTRACT

Due to colloidal instability even with protective coatings, nanoparticles tend to aggregate in complex environments and possibly interact with biota. In this study, visualization of quantum dots (QDs) interaction with rainbow trout (*Oncorhynchus mykiss*) embryos was performed. Studies on zebrafish (*Danio rerio*) and pearl gourami (*Trichogaster leerii*) embryos have shown that QDs interact with embryos in a general manner and their affects are independent on the type of the embryo. It was demonstrated that carboxylated CdSe/ZnS QDs (4 nM) were aggregating in accumulation media and formed agglomerates on the surface of fish embryos under 1–12 days incubation in deep-well water. Detailed analysis of QDs distribution on fish embryos surface and investigation of the penetration of QDs through embryo's membrane showed that the chorion protects embryos from the penetration through the chorion and the accumulation of nanoparticles inside the embryos. Confocal microscopy and spectroscopy studies on rainbow trout embryos demonstrated that QDs cause chorion damage, due to QDs aggregation on the surface of chorion, even the formation of the agglomerates at the outer part of the embryos and/or with the mucus were detected. Aggregation of QDs and formation of agglomerates on the outer part of the embryo's membrane caused the intervention of the aggregates to the chorion and even partially destroyed the embryo's chorion. The incorporation of QDs in chorion was confirmed by two methods: in living

* Correspondence to: R. Rotomskis, Laboratory of Biomedical Physics, National Cancer Institute, Baublio st. 3b, LT-08660 Vilnius, Lithuania.
** Correspondence to: Ž. Jurgelėnė, Nature Research Centre, Akademijos str. 2, LT-08412 Vilnius, Lithuania.
E-mail addresses: ricardas.rotomskis@nvi.lt, (R. Rotomskis), zivile.jurgelene@gmail.com. (Ž. Jurgelėnė).

https://doi.org/10.1016/j.scitotenv.2018.04.206
0048-9697/© 2018 Elsevier B.V. All rights reserved.

embryos from a 3D reconstruction view, and in slices of embryos from a histology view. The damage of chorion integrity might have adverse effects on embryonic development. Moreover, for the first time the toxic effect of QDs was separated from the heavy metal toxicity, which is most commonly discussed in the literature to the toxicity of the QDs.

© 2018 Elsevier B.V. All rights reserved.

1. Introduction

Novel materials at the nanoscale with exciting functional properties presents unique applications in electronics, personal care products, biotechnology and medicine (Nel et al., 2006; Wehmas et al., 2015). Despite the various benefits, the extensive use of nanoparticles (NPs) may lead to the release, transport, and accumulation of these NPs in the aquatic environment (King-Heiden et al., 2009). Although the concentration of NPs in the aquatic environment is unknown, the ecotoxicological impact of NPs is an emerging problem due to their nano-specific biofunctional properties, and physico-chemical transformation in the environment (Rocha et al., 2017). Different processes influence the environmental behaviour and fate of NPs, such as surface coating changes, homo– and hetero-aggregation, disaggregation/deagglomeration, diffusion, interaction with macromolecules and/or organisms and biological transformation (Rocha et al., 2017).

Fish are important model organisms due to rapid embryonic development and short generation time, and are considered as an alternative vertebrate model for screening drug safety, elucidating mechanisms of human diseases, and assessing environmental health (Powers, 1989; Rocha et al., 2015). The early development stages (embryos and larvae) are more susceptible to NPs impact when compared to the mature stage (Rocha et al., 2017). After fertilization, teleost fish eggs are surrounded by chorion that protects the developing organism from negative environmental impact and also plays a role in gas exchange and in a selective transport of small electrolytes and water into the embryos. This protective function, morphology and molecular composition of chorion during development is already described in the literature (Henn, 2011; Jaramillo et al., 2015). Additionally, the interest of the chorion's ability to protect the embryo until hatching has grown (Kashiwada, 2006; Lee et al., 2007; Asharani et al., 2008; Browning et al., 2009; Fent et al., 2010; Osborne et al., 2013; Kang et al., 2015).

Kashiwada (2006) detected silica fluorescent particles (39.4–42 nm, 1 mg/L) adsorbed on the chorion and accumulated in the oil droplets in medaka (*Oryzias latipes*) embryos after 24-hour exposure. Lee et al. (2007) and Browning et al. (2009) noticed that single Ag NPs (5–46 and 11.6 ± 0.9 nm, respectively) transported in and out of zebrafish (*Danio rerio*) embryos through chorion pore canals, and NPs could be trapped inside chorion pore canals and the inner mass of embryos. Asharani et al. (2008) demonstrated that Ag NPs could be entered through the chorion of the zebrafish and could be distributed in the brain, heart, yolk, and blood of embryos. According to Kang et al. (2015), the carbon QDs entered the zebrafish embryos and larvae and then concentrated in different tissues. Meanwhile, Fent et al. (2010) study's results showed that fluorescent silica NPs (~60 and ~200 nm) formed clumps and adsorbed on the chorion of zebrafish embryos. Osborne et al. (2013) demonstrated that Ag NPs (10, 35 nm) and TiO_2 NPs (4, 10, 30, and 134 nm) were appearing as aggregates, located in the chorion of the zebrafish with no evidence of penetration into embryo's tissue. Böhme et al. (2017) showed that Ag, Au, and Al_2O_3 NPs were accumulated at the chorion of zebrafish embryos. Consequently, the data on NPs interaction with fish protective barriers, were limited and contradictory concerning the passage through the chorion as well as retention on its surface.

One type of NPs are nanometer-sized (2–10 nm) semiconductor nanostructured materials, also known as quantum dots (QDs) (Alivisatos, 1996), which could be useful to examining the interaction of NPs and organism. QDs have a range of unique properties making them interesting for many photo-physical applications. The main application areas for QDs are healthcare, quantum computing, quantum optics, optoelectronics, energy, and security and surveillance. Additionally, the key products in the global QDs market are QDs solar cells, medical devices, lasers, lighting, LED displays and sensors (Piccinno et al., 2012). Hence, understanding the impact mechanism of QDs in biological systems is essential for the realization of their safe application. However, it should be noted that there is very limited information regarding their accumulation, distribution, and penetration abilities in fish embryos, and potential toxicity to living organisms in the initial stages of embryogenesis (King-Heiden et al., 2009; Rocha et al., 2017).

There is little information regarding QDs aggregation on the chorion of fish embryos. Zolotarev et al. (2012), Petushkova et al. (2015) noticed the formation of large structure of the QDs particles on the chorion surface of zebrafish embryos after 48 h of exposure. However, they did not mention and investigate the aggregation of QDs. Meanwhile, King-Heiden et al. (2009) noted that during the aggregation process in test-water, the QDs hydrodynamic diameters increased. Most studies are concentrated on the toxicity related to the QDs degradation products, and the release of toxic metals, such as Cd from QDs (Ipe et al., 2005; Ribeiro et al., 2012; Katsumiti et al., 2014; Rocha et al., 2015; Santana et al., 2015). The question about nanotoxicity of QDs is still open: heavy-metal toxicity or specific nanotoxicity of QDs have an influence on the early stage of fish embryogenesis.

No one else explores the QDs aggregate's migration and translocation through the embryo chorion of fish. Furthermore, there is no data on how QDs aggregates are distributed in the chorion of embryos. Additionally, there is no detailed evidence of QDs inside embryos. No study has demonstrated that QDs aggregate in chorion of living embryos. Furthermore, most visualization methods require the death of an animal (Böhme et al., 2017; Brun et al., 2014; Chen et al., 2011; Lee and An, 2014). No data is available on the use of several methods for confirming the distribution of QDs aggregates in an organism. Although confocal microscopy is widely used in biomedical research (Bijeesh et al., 2017), its application in assessing the impact of QDs and 3D reconstruction images in living organism are still scarce. Especially, it would be important to investigate the impacts of QDs aggregates on the embryonic development of fish. Detailed knowledge on the penetration abilities of QDs in aquatic organisms and their ecotoxicity effects is required in the environmental health assessment context.

The aims of the present study were to: assess the stability of carboxylated CdSe/ZnS QDs; examine the distribution of QDs in fish embryos; determine the penetration and translocation abilities of QDs aggregates through the chorion; investigate the effects of QDs on fish embryos; and to explain the possible mechanisms of QDs impact on the test-organism.

2. Materials and methods

2.1. Materials and reagents

For investigations of NPs penetration and translocation abilities in fish embryos, commercially available semiconductor quantum dots (QDs) (CdSe/ZnS-COOH cat. No. A10200) were purchased from Life Technologies (USA). QDs are CdSe/ZnS negative-charged (covered with carboxyl groups), and coated with a polymer layer that allows

facile dispersion of QDs in aqueous solutions with retention of their optical properties. These QDs have a strong photoluminescence (PL) in red spectral region, with a PL peak of 625 nm.

A volume of 100 μL of 8 μM of QDs (stock solution) was dissolved in deep-well water to achieve the final QDs concentration of 4 nM in fish embryos incubation water. Chemical characteristics of the deep-well water are presented in Table 1 (ISO 15586:2003; ISO 6332, 1988; ISO 5814, 1990; ISO 14911, 1998; ISO 10304-1, 2007; ISO 9963-1, 1994).

2.2. Fish embryos exposure

Bioassay was undertaken under controlled laboratory conditions. The laboratory treatments were carried out in an environmental chamber (Bronson PGC-660, Zaltbommel, The Netherlands). The studies were carried out with non-protected life-stages in accordance with EU Directive 2010/63/EU. Dissolved oxygen in the tanks, temperature and pH were measured at 24-hour intervals with a hand-held multi-meter (WTW Multi 340i/SET, Germany). Deep-well water was used as the dilution water in all experiments (Table 1). The mean pH of dilution water was 8.0, and the oxygen concentration was 10 mg/L.

The experiment was done with fertilized fish eggs (embryos) from different ecological groups: pearl gourami (*Trichogaster leerii*), zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*) (at 4 nM QDs concentration). The embryos of these fish have different egg sizes and optical transparency. These properties were important for confocal microscopy. The pearl gourami and zebrafish are aquarium species. In contrast, the rainbow trout is economically valuable fish and naturally found in Lithuania.

The main study was done with rainbow trout embryos, because literature data shows that rainbow trout embryos are very sensitive to pollution and are established model systems for standard toxicological testing of chemicals (Hrovat et al., 2009). Furthermore, the embryos of rainbow trout are relatively large objects for confocal imaging. An additional study was done with other fish like pearl gourami, and zebrafish embryos to visualize the QDs distribution in the whole embryo.

2.2.1. Rainbow trout

The experiments were conducted for three years during spring. Rainbow trout *O. mykiss* (Walbaum, 1792) embryos at the eyed-egg stage after artificial fertilization (at stage 20 of embryogenesis (Ballard, 1973)) were obtained from the Simnas hatchery (Lithuania). The temperature was maintained at 10 ± 0.5 °C.

Studies with embryos and QDs (4 nM) were performed in three replications ($N = 20$ in each group) in the dark under static conditions (static non-renewal experiment) (ISO 7346-1, 1996). The experiment was began with rainbow trout embryos 24–26-day post fertilization at stage 20, when their heartbeat, eye pigmentation, and vitelline veins become visible through the chorion, and the experiment was ended 36-day post fertilization at the 23rd stage till hatching (Ballard, 1973). Sampling was based on embryos hatching. Embryo samples for comet assay were collected after one day of exposure and samples of larvae (one day old) were collected immediately after hatching (embryos started to hatch after 11 days of exposure to QDs). Rainbow trout embryos were taken from the tanks at 24 h' intervals (the duration of experiment was 12 days) to visualize the QDs localization and the impact to the chorion of embryos.

Rainbow trout embryos (eight days before hatching; 28-day post fertilization) were treated with clay (0, 0.375, 0.750, 1.5, 3.0, 6.0 and 12.0 g/L) for eight days (three replications ($N = 30$ in each group)) under static conditions (ISO 7346-1, 1996) to identify a possible impact of clay particles to embryos. The effect of clay on the biological parameters: survival (%) and heart rate (HR, counts/min) of embryos were recorded. Survival was recorded at 24-hour intervals. HR of embryos was measured for 10 embryos individually, and the mean value for 10 embryos was calculated. Samples of embryos were taken upon days 1, 4 and 8 after the exposure started.

Embryo samples for Cd analyses were collected at 31 (four days before hatching) and 35-day post fertilization (one day before hatching). The duration of experiment was four days. Studies with embryos and QDs for Cd analyses were performed in three replications ($N = 30$ in each group) in the dark under static conditions (static non-renewal experiment) (ISO 7346-1, 1996).

Table 1
Chemical characteristics (mg/L) of the deep-well water.

Metals		Cations		Anions	
Mn	0.068	Na^+	3.2	Cl^-	3.7
Zn	0.0128	K^+	1.2	SO_4^{2-}	18.4
Cu	<0.001	Ca^{2+}	70.1	HCO_3^-	258
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		

2.2.2. Zebrafish and pearl gourami

The study was done with zebrafish embryos (*D. rerio*) (Hamilton, 1822) and pearl gourami (*T. leerii*) (Bleeker, 1852) embryos. The fish embryos were treated with 4 nM QDs (the mean pH was 8.0, temperature was maintained at 27 ± 0.5 °C, and the photoperiod was 12 h light–12 h dark cycle) and QDs distribution in the whole embryo was investigated after one day in pearl gourami and after one to three days post fertilization in zebrafish. Additionally, after one day of exposure to QDs, the chorion of pearl gourami embryos was visualized after hatching. Pearl gourami and zebrafish samples were taken from the tanks at 24-hour intervals. The duration of experiment for pearl gourami and zebrafish was one day and three days, respectively.

The laboratory treatments with pearl gourami and zebrafish embryos were carried out under semi static conditions (ISO 12890, 1999). Control water and test solutions in wells were changed at 24-hour intervals to maintain oxygen in incubation water. Studies with embryos were performed in three replications (a 6-well plate along with 10 mL of deep-well water and 15 embryos in each well was used).

To the get the zebrafish and pearl gourami embryos, the sexually mature fish were used with an age from 6 to 12 months. Fish were put into deep-well water in an aquarium with conditions (27 ± 0.5 °C temperature, 12-hour daylight, water filtration, and airing). Males and females of zebrafish were put with a ratio 1:2 in a spawning grid into the aquarium. At 4–5 h after spawning, fishes were removed from the spawning aquarium, and embryos were collected. Pearl gourami embryos were collected from the top of the spawning aquarium.

2.3. Instrumentation

2.3.1. Confocal fluorescence microscopy and visualization

The localization of QDs in fish embryos was observed using the Nikon ECLIPSE TE2000-S C1 Plus confocal microscope (Nikon, Japan), and by scanning with the argon ion laser (488 nm). Imaging was performed using a 4×/0.1 NA, 10×/0.25 NA, 20×/0.50 NA and 60×/1.4 NA objectives (Nikon, Japan). The three-channel RGB detector (band-pass filters 500–530 nm and 570–640 nm for green and red channels, respectively) were used. QDs were excited at 488 nm.

2.3.2. Spectroscopic measurements

The 32-channel spectral detector (C1si, Nikon, Japan), connected to the ECLIPSE TE2000-S microscope, was used to investigate the PL spectra of QDs and autofluorescence of embryos' tissues. Image processing was performed using the Nikon EZ-C1 Bronze version 3.80 and ImageJ 1.46 software. Additionally, the PL spectra of QDs solutions was registered using a spectrofluorimeter (Cary Eclipse, Varian, USA). All measurements ($N = 10$) were carried out using 1 cm plastic cuvettes.

2.3.3. Atomic absorption spectrophotometer (AAS)

The digestion method was used for Cd analysis in rainbow trout embryos after 1 and 4 days of exposure to QDs (Thomas and Mohaideen, 2015). The concentration of Cd in the top and bottom of tanks at the end of the experiment as well as in the whole body of the fish embryos was analysed according to the analysis method ISO 15586: 2003 by an AAS SHIMADZU AA-7000 (Japan). This spectrometer was equipped with a graphite furnace atomizer GFA-7000 and auto-sampler ASC-7000 (measured wavelength 185 to 900 ± 0.3 nm, high-speed deuterium lamp 185 to 430 nm, heating temperature range 50 to 3000 °C, and repeatability 2.5%). The concentration of Cd standard (Sigma, USA) for AAS is 1000 mg/L, and its detection limit is 300 ng/L. The results of total Cd concentration in embryos were presented as ng Cd/g wet weight. The Cd concentration in the whole embryo of rainbow trout (10 individuals per replicate) and total Cd concentration in incubation water during experiments were analysed in three replications. Sampled organisms were dried on absorbent paper and were weighed before being stored at −18 °C until Cd analysis. Cd analysis involves all concentrations of Cd in the QDs structures.

2.3.4. QDs and clay particle analysis

Dynamic light scattering (DLS) technique was used for analysis of QDs hydrodynamic diameters in deep-well water. All experiments were performed with Brookhaven ZetaPALS Zeta Potential Analyzer (Brookhaven Instruments, USA). DLS analysis of the QDs dispersed in deep-well water revealed a polydisperse size distribution of nanoparticles, which had hydrodynamic diameters from 50 nm to 100 nm after preparation and increased to 0.6–1.2 μm in diameter during one week and to 1.8–2.8 μm after few months.

The size of the clay particles was determined using a Fritsch Laser Particle Sizer "Analysette 22" (Fritsch GmbH, Idar-Oberstein, Germany) at the Open Access Centre of the Nature Research Centre (Lithuania). The clay particles varied from 90 to 1920 nm.

2.3.5. Malformations analysis

Morphological malformations of rainbow trout embryos were analysed by using a stereomicroscope (Meiji Techno RZ Series, Japan) and visible malformations were photographed with a Nikon Cool pixel 995 digital camera, Japan. The embryo did not remove from their chorion to further investigate the development malformations.

2.3.6. Comet assay

The comet assay was used to assess the possibility of QDs to induce DNA damage in embryos at 27-day post fertilization (one day of exposure) and in newly hatched larvae of rainbow trout (one day after hatching). The alkaline comet assay was performed following the procedure of Singh et al. (1988) with some modifications (Fatima et al., 2014). A suspension of cells from individual (gently nipped with tweezers in the heart region and rinsed in PBS) was prepared immediately after treatment of the embryos and larvae as described by Jarvis and Knowles (2003). The envelope (chorion), liquids surrounding the embryo and yolk sac of larvae were removed before sample preparation. 11-day test was carried out with rainbow trout embryos (seven specimens in QDs and control groups) for evaluating DNA damage. Cell viability was measured by trypan blue exclusion method (Anderson and Wild, 1994). Only cell suspensions with viability >80% were used. The slides were stained with ethidium bromide, placed under a glass cover and analysed by fluorescence microscopy (Olympus BX51 microscope with an Olympus U-RFL-T fluorescent burner, Tokyo, Japan) and the photos were taken with an Olympus U-CMAD3 (Tokyo, Japan) camera. 100 nuclei of each individual were scored randomly and captured at 40× magnification. Images were analysed using Comet assay IV version 4.2 software and percentage of DNA in the tail (% Tail DNA) was assessed.

2.3.7. Histology assay

Rainbow trout embryos (after eight-day exposure to QDs at 32-day post fertilization) were prepared using the standard paraffin embedding technique for microscopical examination. They were fixed in 4% formaldehyde solution for 24 h and were embedded in paraffin blocks for cutting with the microtome. The unstained slices were used for fluorescence microscopy, and they were imaged using a confocal microscope.

2.4. Statistics

From the data collected, means and standard deviations were calculated for each of the studied parameters of rainbow trout embryos. The toxicity data (heart rate and survival) do not follow a normal distribution (Kolmogorov-Smirnov and Shapiro-Wilk normality test). Differences between the evaluated characteristics studied were tested by nonparametric Kruskal-Wallis test using STATISTICA 7.0 (StatSoft Inc., Tulsa, Oklahoma, USA) software. Differences were accepted as significant at the 95% level of confidence ($p < 0.05$).

Comet assay data follow a normal distribution (Kolmogorov-Smirnov and Shapiro-Wilk normality test), and this data were analysed by *t*-test using statistical analysis software GraphPad Prism® 5.01 (GraphPad Software Inc., San Diego, CA, USA).

3. Results and discussion

3.1. QDs aggregation and agglomeration in the incubation media

The image of rainbow trout embryos in incubation deep-well water (QDs concentration 4 nM) at the beginning of incubation – A, and at the end of experiment (after 10 days of incubation) – B - are presented in Fig. 1. The incubation water is transparent and homogeneous (Fig. 1A) and the homogeneous red PL of QDs under illumination with blue light lamp (Fig. 1C) were detected, indicating that suspended QDs are homogeneously distributed in the incubation water at the beginning of the experiment.

Huge orange/brown-coloured nonhomogeneous sediments covering the bottom of the incubation tank and embryos after 10 days of treatment with QDs were observed (Fig. 1B). These different size sediments covering the embryos and floating in the incubation water exhibit the red/pink-coloured PL of QDs under UV irradiation at the 10th day of incubation (Fig. 1D). It shows that QDs in the incubation media are forming big structures with slightly different optical properties (Fig.1E).

Decreasing fluorescence intensity and appearance of sediments on the bottom of the cuvette were detected after 24 h of QDs incubation in deep-well water (Fig. S1). Notable restoration of PL intensity of QDs was observed after careful pipetting of the QDs solution. It is clearly shown, that QDs in deep-well water start to aggregate, and huge orange coloured pellets appear on the bottom of the cuvette which is similar to the case of QDs incubation with fish embryos (Fig. 1B, D). The PL intensity of QDs suspended in deep-well water decreased drastically. However, no PL band shifting, no PL spectra shape changes were detected keeping the QDs one week in the deep-well water. It seems, that presence of ions in deep-water solution affects the colloidal instability and aggregation of QDs were detected (Fig. S1), but no spectroscopic changes of the photoluminescence of QDs occur.

Slightly different situation was observed when embryos were incubated with QDs dissolved in deep-well water. The widening of the PL band of QDs (the full width at half maximum of PL of QDs about 25 nm at the beginning of the incubation was increased till about 30 nm at the end of the incubation), and negligible shift of QDs PL band maximum from 625 nm to 628 nm (Fig. 1E) after prolonged incubation was detected. A widening and negligible shift of QDs PL band maximum during the incubation period (Fig. 1E) could be explained by the interaction and aggregation of QDs (Kulvietis et al., 2011).

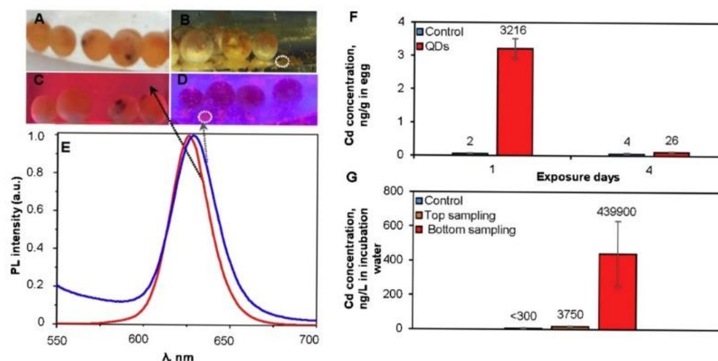


Fig. 1. The image of rainbow trout embryos after treatment with QDs (4 nM): A - white light image taken at the beginning of the experiment and B - white light image taken at the end of experiment (after 10 days of incubation). Aggregates of QDs appear as coloured sediments on the image. Photoluminescence spectra (E) of QDs in incubation water: C - fluorescence image at the beginning of the experiment and D - fluorescence image at the end of the experiment (blue irradiation). F - Cd concentration (ng/g) in embryos after exposure to QDs depended on the duration of exposures (one day after exposure at 31-day post fertilization and four days of exposure at 35-day post fertilization). G - Cd concentration (ng/L) in incubation water after long-term exposure of embryos to QDs depending on the sampling point. White circles denote QDs aggregates in incubation water, which were taken out to measured PL spectra. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The changes of hydrodynamic radius, aggregation and/or agglomeration of QDs in aqueous media were rarely mentioned in some papers on the investigations of QDs interaction with aquatic organisms (Gagné et al., 2008a; King-Heiden et al., 2009; Zolotarev et al., 2012). King-Heiden et al. (2009) studied the toxicity of CdSe/ZnS QDs functionalized with a different coating on zebrafish embryos and larvae. Authors noted that QDs hydrodynamic diameter changes indicating possible aggregation (King-Heiden et al., 2009). Gagné et al. (2008a) performed experiments with freshwater mussels at moderate and high concentrations of CdTe QDs and observed their aggregation. Similar to this study, Zolotarev et al. (2012) after one or two days of incubation also noticed QDs coagulation in solution (got stuck to each other), and deposition of QDs aggregates on zebrafish embryo surface.

The additional information about aggregation and/or agglomeration of QDs was obtained from Cd concentration measurements in the incubation media from the top and bottom of incubation tanks at the end of the experiment. The content of Cd measured in the incubation media as well in the embryos reflects the QDs concentration and distribution changes over time in volume. It was found (Fig. 1G), that almost all Cd were detected in the sediment, only <1% of the Cd was detected in the incubation water collected from the top of the tank. It shows that all QDs formed aggregates/agglomerates are distributed at the bottom layer of the incubation water.

The estimations of Cd concentration in the embryos (10 individuals per replicate) during experiments were analysed in three replications. The values of Cd concentrations measured in rainbow trout embryos after one and four-day exposure period to 4 nM of QDs are presented in Fig. 1F. After one day of incubation, very high concentrations of Cd in embryos were detected suggesting that a lot of QDs are in contact with biological material. However, the concentration of Cd in contact with embryos after four days of incubation had decreased more than a 100-fold. This indicated that after four-day incubation, QDs are separating from the surface of the embryo (Fig. 1C). The dark embryos and red fluorescing pellet floated around the embryos and laid at the bottom of the incubation tank. This clearly shows that decrease of the concentration of Cd in the embryo is related to the formation agglomerates of QDs with mucus on the embryo's surface and separating them from embryos to the incubation water as it is seen in Fig. 1D. It seems that QDs did not get into the embryo.

3.2. QDs and fish embryos imaging

Data on the levels of Cd accumulation in fish embryos is scarce and there is not enough of it prove the accumulation and distribution of the QDs in embryos, therefore the fluorescence confocal microscopy was used for further investigations of the QDs accumulation process in embryos.

The zebrafish and pearl gourami embryos are small and can be successfully used to visualize QDs distribution in the whole embryo using confocal fluorescence imaging (Fig. 2A, C).

Confocal fluorescence imaging indicated that QDs (red colour presenting the PL of the QDs) are located on the surface of zebrafish and pearl gourami embryo (bright field image of embryos after exposure to QDs) (Fig. 2A and C). A stack of 2D images taken at different focal planes along the z axis (Fig. 2A) clearly shows that red fluorescence of QDs is distributed around the zebrafish embryos and might be inserted into the chorion. The pearl gourami embryo after one day of the exposure to QDs and chorion of embryos after hatching was visualized (Fig. 2C (3–6)). The red fluorescence of QDs related to the chorion was detected; however, no red fluorescence of QDs were detected inside of the embryos.

The embryos of rainbow trout are relatively large objects for confocal imaging and because of the scattering of light, it is difficult to penetrate into the deeper layers of the embryos to get information about distribution of QDs inside embryos. The tissues of the embryo are turbid and almost non-transparent with strong light-scattering properties. Consequently, only a small part of the embryo was imaged (Fig. 2B). Nevertheless, according to the stack of 2D images taken at different focal planes along the z axis clearly shows that red fluorescence of the QDs is distributed in the external tissues (white dashed circles) of the embryo (Fig. 2B) and no red photoluminescence of QDs inside of body of embryos was detected.

3.3. The comparison 3D reconstruction of embryos of zebrafish and rainbow trout

For better understanding of the distribution of the QDs in the chorion of embryos, 3D reconstructions were made from a stack of 2D

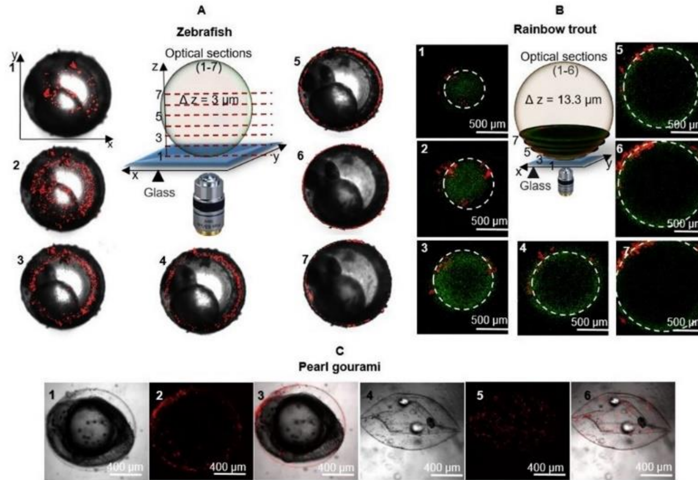


Fig. 2. Imaging scheme of zebrafish (A) and rainbow trout (B) embryos after one, two and four-day exposure to QDs (4 nM), respectively and a stack of 2D images taken at different focal planes 1–7 along the z axis, respectively. Pearl gourami embryo (C 1 - 3) and chorion (C 4 - 6) images of bright field (C 1 and C 4), QDs photoluminescence (C 2 and C 4), and merging of bright field and QDs photoluminescence (C 3 - embryo and C 6 - chorion). Green colour (500–530 nm) represents the autofluorescence of embryo tissues; red colour (569–640 nm) represents QDs photoluminescence. QDs photoluminescence was detected in the external tissues of embryos after exposure to QDs (4 nM). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

images, which were acquired from different optical sections of the specimen.

Depending on the objective, up to 200 2D images with a step size (Δz) of 4–13.3 μm for 3D reconstructions were used. Originally, each 2D image was composed of autofluorescence of embryos (green colour) and QDs photoluminescence (red colour) signals.

3D autofluorescence of the zebrafish (Fig. 3A) and rainbow trout (Fig. 3B) embryo tissues' surface formed a pan-like structure because of the embryo spherical shape with the bottom flattened by the weight of the embryo. A vertical cut-off the isosurface clearly shows QDs photoluminescence inside the autofluorescence 3D isosurface (Fig. 3). Furthermore, due to the relatively small dimension of the embryo a significant PL signal coming from the internal region of the embryo was observed in the inner side of the whole pan-like isosurface structure. As a result, some of the embryos internal structures presented as a set of irregularly distributed green structures. However, there was no QDs

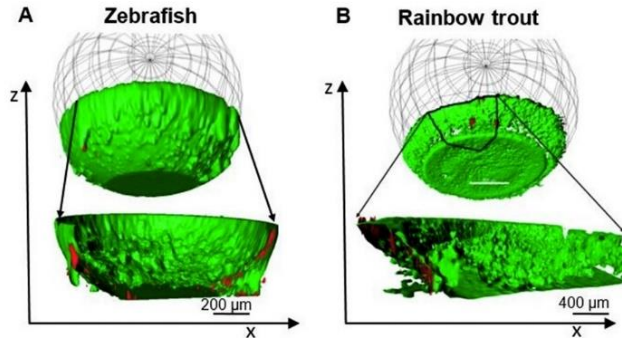


Fig. 3. The 3D reconstruction of zebrafish (A) and rainbow trout (B) embryo fragments after two and four days of exposure to QDs (4 nM). Green colour (500–530 nm) represents the autofluorescence of embryo tissues; red colour (569–640 nm) represents QDs photoluminescence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

photoluminescence detected in this region (Fig. 3). Data shows that QDs photoluminescence (red colour) was detectable only in the region of autofluorescence of external embryo tissues (green colour) (Fig. 3).

3.4. QDs impact on the chorion of rainbow trout embryos

It was detected that QDs are forming aggregates of different sizes on the surface of rainbow trout embryos (chorion) after one - two days treatment with QDs (Fig. 4B). The green colour represents the autofluorescence of the embryonic tissues, and the red colour represents the PL of QDs (Fig. 4). Our results show that QDs or small QDs aggregates formed in the incubation water attached to the surface of the embryos forming aggregation centres on the surface of embryo (Fig. 4B). After three - four days of incubation to QDs, the number of the aggregates attached to the surface of the chorion increased. (Fig. 4C). Furthermore, with the increase of the incubation time, drastic changes in the outer layer of the chorion were detected (Fig. 4D). QDs aggregates intervened into the embryo chorion and caused a disruption of the chorion surface (Fig. 4D). The disintegration of the outer part of the chorion membrane and separation of QDs agglomerates with the mucus from the embryo were detected after incubation four - six days (Fig. 4D).

Moreover, before the hatching of embryos, the destruction of the chorion was detected (Fig. 4E). QDs aggregates damaged chorion integrity of the rainbow trout embryo after 8–12 days of exposure.

The formation of a mucus layer of the chorion of fish embryos is a well-known process (Songe et al., 2016). The mucous layer has been reported as a barrier that prevents penetration of chemicals and bacteria into the organism (Vatsos et al., 2006; Villarreal et al., 2014). We can predict that the mucus on the chorion helps to attach the QDs to the surface of the embryo's chorion and after prolonged incubation time to initiate the formation of aggregates/agglomerates of QDs on the surface of embryos. Moreover, under prolonged time of incubation the QDs aggregates with mucus separated from the surface of chorion.

It was very difficult identified which part of the chorion was separated from the embryos, because to our knowledge, the separation of mucus and chorion body cannot be determined by optical methods. Visualization methods applied in our study showed mucus and chorion together, because green colour (500–530 nm) represents the autofluorescence of all embryos tissues. However, it seems that the agglomerates on the surface of the chorion cause its disintegration and as a consequence destroys the integrity of membrane (Fig. 5).

The opening of the chorion (where a lot of QDs aggregates are concentrated) is clearly seen in the 3D reconstruction of the image of the chorion of rainbow trout embryos. According to the 3D reconstruction detailed analysis, damaging of the embryos surface could be easily visualized (Fig. 5). This might occur during the embryos hatching period as a result of a natural process or by QDs aggregates damage to the chorion integrity.

We can conclude from our experiments that the increasing of size and amount of QDs aggregates/agglomerates to initiate mucus of the chorion separation from the embryo and caused the damage of chorion integrity. These changes could induce the damage of chorion due to QDs aggregates induced mechanical stress.

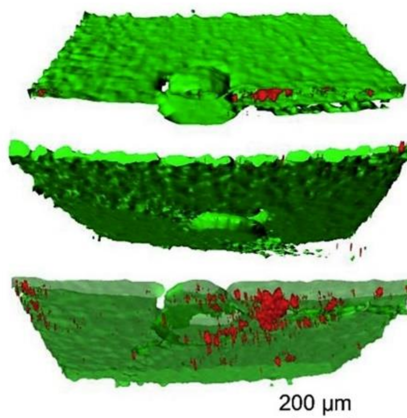


Fig. 5. The 3D reconstruction of the embryo fragment of rainbow trout after 10-day exposure to QDs (4 nM) at 34-day post fertilization. Green colour (500–530 nm) represents the autofluorescence of embryo tissues; red colour (569–640 nm) represents QDs photoluminescence. Split of the chorion by QDs aggregates induced by depletion of the chorion. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

For the first time our study at showed that confocal fluorescence microscopy is a valuable tool for detection and visualization of Cd-based carboxylated CdSe/ZnS QDs in test organisms such as rainbow trout, zebrafish and pearl gourami embryos. According to 2D images, QDs photoluminescence was detected in the external tissues of zebrafish, rainbow trout and pearl gourami embryos, but not in the central part of these embryos (Fig. 2). This confirms that QDs aggregates did not penetrate the chorion after 10–12-day incubation.

Also, the total Cd concentration in embryos indicated that after four days of embryo treatment with QDs, the Cd concentration declined in the embryo (Fig. 1F). It means that the embryos have protection mechanisms to remove QDs from their surface. However, in these surface areas, the chorion lost solidity and QDs aggregates induced a defragmentation and damage of the outer layer of the chorion (Fig. 4E) that should be studied further. Meanwhile, NPs could interact with proteins on and/or in cells, causing altered protein conformation, disruption of plasma membrane integrity, and production of harmful reactive oxygen species (Tsoi et al., 2013; Strtak et al., 2017).

However, data for the association of QDs to the rainbow trout embryos or specific compartments of the embryo are scarce. In recent studies, mainly electron microscopy (Ag NPs, Asharani et al., 2008), fluorescence microscopy (Ru@SiO_2 NPs, Fent et al., 2010; Co_3O_3, CuO,

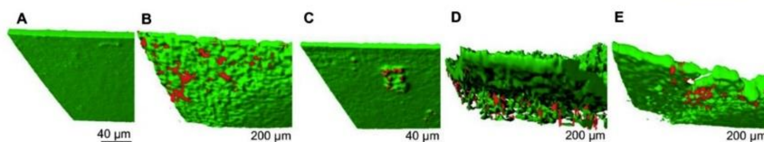


Fig. 4. The 3D reconstruction of the chorion fragments of the rainbow trout embryo after exposure to QDs (4 nM) after 24–36-day post fertilization. Green colour (500–530 nm) represents the autofluorescence of embryo tissues; red colour (569–640 nm) represents QDs photoluminescence. A – embryo chorion in control. B - adsorption of QDs aggregates at the chorion surface. C - overlay of QDs aggregates with chorion surface. D - the QDs aggregates induced defragmentation of the outer layer of the chorion. E - damage of the chorion integrity by QDs aggregates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

NiO, ZnO NPs, Lin et al., 2011), inductively coupled plasma mass spectrometry (Al_2O_3, Ag, and Au NPs, Böhme et al., 2017; ZnO NPs, Brun et al., 2014; Cu_2O, $CuCl_2$ NPs, Chen et al., 2011), and intravital multiphoton laser scanning microscope (CdSe/ZnS QDs, Lee and An, 2014) have been used for zebrafish embryo and larvae visualization. No studies were undertaken to investigate the damage to the chorion of the embryo by various visualization techniques, but some other studies have shown cell necrosis in early life stages of zebrafish in the blastoderm and in the yolk syncytial layer (Osborne et al., 2013).

3.5. Histological slice of rainbow trout embryos and QDs visualization

In addition, the incorporation of QDs into the chorion was examined by the histological investigation in slices of embryos (Fig. 6) and comparing them with the 3D reconstruction view in living embryos (Figs. 2, 3). Histology of rainbow trout embryos exposed to QDs for 8 days revealed that QDs aggregates were detected only on the chorion surface of the embryo (Fig. 6). As it is seen from the histological analysis of the embryo, Fig. 6A, the photoluminescence of QDs inside of the rainbow trout embryo (perivitelline space, yolk sac, etc.) was not detected. However, the photoluminescence of QDs aggregates were detected on the chorion outer layer (Fig. 6C, red colour).

The PL spectra measurements on the slices of the histological samples of fish embryos incubated with QDs (Fig. 6D, E) confirmed the evidence that red spots detected in the microscopic images are the PL of the QDs aggregates. PL spectra (Fig. 6D) measured at the outer layer of the chorion histological slice (marked on the microscopic image (Fig. 6C) as red squares) coincided with the QDs photoluminescence spectra measured in the incubation water and confirm that the red colour on the chorion surface was PL of QDs accumulated at the chorion outer layer.

3.6. Malformations of rainbow trout embryos

Although penetration and accumulation of QDs inside embryos were not observed, we found several damaged embryos (2.4%) in three replications ($N = 20$ in each group). Fig. 7 shows abnormally developing embryos in QDs incubation water. In the present study, QDs induced spinal curvature and blood clots and head hatching (Fig. 7). This study's results showed the QDs exposure also caused blood clots in the embryos heart area (Fig. 7B), which is an open area for further investigations.

Aggregates of NPs could clog the chorionic pores and influenced nutrient transportation, which might have negative effects on embryonic development (Celá et al., 2014). Pericardial edema was the main type of malformation caused by QDs; this could impact embryo cardiac function (Duan et al., 2013). Me did not find data about malformations of rainbow trout embryos induced by QDs. QDs could induce zebrafish embryos developmental malformations such as eyespots and melanin developmental inhibitions (Zhang et al., 2012b), and disintegrated embryos (Duan et al., 2013; George et al., 2011). Other malformations like pericardial edema, yolk sac edema, curvature of the spine, craniofacial malformation, opaque yolk, vitelline cyst, somites decrease and tail malformations were seen in zebrafish larvae after exposure of the embryos to QDs (Duan et al., 2013; George et al., 2011; King-Heiden et al., 2009; Zhang et al., 2012a, 2013; Zolotarev et al., 2012; Wiecinski et al., 2013). According to Ługowska and Sarnowski (2011), head hatching was less successful as compared to the tail hatching. In addition, head hatching usually caused larvae death and/or body malformations. The decrease of the hatching rate in zebrafish after exposure to QDs was observed by Duan et al., 2013, George et al., 2011, King-Heiden et al., 2009, Zhang et al., 2012a, 2013 and Zolotarev et al., 2012.

3.7. DNA damage to rainbow trout embryos and larvae

Comet assay showed significantly increased DNA damage (percentage of DNA in the tail) in rainbow trout embryos after one day of exposure to QDs (27-day post fertilization) and in newly hatched rainbow trout larvae after 11 days of exposure to QDs (one-day post hatching) (Fig. 8).

Several *in vivo* and *in vitro* studies indicate that exposure to Cd released from QDs is the primary determinant of CdSe/ZnS QDs genotoxicity in organisms (Aye et al., 2013; Brunetti et al., 2013;

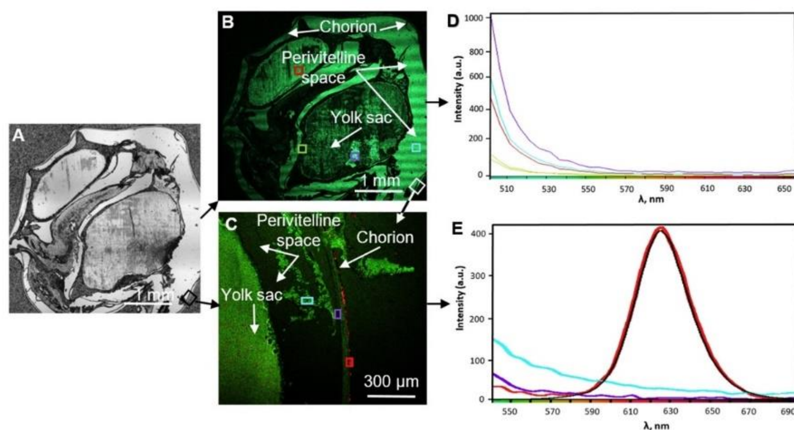


Fig. 6. Histological images and photoluminescence spectra of the rainbow trout after eight-day exposure to QDs (4 nM) at 32-day post fertilization. A - bright field image of the rainbow trout embryos. B and C fluorescence images of the histological slice of the embryo. Green colour (500–530 nm) represents the autofluorescence of embryo tissues; red colour (569–640 nm) represents the photoluminescence of QDs. D and E photoluminescence spectra measured at selected areas (different coloured squares in B and C images) of the histological slice. Black line is the photoluminescence spectra of QDs in incubation water (E). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

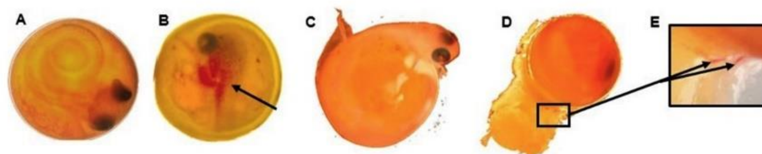


Fig. 7. Developmental disorders of rainbow trout embryos exposed to QDs after 10 days of exposure to QDs (4 nM) at 34-day post fertilization: spinal curvature (A) and blood clot (arrow showed) (B) and head hatching of embryos (C). The output of the yolk from the dead embryos (D) with blood clot (arrows showed) (E).

Galeone et al., 2012). A significant genotoxicity induction was detected in rainbow trout embryos as a result of QDs exposure (Cibulskaitė et al., 2016). Significant induction of cytotoxicity was not observed (Cibulskaitė et al., 2016). Previously, genotoxicity effects of CdS QDs in fish cell lines (RTG-2), of CdTe QDs in rainbow trout hepatocytes, and in zebrafish liver cells have been reported by Munari et al. (2014), Gagné et al. (2008b), Tang et al. (2013), respectively.

3.8. The possible mechanisms of QDs impact to embryos

In this study, Cd did not release from QDs into the incubation water (Fig. 1E; and Fig. 6D, E, Fig. S1). Likewise, this experiment did not detect any evidence for *in vivo* QDs degradation and penetration and accumulation of QDs in embryos was not observed (Figs. 1–6). Nevertheless, QDs adsorption to the chorion of the embryo was detected (Figs. 1–6). QDs slightly disturbed the embryo in toxicological view (Figs. 7 and 8).

Our study showed chorion damage caused by QDs aggregates induced mechanical stress. The mechanic damage is shown in Figs. 4 and 5, the chorion became nonhomogeneous in the beginning of exposure to QDs, when QDs aggregate stuck into the chorion (Fig. 4C). Furthermore, QDs aggregates induced adverse effects on chorion integrity (Figs. 4D, E and 5).

Our result showed that QDs covering the chorion of embryo such as clay. The clay is the example of negative impact to the embryo due to a fine size fraction of materials. The toxicity could be related to the negative developmental conditions of embryos. We did an experiment with clay (0, 0.375, 0.750, 1.5, 3.0, 6.0 and 12.0 g/L) to make sure that the effects of QDs aggregates to embryos are related to mechanical impact of QDs (Fig. 9).

The result indicated that with increasing dosages and exposure, the survival of rainbow trout did not significantly ($p < 0.05$) decrease compared to that of the control (data not showed). However, a slight decrease in survival was observed after four and eight days of exposure. After four days of exposure at 6.0 g/L concentration nine embryos died from three replicates ($N = 90$), and after eight days of exposure at 12.0 g/L concentration died 10 embryos from three replicates ($N = 90$). Little is known about the sensitivity of the embryos to fine size fraction of materials.

Survival to eyed stages of development of Atlantic salmon (*Salmo salar*) were most strongly affected by particles of silts and clays (<0.063 mm) (Julien and Bergeron, 2006; Lapointe et al., 2004). In addition, the results revealed that exposure to clay led to embryos bradycardia after one day and four days of exposure, but later (after eight days of exposure) it recovers to the control (Fig. 9). This tendency was observed in our previous research (Cibulskaitė et al., 2016), in which rainbow trout embryos exposed to QDs for 12 days, results showed that the survival of embryos in QDs did not significantly ($p < 0.05$) differ from the control, and the HR of embryos exposed to QDs during the 12, eight and four days before hatching was significantly ($p < 0.05$) lower when compared to the control (Cibulskaitė et al., 2016). The bradycardia of embryos was also found by Zhang et al. (2012b) and Duan et al. (2013).

This study observed several development malformations of the embryo (Fig. 7), which indicated the changes of physiologic function due to restricted the gas exchange and nutrient transportation. For this reason, QDs aggregates could have physically blocked the micro-pore canals of the embryo chorion like a clay (Julien and Bergeron, 2006). Therefore, the toxicity of QDs could be compared with the impact of clay. Possibly, toxicity was associated with mechanical stress to embryos or/and gas exchange changes due to embryo surface covering with QDs aggregate. According to Julien and Bergeron (2006), Lapointe et al. (2004) and Grieg et al. (2005), clay adheres to embryos and forms a thin coating of sediment. Thin coating around the embryo created by clay-sized sediment restricted the availability of oxygen to the incubating embryos through the chorion (Julien and Bergeron, 2006). Hence, QDs aggregates like particles of clay did not penetrate through the chorion, and the observed damages were a physiological response of organism.

Disturbed gas exchange would be expected to produce toxicity and genotoxicity endpoints in embryos (Cibulskaitė et al., 2016). Hypoxia-induced reactive oxygen species (ROS) may be a physiological response to oxygen deficiency. However, hypoxia-induced ROS production remains unclear. Several studies reported hypoxia-induced genetic instability in organisms and cell cultures (Snyder and Diehl, 2005; Kim et al.,

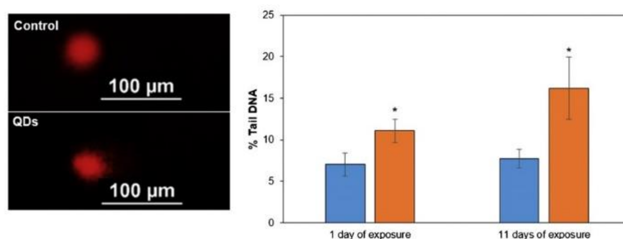


Fig. 8. The percentage of DNA in the comet tail (% Tail DNA, mean ± SD, $N = 7$) of embryos after one day of exposure (27-day post fertilization) and one-day old rainbow trout larvae (one day after hatching) at the end of the experiment (after 11 days of incubation to 4 nM QDs).

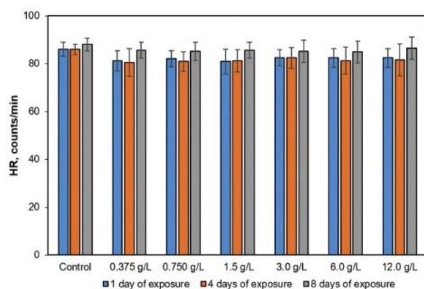


Fig. 9. Effect of clay (0, 0.375, 0.750, 1.5, 3.0, 6.0 and 12.0 g/L) on the heart rate (HR) of rainbow trout embryos (28–36-day post fertilization) depending on the duration of exposure (means ± SD).

2007), and concluded that hypoxia alone may act as genotoxic agent. Several reports have shown that hypoxia is a mutagen, teratogen, because it affects fish embryonic development (Shang and Wu, 2004; Shang et al., 2006). Hypoxia is known to induce mortality, malformation, delay fish (*D. rerio*) embryonic development, hatching, disrupt the apoptotic pattern, balance of sex hormones, affect sex differentiation and sex ratio (Shang and Wu, 2004; Shang et al., 2006). However, hypoxia inducing development impairments in fish, remains unclear (Wu, 2009). In many studies, oxidative stress and inflammatory response is proposed as key mechanisms for understanding nanoparticles-induced toxicity and genotoxicity (Magdolenova et al., 2014; Xiao et al., 2016). Nevertheless, such findings are hypothesis-generating and require further detailed study. There is a need for studies that characterize an organism's mechanisms to long-term exposures to QDs. Duan et al. (2013) showed that exposure to CdTe QDs caused an increase in mortality of zebrafish embryos and larvae in a dose- and timed-dependent manner. The toxicity was influenced by the QDs coating and was more potent in causing mortality than an equivalent amount of Cd^{2+} (King-Heiden et al., 2009). Zhang et al. (2012a, 2013) showed that LC50 120 h for zebrafish larvae is 1.98 mg/L of CdSe-MPA QDs, 185.9 nM of CdTe-TGA QDs and 22.31 mg/L of CdTe-TGA QDs.

Various QDs are known to be toxic to cells (Clift et al., 2011; Soenen et al., 2014; Luo et al., 2013) and different animal species, such as *Hydra vulgaris* (Ambrosone et al., 2012), zebrafish (Zhang et al., 2012a, 2013), and mice (Wang et al., 2016). However, the toxicity of QDs and the mechanism of QDs toxicity during the sensitive embryonic period are unclear, especially in aquatic organisms. Most of these studies indicate that toxicity was associated with QDs instability and metal leakage.

Our results lead to the conclusion that severe effect of QDs toxicity to embryos could be related with destruction of QDs and that heavy metal leakage seems to be the main source of toxicity. The stable QDs can induce only small damages to the chorion of embryos in comparison with the influence of the environment media in natural conditions.

4. Conclusions

Present study showed that carboxylated CdSe/ZnS QDs formed aggregates in deep-well water used for the incubation of embryos. QDs agglomerates also observed on the surface of fish embryos. They could have formed due to the interaction between QDs and mucus excreted by embryos. On the basis of spectroscopic and microscopic investigations on fish embryos *in vivo* and on histological samples and of 3D reconstruction on the results of fish embryo confocal microscopy, the process of interaction of QDs with embryos, accumulation of them in the chorion of embryos and partial disintegration of the embryos membrane was investigated. The chorion of embryos attracted QDs aggregates so that the QDs aggregates and/or formed agglomerates with mucus intervened into the embryo chorion, and caused the abruption of some parts of the chorion and could damage the chorion integrity after 10–12 days of exposure to QDs.

However, QDs aggregates did not translocate into the perivitelline space of rainbow trout (*O. mykiss*), zebrafish (*D. rerio*) or pearl gourami (*T. leerii*) embryos. Therefore, we can state that the chorion of rainbow trout, zebrafish and pearl gourami embryos is a protective barrier against QDs.

The damage of chorion integrity can induce blood clots in the heart area of rainbow trout embryos, provoke head hatching of embryos, and cause possible alterations of fish development. Results of toxicity and genotoxicity studies allows us to assume that hypoxia caused by impact of QDs aggregations could be one of the factors inducing changes of physiologic function in developing fish. However, further investigation must be undertaken to confirm this presumption.

Our study showed that the toxic effects of QDs on biological parameters of fish in its early stages was not related to the release of heavy metal ions, but mainly related to QDs nanotoxicity itself. Despite the rapid increase in nanomaterial production, it is necessary to better understand the specific nanotoxicologic properties of NPs and their potential impacts on health, safety and the environment.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.04.206.

Acknowledgment

This research was supported by the research infrastructure Open Access Centre for Nature Research and the National Cancer Institute at the initiative of the Open R&D Lithuania network. Genotoxicity studies were performed under Nature Research Centre program 3.

Funding

This work was funded by the Research Council of Lithuania, Project No MIP-108/2015.

Declaration of interest

The authors declare that they have no conflict of interest.

References

Alivisatos, A.P., 1996. Semiconductor clusters, nanocrystals, and quantum dots. Science 271 (5251), 933–937.
Ambrosone, A., Mattera, L., Marchesano, V., Quarta, A., et al., 2012. Mechanisms underlying toxicity induced by CdTe quantum dots determined in an invertebrate model organism. Biomaterials 33, 1991–2000.
Anderson, S.L., Wild, G.C., 1994. Linking genotoxic responses and reproductive success in ecotoxicology. Environ. Health Perspect. 102, 9–12.
Asharani, P.V., Wu, Y.L., Gong, Z., Valiyaveettil, S., 2008. Toxicity of silver nanoparticles in zebrafish models. Nanotechnology 19 (25):255102. https://doi.org/10.1088/0957-4484/19/25/255102.
Aye, M., Di Giorgio, C., Berque-Bestel, I., Aime, A., et al., 2013. Genotoxic and mutagenic effects of lipid-coated CdSe/ZnS quantum dots. Mutat. Res. 750 (1–2), 129–138.
Ballard, W.W., 1973. Normal embryonic stages for salmonid fishes, based on *Salmo gairdneri Richardson* and *Salvelinus fontinalis* (Mitchill). J. Exp. Zool. 184, 7–26.
Bijeesh, M.M., Shakhi, P.K., Arunkarthick, S., Varier, G.K., Nandakumar, P., 2017. Confocal imaging of single BaTiO3 nanoparticles by two-photon photothermal microscopy. Sci. Rep. 7 (1643).
Böhme, S., Baccaro, M., Schmidt, M., Potthoff, A., Stärk, H.-J., Reemtsma, T., Kühnel, D., 2017. Metal uptake and distribution in the zebrafish (*Danio rerio*) embryo: differences between nanoparticles and metal ions. Environ. Sci. Nano 2017 (4), 1005–1015.
Browning, L.M., Lee, K.J., Huang, T., Nallathamby, P.D., et al., 2009. Random walk of single gold nanoparticles in zebrafish embryos leading to stochastic toxic effects on embryonic developments. Nano 1, 138–152.
Brun, N.R., Lenz, M., Wehrli, B., Fent, K., 2014. Comparative effects of zinc oxide nanoparticles and dissolved zinc on zebrafish embryos and eleuthero-embryos: importance of zinc ions. Sci. Total Environ. 476, 657–666.

Brunetti, V., Chibli, H., Fiammengo, R., Galeone, A., et al., 2013. InP/ZnS as a safer alternative to CdSe/ZnS core/shell quantum dots: *in vitro* and *in vivo* toxicity assessment. Nano 5 (1), 307–331.

Celá, P., Veselá, B., Matalová, E., Večeřa, Z., Buchtová, M., 2014. Embryonic toxicity of nanoparticles. Cells Tissues Organs 199, 1–23.

Chen, D., Zhang, D., Jimmy, C.Y., Chan, K.M., 2011. Effects of Cu_2O nanoparticle and $CuCl_2$ on zebrafish larvae and a liver cell-line. Aquat. Toxicol. 105 (3), 344–354.

Cibulskaitė, Ž., Stankevičiūtė, M., Kazlauskienė, N., Baršienė, J., Kulvietis, V., Rotomskis, R., 2016. Long-term toxicity and geno-cytotoxicity of quantum dots to rainbow trout *Oncorhynchus mykiss* embryos. 13th International Conference on Protection and Restoration of the Environment, 3rd to 8th July, 2016. Grafima Publications, Mykonos Island, Greece, pp. 460–470.

Clift, M.J., Brandenberger, C., Rothen-Rutishauser, B., Brown, D.M., Stone, V., 2011. The uptake and intracellular fate of a series of different surface coated quantum dots *in vitro*. Toxicology 286, 58–68.

Duan, J., Yu, Y., Li, Y., Yu, Y., et al., 2013. Developmental toxicity of CdTe QDs in zebrafish embryos and larvae. J. Nanopart. Res. 15, 1700.

European Parliament and of the Council of the European Union, 2010. Directive 2010/63/EU of 22 September 2010 on the Protection of Animals Used for Scientific Purposes. Off. J. Eur. Union L276, 233–279.

Fatima, M., Usmani, N., Hossain, M.M., Siddiqui, M.F., Zafeer, M.F., Firdaus, F., Ahmad, S., 2014. Assessment of genotoxic induction and deterioration of fish quality in commercial species due to heavy-metal exposure in an urban reservoir. Arch. Environ. Contam. Toxicol. 1–11.

Fent, K., Weisbrod, C.J., Wirth-Heller, A., Pieles, U., 2010. Assessment of uptake and toxicity of fluorescent silica nanoparticles in zebrafish (*Danio rerio*) early life stages. Aquat. Toxicol. 100, 218–228.

Gagné, F., Auclair, J., Turcotte, P., Fournier, M., et al., 2008a. Ecotoxicity of CdTe quantum dots to freshwater mussels: impacts on immune system, oxidative stress and genotoxicity. Aquat. Toxicol. 86 (3), 333–340.

Gagné, F., Maysinger, D., André, C., Blaise, C., 2008b. Cytotoxicity of aged cadmium-telluride quantum dots to rainbow trout hepatocytes. Nanotoxicology 2 (3), 113–120.

Galeone, A., Vecchio, G., Malvindi, M.A., Brunetti, V., et al., 2012. *In vivo* assessment of CdSe-ZnS quantum dots: coating dependent bioaccumulation and genotoxicity. Nano 4, 6401–6407.

George, S., Xia, T., Rallo, R., Zhao, Y., et al., 2011. Use of a high-throughput screening approach coupled with *in vivo* zebrafish embryo screening to develop hazard ranking for engineered nanomaterials. ACS Nano 5 (3), 1805–1817.

Grieg, S.M., Sear, D.A., Smallman, D., Carling, P.A., 2005. Impact of clay particles on the cutaneous exchange of oxygen across the chorion of Atlantic salmon eggs. J. Fish Biol. 66, 1681–1691.

Henn, K., 2011. Limits of the Fish Embryo Toxicity Test with *Danio rerio* as an Alternative to the Acute Fish Toxicity Test. Dissertation (Germany).

Hrovat, M., Segner, H., Jeram, S., 2009. Variability of *in vivo* fish acute toxicity data. Regul. Toxicol. Pharmacol. 54, 294–300.

Ipe, B.I., Lehnig, M., Niemeyer, C.M., 2005. On the generation of free radical species from quantum dots. Small 1, 706–709.

ISO 10304-1, 2007. Water Quality. Determination of Dissolved Anions by Liquid Chromatography of Ions. Determination of Bromide, Chloride, Fluoride, Nitrate, Nitrite, Phosphate and Sulfate.

ISO 12890, 1999. Water Quality–Determination of Toxicity to Embryos and Larvae of Freshwater Fish–Semi-static Method.

ISO 14911, 1998. Water Quality. Determination of Dissolved Li^+, Na^+, $NH_4{}^+$, K^+, Mn^{2+}, Ca^{2+}, Mg^{2+}, Sr^{2+} and Ba^{2+} Using Ion Chromatography—Method for Water and Waste Water.

ISO 15586, 2003. Water Quality. Determination of Trace Elements Using Atomic Absorption Spectrometry with Graphite Furnace.

ISO 5814, 1990. Water Quality. Determination of Dissolved Oxygen— Electrotechnical Probe Method.

ISO 6332, 1988. Water Quality. Determination of Iron—Spectrometric Method Using 1.10-Phenanthrolinxe.

ISO 7346-1, 1996. Water Quality–Determination of the Acute Lethal Toxicity of Substances to a Freshwater Fish (*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae))–Part 1: Static Method.

ISO 9963-1, 1994. Water Quality. Determination of Alkalinity—Part 1: Determination of Total and Composite Alkalinity.

Jaramillo, R., Garrido, O., Goicoechea, O., Molinari, E., 2015. Comparative study of ultrastructural changes on morphology of chorion during development of two salmonid species (*Salmo salar* and *Oncorhynchus kisutch*). Glob. J. Mar. Sci. 1, 1–9.

Jarvis, R.B., Knowles, J.F., 2003. DNA damage in zebrafish larvae induced by exposure to low-dose rate gamma-radiation: detection by the alkaline comet assay. Mutat. Res. 541, 63–69.

Julien, H.P., Bergeron, N.E., 2006. Effect of fine sediment infiltration during the incubation period on Atlantic salmon (*Salmo salar*) embryo survival. Hydrobiologia 563, 61–71.

Kang, Y.F., Li, Y.H., Fang, Y.W., Xu, Y., et al., 2015. Carbon quantum dots for zebrafish fluorescence imaging. Sci. Rep. 5:11835. https://doi.org/10.1038/srep11835.

Kashiwada, S., 2006. Distribution of nanoparticles in the see-through medaka (*Oryzias latipes*). Environ. Health Perspect. 114 (11) (1697–1602).

Katsumiti, A., Gilliland, D., Arostegui, I., Cajaraville, M.P., 2014. Cytotoxicity and cellular mechanisms involved in the toxicity of CdS quantum dots in hemocytes and gill cells of the mussel *Mytilus galloprovincialis*. Aquat. Toxicol. 153, 39–52.

Kim, B.M., Choi, J.Y., Kim, Y.J., Woo, H.D., Chung, H.W., 2007. Reoxygenation following hypoxia activates DNA-damage checkpoint signaling pathways that suppress cell-cycle progression in cultured human lymphocytes. FEBS Lett. 581 (16), 3005–3012.

King-Heiden, T.C., Wiecinski, P.N., Mangham, A.N., Met, K.M., et al., 2009. Quantum dot nanotoxicity assessment using the zebrafish embryo. Environ. Sci. Technol. 43 (5), 1605–1611.

Kulvietis, V., Streckytė, G., Rotomskis, R., 2011. Spectroscopic investigations of CdTe quantum dot stability in different aqueous media. Lith. J. Phys. 51 (2), 163–171.

Lapointe, M.F., Bergeron, N.E., Bérubé, F., Pouliot, M.A., Johnston, P., 2004. Interactive effects of substrate sand and silt contents, redd-scale hydraulic gradients and interstitial velocities on egg to emergence survival of Atlantic salmon (*Salmo salar*). Can. J. Fish. Aquat. Sci. 61, 2271–2277.

Lee, W.-M., An, Y.-J., 2014. Evidence of three-level trophic transfer of quantum dots in an aquatic food chain by using bioimaging. Nanotoxicology 9, 407–412.

Lee, K.L., Nallathamby, P.D., Browning, L.M., Osgood, C.J., Xu, X.H.N., 2007. *In vivo* imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos. ACS Nano 1, 133–143.

Lin, S., Zhao, Y., Xia, T., Meng, H., et al., 2011. High content screening in zebrafish speeds up hazard ranking of transition metal oxide nanoparticles. ACS Nano 5 (9), 7284–7295.

Ługowska, K., Sarnowski, P., 2011. Heads or tails - fish hatching. Acta Ichthyol. Piscat. 41 (1), 13–17.

Luo, Y.H., Wu, S.B., Wei, Y.H., Chen, Y.C., et al., 2013. Cadmium-based quantum dot induced autophagy formation for cell survival via oxidative stress. Chem. Res. Toxicol. 26, 662–673.

Magdolenova, Z., Collins, A., Kumar, A., et al., 2014. Mechanisms of genotoxicity. A review of *in vitro* and *in vivo* studies with engineered nanoparticles. Nanotoxicology 8 (3), 233–278.

Munari, M., Sturve, J., Frenzilli, G., Sanders, M.B., et al., 2014. Genotoxic effects of CdS quantum dots and Ag2S nanoparticles in fish cell lines (RTG-2). Mutat. Res. Genet. Toxicol. Environ. Mutagen. 775–776, 89–93.

Nel, A., Xia, T., Mädler, L., Li, N., 2006. Toxic potential of materials at the nano level. Science 311, 622–627.

Osborne, O.J., Johnston, B.D., Moger, J., Balousha, M., et al., 2013. Effects of particle size and coating on nanoscale Ag and TiO_2 exposure in zebrafish (*Danio rerio*) embryos. Nanotoxicology 7 (8), 1315–1324.

Petushkova, N.A., Kuznetsova, G.P., Larina, O.V., et al., 2015. One-dimensional proteomic profiling of *Danio rerio* embryo vitellogenin to estimate quantum dot toxicity. Proteome Sci. 13, 17.

Piccinno, F., Gottschalk, F., Seeger, S., Nowack, B., 2012. Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. J. Nanopart. Res. 14 (9), 1–11.

Powers, D.A., 1989. Fish as model systems. Science 20, 352–358.

Ribeiro, D.S.M., Frigerio, C., Santos, J.L.M., Prior, J.A.V., 2012. Photoactivation by visible light of CdTe quantum dots for inline generation of reactive oxygen species in an automated multipumping flow system. Anal. Chim. Acta 735, 69–75.

Rocha, T.L., Gomes, T., Mestre, N.C., Cardoso, C., Joao, M., 2015. Tissue specificresponces of cadmium-based quantum dots in the marine mussel *Mytilus galloprovincialis*. Aquat. Toxicol. 169, 10–18.

Rocha, T.L., Mestre, N.C., Saboia-Morais, S.M.T., Bebianno, M.J., 2017. Environmental behaviour and ecotoxicity of quantum dots at various trophic levels: a review. Environ. Int. 98, 1–17.

Santana, R.M.M., Oliveira, T.D., Rodrigues, S.S.M., Frigerio, C., et al., 2015. Enhancing reactive species generation upon photo-activation of CdTe quantum dots for the chemiluminometric determination of unreacted reagent in $UV/S_2O_8^2$ drug degradation process. Talanta 135, 27–33.

Shang, E.H., Wu, R.S., 2004. Aquatic hypoxia is a teratogen and affects fish embryonic development. Environ. Sci. Technol. 38 (18), 4763–4767.

Shang, E.H., Yu, R.M., Wu, R.S., 2006. Hypoxia affects sex differentiation and development, leading to a male-dominated population in zebrafish (*Danio rerio*). Environ. Sci. Technol. 40 (9), 3118–3122.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191.

Snyder, R.D., Diehl, M.S., 2005. Hypoxia-induced micronucleus formation in mice. Drug Chem. Toxicol. 28, 373–378.

Soenen, S.J., Manshian, B.B., Aubert, T., Himmelreich, U., et al., 2014. Cytotoxicity of cadmium-free quantum dots and their use in cell bioimaging. Chem. Res. Toxicol. 27, 1050–1059.

Songe, M.M., Willems, A., Wiik-Nielsen, J., Thoen, E., Evensen, O., van West, P., Skaar, I., 2016. Saprolegnia diclina IIIA and S. parasitica employ different infection strategies when colonizing eggs of Atlantic salmon, Salmo salar L. J. Fish Dis 39 (3), 343–352.

Strtak, A., Sathiamoorthy, S., Tang, P.S., Tsoi, K.M., et al., 2017. Yeast populations evolve to resist CdSe quantum dot toxicity. Bioconjug. Chem. https://doi.org/10.1021/acs.bioconjchem.7b00056.

Tang, S., Cai, Q., Chibli, H., Allagadda, V., et al., 2013. Cadmium sulfate and CdTe-quantum dots alter DNA repair in zebrafish (*Danio rerio*) liver cells. Toxicol. Appl. Pharmacol. 272 (2), 443–452.

Thomas, S., Mohaideen, J.A., 2015. Determination of some heavy metals in fish, water, and sediments from the bay of Bengal. Int. J. Chem. Sci. 13 (1), 53–62.

Tsoi, K.M., Dai, Q., Alman, B.A., Chan, W.C.W., 2013. Are quantum dots toxic? Exploring the discrepancy between cell culture and animal studies. Acc. Chem. Res. 46, 662–671.

Vatsos, I.N., Thompson, K.D., Adams, A., 2006. Colonization of rainbow trout, *Oncorhynchus mykiss* (Walbaum), eggs by *Flavobacterium psychrophilum*, the causative agent of rainbow trout fry syndrome. J. Fish Dis. 29, 441–444.

Villarreal, F.D., Das, G.K., Abid, A., Kennedy, I.M., Kültz, D., 2014. Sublethal effects of CuO nanoparticles on Mozambique tilapia (*Oreochromis mossambicus*) are modulated by environmental salinity. PLoS One 9 (2), e88723.

Wang, X., Tian, J., Yong, K., Zhu, X., et al., 2016. Immunotoxicity assessment of CdSe/ZnS quantum dots in macrophages, lymphocytes and BALB/c mice. J. Nanobiotechnol. 14:10. https://doi.org/10.1186/s12951-016-0162-4.

Wehmas, L.C., Anders, C., Chess, J., Punnoose, A., et al., 2015. Comparative metal oxide nanoparticle toxicity using embryonic zebrafish. Toxicol. Rep. 2, 702–715.

Wiecinski, P.N., Metz, K.M., King-Heiden, T.C., Louis, K.M., et al., 2013. Toxicity of oxidatively degraded quantum dots. Environ. Sci. Technol. 47 (16):9132–9139. https://doi.org/10.1021/es304987r.
Wu, R.S.S., 2009. Effects of hypoxia on fish reproduction and development. Fish Physiol. 27, 79–141 (Hypoxia).
Xiao, Y.-Y., Liu, L., Chen, Y., Zeng, Y.-L., Liu, M.-Z., Jin, L., 2016. Developmental toxicity of carbon quantum dots to the embryos/larvae of rare minnow (*Gobiocypris rarus*). Biomed. Res. Int. 2016, 4016402.
Zhang, W., Lin, K., Miao, Y., Dong, Q., et al., 2012a. Toxicity assessment of zebrafish following exposure to CdTe QDs. J. Hazard. Mater. 213–214, 413–420.
Zhang, W., Lin, K., Sun, X., Dong, Q., et al., 2012b. Toxicological effect of MPA-CdSe QDs exposure on zebrafish embryo and larvae. Chemosphere 89, 52–59.
Zhang, W., Miao, Y., Lin, K., Chen, L., et al., 2013. Toxic effects of copper ion in zebrafish in the joint presence of CdTe QDs. Environ. Pollut. 176 (C), 158–164.
Zolotarev, K.V., Kashirtseva, V.N., Mishin, A.V., Belyaeva, N.F., et al., 2012. Assessment of toxicity of Cdse/Cds/Zns/S,S-dihydrolipoic acid/polyacrylic acid quantum dots at *Danio rerio* embryos and larvae. ISRN Nanotechnol. 2012:1–5. https://doi.org/10.5402/2012/914636 Article number 914636.

IV

Responses of biomarkers in Atlantic salmon (*Salmo salar*) following exposure to environmentally relevant concentrations of complex metal mixture (Zn, Cu, Ni, Cr, Pb, Cd). Part II.

Stankevičiūtė M, Sauliutė G, Makaras T, Markuckas A, Virbickas T, Baršienė J.

Ecotoxicology 2018, https://doi.org/10.1007/s10646-018-1960-2

Reprinted with permission from Springer Nature

Ecotoxicology
https://doi.org/10.1007/s10646-018-1960-2

Responses of biomarkers in Atlantic salmon (*Salmo salar*) following exposure to environmentally relevant concentrations of complex metal mixture (Zn, Cu, Ni, Cr, Pb, Cd). Part II

Milda Stankevičiūtė[1] · Gintarė Sauliutė[1] · Tomas Makaras[1] · Arvydas Markuckas[2] · Tomas Virbickas[1] · Janina Baršienė[1]

Accepted: 25 June 2018
© Springer Science+Business Media, LLC, part of Springer Nature 2018

Abstract

The aim of this research was to assess interactions between metals at low exposure concentrations (Maximum-Permissible-Concentrations accepted for the inland waters in EU) and to assess possible influence of background exposure (10-times reduced concentration of a single metal) on toxicological significance of selected biomarkers in *Salmo salar* after treatment with metal mixture (Zn – 0.1, Cu – 0.01, Ni – 0.01, Cr – 0.01, Pb – 0.005 and Cd – 0.005 mg/L). The tissue-specific bioaccumulation, genotoxicity and cytotoxicity responses (erythrocytic nuclear abnormalities assay) in peripheral blood, kidneys, gills and liver erythrocytes of fish to metal mixtures were assessed after 14 days treatment. Treatment with primary mixture (MIX) or two variants of this mixture (Cr↓ (10 times reduced Cr^{6+} concentration) and Cu↓ (10 times reduced Cu^{2+} concentration)) induced the strongest responses in genotoxicity and cytotoxicity endpoints. Exposure to these mixtures highly affected Zn, Cu and Cd bioaccumulation in liver tissue. The highest amount of Ni accumulated was measured after Cd↓ treatment in all tissues. Treatments with reduced concentration of non-essential metal resulted in an increased accumulation of Pb, Ni, or Cd; treatments with reduced concentration of essential metal resulted in a reduced accumulation of certain metals (especially Cd and Pb) in tissues compared between treatments. Glucose content in blood and behavioural endpoints were evaluated after short-term exposure to metal mixtures (MIX, Cr↓, Cu↓). Significant increase in blood glucose concentration was measured after all treatments. These metal mixtures elicit significant behavioural alterations in fish. Consequently, this research revealed a significant influence of background exposure considering mixture toxicity.

Highlights

- 10-fold reduction of mixtures components highly affected bioaccumulation of several metals in analysed tissues of *Salmo salar*.
- Significant influence of background exposure considering mixture toxicity was detected.
- Exposure to metal mixtures at environmentally relevant concentrations elicited genotoxicity and cytotoxicity.
- Alterations in glucose content in blood and behavioural responses were observed.

Keywords Genotoxicity · cytotoxicity · *Salmo salar* · metals · bioaccumulation · behavioural responses

Introduction

As reported by European Environment Agency (EEA 2012) different chemicals, such as metals, industrial chemicals, pesticides and other pollutants are responsible for poor chemical status of surface water bodies across Europe. Metals are the main pollutants of any industrial or domestic discharges, and they are assumed as most hazardous in the toxicological studies (Javed and Usmani 2017). Heavy metals accounts for 20% of river water bodies in poor

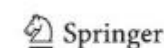
Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10646-018-1960-2) contains supplementary material, which is available to authorized users.

✉ Milda Stankevičiūtė
milda.stan@gmail.com

[1] Nature Research Centre, Akademijos str. 2, Vilnius 08412, Lithuania

[2] Department of Biochemistry and Molecular Biology, Life Sciences Center, Vilnius University, Saulėtekio av. 7, Vilnius 10223, Lithuania

Published online: 10 July 2018



status; are the major pollutant (accounting for 60%) in lakes and accounts for 50% of coastal water bodies in poor status (EEA 2012). Aquatic organisms are exposed to a number of various pollutants covered by different EU regulations, while mixture effects are not currently regulated. Several researches emphasized the necessity of studies related to interactions of low concentration toxic metals and essential metals mixtures (Cobbina et al. 2015; Nys et al. 2017). As concluded by Nys with co-authors (2017), the understanding about metal mixture toxicity and their interactions is far from fully investigated, neither mechanism are fully understood nor performance of mixtures risk assessment. Future progress in mixture toxicology, must be able to provide experimental data for many more mixture elements, move in the direction of probabilistic exposure, ensure that data and methods are useful in the assessment of mixtures and provide a better perception of whether an assessment is too cautious or insufficiently protective (Evans et al. 2016).

Pollution of freshwater from industrial, domestic, agriculture sources and man-made barriers to movement are the major factors responsible of the decline of Atlantic salmon (*S. salar*) populations in Europe. Historically, *S. salar* is a keystone migratory species with a North Atlantic distribution, with presence in all countries whose rivers enter the North Atlantic (Hendry and Cragg-Hine 2003). The Atlantic salmon is included in annexes II and V of the European Union (EU) Habitats Directive as a species of European significance. Nevertheless, development of salmon populations since the mid-1990s has been encouraging (HELCOM 2011). The species is an economically (subsistence, recreational and commercial fisheries) and ecologically important fish species throughout the European region (Kulmala et al. 2012). Moreover, *S. salar* is a non-model fish that less frequently used in behavioural toxicity tests and geno-cytotoxicity assays. Therefore, there is a growing interest in the application of behavioural analysis of Atlantic salmon to toxicity assessment.

Numerous studies have demonstrated the impact of individual metal ions on fish species integrating the effects of contaminants across different levels of biological organization (Price 2013). Metals in combination may exhibit synergistic, additive or antagonistic effects on fish survival, growth and reproduction (Driessnack et al. 2017a, Stankevičiūtė et al. 2016). As stated by Javed and Usmani (2017), adverse effects of metals on fish are multidirectional, inducing many disturbances in the physiological and chemical process. Metals are known to affect fish condition factor and organosomatic indices, particularly hepatosomatic index (Stankevičiūtė et al. 2016; Javed and Usmani 2017). Gills and liver were found to be target tissues for metals bioaccumulation, however, only several studies recorded bioaccumulation in kidneys (Stankevičiūtė et al. 2017; Javed and Usmani 2017). Biochemical disorders, genotoxicity, cytotoxicity and behavioural changes after single metal or binary-metal mixture exposure also were reported (Sampaio et al. 2008; Zhu et al. 2011; Stankevičiūtė et al. 2016). However, little concern was given to understand the interaction and the effects to fish behaviour over the short period caused by combined trace metals and due to variation in their concentrations in the mixture. Different activity patterns in swimming behaviour of fish may suggest about possible synergistic or antagonistic actions of contaminants.

As concluded by Javed and Usmani (2017), battery of biomarkers must be used to examine the health of aquatic organisms. Kroon with co-authors (2017) indicated the suitable fish bioaccumulation markers and biomarkers of exposure. Gills, liver and muscle tissues were indicated as the most important tissues for metal bioaccumulation analysis and atomic absorption spectroscopy as one of the most common methods used to measure metal accumulation. Micronuclei together with other nuclear abnormalities as irreversible genotoxic events were emphasized as suitable biomarkers of metal exposure (Kroon et al. 2017). Glucose level in fish blood is one of the main indicators of stress measured in the most scientific research, which indicates the health condition of fish (Bartonkova et al. 2016). Metallothioneins (MT) as a biomarker of metal exposure are involved in the detoxification process of metals (Fabrin et al. 2018). The behavioural endpoints such as average, maximum or angular velocity are very informative parameters for behavioural pattern assessment. Velocity is a derivative ratio between the time spend moving and the distance travel (Melvin et al. 2017). Moreover, when determining the baseline behaviour of fish, the average velocity is more stable in relation to time then other endpoints (Makaras et al. 2018). In addition, we chose movement duration as another endpoint, which also may indicate such changes in fish movement behaviour.

This study was designed to evaluate toxicological significance of biological effects (change in the glucose, metallothionein content, genotoxicity, cytotoxicity, bioaccumulation in the different tissues and behavioural responses) caused by metal (Zn, Cu, Ni, Cr, Pb, Cd) mixture in Atlantic salmon tissues by testing the mixture as a whole (whole mixture approach) and by reducing environmentally realistic concentration of single metal in the mixture by 10-times (represent the possible influence of background exposure in the aquatic environment). This is a follow-up study of previous *S. salar* experimental research were specific toxicity endpoints such as genotoxicity and cytotoxicity were identified and recognized as suitable biomarkers to detect toxicity induced by metal mixture at low exposure concentrations. Moreover, the exposure-response relation and time necessary to attain the steady-state concentrations of metals in tissues of Atlantic salmon was established in

the previous study (Stankevičiūtė et al. 2017). The time needed to attain steady-state concentrations of metals in most of the tissues was 14 days. However, it was impossible to identify the toxicant responsible for genotoxicity and cytotoxicity outcome. The current experimental research was conducted to evaluate the effects, which arises due to interactions between metals in the mixture at steady-state condition and to assess possible influence of background exposure on chosen toxicological endpoints.

The objectives of this research are to disclose whether interactions at low metal exposure concentrations (Maximum-Permissible-Concentrations (MPC) accepted for the inland waters in EU) are likely to occur and elicit toxicologically significant effects when existing in mixture: (1) on bioaccumulation of metals in different tissues (gills, liver, kidneys and muscle) of Atlantic salmon, (2) on genotoxicity and cytotoxicity endpoints, (3) on behavioural responses and locomotor activity, (4) on glucose and metallothionein contents.

Material and methods

Study animals

The test was conducted on hatchery-reared one-year-old Atlantic salmon (*Salmo salar* Linnaeus, 1758) smolts, average total weight 45.4 ± 4.38 g and average total length 172.5 ± 6.62 mm (mean ± SD, $N = 142$). The fish was obtained from Meškerinė fish hatchery (Švenčionys District, Lithuania) and 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 rate 1 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 fish started to swim freely in all directions and they feed well.

General laboratory procedures

I Experiment (genotoxicity, cytotoxicity, metallothionein determination and bioaccumulation)

The experiment was conducted under semi-static rotating water-current conditions in polyethylene (PE) tanks of 35-L total volume filled to a level of 30 L with continuously aerated deep-well water. Test solutions and clean water were renewed every day. The animals were fed before the transfer. Test fish were transferred into other tanks with the same amount of freshly prepared solutions. Empty tanks were washed.

II Experiment (glucose level measurement and behavioural assay)

Each test fish was placed into an approximately 80 L experimental 5 mm thick glass aquarium divided into eight equal 210 × 310 × 130 mm sized sections. Sections were filled with 3 L of continuously aerated deep-well water, with an air stone separated from the main testing area by a baffle (1.5 mm mesh size). Each section was covered with a translucent polymeric glass to prevent fish from jumping out of the aquarium.

Preparation of test solutions

Reagent grade metal salts («REACHIM» Company, Russia) were used as the toxicants. For each metal, stock solutions 1000 mg/L were prepared by dissolving necessary amount of the salts in distilled water, the final concentration being recalculated according to the amount of metals ions. These dilutions were further diluted. For each test concentration, 1000 mL complex solution was made up in a HDPE bottle. Each solution was acidified with reagent-grade nitric acid (final concentration 0.5% v/v). Concentrated stock solutions were prepared one day prior to the test. Fish were transferred to tanks after concentrated stock solutions were diluted in the deep-well water.

Test procedures

Experimental design including types of performed treatments, biomarkers, number of replicates, exposure duration and number of animals used in this research, is presented in Table 1. Test fish were exposed for 14 days period to a six metal (Zn, Cu, Ni, Cr, Pb and Cd) mixture (herein after referred to as MIX) at a concentration corresponding to maximum-permissible-concentrations (MPC) accepted for the inland waters in EU (Directive 2008/105/EC) (Table 3). Other treatments were performed by 10-times reducing MPC of single metal in the mixture (MIX) made of 6 metals, while other 5 metals concentrations remain constant (e.g., Zn↓ (metal with 10-times reduced concentration in MIX), while Cu, Ni, Cr, Pb, Cd concentrations remain constant (herein after referred to as Zn↓) and etc.).

During behavioural and glucose level assays, the test solution (MIX, Cu↓ and Cr↓ mixtures) was slowly injected (about 5 s) into the aquarium aeration zone through silicon tubes attached to the aquarium. Each of metal mixtures solutions was prepared up to 3 mL of volume containing the necessary amount of test concentration. The ratio between prepared test solution (3 mL) and dilution water (3 L) was

Table 1 The design of experiments (biomarkers, type of treatments, exposure duration, number of replicates and animals used in research)

Experiment	Biomarkers	Treatment Number of animals per replicate/replicates								Exposure duration	Number of animals (*N*)
		Control	MIX	Zn↓	Cu↓	Ni↓	Cr↓	Pb↓	Cd↓		
I	Bioaccumulation ENAs	7/1	7/1	7/1	7/1	7/1	7/1	7/1	7/1	14 days	56
	MT	7/1	7/1	–	–	–	–	–	–	14 days	14
II	Behavioural assay (*Acclimation-control test*)	8/1 (+8/2*)	–	–	–	–	–	–	–	4 h	72
	Glucose and behavioural assay	8/2	8/2	–	8/2	–	8/2	–	–	4 h (2** h)	
		Total number of animals used in research									142

*The same replicates were used for glucose and behavioural assay

**Fish movements were recorded for 4 h—the first 2 h acclimation period, the second 2 h control and exposure period

Table 2 Chemical and physical characteristics of the deep-well water (all values are in mg/L, unless otherwise noted)

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				

only 0.001 part, which cannot cause any changes in water flow speed and direction or even in water pressure that fish might be able to feel these differences.

A total of 70 *S. salar* fish were dissected for the collection of samples for bioaccumulation, erythrocytic nuclear abnormalities (ENAs) and metallothionein (MT) assays (Table 1). After the behavioural testing and glucose sampling, 72 fish were transferred to holding tanks (1000-L volume) supplied with flow-through aerated metal-free deep-well water and left to recover. All experiments on fish were conducted in accordance with local and EU regulations (Directive 2010/63/EU).

Physico-chemical analyses

The main physico-chemical parameters of the water (temperature, dissolved O_2, pH and conductivity) were measured routinely with a hand-held multi-meter (WTW Multi 340i/SET, Germany). Deep-well water was analysed for a number of standard physical and chemical properties, such as alkalinity, chlorides, nitrates, ammonia, metals according to standard analytical methods (ISO 15586:2003; ISO 6332:1988; ISO 5814 1990; ISO 10523:2008; ISO 14911:1998; ISO 10304-1:2007; ISO 9963-1:1994) (Table 2).

Designed nominal metal concentrations in the tanks ($N = 4$) were checked during blank tests (without fish) with an atomic absorption spectrophotometer (SHIMADZU AA-6800, Japan) by graphite furnace technique using proprietary software. Each water sample was acidified with reagent-grade nitric acid (final concentration 0.5% v/v) and analysed in triplicate. During blank test, metals concentrations were measured routinely to identify possible contamination by and/or sorption of metals in tanks. No significant change with time in metals concentrations was found.

Biomarker analyses

Metal bioaccumulation analysis

After the testing was completed, all fish (of control and metal-exposed groups) were sacrificed. 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 (~3 g), gills (whole organ), liver (whole organ) and kidneys (whole organ); organs were weighed to an accuracy of ±0.001 g. Fish samples were hot air oven-dried at 85 °C for 24 h until reached constant weight, predigested tightly in a concentrated ultrapure HNO_3 (60%) and H_2O_2 (30%) (Lach-Ner, Chempur, respectively) at a ratio of 5:1 v/v for 8 h at a room temperature and then microwave-digested quickly (Jia et al. 2005). After cooling

solutions were filtered through a 0.45 μm glass filter and diluted with deionized water. Metal concentrations were measured by atomic absorption spectrophotometry on Varian Spectr AA 55 (USA) with a graphite furnace technique in accordance with standardized procedure ISO 15586:2003 final concentration being expressed as mg/kg of wet weight. Accuracy of analytical procedure was checked using certified reference material fish homogenate (IAEA–407). Recoveries were in acceptable range (within 10%) of the certified values.

Metallothionein determination

Metallothionein content determination was assayed according to the method of Peixoto et al. (2003). For metallothionein level assays, the liver and kidney were removed, weighted and frozen (−80 °C). The organs were homogenized with Potter-Elvehjem homogenizer in 4 volumes of 20 mM Tris-HCl buffer, pH 8.6, containing 0.5 mM PMSF and 0.01% β-mercaptoethanol. The homogenate was then centrifuged at 17,000×*g* for 30 min at 4 °C. Aliquots of 1 ml of supernatant containing metallothioneins were added with 1.05 ml of cold (−20 °C) absolute ethanol and 80 μl chloroform. The samples were centrifuged at 6000 × *g* for 10 min at 4 °C. The collected supernatant was combined with three volumes of cold ethanol (−20 °C), maintained at −20 °C for 1 h and centrifuged at 6000×*g* for 10 min at 4 °C. The metallothionein-containing pellets were then rinsed with 1 ml of 87% ethanol and 1% chloroform mix and centrifuged at 6000×*g* for 10 min at 4 °C. The metallothionein content in the pellet was evaluated using the colorimetric method with DTNB reagent. The pellet was suspended in 150 μl 0.25 M NaCl and subsequently 150 μl 1 N HCl containing 4 mM EDTA was added to the sample. 4.2 ml 2 M NaCl containing 0.43 mM DTNB buffered with 0.2 M Na-phosphate, pH 8.0 was then added to the sample at room temperature. The sample was centrifuged at 3000×*g* for 5 min at room temperature. The supernatant absorbance was evaluated at 412 nm. Metallothionein concentration was estimated using molar absorption coefficient at 412 nm 14140 $M^{-1}cm^{-1}$ (Eyer et al. 2003) and expressed as micrograms of SH groups per gram of wet weight.

Erythrocytic nuclear abnormalities (ENAs) analysis in in vivo assay

Erytrocytic nuclear abnormalities (ENAs) analysis was performed in peripheral blood, gills, kidneys and liver erythrocytes. Blood was immediately taken from the caudal vein. A drop of blood was directly smeared on microscopic slides and air-dried. After the sacrifice, small pieces of cephalic kidneys, liver and gills were dissected, softly dragged along clean slide and allowed to dry for 1–2 h (Baršienė et al. 2006). Dried smears were fixed in methanol for 10 min. and were stained with 10% Giemsa solution in phosphate buffer pH = 6.8 for 8 min. (Baršienė et al. 2004). The stained slides were analysed under bright-field microscopes Olympus BX51 (Tokyo, Japan) using an immersion objective (1000×) and the photos were taken with an Olympus U-CMAD3 (Tokyo, Japan) camera. 4000 erythrocytes with intact cellular and nuclear membrane per fish were evaluated using blind scoring by a single observer. Total 28,000 erythrocytes were analysed in each treatment group. Final results were expressed as the mean value (‰) of sums of analysed individual lesions scored in 1000 erythrocytes per fish sampled from every study group. The formation of micronuclei (MN), nuclear buds (NB), nuclear buds on filament (NBf), 8-shaped nuclei and fragmented-apoptotic (FA), kidney-shaped, blebbed (BL), vacuolated (VacNuc) erythrocytes were identified using criteria described by Fenech et al. (2003) and Baršienė et al. (2014). The photos of erythrocytic nuclear abnormalities in *S. salar* blood were presented in our previous study (Stankevičiūtė et al. 2017). The genotoxicity levels were assessed as the sum of the frequencies of micronuclei, nuclear buds and nuclear buds on filament (MN + NB + NBf). Cytotoxicity levels were assessed as the sum of the frequencies of fragmented-apoptotic and 8-shaped erythrocytes (FA + 8-shaped).

Blood sampling and glucose measurement

Peripheral blood of each fish for glucose (Glu) evaluation was collected after 2 h of exposure to clean water (control) and to metal mixtures. Blood samples from fish were taken from the caudal vein through disposable syringe (1 mL volume) by drawing approximately 0.1 mL of blood and then transferred to heparinized blood collection tubes. Concentrations of Glu in fish blood were determined by using the automatic Glucose Analyser (EKSAN-Gm, ANALITA, Joint-Stock Company Ltd, LITHUANIA). The minimum detection limit of the blood Glu method is from 2 to 30 mmol/L and the error for repeated measurements (precision) is ≤5%. Minimal blood sample volume per measurement is 50 μL.

Behavioural assay

Acclimation-control test The acclimation-control period tests were performed to collect data on the locomotor activity dynamics in *S. salar* juveniles during the 4 h long observation and to establish the adequate acclimation duration and the control (baseline) level of fish locomotion. Behavioural test was used to establish the effect of metal mixture on fish over short period of time.

Behavioural endpoints of fish such as average velocity (cm/s), average movement duration (%) were recorded for 1 min at selected intervals (every 10 min) for 4 h using a digital video camera (PANASONIC HC-V770 Wi-Fi, 12.76 MP). The video camera was positioned 3 m above the test aquarium. The researcher left the place immediately after the start of video recording. We have checked on how fish respond to control solution (3 mL of dilution water) being introduced into the tank through the aeration zone during the acclimation-control after 2 h of acclimation period. No statistical significant changes in fish response (according to average speed, movement duration) were determined. All the obtained data were analysed using the ETHOVISION XT 12 (Noldus Technologies, Inc. The Netherlands) software.

Chemical exposure test Treatment with MIX or two variants of this mixture (Cr↓ (10 times reduced Cr^{6+} concentration) and Cu↓ (10 times reduced Cu^{2+} concentration)) induced the strongest responses in geno- and cytotoxicity endpoints. Moreover, exposure to these mixtures highly affected bioaccumulation of several metals (Zn, Cu and Cd) in liver tissue. Consequently, these three mixtures were chosen to establish the effect of metal mixture on fish behaviour over short period of time. In this test, we used the same experimental procedure as in the acclimation-control test. Based on the acclimation-control test results, the acclimation period was set for 2 h. After 2 h of acclimation, the test solution (MIX, Cu↓ and Cr↓ mixtures) was slowly injected (about 5 s) into the aquarium aeration zone through silicon tubes attached to the aquarium. Fish movements were recorded immediately after the start (1 min) of exposure (every 10 min) for 2 h. The tracked video data were further processed using the same software.

Data analyses

All the data obtained were analysed using STATISTICA 7.0 (StatSoft Inc., Tulsa, Oklahoma, USA) or GraphPad Prism® 5.01 (GraphPad Software Inc., San Diego, CA, USA) software. Since the most of geno- and cytotoxicity data do not follow a normal distribution (Kolmogorov-Smirnov and Shapiro-Wilk normality test), these data were analysed by the nonparametric Kruskal-Wallis test (for comparison of differences between groups). Metal bioaccumulation, MT and glucose content data follow a normal distribution (Kolmogorov-Smirnov and Shapiro-Wilk normality test). Data for metal contents in tissues were evaluated by two-way ANOVA followed by Bonferroni post hoc test, MT levels and glucose content in blood were analysed by one-way ANOVA. The results were expressed as mean ± standard error or standard deviation. The level of significance was established at $p < 0.05$. A discriminant function analysis (DA) was performed to determine which metal concentration and ENAs coefficients distinguished treatments from each other and to identify any statistical similarity among data due to overlapping of statistical ellipses. Functions 1 and 2 explain the highest proportion of the total variability in the data. Only functions that presented $p < 0.05$ and eigenvalues greater than 1 were considered statistically significant. Ellipses show 95% confidence intervals and if they did not overlap indicate differences between treatments. Scatterplots of discriminant functions using metal concentrations in specific tissue and ENAs data in peripheral blood were included in the results section, while results of discriminant function analysis (eigenvalue, cum. proportion, chi-sqr., df, standardized discriminant function coefficients and means of canonical variables) and DA results utilizing metal concentrations in certain tissue and ENAs data in the same tissue erythrocytes are presented in Supplement 1 (Table 5–7, Fig. 1).

Behavioural data were checked for normality (Kolmogorov-Smirnov tests) and homogeneity of variances (Levene's test), the data which were not normally distributed or not homogeneous were transformed. The arcsine transformation was applied to data intended for use in a statistical methods designed for parametric data. Nested ANOVA test followed by Tukey post hoc test was used to analyse the data collected over 4 h acclimatization-control observational timeframes to identify significant differences ($p < 0.05$) between three control groups variables at the same time point. Nested Repeated Measures ANOVA was used to establish significant differences ($p < 0.05$) between mean values of all three control groups at selected time intervals. Nested ANOVA was applied to test the significance between treatment group and control group at the same time points and Nested Repeated Measures ANOVA was used for comparison between treatment and control groups at 1 h and 2 h treatment intervals. The Tukey's HSD test was used to test differences among samples mean for significance at level $p < 0.05$.

Results

Physico-chemical analyses

Water quality parameters of the deep-well water, which was used for the experiments, are presented in Table 2. The data indicate that metals concentrations are at low limits in the water used for the experiments. After the addition of metals, mean measured concentrations in the experimental tanks were within 5–20% of the target (Table 3). Metal concentrations in control water are presented in Table 2.

Table 3 Metals and their test waterborne concentrations (mg/L) in test media

Metal	Source	Concentration (mg/L)			
		MIX (MPC)	MIX Measured (mean ± SD)	Metal↓ (with 10-times reduced concentration)	Metal↓ Measured (mean ± SD)
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

Metal bioaccumulation

The levels of accumulation for Zn, Cu, Ni, Cr, Pb and Cd (means ± SD (mg/kg of w/w)) in gills, liver, kidneys and muscle of Atlantic salmon after treatment with metal mixtures are presented in Fig. 1. Variation in concentration of accumulated metal in tissue was noticed between treatments. Also, different organs exhibited different patterns in metal bioaccumulation. For instance, the accumulation of Cu and Zn in gills and muscle tissues was similar between all treatments. No significant differences in accumulated amount of these metals were measured compared to control group and between treatments. Except for Cu concentration, which increased significantly in muscle tissue after Ni↓ treatment compared to control, Zn↓ and Cu↓ mixtures. In liver, Zn and Cu accumulation was the most intensive after treatment with Cu↓ and Cr↓ mixtures, respectively. The amounts of Zn and Cu accumulated in liver were significantly higher in comparison to control and other treatments groups. The results demonstrated that kidneys contained the highest concentrations of Zn and Cu after treatment with MIX and Pb↓ mixture, respectively. A significant decrease in accumulated Zn and Cu amounts in liver and kidneys was noticed after treatment with several mixtures with reduced concentration of a single metal in comparison to MIX (Fig. 1).

The highest mean concentrations of Ni were measured after Cd↓ mixture treatment in all tissues investigated, particularly in kidneys. Significant elevation of Ni concentration in gills, liver and kidneys were measured between almost all treatments groups in comparison to control. Significant decrease of mean Ni concentration in liver were measured after Cu↓ treatment, in gills – after Cu↓, Cr↓ treatments, in kidneys – after Zn↓, Cu↓, Cr↓ treatments and in muscle – after Zn↓, Pb↓ treatments in comparison to MIX treatment.

The data on Cr distribution in investigated tissues showed a wide variety depending on performed treatment. Significantly increased Cr concentration in gills and kidneys were noted after treatment with Ni↓ mixture in comparison to control and other mixtures treatment. The highest and significant amount of Cr accumulated in liver and muscle tissues was detected after treatment with Cd↓ and Cu↓ mixtures, respectively. Significantly reduced concentration of Cr in gills was measured after treatment with Pb↓ and Cd↓ mixtures in comparison to all other variants of MIX.

Lead concentration was very unevenly distributed in analysed tissues after treatment with variants of MIX. When compared to the control, the highest and significant accumulation of Pb was measured after treatment with MIX and Cd↓ mixtures in muscle and after treatment with Cd↓ mixture in kidneys. While, in liver – after treatment with Ni↓ and Cd↓ mixtures, in gills – after treatment with MIX, Zn↓ and Cd↓. Significant decrease in accumulated Pb amount was detected in gills after treatment with Cu↓, Ni↓ and Cr↓ mixtures in comparison with MIX treatment, also such decreases, but not significant were noticed in other tissues investigated (except for liver after treatment with Ni↓ mixture).

Bioaccumulation of Cd showed the highest increase after treatment with Pb↓ mixture in gills and muscle, in liver and kidneys – after treatment with Cr↓ and Ni↓, respectively. When compared to MIX treatment, significantly higher Cd concentration was measured after Cr↓ and Ni↓ treatments in liver and kidneys, respectively. When compared to control, significant bioaccumulation of Cd was detected almost after all variants of metal treatments in gills, liver and kidneys. No significant increase in Cd concentration was measured in muscle tissue after all treatments performed.

As shown in Table 4, the amount of accumulated metal in tissue differs depending on performed treatment. Considering the sequences of accumulated metal amount in different treatments, such trend was detected: the first places of sequences were occupied by treatments with reduced concentration of toxic metal (mostly Cd), while treatments with reduced concentration of essential metal (e.g. Cu, Cr and Zn) mostly occurred in the last places of the sequences.

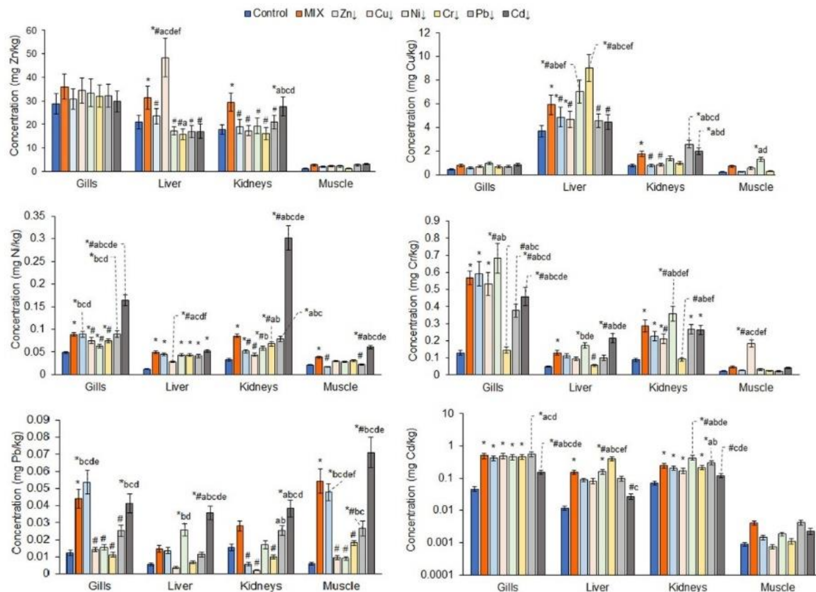


Fig. 1 Mean concentration of accumulated metals in *S. salar* tissues after treatments with complex metal mixture and variants of this mixture with 10-times reduced concentration of a single metal in the mixture (mean ± SD, $n = 7$). Asterisks (*) denote significant differences from control group, # – from MIX, **a** from Zn↓; **b** from Cu↓; **c** from Ni↓; **d** from Cr↓; **e** from Pb↓; **f** from Cd↓ ($p < 0.05$). Bioaccumulation data after MIX treatment are from the previous study (Stankevičiūtė et al. 2017)

Metallothioneins induction

Metallothionein content in *S. salar* liver and kidneys is presented on Fig. 2. Liver and kidneys MT did not show statistically significant differences between the control and exposed group (MIX). However, hepatic MT level increased 1.34-fold after MIX treatment. Consequently, no further investigation of MT content after treatment with MIX variants was performed.

ENAs frequencies

The results obtained using nuclear abnormalities analysis in erythrocytes of *S. salar* exposed to different metal mixtures and their control is presented in Fig. 3. The ENAs such as MN, BL, VacNuc, exhibited the highest frequencies in most of the treatments. The micronuclei showed the highest frequencies in Cr↓ or Cu↓ treatments, followed by MIX and Ni↓. The ENAs NBf, NB and VacNuc, showed the highest induction after treatment with MIX in gills and kidneys erythrocytes. The highest BL and Kidney-shaped frequencies were measured in Cd↓ treatment in all tissues, except gills erythrocytes for BL and kidneys – for Kidney-shaped frequencies. The highest induction of 8-shaped frequencies was measured in Cr↓, Cu↓ or Zn↓ treatments, depending on analysed tissue. FA and NBf exhibited the lowest frequencies in most of the treatments (Fig. 3). The highest induction of Total ENAs was detected in Cr↓ treatment, except in gills erythrocytes – after treatment with MIX. Significant differences of total ENAs induction was measured after Cr↓ treatment in all analysed tissues erythrocytes, after Cu↓ treatment in blood and after MIX, Pb↓ treatments in gills erythrocytes in comparison to control. Significant differences of separate ENAs induction, such as MN, Kidney-shaped and 8-shaped frequencies were measured in several treatments in blood; such as MN, NB, VacNuc and FA in several treatments in gills; such as MN in Cr↓ treatment in liver erythrocytes. No significant differences of separate ENAs induction were measured in kidneys erythrocytes in any treatment performed.

Table 4 The sequence of metal amount accumulated in *S. salar* tissues after performed metal treatments

Metal	Tissue	Sequence
Zn	Gills	MIX > Cu↓ > Ni↓ > Pb↓ > Cr↓ > Zn↓ > Cd↓
Cu		Ni↓ > Cd↓ > MIX > Cu↓ > Pb↓ > Cr↓ > Zn↓
Ni		Cd↓ > Zn↓ > Pb↓ > MIX > Cr↓ > Cu↓ > Ni↓
Cr		Ni↓ > Zn↓ > MIX > Cu↓ > Cd↓ > Pb↓ > Cr↓
Pb		Zn↓ > MIX > Cd↓ > Pb↓ > Ni↓ > Cu↓ > Cr↓
Cd		Pb↓ > MIX > Cu↓ > Cr↓ > Ni↓ > Zn↓ > Cd↓
Zn	Liver	Cu↓ > MIX > Zn↓ > Ni↓ > Cd↓ > Pb↓ > Cr↓
Cu		Cr↓ > Ni↓ > MIX > Zn↓ > Cu↓ > Pb↓ > Cd↓
Ni		Cd↓ > MIX > Zn↓ > Ni↓ > Cr↓ > Pb↓ > Cu↓
Cr		Cd↓ > Ni↓ > MIX > Zn↓ > Pb↓ > Cu↓ > Cr↓
Pb		Cd↓ > Ni↓ > MIX > Zn↓ > Pb↓ > Cr↓ > Cu↓
Cd		Cr↓ > Ni↓ > MIX > Pb↓ > Zn↓ > Cu↓ > Cd↓
Zn	Kidneys	MIX > Cd↓ > Pb↓ > Ni↓ > Zn↓ > Cu↓ > Cr↓
Cu		Pb↓ > Cd↓ > MIX > Ni↓ > Cr↓ > Cu↓ > Zn↓
Ni		Cd↓ > MIX > Pb↓ > Cr↓ > Ni↓ > Zn↓ > Cu↓
Cr		Ni↓ > MIX > Pb↓ > Cd↓ > Zn↓ > Cu↓ > Cr↓
Pb		Cd↓ > MIX > Pb↓ > Ni↓ > Cr↓ > Zn↓ > Cu↓
Cd		Ni↓ > Pb↓ > MIX > Cr↓ > Zn↓ > Cu↓ > Cd↓
Zn	Muscle	Cd↓ > Pb↓ > MIX > Cu↓ > Ni↓ > Zn↓ > Cr↓
Cu		Ni↓ > Cd↓ > MIX > Cu↓ > Pb↓ > Cr↓ > Zn↓
Ni		Cd↓ > MIX > Cr↓ > Cu↓ > Ni↓ > Pb↓ > Zn↓
Cr		Cu↓ > MIX > Cd↓ > Ni↓ > Zn↓ > Cr↓ > Pb↓
Pb		Cd↓ > MIX > Zn↓ > Pb↓ > Cr↓ > Cu↓ > Ni↓
Cd		Pb↓ > MIX > Cd↓ > Ni↓ > Zn↓ > Cr↓ > Cu↓

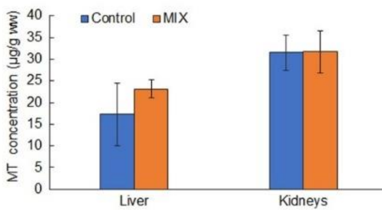


Fig. 2 Metallothionein (MT) content (mean ± SD, $n = 7$) in *S. salar* liver and kidneys

Summed genotoxicity and cytotoxicity levels in erythrocytes of different tissues are shown in Fig. 4. The highest level of genotoxicity in peripheral blood and liver erythrocytes was detected after Cr↓ treatment, in gills – after MIX and Cr↓ treatments and in kidneys after treatment with MIX and Cu↓. The highest cytotoxicity level was detected after Cr↓ and Cu↓ treatment in peripheral blood, after Cr↓ treatment in kidneys; after Pb↓ treatment in gills and after Cu↓ treatment in liver erythrocytes. Significantly increased genotoxicity levels were measured in Cr↓ and Cu↓ treatments in peripheral blood erythrocytes, after Cr↓ treatment in gills and liver and after MIX and Cu↓ treatments in kidneys erythrocytes compared to control level. Significant cytotoxicity level was detected after Cr↓ and Cu↓ treatments in peripheral blood and after Pb↓ treatment in gills erythrocytes compared to control level.

Patterns of biomarkers response to treatments

The discriminant analysis using data of metal concentration in specific tissue and ENAs in peripheral blood after different treatments indicated five significant functions in gills and liver, while six – in kidneys and muscle tissues (supplement 1: Tables 1–4). The first two discriminant functions have the highest eigenvalues and explain the largest proportion of the total variability in the data (supplement 1: Tables 1–4). Scatterplots of function 1 versus function 2 are presented in Fig. 5a–d. Figure 5b, c shows that, data of Cd↓ treatment were entirely separated from other treatments data in liver and kidneys. Moreover, a clear separation of Cd↓ treatment from other treatments was noted in gills and muscle tissues (Fig. 5a, d).

Discriminant function analysis indicated Cu, Ni, Cr, Pb, Cd in gills and MN, NBf, Vac-Nuc endpoints in peripheral blood as variables, which significantly ($p < 0.03$) contributed to separation of treatments. Ni, Pb and Cr, Cd, MN indicated the highest coefficients (1.03, 0.60 and −1.06, −0.67, −0.46, respectively) in function 1 and function 2, respectively (Supplement 1: Table 1). In liver, all metals, except Cu and ENAs in peripheral blood such as NB, 8-shaped and FA were the reasons for the separation of treatments. Pb, NB and 8-shaped presented the highest coefficients (−1.67, −0.81, −0.84, respectively) in function 1, while Cd had the highest coefficient (−0.85) in function 2 (supplement 1: Table 2). In kidneys, all metals and NB data were significantly contributed to separation between treatments. Ni and Pb indicated the highest coefficients (−1.09, −0.80, respectively) in function 1, while Cr and Cu (−0.85, −0.72) – in function 2 (supplement 1: Table 3). In muscle, all metals and MN, NB, NBf were significantly contributed to separation between treatments. Pb and Zn showed the highest coefficients (0.84, 0.82, respectively) in function 1, while Cr and Cu (1.16 and 0.53) – in function 2.

The similarities were detected between data of MIX, Zn↓ and Pb↓ treatments in gills and liver tissues; between data of MIX and Pb↓ treatments in kidneys, they ellipses were overlapped (Fig. 5a–c). Data of all treatments were separated from the control data in gills and liver tissues. Similar DA results were obtained using metal concentration and ENAs data in the same tissue, these results are presented in supplement 1 (Fig. 1).

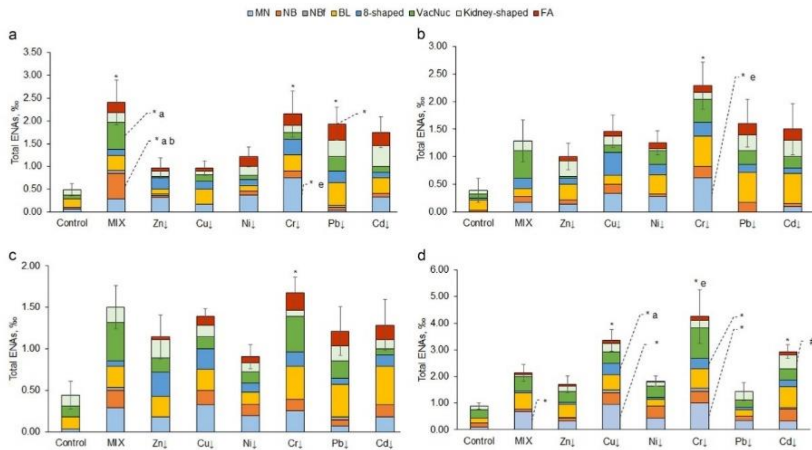


Fig. 3 Total number of ENAs in: **a** gills, **b** liver, **c** kidneys and **d** peripheral blood erythrocytes (mean ± SEM, $n = 7$). Asterisks (*) denote significant differences from control group, # – from MIX, **a** from Zn↓; **b** from Cu↓; **c** from Ni↓; **d** from Cr↓; **e** from Pb↓; **f** from Cd↓ ($p < 0.05$). ENAs data after MIX treatment are from the previous study (Stankevičiūtė et al. 2017)

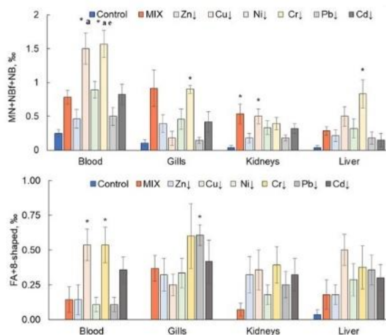


Fig. 4 Summed genotoxicity (MN + NBf + NB) and cytotoxicity (FA + 8-shaped) responses in *S. salar* erythrocytes (mean ± SEM, $n = 7$). Asterisks (*) denote significant differences from control group, # – from MIX, **a** from Zn↓; **b** from Cu↓; **c** from Ni↓; **d** from Cr↓; **e** from Pb↓; **f** from Cd↓ ($p < 0.05$). ENAs data after MIX treatment are from the previous study (Stankevičiūtė et al. 2017)

Blood glucose level

Blood glucose levels were observed to increase significantly after 120 min treatment with MIX, Cu↓ and Cr↓ mixtures in comparison to control (Fig. 6). However, the amount of blood glucose in fish did not differ between mixtures investigated.

Behavioural alterations

The Acclimation-control test

The statistical data analysis of acclimation-control period showed that all three control groups (control group consisted of 8 fish individuals) did not differ between each other at the same time points ($p > 0.05$) (data not shown). In this case, overall mean values at different time points corresponding to all three control groups were determined for further behavioural data analysis. The analysis of the behavioural data of *S. salar* over time showed behavioural patterns of the fish during the acclimation-control period (Fig. 7). According to average velocity obtained data (Fig. 7a), fish swimming activity were higher at the beginning of the test compared to time points after 70 min ($p < 0.05$), where fish activity is used to decrease. The average movement duration (%) (arcsine data transformation was used to perform statistical analysis) data revealed a similar tendency of behavioural pattern in fish activity (the increase of activity at the beginning of the test and a significant decrease after 70 min ($p < 0.05$) of control exposure) (Fig. 7b). The variation between data time points for both behavioural endpoints were the most stable after 2 h: for average velocity ($F(12,14) = 1.32$, $p = 0.214$) and

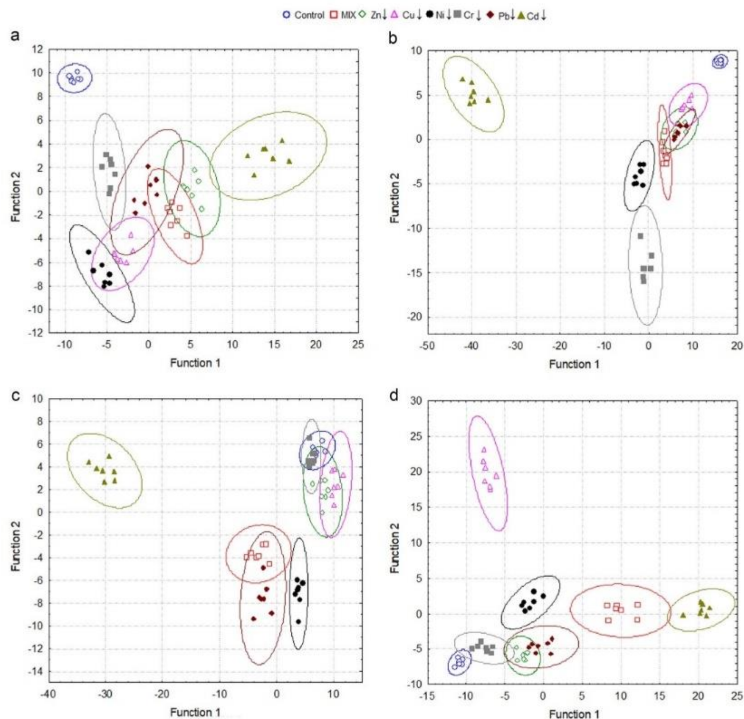


Fig. 5 Scatterplots of discriminant function 1 versus discriminant function 2 using metal concentration data in specific tissue (**a** gills, **b** liver, **c** kidneys, **d** muscle) and erythrocytic nuclear abnormalities in peripheral blood (ellipses show 95% confidence intervals)

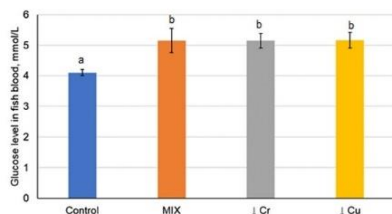


Fig. 6 Blood glucose values of *S. salar* after 2 h metal mixture treatments (mean ± SEM, $n = 16$). Letters denote significant differences between groups

movement duration ($F(12,25) = 1.07$, $p = 0.383$). This period (after 2 h) was set up as the control (baseline) period for the metal mixtures toxicity tests. In this test, differences in fish behaviour throughout time may indicate the acclimation process of fish to the new test environment. Increased activity of fish after transporting to the test aquariums could be interpreted as "anxious behaviour" for fish, while after few hour of acclimation, activity is slowly decreased and remained more stable in relation to time, which could be described as baseline behaviour "control level" established under the laboratory condition.

Metal mixtures exposure

The obtained data demonstrates changes in locomotor activity patterns of the *S. salar* exposed to different metal

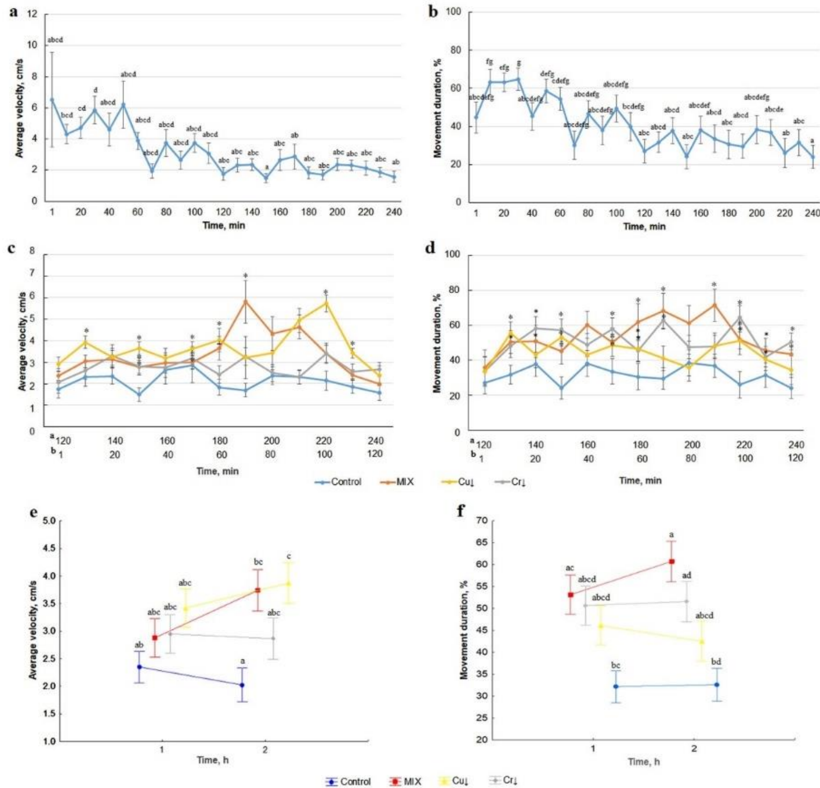


Fig. 7 Average velocity (cm/s) and average movement duration (%) of *S. salar* recorded for 4 h during **a**, **b** acclimatization-control period (mean ± SEM, $n = 24$), **c**, **d** exposure period (after 2 h of acclimation for 2 h of exposure, $n = 16$) to MIX, Cu↓ and Cr↓ solutions and **e**, **f** comparison between treatment and control groups at 1 h and 2 h treatment intervals (mean ± SEM, $n = 16$). Data were acquired at 10 min intervals. Horizontal axis (a) indicates the time sequence after acclimatization period and (b) indicates the time sequence after the start of exposure. Letters represent groups that differ significantly at level $p < 0.05$. Asterisks (*) denote significant differences between treatment and control group at the same time points ($p < 0.05$)

mixture concentrations at selected test-intervals (every 10 min for 2 h of exposure) (Fig. 7c, d). In comparison with the control period at the same time points, MIX exposure was determined to induce a significant increase in the average velocity of the fish after 30 ($p = 0.014$), 50 ($p = 0.044$) and 70 min ($p < 0.001$) of exposure (Fig. 7c). The peak of the response to MIX treatment was observed after 70 min of exposure. Fish exposed to Cu↓ showed significant difference compared to control in response after 10 min ($p = 0.006$), 30 min ($p > 0.001$), 50 min ($p = 0.002$), 60 min ($p = 0.025$) and reached a peak within 100 min ($p < 0.001$) of exposure, at 110 min ($p = 0.003$) fish activity decreased. Only two statistically significant values in response to Cr↓ exposure were established at 30 ($p = 0.005$) and 50 ($p = 0.006$) min of exposure indicating smaller effect of Cr↓ toxicity to rainbow trout fish behaviour.

The average movement duration (%) (arcsine data transformation for statistical analysis was applied) data

Table 5 Effects analysis between variables for average velocity endpoint

Effect	SS	df	MS	F	p
Intercept	1264.9	1	1264.9	461.78	0.000
Treatment	47.44	3	15.81	5.77	0.0015
Replicate(treatment)	15.20	5	3.04	1.11	0.364
Error	172.56	63	2.74		
Hour	1.55	1	1.55	1.06	0.307
Hour × treatment	7.93	3	2.64	1.81	0.154
Hour × replicate (treatment)	3.81	5	0.763	0.523	0.758
Error	91.84	63	1.46		

Table 6 Effects analysis between variables for movement duration (data were arcsine transformed for statistical evaluation)

Effect	SS	df	MS	F	p
Intercept	75.66	1	75.66	929.6	0.000
Treatment	1.95	3	0.651	8.00	0.0001
Replicate(treatment)	0.588	5	0.118	1.44	0.221
Error	5.13	63	0.081		
Hour	0.008	1	0.008	0.512	0.477
Hour × treatment	0.057	3	0.019	1.15	0.334
Hour × replicate(treatment)	0.038	5	0.008	0.462	0.803
Error	1.03	63	0.016		

showed rather different results in fish response to MIX, Cu↓ and Cr↓ treatments compared to average velocity data (Fig. 7d). Significant increases ($p < 0.05$) in swimming activity of salmon juveniles was determined almost at every time points, except at the beginning of the test (1 min), 40 and 80 min ($p > 0.05$) of exposure. The highest data values was observed to MIX exposure showing a steady increase in fish locomotor activity over time, however, no clear peak in response for all three treatments based on this endpoint was observed.

The behavioural data of fish to the effects of these metal mixtures revealed significant differences between control and treatment groups at 1 and 2 h time intervals (Fig. 7e, f). The differences in both endpoints between treatments was found to be significant for average velocity ($F(3,63) = 5.77$, $p = 0.0015$) and for average movement duration ($F(3,63) = 8.00$, $p < 0.001$) (Tables 5 and 6).

Based on average velocity data, the control group after 1 h of exposure did not significantly differ ($p > 0.05$) any treatment groups. The MIX and Cu↓ treatment group after 2 h of exposure differed from control (2 h) showing a significance at level $p = 0.041$ and $p = 0.021$, respectively, although the treatment groups did not differ ($p > 0.05$) between each other after 1 and 2 h of exposure. Data obtained from movement duration in fish showed an opposite results: Cu↓ treatment group did not differ from control after 1 and 2 h of exposure, while fish exposed to MIX showed a significance in response compared to control after 1 h and 2 h ($p < 0.001$ and $p = 0.018$, respectively) of exposure.

In summary, the behavioural patterns of *S. salar* to metal mixtures were rather different. Fish exposed to MIX and Cu↓ showed a presence of the different peak over time in average velocity endpoint, while no peak in response of fish exposed to Cr↓ was observed. Mean values of the response to Cr↓ exposure was apparently much lower than MIX and Cu↓ and did not significantly differ from control ($p = 0.237$). However, the results of movement duration endpoint showed no significant difference between control and Cu↓ treatment ($p = 0.068$). Only MIX treatment was found to be a significant compared to control for both endpoints: average velocity ($p = 0.022$) and movement duration ($p < 0.001$). The meaningful results in swimming activity of salmon juveniles to mixtures treatment were observed after 10 min of exposure, while at different time points indicating the significant peaks of the behavioural response. Moreover, this behavioural study showed the rapidness of the behavioural response of fish juveniles to metal mixtures treatment indicating the sensitivity of *S. salar* species to water contamination.

In addition, the response patterns of fish based on average velocity data (to MIX and Cu↓ exposure) could be characterized in the following order: (1) increased velocity, (2) peak of velocity, (3) decreased velocity.

Discussion

The present study examined tissue-specific metal accumulation (Cu, Zn, Ni, Cr, Pb and Cd) and geno-cytotoxicity responses in *S. salar* after treatment with complex metal mixture (MIX) and six variants of this mixture with 10-times reduced concentration of a single metal in the mixture. The concentrations for waterborne metals (Cu, Zn, Ni, Cr, Pb and Cd) used in the current study are environmentally relevant, while 10-times reduced concentration of a single metal in the mixture represent the possible influence of background exposure in the aquatic environment. The results of this study demonstrated that reduction of even low (MPC) metal concentration in mixture had a significant effect on metals distribution in tissues and affects the frequencies of genotoxicity and cytotoxicity endpoints. Furthermore, metal mixtures (MIX, Cu↓ and Cr↓) elicit behavioural alterations in *S. salar*, such as changes in locomotor activity and rise blood glucose level. Exposure to toxic and essential metals mixture with slight change of a single metal concentration resulted in different risk of

toxicity, which can apparently be influenced by metal-metal interactions in both outside and inside the organism.

Driessnack with co-authors (2017a) emphasized that understanding of the harmful effects of metal exposure mainly occurs from researches performed with individual metals. However, metals exist in complex composition in aquatic environment and toxicity evaluation for individual chemicals leads to misrepresentation of risk of chemical mixtures. In the current study, experimental data on metal bioaccumulation in fish muscle, liver, kidneys and gills following exposure to complex metal mixtures provide valuable information to develop a multimetal toxicity models that predict the toxicity of mixtures to aquatic organisms. Muscle tissue exhibited the lowest and less significant variations in accumulated metals amount after treatment with MIX variants with 10-time reduced concentration of a single metal. The bioaccumulation pattern of Pb in muscle was most affected by reduction of metal concentration in comparison to other metals accumulation in muscle tissue. Cu↓ treatment markedly affected accumulated amount of Zn in liver (increased 1.5-fold) and Cr in muscle (increased 4-fold) compared to MIX treatment. Moreover, Cr↓ treatment 1.5-fold increased Cu accumulation and 2.6-fold Cd accumulation in liver. However, treatments with reduced metal concentration induced the lowest variation in the amount of accumulated Cd in tissues, compared to accumulated amount changes of other metals. The highest amount of Ni accumulated was measured after Cd↓ treatment in all tissues. However, accumulation of other metals did not reveal such clear dependence on performed treatment. Patterns of metal bioaccumulation suggested the existence of competitive metals interaction in complex mixture even at low exposure levels. Several researches are made on tissue-specific metals accumulation in fish influenced by the interaction between binary metal mixtures (Driessnack et al. 2016, 2017a, b). Tissue-specific accumulation of Cu and Ni in fathead minnow (*Pimephales promelas*) was not affected by the interaction of these metals in the mixture (Driessnack et al. 2017a). While, Cu and Cd accumulation significantly increased in gills and liver in fish co-exposed to Cu and Cd mixture, however, interaction between metal accumulation was observed only in liver (Cd significantly reduced Cu accumulation) (Driessnack et al. 2016). Cd and Zn co-exposure decreased Cd accumulation in gills and liver of fathead minnow (Driessnack et al. 2017b). Experimental studies of binary metal mixture interactions using salmonid fish reported decreased Zn accumulation in gills of *O. mykiss* during Zn/Cd co-exposure, but Zn/Cu mixture did not have such an effect (Saibu et al. 2018). In this study, slight increase in MT content in liver and kidneys of *S. salar* was detected after MIX treatment. This indicates that, treatment with complex metal mixture at MPC causes the protective physiological reactions of the organism. 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). Therefore, a large amount of accumulated Zn, Cu or Cd after Cu↓, Ni↓ or Cr↓ treatments may result in a higher MT levels in liver or kidneys tissues compared to MIX treatment.

High concentrations of individual metals have a potential genotoxicity and cytotoxicity effects. However, genocytotoxicity responses to metal mixtures at environmentally relevant concentrations are scarcely investigated. Particularly, changes in biological responses and their toxicological significance after reduction of concentration of single metal in the mixture have not been investigated. The results of the present study showed that genotoxicity of mixture depend on the concentrations of metals in the mixture. Discriminant function analysis indicated that data of Cd↓ treatment were entirely separated from other treatments and control data. Ni, Pb followed by Cr contributed most to the separation between treatments in gills and kidneys tissues, Pb, Cd – in liver and Pb, Cr – in muscle. Considering ENAs data, MN, NB and NBf contributed most to the discriminatory power of functions. Similarities were noticed between MIX, Zn↓ or Pb↓ treatments data. Considering frequencies of single genotoxicity endpoints, such as MN, NB and NBf, the highest frequencies were detected after Cr↓ treatment in most cases, fallowing by MIX and Cu↓ treatments. The frequencies of cytotoxicity endpoints, showed the highest induction after Cr↓, Cu↓ or Pb↓ treatment. Moreover, significant elevation in total ENAs in peripheral blood was measured after Cd↓ treatment. It is important to emphasize, that mixture with reduced concentration of Cr caused the highest induction of genotoxicity and cytotoxicity endpoints frequencies. While, metal bioaccumulation data shows that treatment with reduced Cr resulted in a decreased accumulation of nonessential metals such as Cd or Pb in tissues, except increased Cd bioaccumulation in liver. These results may suggest occurrence of hermetic-like responses, that some of the metals in a mixture induce hormesis. Exposure to low concentrations of Cr may produce a beneficial or stimulatory effect. Perez-Benito (2006) emphasized hormetic effect of low Cr (VI) concentrations such as an increased lifespan of exposed fish. Antioxidant effect of Cr, Pb and Cd mixture at environmentally relevant concentrations due to significantly altered levels of SOD and CAT in different tissues of *Cyprinus carpio* was reported by Rajeshkumar with co-authors (2017). As concluded by Calabrese and Mattson (2011) low concentration hormetic stimulation indicates an adaptive response that represent an environmentally-induced altered phenotype and allows a quantitative estimate of biological plasticity. In the present study, changes of the geno- and cytotoxic endpoints frequencies following

treatments with different concentration of a single metal in the mixtures may be related to variations of antioxidant defense enzyme activities leading to bioprotective functions. This presumption may be considered as a possible mechanism for the hormetic effects. However, more research must be conducted to predict hormesis in chemical mixtures.

Considering the sequences of accumulated metal amount after performed treatments, such trends were detected: (1) the first places of sequences were occupied by treatments with reduced concentration of non-essential metal (mostly Cd, following by mixture with reduced Pb concentration) indicating the increased accumulation of certain metals (Pb, Ni, Cd) in tissues after reduction of concentration of non-essential metal in the mixture, (2) while treatments with reduced concentration of essential metal (e.g. Cu, Cr and Zn) mostly occurred in the last places of the sequences indicating reduced accumulation of certain metals (especially Cd and Pb) in tissues compared between performed treatments. Notwithstanding, the lowest genotoxicity responses in all tissues were mostly measured after treatment with mixtures with reduced concentration of Zn or Pb. Moreover, DA analysis revealed similarities between these treatments data. Such clear trend was not observed considering cytotoxicity endpoints levels. Each metal in the mixture may provoke specific toxicity endpoints due to their involvement in a spectrum of metabolic pathways in the biological systems (Wah Chu and Chow 2002). Experimental study showed, that metals with higher covalent index such as Pb and Cu, elicit synergistic effect, while metals with low covalent index (Cd, Ni and Zn) have a volatile impact (Wah Chu and Chow 2002). Also, metal with low covalent index, such as Zn, have neutralizing effect on other metals with lower covalent index, e.g., Cd, Ni toxicity (Wah Chu and Chow 2002). Cobbina with co-authors (2015) showed that exposure to metal mixtures at low dose influences homeostatic regulation of toxic and essential metals. A literature review survey on single metal genotoxicity at low concentrations showed that some of metals comprising mixture in this study singly were genotoxic to certain fish species even at MPC we used. According to the research data 0.01 mg/L of Cu causes different responses depending on fish species and tissues. Zhu with co-authors (2004) study shows, that individually Cu at 0.01, 0.1 and 1 mg/L did not induce significant MN formation in carp (*Cyprinus carpio*) blood. Copper (0.01 mg/L) did not cause increase in micronucleus and binuclei in *Carassius gibelio* blood, gills and liver cells, but induce micronuclei formation in *C. carpio* gills (Cavas et al. 2005). Cadmium at 0.01 mg/L is known to induce MN formation in carp blood, while exposure to Cd at 0.001 mg/L concentrations did not significantly induce MN frequencies (Zhu et al. 2004). However, cadmium (0.005 mg/L) treatment caused binuclei formation in liver of *C. carpio* and micronuclei and binuclei formation in gills and liver of *Corydoras paleatus*, respectively. Blood, liver and gills of *C. gibelio* was not sensitive to Cd exposure at 0.005 mg/L considering micronuclei and binuclei formation (Cavas et al. 2005). While, all tested Cr concentrations (0.001, 0.01, 0.1 mg/L) induced significant MN frequencies in carp erythrocytes in comparison to control level (Zhu et al. 2004). DNA damage increased in both blood cells and gills of *Prochilodus lineatus* exposed to Ni concentrations (25, 250 and 2500 μg L(−1)) (Palermo et al. 2015). Cadmium, Cr and Cu are able to induce genotoxic effects at environmentally relevant concentration in Argentinean silverside (*Odontesthes bonariensis*) gills and liver cells (Gasulla et al. 2016). Considering metal mixture genotoxicity to fish at low concentrations, fewer researches are made (Valskienė et al. 2015; Güner and Muranlı 2011; Zhang et al. 2008; Zhu et al. 2004).

In the present study, glucose blood parameter was used to identify possible stress in fish caused by metal mixtures. Blood glucose levels were observed to increase significantly after treatment with MIX, Cr↓ and Cu↓ mixtures. The response to a stress in fish is characterized by a series of biochemical and physiological changes, which results in releasing stress hormones (e.g. cortisol and glucose) into the bloodstream (Randall and Perry 1992). These changes enhance the tolerance of an organism to cope with real or perceived stress in order to maintain its normal or homeostatic state (Barton 2002). Stress is also could be described as non-specific response of organism to any environmental stimuli (Wu et al. 2015).

Little concern was given to understand the interaction and the effects caused by combined trace metals and its concentrations in complex mixture and how it may affect fish behaviour over the short period. Different activity patterns in swimming behaviour of fish may suggest about possible synergistic or antagonistic actions of contaminants. Analysed behavioural data suggest that Cr^{6+} presence in the mixture at different concentrations may have a role in fish behavioural response. According to average velocity data, it was found that Cr↓ mixture (10 times reduced Cr^{6+} concentration) exhibited a weaker effect on fish behaviour compared to the Cu↓ mixture (10 times reduced Cu^{2+} concentration) exposure. Data based on recorded movement duration of the same fish showed a different significance of the response to mixtures. These differences between behavioural endpoints could be discussed from the different perspectives reflecting on different mechanism of the toxicity of mixtures in fish behaviour. The presence of Cr^{6+} in the mixture may cause more severe effect on fish compared to its reduced concentration in mixture, as a result causing abnormal swimming behaviour of fish. Thus, the erratic swimming performance of fish during the exposure may be

'unseen' by recorded duration of fish movement. When fish swim at the low speed for a longer duration, the time spent moving is increasing, while fish swim at the high speed for very short period, time spent moving will be estimated lower for this period. An increased fish movement duration but at low swimming velocity may indicate a better tolerance, regardless of the chemical exposure. In this case, for better understanding of the toxicity on fish more behavioural parameters could be combined to indicate behavioural complexity and occurrences of erratic movements.

Related research on fish behaviour toxicology indicated that, Cr^{6+} considered being highly toxic for fish due to its high solubility and mobility (US EPA 1998, 2002). Mishra and Mohanty (2008) showed that teleost fish (*Channa punctatus*) exposed to Cr^{6+} displayed erratic swimming and became lethargic at the concentration of 20 and 40 mg/L. Moreover, the changes in gills such as epithelium necrosis, desquamation and aneurism were observed, which might have a direct impact on fish behaviour. Svecevičius (2007, 2009) noted that that changes in locomotor activity of rainbow trout (*Oncorhynchus mykiss*) adults exposed to Cr^{6+} solution detected at very low concentration of 0.059 mg/L within 15 min of exposure, while the presence of avoidance response was established at even lower concentration of 0.003 mg/L. In other cases, Cu ions may interact with olfactory neurons relatively fast (e.g., within minutes), causing chemosensory irritation in salmonids, which evokes avoidance behaviour in fish (Tierney et al. 2010). However, in long-term exposure of Cu, fish may lose the ability to detect and respond to chemical contaminants in ambient water (Sandahl et al. 2007). Baldwin et al. (2011) established that Cu exposure disrupted the olfactory responsiveness of salmonids (*O. mykiss* and *O. kisutch*) to amino acids at the concentrations 0.005 and 0.02 mg/L, respectively after 3 h of exposure.

Conclusions

The current study examined the effects of exposure to metal mixture low waterborne concentration and importance of 10-fold reduction of single mixture component on tissue-specific metal accumulation, genotoxicity and cytotoxicity, glucose content in blood and behavioural responses in fish. This study data from individual and biochemical levels shows that metal mixture alters stress and swimming behaviour in *S. salar*. The most severe genotoxicity and cytotoxicity endpoints were detected after treatments with MIX, Cr↓ (10 times reduced Cr^{6+} concentration) and Cu↓ (10 times reduced Cu^{2+} concentration) mixtures. Consequently, this research revealed the significant influence of background exposure on chosen toxicological endpoints, indicating that minor changes in a single metal concentration in complex mixture can markedly increase the risk of toxicity. The results of the study showed that interactions at low metal exposure concentrations are likely to occur and elicit toxicologically significant effects. Therefore, environmental risk assessments addressing individual substances are insufficiently protective for such complex mixtures. Furthermore, low concentrations of metals in mixture should be considered during environment risk assessment, likewise possible background exposure in the aquatic environment.

The results suggested that due to discharges of metals at MPC into the aquatic environment, the occurrence of threats to early development stages and juvenile *S. salar* health is a likely scenario. However, further studies with more focus on toxicological significance of the biological effects induced by environmentally relevant metal mixture concentrations and background exposure in fish species of various ecological groups is therefore suggested to ensure that these results are not species-specific. Furthermore, long-term behavioural analysis can provide information on possible acclimation of fish as a result of long-term exposure to low metals concentrations.

Acknowledgements This research was supported by the research infrastructure Open Access Centre for Nature Research at the initiative of the Open R&D Lithuania network. This study was funded by the Research Council of Lithuania through the project ACTIS S-MIP-17-10. Metallothionein determination was funded by the Research Council of Lithuania, Project No. MIP-108/2015. We are thankful to Dalia Baršytė Lovejoy (Toronto University) for the language check. We are thankful to Liutauras Savičius for the production of the experimental aquariums for behavioural assay. The authors are grateful to Dr Kęstutis Arbačiauskas and Dr Eglė Šidagytė for the suggestions in data analysis.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. LT 61-13-005; SR-432.

References

Baldwin DH, Tatara CP, Scholz NL (2011) Copper-induced olfactory toxicity in salmon and steelhead: Extrapolation across species and rearing environments. Aquat Toxicol 101:295–297. https://doi.org/10.1016/j.aquatox.2010.08.011

Baršienė J, Lazutka J, Syvokienė J, Dedonytė V, Rybakovas A, Bagdonas E, Bjornstad A, Andersen OK (2004) Analysis of micronuclei in blue mussels and fish from the Baltic and the North Seas. Environ Toxicol 19(4):365–371. https://doi.org/10.1002/tox.20031

Baršienė J, Dedonytė V, Rybakovas A, Andreikenaitė L, Andersen OK (2006) Investigation of micronuclei and other nuclear abnormalities in peripheral blood and kidney of marine fish

treated with crude oil. Aquat Toxicol 78S:S99–S104. https://doi.org/10.1016/j.aquatox.2006.02.022

Baršienė J, Butrimavičienė L, Grygiel W, Lang T, Michailovas A, Jackūnas T (2014) Environmental genotoxicity and cytotoxicity in flounder (*Platichthys flesus*), herring (*Clupea harengus*) and Atlantic cod (*Gadus morhua*) from chemical munitions dumping zones in the southern Baltic Sea. Mar Environ Res 96:56–67. https://doi.org/10.1016/j.marenvres.2013.08.012

Bartonkova J, Hyršl P, Vojtek L (2016) Glucose determination in fish plasma by two different moderate methods. Acta Vet Brno 85:349–353. https://doi.org/10.2754/avb201685040349

Barton B (2002) Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. Integ Comp Biol 42:517–525. https://doi.org/10.1093/icb/42.3.517

Calabrese EJ, Mattson MP (2011) Hormesis provides a generalized quantitative estimate of biological plasticity. J Cell Commun Signal 5(1):25–38. https://doi.org/10.1007/s12079-011-0119-1

Cavas T, Garanko NN, Arkhipchuk VV (2005) Induction of micronuclei and binuclei in blood, gill and liver cells of fishes subchronically exposed to cadmium chloride and copper sulphate. Food Chem Toxicol 43(4):569–574. https://doi.org/10.1016/j.fct.2004.12.014

Cobbina SJ, Chen Y, Zhou Z, Wu X, Feng W, Wang W, Mao G, Xu H, Zhang Z, Wu X, Yang L (2015) Low concentration toxic metal mixture interactions: Effects on essential and non-essential metals in brain, liver, and kidneys of mice on sub-chronic exposure. Chemosphere 132:79–86. https://doi.org/10.1016/j.chemosphere.2015.03.013

Directive 2008/105/EC of the European Parliament and of the Council of 496 16 December 2008 on environmental quality standards in the field of water 497 policy, amending and subsequently repealing Council Directives 82/176/EEC, 498 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 499 2000/60/EC of the European Parliament and of the Council. Official Journal of the European Communities L 500 348, p 0084–0097. Accessed 24 Dec 2008

Directive 2008/105/EC of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. L. 276

Driessnack MK, Matthew AL, Raine JC, Niyogi S (2016) Interactive effects of chronic waterborne copper and cadmium exposure on tissue-specific metal accumulation and reproduction in fathead minnow (*Pimephales promelas*). Comp Biochem Physiol C 179:165–173. https://doi.org/10.1016/j.cbpc.2015.10.009

Driessnack MK, Jamwal A, Niyogi S (2017a) Effects of chronic exposure to waterborne copper and nickel in binary mixture on tissue-specific metal accumulation and reproduction in fathead minnow (*Pimephales promelas*). Chemosphere 185:964–974. https://doi.org/10.1016/j.chemosphere.2017.07.100

Driessnack MK, Jamwal A, Niyogi S (2017b) Effects of chronic waterborne cadmium and zinc interactions on tissue-specific metal accumulation and reproduction in fathead minnow (*Pimephales promelas*). Ecotoxicol Environ Saf 140:65–75. https://doi.org/10.1016/j.ecoenv.2017.02.023

Eyer P, Worek F, Kiderlen D, Sinko G, Stuglin A, Simeon-Rudolf V, Reiner E (2003) Molar absorption coefficients for the reduced Ellman reagent: reassessment. Anal Biochem 312:224–227. https://doi.org/10.1016/S0003-2697(02)00506-7

EEA (2012) European waters—assessment of status and pressures. Report No 8/2012, ISSN 1725-9177

Evans RM, Martin OV, Faust M, Kortenkamp A (2016) Should the scope of human mixture risk assessment span legislative/regulatory silos for chemicals? Sci Total Environ 543:757–764. https://doi.org/10.1016/j.scitotenv.2015.10.162

Fabrin TMC, Diamante NA, Mota TFM, Ghisi NC, Prioli SMAP, Prioli AJ (2018) Performance of biomarkers metallothionein and ethoxyresorufin O-deethylase in aquatic environments: a meta-analytic approach. Chemosphere 205:339–349. https://doi.org/10.1016/j.chemosphere.2018.04.069

Fenech M, Chang WP, Kirsch-Volders M, Holland N, Bonassi S, Zeiger E (2003) HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutat Res 534:65–75. https://doi.org/10.1016/S1383-5718(02)00249-8

Gasulla J, Picco SJ, Carriquiriborde P, Dulout FN, Ronco AE, de Luca JC (2016) Genotoxic Effects Induced by Cd($^{+2}$), Cr($^{+6}$), Cu($^{+2}$) in the Gill and Liver of *Odontesthes bonariensis* (Piscies, Atherinopsidae). Bull Environ Contam Toxicol 96(5):591–595. https://doi.org/10.1007/s00128-016-1774-y

Güner U, Muranlı FDG (2011) Micronucleus test, nuclear abnormalities and accumulation of Cu and Cd on Gambusia affinis (Baird & Girard, 1853). Turk. Turk J Fish Aquat Sci 11:615–622. https://doi.org/10.4194/1303-2712-v11_4_16

HELCOM (2011) Salmon and Sea Trout Populations and Rivers in the Baltic Sea—HELCOM assessment of salmon (*Salmo salar*) and sea trout (*Salmo trutta*) populations and habitats in rivers flowing to the Baltic Sea. Balt Sea Environ Proc No. 126A

Hendry K, Cragg-Hine D (2003) Ecology of the Atlantic Salmon. Conserving Natura 2000 Rivers Ecology Series No. 7. English Nature, Peterborough

Hylland K, Haux C, Hogstrand C (1992) Hepatic metallothionein and heavy metals in dad limanda limanda from the German Bight. Mar Ecol Prog Ser 91:89–96. https://doi.org/10.7773/cm.v31i12.89

ISO 6332 (1988) Water quality. Determination of iron—spectrometric method using 1.10-phenanthroline. ISO, the InternationalOrganization for Standardization

ISO 5814 (1990) Water quality. Determination of dissolved oxygen—electrotechnical probe method. SO, theInternational Organization for Standardization

ISO 9963-1 (1994) Water quality. Determination of alkalinity—Part 1: determination of total and composite alkalinity. ISO, the International Organization for Standardization

ISO 14911 (1998) Water quality. Determination of dissolved Li^+, Na^+, NH_4^+, K^+, Mn^{2+}, Ca^{2+}, Mg^{2+}, Sr^{2+} and Ba^{2+} using ion chromatography—method for water and waste water. ISO, the International Organization for Standardization

ISO 15586 (2003) Water quality. Determination of trace elements using atomic absorption spectrometry with graphite furnace. International Organization for Standardization, Geneva

ISO 10304-1 (2007) Water quality. Determination of dissolved anions by liquid chromatography of ions. Determination of bromide, chloride, fluoride, nitrate, nitrite, phosphate and sulfate. ISO, the International Organization for Standardization

ISO 10523 (2008) Water quality. Determination of pH. ISO, the International Organization for Standardization

Jia H, Ren H, Satoh S, Endo H, Hayashi T (2005) Comparison of pretreatment condition of cadmium in fish sample and diet by microwave digestion method for ICP-AES. J Tokyo Univ Mar Sci Technol 1:41–46

Javed M, Usmani N (2017) An overview of the adverse effects of heavy metal contamination on fish Health. Proc Natl Acad Sci India Sect B https://doi.org/10.1007/s40011-017-0875-7

Knapen D, Reynders H, Bervoets L, Verheyen E, Blust R (2007) Metallothionein gene and protein expression as a biomarker for metal pollution in natural gudgeon populations. Aquat Toxicol 82:163–172. https://doi.org/10.1016/j.aquatox.2007.02.008

Kroon F, Streten C, Harries S (2017) A protocol for identifying suitable biomarkers to assess fish health: a systematic review. PLoS One 12(4):e0174762. https://doi.org/10.1371/journal.pone.0174762

Kulmala S, Haapasaari P, Karjalainen TP, Kuikka S, Pakarinenm T, Parkkila K, Romakkaniemi A et al. (2012) Ecosystem services provided by the Baltic salmon—a regional perspective to the socio-economic benefits associated with a keystone species, In Socio-economic importance of ecosystem services in the Nordic Countries—synthesis in the context of The Economics of Ecosystems and Biodiversity (TEEB), Copenhagen Nordic Council of Ministers, p 266–276

Makaras T, Svecevičius G, Kazlauskienė N, Montvydienė D (2018) Rapid detection of sublethal toxicity using locomotor activity of rainbow trout juveniles. Bull Environ Contam Toxicol 100:221–227. https://doi.org/10.1007/s00128-017-2244-x

Melvin SD, Petit MA, Duvignacg MC, Sumpter JP (2017) Towards improved behavioural testing in aquatic toxicology: acclimation and observation times are important factors when designing behavioural tests with fish. Chemosphere 180:430–436. https://doi.org/10.1016/j.chemosphere.2017.04.058

Min EY, Ahn TY, Ju-Chan Kang (2016) Bioaccumulation, alterations of metallothionein, and antioxidant enzymes in the mullet Mugil cephalus exposed to hexavalent chromium. Can J Fish Aquat Sci 19:19. https://doi.org/10.1186/s41240-016-0020-1

Mishra AK, Mohanty B (2008) Acute toxicity impacts of hexavalent chromium on behavior and histopathology of gill, kidney and liver of the freshwater fish, Channa punctatus (Bloch). Environ Toxicol Pharmacol 26(2):136–141. https://doi.org/10.1016/j.etap.2008.02.010

Nys C, Versieren L, Cordery KI, Blust R, Smolders E, De Schamphelaere KAC (2017) Systematic evaluation of chronic metal-mixture toxicity to three species and implications for risk assessment. Environ Sci Technol 51(8):4615–4623. https://doi.org/10.1021/acs.est.6b05688

Palermo FF, Risso WE, Simonato JD, Martinez CBR (2015) Bioaccumulation of nickel and its biochemical and genotoxic effects on juveniles of the neotropical fish Prochilodus lineatus. Ecotoxicol Environ Saf 116:19–28. https://doi.org/10.1016/j.ecoenv.2015.02.032

Perez-Benito JF (2006) Effects of chromium (VI) and vanadium (V) on the lifespan of fish. J Trace Elem Med Biol 20(3):161–170. https://doi.org/10.1016/j.jtemb.2006.04.001

Peixoto NC, Roza T, Flores EMM, Pereira ME (2003) Effects of zinc and cadmium on $HgCl_2$-δ-ALA-D inhibition and Hg levels in tissues of suckling rats. Toxicol Lett 146:17–25. https://doi.org/10.1016/j.toxlet.2003.08.006

Price MHH (2013) Sub-lethal metal toxicity effects on salmonids: a review. Report prepared for Skeena Wild Conservation Trust. Smithers, BC p 64

Rajeshkumar S, Liu Y, Ma J, Duan HY, Li X (2017) Effects of exposure to multiple heavy metals onbiochemical and histopathological alterations in common carp, Cyprinus carpio L. Fish Shellfish Immunol 70:461–472. https://doi.org/10.1016/j.fsi.2017.08.013

Randall DJ, Perry SF (1992) Catecholamine. In: Hoar WS, Randall DJ, Farrell TP (eds) Fish physiology 12. Academic Press, New York, NY

Saibu Y, Jamwal A, Feng R, Peak D, Niyogi S (2018) Distribution and speciation of zinc in the gills of rainbow trout (*Oncorhynchus mykiss*) during acute waterborne zinc exposure: interactions with cadmium or copper. Comp Biochem Physiol C 206–207:23–31. https://doi.org/10.1016/j.cbpc.2018.02.004

Sampaio FG, Boijink CDL, Oba ET, Santos LRBD, Kalinin AL (2008) Antioxidant defenses and biochemical changes in pacu (*Piaractus mesopotamicus*) in response to single and combined copper and hypoxia exposure. Comp Biochem Physiol, Part C 147:43–51. https://doi.org/10.1016/j.cbpc.2007.07.009

Sandahl JF, Baldwin DH, Jenkins JJ, Scholz NL (2007) A sensory system at the interface between urban storm water runoff and salmon survival. Environ Sci Tech 41:2998–3004. https://doi.org/10.1021/es062287r

Stankevičiūtė M, Butrimavičienė L, Valskienė R, Greiciūnaitė J, Baršienė J, Vosylienė Z, Svecevičius G (2016) Analysis of nuclear abnormalities in erythrocytes of rainbow trout (*Oncorhynchus mykiss*) treated with Cu and Zn and after 4-, 8-, and 12-day depuration (post-treatment recovery). Mutat Res Genet Toxicol Environ Mutagen 797:26–35. https://doi.org/10.1016/j.mrgentox.2016.01.003

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. https://doi.org/10.1007/s10646-017-1833-0

Svecevičius G (2007) Avoidance response of rainbow trout *Oncorhynchus mykiss* to hexavalent chromium solutions. Bull Environ Contam Toxicol 79:596–600. https://doi.org/10.1007/s00128-003-0154-6

Svecevičius G (2009) Use of behavioral responses of rainbow trout *Oncorhynchus mykiss* in identyfing sublethal exposure to hexavalent chromium. Bull Environ Contam Toxicol 82:564–568. https://doi.org/10.1007/s00128-009-9657-0

Tierney KB, Baldwin DH, Hara TJ, Ross PS, Scholz NL, Kennedy CJ (2010) Olfactory toxicity in fishes. Aquat Toxicol 96:2–26. https://doi.org/10.1016/j.aquatox.2009.09.019

US EPA (1998) Toxicological review of hexavalent chromium. U.S. Environmental Protection Agency, Washington DC, p 70

US EPA (2002) Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. U.S. Environmental Protection Agency, 5th edn. Office of Water, Washington, DC

Valskienė R, Stankevičiūtė M, Butrimavičienė L, Greiciūnaitė J, Svecevičius G (2015) Induction of nuclear abnormalities in rainbow trout (*Oncorhynchus mykiss*) after exposure to a model mixture of heavy metals (Zn, Cu, Ni, Cr, Cd, Pb) at maximum permissible concentration. Proceedings of the 18th Conference for Junior Researchers "Science – Future of Lithuania", aplinka 15:100–105

Velma V, Vutukuru SS, Tchounwou PB (2009) Ecotoxicology of hexavalent chromium in freshwater fish: a critical review. Rev Environ Health 24(2):129–145. https://doi.org/10.1515/REVEH.2009.24.2.129

Wah Chu K, Chow KL (2002) Synergistic toxicity of multiple heavy metals is revealed by a biological assay using a nematode and its transgenic derivative. Aquat Toxicol 61:53–64. https://doi.org/10.1016/S0166-445X(02)00017-6

Wu H, Aoki A, Arimoto T, Nakano T, Ohnuki H, Murata M, Ren H, Endo H (2015) Fish stress become visible: A new attempt to use biosensor for real-time monitoring fish stress. Biosens Bioelectron 67:503–510. https://doi.org/10.1016/j.bios.2014.09.015

Zhang Y, Wang YJ, Yu RL, Zhang S, Wu ZB (2008) Effects of heavy metals Cd^{2+}, Pb^{2+} and Zn^{2+} on DNA damage of loach *Misgurnus anguillicaudatus*. Front Biol China 3(1):50–54. https://doi.org/10.1007/s11515-008-0012-3

Zhu B, Wu ZF, Li J, Wang GX (2011) Single and joint action toxicity of heavy metals on early developmental stages of Chinese rare minnow (Gobiocypris rarus). Ecotoxicol Environ Saf 74 (8):2193–2202. https://doi.org/10.1016/j.ecoenv.2011.07.033

Zhu Y, Wang J, Bai Y, Zhang R (2004) Cadmium, chromium, and copper induce polychromatocyte micronuclei in carp (*Cyprinus carpio* L.). Bull Environ Contam Toxicol 72(1):78–86. https://doi.org/10.1007/s00128-003-0243-6

Responses of biomarkers in Atlantic salmon (*Salmo salar*) following exposure to environmentally relevant concentrations of complex metal mixture (Zn, Cu, Ni, Cr, Pb, Cd). Part II

Ecotoxicology

Milda Stankevičiūtė * [a], Gintarė Sauliutė [a], Tomas Makaras [a], Arvydas Markuckas [b], Tomas Virbickas [a], Janina Baršienė [a]

[a]Nature Research Centre, Akademijos str. 2, 08412 Vilnius, Lithuania

[b]Vilnius University, Life Sciences Center, Department of Biochemistry and Molecular Biology, Saulėtekio av. 7, 10223 Vilnius, Lithuania

***Corresponding author – Milda Stankevičiūtė,** Email: milda.stan@gmail.com, ORCID: 0000-0001-8906-3814

Table 1 Results of discriminant function analysis on metal concentrations in gills and ENAs in peripheral blood of *S. salar* exposed to complex metal mixtures and variants of this mixtures with 10-times reduced concentration of single metal in the mixture

Wilks' lambda: 0.00000	$p < 0.0000$				
Discriminant function	1	2	3	4	5
Eigenvalue	55.55	26.76	15.66	5.17	1.19
Cum. Proportion	0.53	0.78	0.93	0.97	0.99
Chi-Sqr.	603.0*	425.5*	279.3*	155.5*	75.4*
Degrees of freedom	98	78	60	44	30
Variables	Standardized discriminant function coefficients				
Zn	-0.21	-0.24	-0.21	0.12	0.41
Cu	-0.13	-0.27	-0.39	-0.31	0.63
Ni	1.03	0.002	-0.54	0.09	-0.01
Cr	0.17	-1.06	0.01	-0.45	-0.35
Pb	0.60	0.35	0.84	0.15	0.04
Cd	-0.03	-0.67	0.38	0.74	0.25
MN	0.23	-0.46	-0.72	0.38	-0.13
NB	-0.23	-0.26	-0.31	-0.14	-0.08
NBf	0.30	-0.46	-0.16	0.26	-0.60
BL	-0.05	0.41	0.59	0.22	-0.16
8-shaped	0.02	-0.15	-0.10	0.19	-0.43
VacNuc	-0.22	-0.42	-0.04	-0.01	-0.31
Kidney-shaped	-0.08	-0.17	-0.31	0.01	0.45
FA	0.20	0.43	0.03	-0.27	-0.13
Means of canonical variables					
Control	-8.87	9.54	0.63	-2.11	-0.13
MIX	3.12	-2.10	3.86	0.68	0.66
Zn↓	5.14	0.23	6.88	0.10	-1.20
Cu↓	-3.11	-5.28	-2.73	0.65	-1.45
Ni↓	-5.60	-6.96	-0.94	-3.37	0.58
Cr↓	-4.70	1.65	-3.33	3.58	-0.38
Pb↓	-0.23	-0.06	0.63	1.94	1.91
Cd↓	14.24	2.98	-5.00	-1.47	0.02

*Significant result

Table 2 Results of discriminant function analysis on metal concentrations in liver and ENAs in peripheral blood of *S. salar* exposed to complex metal mixtures and variants of this mixtures with 10-times reduced concentration of single metal in the mixture

Wilks' lambda: 0.00000	p < 0.0000				
Discriminant function	1	2	3	4	5
Eigenvalue	294.34	49.56	19.25	7.01	1.54
Cum. Proportion	0.79	0.92	0.97	0.99	0.10
Chi-Sqr.	716.9*	466.6*	294.0*	161.6*	70.1*
Degrees of freedom	98	78	60	44	30
Variables	Standardized discriminant function coefficients				
Zn	0.003	0.27	-0.39	0.99	0.12
Cu	-0.09	-0.48	-0.06	0.11	-0.36
Ni	-0.26	-0.33	0.82	0.18	0.50
Cr	-0.79	-0.11	0.35	0.10	-0.68
Pb	-1.67	0.17	-0.45	0.01	0.08
Cd	-0.72	-0.85	-0.54	0.04	-0.08
MN	0.05	-0.22	0.18	0.03	0.06
NB	-0.81	-0.11	-0.66	0.15	-0.26
NBf	0.61	-0.17	-0.07	-0.22	0.09
BL	0.20	0.36	-0.27	0.05	0.07
8-shaped	-0.84	-0.24	-0.63	0.10	-0.004
VacNuc	-0.39	-0.37	-0.24	-0.08	-0.13
Kidney-shaped	0.004	-0.06	0.02	-0.34	0.29
FA	1.01	0.05	0.80	0.18	-0.05
Means of canonical variables					
Control	16.10	8.74	-4.39	-3.44	-0.97
MIX	3.65	-1.44	2.52	2.48	-0.20
Zn↓	6.91	1.20	4.51	-0.07	1.31
Cu↓	8.90	3.85	-4.31	4.89	0.14
Ni↓	-2.12	-4.11	4.92	-0.08	-2.35
Cr↓	-0.71	-14.19	-5.43	-1.30	0.42
Pb↓	6.86	0.84	3.49	-2.06	1.44
Cd↓	-39.59	5.11	-1.30	-0.41	0.22

*Significant result

Table 3 Results of discriminant function analysis on metal concentrations in kidneys and ENAs in peripheral blood of *S. salar* exposed to complex metal mixtures and variants of this mixtures with 10-times reduced concentration of single metal in the mixture

Wilks' lambda: 0.00000	p < 0.0000					
Discriminant function	1	2	3	4	5	6
Eigenvalue	174.81	28.90	9.10	3.98	2.98	1.70
Cum. Proportion	0.79	0.92	0.96	0.98	0.99	1.00
Chi-Sqr.	669.5*	442.0*	292.5*	190.8*	120.2*	59.37*
Degrees of freedom	98	78	60	44	30	18
Variables	Standardized discriminant function coefficients					
Zn	0.07	0.40	-0.37	0.12	0.68	0.53
Cu	-0.17	-0.72	-0.32	-0.41	-0.63	0.14
Ni	-1.09	0.34	0.42	-0.01	-0.09	-0.10
Cr	-0.11	-0.85	0.38	0.62	-0.22	0.20
Pb	-0.80	-0.24	-0.54	-0.03	0.46	-0.08
Cd	0.11	-0.38	0.55	-0.43	0.43	-0.33
MN	0.13	-0.04	0.03	-0.35	0.17	0.68
NB	0.22	0.09	-0.33	-0.45	0.34	-0.20
NBf	0.10	-0.02	0.46	-0.23	0.30	0.20
BL	-0.35	0.01	0.42	0.22	-0.03	0.06
8-shaped	-0.11	0.11	0.08	-0.37	-0.005	0.33
VacNuc	0.22	-0.02	0.24	-0.20	0.54	-0.20
Kidney-shaped	-0.02	0.003	-0.18	-0.11	-0.39	0.05
FA	0.22	0.03	-0.001	0.36	-0.03	0.08
Means of canonical variables						
Control	7.13	5.47	-5.00	1.05	0.04	-1.36
MIX	-3.12	-3.64	-2.61	1.16	2.52	1.89
Zn↓	8.09	1.82	2.16	2.51	-1.04	-0.38
Cu↓	10.38	2.47	2.21	0.11	-1.59	1.90
Ni↓	3.88	-7.15	3.23	0.60	1.38	-1.55
Cr↓	6.12	4.90	1.35	-3.75	1.54	-0.19
Pb↓	-2.37	-7.54	-2.66	-2.00	-2.46	-0.12
Cd↓	-30.11	3.68	1.33	0.32	-0.40	-0.19

*Significant result

Table 4 Results of discriminant function analysis on metal concentrations in muscle and ENAs in peripheral blood of *S. salar* exposed to complex metal mixtures and variants of this mixtures with 10-times reduced concentration of single metal in the mixture

Wilks' lambda: 0.00000			p < 0.0000			
Discriminant function	1	2	3	4	5	6
Eigenvalue	109.41	76.02	24.23	20.05	4.19	1.47
Cum. Proportion	0.46	0.79	0.89	0.97	0.99	1.00
Chi-Sqr.	813.2*	606.2*	415.1*	273.0*	139.0*	66.5*
Degrees of freedom	98	78	60	44	30	18
Variables	Standardized discriminant function coefficients					
Zn	0.82	0.27	0.15	-0.55	0.04	0.27
Cu	0.37	0.53	-0.83	-0.65	0.27	-0.19
Ni	0.71	0.02	-0.34	0.74	-0.31	0.16
Cr	-0.10	1.15	0.32	-0.07	0.02	0.01
Pb	0.84	-0.04	0.61	-0.24	0.51	-0.27
Cd	0.33	0.001	0.33	-0.45	-0.81	-0.08
MN	-0.29	0.002	0.40	0.25	-0.21	-0.60
NB	-0.34	0.25	-0.45	-0.19	0.36	-0.30
NBf	-0.26	0.15	0.12	0.65	-0.33	-0.35
BL	-0.18	0.35	-0.001	0.15	-0.26	-0.03
8-shaped	-0.16	0.11	-0.25	0.004	0.08	-0.12
VacNuc	-0.21	0.46	-0.26	0.25	-0.21	-0.37
Kidney-shaped	0.07	-0.26	0.14	-0.07	0.23	0.39
FA	0.71	-0.33	-0.01	-0.23	0.01	0.22
Means of canonical variables						
Control	-10.85	-6.88	-0.96	2.49	-0.17	1.87
MIX	9.98	0.42	2.83	-2.31	-2.06	-1.18
Zn↓	-2.64	-5.92	6.11	-2.33	3.76	-0.63
Cu↓	-7.15	19.76	2.55	0.98	0.10	0.29
Ni↓	-1.69	1.61	-10.08	-5.13	1.08	-0.48
Cr↓	-7.67	-4.85	-1.51	6.81	-1.18	-1.68
Pb↓	-0.31	-4.68	2.65	-5.08	-2.57	0.87
Cd↓	20.33	0.54	-1.58	4.58	1.05	0.94

*Significant result

Table 5 Results of discriminant function analysis on metal concentrations in gills and ENAs in gills of *S. salar* exposed to complex metal mixtures and variants of this mixtures with 10-times reduced concentration of single metal in the mixture

Wilks' lambda: 0.00000	$p < 0.0000$				
Discriminant function	1	2	3	4	5
Eigenvalue	56.07	29.75	9.30	4.94	2.01
Cum. Proportion	0.54	0.83	0.92	0.97	0.99
Chi-Sqr.	602.3*	424.4*	273.6*	171.0*	92.6*
Degrees of freedom	98	78	60	44	30
Variables	Standardized discriminant function coefficients				
Zn	0.14	-0.14	0.06	-0.09	-0.16
Cu	-0.13	-0.19	0.58	0.25	-0.81
Ni	-0.80	0.48	0.33	-0.31	0.13
Cr	-0.28	-0.84	0.49	0.27	0.32
Pb	-0.79	-0.07	-0.77	0.23	-0.12
Cd	-0.10	-0.83	-0.39	-0.78	0.19
MN	-0.10	-0.05	-0.33	-0.18	-0.09
NB	0.17	-0.001	0.05	0.15	-0.62
NBf	-0.16	-0.15	-0.07	0.11	-0.13
BL	0.23	0.14	-0.06	-0.41	0.53
8-shaped	0.07	-0.24	-0.18	-0.24	0.13
VacNuc	-0.37	-0.53	-0.13	-0.21	-0.37
Kidney-shaped	-0.07	-0.09	-0.15	0.01	-0.004
FA	-0.28	-0.26	0.23	0.13	-0.24
Means of canonical variables					
Control	10.25	7.40	-1.21	2.85	-0.20
MIX	-4.89	-4.90	-2.09	0.49	-2.47
Zn↓	-6.01	-2.35	-4.50	1.76	1.75
Cu↓	3.75	-2.94	2.30	-1.02	1.77
Ni↓	3.67	-6.43	4.35	1.86	-0.46
Cr↓	5.91	2.89	-1.34	-3.29	-0.92
Pb↓	-0.14	-1.39	-0.82	-2.67	0.63
Cd↓	-12.53	7.71	3.32	0.02	-0.09

*Significant result

Table 6 Results of discriminant function analysis on metal concentrations in liver and ENAs in liver of *S. salar* exposed to complex metal mixtures and variants of this mixtures with 10-times reduced concentration of single metal in the mixture

Wilks' lambda: 0.00000	p < 0.0000				
Discriminant function	1	2	3	4	5
Eigenvalue	211.21	51.69	11.95	9.58	2.13
Cum. Proportion	0.74	0.92	0.96	0.99	1.00
Chi-Sqr.	711.8*	473.4*	296.9*	183.0*	78.0*
Degrees of freedom	91	72	55	40	27
Variables	Standardized discriminant function coefficients				
Zn	0.35	0.15	-0.39	-1.09	-0.15
Cu	-0.31	-0.40	-0.02	-0.14	0.28
Ni	-0.56	-0.29	-0.66	0.16	-0.49
Cr	-0.98	-0.24	-0.31	0.12	0.67
Pb	-1.15	0.21	0.38	-0.26	-0.07
Cd	-0.23	-1.04	0.54	-0.33	0.05
MN	-0.20	0.09	-0.71	-0.38	0.19
NB	-0.38	-0.09	0.08	0.33	-0.08
BL	0.27	-0.38	0.16	0.01	-0.04
8-shaped	-0.23	-0.02	-0.13	-0.21	0.15
VacNuc	0.26	0.23	-0.04	0.49	0.45
Kidney-shaped	0.31	0.24	-0.04	-0.20	-0.82
FA	-0.24	-0.41	0.07	-0.20	0.001
Means of canonical variables					
Control	15.72	9.12	4.99	1.75	1.16
MIX	1.92	-1.56	-2.69	-0.62	0.42
Zn↓	3.28	1.03	-2.88	1.50	-1.89
Cu↓	9.87	4.20	-2.22	-6.13	0.06
Ni↓	-4.86	-4.68	-3.36	2.56	2.54
Cr↓	1.98	-14.02	4.59	-1.67	-0.38
Pb↓	4.39	0.28	-0.45	3.51	-1.69
Cd↓	-32.29	5.62	2.02	-0.91	-0.22

*Significant result

Table 7 Results of discriminant function analysis on metal concentrations in kidneys and ENAs in kidneys of *S. salar* exposed to complex metal mixtures and variants of this mixtures with 10-times reduced concentration of single metal in the mixture

Wilks' lambda: 0.00000	$p < 0.0000$					
Discriminant function	1	2	3	4	5	6
Eigenvalue	177.50	32.05	9.43	3.91	2.44	1.16
Cum. Proportion	0.78	0.92	0.97	0.98	0.99	1.00
Chi-Sqr.	650.5*	422.3*	268.4*	165.3*	95.2*	40.8*
Degrees of freedom	98	78	60	44	30	18
Variables	Standardized discriminant function coefficients					
Zn	0.001	0.44	-0.59	-0.52	0.31	-0.51
Cu	0.0003	-0.87	-0.27	0.57	-0.64	-0.10
Ni	-1.15	0.37	0.43	0.05	0.01	-0.05
Cr	-0.10	-0.80	0.09	-0.72	-0.44	0.20
Pb	-0.84	-0.26	-0.48	0.00	0.41	0.23
Cd	-0.08	-0.42	0.78	0.40	0.73	-0.11
MN	0.01	-0.31	0.07	0.11	-0.24	-0.51
NB	0.42	-0.10	-0.32	0.14	-0.01	-0.66
NBf	0.10	0.16	-0.40	-0.48	-0.17	-0.27
BL	0.02	-0.09	-0.02	0.11	-0.34	0.17
8-shaped	0.09	-0.07	0.03	0.15	-0.23	-0.06
VacNuc	-0.07	-0.14	-0.30	-0.06	0.18	-0.46
Kidney-shaped	0.15	0.03	-0.36	-0.12	-0.28	0.14
FA	0.06	-0.02	0.21	0.05	-0.07	-0.19
	Means of canonical variables					
Control	7.51	6.18	-3.84	0.16	0.95	1.59
MIX	-2.92	-3.92	-4.57	-2.04	0.91	-1.45
Zn↓	8.15	2.37	1.75	-1.76	-0.81	0.47
Cu↓	10.86	2.39	1.52	-1.01	-2.33	-0.77
Ni↓	2.90	-7.24	4.01	-0.99	1.93	0.69
Cr↓	5.54	4.46	1.65	3.14	1.44	-1.29
Pb↓	-1.62	-8.19	-1.79	2.76	-1.74	0.58
Cd↓	-30.43	3.94	1.28	-0.27	-0.36	0.18

*Significant result

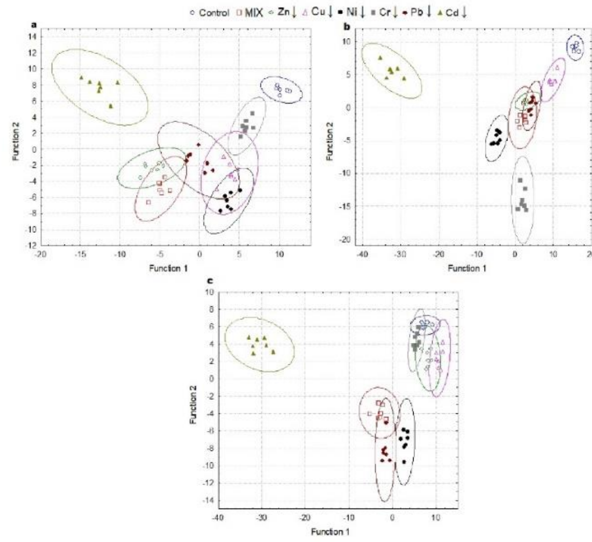


Fig. 1 Scatterplots of discriminant function 1 versus discriminant function 2 using metal concentrations and ENAs data in the same tissue: (a) gills, (b) liver, (c) kidneys

VI

Environmental genotoxicity risk assessment along the transport routes of chemical munitions leading to the dumping areas in the Baltic Sea

Baršienė J, Butrimavičienė L, Grygiel W, Stunžėnas V, Valskienė R, Greiciūnaitė J, Stankevičiūtė M

Marine Pollution Bulletin 2018, 103(1–2): 45–53

Reprinted with permission from Elsevier Ltd.

Marine Pollution Bulletin 103 (2016) 45–53

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Environmental genotoxicity assessment along the transport routes of chemical munitions leading to the dumping areas in the Baltic Sea

Janina Baršienė [a], Laura Butrimavičienė [a], Włodzimierz Grygiel [b], Virmantas Stunžėnas [a], Roberta Valskienė [a], Janina Greiciūnaitė [a], Milda Stankevičiūtė [a,*]

[a] Nature Research Centre, Akademijos 2, 08412, Vilnius, Lithuania
[b] National Marine Fisheries Research Institute in Gdynia, 1 Kollataja Street, 81-332 Gdynia, Poland

ARTICLE INFO

Article history:
Received 15 September 2015
Received in revised form 22 December 2015
Accepted 23 December 2015
Available online 4 January 2016

Keywords:
Genotoxicity
Micronuclei
Nuclear buds
Herring
Chemical munitions
Baltic Sea

ABSTRACT

The frequencies of micronuclei (MN), nuclear buds (NB) and nuclear buds on filament (NBf) were examined in 660 specimens of herring (*Clupea harengus*) collected in 2009–2014 at 65 study stations located mainly along the chemical munition transport routes in the Baltic Sea. The frequency of nuclear abnormalities was strongly increased in herring caught at four stations located close to chemical munition dumping sites, or CWAs — substances (chemical warfare agents) in sediments. Significant increase of MN, NB and NBf was observed in fish caught November 2010–2013 compared to 2009. The most significantly increased genotoxicity responses were recorded in fish caught at stations along CW (chemical weapons) transport routes, close to the Bornholm CW dumping area, in zones with CWAs in sediments and with oil–gas platforms.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Over the last few decades, the problem of sea-dumped chemical warfare agents (CWAs), which after World Wars I and II were accumulated and dumped into the seas in hundreds of thousand tons has attracted international attention (Kaffka, 1995). About fifteen thousand tons of chemical warfare agents have been dumped at different sites of the Baltic Sea (HELCOM, 2013). Chemical munitions east of the Bornholm Island and in the Gotland Deep were dumped in considerably wide areas. The main CWAs were sulphur mustard (63%), arsenic-containing compounds such as Clark and arsine oils, Adamsite (31%) and α-chloroacetophenone (5%) (HELCOM, 1994). These agents were primarily dumped in the form of munitions, mostly in bombs, shells and containers. Munitions were also packed into wooden boxes, which could drift before sinking. Later these wooden boxes with warfare agents were reported to have drifted to the Bornholm Island or the Swedish coastline (HELCOM, 1996).

For a long time, the problem was not getting enough attention, and it was politically sensitive with undefined responsibility. The full extent of munitions dumping operations has remained unlearned and the real picture of dumping locations is still incomplete. Moreover, there are indications that some of chemical weapons (CW) were thrown overboard along transport routes from the Wolgast port to the dumpsites in the Baltic Sea. Designed transport routes to and from the dumping areas were changed, some dumping did not follow procedures and missing records led to the current lack of knowledge on the location of CW (HELCOM, 2013). Unofficial CW dumping sites of the Słupsk Furrow and the Gdansk Deep as well as chemical warfare agents in the sediments of the other locations of the Polish exclusive economic zone (EEZ) were identified (CHEMSEA Findings, 2014; Bełdowski et al., 2015).

Until recently literature, data on the occurrence and fate of CWAs in marine dumpsites were scarce (Missiaen et al., 2010). Potential CWAs accumulation in living marine organisms, their interference with enzyme-controlled biological processes or their potential to modify genetic material remains poorly described. Rusted bombs, shells and containers leak hazardous substances leading to measurable levels of CWAs in different zones of the Baltic Sea. Since fishing with bottom trawls in the southern Baltic is extensive, by-catch and disposal of chemical munitions remains problematic (HELCOM, 2013).

Elevated concentrations of warfare agents were detected in sediments from dumping sites in the southern Adriatic Sea (Amato et al., 2006), in the Baltic Sea (Garnaga and Stankevičius, 2005) and in the North Sea (Tørnes et al., 2002, 2006), suggesting that leakage of CWAs contributed to environmental pollution. Assessment of the Bornholm chemical munition dumpsite showed that pollution of the bottom sediments with warfare agents was patchy and the agents were detected at a significant distance from the shipwrecks (Missiaen et al., 2010).

* Corresponding author at: Nature Research Centre, Institute of Ecology, Akademijos str. 2, 08412, Vilnius, Lithuania.
E-mail address: milda.stan@gmail.com (M. Stankevičiūtė).

http://dx.doi.org/10.1016/j.marpolbul.2015.12.048
0025-326X/© 2015 Elsevier Ltd. All rights reserved.

However, it should be stressed that only few publications, disclosing biological or ecological significance of chemical warfare agents exist (Sanderson et al., 2008, 2010; Della Torre et al., 2010, 2013; Baršienė et al., 2014). Attributed to the leakage of yperite from rusted bomb shells dumped in the southern Adriatic Sea, 3–4-times higher levels of As and Hg were recorded in fillets of two benthic fish species and genotoxicity effects of CWA were described (Della Torre et al., 2010, 2013). The most significantly increased total geno-cytotoxicity levels were described in three fish species collected in 2009–2011 near a known chemical munition dumpsite in the Bornholm Basin. The levels of genotoxicity that was significantly higher than the reference genotoxicity were found in all stations revealing that in the sampling area fish were widely affected (Baršienė et al., 2014).

The main objective of the present study was to evaluate the levels of environmental genotoxicity in herring (*Clupea harengus*), native fish species caught mostly at zones along chemical munition transport routes to the Bornholm, Gotland and Gdansk CW dumping sites. Environmental genotoxicity was assessed at each of the 65 studied stations, including 60 stations located in the Polish EEZ and 5 stations situated close to the Bornholm CW dumpsite, in the Danish EEZ. The study stations were selected considering CW transport and dumping procedures, reported encounters with CWAs and recently discovered sites polluted with warfare materials (HELCOM, 2013; CHEMSEA Findings, 2014). In the Polish waters (the Bornholm area, the Peninsula of Hel, the areas neighbouring with Władysławowo, Kołobrzeg, and other zones), ammunition with warfare materials and poisoning mustard lumps were found on the seabed or on beaches (Szarejko and Namieśnik, 2009). Our study stations were located in these zones; however sadly, the coordinates of most encounters with CWAs in the Polish EEZ are not publically recorded.

The study area is regarded as vital spawning area for commercially important fish species in the Baltic Sea (Köster et al., 2003) and is heavily polluted by various hazardous substances (HELCOM, 2010). Leaking and degrading chemical warfare products in the environment can interact with other pollutants and may lead to unpredictable genotoxicity responses.

The induction of micronuclei (MN), nuclear buds (NB), and nuclear buds on filament (NBf) was used as genotoxicity endpoints in blood erythrocytes of herring. The micronucleus test is a sensitive and fast approach to detect structural and numerical chromosomal alterations induced by clastogenic and aneugenic agents (Heddle et al., 1991). The formation of nuclear buds may reflect an unequal capacity of organisms to expel damaged, amplified, failed replication or improperly condensed DNA, chromosome fragments without telomeres and centromeres from the nucleus (Lindberg et al., 2007).

2. Materials and methods

2.1. Sampling of fish

From December 2009 to February 2014, 660 specimens of herring (*C. harengus*) were collected at 65 study stations located in the Bornholm and the eastern part of the Gotland Basins (the Baltic Sea; Fig. 1). Samples were obtained from research catches carried out by the RVs "Baltica" (617 specimens from 62 stations) and "Walther Herwig III" (43 specimens from three stations) using standard bottom or pelagic trawls.

Most of the study stations for herring were located at the chemical munition transport (to the Bornholm and Gotland dumpsites) route in the Polish EEZ. Study stations (17a, 19a, 21a, 23, 16b and 45d) were most closely located to a known CW dumpsite in southeast or east of the Bornholm Island (Fig. 1). The group of stations in the eastern

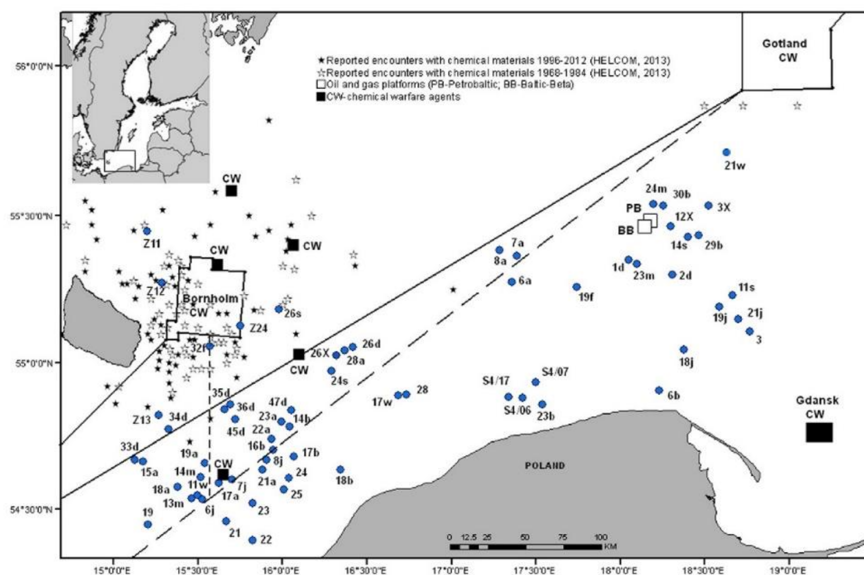


Fig. 1. Location of sampling stations in the Bornholm and the eastern Gotland Basins of the Baltic Sea.

Gotland Basin was located in the area with warfare agents-related detections (CHEMSEA Findings, 2014).

Information about the sampling stations, geographic coordinates of each fish catch-station, depth of trawling and hydrological parameters such as seawater temperature, salinity and oxygen concentrations are presented in Table 1. The dates of sampling surveys, study stations and numbers of collected fish specimens are presented in Table 2. In total, 660 herring specimens were used for the analysis of nuclear abnormalities (NA) in peripheral blood erythrocytes.

Table 1
The list of fish catch-stations and hydrological parameters measurements over the bottom of the Bornholm and the eastern Gotland Basins (data from the r/v "Baltica" surveys and for S4 stations – from the r/v "Walther Herwig III" survey).

Study stations	Latitude	Longitude	Depth of trawling (m)	Temperature at the bottom (°C)	Salinity at the bottom (PSU)	O_2 concentration at the bottom (mg/l)
S4/06	54°52.53'N	17°23.82'E	25	7.42	7.67	10.68
S4/07	54°55.61'N	17°29.62'E	23	7.41	7.68	10.62
S4/17	54°52.43'N	17°23.34'E	23	5.15	7.56	11.08
3	55°06.30'N	18°45.70'E	92	5.168	11.551	1.52
19	54°26.40'N	15°12.00'E	32	6.90	8.13	6.44
21	54°27.20'N	15°40.00'E	51	5.411	9.915	4.81
22	54°22.90'N	15°48.80'E	31	7.732	7.256	7.63
23	54°30.70'N	15°49.20'E	54–56	5.71	9.03	5.48
24	54°36.20'N	16°02.00'E	48	7.67	7.35	7.64
25	54°33.60'N	16°00.20'E	44–47	7.798	7.283	7.77
28	54°52.80'N	16°41.90'E	20	7.46	7.28	7.85
6a	55°15.90'N	17°20.90'E	89	6.56	11.23	3.22
7a	55°21.50'N	17°22.80'E	72	6.18	10.94	2.22
8a	55°23.00'N	17°17.00'E	61	2.45	7.60	8.90
15a	54°39.50'N	15°08.90'E	61	5.01	10.43	8.25
17a	54°34.80'N	15°36.80'E	60	5.59	11.73	5.60
18a	54°33.90'N	15°22.30'E	56	3.58	8.51	8.06
19a	54°38.90'N	15°31.70'E	68	5.91	11.97	4.27
21a	54°36.60'N	15°52.50'E	55	5.55	11.99	3.85
22a	54°43.10'N	15°54.70'E	54	6.03	12.60	3.76
23a	54°47.50'N	15°59.30'E	52	4.94	11.10	5.99
28a	55°02.30'N	16°21.60'E	54	4.35	9.31	5.45
Z11	55°26.50'N	15°11.50'E	57–74	3.43	11.85	4.66
Z12	55°15.80'N	15°17.00'E	65–61	3.22	9.42	5.47
Z13	54°49.20'N	15°15.60'E	50–52	2.70	8.42	5.45
Z24	55°07.40'N	15°45.00'E	66	4.66	12.76	2.81
6b	54°53.80'N	18°13.60'E	25	6.97	7.17	7.78
14b	54°47.50'N	16°00.20'E	51	5.68	9.86	3.66
16b	54°40.70'N	15°52.60'E	54	5.99	11.32	3.00
17b	54°40.50'N	16°04.00'E	48	5.58	10.20	2.47
18b	54°37.60'N	16°20.30'E	34	5.53	8.37	6.26
23b	54°51.30'N	17°31.70'E	23	7.86	7.11	7.79
29b	55°26.00'N	18°28.00'E	87	no data	no data	no data
30b	55°31.80'N	18°13.50'E	79–84	no data	no data	no data
1d	55°21.00'N	18°03.00'E	83	6.41	12.13	2.92
2d	55°18.00'N	18°17.90'E	72–78	5.94	11.11	3.99
26d	55°03.10'N	16°24.60'E	54	2.16	7.93	8.72
33d	54°39.40'N	15°09.10'E	60–61	6.57	12.74	3.72
34d	54°46.20'N	15°19.30'E	69	6.78	13.42	2.64
35d	54°50.30'N	15°38.70'E	76	6.91	14.31	2.36
36d	54°50.70'N	15°41.00'E	76	6.91	14.31	2.36
45d	54°47.80'N	15°42.70'E	74	6.86	14.77	2.72
47d	54°50.20'N	16°03.00'E	51	2.30	7.93	8.92
19f	55°15.30'N	17°43.90'E	35	15.96	7.29	6.40
32f	55°03.20'N	15°33.60'E	54	4.54	12.02	2.58
6j	54°32.30'N	15°29.50'E	58	5.60	12.22	2.69
7j	54°36.00'N	15°42.00'E	59	5.84	12.75	2.80
8j	54°40.30'N	15°56.00'E	54	5.65	10.59	5.30
18j	55°02.40'N	18°21.90'E	68	4.60	10.03	2.40
19j	55°11.30'N	18°35.20'E	88	4.83	10.40	1.33
21j	55°09.00'N	18°41.50'E	93	4.85	10.66	1.07
13m	54°32.50'N	15°29.40'E	57	5.55	10.07	5.28
14m	54°35.60'N	15°30.50'E	62	6.53	7.16	3.63
23m	54°21.80'N	18°05.70'E	81	5.95	10.96	4.43
24m	55°31.70'N	18°09.70'E	78	5.74	10.96	2.53
11s	55°13.50'N	18°40.00'E	40	3.71	7.21	8.23
14s	55°25.40'N	18°24.00'E	50	3.46	7.26	8.43
24s	54°58.20'N	16°17.20'E	30	15.52	7.08	7.09
26s	55°11.00'N	15°57.90'E	70	5.04	11.92	3.69
11w	54°32.40'N	15°29.20'E	58	5.62	12.49	2.65
17w	54°52.80'N	16°41.50'E	20	9.2	7.22	8.38
21w	55°42.40'N	18°37.50'E	99	4.81	10.25	1.18
3X	54°56.50'N	18°37.80'E	76–73	5.06	10.03	2.73
12X	55°27.50'N	18°18.00'E	87–84	7.91	13.33	2.37
26X	55°01.30'N	16°19.00'E	56	6.54	10.91	4.82

Table 2
Materials for the analysis of genotoxicity in peripheral blood erythrocytes of herring specimens collected from different study-stations of the Bornholm and the eastern Gotland basins of the Baltic Sea (data from the Polish r/v "Baltica" surveys in the period November 2010–February 2014 and the German r/v "Walther Herwig III" survey in December 2009 and 2010.

Sampling date, station ID and the number of investigated specimens – in brackets	
December 2009 – S4/06 (21), S4/07 (12), *December 2010* – S4/17 (10)	*November 2010* – 3 (10), 19 (10), 21 (10), 22 (10), 23 (10), 24 (10), 25 (10), 28 (10)
February 2011 – 6a (10), 7a (10), 8a (10), 15a (10), 17a (10), 18a (10), 19a (10), 21a (10), 22a (10), 23a (10), 28a (10)	*June 2011* – Z11 (10), Z12 (10), Z13 (10), Z24 (10)
November 2011 – 6b (9), 14b (10), 16b (10), 17b (10), 18b (10), 23b (10), 29b (10), 30b (10)	*February 2012* – 1d (10), 2d (10), 26d (10), 33d (9), 34d (10), 35d (10), 36d (10), 45d (10), 47d (10)
September 2012 – 19f (10), 32f (10)	*November 2012* – 6j (10), 7j (10), 8j (10), 18j (10), 19j (10), 21j (10)
February 2013 – 13m (10), 14m (9), 23m (10), 24m (10)	*September 2013* – 11s (10), 14s (10), 24s (10), 26s (10)
November 2013 – 11w (10), 17w (10), 21w (10)	*February 2014* – 3X (10), 12X (10), 26X (10)
Total	660 specimens from 65 stations

2.2. Sample preparation and analysis

Sample preparation was performed immediately after catching herring. Only living specimens in good condition were processed for further analysis. A drop of blood (taken from the caudal vein of fish using a heparinized syringe) was directly smeared on glass slides and air-dried. Smears were fixed in methanol for 10 min. and later were stained with 5% Giemsa solution in phosphate buffer pH = 6.8 for 8 min. The stained slides were analysed under light microscopes Olympus BX51 or Nikon Eclipse 50*i* at final magnification of 1000×. Blind scoring of micronuclei and both types of nuclear buds were performed on coded slides. Micronuclei (MN) were identified according to the following criteria: (1) round and ovoid-shaped non-refractory particles in the cytoplasm: (2) colour and structure similar to chromatin; (3) diameter of 1/3–1/20 of the main nucleus: and (4) particles completely separated from the main nucleus (Heddle et al., 1991).

Nuclear buds were identified using criteria described by Fenech et al. (2003). Common nuclear buds (NB) were characterized as extruded nuclear material and nuclear buds on filament (NBf) were identified as extruded nuclear material conjugated to the main nucleus by thin nucleoplasmic connection. The morphological features of studied nuclear abnormalities (NA) were presented in our previous study (Baršienė et al., 2015). For each studied specimen of fish, 4000 intact erythrocytes were analysed. Final results were expressed as the mean value (in ‰) of sums of the analysed individual lesions scored in 1000 cells per fish collected from every study station (Baršienė et al., 2004).

2.3. Statistical analyses

Statistical analyses were carried out using the GraphPad PRISM 5.0 statistical package. Means and standard errors were calculated for herring from each station studied. The non-parametric Mann–Whitney *U*-test was used to compare the frequencies of nuclear abnormalities observed in herring at the reference and other stations. Spearman's correlation analysis was performed to identify possible relationships between nuclear abnormalities and biometrical measurements in fish, or between MN, NBf and NB responses in herring from different study stations located in the Bornholm and the eastern Gotland basins of the Baltic Sea.

3. Results

3.1. Biomarker responses in herring from studied stations

Analysis of genotoxicity biomarkers in herring blood erythrocytes showed a strongly increased frequency of MN, NB or NBf in fish, caught at four stations (7j, 11s, 14s and 21w) of 65 analysed stations (Table 3). The station 7j is located in the Bornholm Basin; the other three stations are located in the eastern Gotland Basin. Since in these stations one or two specimens with extremely high responses were found, all further results are presented without MN, NB or NBf data determined in these specimens.

In blood erythrocytes of herring from all 65 stations, the frequency of micronuclei (MN/1000 erythrocytes) varied from 0.08‰ in fish from station 18j (0.09‰ in S4/17) to 3.45‰ in fish from station 16b. Frequencies of NB varied from 0.21‰ in fish from station 19 to 2.73‰ in herring from station 15a. NBf ranged from 0.04‰ in herring from station S4/06 to 1.85‰ – from station 45d.

The gradients of analysed nuclear abnormalities (MN, NB, NBf) and induction of genotoxicity are presented only for the responses higher than 1.0 NA/1000 erythrocytes (10-fold of the reference levels) in herring collected at 65 study stations. There were 10 stations for MN, 20 stations for NB and 9 stations – for NBf responses higher than 1.0‰. The highest levels of MN, NB and NBf were observed in fish caught in the Bornholm Basin. Genotoxicity responses among therein-collected specimens were higher than 1.0‰ (NA/1000 erythrocytes) and were found in the majority (70–85%) of analysed fish (Table 4). In general, extremely high levels of MN were detected in herring from closely to CW located stations (17a, 21a, 7j, also in 13m and 11w). High levels of NB incidence were observed in CW dumping (stations 17a, 21a, 11w, 7j) and transport route (stations 6a, 7a and 8a) areas, also in oil and gas exploitation zone (stations – 19j, 3, 21j).

In herring with the highest responses (10-fold of the reference levels) the disproportionate induction of MN, NB and NBf frequencies was observed. The prevalence of MN > NB and MN > NBf was found in fish caught mainly at the Bornholm area of CW transport routes. Higher induction of NB than MN was found in fish from 19 study stations; however higher MN compared to NB was detected only in six study stations. The most significant disproportionate induction of NB > MN and NBf > MN was determined in herring collected from the eastern Gotland Basin and it was less expressed in the fish from the Bornholm zone of CW transport route. The higher levels of NB compared to NBf were determined in herring from 19 study stations located mainly in the Bornholm area, in which was also found the prevalence of NBf > NB (Table 5). It is noteworthy to point out, that disproportionate induction of NBf > NB and NB > NBf was in fish from areas of the closely located

Table 3
Highly increased frequencies of MN, NB and NBf in herring blood erythrocytes.

Parameter	Station 7j	Station 11s	Station 14s	Station 21w
MN, ‰	11.08	40.55	40.98	6.40
NB, ‰	8.73	20.40	20.55	20.78
NBf, ‰	4.63	20.50	0.58	4.48

Table 4
Gradients of genotoxicity response in herring collected at different stations (data only for study groups showing responses over 1.0 for the measured NA/1000 erythrocytes).

Biomarker	Values over	Gradient of responses per stations
MN	1.0 (10 stations)	**16b > 33d > 13m > 8a > 7j > 21a > 17a > 14s > 11w = 12X**
NB	1.0 (20 stations)	**15a > 17a > 19j > 11w > 19a > 28a > 3 > 8a = 18a > 24 > 29b > 18j > 28 = 7a > 21a > 23a > 6a > 22a > 21j = 3X**
NBf	1.0 (9 stations)	**45d > 29b > 35d > 19j > 19f = Z24 > 33d > 34d > 24m**

Table 5
Comparison of genotoxicity responses in herring collected at stations showing responses over 1.0 for the measured NA/1000 erythrocytes (bolded stations where the differences between responses higher than 5-times).

Biomarkers	Responses over	Stations, in which responses were higher in times
MN > NB	MN > 1.0 (6 stations)	**13 m – 7.40**; 33d – 3.66; 16b – 3.45; 14s – 1.77; 7j – 1.47; 12X – 1.13 times
MN > NBf	MN > 1.0 (11 stations)	**16b – 6.90; 17a – 5.14**; 21a – 4.88; 13 m – 4.48; 8a – 4.34; 11w – 2.76; 12X – 2.19; 33d – 2.02; 6a – 1.87; 14 s – 1.86; 7j – 1.75 times
NB > MN	NB > 1.0 (19 stations)	**18j – 15.38; 19j – 6.18**; 24 – 4.13; 15a – 2.97; 18a – 2.87; 28 – 2.75; 28a – 2.63; 29b – 2.17; 19a – 2.07; 23a – 1.93; 3 – 1.88; 22a – 1.69; 3X – 1.67; 17a – 1.65; 11w – 1.62; 7a – 1.34; 21j – 1.19; 6a – 1.14; 8a – 1.07 times
NBf > MN	NBf > 1.0 (8 stations)	**35d – 6.09; 24 m – 5.72**; 19j – 4.93; 34d – 4.91; 45d – 3.85; 29b – 2.75; 36d – 2.0; Z24 – 1.77 times
NBf > NB	NBf > 1.0 (7 stations)	35d – 2.33; 45d – 2.31; 34d – 2.05; 24 m – 1.87; 33d – 1.82; Z24 – 1.71; 29b – 1.27 times
NB > NBf	NB > 1.0 (19 stations)	**24 – 10.15**; **21a – 5.57; 23a – 5.52; 28a – 5.07; 22a – 5.10**; 15a – 4.79; 8a – 4.66; 11w – 4,47; 18j – 4.39; 28 – 4.17; 19a – 3.97; 18a – 3.46; 17a – 3.12; 3X – 3.0; 3 – 2.84; 7a – 2.63; 21j – 2.44; 6a – 2.13; 19j – 1.25 times

stations. Similar tendency was observed in cases NB > MN and in MN > NBf (Table 5).

Considering a large number of studied stations, for analysis of MN, NB and NBf levels in herring, we subdivided the study areas into two groups. One group consists of 41 stations located in the Bornholm area and the other group of 24 stations situated closer to the Gotland CW dumping area.

In the Bornholm area, the MN frequency lower than the background assessment criteria level BAC P90 (in herring equals to 0.39‰) was detected in 12 stations (26d < 17b < 14b < 34d = 35d = 8j = 26X < Z13 < 24 < 47d < 14m < 23). In five stations (13m < 19 < 26s < 26d = 24s) NB frequency was below P90 (0.40‰) and in eight stations (22 < 19 < 24 < 17w < 17a = 23a < 22a < 21a) NBf levels were lower than the P90 value (0.25‰). The frequency of genotoxicity biomarker responses higher than 1.0‰ was recorded for MN in herring at the sequence of the stations 16b > 33d > 13m > 21a > 17a > 21w, for NB – 15a > 17a > 11w > 19a > 28a > 18a > 24 > 28 > 21a > 23a > 22a, and for NBf – in gradient of 45d > 35d > Z24 > 33d > 34d (Fig. 2).

Analysis of genotoxicity parameters in herring from the eastern Gotland Basin showed responses higher than 1.0‰ only in three stations (8a > 14s > 12X) for MN, in nine stations (19j > 3 > 8a > 29b > 18j > 7a > 6a > 21j = 3X) for NB and in four stations (29b > 19j > 19f > 24m) for NBf out of 24 studied stations. The responses lower than P90 were recorded in nine stations (18j < S4/17 < S4/07 < 24m < S4/06 = 23b < 1d = 19j) for MN, in two stations (S4/06 < S4/07) for NB and in four stations (S4/06 < S4/17 < S4/07 < 6b) for NBf (Fig. 3).

The analysis of time-related genotoxicity changes in the study area was performed after subdivision of results into three groups according to the fish sampling dates. The first group consists of 27 stations where herring was collected February 2011–2014. Second group consists of 28 stations, in which herring was sampled on 6th of December 2009, 7th of December 2010 and also at the end of November 2010–2013. The third group includes fish caught June 2011, September 2012 and 2013 (10 stations).

In the first group, overwhelming 1.0‰ frequency of MN values was found in herring from six stations, NBf – from five and NB – from 12 stations out of 27 studied. Extremely high NB (2.73‰) and MN (2.38‰) frequencies were found in the fish caught at the same location (stations 15a and 33d) and NBf frequency of 1.86‰ – in station 45d. All three genotoxicity parameters showed the highest responses (4.22‰, 4.21‰ and 3.18‰) in the fish from the same stations, respectively. The lowest NB responses (0.20‰) there were detected in the fish from station 13 m, MN (0.15‰) – from station 26d and NBf (0.21‰) – from stations 17a and 23a. In herring collected February 2011–2014, the decrease of MN and NB was observed in the year of 2012, except only fish from station 33d, in 2013 (except station 13 m) and in 2014. A marked increase of NBf induction was found in 2012 and 2013 compared to responses in 2011 (Fig. 4).

In the second group of herring, increased genotoxicity responses were found in the fish sampled in late November 2010–2013 compared to the responses in fish caught at station S4/06 in early December 2009, with exceptions for the responses in the fish from some single stations. The frequency of MN in herring from station S4/07 and NBf from

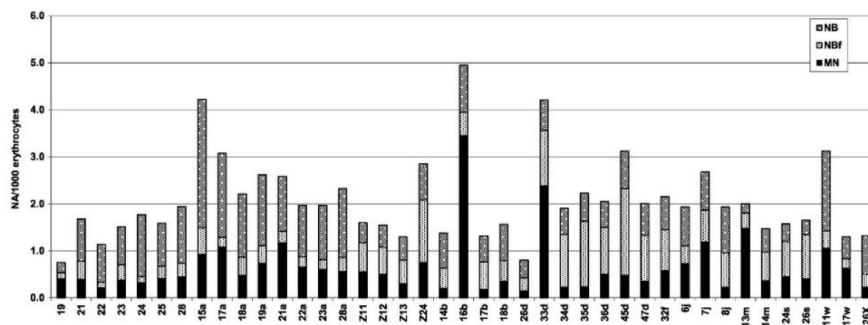


Fig. 2. MN, NBf and NB levels in herring, collected from 41 stations located in the Bornholm area.

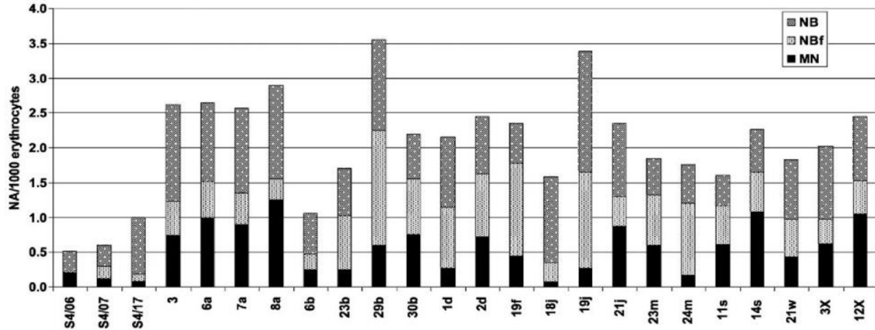


Fig. 3. MN, NB and NBf levels in herring from the eastern part of the Gotland Basin.

stations S4/06 and S4/07 were at the reference levels. Extremely high induction of MN was found in the fish caught from the Bornholm zone at station 16b (Fig. 5).

In the third group of herring, caught in June and September, there were no temporal differences. The highest MN incidences were in the specimens caught at station 14 s, NB – at station Z24 and NBf – at station 19f (Fig. 6).

The Spearman correlation analysis of MN, NBf and NB frequencies and biometrical variables (fish total length, total weight, liver weight, age and condition index (CI)) in herring showed a positive correlation only in some studied stations. In the fish from stations 7a, 17a and 23a a strong correlation was detected between liver weight and MN (7a – $r_s = 0.714$; 17a – $r_s = 0.847$; 23a – $r_s = 0.817$), as well between liver weight and NB (7a – $r_s = 0.717$; 17a – $r_s = 0.803$; 23a – $r_s = 0.656$). A strong positive correlation was recorded between age and MN (15a – $r_s = 0.798$; 19a – $r_s = 0.722$; 21j – $r_s = 0.770$), NB (18b – $r_s = 0.799$), NBf (1d – $r_s = 0.818$) also between CI and NB (19j – $r_s = 0.816$), NBf – (19j – $r_s = 0.769$).

4. Discussion

This paper presents data on MN, NB and NBf levels in the Baltic native fish *C. harengus* collected mostly from the Polish EEZ. To our knowledge, the study represents the first attempt to evaluate environmental genotoxicity using three genotoxicity endpoints in the marine environment. In addition, our study is describing the pattern of MN, NB and NBf responses in the fish living in zones, where chemical warfare materials were dumped or close to the chemical munitions transportation route.

After the Second World War, at least 40,000 t of chemical munitions were transported from Germany and dumped in the Baltic Sea. The locations of three main dumping areas – east of the Bornholm Island, southeast of the Gotland Island, and south of the Little Belt are well known. The Bornholm Basin chemical munitions dumpsite covers a large area, where about 11,000 t of chemical warfare agents were dumped east of the island of Bornholm. The main types of warfare agents dumped in the Bornholm Basin (1945–1965) include sulphur mustard, phosgene, α-chloroacetophenone as well as arsenic-

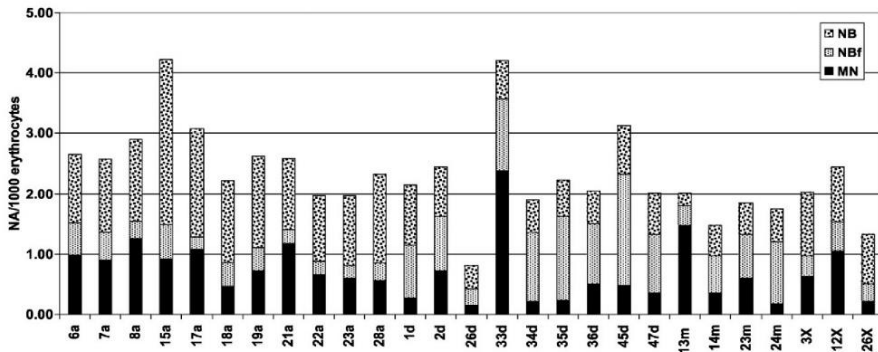


Fig. 4. The levels of MN, NBf and NB in herring caught at 27 stations in February 2011 (a), 2012 (d), 2013 (m) and 2014 (X).

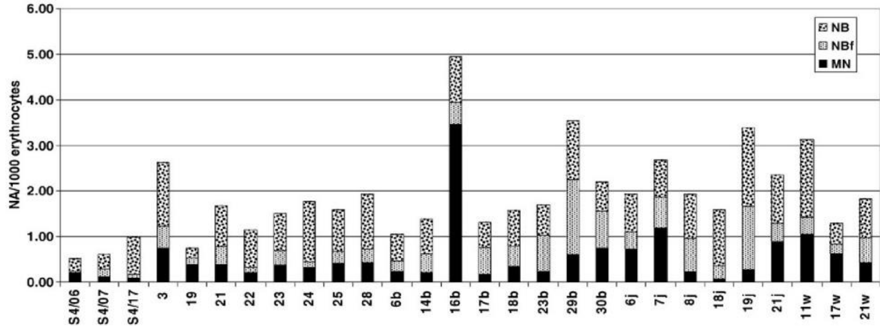


Fig. 5. The levels of MN, NBf and NB in herring caught at 28 stations in December 2009 (S4/06, S4/07) and 2010 (S4/17), in November 2010 (3–28), 2011 (b), 2012 (j) and 2013 (w).

containing compounds such as Adamsite (diphenylaminechloroarsine, DM), Clark I (diphenylarsine chloride, DA), Clark II (diphenylarsine cyanide, DC) and arsine oil (HELCOM, 1993; Politz, 1994). Arsine oil is a technical mixture of Clark I (35%), phenyldichloroarsine (PDCA; 50%), trichloroarsine (TCA; 5%) and triphenylarsine (TPA; 5%). In 1947, 2000 t of chemical warfare materials, including sulphur mustard, Clark, Adamsite, α-chloroacetophenone and Tabun were shipped by the 'Elbing IV' and 'Elbing VIII' from the loading port of Wolgast in Germany and dumped in the Gotland Deep areas (HELCOM, 1994, 2013).

At present, it is clear that chemical munitions were scattered not only in the designed areas, but also closeby while some were thrown overboard *en route* from the storage to the dumping sites. Most of the transport routes were located in the Polish EEZ. Therefore, scattered warfare munitions were likely to have been spread over a large area during dumping *en route*, and might thus become encountered in fishing trawls. On the other hand, due to the increasing offshore technical marine activities, like installation of pipelines and sea cables, and wind farms construction, the risk of encountering dumped chemical munitions is increasing (HELCOM, 2013; Bełdowski et al., 2015).

The results of the present study revealed most significantly increased MN, NB and NBf incidences in herring collected in 2010–2014 at stations located along the chemical munitions transport routes, close to the Bornholm CW dumping area and in zones with CWAs-related substances in sediments. Most of the highest genotoxicity associated stations, which were top five places in the gradients of MN, NB and NBf were located along the chemical munitions transport routes. Some stations located in zones of the eastern Gotland Basin, where arsenic was detected in sediments (CHEMSEA Findings, 2014), and potentially could be polluted from the oil-gas platforms located nearby the investigated stations. Station Z24 is situated in the Bornholm CW dumping area.

Amato et al. (2006) demonstrated 10-fold higher concentrations (29.7 mg/kg dw) of total As in blackbelly rosefish (*Helicolenus dactylopterus*) specimens from a CWA dumpsite in the southern Adriatic Sea, than fish from reference conditions. Della Torre et al. (2010) confirmed the data indicating release of residues of yperite from chemical munitions dumped in the Adriatic Sea, demonstrated CWAs accumulation in fish tissues and DNA damage in gills of European conger (*Conger conger*) caught at the dumping area.

It is important to stress, that the highest levels of MN, NB and NBf were recorded in the fish caught at closely to each other located stations of the study area. Very high levels of MN were detected in herring from stations 17a, 21a, 7j, 13m and 11w; of NB — in the same stations, also in stations 19j, 3, 21j, as well as in stations 6a, 7a and 8a. Moreover, disparity between MN, NB and NBf frequencies was observed in herring, caught mainly at stations with the highest responses of measured biomarkers. The pattern of genotoxicity responses we have recorded obviously is due to the occurrence and distribution of genotoxic substances. Different mixtures of pollutants can provoke different levels of genotoxicity in the study area and especially in certain sites potentially impacted by chemical warfare agents.

Increased genotoxicity levels were recorded in herring caught at stations located along the warfare munitions transport routes in the Bornholm Basin. It should be pointed out, that in the zone of stations 17a, 21a and 7j dumped chemical warfare material was measured

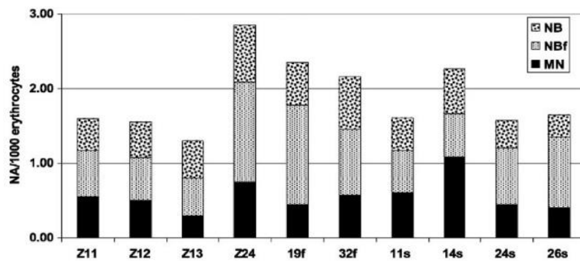


Fig. 6. The levels of MN, NBf and NB in herring caught at 10 stations in June 2011 (Z), September 2012 (f) and 2013 (s).

(Andrulewicz, 1996) and specificity in PCBs profiles has been described in sediments from station located nearby (Szlinder-Richert et al., 2012). Measurements of PCB concentrations during the period 1997–2006 in fish inhabiting the Kołobrzeg–Darłowo area (our study stations were located there) have shown elevated concentrations of PCBs (congeners 101, 118, 153, 138 and 180), which were determined in sprat and in cod (Szlinder-Richert et al., 2009a). In 2002–2006, elevated concentrations of polychlorinated dibenzofurans (PCDFs), and dioxin-like polychlorinated biphenyls (dl-PCBs) have been observed in two out of 72 herring samples collected from the southern Baltic Sea region. In the same region, the level of seven polybrominated diphenyl ether (PBDE) congeners in fish species was low (Szlinder-Richert et al., 2009b, 2010).

The study stations 19j, 3 and 21j are located in the Gdansk Deep. In sediments of this study zone, increased concentrations of arsenic were detected (CHEMSEA Findings, 2014). Furthermore, there is oil and gas exploitation in this area. In flounder caught at the Władysławowo fishing ground and from the Gulf of Gdansk approximately 2-times higher concentrations of 1-OH pyrene in bile, sum of seven PCBs, and sum of all studied persistent pollutants than in the fish from the reference site, i.e. the Ustka-Łeba fishing ground were detected (Dąbrowska et al., 2014). Furthermore, increased concentrations of polybrominated diphenyl ether congeners BDE-47 and BDE-100 have been detected in muscles of flounder collected in February 2010 from the Władysławowo fishing ground (Waszak et al., 2012). Genotoxicity of BDE-47 in marine fish has been recorded in laboratory exposures (Baršienė et al., 2005; Bolognesi et al., 2006). Moreover, DNA damage in flounder positively correlated with lipid-based concentrations of PCB 118 in muscles as compared to the reference site (Dąbrowska et al., 2014).

All three genotoxicity parameters showed the highest responses in the fish from station 15a (4.22‰; February 2010) and from station 33d (4.21‰; February 2011). Both stations are located *en route* of the chemical munitions and there is information on warfare ammunition dumped closely to these stations (Andrulewicz, 1996). However, in close vicinity, very low levels of MN, NB and NBf responses we found in herring caught in 2009 from station B03/46. High levels of MN, NB and NBf induction were registered in herring caught at stations located around the Bornholm CW dumping zone. Many CW-fishing incidences are known to occur in the zone (HELCOM, 2013) and pollution with CWAs cannot be excluded.

The highest concentrations of active parental chemical warfare agents (Clark I, Adamsite) and arsenic oil consisting of TPA and TCA were detected in sediments and pore water at stations CWA16 and CWA19 (Sanderson et al., 2012) and CWA17 (Sanderson et al., 2014), along the Nord Stream pipeline route in the Bornholm Deep. These stations were located in the area of our study stations (15a, 33d, Z13 and 34d) showing high genotoxicity levels. The measurements of chemical warfare agent (CWA) concentrations in sediments and pore water collected in 2008–2012 revealed degradation products of CWAs in CWA5-CWA13 stations (Sanderson et al., 2014). Z12 station, where high levels were detected evaluating MN and NBf responses in herring is located between stations CWA5 and CWA10. Since in the studies of Sanderson et al. (2014) and Fauser et al. (2013) there was not geographical coordinates of study stations presented, the correct collation of location of their and our study stations is difficult. In principle, all reliable scientific data in marine environment should be connected to geographical coordinates of sample collection.

Munitions containing CWA's are more spread out of designated areas than expected (Bełdowski et al., 2015). Fifty-five sediment samples out of 175 analysed were positive for CWAs and contained at least one of CWA's targets chemical. Pollution with arsenic containing and mustard related CWA were detected in sediments of the Słupsk Furrow and even in the reference area, where theoretically they should not exist. Our study stations 28a, 26d, 24s and 26X are located in the Słupsk Furrow, station 18j — at the reference area. Extremely high incidences of nuclear buds were determined in herring from stations 28a, 26X and 18j. Extremely high and high NBf levels were found in fish caught, respectively at station 24s and at stations 28a, 26d, 26X and 18j.

Overall, our study results indicated high and extremely high genotoxicity zones for herring caught at stations located along the CW transportation routes, close to the Bornholm CW dumping area and in zones where arsenic in sediments, also sulphur mustard- and triphenylarsine-related substances were detected (CHEMSEA Findings, 2014). Low genotoxicity zones were mostly located at a distance from the CW transport routes and further from areas where CW incidents were registered and presented in HELCOM (2013).

Time-related analysis revealed statistically significant increases of MN, NB and NBf incidences in herring caught in November 2010–2013 compared to the responses in 2009. Within the period of February 2011–2014, the decrease of MN and NB was recorded in 2012–2014 and increased NBf incidences – in 2012 and 2013 compared to the responses in 2011. The comparatively low influence of environmental and biological factors estimated in the present and previous our studies (Baršienė et al., 2012a, 2014) on genotoxicity in fish most likely indicates that the source of the elevated genotoxicity responses is environmental pollution. The increased distribution of pollution during construction of gas pipelines and cables, as well as implementation of the mine clearance programme in 2008–2011 (Möller, 2011) or extensive military exercises could be suspected contributors. Data of this study have provided evidence that the emergence of increased genotoxicity responses may be concurrent with the above-mentioned pan-Baltic interventions. There is a necessity to monitor genotoxicity impacts of CWAs on marine organisms and to determine the ecological significance in the areas of CW dumpsites and also including CW lost along their transport route.

5. Conclusions

The induction of MN, NB and NBf was analysed in herring *C. harengus* caught in 2009–2014 at 65 study stations located mostly at zones along chemical munitions transport routes to the Bornholm, Gotland and Gdansk CW dumping sites. The highest genotoxicity levels were recorded in herring caught at stations along the CW transport routes, close to the Bornholm CW dumping site, in zones with CWAs-related substances in sediments and in nearby to oil-gas platforms. Disparity between MN, NB and NBf incidences and the highest genotoxicity response levels were detected in the fish caught at closely to each other located stations of the study area. Genotoxicity levels were found to be lower at stations located further away from the known pollution by chemical warfare agents. Time-related analysis revealed statistically significant increases of MN, NB and NBf incidences in herring caught in 2012–2014 compared to the responses in 2009 or in 2011.

Conflict of interests

None.

Acknowledgements

The Research Council of Lithuania through the GENOTOX-CG project MIP-33/2012 funded this study. The authors would like to thank Thomas Lang (Thünen Institute of Fisheries Ecology, Cuxhaven, Germany) and Aleksandras Rybakovas (Nature Research Centre, Vilnius) for collecting samples from stations S4/06, S4/07, S4/17 and B03/46 during *Walther Herwig III* cruises. We are thankful to Dalia Baršytė Lovejoy (Toronto University) for the language check.

References

Amato, E., Alcaro, L., Corsi, I., Torre, C.D., Farchi, C., Focardi, S., Marino, G., Tursi, A., 2006. An integrated ecotoxicological approach to assess the effects of pollutants released by unexploded chemical ordnance dumped in the southern Adriatic (Mediterranean Sea). Mar. Biol. 149, 17–23.

Andrulewicz, E., 1996. War gases and ammunition in the Polish Economic Zone of the Baltic Sea. In: Kaffka, A.V. (Ed.), Sea-Dumped Chemical Weapons: Aspects, Problems and Solutions. NATO ASI Series 7, pp. 9–15.

Baršienė, J., Lazutka, J., Šyvokienė, J., Dedonytė, V., Rybakovas, A., Bjornstad, A., Andersen, O.K., 2004. Analysis of micronuclei in blue mussels and fish from the Baltic and the North Seas. Environ. Toxicol. 19, 365–371.

Baršienė, J., Dedonytė, V., Rybakovas, A., Andersen, O.-K., 2005. Induction of micronuclei in Atlantic cod (*Gadus morhua*) and turbot (*Scophthalmus maximus*) after treatment with bisphenol A, diallyl phthalate and tetrabromodiphenyl ether-47. Ekologija 4, 1–7.

Baršienė, J., Rybakovas, A., Lang, T., Grygiel, W., Andreikėnaitė, L., Michailovas, A., 2012a. Risk of environmental genotoxicity in the Baltic Sea over the period of 2009–2011 assessed by micronuclei frequencies in blood erythrocytes of flounder (*Platichthys flesus*), herring (*Clupea harengus*) and eelpout (*Zoarces viviparus*). Mar. Environ. Res. 77, 35–42.

Baršienė, J., Butrimavičienė, L., Grygiel, W., Lang, T., Michailovas, A., Jackūnas, T., 2014. Environmental genotoxicity and cytotoxicity in flounder (*Platichthys flesus*), herring (*Clupea harengus*) and Atlantic cod (*Gadus morhua*) from chemical munitions dumping zones in the southern Baltic Sea. Mar. Environ. Res. 96, 56–67.

Baršienė, J., Butrimavičienė, L., Michailovas, A., Grygiel, W., 2015. Assessing the environmental genotoxicity risk in the Baltic Sea: frequencies of nuclear buds in blood erythrocytes of three native fish species. Environ. Monit. Assess. 187 (1), 4078.

Bełdowski, J., Klusek, Z., Szubska, M., Turja, R., Bulczak, A.I., Rak, D., Brenner, M., Lang, T., Kotwicki, L., Grzelak, K., Jakacki, J., Fricke, N., Östin, A., Olsson, U., Fabisiak, J., Garnaga, G., Nyholm, J.R., Majewski, P., Broeg, K., Söderström, M., Vanninen, P., Popiel, S., Nawała, J., Lehtonen, K., Berglind, R., Schmidt, B., 2015. Chemical munitions search & assessment — an evaluation of the dumped munitions problem in the Baltic Sea. Deep-Sea Res. II.

Bolognesi, C., Perrone, E., Roggieri, P., Pampanin, D.M., Sciutto, A., 2006. Assessment of micronuclei induction in peripheral erythrocytes of fish exposed to xenobiotics under controlled conditions. Aquat. Toxicol. 78S, S93–S98.

CHEMSEA Findings, 2014. Beldowski, J., Fabisiak, J., Popiel, S., Ôstin, A., Olsson, U., Vanninen, P., Latsumaki, A., Lang, T., Fricke, N., Brenner, M., Berglind, R., Baršienė, J. et al., Gdansk, Institute of Oceanology, Polish Academy of Sciences, (86 pp.).

Dąbrowska, H., Kopko, O., Góra, A., Waszak, I., Walkusz-Miotk, J., 2014. DNA damage, EROD activity, condition indices, and their linkages with contaminants in female flounder (*Platichthys flesus*) from the southern Baltic Sea. Sci. Total Environ. 496, 488–498.

Della Torre, C., Petochi, T., Corsi, I., Dinardo, M.M., Baroni, D., Alcaro, L., Focardi, S., Tursi, A., Marino, G., Frigeri, A., Amato, E., 2010. DNA damage, severe organ lesions and high muscle levels of As and Hg in two benthic fish species from a chemical warfare agent dumping site in the Mediterranean Sea. Sci. Total Environ. 408, 2136–2145.

Della Torre, C., Petochi, T., Farchi, C., Corsi, I., Dinardo, Sammarini, V., Alcaro, L., Mechelli, L., Focardi, S., Tursi, A., Marino, G., Amato, E., 2013. Environmental hazard of yperite released at sea: sublethal toxic effects on fish. J. Hazard. Mater. 248-249, 246–253.

Fauser, P., Sanderson, H., Hedegaard, R.V., Sloth, J.J., Larsen, M.M., Krongaard, T., Bossi, R., Larsen, J.B., 2013. Occurrence and sorption properties of arsenicals in marine sediments. Environ. Monit. Assess. 185, 4679–4691.

Fenech, M., Chang, W.P., Kirsch-Volders, M., Holland, N., Bonassi, S., Zeiger, E., 2003. HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutat. Res. 534, 65–75.

Garnaga, G., Stankevičius, A., 2005. Arsenic and other environmental parameters at the chemical munitions dumpsite in the Lithuanian economic zone of the Baltic Sea. Environ. Res. Eng. Manag. 33, 24–31.

Heddle, J.A., Cimino, M.C., Hayashi, M., Romagna, F., Shelby, M.D., Tucker, J.D., Vanparys, P., MacGregor, J.T., 1991. Micronuclei as an index of cytogenetic damage: past, present, and future. Environ. Mol. Mutagen. 18, 277–291.

HELCOM, 1993. Complex Analysis of the Hazard Related to the Captured German Chemical Weapon Dumped in the Baltic Sea (HELCOM CHEMU 2/2/1/Rev. 1, 27).

HELCOM, 1994. Report on Chemical Munitions Dumped in the Baltic Sea, Report to the 15th Meeting of Helsinki Commission. Danish Environmental Protection Agency.

HELCOM, 1996. Third periodic assessment of the state of the marine environment of the Baltic Sea, 1989–1993. Executive Summary & Background Document. Baltic Sea Environment Proceedings.

HELCOM, 2010. Hazardous substances in the Baltic Sea. An integrated thematic assessment of hazardous substances in the Baltic Sea. Baltic Sea Environmental Process Series 120B.

HELCOM, 2013. Chemical munitions dumped in the Baltic Sea. Report of the *Ad Hoc* Expert Group to Update and Review the Existing Information on Dumped Chemical Munitions in the Baltic Sea (HELCOM MUNI). Baltic Sea Environment Proceeding (BSEP) 142, p. 128.

Kaffka, A.V., 1995. Sea-Dumped Chemical Weapons: Aspects, Problems and Solutions. p. 170.

Köster, F.W., Möllmann, C., Neuenfeldt, S., Vinther, M., St. John, M.A., Tomkiewicz, J., Voss, R., Hinrichsen, H.-H., MacKenzie, B., Kraus, G., Schnack, D., 2003. Fish stock development in the Central Baltic Sea (1974–1999) in relation to variability in the physical environment. ICES Mar. Sci. Symp. 219, 294–306.

Lindberg, H.K., Wang, X., Järventaus, H., Falck, G.C.M., Norppa, H., Fenech, M., 2007. Origin of nuclear buds and micronuclei in normal and folate-deprived human lymphocytes. Mutat. Res. 617, 33–45.

Missiaen, T., Söderström, M., Popescu, I., Vanninen, P., 2010. Evaluation of a chemical munition dumpsite in the Baltic Sea based on geophysical and chemical investigations. Sci. Total Environ. 408, 3536–3553.

Möller, G., 2011. How to maximize the efficiency and focus the national and multinational Mine Clearance activities to the areas most needed. Third International Dialogue on Underwater Munitions, April 13–15, 2011, Sopot, Poland, p. 16.

Politz, F., 1994. Zeitbombe Ostsee — Das Giftgas-Erbe auf dem Meerersgrund. Chr. Links Verlag — LinksDruck GmbH, Berlin (134 pp. (in German)).

Sanderson, H., Fauser, P., Thomsen, M., Sørensen, P.B., 2008. Screening level fish community risk assessment of chemical warfare agents in the Baltic Sea. J. Hazard. Mater. 154, 846–857.

Sanderson, H., Fauser, P., Thomsen, M., Vanninen, P., Savin, Y., Hirvonen, A., Missiaen, T., Polyak, Y., Paka, V., Niiranen, S., Borodin, P., Medvedeva, N., Feller, P., Soderstrom, M., Khalikov, I., Zhurbas, V., Gress, A., 2010. Feature: risk assessment of chemical munitions dumped in the Baltic Sea in 1947. Environ. Sci. Technol. 44, 4389–4394.

Sanderson, H., Fauser, P., Thomsen, M., Larsen, J.B., 2012. Weight-of-evidence environmental risk assessment of dumped chemical weapons after WWII along the Nord-Stream gas pipeline in the Bornholm Deep. J. Hazard. Mater. 215–216, 217–226.

Sanderson, H., Fauser, P., Rahbek, M., Larsen, J.B., 2014. Review of environmental exposure concentrations of chemical warfare agent residues and associated the fish community risk following the construction and completion of the Nord Stream gas pipeline between Russia and Germany. J. Hazard. Mater. 279, 518–526.

Szarejko, A., Namieśnik, J., 2009. The Baltic Sea as a dumping site of chemical munitions and chemical warfare agents. Chem. Ecol. 25 (1), 13–26.

Szlinder-Richert, J., Barska, I., Mazerski, J., Usydus, Z., 2009a. PCBs in fish from the southern Baltic Sea: levels, bioaccumulation features, and temporal trends during the period from 1997 to 2006. Mar. Pollut. Bull. 58, 85–92.

Szlinder-Richert, J., Barska, I., Usydus, Z., Ruczyńska, W., Grabic, R., 2009b. Investigation of PCDD/Fs and dl-PCBs in fish from the southern Baltic Sea during the 2002–2006 period. Chemosphere 7a, 1509–1515.

Szlinder-Richert, J., Barska, I., Usydus, Z., Grabic, R., 2010. Polybrominated diphenyl ethers (PBDE) in selected fish species from the southern Baltic Sea. Chemosphere 78, 695–700.

Szlinder-Richert, J., Usydus, Z., Drgas, A., 2012. Persistent organic pollutants in sediment from the southern Baltic: risk assessment. J. Environ. Monit. 14, 2100–2107.

Tørnes, J.A., Voie, Ø.A., Ljønes, M., Opstad, A.M., Bjerkeseth, L.H., Hussain, F., 2002. Investigation and Risk Assessment of Ships Loaded with Chemical Ammunition Scuttled in Skagerrak TA-1907/2002.

Tørnes, J.A., Opstad, A.M., Johnsen, B.A., 2006. Determination of organoarsenic warfare agents in sediment samples from Skagerrak by gas chromatography–mass spectrometry. Sci. Total Environ. 356 (1–3), 235–246.

Waszak, I., Dabrowska, H., Góra, A., 2012. Bioaccumulation of polybrominated diphenyl ethers (PBDEs) in flounder (*Platichthys flesus*) in the southern Baltic Sea. Mar. Environ. Res. 79, 132–141.

VII

Environmental genotoxicity and cytotoxicity levels in herring (*Clupea harengus*), flounder (*Platichthys flesus*) and cod (*Gadus morhua*) inhabiting the Gdansk Basin of the Baltic Sea

Valskienė R, Baršienė J, Butrimavičienė L, Grygiel W, Stunžėnas V, Jokšas K, Stankevičiūtė M

Marine Pollution Bulletin 2018, 133: 65–76

Reprinted with permission from Elsevier Ltd.

Marine Pollution Bulletin 133 (2018) 65–76

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Environmental genotoxicity and cytotoxicity levels in herring (*Clupea harengus*), flounder (*Platichthys flesus*) and cod (*Gadus morhua*) inhabiting the Gdansk Basin of the Baltic Sea

Roberta Valskienė[a,*], Janina Baršienė[a], Laura Butrimavičienė[a], Wlodzimierz Grygiel[b], Virmantas Stunžėnas[a], Kęstutis Jokšas[a], Milda Stankevičiūtė[a]

[a] Nature Research Centre, Akademijos 2, 08412 Vilnius, Lithuania

[b] National Marine Fisheries Research Institute, 1 Kollataja Street, 81-332 Gdynia, Poland

ARTICLE INFO

Keywords:
Genotoxicity and cytotoxicity
Herring
Flounder
Cod
Dumped munitions
Baltic Sea

ABSTRACT

Frequencies of eight nuclear abnormalities (NAs) reflecting environmental genotoxicity and cytotoxicity, were examined in 739 specimens of herring (*Clupea harengus*), flounder (*Platichthys flesus*) and cod (*Gadus morhua*) collected between 2009 and 2017 at 50 study stations located in the Gulf of Gdansk and the southern part of the Gdansk Deep (the Baltic Sea). The highest levels of geno-cytotoxicity were recorded in fish caught at stations located in close proximity to chemical and conventional munitions dumping sites or in zones polluted by Chemical Warfare Agents (CWAs). The values of NAs were significantly higher (up to 51-fold compared with the reference level) in herring caught at seven stations and in cod from one station located close to the munitions dumping sites. Exceptionally high total genotoxicity (ΣGtox) risk was found for flounder collected from 18 (72.0%) stations, herring caught at 12 (38.7%) stations and cod caught at four (17.4%) studied stations.

1. Introduction

The Gdansk Basin is classified as having a moderate or poor health status due to the results obtained by the integrated HELCOM CHASE confidence assessment (HELCOM, 2010; Dąbrowska et al., 2017). Within the last decade, literature has specified that this area is polluted by increased concentrations of a wide variety of genotoxins, including metals, polycyclic aromatic hydrocarbons and other organic pollutants (Polak-Juszczak, 2012; Kobusińska et al., 2014; Koniecko et al., 2014; Staniszewska et al., 2011, 2014; Zaborska, 2014; Bełdowski et al., 2014; Jędruch et al., 2015). About 60 tons of conventional munitions have been dumped in the Gdansk Deep (HELCOM, 2013). Most of them were packed in wooden boxes and metal containers, which are exposed over time to salt water, bottom water currents and human activities such as fishing with trawls; these activities may cause the release of contaminants (HELCOM, 2013). It is known that conventional munitions with different types of explosives (e.g., 2,4,6-Trinitrotoluene = TNT) have fuses which contain metals such as mercury (II) fulminate and lead (II) azide (HELCOM, 2013).

Furthermore, the existence of an unofficial chemical munitions dumpsite in the Gdansk Deep was verified during 2011–2014 period by Chemical Munitions, Search and Assessment project (CHEMSEA Findings, 2014). Further, Chemical warfare (CW) may contribute to increased mercury concentration in the Gdansk area, since it is known that many bombs and shells were filled with arsenic-containing and mustard-related CWAs and dumped in different sites of the Baltic Sea (HELCOM, 2013). CWA degradation products have been detected in the sediments from known dumpsites of CW and in other sites of the Baltic Sea (CHEMSEA Findings, 2014). In addition, the highest amounts of total arsenic were measured in the Gdansk Deep and Bornholm Deep (CHEMSEA Findings, 2014; Bełdowski et al., 2016a). The chemical warfare substances, as well as arsenic genotoxins can cause lethal or chronic toxic effects in marine organisms such as fish, or affect entire food webs (HELCOM, 2013). However, there is a lack of information about biological effects of CWAs in marine organisms. Ecological risks of CWAs for living marine organisms are poorly studied, and only a few scientific papers have been published about geno-cytotoxicity effects, biochemical markers and distribution of CWAs (Sanderson et al., 2008, 2010; Della Torre et al., 2010, 2013; Baršienė et al., 2014, 2016; Bełdowski et al., 2016b).

Della Torre et al. (2010, 2013) have identified DNA damage (Comet assay, quantified as 28.77% and 28.51% DNA migrated in the tail) in gills of conger fished at a CW dumping site in the Mediterranean Sea and Southern Adriatic Sea. Pathology of the head kidney, decreased

* Corresponding author at: Nature Research Centre, Institute of Ecology, Akademijos str. 2, 08412 Vilnius, Lithuania.
E-mail address: roberta.bucyte@gmail.com (R. Valskienė).

https://doi.org/10.1016/j.marpolbul.2018.05.023
Received 9 August 2017; Received in revised form 10 May 2018; Accepted 12 May 2018
0025-326X/ © 2018 Elsevier Ltd. All rights reserved.

stability of lysosomal membrane and genotoxicity effects were detected in benthic fish from dumpsites of chemical munitions, mainly located at the eastern part of the Bornholm Island zone compared with the reference sites (CHEMSEA Findings, 2014; Baršienė et al., 2014). High and extremely high genotoxicity levels were recorded in fish species caught along CW transportation routes, close to the Bornholm CW dumping area and in zones where arsenic and other CWA containing substances were detected in sediments (Baršienė et al., 2016).

In recent years, more attention has been devoted to the Good Environmental Status (GES) for marine environment sustainability, and various initiatives and projects have been designed to restore or maintain it. An international workshop on marine integrated contaminant monitoring (ICON) focused on chemical analyses and biological effects in fish, mussels, and gastropods in European coastal and offshore areas (Iceland, North Sea, Baltic, Wadden Sea, Seine estuary and the western Mediterranean). The aim of the ICON project was to develop an integrated monitoring database which would be suitable for the assessment of GES for Descriptor 8 of the European Union (EU) Marine Strategy Framework Directive (MSFD) (Vethaak et al., 2017). The genotoxicity impacts based on ICES/OSPAR Background Assessment Criteria (BAC) were included in the GES assessment (ICES, 2011; Baršienė et al., 2012a; Hylland et al., 2017).

The aim of this study was to assess: a) long-term (2009–2017 period) environmental genotoxicity and cytotoxicity and possible species-specific responses in the offshore part of the Gulf of Gdansk and the southern part of the Gdansk Deep using three common Baltic Sea fish species (herring, flounder and cod); b) environmental geno- and cytotoxicity levels in fish caught in newly verified CW and conventional munitions dumpsites. In the present study, a total of eight nuclear abnormalities (NAs) were examined in fish blood erythrocytes. The total genotoxicity (ΣGtox) and total cytotoxicity (ΣCytox) levels were assessed as the sum of the frequencies of genotoxicity (MN + NB + NBf + BNb) and cytotoxicity (FA + BN + 8-shaped) endpoints. Genotoxicity risk assessment (based on BAC) in herring (*Clupea harengus*), flounder (*Platichthys flesus*) and cod (*Gadus morhua*) was performed at 50 study stations for the first time in the Gdansk Basin.

2. Materials and methods

2.1. Sampling of fish

Herring (*Clupea harengus*), flounder (*Platichthys flesus*) and cod (*Gadus morhua*) were collected from December 2009 to March 2017 at 50 study stations, located in the Gdansk Basin, more precisely in the Gulf of Gdansk and in the southern part of the Gdansk Deep of the Baltic Sea (Fig. 1).

The list of the fish sampling stations, geographic coordinates of each trawling station, depth of trawling (m) and hydrological parameters such as seawater temperature (°C), salinity (PSU) and O_2 concentration (mg/l) are presented in Table 1. Samples were obtained from research surveys carried out mainly by the Polish RV "Baltica" and partly by the German RV "Walther Herwig III" (the material collected in December 2009 and 2011 from S and B stations) using standard bottom or pelagic small meshed trawls.

Ten study stations (B15/14, B15/15, 4a, 2s, 4w, 1f, 7f, 6F, 7K and 1M; marked by a grey circle in Fig. 1) were located close to the chemical munitions dumping site discovered during CHEMSEA project implementation (CHEMSEA Findings, 2014). Nineteen (S1/10, S1/11, S1/12, S1/13, S1/14, S3/21, S3/22, S3/24, S3/27, 3a, 4s, 5s, 1w, 24n, 1B, 9K, 41P, 50P, 49T) of the analysed stations in the Gdansk Basin were located in zones where dumpsites of conventional munitions (marked with rhombus in Fig. 1) were reported. The reference stations for cod and flounder are 4M and 50X, respectively, whereas for herring two (50X and 40M) reference stations were selected. Reference station 50X was used for statistical analysis with data until February 2014 while reference station 40M – with data from September 2014 to March 2017. In general, control stations were chosen depending on low ΣGtox risk levels.

The analysis of environmental genotoxicity and cytotoxicity was performed 739 fish specimens (herring – 305, flounder – 214 and cod – 220 specimens; Table 2).

2.2. Sample preparation and analysis

Blood samples were taken immediately after catching the fish and only from specimens in good condition. Average total body weight and length were assessed for herring (60.54 ± 15.48 g and 20.49 ± 1.34 cm, respectively), flounder (243.95 ± 59.0 g and 27.16 ± 1.68 cm) and cod (541.87 ± 157.35 g and 38.40 ± 3.84 cm (mean ± SD)). A drop of blood (taken from the caudal vein of fish using a heparinized syringe) was directly smeared on glass slides and air-dried. Smears were fixed in methanol for 10 min. and stained with 5% Giemsa solution in phosphate buffer (pH = 6.8) for 8 min. The stained slides were analysed under the light microscopes Olympus BX51 or Nikon Eclipse 50*i* at final magnification of 1000 ×. Blind scoring of micronuclei and other nuclear abnormalities of the erythrocytes were performed on coded slides.

In total, eight nuclear abnormalities (NAs) were examined in fish blood erythrocytes. Micronuclei (MN), nuclear bud (NB), nuclear bud on filament (NBf) and bi-nucleated erythrocyte with nucleoplasmic bridge (BNb) were used as genotoxicity endpoints. Induction of fragmented (Frag), apoptotic (Apop), bi-nucleated (BN) and 8-shaped nuclei erythrocytes was applied as cytotoxicity endpoints (Fig. 2). In some cases, due to low frequencies of Apop and Frag erythrocytes, their induction was shown as a sum of both responses (FA). Considering the large extent of the study, the results are presented as the sum of the frequencies of four genotoxicity endpoints investigated biomarker responses in the fish blood erythrocytes. The total genotoxicity (ΣGtox) and total cytotoxicity (ΣCytox) levels assessed as the sum of the frequencies of four genotoxicity (MN + NB + NBf + BNb) and four cytotoxicity (FA + BN + 8-shaped) endpoints.

The morphological features of the studied NAs were presented in our previous study (Baršienė et al., 2014). Micronuclei (MN) were identified according to the following criteria: (1) round and ovoid-shaped non-refractory particles in the cytoplasm, (2) colour and structure similar to chromatin, (3) diameter of 1/3e1/20 of the main nucleus, (4) particles completely separated from the main nucleus (Heddle et al., 1991). Nuclear buds (NB) and nuclear buds on filament (NBf) were characterized as extruded nuclear material conjugated to the main nucleus by nucleoplasmic connection. Nucleoplasmic bridges in bi-nucleated cells (BNb) are described as a continuous nucleoplasmic link between two nuclei with the same staining and focusing pattern as the nuclei and the width, which may vary but does not exceed one-fourth of the diameter of the nuclei. Fragmented-apoptotic cells (FA) in early stages were identified by the presence of chromatin condensation within the nucleus and intact cytoplasmic and nuclear boundaries, while late apoptotic cells exhibit nuclear fragmentation into smaller nuclear bodies within an intact cytoplasm/cytoplasmic membrane. Nuclei of bi-nucleated cells (BN) cannot overlap, must be approximately equal in size, must have similar staining pattern and intensity, should have intact nuclear membranes and be located within the same cytoplasm membrane (Fenech et al., 2003). Eight-shaped erythrocytes were evaluated also as cytotoxicity markers (Baršienė et al., 2014). For each studied specimen of fish, 4000 intact erythrocytes were analysed. Final results were expressed as the mean value (in ‰) of sums of the analysed individual lesions scored in 1000 cells per fish collected from every study station.

2.3. Environmental genotoxicity risk assessment in fish

The environmental ΣGtox risk, in each of the 50 studied stations was

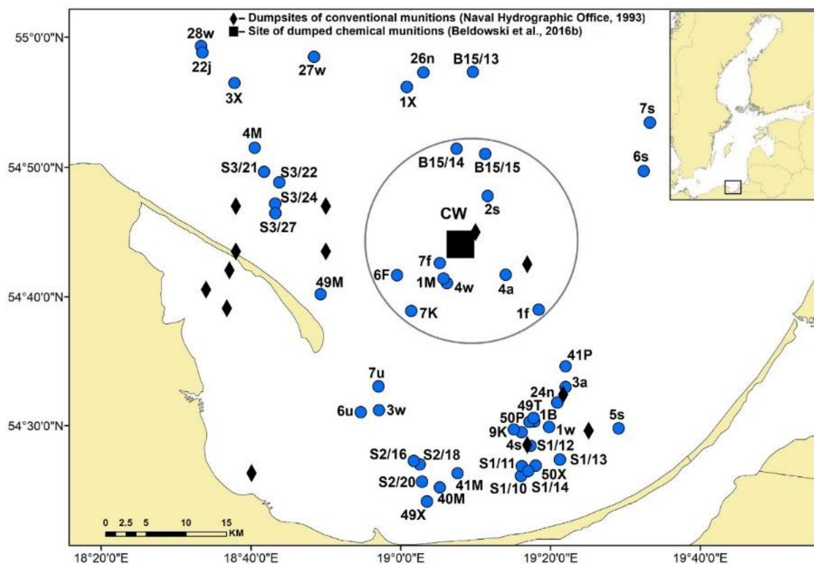


Fig. 1. Location of sampling stations in the Gulf of Gdansk and the southern part of the Gdansk Deep (Baltic Sea).

assessed, based on the established background (BAC) as the sum ΣGtox of investigated biomarker responses such as MN, NB, NBf and BNb in studied fish species. The BAC value for ΣGtox in herring is 0.85‰; in flounder – 0.40‰; in cod – 0.55‰ (MN + NB + NBf + BNb/1000 erythrocytes). The background level of ΣGtox frequencies was calculated in the same way as for MN, following methodology presented in the ICES background document for genotoxicity assessment in marine organisms (Baršienė et al., 2012a). The empirical 90% percentile (P90) values were determined in fish collected from 2001 to 2010 in the reference sites Kvädofjarden, Palanga, Leba, Pärnu, as well as from 1a-1, 2a-1 and 2b-1 in the Gulf of Finland; B03/45, B03/46, B11/03, B11/04 and SFI4/06 in the southern Baltic Sea. These zones are characterized by having unknown local sources of contamination and no impact from urban or industrial activity. In general, an elevated ΣGtox frequency lies above the P90 percentile, whereas the majority of values below the P90 value belong to individuals that are unexposed, weakly-exposed, non-responding or adapted to stressful conditions (Baršienė et al., 2012b).

The study stations were evaluated for the genotoxicity risk was based on the frequency of ΣGtox exceeding its BAC value, using a 5-grade scale. The grades are: low, moderate, increased, high, and exceptionally high, which correspond to ΣGtox > BAC occurring in 0.0–19, 20–39, 40–59, 60–79, and 80–100% of specimens, respectively. Environmental genotoxicity risk zones were mapped utilizing the program ArcGIS Desktop and the ArcMap application. Geographical coordinates system: GCS WGS 1984, Projection: Mercator.

2.4. Statistical analysis

Geno- and cytotoxicity data do not follow a normal distribution (Kolmogorov–Smirnov test and Shapiro–Wilk test). The non-parametric Mann-Whitney *U* test was used to compare NAs frequencies between investigated and reference stations. The mean and standard errors were calculated for any fish from each station studied. The level of significance was established at $p < 0.05$. The statistical analysis was carried out using the GraphPad Software Inc., San Diego, CA, USA (Prism® 5.01).

3. Results

3.1. Environmental genotoxicity and cytotoxicity levels in herring

In blood erythrocytes of herring, the ΣGtox levels varied from 0.54‰ at station S3/22 and 0.58‰ in specimens at station 50X to 3.35‰ at station 41P. The highest ΣGtox response (2.31‰–3.35‰) was found in fish inhabiting stations 1B < 26n < 3a < 4s < 7K < 6F < 4a < 41P (Fig. 3). The ΣCytox levels were lower than ΣGtox and ranged from 0.0‰ in fish at stations S1/12 and S1/13 to 1.70‰ at station 7f (located close to CW dumpsite). High ΣCytox responses (0.70‰–1.45‰) recorded at stations 26n < 1B < 1f < 1w < 6F < 7K (Fig. 3). In general, the highest ΣGtox or ΣCytox levels in herring erythrocytes were reported in stations located close to chemical and conventional munitions dumpsites (7f, 4a, 6F, 7K and 3a, 4s, 1w, 1B, 41P, respectively).

Cytotoxicity responses were identified mainly as 8-shaped nuclei erythrocytes in fishes caught in 2014–2017, and fragmented nuclei of erythrocytes were found in herring specimens with strongly elevated values of nuclear damage (stations 4s, 1w, 3w and 1B; Table 3).

The herring specimens collected in seven study stations displayed significantly increased frequencies of MN, NB, NBf, BNb and Frag. Moreover, strongly increased (up to 51-fold compared to the reference level) genotoxicity values were measured in herring caught at seven stations (1f – 16.13‰; 7f – 14.23‰; 4s – 72.58‰; 1w – 29.98‰; 3w –

Table 1
The list of fish catch-stations with their hydrological parameters measurements over the bottom of the study stations located in the Gdansk Basin (data from the RV "Baltica" surveys and for S and B stations – from the RV "Walther Herwig III" survey).

Study stations	Latitude	Longitude	Depth of trawling (m)	Temperature at the seabed (°C)	Salinity at the seabed (PSU)	O_2 concentration at the seabed (mg/l)
S1/10	54°26.09′N	19°16.04′E	64	6.81	10.76	2.26
S1/11	54°26.84′N	19°16.15′E	53	6.74	9.85	4.81
S1/12	54°28.44′N	19°17.33′E	56	6.59	10.10	3.15
S1/13	54°27.36′N	19°21.25′E	52	6.52	9.32	4.98
S1/14	54°25.89′N	19°16.62′E	58	6.60	10.08	2.7
S2/16	54°26.76′N	19°01.44′E	46	7.43	7.95	9.03
S2/18	54°25.65′N	19°02.83′E	60	6.71	10.04	3.01
S2/20	54°26.99′N	19°02.54′E	67	6.99	10.11	4.13
S3/21	54°49.65′N	18°41.75′E	36	7.90	7.61	10.49
S3/22	54°48.83′N	18°43.76′E	49	7.77	8.34	8.05
S3/24	54°47.20′N	18°43.23′E	61	6.93	9.28	5.28
S3/27	54°46.28′N	18°43.15′E	56	7.30	8.51	7.54
3a	54°33.00′N	19°22.00′E	75	1.93	7.21	9.02
4a	54°41.70′N	19°14.00′E	93	6.39	11.26	1.80
B15/13	54°56.81′N	19°08.98′E	101	5.06	10.89	0.30
B15/14	54°50.86′N	19°06.86′E	101	5.01	10.93	0.27
B15/15	54°50.62′N	19°11.16′E	98	4.99	10.94	0.36
1f	54°39.00′N	19°18.40′E	31	11.50	7.33	6.33
7f	54°42.60′N	19°05.20′E	43	5.05	7.42	7.36
22j	54°58.50′N	18°33.30′E	70	4.71	10.37	2.07
2s	54°47.80′N	19°11.60′E	80	4.19	9.65	3.11
4s	54°29.50′N	19°16.10′E	60	3.36	8.18	5.36
5s	54°29.80′N	19°29.10′E	50	2.45	7.44	7.07
6s	54°49.72′N	18°92.45′E	50	3.70	7.35	7.53
7s	54°53.45′N	18°93.25′E	30	13.69	7.05	6.94
1w	54°29.9′N	19°19.80′E	70	4.12	9.94	2.80
3w	54°31.2′N	18°57.10′E	64	4.11	9.39	4.15
4w	54°41.4′N	19°06.10′E	92	4.64	10.62	1.48
27w	54°58.5′N	18°48.40′E	95	4.45	10.7	2.50
28w	54°58.8′N	18°32.80′E	72	4.17	9.90	2.59
1X	54°56.20′N	19°00.80′E	102	7.04	12.22	2.93
3X	54°56.50′N	18°37.80′E	76	5.06	10.03	2.73
49X	54°24.10′N	19°03.30′E	32	2.67	7.89	10.06
50X	54°26.90′N	19°18.00′E	63	5.01	10.27	1.49
6u	54°31.06′N	18°54.66′E	35	4.60	8.40	4.59
7u	54°33.05′N	18°57.05′E	40	4.80	8.40	4.70
24n	54°31.80′N	19°20.90′E	71	No data	No data	No data
26n	54°57.30′N	19°03.00′E	102	No data	No data	No data
1B	54°30.20′N	19°17.50′E	72	No data	No data	No data
6F	54°41.67′N	18°59.51′E	91–88	6.2	11.4	2.19
7K	54°38.90′N	19°01.40′E	82	6.69	12.25	1.42
9K	54°29.70′N	19°15.10′E	71	8.53	7.59	7.27
1M	54°41.40′N	19°05.70′E	92	6.98	12.48	1.15
4M	54°51.50′N	18°40.50′E	59	5.34	9.01	7.71
40M	54°25.20′N	19°05.20′E	41	3.72	7.88	8.43
41M	54°26.30′N	19°07.60′E	55	5.51	9.74	4.31
49M	54°40.20′N	18°49.30′E	67	3.59	7.99	8.99
41P	54°34.60′N	19°22.00′E	80	8.48	12.63	3.07
50P	54°30.30′N	19°17.20′E	72	8.78	7.36	7.55
49T	54°30.60′N	19°17.70′E	72	5.35	8.92	5.19

17.13‰; 1B – 51.35‰; 6F – 8.90‰) and ΣCytox level – in herring from four stations (4s – 20.30‰; 1w – 3.80‰; 3w – 3.18‰; 1B – 5.28‰).

Since in these stations, one or two specimens with extremely high responses were found, all further results are presented without these frequencies of MN, NB, NBf, BNb, Frag or Apop in herring specimens (Table 3).

A separate analysis of nuclear lesion responses showed that the frequency of micronuclei (MN/1000 erythrocytes) varied from 0.13‰ in herring at station 40X to 1.90‰ at station 7K. The values of NB changed from 0.17‰ in herring at station S3/24 to 1.80‰ at station 4a. The frequency of NBf ranged from 0.04‰ (station S3/22) to 0.83‰ and 0.90‰ (stations 2s and 4s, respectively) (Fig. 4). BNb were not detected at 20 out of 31 investigated stations. Very low frequencies of BNb (0.03‰ and 0.13‰) were detected in herring at 11 stations.

3.2. *Environmental genotoxicity and cytotoxicity levels in flounder*

Analysis of geno-cytotoxicity in flounder did not reveal strongly elevated responses compared to the reference station. The highest ΣGtox levels were recorded in flounder caught from December 2009 to November 2013 (w stations), except for those collected from station 9K. At 25 investigated stations, ΣCytox responses were lower than ΣGtox, except six stations where these responses were higher than others (Fig. 5). In general, ΣGtox is higher than ΣCytox in flounder collected in December 2009–November 2013 and in most recent samplings (November 2016–March 2017), in contrast to a higher ΣCytox, that was detected from 2014 to 2016 (Fig. 5). It should be pointed that two (4a and 1M) out of 25 analysed stations were located close to the chemical munitions dumping site (see Fig. 1). The highest ΣCytox level (1.38‰) was recorded at station 1M, while the highest ΣGtox level (1.94‰) was recorded at station 4a (Fig. 5).

The analysis of separate genotoxicity biomarker responses (MN, NB and NBf) revealed a low induction of MN in flounder caught at the reference station 50X and at stations 41M < 1X = 1M < 4M (0.05‰–0.13‰). The highest values of MN were recorded in flounder caught at stations 4a (0.80‰), 1w (0.75‰) and 41P (0.73‰). In these

Table 2

The material for the analysis of genotoxicity and cytotoxicity in peripheral blood erythrocytes of herring, flounder and cod collected in the Gdansk Basin of the Baltic Sea (data from the Polish RV "Baltica" surveys in February 2011–March 2017 and the German RV "Walther Herwig III" survey in December 2009 and in December 2011).

Sampling date	Herring sampling stations (number of specimens)	Flounder sampling stations (number of specimens)	Cod sampling stations (number of specimens)
December 2009	S1/10 (10), S1/12 (5), S1/13 (11), S2/18 (8), S2/20 (21), S3/21 (5), S3/22 (6), S3/24 (16)	S1/10 (10), S1/11 (4), S1/14 (6), S2/16 (5), S2/18 (9), S3/27 (5)	–
February 2011	3a (10), 4a (10)	3a (10), 4a (10)	–
December 2011	–	–	B15/13 (20), B15/14 (11), B15/15 (10)
September 2012	1f (10), 7f (10)	–	–
November 2012	–	22j (10)	–
September 2013	2s (10), 4s (9), 5s (4)	6s (5), 7s (5)	2s (10)
November 2013	1w (10), 3w (10), 27w (10)	1w (10), 3w (10), 28w (5)	1w (10), 3w (10), 4w (10)
February 2014	3X (10), 50X (10)	1X (10), 50X (10)	1X (10), 49X (10)
September 2014	7u (10)	–	6u (5), 7u (5)
November 2014	24n (10), 26n (10)	24n (10)	24n (10), 26n (10)
February 2015	1B (10)	1B (10)	1B (10)
September 2015	6F (10)	–	6F (10)
November 2015	7K (10)	9K (10)	7K (10)
February 2016	1M (10), 40M (10)	1M (10), 4M (10), 41M (10)	1M (4), 4M (10), 40M (10), 49M (5)
October 2016	41P (10), 50P (10)	41P (10), 50P (10)	41P (10), 50P (10)
March 2017	49T (10)	49T (10)	49T (10)
Total	31 stations, 305 specimens	25 stations, 214 specimens	23 stations, 220 specimens

study stations the level of MN was up to 16-fold higher than the reference level (0.05‰) (Fig. 5).

The frequency of nuclear buds (NB) varied from 0.08‰ in flounder caught at station 1X to 1.43‰ at station 41P. A higher induction of NB was noted in flounder collected from 2009 to 2012, compared to later samplings. In eleven stations (S2/16, S2/18, S3/27, 3a, 4a, 7s, 1w, 28w, 1B, 41M, 41P) NBf were not recorded, while in fourteen stations (S1/10, S1/11, S1/14, 22j, 6s, 3w, 1X, 50X, 24n, 9K, 1M, 4M, 50P, 49T), NBf values ranged between 0.03‰ and 0.06‰, and the highest response was 0.23‰. The induction of BNb was not detected in 15 stations, while in other ten stations the frequencies of BNb were very low and ranged between 0.03‰ and 0.08‰ (Fig. 6).

The induction of 8-shaped erythrocytes was the predominant cytotoxicity response. The highest frequency of such type of damaged erythrocytes (1.20‰) was found in station 1M, while the lowest one was found at stations 3a (0.21‰) and 4a (0.22‰).

3.3. Environmental genotoxicity and cytotoxicity in cod

The ΣGtox levels in blood erythrocytes of cod specimens varied from 0.10‰ (station 4M) to 2.63‰ and 3.22‰ in stations 6F and 7K which are located in close proximity to the dumping site of chemical munitions. The ΣGtox responses over 1.0‰ were recorded in cod caught at stations B15/13, 49X, 26n, 7u and 6u. Instead low levels of ΣGtox responses (0.10‰, 0.20‰, 0.31‰, 0.32‰, 0.46‰ and 0.50‰) were recorded in fish from stations 4M < 40M < 1M < B15/14 < 49M < 1K (Fig. 7).

The ΣCytox (FA + BN + 8-shaped) levels ranged from 0.19‰ at station 4M to 2.00‰ – at station 7K. Very low responses (0.23‰) were detected in stations 1X and 49X. The results show high frequencies (over 1.0‰) of cytotoxicity parameters at stations 7K > 6u > 7u (Fig. 6). A strongly increased cytotoxicity level (5.00‰) in outlier specimens measured at station 6F. In summary, the highest geno-cytotoxicity responses in cod erythrocytes were reported in four (6u, 7u,

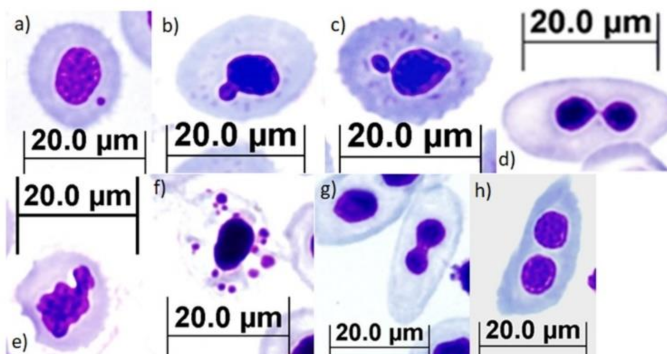


Fig. 2. Nuclear abnormalities in peripheral blood: a) erythrocyte with micronucleus (MN) in herring, b) erythrocyte with nuclear bud (NB) in cod, c) nuclear bud on filament (NBf) in cod, d) bi-nucleated erythrocyte with nucleoplasmic bridge (BNb) in herring, e) fragmented (Frag) erythrocyte in herring, f) apoptotic (Apop) erythrocyte in herring, g) 8-shaped nuclei erythrocyte in founder, h) bi-nucleated (BN) erythrocyte in flounder.

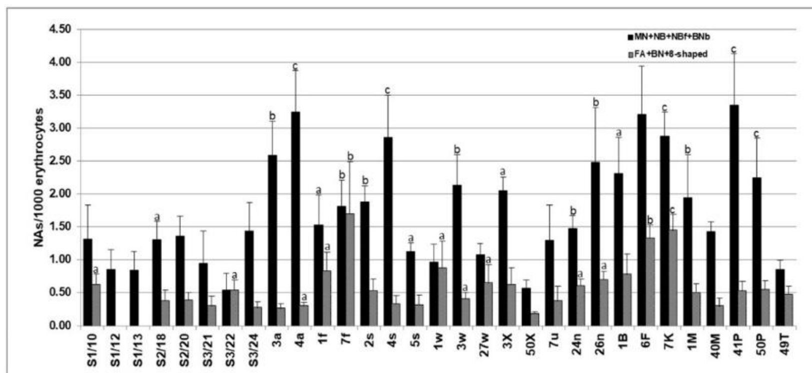


Fig. 3. The levels of ΣGtox (MN + NB + NBf + BNb) and ΣCytox (FA + BN + 8-shaped) in blood erythrocytes of herring collected in the Gdansk Basin of the Baltic Sea in 2009–2017. Letters indicate statistically significant differences between study stations and the reference stations (50X and 40M) (Mann-Whitney *U* test); $^{a}p < 0.01$, $^{b}p < 0.001$, $^{c}p < 0.0001$.

6F and 7K) stations. It should be pointed that stations 6F and 7K are located close to the dumpsite of chemical munitions (Fig. 1).

The measurement of environmental genotoxicity in cod, well-accepted distinct biomarker response, revealed exceptionally high levels in specimens collected in September and November 2015 at stations 7K and 6F. The frequencies of MN reached 1.15‰ in the fish from 6F and 1.00‰ – in 7K, NB – 1.28‰ and 1.50‰, while in nearby located station 49M the frequency of MN was equal to 0.04‰. Low genotoxicity responses were recorded in cod sampled at stations M (Fig. 8).

The predominant cytotoxicity response was the induction of 8-shaped nuclei erythrocytes; however, the major response of fragmented-apoptotic (FA) cells was recorded in cod from stations 7K, 6F, 2s and B15/15, which are located close to the dumping site of chemical munitions (Fig. 8). The highest frequency of fragmented and 8-shaped nuclei cells was recorded at station 7K (Fig. 8).

Cod specimens with strongly increased frequencies of fragmented (3.25‰ ± 2.86) and apoptotic (1.58‰ ± 1.27) erythrocytes were caught at station 6F. All further results will be presented without the specimens with strongly increased frequencies of Frag and Apop at station 6F. In other studied specimens of cod at station 6F, the frequencies of fragmented erythrocytes were 0.39‰ ± 0.11, and apoptotic frequencies – 0.31‰ ± 0.08.

3.4. Comparison between studied fish species

Analysis of geno-cytotoxicity responses in different fish species caught at same study stations showed higher ΣGtox levels in herring than in cod or flounder, collected from 13 out of 20 studied stations. In contrast, environmental ΣCytox is higher in flounder and cod than in herring (Table 4).

ΣGtox was higher than ΣCytox at most of studied stations. An opposite situation was recorded only at nine stations (2s, 3w, 1X, 24n, 1B, 6F, 1M, 4M, 40M and 41P), predominantly for flounder and cod (Fig. 9).

A higher (1.9-fold to 6.3-fold) ΣGtox response was determined for herring in 45.46% of the analysed stations (24n < 3w < 2s < 1B < 1M) compared to cod, except in one station (6F) where ΣGtox response was 4.1-fold higher in cod than in

Table 3
Strongly increased frequencies of MN, NB, NBf, BNb and Frag in herring blood erythrocytes. Data (mean ‰ ± SE) with these specimens are presented on the top of the Table, on the bottom – data without these specimens.

Parameter	Stations						
	1f	7f	4s	1w	3w	1B	6F
The values of nuclear abnormalities with strongly increased frequencies							
MN	10.38 ± 7.96	9.90 ± 8.01	50.90 ± 49.90	6.55 ± 4.08	4.85 ± 3.70	8.70 ± 7.71	7.45 ± 2.41
NB	3.98 ± 2.45	3.10 ± 1.93	20.73 ± 19.92	15.85 ± 12.10	11.55 ± 10.94	42.55 ± 41.72	
NBf	1.75 ± 1.00	1.15 ± 0.47	1.00 ± 0.30	6.58 ± 4.60	0.73 ± 0.37		
BNb				1.00 ± 1.00			
Frag			20.10 ± 19.99	2.30 ± 1.99	2.68 ± 2.59	4.60 ± 4.60	
The values of nuclear abnormalities without strongly increased frequencies							
MN	0.42 ± 0.16	1.00 ± 0.31	1.00 ± 0.16	0.44 ± 0.27	1.17 ± 0.43	1.00 ± 0.38	1.65 ± 0.62
NB	0.53 ± 0.24	1.22 ± 0.51	0.81 ± 0.32	0.31 ± 0.08	0.61 ± 0.18	0.83 ± 0.22	1.03 ± 0.21
NBf	0.56 ± 0.15	0.72 ± 0.21	0.90 ± 0.32	0.22 ± 0.03	0.36 ± 0.08	0.43 ± 0.15	0.40 ± 0.11
BNb	0.03 ± 0.03	0.08 ± 0.05	0.05 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.10	0.03 ± 0.03
Frag	0.28 ± 0.25	1.13 ± 0.77	0.11 ± 0.06	0.33 ± 0.33	0.08 ± 0.04	0.00 ± 0.00	0.33 ± 0.05
Apop	0.03 ± 0.03	0.05 ± 0.05	0.08 ± 0.05	0.00 ± 0.00	0.08 ± 0.05	0.10 ± 0.10	0.33 ± 0.11
8-shaped	0.48 ± 0.11	0.48 ± 0.20	0.13 ± 0.07	0.56 ± 0.14	0.22 ± 0.07	0.68 ± 0.28	0.63 ± 0.20
BN	0.05 ± 0.03	0.08 ± 0.05	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.03	0.00 ± 0.00	0.00 ± 0.00

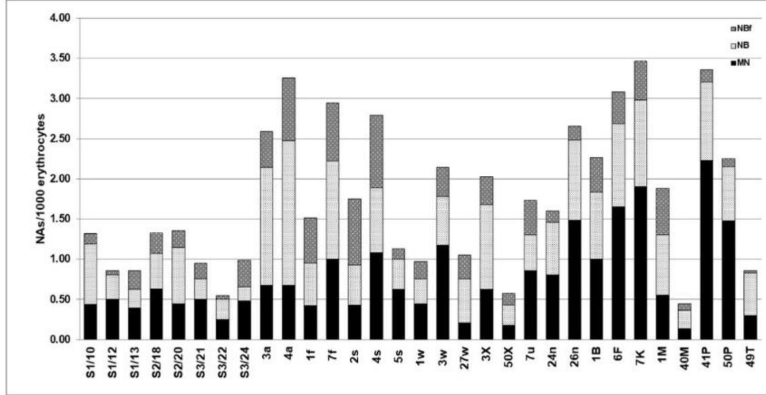


Fig. 4. The frequency of MN, NB and NBf in blood erythrocytes of herring collected from the Gdansk Basin of the Baltic Sea.

herring. The same tendency was found for herring and flounder. The ΣGtox response in herring was 2.3-fold to 3.9-fold higher than in flounder in 44.44% of the analysed stations (3w < 1M < 24n < 1B). In flounder, significantly higher ΣCytox response was found in two stations: 1M (2.4-fold higher than in cod; 2.8-fold higher than in herring) and 1X (3.11-fold higher than in cod). The most sensitive bioindicator is herring, followed by flounder and cod (Fig. 9).

3.5. Environmental genotoxicity risk assessment in fish

The assessment of ΣGtox risk levels in herring, flounder and cod indicated an exceptionally high risk for studied fish species caught at 26 out of 50 studied stations (52.00%). Flounder sampled at 18 (72.0%), herring caught at 12 (38.7%) and cod caught at four studied stations (17.4%) were classified as living in areas with an exceptionally high genotoxicity risk.

A background level of ΣGtox was found at three for herring, at one for flounder and at 7 stations for cod (Fig. 10). Assessing the numbers of fish inhabiting the stations characterized as low ΣGtox risk, it should be stressed that only two stations (S3/22 and 50X) for herring and two stations (B15/14 and 40M) for cod were attributed as completely safe zones.

In close proximity to the chemical munitions dumping site, high or exceptionally high ΣGtox risk for herring and flounder was detected at all analysed stations (at seven and two stations, respectively); while for cod exceptionally high ΣGtox risk level was determined at two out of seven analysed stations. In zones of conventional munitions dumpsites, high and exceptionally high ΣGtox risk for herring at seven stations and

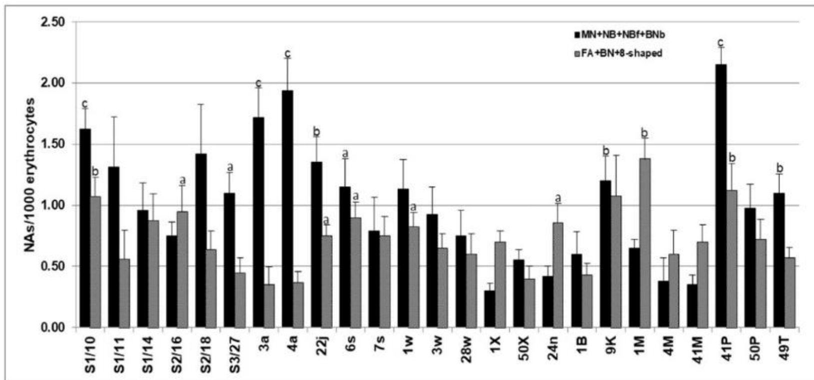


Fig. 5. The levels of ΣGtox (MN + NB + NBf + BNb) and ΣCytox (FA + BN + 8-shaped) in blood erythrocytes of flounder collected in 2009–2017 from the Gdansk Basin of the Baltic Sea. Letters indicate statistically significant differences between study stations and the reference station 50X; [a]$p < 0.01$, [b]$p < 0.001$, [c]$p < 0.0001$.

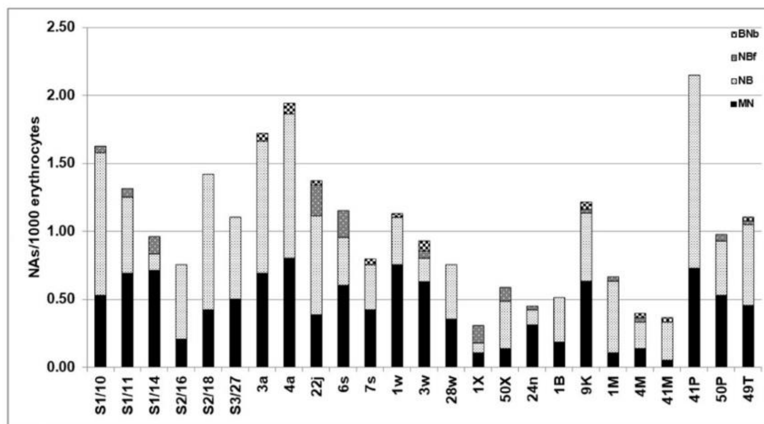


Fig. 6. The frequency of MN, NB, NBf and BNb in blood erythrocytes of flounder caught in 2009–2017 at the Gdansk Basin of the Baltic Sea.

for flounder at ten stations of all analysed 19 stations was indicated (Fig. 10).

4. Discussion

This study revealed strongly increased frequencies of separate genotoxicity endpoints (compared with the reference station) in specimens of herring caught at stations 1f, 7f, 4s, 1w, 3w, 1B and 6F as well as cytotoxicity responses – at stations 4s, 1w, 3w, 1B. It is noteworthy that the most of these stations are located in close proximity to chemical munitions and conventional munitions dumpsites. Despite the least geno-cytotoxicity responses in cod specimens compared with herring and flounder responses, strongly increased values of fragmented (Frag) and apoptotic (Apop) nuclei erythrocytes were measured in station 6F. All further results in this study were presented without the strongly increased frequencies of those genotoxicity and cytotoxicity parameters in herring and cod specimens. It should be noted that without this data separate geno-cytotoxicity responses in cod caught at 6F station were still high and even MN and NB values reached over 1‰. Moreover, at station 7K six out of eight endpoints showed the highest values, compared with all other stations of cod.

The specific nature of currents and pollutants in sediments may contribute to increased geno-cytotoxicity levels in studied areas. The currents in the Gdansk Deep are mostly directed eastward and slightly southward and they may influence high genotoxicity response in herring and cod at stations 4a and 1f. The separate monthly flow in July and October was westward (Bulczak et al., 2016) possibly contributing to increased nuclear damage in herring and cod at station 6F (September 2015), 7K (November 2015) and at station 7f (September 2012). Moreover, extremely high responses in herring were found during these

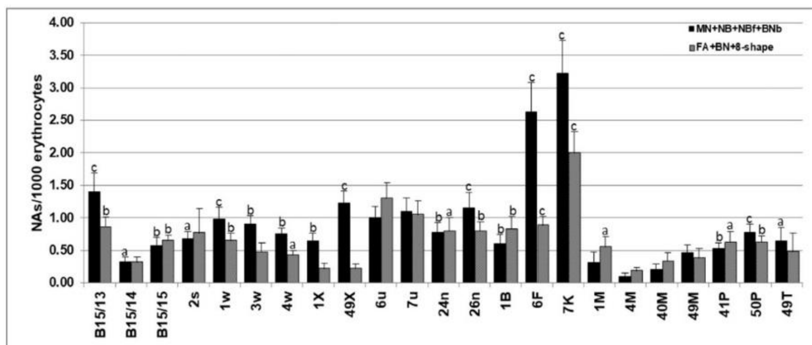


Fig. 7. The levels of ΣGtox (MN + NB + NBf + BNb) and ΣCytox (FA + BN + 8-shaped) in cod caught in 2009–2017 from the Gdansk Basin of the Baltic Sea. Letters indicate statistically significant differences between study stations and the reference station 4M (Mann-Whitney U test); $^{a}p < 0.01$, $^{b}p < 0.001$, $^{c}p < 0.0001$).

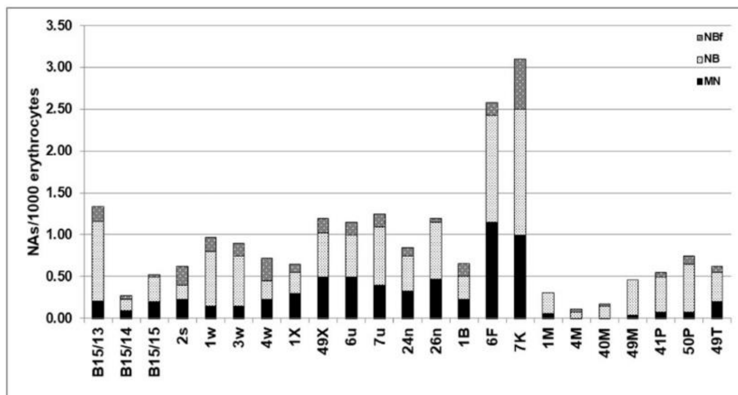


Fig. 8. The frequency of MN, NB and NBf in blood erythrocytes of cod caught in 2009–2017 at 23 stations located in the Gdansk Basin of the Baltic Sea.

samplings.

Published scientific literature data indicated Gdansk Basin pollution by increased concentrations of As as well as Pb, Hg and Cd (Polak-Juszczak, 2012; Staniszewska et al., 2011, 2014; Zaborska, 2014; Bełdowski et al., 2014; Jędruch et al., 2015). The measurements of triphenylarsine, phenyldichlorarsine-related compounds in the Gdansk Deep evidently showed the leakage in sediments due to CWs damage and corrosion (CHEMSEA Findings, 2014). The CWAs mixtures that exist in the environment may have harmful impacts on organisms, since their toxicity is equal to their precursors (Missiaen et al., 2010).

In the Gdansk Basin a very high ΣGtox (2.48‰) and ΣCytox (0.70‰) responses for herring were found in station 26n, which is located outside chemical and conventional munitions dumpsites. Bełdowski et al. (2016a) measured mustard (yperite) related CWA compounds in sediments of the southern Baltic Sea. One site was located at the same area as our station 26n. Bełdowski et al. (2016b) have detected high CWA concentrations in sediments in the Bornholm CWA dumpsite and described hotspot sites. Moreover, caged mussels in those areas revealed markedly lower lysosomal membrane stability, significantly elevated activities of antioxidant defence enzymes and significantly increased cytotoxicity levels in comparison to the specimens from the reference site.

In the study area, a wide variety and distribution of other genotoxins, including metals, polycyclic aromatic hydrocarbons and organic pollutants exist in the study area (Polak-Juszczak, 2012; Kobusińska et al., 2014; Koniecko et al., 2014; Staniszewska et al., 2011, 2014; Zaborska, 2014; Bełdowski et al., 2014; Jędruch et al., 2015). In this study, high ΣCytox responses for cod were detected in stations 6u (1.30‰) and 7u (1.05‰), which are located close to the station 3w (where strongly increased genotoxicity and cytotoxicity responses were detected in herring). In the zone near these stations, Kobusińska et al. (2014) demonstrated increased concentrations of pentachlorphenol (PCP) in sediments, also, toxicity units by Microtox test was at toxic level.

Scientific literature data pointed out that Gdansk Basin is mostly polluted by benz[*a*]anthracene, cesium-137, mercury, PCB and the region was assigned a moderate or poor health status (HELCOM, 2010). Dąbrowska et al. (2017) have reported that concentrations of CB-118, heptachlor, PBDE, Hg and p,p′-DDT are greater than EACs (Environmental Assessment Criteria), therefore, Good Environmental Status (GES) cannot be assigned to the Gdansk Basin. The relationship between organic and metal contaminants in sediments and the biological effects of these contaminant on flounder and mussels was found. A principal component assay was used explaining 34.8% of the total variance and showed positive linkage between DNA strand breakage, EROD activity and contaminants in flounder caught in the study area (Dąbrowska et al., 2017). Moreover, in autumn 2002, environmental genotoxicity studies in different sites located in the Swedish, Lithuanian, Polish and German EEZ showed the highest MN levels in flounder collected at the southern Gdansk Deep zone. The frequency of MN in fishes collected closely to our station S3/27 was 5–6 times higher than in ones living in the reference Kvadofjarden site (HELCOM, 2010). The comparison of ΣGtox responses between three studied fish species caught at the same study stations in the Gdansk Basin indicated herring as the most sensitive bioindicator (in 65% of the studied stations) followed by flounder and cod. The findings of the present study showed the higher ΣGtox and ΣCytox in herring compared to other two species at most stations, while an opposite situation was found in other stations. As a confounding factor, it should be emphasized, that strong positive correlation (Spearman correlation and regression analysis)

Table 4
Results of comparison between ΣGtox and ΣCytox responses in fish species collected at the same study station (analysis in 20 study stations; Her – herring, Fl – flounder).

Comparison between species	Her > Cod	Her > Fl	Fl > Her	Fl > Cod	Cod > Her	Cod > Fl
ΣGtox	2s, 3w, 7u, 24n, 26n, 1B, 1M, 40M, 41P, 50P, 49T	3a, 4a, 3w, 24n, 1B, 1M, 41P, 50P	S2/18, 1w, 50X, 49T	1w, 4M, 41P, 50P, 49T	1u, 6F, 7K	1X
ΣCytox	1w, 3w, 1B, 6F	4a, 1w, 3w, 1B	S2/18, 3a, 50X, 24n, 1M, 41P, 50P, 49T	1X, 24n, 1M, 4M, 41P, 50P, 49T	2s, 1u, 7u, 26n, 7K, 40M, 41P, 50P	1u

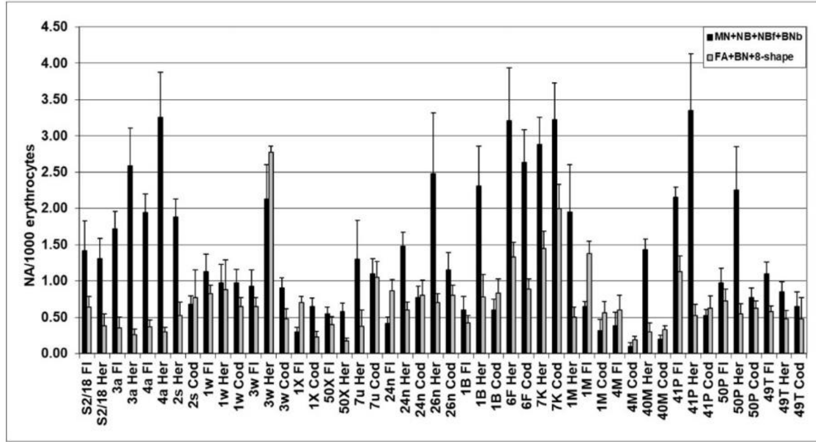


Fig. 9. The levels of ΣGtox (MN + NB + NBf + BNb) and ΣCytox (FA + BN + 8-shaped) in herring, flounder and cod caught at the same study stations located in the Gdansk Basin of the Baltic Sea in 2009–2017.

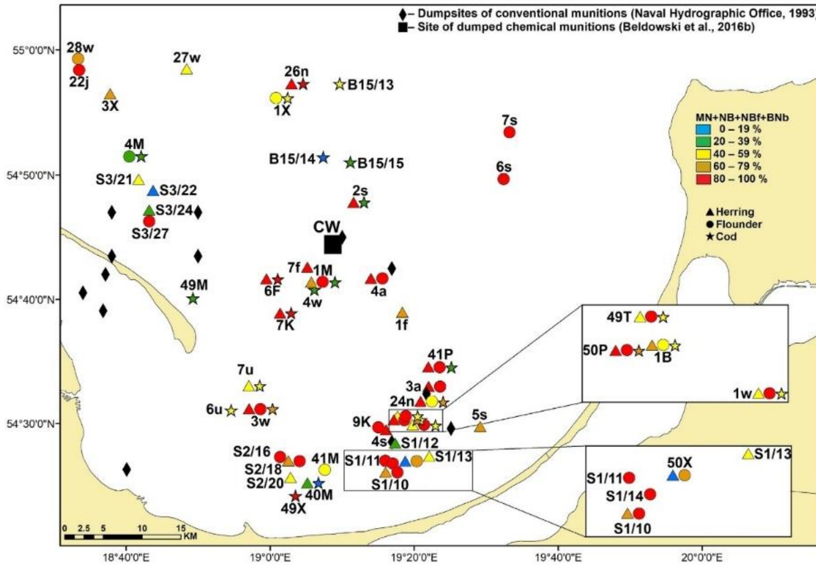


Fig. 10. Results of environmental ΣGtox (MN + NB + NBf + BNb) in herring, flounder and cod collected from 50 study stations in the Gulf of Gdansk and in the southern part of the Gdansk Deep (the Baltic Sea) in 2009–2017.

between geno-cytotoxicity and environmental or biological variables was detected only in three analysed fish species collected from several study stations in the Bornholm CW dumping area (Baršienė et al., 2014).

The highest ΣGtox and ΣCytox levels were recorded at 10 sampling stations close to CW and at 19 stations close to conventional munitions dumpsites: for herring at nine stations (3a, 4a, 1f, 4s, 1w, 1B, 6F, 7K and 41P), for flounder – at six stations (3a, 4a, 9K, 1M and 41P) and for cod – at two stations (6F and 7K). Della Torre et al. (2010, 2013) investigated genotoxicity effects in European conger (*Conger conger*) gills, which is one of the target tissues of CWAs. The study reported deep dermal, external and internal organ damage in liver and spleen and significantly high EROD activity in blackbelly rosefish (*Helicolenus dactylopterus*) and European conger, caught near CW dumping site in the Southern Adriatic Sea. Furthermore, 3–4 times higher levels of As and Hg were determined in fish fillets suggesting the leakage of yperite and other chemical materials from rusted bombshells (Della Torre et al., 2010). Analysis of sediments from the Gdansk Basin showed the highest amounts of arsenic (As) in half of the thirty samples in the Gdansk Deep; the mean arsenic concentration (15–25 μg/g) is clearly higher than the reference area (0.3–5 μg/g) (Bełdowski et al., 2016a). Therefore, earlier literature data may clarify the exceptionally high genotoxicity risk for herring, flounder and cod caught at stations located close to proximity to the existing chemical and conventional munitions dumping sites or in zones contaminated by CWAs in the Gdansk Basin of the Baltic Sea.

The assessment of ΣGtox risk levels in herring, flounder and cod in the Gdansk Basin indicated an exceptionally high risk for fish species caught at 52.0% of the studied stations. Herring, flounder and cod showed an exceptionally high ΣGtox risk in 38.7%, 72% and 17.4% stations, respectively. Likewise, in our earlier studies in the Bornholm Basin an extremely high genotoxicity risk was determined at 29 out of 31 studied stations for herring, at 21 out of 24 for flounder and at 5 out of 10 for cod. The most significantly increased geno-cytotoxicity levels were recorded in fish caught close to proximity to the Bornholm CW dumping area and in zones with a high content of arsenic and other CWA containing substances in sediments (Baršienė et al., 2014). The analysis of herring caught along the CW transportation routes in the southern Baltic Sea showed an extremely high genotoxicity risk in 27.7% out of 65 analysed stations (Baršienė et al., 2016). Nevertheless, a significant shortage of scientific information exists on the integrated assessment of pollutants and their biological impacts in the southern Baltic Sea. Although we found that the greatest geno and cytotoxic damage was found in the areas in close proximity to the CW or conventional munitions dumping sites. It should be emphasized, that in the studied areas other types of pollution: metals, polycyclic aromatic hydrocarbons and other organic pollutants; may also contribute to the effects seen. Further studies in the Gdansk marine area should include more careful analysis on the integrated assessment of marine species bioaccumulation of hazardous substances and their geno-cytotoxicity effects.

5. Conclusions

Long-term (2009–2017) environmental genotoxicity and cytotoxicity studies showed increased responses in fish species inhabiting zones located close to chemical and conventional munitions dumpsites. The interspecies comparison of ΣGtox responses in fish caught at the same study station indicated herring as the most sensitive bioindicator species, followed by flounder and cod. The higher ΣGtox level in herring than in cod and flounder were determined in 65% of the studied stations.

For the first time, the genotoxicity risk based on ICES/OSPAR Background Assessment Criteria (BAC) was evaluated for herring, flounder and cod caught in the Gdansk Basin. The exceptionally high ΣGtox risk for analysed fish species was characterized at 26 out of all studied stations. All analysed stations close to chemical munitions dumping site indicated high and exceptionally high ΣGtox risk for herring and flounder, whereas for cod - exceptionally high ΣGtox risk was detected at 28.6% of analysed stations. In conventional munitions dumpsites, high and exceptionally high ΣGtox risk was identified at seven stations for herring and at ten stations for flounder of all analysed 19 stations. Consequently, Good Environmental Status (GES) level cannot be achieved in zones where high and exceptionally high ΣGtox risk was indicated in the Gdansk Basin.

Conflict of interests

None.

Acknowledgements

The Research Council of Lithuania through the GENOTOX-CG project MIP-33/2012 funded this study. The authors would like to thank Thomas Lang (Thünen Institute of Fisheries Ecology, Cuxhaven, Germany) and Aleksandras Rybakovas (Nature Research Centre, Vilnius) for collecting samples from stations S (December 2009) and B15 (December 2011) during RV Walther Herwig III cruises. We are thankful to Dalia Barsyte Lovejoy and Sabine Lovejoy (University of Toronto) for the language editing.

References

Baršienė, J., Lyons, B., Rybakovas, A., Martinez-Gomez, C., Andreikėnaitė, L., Brooks, S., Maes, T., 2012a. Background document: micronucleus assay as a tool for assessing cytogenetic/DNA damage in marine organisms. ICES Cooperative Research Report No 315. In: Integrated Marine Environmental Monitoring of Chemicals and Their Effects, pp. 71–83.

Baršienė, J., Rybakovas, A., Lang, T., Grygiel, W., Andreikėnaitė, L., Michailovas, A., 2012b. Risk of environmental genotoxicity in the Baltic Sea over the period of 2009–2011 assessed by micronuclei frequencies in blood erythrocytes of flounder (*Platichthys flesus*), herring (*Clupea harengus*) and eelpout (*Zoarces viviparus*). Mar. Environ. Res. 77, 35–42.

Baršienė, J., Butrimavičienė, L., Grygiel, W., Lang, T., Michailovas, A., Jackūnas, T., 2014. Environmental genotoxicity and cytotoxicity in flounder (*Platichthys flesus*), herring (*Clupea harengus*) and Atlantic cod (*Gadus morhua*) from chemical munitions dumping zones in the southern Baltic Sea. Mar. Environ. Res. 96, 56–67.

Baršienė, J., Butrimavičienė, L., Grygiel, W., Stunžėnas, V., Valskienė, R., Greiciūnaitė, J., Stankevičiūtė, M., 2016. Environmental genotoxicity assessment along the transport routes of chemical munitions leading to the dumping areas in the Baltic Sea. Mar. Pollut. Bull. 103 (1–2), 45–53.

Bełdowski, J., Miotk, M., Bełdowska, M., Pempkowiak, J., 2014. Total, methyl and organic mercury in sediments of the Southern Baltic Sea. Mar. Pollut. Bull. 87, 388–395.

Bełdowski, J., Szubska, M., Emelyanov, E., Garnaga, G., Drzewińska, A., Bełdowska, M., Vanninen, P., Östin, A., Fabisiak, J., 2016a. Arsenic concentrations in Baltic Sea sediments close to chemical munition dumpsites. Deep-Sea Res. II Top. Stud. Oceanogr. 128, 114–122.

Bełdowski, J., Klusek, Z., Szubska, M., Turja, R., Bulczak, A.I., Rak, D., Brenner, M., Lang, T., Kotwicki, L., Grzelak, K., Jakacki, J., Fricke, N., Östin, A., Olsson, U., Fabisiak, J., Garnaga, G., Nyholm, J.R., Majewski, P., Broeg, K., Söderström, M., Vanninen, P., Popiel, S., Nawała, J., Lehtonen, K., Berglind, R., Schmidt, B., 2016b. Chemical munitions search & assessment – an evaluation of the dumped munitions problem in the Baltic Sea. Deep-Sea Res. II Top. Stud. Oceanogr. 128, 85–95.

Bulczak, A.I., Rak, D., Schmidt, B., Bełdowski, J., 2016. Observations of near-bottom currents in Bornholm Basin, Slupsk Furrow and Gdansk Deep. Deep-Sea Res. II Top. Stud. Oceanogr. 128, 96–113.

CHEMSEA Findings, 2014. In: Bełdowski, J., Fabisiak, J., Popiel, S., Őstin, A., Olsson, U., Vanninen, P., Latsumaki, A., Lang, T., Fricke, N., Brenner, M., Berglind, R., Baršienė, J. (Eds.), Gdansk, Institute of Oceanology. Polish Academy of Sciences (86 pp.).

Dąbrowska, H., Kopko, O., Lehtonen, K.K., Lang, T., Waszak, I., Balode, M., Strode, E., 2017. An integrated assessment of pollution and biological effects in flounder, mussels and sediment in the southern Baltic Sea coastal area. Environ. Sci. Pollut. Res. 24 (4), 3626–3639.

Della Torre, C., Petochi, T., Corsi, I., Dinardo, M.M., Baroni, D., Alcaro, L., Focardi, S., Tursi, A., Marino, G., Frigeri, A., Amato, E., 2010. DNA damage, severe organ lesions and high muscle levels of As and Hg in two benthic fish species from a chemical warfare agent dumping site in the Mediterranean Sea. Sci. Total Environ. 408, 2136–2145.

Della Torre, C., Petochi, T., Farchi, C., Corsi, I., Dinardo, Sammarini, V., Alcaro, L., Mechelli, L., Focardi, S., Tursi, A., Marino, G., Amato, E., 2013. Environmental hazard of yperite released at sea: sublethal toxic effects on fish. J. Hazard. Mater. 248–249, 246–253.

Fenech, M., Chang, W.P., Kirsch-Volders, M., Holland, N., Bonassi, S., Zeiger, E., 2003. HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutat. Res. 534, 65–75.

Heddle, J.A., Cimino, M.C., Hayashi, M., Romagna, F., Shelby, M.D., Tucker, J.D., Vanparys, Ph., MacGregor, J.T., 1991. Micronuclei as an index of cytogenetic damage: past, present, and future. Environ. Mol. Mutagen. 18, 277–291.

HELCOM, 2010. Hazardous substances in the Baltic Sea. An integrated thematic assessment of hazardous substances in the Baltic Sea. In: Baltic Sea Environmental Process Series, pp. 120B.

HELCOM, 2013. Chemical Munitions Dumped in the Baltic Sea. Report of the *ad hoc* Expert Group to Update and Review the Existing Information on Dumped Chemical Munitions in the Baltic Sea (HELCOM MUNI). Baltic Sea Environment Proceeding (BSEP) 142. pp. 128.

Hylland, K., Robinson, C.D., Burgeot, T., Martínez-Gomez, C., Lang, T., Svavarsson, J., Thain, J.E., Vethaak, A.D., Gubbins, M.J., 2017. Integrated chemical and biological assessment of contaminant impacts in selected European coastal and offshore marine areas. Mar. Environ. Res. 124, 130–138.

ICES, 2011. Report of the Study Group on Integrated Monitoring of Contaminants and Biological Effects (SGIMC), 14e18 March 2011, Copenhagen, Denmark. ICES CM 2011/ACOM: 30. (265 pp.).

Jędruch, A., Bełdowski, J., Bełdowska, M., 2015. Long-term changes and distribution of mercury concentrations in surface sediments of the Gdansk Basin (Southern Baltic Sea). J. Soils Sediments 15 (12), 2487–2497.

Kobusińska, M., Skauradszun, M., Niemirycz, E., 2014. Factors determining the accumulation of pentachlorophenol – a precursor of dioxins in bottom sediments of the Gulf of Gdańsk (Baltic Sea). Oceanol. Hydrobiol. Stud. 43, 154–164.

Koniecko, I., Staniszewska, M., Falkowska, L., Burska, D., Kielczewska, J., Jasinska, A., 2014. Alkylphenols in surface sediments of the gulf of Gdansk (Baltic Sea). Water Air Soil Pollut. 225, 2040.

Missiaen, T., Söderström, M., Popescu, I., Vanninen, P., 2010. Evaluation of a chemical munition dumpsite in the Baltic Sea based on geophysical and chemical investigations. Sci. Total Environ. 408, 3536–3553.

Polak-Juszczak, L., 2012. Bioaccumulation of mercury in the trophic chain of flatfish from the Baltic Sea. Chemosphere 89, 585–591.

Sanderson, H., Fauser, P., Thomsen, M., Sørensen, P.B., 2008. Screening level fish community risk assessment of chemical warfare agents in the Baltic Sea. J. Hazard. Mater. 154, 846–857.

Sanderson, H., Fauser, P., Thomsen, M., Vanninen, P., Savin, Y., Hirvonen, A., Missiaen, T., Polyak, Y., Paka, V., Niiranen, S., Borodin, P., Medvedeva, N., Feller, P., Soderstrom, M., Khalikov, I., Zhurbas, V., Gress, A., 2010. Feature: risk assessment of chemical munitions dumped in the Baltic Sea in 1947. Environ. Sci. Technol. 44, 4389–4394.

Staniszewska, M., Burska, D., Sapota, G., Bogdaniuk, M., Borowiec, K., Nosarzewska, I., Bolałek, J., 2011. The relationship between the concentrations and distribution of organic pollutants and black carbon content in benthic sediments in the Gulf of Gdańsk, Baltic Sea. Mar. Pollut. Bull. 62, 1464–1475.

Staniszewska, M., Falkowska, L., Grabowski, P., Kwaśniak, J., Mudrak-Cegiołka, S., Reindl, A.R., Sokołowski, A., Szumiło, E., Zgrundo, A., 2014. Bisphenol a, 4-tert-octylphenol, and 4-nonylphenol in the gulf of Gdańsk (southern Baltic). Arch. Environ. Contam. Toxicol. 67, 335–347.

Vethaak, A.D., Davies, I.M., Thain, J.E., Gubbins, M.J., Martínez-Gömez, C., Robinson, C.D., Moffat, C.F., Burgeot, T., Maes, T., Wosniok, W., Giltrap, M., Lang, T., Hylland, K., 2017. Integrated indicator framework and methodology for monitoring and assessment of hazardous substances and their effects in the marine environment. Mar. Environ. Res. 124, 10–20.

Zaborska, A., 2014. Anthropogenic lead concentrations and sources in Baltic Sea sediments based on lead isotopic composition. Mar. Pollut. Bull. 85, 99–113.

VIII

Environmental genotoxicity and risk assessment in the Gulf of Riga (Baltic Sea) using fish, bivalves and crustaceans

Butrimavičienė L, Baršienė J, Greiciūnaitė J, Stankevičiūtė M, Valskienė R

Environmental Science and Pollution Research 2018,
https://doi.org/10.1007/s11356-018-2516-y

Reprinted with permission from Springer Nature

Environmental Science and Pollution Research
https://doi.org/10.1007/s11356-018-2516-y

RESEARCH ARTICLE

Environmental genotoxicity and risk assessment in the Gulf of Riga (Baltic Sea) using fish, bivalves, and crustaceans

Laura Butrimavičienė[1] · Janina Baršienė[1] · Janina Greiciūnaitė[1] · Milda Stankevičiūtė[1] · Roberta Valskienė[1]

Received: 15 March 2018 / Accepted: 6 June 2018
© Springer-Verlag GmbH Germany, part of Springer Nature 2018

Abstract

Environmental genotoxicity in the Gulf of Riga was assessed using different bioindicators (fish, clams, and isopods) collected from 14 study stations. Comparison of genotoxicity responses (micronuclei (MN) and nuclear buds (NB)) in blood erythrocytes of herring (*Clupea harengus*), eelpout (*Zoarces viviparous*), and flounder (*Platichthys flesus*) revealed the species- and site-specific differences. For the first time, the analysis of genotoxicity was carried out in gill cells of isopods *Saduria entomon*. The highest inductions of MN and NB in gill cells of investigated *S. entomon* and clams (*Macoma balthica*) were evaluated in specimens from station 111A (offshore zone). In fish, the highest incidences of MN were measured in eelpout and in herring collected in the southern part of Gulf of Riga (station GOR3/41S). Moreover, in the southern coastal area, the assessment of genotoxicity risk (according to micronuclei levels) indicated exceptionally high risk for flounder, eelpout, and clams.

Keywords Genotoxicity · Fish · Clams · Isopods · Baltic Sea · Risk assessment

Introduction

Large amounts of hazardous substances from various anthropogenic sources are affecting the Baltic Sea. Due to limited water exchange, shallowness, riverine inputs, dense human populations, high levels of industrialization, and agriculture along the Baltic Sea shores, Baltic Sea Bays are suffering from pollution, eutrophication, no exception to the Gulf of Riga, which is the third of the largest bay in the Baltic Sea. Timely operative identification and effective reduction of existing pollution and the other negative impacts are the major aspects in the vision of the healthy Baltic Sea environment (HELCOM 2010a).

Many of the hazardous substances discharged from the industries, agriculture, or municipal activities can pose a genetic risk to marine organisms (Baršienė et al. 2004, 2006a, 2006b, 2012a, 2012b, 2013; Bolognesi and Hayashi 2011; Marigomez et al. 2013; Turja et al. 2014). Genotoxic compounds, due to their capacity to damage the structure on the DNA molecule, provoke concomitant adverse effects disturbing biological integrity and the whole ecosystem (Shugart 2000). In order to assess both clastogenic and aneugenic effects of contaminants in various organisms, many studies have applied the micronucleus test, which has served as an index of cytogenetic damage for many years (Hayashi 2016). There are several possible genetic mechanisms by which exposure to genotoxins directly or indirectly induces micronuclei (MN) formation; one of them is budding process (Cheong et al. 2013). Serrano-García and Montero-Montoya (2001) found that nuclei buds (NBs) were consistently formed where micronuclei were found. Finally, different mechanistic origin of NB and MN, according to Lindberg et al. (2007), cannot be excluded. Induction and disproportion of frequencies of various nuclear abnormalities in various aquatic species may be affected by various toxic compounds, their concentrations, exposure time, biotic and abiotic factors, or other

Highlights

• First time study on environmental genotoxicity in the Gulf of Riga.
• Organisms from different phyla of Mollusca, Arthropoda, and Chordata were used for genotoxicity (MN and NB incidences) determination.
• First time isopod *Saduria entomon* was used as environmental genotoxicity bioindicator.
• The assessment of genotoxicity risk in the southern coastal area indicated exceptionally high risk for flounder, eelpout, and clams.
• The highest genotoxicity risk was observed in eight study stations out of 14 investigated.

Responsible editor: Cinta Porte

✉ Laura Butrimavičienė
laura@ekoi.lt

[1] Nature Research Centre, Institute of Ecology, Akademijos Str. 2, LT-08412 Vilnius, Lithuania

Published online: 21 June 2018

individual characteristics (Carrasco et al. 1990; Pinto-Silva et al. 2005; Del Barga et al. 2006; Baršienė et al. 2008; Smolarz and Berger 2009; Bolognesi and Hayashi 2011; Negreiros et al. 2011; Taylor and Maher 2013; Della-Torre et al. 2015).

The Baltic clam (*Macoma balthica*) is one of the most common bottom-dwelling species in the Baltic Sea and a significant element of the food web. The Baltic clam plays the main role in sediment reworking and bioturbation. The important feature of these filter-feeding organisms is to maintain the integrity of overall healthy ecosystem (Michaud et al. 2006). Bivalves are especially appropriate marine environmental genotoxicity bioindicators due to their response to low concentrations of genotoxic substances, which can be present in chronically polluted habitats (Baršienė et al. 2012a). As a sentinel organism, *M. balthica* has been used only in few environmental geno- and cytotoxicity investigations in the Baltic Sea (Baršienė et al. 2008; Smolarz and Bradtke 2011) and in laboratory examinations (Smolarz and Berger 2009). A significantly increased MN incidences and frequencies of other nuclear abnormalities have been found in *M. balthica* collected from Lithuanian waters of the Baltic Sea affected by pollution from intensive navigation (Baršienė et al. 2008). Sediment-dwelling *M. balthica* was used as a bioindicator on the assessment of ecological risk at the Gdynia dumping sites. Significantly increased MN values were observed in individuals from the stations, which were located in the Gdynia dumping sites (Michałek et al. 2014). Time-related cytotoxicity and genotoxicity effects have been described in gill cells of *M. balthica* exposed to different concentrations of brominated flame-retardant HBCDD (hexabromocyclododecane) (Smolarz and Berger 2009).

In the Gulf of Riga, herring (*Clupea harengus*) is the most important commercial fish species and dominates the catches at about 90% of total values. The stock is mostly stationary (Kotta et al. 2008). Flounder (*Platichthys flesus*) and eelpout (*Zoarces viviparous*) in the Gulf of Riga consume a broad spectrum of benthic organisms, including bivalves *M. balthica* (Urtans 1990). Fish as bioindicators were used in environmental genotoxicity studies in different areas of the Baltic Sea and in many investigations (Baršienė et al. 2012b, 2014, 2015, 2016).

The largest crustaceans *Saduria entomon* are an important food item for many commercial fish species in the Baltic Sea. This stationary species is well adapted to a long period of hypoxia and widely distributed in the Baltic Sea waters (Kvach 2009). Moreover, *S. entomon* regulates the abundance of clams *M. balthica* (Ejdung and Bonsdorff 1992). There are no data on genotoxicity in crustaceans *S. entomon* species. However, the recent investigation has shown that microcrustaceans Daphnids (*Daphnia magna*) and Copepods (*Acanthocyclops robustus*) from subphylum Crustacea can serve as suitable bioindicators for genotoxicity assessment using MN test (Barka et al. 2016).

This study is the first attempt to assess environmental genotoxicity in the Gulf of Riga by analysis of bioindicators from the Mollusca, Arthropoda, and Chordata phyla. The levels of nuclear abnormalities (micronuclei and nuclear buds) were evaluated in peripheral blood erythrocytes of *P. flesus*, *C. harengus*, and *Z. viviparous*, in gill cells of *M. balthica* and for the first time in gill cells of *S. entomon*. Environmental genotoxicity risk in the Gulf of Riga was assessed using MN responses in three fish species and clams.

Materials and methods

Sampling and sample preparation

Blood samples were collected from 88 *Platichthys flesus*, 89 *Clupea harengus*, and 22 *Zoarces viviparous* specimens caught during r/v "Walther Herwig" sampling cruise using standard bottom or pelagic trawls in December 2010. Gills were dissected from 84 of *Macoma balthica* and 42 of *Saduria entomon* specimens sampled with the Van Veen grab (0.1 m^2) according to the HELCOM guidelines during r/v "Aranda" sampling campaign in September 2010. Specimens of fish *P. flesus* weight range 113–360 g and length range 20–31 cm; of *C. harengus*, 25–58 g and 16–20 cm; and of *Z. viviparous*, 38–123 g and 21–30 cm. The shell length of clams ranged between 1.4 and 2.7 cm, the whole body of isopods, 1.7 and 7.4 cm.

The location, of the total 14 investigated stations of the Gulf of Riga, is shown in Fig. 1. Stations 163B-S, GRF1-S, GOR3/42-S, GOR3/41-S, and 167B-S were assigned as southern coastal area; stations GOR1/31-E, GOR1/30-E, 107-E, and NL5A-E, as eastern coastal area; stations GRF6A-W, GOR2/37-W, GOR2/40-W, and GOR2/35-W, as western coastal area of the Gulf of Riga; and station 111A, as offshore zone of the Gulf of Riga.

The list of study stations, geographic coordinates, depth of trawling, number of collected specimens, seawater temperature, and salinity is presented in Table 1.

The preparation of slides and investigation of the micronuclei and nuclear buds were carried out following the earlier described method (Baršienė et al. 2004, 2012a). Blood samples from fish were taken immediately after catching. A drop of blood (taken from the caudal vein of fish using a heparinized syringe) was directly smeared on glass slides. Gills from clams and isopods were dissected and placed on slides, gently nipped for 2–3 min with tweezers until the cells spread within the drop. The cell suspension was then softly smeared on the whole surface. Microscopic slides were air-dried, fixed in methanol for 10 min, and stained with 5% Giemsa solution in phosphate buffer (pH 6.8). Blind scoring of nuclear abnormalities was performed on coded slides under the light microscopes

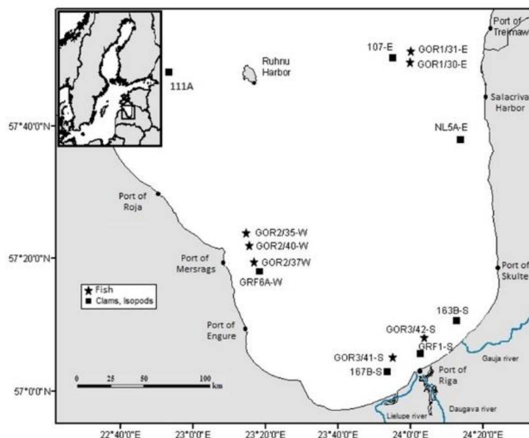


Fig. 1 Study stations in the Gulf of Riga

(Olympus BX51 and Nikon Eclipse 50i) at a final magnification of × 1000. For each studied specimen of fish, 4000 intact peripheral erythrocytes; for mussels, 1000 gill cells; and for isopods all gill cells with intact cytoplasm were scored. Final results were expressed as the mean value (per mille, ‰) of sums of the analyzed individual lesions scored in 1000 cells per fish, bivalve, or crustacean collected from each study location. Micronuclei and nuclear buds were identified using criteria described for fish erythrocytes and bivalves' gill cells in previously presented publications (Baršienė et al. 2012a, 2013, 2015). Micronucleus is smaller than 1/3 of the main nucleus, round- or ovoid-shaped, usually at the same color and not connected to the main nucleus. Cells with large evagination of nuclear membrane, which forms narrow nucleoplasmic connection with the main nuclei, as well as buds on thin nucleoplasmic filament were recorded as NB. The morphological features of studied nuclear abnormalities are shown in Fig. 2.

Table 1 The list of bioindicator catch stations and hydrological parameter measurements over the bottom of the Gulf of Riga (data for clams and isopods from the r/v "Aranda" surveys and for fish from the r/v "Walther Herwig III" survey)

Station code	Coordinates		Depth of trawling (m)	Bioindicators and number of specimens (*n*)	Temperature (°C)	Salinity (PSU)
	Latitude	Longitude				
167B-S	N57.0329	E023.5324	17.2	Clams (12)/isopods (4)	16.5 at the sea surface	5.0 at the sea surface
GRF1-S	N57.0670	E024.0340	15.4	Clams (12)/isopods (6)	16.3 at the sea surface	4.9 at the sea surface
163B-S	N57.1101	E024.1302	17	Clams (12)	16.1 at the sea surface	5.0 at the sea surface
GRF6A-W	N57.1844	E023.1741	26.5	Clams (12)/isopods (8)	16.1 at the sea surface	5.0 at the sea surface
NL5A-E	N57.3798	E024.1400	23.9	Clams (12) /isopods (8)	14.4 at the sea surface	5.1 at the sea surface
107-E	N57.509900	E023.5497	27.7	Clams (12)/isopods (8)	15.3 at the sea surface	5.1 at the sea surface
111A	N57.4826	E022.5146	28.7	Clams (12)/isopods (8)	16.1 at the sea surface	5.1 at the sea surface
GOR1/30-E	N57.5031	E24.0011	30	Flounder (14), herring (19), eelpout (10)	5.92 at the bottom	5.63 at the bottom
GOR1/31-E	N57.5092	E24.0033	31	Flounder (14), herring (10)	5.98 at the bottom	5.65 at the bottom
GOR2/35-W	N57.2296	E23.1357	26	Flounder (15), herring (21), eelpout (5)	5.80 at the bottom	6.15 at the bottom
GOR2/37-W	N57.1887	E23.1589	27	Flounder (9), herring (10)	5.50 at the bottom	6.02 at the bottom
GOR2/40-W	N57.2235	E23.1383	27	Flounder (5)	3.86 at the bottom	4.52 at the bottom
GOR3/41-S	N57.0451	E23.5483	25	Flounder (15), herring (23), eelpout (7)	4.52 at the bottom	4.89 at the bottom
GOR3/42-S	N57.0829	E24.0243	50	Flounder (16), herring (6)	7.73 at the bottom	9.74 at the bottom

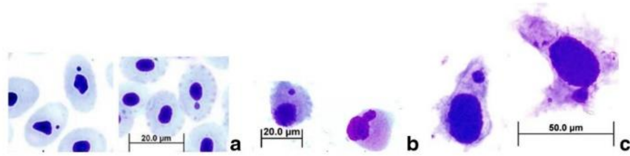


Fig. 2 Micronuclei and nuclear buds in fish (**a**), in clams (**b**), and in isopods (**c**)

The environmental genotoxicity risk assessment in fish and clams

The risk in each of the studied stations was assessed on the basis of the established background response (BAC) of MN in studied fish and clam species. Methodology of BAC calculation and reported BAC levels for different Baltic Sea organisms is presented in the article by Baršienė et al. (2012c). The BAC in herring consists of 0.39 MN/1000 erythrocytes; in flounder, 0.23 MN/1000 erythrocytes; in eelpout, 0.38 MN/1000 erythrocytes; and in clams, 2.90 MN/1000 gill cells. In general, an elevated MN frequency lies above the P90 percentile. The 90th percentile (P90) values were calculated for those stations/areas which were considered reference stations (i.e., no known local sources of contamination or those areas that were not considered unequivocally as reference sites but were less influenced by human and industrial activity). P90 separates the upper 10% of all values in the group from the lower 90% (Baršienė et al. 2012c).

The stations were grouped according to a 5-grade scale, i.e., 0.0–19% of specimens with MN frequencies higher than their BAC were indicated as low; 20–39%, as moderate; 40–59%, as increased; 60–79%, as high; and 80–100%, as exceptionally high genotoxicity risk level. Environmental genotoxicity risk zones (indicated as MN responses in 5-grade risk scales) were mapped to the GIS system utilizing the program ArcGIS Desktop and the ArcMap application (Fig. 7). Geographical coordinates system GCS WGS 1986, Projection: Mercator was used. Since, at some stations, two or three species were collected, results from the flounder analysis are marked exactly under the geographical coordinates of the fish sampling. The other species are marked closely to the flounder.

Statistical analysis

The mean values of MN and NB, standard errors, and P values were calculated for all investigated bioindicators from each station using GraphPad Prism® 5.01 (GraphPad Software Inc., San Diego, CA, USA) statistical package. The level of significance was established at $p < 0.05$. Non-parametric one-way (ANOVA) followed by Dunn's Multiple Comparison Test was used to compare frequencies of MN and NB between study stations. We used STATISTICA 7.0 (StatSoft Inc., Tulsa, Oklahoma, USA) principal component analysis (PCA) to obtain an integrated view of the relationships between the investigated variables (MN and NB) and studied species in the investigated Baltic Sea area.

Results

Environmental genotoxicity responses in flounder, herring, and eelpout

The highest frequency of micronuclei (MN/1000 cells) was detected in eelpout caught at station GOR3/41-S and was equal to 0.68‰. A slightly lower level of MN (0.61‰) was detected in flounder from station GOR2/37-W. The value of MN in eelpout from station GOR1/30-E was equal to 0.51‰. The highest level of MN in herring was recorded at station GOR3/41-S (0.38‰). In herring, collected at station GOR3/42-S, the lowest MN frequency (0.16‰) in comparison to other fish species was found. The lowest MN incidences (0.23‰) in flounder were in fish from station GOR1/31-E, while in eelpout it was equal to 0.45‰ and was determined in specimens from station GOR2/35-W (Fig. 3).

In all three fish species caught at station GOR1/30-E, the highest induction of nuclear buds (NB) was determined with the gradient of NB responses in fish—eelpout (0.37‰) < herring (0.47‰) < flounder (0.84‰). The reference level of NB (0.1‰) was found in flounder from station GOR2/40-W. In herring from station GOR1/31-E and in eelpout from station GOR2/35-W, the frequency of NB was equal to 0.32 and 0.35‰ respectively (Fig. 2). Significant differences between analyzed genotoxicity parameters in the fish species caught at different stations were recorded only in NB responses of flounder ($p < 0.05$). Significant increment of NB values was registered in flounder caught at station GOR1/30-E compared to all other six study stations located in the Gulf of Riga.

Comparison of genotoxicity parameters MN and NB data in fish revealed the species- and site-specific differences.

Environmental genotoxicity responses in *M. balthica* and *S. entomon*

There was a high variability in the frequencies of nuclear abnormalities among the groups of isopods and quite similar levels of determined responses in clams from sampled stations. There

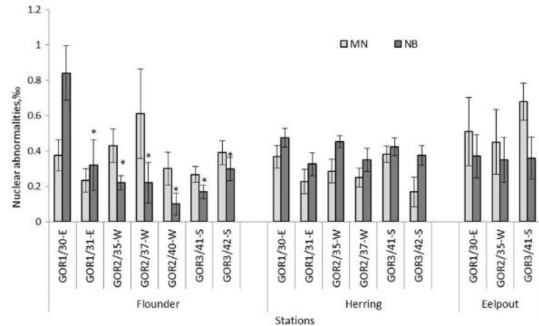


Fig. 3 Frequencies of nuclear abnormalities (micronuclei (MN) and nuclear buds (NB)) in blood erythrocytes of flounder, herring, and eelpout caught at stations of the Gulf of Riga (mean in ‰ ± SE). * Significant differences from station GOR1/30-E

were no significant differences in genotoxicity responses (MN and NB) of both bioindicators between investigated stations.

The highest induction of micronuclei and nuclear buds in gill cells of *M. balthica* and *S. entomon* was recorded in specimens from station 111A. The frequency of MN in clams was equal to 6.1‰, in isopods, 4.59‰. Both species caught at the same station revealed the highest values of nuclear buds (4.69‰ in clams, 6.09‰ in isopods). Slightly lower levels of MN in *M. balthica*, 5.04‰ (GRF1-S), and in isopods (3.40‰) were detected in specimens from station GRF6A-W. The lowest MN incidences (0.94‰) were registered in *S. entomon* from station 107-E. Moreover, isopods from station 107-E revealed the lowest level of NB (0.72‰). Mussels collected from stations 107-E, NL5A-E, and 163B-S showed a quite similar level of MN, with values varied between 3.56 and 3.88‰. The frequencies of MN in clams from stations GRF6A-W and 167B-S were equal to 4.21 and 4.23‰. High genotoxicity levels were recorded for NB values in *M. balthica* from station NL5A-E (4.04‰) and in isopods from station GRF1-S (3.75‰) (Fig. 4). In station GRF6A-W, the lowest level of the NB in clam gills was found. The frequencies of the nuclear abnormalities in clams inhabiting the other stations varied between 3.04 and 3.52‰. In isopods, from stations 167B-S and GRF6A-W, the frequencies of NB were equal to 2.5‰. Slightly higher values (2.66‰) were measured in an isopod group from station NL5A-E (Fig. 4).

Principal component analysis

Two factors (factor 1 and factor 2) accounting for 100% of both (MN and NB) biomarker data sets for different stations in different fish species (flounder, eelpout, and herring) were used. The first factor (MN) accounted for 50.29% of the total variance, and the results show that eelpout sampled from station GOR3/41-S and flounder caught at station GOR2/37-W were clearly different from those of other sites by its location on the positive side of factor 1 (Fig. 5). On the positive side of factor 1 were all study stations of eelpout catches. The high levels of MN and low levels of NB in eelpout defined this distribution. On the contrary, flounders from stations GOR3/42-S, GOR2/35-W, GOR3/41-S, and GOR2/40-W were on the positive side of factor 1, but in the negative side of factor 2, due to their high levels of MN.

The second factor (NB) explained 49.71% of the total variance. The flounders from station GOR1/30-E were located on the positive side of factor 2 and had the high component loadings for this factor, which was characterized by the high frequency of NB in fish erythrocytes. On the contrary, flounders collected from station GOR1/31-E were on the negative side of factor 2. On the positive side of factor 2 were located two stations where herring have been caught. In herrings from stations GOR1/30-E and GOR3/41-S, high induction in NB was noted. Herring groups from stations GOR2/37-W, GOR1/31-E, and GOR3/42-S were located on the negative side of factor 2.

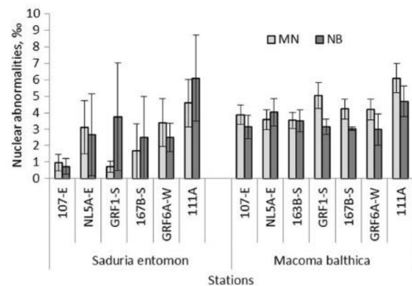


Fig. 4 Frequencies of nuclear abnormalities (micronuclei (MN) and nuclear buds (NB)) in gill cells of isopods *Saduria entomon* and clams *Macoma balthica* sampled at different sites of the Gulf of Riga

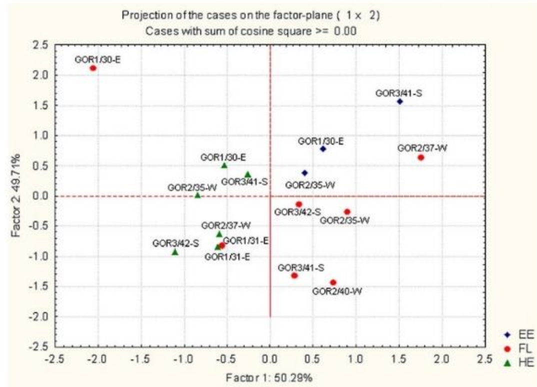


Fig. 5 PCA results for eelpout (EE), flounder (FL), and herring (HE) from various sampling stations and according to analyzed genotoxicity biomarkers: micronuclei and nuclear buds

M. balthica and *S. entomon* genotoxicity data for different stations revealed that the component (factor 1—MN) accounted for 80.14% of the total variance (Fig. 6). The mussels from stations 107-E, 167B-S, GRF6A-W, GRF1, and 111A were located on the positive side of factor 1 and indicated the high factor loadings (according to high frequency of micronuclei). Clams from stations 163B-S and NL5A-E and isopods from 111A were on the positive side of factor 1 and in the negative side of factor 2, due to their high levels of NB. The second factor (factor 2—NB) explained 19.86% of the total variance. Specimens of *S. entomon* with the highest values of NB caught at stations 167B-S and GRF1-S were located on the negative side of factor 2. While, isopods from stations 107-E, NL5A-E, and GRF6A-W were on the positive side, the high levels of MN and low levels of NB defined this separation.

Environmental genotoxicity risk assessment in fish and clams

The environmental genotoxicity risk was assessed using four different bioindicators (three fish species and clams) collected in the Gulf of Riga. In the southern coastal area (163B-S, GRF1-S, GOR3/42-S, GOR3/44-S, GOR3/41-S, GOR3/43, and 167B-S station), the assessment of genotoxicity risk

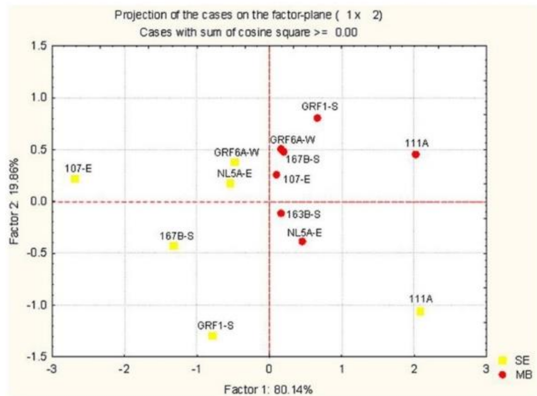


Fig. 6 PCA results for *Saduria entomon* (SE) and *Macoma balthica* (MB) from various sampling sites and according two genotoxicity biomarkers: micronuclei and nuclear buds

(according to MN levels) indicated exceptionally high risk for three species (flounder, eelpout, and clams) (Fig. 7). In the western coastal area (GRF6A-W, GOR2/37-W, GOR2/40-W, and GOR2/35-W station), exceptionally high risk level was recorded for two species (flounder and clams) out of four species investigated. Moreover, exceptionally high risk level was measured in clams collected in offshore zone (station 111A). High genotoxicity risk was identified for flounder and clams, collected from the eastern coastal area (GOR1/31-E, GOR1/30-E, 107-E, and NL5A-E station).

Analysis of MN levels revealed an exceptionally high risk for flounder inhabiting four out of seven stations, for eelpout one out of three stations, and for clams collected from four out of seven study stations. Two stations for flounder and for clams were attributed to have high risk of genotoxicity level. Slightly increased level of genotoxicity risk was identified for two herring stations (out of six stations) and for one station for flounder and eelpout. Moderate risk level was identified in four herring stations. Low genotoxicity risk level, according to four different bioindicators, was attributed for eelpout and only in one station.

Discussion

The present study provides the first data set on genotoxicity responses at various stations of the Gulf of Riga. The endpoints of genotoxicity (induction of micronuclei and nuclear buds) were analyzed in flounder, herring, and eelpout blood erythrocytes. Erythrocytes are especially relevant for the assessment of genotoxicity as they can act as carriers of pollutants via blood circulation and are directly affected by mutagenic, genotoxic, and carcinogenic compounds. Gills of bivalves (*M. balthica*) were used as target tissue for environmental genotoxicity assessment of hazardous substances in the Baltic Sea.

For the first time, the analysis of environmental genotoxicity in crustaceans collected at various stations of Gulf of Riga was additionally carried out. Similarities of genotoxicity responses (MN and NB) in gill cells between crustaceans and the clams (station 111A), and the common prevalence of *S. entomon* in the Baltic and even in other sea waters, have strengthened them to be a prospective environmental genotoxicity bioindicator in future investigations.

The results from the study showed that the major factor triggering the increase of environmental genotoxicity in aquatic organisms of the Gulf of Riga could be suspected the riverborne pollution from Daugava, Lielupe, and Gauja Rivers. PCBs, DDT, and related compounds, hexachlorobenzene (HCB), bis(4-chlorophenyl) sulfone (BCPS), and two hexachlorocyclohexane (HCH) isomers, were found in fish from the rivers Daugava and Lielupe (Valters et al. 1999). According to HELCOM (2015) data, there were registered increased concentrations of flow-weighted nitrogen and phosphorus in the biggest Latvian River Daugava (1994–2010); a significant increase in direct waterborne nitrogen inputs from Latvia to the Baltic Sea was presented. In bivalves collected from the River Gauja, there were found high concentrations of Cd and Hg (Kalvane and Veidemane 2013). Moreover, southern coastal area of Gulf of Riga could be mostly affected by genotoxins inputs from the Riga city and port.

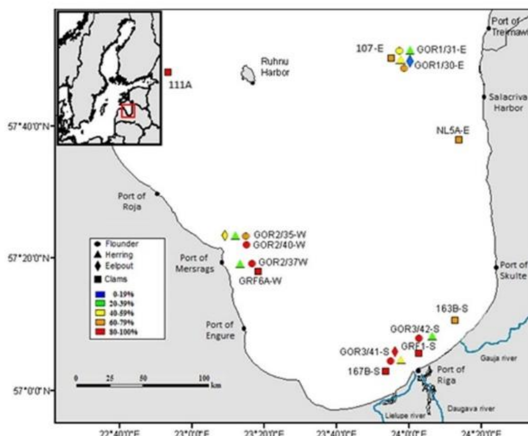


Fig. 7 Results of environmental genotoxicity risk assessment (according to MN levels) in different fish species and in clams collected from 14 study stations in the Gulf of Riga

In our study, the highest frequencies of MN were recorded in herring, and in eelpout caught at station GOR3/41-S, which was located in front of the estuary of River Daugava. In the sediments of the River Daugava, elevated levels of trace metals (Hg, Cd, Pb, Cu, Zn, and Mn) were determined. Meanwhile, in the sediments of the River Lielupe, there were found the highest levels of Ni (Seisuma and Kulikova 1999). Metal (Cd, Pb, and Zn) pollution in the River Daugava was presented by Yurkovskis and Poikāne (2008). Site-specific elevation of environmental genotoxicity and cytotoxicity in different fish species collected closely to the estuaries of the rivers was presented by various researchers (Rybakovas et al. 2009; Capela et al. 2016; Kračun-Kolarević et al. 2016).

During our investigation, high frequencies of MN incidences were found in clams from southern part of the Gulf of Riga (station GRF1-S). According to Barda et al. (2015), significant concentration of algal hepatotoxin nodularin-R (NOD-R) was detected in *M. balthica* specimens collected from the mouth of the River Daugava. Induction of MN and other nuclear abnormalities after exposure with biotoxins in fish (Del Barga et al. 2006; Zhang et al. 2013) and bivalves (Pinto-Silva et al. 2003, 2005) has been presented.

Genotoxicity risk assessment indicated exceptionally high risk for three species (flounder, eelpout, and clams) collected in the southern coastal area (stations—163B-S, GOR3/42-S, GRF1-S, GOR3/41-S, and 167B-S). In sediments collected from the southern part of Gulf of Riga in 2009, elevation of Cd concentration was found (Sesuma and Kulikova 2012). Genotoxicity effects of heavy metals in fish and bivalves have been described in many experimental and field studies (Taylor and Maher 2013; Canalejo et al. 2016; Stankevičiūtė et al. 2016). According to the HELCOM data (2010b), in blue mussels from the southern coast area of Gulf of Riga, there were found particularly high concentrations of dioxin-like congener CB-118. Potential of dioxins to induce genotoxicity in different marine organisms was determined experimentally (Canesi et al. 2014; Della Torre et al. 2015).

Exceptionally high risk level was recorded in the western coastal area investigated stations for two species (flounder and clams) out of four species investigated. Bioindicators from the western stations of the Gulf of Riga could be under the impact of navigation pollution from small ports (Engure, Mersrags, Roja) as well as due to the agricultural activities. Moreover, in the bivalves *M. balthica* from the western coastal area of Gulf of Riga (Engure and Mersrags ports zone), there were detected significant concentrations of NOD-R (Barda et al. 2015). According to the data of the Ministry of Transport of Latvia, Mersrag's port is one of the fastest growing ports among the small ports of Latvia. Statistics of the Latvian Maritime Administration (2012) identified 30 maritime accidents of liquid bulk vessels in Latvia's territorial waters or vessels sailing under the Latvian flag during the period of 1993–2010. There were determined elevated concentrations of Hg in sediments from the Roja station in 2007 (Seisuma and Kulikova 2012). Furthermore, from the same Roja area, there were measured higher concentrations of Pb in the liver of eelpouts (*Z. viviparous*) (Voigt 2014). There were found higher concentrations of metals (eight times for Cd and two times for Mn) in sediments collected (in May 2010) from the station, which was located near the Engure port (Putna et al. 2014). Environmental genotoxicity arising from intensive navigation activities and pollution from harbors already has been reported (Baršienė 2002; Baršienė et al. 2006b; Magni et al. 2006; Bocchetti et al. 2008; Martinovic et al. 2015; Gherras Touahri et al. 2016).

Genotoxicity analysis has shown that especially high level of nuclear buds (NB) was determined in all fish species caught at station GOR1/30-E (eastern coastal area of the Gulf of Riga). Meanwhile, bioindicators from the close station GOR1/31-E (herring and flounder) and outlying station 107-E (isopods) revealed low frequencies of analyzed nuclear abnormalities. Genotoxicity levels in *S. entomon* collected from 107-E station could be assigned as reference ones; in isopods from 107-E, there were registered the lowest MN (0.94‰) and NB (0.72‰) incidences out of all investigated stations from Riga Bay.

The highest values of MN and NB were determined in clams and isopods from station 111A, which is located on the open sea in Gulf of Riga (offshore sea zone). Chromatin extrusion parameters such as NB can correctly reflect the levels of environmental contamination in the field studies and are able to indicate the overall fish health. Increased NB frequency was described in flounder (*P. flesus*) from the Gulf of Gdansk, in eelpout (*Z. viviparus*) from the Estonian (Kreitsberg et al. 2012), and also in southern Baltic Sea zones (Rybakovas et al. 2009). High levels of NB in erythrocytes were found in fish caught at oil and gas platform zones (Rybakovas et al. 2009), aluminum smelter zone (Baršienė et al. 2010), and in mussels after oil spill (Baršienė et al. 2012a).

Berezina et al. (2013) presented data on the lowest quality of sediments collected in September 2010 from the same station 111A by using an amphipod *Gmelinoides fasciatus* bioassay study. In *M. balthica* (collected in May 2001) from the northernmost part of the Eastern Gotland Basin (station EGB2_01 is situated near the entrance to the Gulf of Riga), the highest concentrations of a-HCH, c-HCH, CB 118, and DDD (lw) were found (Pikkarainen 2007). In fish from the west coast of Riga Bay (our stations 111A and GRF6A-W are situated), high ecotoxicological threshold level concentrations of dioxin-like congener (CB-118) were measured (HELCOM 2010b). Higher induction of MN was in native and caged blue mussels *M. galloprovincialis* individuals at sites polluted by heavy metals, PAHs, PCBs, phthalates, and nonylphenol ethoxylates in comparison to the pre-exposure group mussels (Gorbi et al. 2008; Marigomez et al. 2013).

PCA was applied to obtain an integrated view of the relationships between two investigated variables (MN and NB).

The first factor (MN) accounted for 50.29% of the whole variance in fish and was more promising in invertebrates—80.14%—while the second one (factor 2—NB) explained only 19.86% of the total variance in invertebrates, but in fish was equal to 49.71%. The frequency of MN that assesses DNA damage of both clastogenic and aneugenic potential of pollution in analyzed organisms was proved to be a more efficient biomarker in this study, especially in invertebrates. Experimental studies have revealed a negative correlation between MN, NB and biological parameters and gross morphometric indices in fish (Stankevičiūtė et al. 2016). Connection between the biological parameters, indexes, and DNA damage in flounder was demonstrated under environmental studies of the Baltic Sea (Dabrowska et al. 2014). Genotoxic effects caused by pollutants, leading to changes in biological parameters and indices, may affect health at individual and at population levels in time.

Increased frequency of MN and NB in organisms from investigated sites of the Gulf of Riga indicates the presence of pollution with genotoxic agents, which are able to promote mutagenic damage in erythrocytes of fish and gill cells of invertebrates. However, the potential effects of other environmental stressors (biotic and abiotic) cannot be excluded. Individual responses of each bioindicator's species to the various stressors can provoke differences in the genotoxicity level as well.

Multi-species assessment of DNA damage in various tissues of the target species will uncover a pattern of early responses as well as short- or long-term adaptations to chronically produced pollution at low concentrations of contaminants. It is important to improve monitoring strategy using validated relevant bioindicators; however, research of the new target species in environmental genotoxicity assessment is required.

Conclusion

For the first time, the level of environmental genotoxicity (MN and NB values) and the risk assessment (according to MN) in Gulf of Riga in different types of investigated organisms was evaluated. Site- and species-specific differences in genotoxicity responses have been found. According to elevated MN responses in fish (herring and eelpout) and the risk assessment (there were found exceptionally high risk levels for flounder, eelpout, and clams), southern coastal area of Gulf of Riga could be considered as the most affected by genotoxin's pollution. The highest induction of NB in all investigated fish species was at station GOR1/30-E (eastern coastal area). Clams and for the first time in environmental genotoxicity assessment used, isopods revealed the highest levels of both genotoxicity endpoints at station 111A (offshore zone). The highest genotoxicity risk was determined in 8 out of 14 study stations investigated in the Gulf of Riga.

Acknowledgements The authors would like to thank Thomas Lang (Thünen Institute of Fisheries Ecology, Cuxhaven, Germany), Kari Lehtonen (Finnish Environment Institute, Helsinki, Finland), and Aleksandras Rybakovas (Nature Research Centre, Vilnius) for collecting fish samples from the study stations during Walther Herwig III cruises. We are thankful to Dalia Baršytė Lovejoy (Toronto University) for the language check.

Funding information This study was funded mainly by BONUS BEAST project (FP/2007e2013 under grant agreement no. 217246).

Compliance with ethical standards

Conflict of interest The authors confirm that they have no conflict of interest.

References

Barda I, Kankaanpaa H, Purina I, Balode M, Sjovall O, Meriluoto J (2015) Bioaccumulation of hepatotoxins—a considerable risk in the Latvian environment. Environ Pollut 196:313–320. https://doi.org/10.1016/j.envpol.2014.10.024

Barka S, Ouanes Z, Gharbi A, Gdara I, Mouelhi S, Hamza-Chaffai A (2016) Monitoring genotoxicity in freshwater microcrustaceans: a new application of the micronucleus assay. Mut Res 803-804:27–33. https://doi.org/10.1016/j.mrgentox.2016.05.002

Baršienė J (2002) Genotoxic impacts in Klaipėda port and Būtingė oil terminal areas (Baltic Sea). Mar Environ Res 54:475–479. https://doi.org/10.1016/S0141-1136(02)00160-5

Baršienė J, Lazutka J, Šyvokienė J, Dedonytė V, Rybakovas A, Bjornstad A, Andersen OK (2004) Analysis of micronuclei in blue mussels and fish from the Baltic and the North Seas. Environ Toxicol 19: 365–371. https://doi.org/10.1002/tox.20031

Baršienė J, Schiedek D, Rybakovas A, Šyvokienė J, Kopecka J, Förlin L (2006a) Cytogenetic and cytotoxic effects in gill cells of the blue mussel *Mytilus* spp. from different zones of the Baltic Sea. Mar Pollut Bull 53:469–478. https://doi.org/10.1016/j.marpolbul.2005.11.015

Baršienė J, Lehtonen K, Koehler A, Broeg K, Vourinen PJ, Lang T, Pempkowiak J, Šyvokienė J, Dedonytė V, Rybakovas A, Repečka R, Vountisjarvi H, Kopecka J (2006b) Biomarker responses in flounder (*Platichthys flesus*) and mussel (*Mytilus edulis*) in the Klaipėda-Būtingė area (Baltic Sea). Mar Pollut Bull 53:422–436. https://doi.org/10.1016/j.marpolbul.2006.03.009

Baršienė J, Andreikėnaitė L, Garnaga G, Rybakovas A (2008) Genotoxic and cytotoxic effects in the bivalve mollusks *Macoma balthica* and *Mytilus edulis* from the Baltic Sea micronuclei (MN), nuclear buds (NB), fragmented-apoptotic (FA) and bi-nucleated (BN) cells. Ekologija 54:44–50

Baršienė J, Bjornstad A, Rybakovas A, Šyvokienė J, Andreikėnaitė L (2010) Environmental genotoxicity and cytotoxicity studies in mussels and fish inhabiting northern Atlantic zones impacted by aluminum industry. Ekologija 56:116–123

Baršienė J, Rybakovas A, Garnaga G, Andreikėnaitė L (2012a) Environmental genotoxicity and cytotoxicity studies in mussels before and after the oil spill in marine oil terminal (Baltic Sea). Environ Monit Assess 184:2067–2078. https://doi.org/10.1007/s10661-011-2100-0

Baršienė J, Rybakovas A, Lang T, Grygiel W, Andreikėnaitė L, Michailovas A (2012b) Risk of environmental genotoxicity in the Baltic Sea over the period of 2009–2011 assessed by micronuclei frequencies in blood erythrocytes of flounder (*Platichthys flesus*), herring (*Clupea harengus*) and eelpout (*Zoarces viviparus*). Mar Environ Res 77:35–42. https://doi.org/10.1016/j.marenvres.2012.01.004

Baršienė J, Lyons B, Rybakovas A, Martinez- Gómez C, Andreikenaitė L, Brooks S, Maes T (2012c) Background document: micronucleus assay as a tool for assessing cytogenetic/DNA damage in marine organisms (p. 71–83) in Davies IM and Vethaak AD (Eds). Integrated marine environmental monitoring of chemicals and their effects. ICES Cooperative Research Report Rapport No. 315, 277 p

Baršienė J, Rybakovas A, Lang T, Andreikėnaitė L, Michailovas A (2013) Environmental genotoxicity and cytotoxicity levels in fish from the North Sea offshore and Atlantic coastal waters. Mar Pollut Bul 68:106–116. https://doi.org/10.1016/j.marpolbul.2012.12.011

Baršienė J, Butrimavičienė L, Grygiel W, Lang T, Michailovas A, Jackūnas T (2014) Environmental genotoxicity and cytotoxicity in flounder (*Platichthys flesus*), herring (*Clupea harengus*) and Atlantic cod (*Gadus morhua*) from chemical munitions dumping zones in the southern Baltic Sea. Mar Environ Res 96:56–67. https://doi.org/10.1016/j.marenvres.2013.08.012

Baršienė J, Butrimavičienė L, Michailovas A, Grygiel W (2015) Assessing the environmental genotoxicity risk in the Baltic Sea: frequencies of nuclear buds in blood erythrocytes of three native fish species. Environ Monit Assess 187:4078. https://doi.org/10.1007/s10661-014-4078-x

Baršienė J, Butrimavičienė L, Grygiel W, Stunžėnas V, Valskienė R, Greiciūnaitė J, Stankevičiūtė M (2016) Environmental genotoxicity assessment along the transport routes of chemical munitions leading to the dumping areas in the Baltic Sea. Mar Pollut Bull 103:45–53. https://doi.org/10.1016/j.marpolbul.2015.12.048

Berezina NA, Strode E, Lehtonen KK, Balode M, Golubkov SM (2013) Sediment quality assessment using *Gmelinoides fasciatus* and *Monoporeia affinis* (Amphipoda, Gammaridea) in the northeastern Baltic Sea. Crustaceana 86:780–801. https://doi.org/10.1163/15685403-00003215

Bocchetti R, Fattorini D, Pisanelli B, Macchia S, Oliviero L, Pilato F, Pellegrini D, Regoli F (2008) Contaminant accumulation and biomarker responses in caged mussels, *Mytilus galloprovincialis*, to evaluate bioavailability and toxicological effects of remobilized chemicals during dredging and disposal operations in harbour areas. Aquat Toxicol 89:257–266. https://doi.org/10.1016/j.aquatox.2008.07.011

Bolognesi C, Hayashi M (2011) Micronucleus assay in aquatic animals. Mutagenesis 26:205–213. https://doi.org/10.1093/mutage/geq073

Canesi L, Frenzilli G, Balbi T, Bernardeschi M, Ciacci C, Corsolini S, Della Torre C, Fabbri R, Faleri C, Focardi S, Guidi P, Kočan A, Marcomini A, Mariottini M, Nigro M, Pozo-Gallardo K, Rocco L, Scarcelli V, Smerilli A, Corsi I (2014) Interactive effects of n-TiO2 and 2,3,7,8-TCDD on the marine bivalve *Mytilus galloprovincialis*. Aquat Toxicol 153:53–65. https://doi.org/10.1016/j.aquatox.2013.11.002

Capela R, Raimundo J, Santos MM, Caetano M, Micaelo C, Vale C, Guimarães L, Reis-Henriques MA (2016) The use of biomarkers as integrative tools for transitional water bodies monitoring in the water framework directive context—a holistic approach in Minho river transitional waters. Sci Total Environ 539:85–96. https://doi.org/10.1016/j.scitotenv.2015.08.113

Carrasco KR, Tilbury KL, Myers MS (1990) Assessment of the piscine micronucleus test as an *in situ* biological indicator of chemical contaminant effects. Can J Fish Aquat Sci 47:2123–2136. https://doi.org/10.1139/f90-237

Canalejo A, Diaz-de-Alba M, Granado-Castro MD, Cordoba F, Espada-Bellido E, Galindo-Riaño MD, Torronteras R (2016) Early genotoxic response and accumulation induced by waterborne copper, lead, and arsenic in European seabass, *Dicentrarchus labrax*. Environ Sci Pollut Res 23:3256–3266. https://doi.org/10.1007/s11356-015-5435-1

Cheong HS, Seth I, Joiner MC, Tucker JD (2013) Relationships among micronuclei, nucleoplasmic bridges and nuclear buds within individual cells in the cytokinesis-block micronucleus assay. Mutagenesis 28(4):433–440. https://doi.org/10.1093/mutage/get020

Dabrowska H, Kopko O, Góra A, Waszak I, Walkusz-Miotk J (2014) DNA damage, EROD activity, condition indices, and their linkages with contaminants in female flounder (Platichthys flesus) from the southern Baltic Sea. Sci Total Environ 496:488–498. https://doi.org/10.1016/j.scitotenv.2014.07.079

Del Barga I, Frenzilli G, Scarcelli V, Nigro M, Malmvärn A, Asplund L, Förlin L, Sturve J (2006) Effects of algal extracts (Polysiphonia fucoides) on rainbow trout (*Oncorhynchus mykiss*): a biomarker approach. Mar Environ Res 62:S283–S286. https://doi.org/10.1016/j.marenvres.2006.04.017

Della Torre C, Buonocore F, Frenzilli G, Corsolini S, Brunelli A, Guidi P, Kocan A, Mariottini M, Mottola F, Nigro M, Pozo K, Randelli E, Vannuccini ML, Picchietti S, Santonastaso M, Scarcelli V, Focardi S, Marcomini A, Rocco L, Scapigliati G, Corsi I (2015) Influence of titanium dioxide nanoparticles on 2,3,7,8-tetrachlorodibenzo-p-dioxin bioconcentration and toxicity in the marine fish European sea bass (*Dicentrarchus labrax*). Environ Pollut 196:185–193. https://doi.org/10.1016/j.envpol.2014.09.020

Ejdung G, Bonsdorff E (1992) Predation on the bivalve *Macoma balthica* by the isopod *Saduria entomon*: laboratory and field experiments. Mar Ecol Prog Ser 88:207–214

Gherras Touahri H, Boutiba Z, Benguedda W, Shaposhnikov S (2016) Active biomonitoring of mussels Mytilus galloprovincialis with integrated use of micronucleus assay and physiological indices to assess harbor pollution. Mar Pollut Bull 110:52–64. https://doi.org/10.1016/j.marpolbul.2016.06.029

Gorbi S, Lamberti CV, Notti A, Benedetti M, Fattorini D, Moltedo G, Regoli F (2008) An ecotoxicological protocol with caged mussels, *Mytilus galloprovincialis*, for monitoring the impact of an offshore platform in the Adriatic Sea. Mar Environ Res 65:34–49. https://doi.org/10.1016/j.marenvres.2007.07.006

Hayashi M (2016) The micronucleus test–most widely used *in vivo* genotoxicity test. Genes and Environment 38:18. https://doi.org/10.1186/s41021-016-0044-x

HELCOM (2010a) Hazardous substances in the Baltic Sea - An integrated thematic assessment of hazardous substances in the Baltic Sea Baltic Sea Environment Proceedings 120B

HELCOM (2010b) Ecosystem health of the Baltic Sea 2003-2007: HELCOM initial holistic assessment. Baltic Sea Environment Proceedings 122, 1-63

HELCOM (2015) Updated Fifth Baltic Sea pollution load compilation (PLC-5.5). Baltic Sea Environment Proceedings No. 145, 1-71-73

Kalvane I, Veidemane K. (eds.). (2013) Final report on assessment of the quality status of the transboundary water bodies (coastal, lakes, rivers) in Gauja/Koiva river basin district, p. 59–60

Kotta J, Lauringson V, Martin G, Simm M, Kotta I, Herku¨l K, Ojaveer H (2008) Gulf of Riga and Pärnu Bay. Ecology of Baltic Coastal Waters, Ecological Studies 197: 217–243. https://doi.org/10.1007/978-3-540-73524-3_10

Kračun-Kolarević M, Kolarević S, Jovanović J, Marković V, Ilić M, Simonović P, Simić V (2016) Evaluation of genotoxic potential throughout the upper and middle stretches of Adige river basin. SciTotal Environ 571:1383–1391. https://doi.org/10.1016/j.scitotenv.2016.07.099

Kreitsberg R, Tuvikene A, Baršienė J, Fricke NF, Rybakovas A, Andreikėnaitė L, Rumvolt K, Vilbaste S (2012) Biomarkers of environmental contaminants in the coastal waters of Estonia (Baltic Sea): effects on eelpouts (*Zoarces viviparus*). J Environ Monit 14: 2298–2308. https://doi.org/10.1039/C2EM30285C

Kvach Y (2009) First report of Saduria (Mesidotea) entomon (Linnaeus, 1758) (Isopoda: Chaetiliidae) in the Black Sea. Aquat Invas 4:393–395. https://doi.org/10.3391/ai.2009.4.2.17

Latvian Maritime Administration (2012) The Internet site of Latvian Maritime Administration. Available at: http://www.jurasadministracija.lv/index.php?action=145

Lindberg HK, Wang X, Järventaus H, Falck GC, Norppa H, Fenech M (2007) Origin of nuclear buds and micronuclei in normal and folate-

deprived human lymphocytes. Mut Res 617:33–45. https://doi.org/10.1016/j.mrfmmm.2006.12.002

Magni P, De Falco G, Falugi C, Franzoni M, Monteverde M, Perrone E, Sgro M, Bolognesi C (2006) Genotoxicity biomarkers and acetylcholinesterase activity in natural populations of *Mytilus galloprovincialis* along a pollution gradient in the Gulf of Oristano (Sardinia, western Mediterranean). Environl Pollut 142:65–72. https://doi.org/10.1016/j.envpol.2005.09.018

Marigomez I, Zorita I, Izagirre U, Ortiz-Zarragoitia M, Navarro P, Etxebarria N, Orbea A, Soto M, Cajaraville MP (2013) Combined use of native and caged mussels to assess biological effects of pollution through the integrative biomarker approach. Aquat Toxicol 136–137:32–48. https://doi.org/10.1016/j.aquatox.2013.03.008

Martinovic R, Kolarevic S, Kracun-Kolarevic M, Kostic J, Markovic S, Gacic Z, Kljajic Z, Vukovic-Gacic B (2015) Genotoxic potential and heart rate disorders in the Mediterranean mussel *Mytilus galloprovincialis* exposed to Superdispersant-25 and dispersed diesel oil. Mar Environ Res 108:83–90. https://doi.org/10.1016/j.marenvres.2015.05.001

Michałek M, Osowiecki A, Błeńska M, Smolarz K (2014) Report on the assessment of ecological risk at the dumping sites: "application of ecosystem principles for the location and management of offshore dumping sites in SE Baltic Region (ECODUMP)". Department of Aquatic Ecology, Maritime Institute in Gdańsk

Michaud E, Desrosiers G, Mermillod-Blondin F, Sundby B, Stora G (2006) The functional group approach to bioturbation: II. The effects of the *Macoma balthica* community on fluxes of nutrients and dissolved organic carbon across the sediment-water interface. J Exp Mar Biol Ecol 337:178–189. https://doi.org/10.1016/j.jembe.2006.06.025

Negreiros LA, Silva BF, Paulino MG, Fernandes MN, Chippari-Gomes AR (2011) Effects of hypoxia and petroleum on the genotoxic and morphological parameters of *Hippocampus reidi*. Comp Bioch Physiol C: Toxicol Pharmacol 153:408–414. https://doi.org/10.1016/j.cbpc.2011.02.001

Pikkarainen AL (2007) Polychlorinated biphenyls and organochlorine pesticides in Baltic Sea sediments and bivalves. Chemosphere 68: 17–24. https://doi.org/10.1016/j.chemosphere.2006.12.079

Pinto-Silva C, Ferreira JF, Costa RH, Belli Filho P, Creppy EE, Matias WG (2003) Micronucleus induction in mussels exposed to okadaic acid. Toxicon 41(1):93–97

Pinto-Silva C, Creppy EE, Matias WG (2005) Micronucleus test in mussels *Perna perna* fed with the toxic dinoflagellate *Prorocentrum lima*. Arch Toxicol 79:422–426. https://doi.org/10.1007/s00204-004-0645-1

Putna I, Strode E, Bārda I, Purina I, Rimša E, Jansons M, Balode M, Strāke S (2014) Sediment quality of the ecoregion Engure, Gulf of Riga, assessed by using ecotoxicity tests and biomarker responses. Proceedings of the Latvian academy of sciences 68:101–111. https://doi.org/10.2478/prolas-2014-0009

Rybakovas A, Baršienė J, Lang T (2009) Environmental genotoxicity and cytotoxicity in the offshore zones of the Baltic and North Seas. Mar Environ Res 68:246–256. https://doi.org/10.1016/j.marenvres.2009.06.014

Seisuma ZK, Kulikova I (1999) Heavy metals in the sediments of the Gulf of Riga both in the shallow coastal zone and the river mouths volume 12. Special Publication. Proceedings of the Fifth Baltic Marine Geological Conference, Vilnius, ACADEMIA, 129 p

Seisuma ZK, Kulikova I (2012) Distribution of metal concentrations in sediments of the coastal zone of the Gulf of Riga and open part of the Baltic Sea. Oceanology 52(6):780–784

Serrano-García L, Montero-Montoya R (2001) Micronuclei and chromatid buds are the result of related genotoxic events. Environl Mol Mutag 38(1):38–45. https://doi.org/10.1002/em.1048

Shugart LR (2000) DNA damage as a biomarker of exposure. Ecotoxicology 9:329–340

Smolarz K, Berger A (2009) Long-term toxicity of hexabromocyclododecane (HBCDD) to the benthic clam *Macoma balthica* (L.) from the Baltic Sea. Aquat Toxicol 95:239–247. https://doi.org/10.1016/j.aquatox.2009.09.010

Smolarz K, Bradtke K (2011) Bioindicative potential of shell abnormalities occurring in the clam *Macoma balthica* (L.) from the Baltic Sea. Mar Pollut Bull 62:1421–1426. https://doi.org/10.1016/j.marpolbul.2011.04.031

Stankevičiūtė M, Butrimavičienė L, Valskienė R, Greiciūnaitė J, Baršienė J, Vosylienė MZ, Svecevičius G (2016) Analysis of nuclear abnormalities in erythrocytes of rainbow trout (Oncorhynchus mykiss) treated with Cu and Zn and after 4-, 8-, and 12-day depuration (post-treatment recovery). Mutat Res Genet Toxicol Environ Mutagen 797:26–35. https://doi.org/10.1016/j.mrgentox.2016.01.003

Taylor AM, Maher WA (2013) Exposure-dose-response of *Tellina deltoidalis* to metal-contaminated estuarine sediments: 1. Cadmium spiked sediments. Comp Biochem Physiol 158:44–55. https://doi.org/10.1016/j.cbpc.2013.04.005

Turja R, Höher N, Snoeijs P, Baršienė J, Butrimavičienė L, Kuznetsova T, Kholodkevich SV (2014) A multibiomarker approach to the assessment of pollution impacts in two Baltic Sea coastal areas in Sweden using caged mussels (*Mytilus trossulus*). Sci Total Environ 473–474: 398–409. https://doi.org/10.1016/j.scitotenv.2013.12.038

Urtans E (1990) Characteristics of food of smelt, *Osmerus eperlanus* L., and eelpout (*Zoarces viviparus* L.) in the Gulf of Riga. Fischerei-Forschung 28: 34–38 (in Russian)

Valters K, Olsson A, Vitinsh M, Bergman A (1999) Contamination sources in Latvia: levels of organochlorines in perch (Perca fluviatilis) from rivers Daugava and Lielupe. Journal of the Human Environment 28(4):335–340

Voigt HR (2014) Concentrations of lead in some coastal fishes from the Baltic Sea. Estonian Journal of Ecology 63:39–52. https://doi.org/10.3176/eco.2014.1.04

Yurkovskis A, Poikāne R (2008) Biogeochemical, physical and anthropogenic transformations in the Daugava River estuary and plume, and the open Gulf of Riga (Baltic Sea) indicated by major and trace elements. J Mar Syst 70(1–2):77–96. https://doi.org/10.1016/j.jmarsys.2007.03.003

Zhang J, Cowie G, Naqvi SWA (2013) Hypoxia in the changing marine environment. Environ Res Lett 8:3. https://doi.org/10.1088/1748-9326/8/1/015025

IX

Induction of nuclear abnormalities in rainbow trout (*Oncorhynchus mykiss*) after exposure to model mixture of heavy metals (Zn, Cu, Ni, Cr, Cd, Pb) at maximum premissible concentration

Valskienė R, Stankevičiūtė M, Butrimavičienė L, Greiciūnaitė J, Svecevičius G

Proceedings of the 18th Conference for Junior Researchers "Science – Future of Lithuania" 2015, ISSN 2029-5456. Vilnius, Technika. p. 100–105

18-osios Lietuvos jaunųjų mokslininkų konferencijos „Mokslas – Lietuvos ateitis“ teminė konferencija
Proceedings of the 18th Conference for Junior Researchers "Science – Future of Lithuania"

APLINKOS APSAUGOS INŽINERIJA / ENVIRONMENTAL PROTECTION ENGINEERING

ISSN 2029-5456 / eISSN 2029-7149
2015 m. balandžio 9 d. Vilnius
9 April 2015, Vilnius, Lithuania
ISBN 978-609-457-849-6 / eISBN 978-609-457-850-2
Article Number: *aplinka.15*

Aplinkos apsaugos inžinerija
Environmental protection engineering

http://jmk.aainz.vgtu.lt

INDUCTION OF NUCLEAR ABNORMALITIES IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) AFTER EXPOSURE TO A MODEL MIXTURE OF HEAVY METALS (ZN, CU, NI, CR, CD, PB) AT MAXIMUM PERMISSIBLE CONCENTRATION

Roberta Valskienė[1], Milda Stankevičiūtė[2], Laura Butrimavičienė[3], Janina Greiciūnaitė[4], Gintaras Svecevičius[5]

[1, 2, 3, 4, 5] *Nature Research Centre, Institute of Ecology*
E-mails: [1]roberta.valskiene@gamtostyrimai.lt; [2]milda.stan@gmail.com; [3]laura@ekoi.lt; [4]j.greiciunaite@gmail.com; [5]gintaras.svecevicius@takas.lt;

Abstract. Genotoxicity and cytotoxicity responses were assessed in rainbow trout (*Oncorhynchus mykiss*) after 14-day exposure to heavy metal model mixture (HMMM) at concentrations corresponding to Lithuanian inland water standards or Maximum-Permissible-Concentration (MPC) (Zn-0.1, Cu-0.01, Ni-0.01, Cr-0.01, Pb-0.005 and Cd-0.005 mg/l, respectively) accepted for receiving water bodies. Ten different types of nuclear abnormalities were analyzed in blood, kidney and liver erythrocytes of the fish. The results revealed statistically increased levels of MN, 8-shaped, BN, MN+NB+BNb and 8+BN+RIPeryt in blood; 8-shaped BN, RIPeryt, VACnuc, MN+NB+BNb and 8+BN+RIPeryt in the kidney; RIPeryt and 8+BN+RIPeryt in liver erythrocytes. HMMM did not increase the frequency of BNb, NBf, NB, BL and FA.

Keywords: micronuclei, nuclear abnormalities, rainbow trout, heavy metal mixture.

Introduction

Heavy metals (HMs) are important environmental pollutants because of their toxicity, persistence, bioaccumulation and magnification in organisms (Roy 2010). Aquatic ecosystem pollution by HMs has growing trends and is a serious global problem (Malik *et al.* 2010). Heavy metals are common persistent aquatic pollutants and are assigned to priority hazardous substances (EUR-Lex 2008; US EPA 2009).

In the natural environments, fish are exposed to different HM mixtures, which are usually more toxic than individual ones due to synergistic interactions between metals (Jezierska, Sarnowski 2002). However, HMs can act as genotoxins, inducing genomic instability at very low concentrations. Genotoxicity of metals arises from their ability to cause the formation of highly reactive oxygen species (ROS) and electrophilic free-radical metabolites that cause DNA damage (Waisberg *et al.* 2003; Jiraungkoorskul and Sahaphong 2007). Furthermore, pollutants are usually discharged in complex mixtures; thus, interactions between unknown substances can lead to deviations in genotoxicity responses to pollution (Jha *et al.* 2008).

The frequency of nuclear abnormalities (NAs) in erythrocytes is considered as a reliable indicator of cell damage (in particular, DNA alteration) caused by xenobiotics (Bolognesi and Hayashi 2011). It is also important that nuclear abnormalities may exhibit significant variations, depending upon the type of pollutants, fish species, time of exposure, different areas, seasons, etc. (Fuzinatto *et al.* 2013; Ahmed *et al.* 2013; Okonkwo *et al.* 2011; Omar *et al.* 2012; Wirziger *et al.* 2007; Cavas *et al.* 2005). Therefore, the need to develop tests for monitoring contaminant genotoxicity effects is greatly feasible in ecotoxicology and has led to the development of a variety of tools for detection of genotoxins in aquatic media.

Since many compounds are able to induce genotoxic effects with or without directly damaging DNA, the necessity to measure both clastogenic and aneugenic effects of contaminants in organisms is evident. The micronucleus (MN) test is one of the most frequently

used tests in environmental genotoxicity studies and has served as an index of cytogenetic damage for over 30 years (Fenech *et al.* 2003). This is a sensitive and fast test to detect genomic alterations due to clastogenic effects and impairments of the mitotic spindle. Micronucleus test can be produced from chromosome fragments or an entire chromosome that lags at cell division due to lack of centromere, damage in centromere or defect in cytokinesis (Heddle *et al.* 1991).

Morphological nuclear alterations have, firstly, been described in fish erythrocytes by Carrasco *et al.* (1990) and were suggested as complement markers in genotoxicity surveys. In addition to MN test, other NAs such as nuclear buds, bi-nucleated cells and some other NAs have been successfully used for the assessment of pollutant effects in aquatic organisms (Venier *et al.* 1997; Cavas and Ergene-Gozukara 2003; Dailianis *et al.* 2003; Baršienė *et al.* 2006b, 2006c, 2008; Omar *et al.* 2012).

The present study was conducted in order to determine the induction of ten types of NAs in three tissues (blood, kidney, liver) of rainbow trout exposed to heavy metal model mixture (Zn, Cu, Ni, Cr, Pb and Cd) (HMMM). Different combined tissues are usually used by most researchers to determine DNA damage induced by heavy metals (HM), e.g. gills, liver, kidney, blood (Cavas *et al.* 2005; Talapatra *et al.* 2007; Ahmed *et al.* 2013). The micronucleus test has been mostly applied to fish peripheral blood erythrocytes. The most toxic metals were Pb and Cd, followed by Cu, Cr, Ni and Zn in HMMM that were used in the present study. Guardiola *et al.* (2013) revealed that Pb and Cd are very harmful HMs even at low concentrations when exposed to for a long time period. Some HMs such as Cu, Cr, Ni and Zn are essential nutrients for living organisms; however, at high concentrations they can lead to toxic effects in aquatic organisms.

The goal of the present study was to analyze genotoxicity and cytotoxicity in rainbow trout exposed to HMMM at concentrations corresponding to Lithuanian inland water standards. In this study, we emphasize that HM at Maximum-Permissible-Concentrations (MPC) combined together could lead NAs in fish erythrocytes.

Material and methods

The MN test was conducted on one-year-old rainbow trouts (*Oncorhynchus mykiss* Walbaum 1792) of almost similar size; about 10 g of total weight acclimated to laboratory conditions for two weeks. One group of fish, consisting of 10 specimens, was exposed for 14 days to HMMM solution. Concentration of HM in the mixture was chosen based on accepted Lithuanian inland water standards or MPC (Zn-0.1, Cu-0.01, Ni-0.01, Cr-0.01, Pb-0.005 and Cd-0.005 mg/l, respectively) for receiving water bodies (Svecevičius *et al.* 2014). The control group (nine specimens) was maintained separately in the tank with clean aerated deep-well water. Clean water and HMMM solution was renewed every 24 hours, and the test fish were fed bloodworms before this.

Peripheral blood samples were taken using a syringe from the caudal vein. A drop of blood was smeared on microscopic slides and air-dried. In fish, after the sacrifice, small pieces of cephalic kidney and liver were dissected, softly dragged along clean slides and allowed to dry for one-two hours. Dried blood, kidney and liver smears were subsequently fixed in methanol for 10–15 min, and were stained with 10% Giemsa solution for 8 min. (Baršienė *et al.* 2004). The frequency of ten NAs (micronuclei (MN), bi-nucleated erythrocytes with nucleoplasmic bridge (BNb), nuclear bud on filament (NBf), nuclear buds (NB), blebbed (BL), fragmented-apoptotic (FA), 8-shaped, bi-nucleated (BN), ripped erythrocytes (RIPeryt), vacuolated nuclei (VacNuc) of erythrocytes were evaluated (per 1000 cells) by scoring 4000 erythrocytes at a 1000 × magnification using Olympus BX 51 and Nikon eclipse 50i bright-field microscopes. A total of 12.000 erythrocytes with intact cellular and nuclear membrane were examined for each fish specimen.

The endpoints of HM genotoxicity were assessed as frequencies of MN, BNb, NBf, NB, BL and summed genotoxicity (MN+NB+BNb) in mature (blood) and immature (kidney, liver) erythrocytes of fishes. The cytotoxicity effects were evaluated by scoring frequency of FA, 8-shaped, BN, RIPeryt, VACnuc and summed cytotoxicity (8+BN+RIPeryt) in the same tissues.

Blind scoring of MN and other NAs was performed on coded slides without knowledge about the origin of the samples. MN was identified using the following criteria: round or ovoid-shaped non-refractory particles with color and structure similar to chromatin, with a diameter 1/3–1/20 of the main nucleus and clearly detached from it. Nuclear buds, fragmented-apoptotic, bi-nucleated and other NAs were identified using criteria described by other scientists (Fenech *et al.* 2003; Harabawy *et al.* 2014).

The means of studied NAs, standard errors and significance level (p) values were calculated for each test group using the PRISM statistical package. The non-parametric Mann-Whitney U-test was used to compare

alteration frequencies between the control and exposed fish groups. One-way ANOVA was used to analyze NAs in different tissues of rainbow trout.

Results and discussion

The results of the present study showed potential genotoxicity and cytotoxicity of HMMM studied after 14-day exposure. The frequencies of ten NAs in the blood, kidney and liver of rainbow trout of control and exposed groups were analyzed (presented in Table 1).

The levels of NAs studied after exposure were higher in blood than in kidney erythrocytes, except for induction of BNb and VACnuc. The lowest amounts of NAs were recognized in liver erythrocytes of all analyzed tissues. Five of all parameters (BNb, NBf, NB, BL, FA) did not reveal significant elevated levels, so there will be no further discussion.

The MN frequencies were the highest in blood (1.90 ‰ MN/1000), followed by kidney and liver erythrocytes (0.45 ‰ and 0.39 ‰, respectively), while a control level of MN varied from 0.14 ‰ to 0.50 ‰. The MN frequency in blood erythrocytes in the control group increased significantly ($p = 0.0007$) and were the highest (3.8-fold) in all tissues (3.2-fold in kidney and 1.3-fold in liver erythrocytes). When the frequencies of MN and other genotoxicity parameters were summed up (MN+NB+BNb) and analyzed together, the highest increase in nuclear abnormalities was observed in kidney erythrocytes (4.3-fold) while in blood erythrocytes it was decreased (from 3.8-fold to 3.3-fold).

Results of genotoxicity parameters after treatment were analyzed with the non-parametric Mann-Whitney *U*-test that showed a statistically significant increase ($p = 0.0007$) of MN in blood erythrocytes of the test fish. The MN induction was insignificant in kidney and liver erythrocytes ($p = 0.1005$; $p = 0.8042$, respectively). The values of total genotoxicity (MN+NB+BNb) were significantly increased after exposure to HMMM in two tissues: blood ($p = 0.0077$) and kidney ($p = 0.0479$). The results are presented in Figure 1.

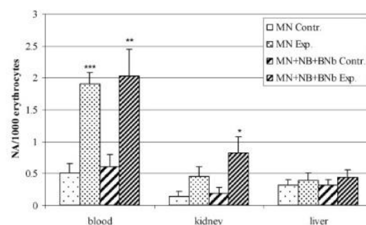


Fig. 1. Micronuclei and summed genotoxicity (MN+NB+BNb), (mean ± SEM, $N = 9–10$) induction in the blood, kidney and liver erythrocytes of rainbow trout

Asterisks (*) denote significant differences between the control and exposed fish groups (* – $p < 0.05$, (** – $p < 0.01$, (*** – $p < 0.001$)

Analysis showed that all cytotoxicity parameters (8-shaped, BN, RIPeryt and VACnuc) taken individually revealed significantly elevated levels in kidney erythrocytes ($p = 0.0409$; $p = 0.0425$; $p = 0.0417$; $p = 0.0114$, respectively). Furthermore, 8-shaped and BN were significant in blood erythrocytes ($p = 0.0316$; $p = 0.0464$, respectively). Three cytotoxicity parameters are presented in Figure 2.

Table 1. Frequencies of nuclear abnormalities in the blood, kidney and liver erythrocytes of rainbow trout (mean ± SEM, $N = 9–10$)

Parameters	Blood		Kidney		Liver	
	Control	Exposed	Control	Exposed	Control	Exposed
MN	0.50 ± 0.15	1.90 ± 0.18	0.14 ± 0.07	0.45 ± 0.16	0.31 ± 0.09	0.39 ± 0.12
BNb	0.03 ± 0.03	0.25 ± 0.17	0.00	0.30 ± 0.20	0.00	0.03 ± 0.03
NBf	0.03 ± 0.03	0.13 ± 0.05	0.00	0.03 ± 0.03	0.00	0.00
NB	0.06 ± 0.04	0.10 ± 0.04	0.03 ± 0.03	0.05 ± 0.03	0.00	0.03 ± 0.03
BL	0.17 ± 0.10	0.08 ± 0.04	0.08 ± 0.04	0.05 ± 0.03	0.00	0.06 ± 0.04
FA	0.00	0.00	0.00	0.00	0.00	0.00
8-shaped	0.31 ± 0.13	0.80 ± 0.22	0.19 ± 0.11	0.67 ± 0.19	0.16 ± 0.08	0.56 ± 0.26
BN	0.14 ± 0.11	2.93 ± 1.78	0.11 ± 0.11	2.40 ± 1.28	0.0	1.19 ± 0.90
RIPeryt	1.44 ± 1.09	13.05 ± 6.60	0.42 ± 1.15	9.75 ± 4.96	0.34 ± 0.24	7.47 ± 4.89
VACnuc	0.28 ± 0.09	0.55 ± 0.19	0.28 ± 0.10	1.55 ± 0.41	0.40 ± 0.15	0.53 ± 0.13
MN+NB+BNb	0.61 ± 0.19	2.03 ± 0.41	0.19 ± 0.09	0.82 ± 0.25	0.31 ± 0.09	0.44 ± 0.12
8+BN+RIPeryt	2.14 ± 1.57	16.75 ± 8.24	1.72 ± 1.36	12.83 ± 6.35	0.50 ± 0.23	9.22 ± 6.03

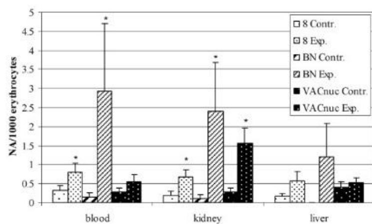


Fig. 2. Induction of 8-shaped, BN and VACnuc (mean ± SEM, $N = 9–10$) erythrocytes in blood, kidney and liver of rainbow trout

Asterisks (*) denote significant differences between the control and exposed fish groups ($p < 0.01$).

Only one parameter (RIPeryt; $p = 0.0264$) of cytotoxicity was significant in liver erythrocytes. The sum of three cytotoxicity parameters (8+BN+RIPeryt) showed significantly elevated levels observed in all tissues ($p < 0.05$) (Figure 3 represents one parameter (RIPeryt) of cytotoxicity).

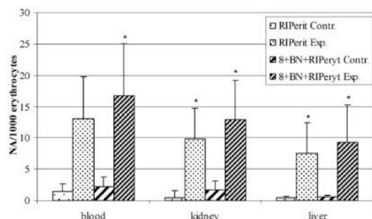


Fig. 3. Induction of RIPeryt and summed cytotoxicity (8+BN+RIPeryt), (mean ± SEM, $N = 9–10$) in rainbow trout blood, kidney and liver erythrocytes

Asterisks (*) denote significant differences between the control and exposed fish groups ($p < 0.01$).

Our study found out that the frequencies of NAs were different in all tissues of rainbow trout. Inter-tissue analysis of HMMM genotoxicity and cytotoxicity showed statistically higher induction of MN ($p < 0.0001$, $F = 21.59$, $R^2 = 0.6878$), MN+NB+BNb ($p < 0.0001$, $F = 8.352$, $R^2 = 0.4601$) and VACnuc ($p = 0.0012$, $F = 4.822$, $R^2 = 0.3298$) (Oneway ANOVA). Our findings are in agreement with the earlier study carried out by Ahmed *et al.* (2013) and Nunes Oss *et al.* (2013). They used a variety of inter-tissues (gills, blood and liver) for heavy metal toxicity research and found that the frequencies of NAs were different in the tissues analyzed. These results could be difficult to compare because of different combination of tissues used. The micronucleus test was mostly applied in peripheral blood erythrocytes of fish (Omar *et al.* 2012; Fuzinatto *et al.* 2013).

In this study, exceptionally rare occurrence of FA erythrocytes can be explained as very weak elimination of micronucleated damaged cells in rainbow trout. As a result, high levels of MN in peripheral blood were found. Besides, the differences between induction of MN in blood and kidney erythrocytes of rainbow trout indicated a weak detoxification of contaminants in the liver and their elimination via the fish kidney (Baršienė *et al.* 2006a).

According to some authors (Güner *et al.* 2011), the use of Cu (0.01 mg/l) and Cd (0.01 mg/l) mixture is more effective in causing total NAs than their individual use in this case for Mosquito fish (*Gambusia affins*). Yet these results are not significantly reliable for MN. The same results were recognized by Cavas *et al.* (2005). Affected with Cu (0.01 mg/l) and Cd (0.005 mg/l), MN frequencies (in blood erythrocytes) were different in three analysed fish species (*Carassius gibelio*, *Cyprinus carpio*, *Corydoras paleatus*) but an increase of MN in the control group was not significantly reliable. Many scientists agree that several NAs in different fish species are better biomarkers for Cu and Cd exposure than MN alone. Bonacker *et al.* (2005) used the CREST technique, a specific cytogenetic approach for the assessment of centromere presence; it disclosed that lead chloride exhibits predominantly aneugenic potency. This would explain why HMMM used in the present study resulted in a significantly reliable increase of MN in blood erythrocytes.

A significant increase in the frequency of MN and BN cells has been detected in the blood and gill cells of fish *Orechromis niloticus* exposed to Cr effluents (Cavas and Ergene-Gozukara 2005). Also the results of Ahmed *et al.* (2013) study showed that Cr (at 1.6; 3.5; 9.0 mg/l doses) causes toxic and tissue (gill, blood and liver) specific genotoxic effects in catfish *Heteropneustes fossilis*. Here, a statistically increased number of BN erythrocytes in blood ($p = 0.0464$) and kidney ($p = 0.0425$) of rainbow trout could be suspected as a result of alterations in chromosome segregation and cytokinesis (Ahmed *et al.* 2013).

Harabavy and Mosleh (2014) demonstrated that exposure of *Oreochromis niloticus* to metal mixture of Cd, Cu, Pb and Zn (1.25 mg/L of each) caused statistically significant formation of MN, BN, VACnuc,

kidney-shaped nuclei and other NAs in peripheral blood erythrocytes.

Generalizing our findings, it is obvious that piscine micronucleus test in peripheral blood is a valid technique for assessment of genotoxic effects caused by different contaminants and their mixtures in rainbow trout. The MN test in fish erythrocytes is a simple, cost-effective and rapid method for detection of genotoxic impacts at low concentrations of DNA reactive and non-DNA reactive compounds. Our study showed that HM in mixture even at maximum permissible concentrations induced genotoxic and cytotoxic alterations in the blood, kidney and liver erythrocytes of rainbow trout (perhaps due to synergistic interactions between metals) – a standard aquatic animal used in the studies of environmental pollutant effects (Bravo *et al.* 2011). The present study also revealed that fish can be potential bioindicators for environmental monitoring. More investigations must be performed on different fish species to confirm the impact of various HMMM on different tissues.

Conclusions

1. Non-parametric Mann-Whitney *U*-test showed a statistically significant increase in genotoxicity parameters in blood (MN: $p = 0.0007$; MN+NB+ BNb: $p = 0.0077$) and kidney (MN+NB+BNb: $p = 0.0479$) erythrocytes.
2. All cytotoxicity parameters showed significantly elevated levels in kidney (8-shaped: $p = 0.0409$; BN: $p = 0.0425$; RIPeryt: $p = 0.0417$; VACnuc: $p = 0.0114$; 8+BN+RIPeryt: $p = 0.0179$); 8-shaped, BN 8+BN+RIPeryt and in blood ($p = 0.0316$; $p = 0.0464$; $p = 0.0283$ respectively); RIPeryt and 8+BN+RIPeryt ($p = 0.0264$; $p = 0.0340$) in liver erythrocytes.
3. Inter-tissue analysis of HMMM genotoxicity and cytotoxicity revealed statistically higher (One-way ANOVA) induction of MN ($p < 0.0001$, $R^2 = 0.6878$), MN+NB+BNb ($p < 0.0001$, $R^2 = 0.4601$) and VACnuc ($p = 0.0012$, $R^2 = 0.3298$).
4. Heavy metals in mixture even at maximum permissible concentrations induce genotoxic and cytotoxic alterations, perhaps due to synergistic interactions between metals.
5. Rainbow trout is an aquatic organism sensitive to HM exposure at low concentrations and can therefore be used as a potential bioindicator for environmental monitoring.

Acknowledgements

We are very thankful to habil. dr. Janina Baršienė for patient guidance, advice and encouragement in preparing the article.

References

Ahmed, M. K.; Kundu, G. K.; Al-Mamun, M. H., Sarkar, S. K.; Akter, M. S.; Khan, M. S. 2013. Chromium (VI) induced acute toxicity and genotoxicity in freshwater stinging catfish, Heteropneustes fossilis, *Ecotoxicology and Environmental Safety* 92: 64–70.

Baršienė, J.; Andreikėnaitė, L.; Rybakovas, A. 2006a. Cytogenetic damage in perch (*Perca fluviatilis* L.) and duck mussel (*Anodonta anatina* L.) exposed to crude oil, *Ekologija* 1: 25–31.

Baršienė, J.; Dedonytė, V.; Rybakovas, A.; Andreikėnaitė, L; Andersen, O. K. 2006b. Investigation of micronuclei and other nuclear abnormalities in peripheral blood and kidney of marine fish treated with crude oil, *Aquatic Toxicology*. 78(1): 99–104.

Baršienė, J.; Lazutka, J.; Šyvokienė, J.; Dedonytė, V.; Rybakovas, A.; Bagdonas, E.; Bjornstad, A.; Andersen, O. K. 2004. Analysis of micronuclei in blue mussels and fish from Baltic and North Seas, *Environmental toxicology* 19: 365–371.

Baršienė, J.; Rybakovas, A.; Forlin, L.; Šyvokienė, J. 2008. Environmental genotoxicity studies in mussels and fish from the Göteborg area of the North Sea, *Acta Zoologica Lituanica* 18(4): 240–247.

Bolognesi, C.; Hayashi, M. 2011. Micronucleus assay in aquatic animals, *Mutagenesis* 26: 205–13.

Bonacker, D.; Stoiber, T.; Bohm, K. J.; Prots, L.; Wang, M.; Unger, E.; Their, R.; Bolt, H. M.; Degen, G. H. 2005. Genotoxicity of inorganic lead salts and disturbance of microtubule function, *Environmental Molecular Mutagenesis* 45: 346–353.

Bravo, C. F.; Curtis, L. R.; Myers, M. S.; Meador, J. P.; Johnson, L. L.; Buzitis, J.; Collier, T. K.; Morrow, J. D.; Laetz, C. A.; Loge, F. J.; Arkoosh, M. R. 2011. Biomarker responses and disease susceptibility in juvenile Rainbow trout Oncorhynchus mykiss fed a high molecular weight PAH mixture, *Environmental Toxicology and Chemistry* 30(3): 704–714.

Carrasco, K. R.; Tilbury, K. L.; Myers, M. S. 1990. Assessment of the piscine micronucleus test as an *in situ* biological indicator of chemical contaminant effects, *Canadian Journal of Fish and Aquatic Science* 47: 2123–2136.

Cavas, T.; Ergene-Gozukara, S. 2003. Micronuclei, nuclear lesions and interphase silver-stained nucleolar organizer regions (AgNORs) as cyto-genotoxicity indicators in *Oreochromis niloticus* exposed to textile mill effluent, *Mutation Research* 538: 81–91.

Cavas, T.; Ergene-Gozukara, S. 2005. Induction of micronuclei and nuclear abnormalities in *Oreochromis niloticus* following exposure to petroleum refinery and chromium processing plant effluents, *Aquatic Toxicology* 74: 264–271.

Cavas, T.; Garanko, N. N.; Arkhipchuk, V .V. 2005. Induction of micronuclei and binuclei in blood, gill and liver cells of fishes subchronically exposed to cadmium chloride and copper sulphate, *Food and Chemical Toxicology* 43: 569–574.

Dailianis, S.; Domouhtsidou, G.P.; Raftopoulou, E.; Kalayianni, M.; Dimitriadis, V. K. 2003. Evaluation of neutral red retention assay, micronucleus test, acetylcholinesterase activity and a signal transduction molecule (cAMP) in tissues of *Mytilus galloprovincialis* (L.), in pollution monitoring, *Marine Environmental Research* 56: 443–470.

EUR-Lex 2008. *Directive on environmental quality standards (Directive 2008/105/EC) Official Journal of the European Union* 51: 84–97.

Fenech, M.; Chang, W. P.; Kirsch-Volders, M.; Holland, N.; Bonassi, S.; Zeiger, E. 2003. HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures, *Mutation Research* 534: 65–75.

Fuzinatto, C. F.; Flohr, L.; Melegari, S. P.; Matias, W. G. 2013. Induction of micronucleus of Oreochromis niloticus exposed to waters from the Cubatão do Sul River, southern Brazil, *Ecotoxicology and Environmental Safety* 98: 103–109.

Guardiola, F. A.; Cuesta, A.; Meseguer, J.; Martínez, S.; Martínez-Sánchez, M. J.; Pérez-Sirvent, C.; Esteban, M. A. 2013. Accumulation, histopathology and immuno-toxicological effects of waterborne cadmium on gilthead seabream (*Sparus aurata*), *Fish & Shellfish Immunology* 35: 792–800.

Güner, U.; Muranli, F. D. G. 2011. Micronucleus Test, Nuclear Abnormalities and Accumulation of Cu and Cd on *Gambusia affinis* (Baird & Girard, 1853), *Turkish Journal of Fisheries and Aquatic Sciences* 11: 615–622.

Harabawy, A. S. A.; Mosleh, Y. Y. I. 2014. The role of vitamins A, C, E and selenium as antioxidants against genotoxicity and cytotoxicity of cadmium, copper, lead and zinc on erythrocytes of Nile tilapia, Oreochromis niloticus, *Ecotoxicology and Environmental Safety* 104: 28–35.

Heddle, J. A.; Cimino, M. C.; Hayashi, M.; Romagna, F.; Shelby, M. D.; Tucker, J. D.; Vanparys, P.; MacGregor, J. T. 1991. Micronuclei as an index of cytogenetic damage: past, present, and future, *Environmental and Molecular Mutagenesis* 18: 277–291.

Jezierska, B.; Sarnowski, P. 2002. The effect of mercury, copper and cadmium during single and combined exposure on oxygen consumption of *Oncorhynchus mykiss* (WAL.) and *Cyprinus carpio* (L.) larvae, *Archives of Polish Fisheries* 10(1): 15–22.

Jha, M. K.; Upadhaya, R. R.; Lee, Jae-chun; Kumar, V. 2008. Treatment of rayon waste effluent for the removal of Zn and Ca using Indion BSR resin, *Desalination* 228: 97–107.

Jiraungkoorskul, W.; Sahaphong, S. 2007. Efficacy of ascorbic acid reducing waterborne copper toxicity in butterfish (Poronotus triacanthus), *Journal of Biological Sciences* 7: 620–625.

Malik, N.; Biswas, A. K.; Qureshi, T. A.; Borana, K.; Virha, R. 2010. Bioaccumulation of heavy metals in fish tissues of a freshwater lake of Bhopal, *Environmental Monitoring and Assessment* 160:267–276.

Nunes Oss, R.; Baroni,V. D.; Duarte, R. M.; Val, A. L.; Almeida Val, V. M. F.; Gomes, L. C. Recovery of fat snook, Centropomus parallelus (Teleostei: Perciformes) after subchronic exposure to copper, *Comparative Biochemistry and Physiology*. Part C. 157: 306–309.

Okonkwo, J.C.; Obiakor, M. O.; Nnabude, P. C. 2011. Micronuclei profile: an index of chromosomal aberrations in freshwater fishes (Synodontis clarias and Tilapia nilotica), *Online Journal of Animal and Feed Research* 1(1): 40–45.

Omar, W. A.; Zaghloulb, K. H.; Abdel-Khaleka, A. A.; Abo-Hegaba, S. 2012. Genotoxic effects of metal pollution in two fish species, Oreochromis niloticus and Mugil cephalus, from highly degraded aquatic habitats, *Mutation Research* 746: 7–14.

Roy, S. P. 2010. Overview of heavy metals and aquatic environment with notes on their recovery, *Ecoscan: an international Quarterly Journal of Environmental Sciences* 4: 235–240.

Svecevičius, G.; Idzeli,s R. L.; Mockutė, E. 2014. Bioaccumulation of heavy metals in different body tissues of gibel carp *Carassius gibelio* exposed to a model mixture (Cu, Zn, Ni, Cr, Pb, Cd) and to nickel separately, *Journal of Environmental Engineering and Landscape Management* 22(4): 292–300.

Talapatra, S. N.; Banerjee, S. K. 2007. Detection of micronucleus and abnormal nucleus in erythrocytes from the gill and kidney of Labeo bata cultivated in sewage-fed fish farms, *Food and Chemical Toxicology* 45: 210–215.

US EPA. 2009. *National recommended water quality criteria*. Office of Water. Office of Science and Technology (4304T). Washington, DC. 25.

Venier, P.; Minisi, S.; Voltan, R.; Ciccotti, E.; Pinna, A. 1997. Formation and persistence of DNA adducts and micronuclei in rainbow trout after treatment with benzo[*a*]pyrene, *Mutation Research* 379: S94.

Waisberg, M.; Joseph, P.; Hale, B., Beyersmann, D. 2003. Molecularandcellular mechanism of cadmium carcinogenesis, *Toxicology* 192: 95–117.

Wirzinger, G.; Weltje, L.; Gercken, J.; Sordyl, H. 2007. Genotoxic damage in field-collected three-spined sticklebacks (*Gasterosteus aculeatus* L.): A suitable biomonitoring tool? *Mutation Research* 628: 19–30.

BRANDUOLIO PAŽAIDŲ SUSIDARYMAS VAIVORYKŠTINIO UPĖTAKIO (*ONCORHYNCHUS MYKISS*) ERITROCITUOSE PAVEIKUS SUNKIŲJŲ METALŲ MODELINIU MIŠINIU (ZN, CU, NI, CR, CD, PB) ESANT DIDŽIAUSIOMS LEISTINOMS KONCENTRACIJOMS

R. Valskienė, M. Stankevičiūtė, L. Butrimavičienė, J. Greiciūnaitė, G. Svecevičius

Santrauka

Genotoksinis bei citotoksinis poveikis vertintas vaivorykštinį upėtakį (*Oncorhynchus mykiss)* 14 parų paveikus sunkiųjų metalų modeliniu mišiniu (SMMM), atitinkančiu didžiausias leistinas sunkiųjų metalų koncentracijas (Zn-0,1, Cu-0,01, Ni-0,01, Cr-0,01, Pb-0,005 ir atitinkamai Cd-0,005 mg/l) vandenų telkiniuose-priimtuvuose. Žuvų kraujo, inkstų bei kepenų eritrocituose buvo vertinti dešimt skirtingų branduolio pažaidų. Gauta statistiškai patikima indukcija MN, 8-shaped, BN, MN+NB+BNb ir 8+BN+RIPeryt kraujo; 8-shaped BN, RIPeryt, VACnuc, MN+NB+BNb ir 8+BN+RIPeryt inkstų; RIPeryt ir 8+BN+RIPeryt kepenų eritrocituose.

Reikšminiai žodžiai: mikrobranduoliai, branduolio pažaidos, vaivorykštinis upėtakis, sunkiųjų metalų mišinys.

X

Experimental studies on the toxicity and geno-cytotoxicity effects of cadmium in embryos and larvae of rainbow trout, *Oncorhynchus mykiss*

Kazlauskienė N, Cibulskaitė Ž, Stankevičiūtė M, Baršienė J

Proceedings of the 13th International Conference on Protection and Restoration of the Environment 2016, ISBN 978-960-6865-94-7. Mykonos island, Greece. p. 449–459

Experimental studies on the toxicity and geno-cytotoxicity effects of cadmium in embryos and larvae of rainbow trout, *Oncorhynchus mykiss*

N. Kazlauskienė, Ž. Cibulskaitė*, M. Stankevičiūtė, J. Baršienė

Nature Research Centre, Akademijos str. 2, LT–08412 Vilnius, Lithuania
*Corresponding author: Email: ziwiliukx@gmail.com

Abstract

Cadmium (Cd) is one of the metals that have irrefutable negative effect to biota. The purpose of the study were: (1) to determine the 96-hour LC_{50} of Cd to rainbow trout *Oncorhynchus mykiss* embryos and larvae; (2) to investigate the toxic effect of Cd (2 μg Cd L^{-1} as $CdCl_2{\cdot}H_2O$) on biological parameters of rainbow trout in early ontogenesis (embryos and larvae) in relation with the duration of exposure; and (3) to evaluate geno-cytotoxicity potential in erythrocytes of the fish embryos and larvae exposure to Cd. Bioassay testing was conducted under controlled laboratory conditions. The 96-hour LC_{50} values of Cd were determined to embryos and larvae. Chronic test on fish at early stages of development (starting from "eye-egg" embryos 12-day before hatching and continued 12-day after hatching and including hatching period) was performed under static conditions. Several endpoints including mortality; function of the cardio-respiratory system; developmental disorders; hatching rate and cytogenetic damage [micronucleus (MN) and other nuclear abnormalities (NA) assays] in embryos and larvae were investigated. Cadmium at sublethal concentration showed a negative effect on the biological parameters of embryos and larvae which is related to the duration of exposure. Various developmental disorders were observed. Long-term exposure of embryos to Cd induced significant formation of MN and pooled cytotoxicity and caused significant increase of all geno-cytotoxicity endpoints analyzed in larvae erythrocytes. This is the first reported attempt to estimate different biological impacts on fish in early ontogenesis and herewith is an important step in understanding the Cd toxicity and geno-cytotoxicity mechanisms.

Keywords: cadmium; fish; embryos; larvae; toxicity; geno-cytotoxicity

1. INTRODUCTION

Heavy metal cadmium (Cd) is widely used in various modern industrial fields electroplating and galvanization processes in the production of pigments in batteries, as a chemical reagent, and in miscellaneous industrial processes and agriculture [1, 2]. Nanotechnology poses yet another risk for toxic Cd, which will now enter the biological realm in a nano-form [3]. The nanocrystal cores of many types of nanoparticles are miscible in water and contain the toxic metal Cd, so possible release of Cd from the nano-form cores presents an environmental concern [4].

Cd toxicity in different freshwater fish species has been extensively investigated by various scientists. Some authors demonstrated the morphological, physiological, hematological, biochemical and immunological changes in adult fish (*Clarias gariepinus*, *Oreochromis mossambicus*) experimentally treated with Cd [5, 2]. In addition, the Cd toxicity effects in developing fish may serve as important bioindicators for this type of pollution [6-9].

Cd has been reported to exert deleterious effects of nephrotoxicity, cytotoxicity, mutagenicity, genotoxicity, immunotoxicity, as well as, clastogenic, teratogenic, carcinogenic impacts in fish [10-

Editors: A. Kungolos, C. Christodoulatos, A. Koutsospyros,
C. Emmanouil, C. Laspidou, Z. Mallios, D. Dermatas
ISBN: 978-960-6865-94-7

13, 6]. Cd that enters aquatic environments accumulates within the tissues of aquatic organisms.

The accumulation in the organisms depends on the concentration, route of absorption, environmental conditions and other intrinsic factors [14]. Cd has numerous adverse effects in fish reproductive processes, such as sexual maturation, spermatogenesis, fertilization success and development of the embryonic and post embryonic stages [15]. During the acute exposure of Cd, the freshwater fish showed some abnormal activities like erratic swimming, equilibrium loss and enhanced surfacing behavior [16]. Moreover, it has adverse effects on cellular defense systems and enzymes induce lipid peroxidation, DNA strand breaks, chromosome aberrations, disruption of DNA repair, and apoptosis [10, 11, and 13].

The molecular mechanisms underlying Cd toxicity and their specific effects on fish are poorly understood [7]. Cd in ionic form gain entry into cells by simple diffusion or through membrane carriers and ion channels [15]. According to Bougas and co-authors [7] this metal can affect the transcription level of genes involved in iron metabolism, vitamin metabolism, blood coagulation, and calcium transport.

In recent years, early fish development studies have gained interest amongst researchers because of their high sensitivity to pollutants and their ecological relevance [15]. Our research focuses on nanoparticle (quantum dots) and heavy metal toxicity mechanisms in developing fish, in particular, the Cd toxicity and geno-cytotoxicity effects in early fish development. The comprehensive investigation of biological impacts in early fish development is an important step in understanding the Cd toxicity and geno-cytotoxicity mechanisms.

The main aims of the study were: (1) to determine the 96-hour LC_{50} of Cd to rainbow trout *Oncorhynchus mykiss* embryos and larvae; (2) to investigate the toxic effect of Cd (2 μg Cd/L as $CdCl_2 \cdot H_2O$) on biological parameters in early development in relation to the duration of exposure; and (3) to evaluate geno-cytotoxicity potential of Cd in erythroblasts of the fish embryos and larvae.

2. MATERIALS AND METHODS

2.1 Test-object

The toxicity study was performed at the Laboratory of Ecology and Physiology of Hydrobionts (Nature Research Centre). Rainbow trout (*Oncorhynchus mykiss*) embryos were obtained as eyed eggs from the Simnas experimental hatchery (Alytus District, Lithuania).

2.2 Bioassay testing

Bioassay testing was carried out under controlled laboratory conditions. The tests on fish embryos and larvae were performed accordance with guidelines presented in ISO standards (ISO 7346-1:1996, ISO 12890:1999). Studies have been carried out with non-protected life-stages accordance with EU Directive 2010/63/EU. Acute test (96-hour) on fish at early stages of development [starting from "eye-egg" embryos (96-hour before hatching) – the first test; hatched larvae at hatching period – the second test (the first test continue); and starting from 1-day old larvae – the third test] were performed under semi static conditions. Control water and test solutions in aquaria were additionally aerated/changed at 24-hour intervals. Chronic tests: 12-day (starting from "eye-egg" embryos 12-day before hatching) on embryos and 24-day (starting from "eye-egg" embryos 12-day before hatching and continued 12-day after hatching and including hatching period) on larvae were performed under static conditions. All tests were performed in the climatic camera (Bronson PGC-660, Germany). Studies with embryos and larvae were performed in two replications (N = 20 in each group).

ISBN: 978-960-6865-94-7

2.3 Water chemical and physical characteristics

Deep-well water was used for dilution and control test [17]. Dissolved oxygen in the glass aquaria, temperature, pH and conductivity were measured routinely with a hand-held multi-meter (WTW Multi 340i/SET, Germany).

2.4 Tests-parameters

Biological parameters as mortality, physiological (cardio-respiratory), such as heart rate (HR, counts/min), gill ventilation frequency (GVF, counts/min), hatching rate (%), blood circulatory system development disorders (%) of embryos and larvae were investigated. Blood circulation system was viewed using stereomicroscope (Meiji Techno RZ Series, Japan), and observed abnormalities and lesions were registered by Nikon Cool pix 995 digital camera (Japan). The number of hatched eggs and the mortality of embryos and larvae were monitored and recorded daily. Other parameters of embryos and larvae were monitored and recorded after 1, 4, 8 and 12 days. Mortalities and hatching rates were then recorded during hatching period (1-6-day). Total number of eggs in each treatment was 20 fertilized eggs (two replications). Unfertilized eggs were approximately 2%.

2.5 The concentration of Cd

Reagent grade cadmium chloride ($CdCl_2 \cdot H_2O$) («REACHIM» Company, Russia) was used as the toxicant and stock solutions were prepared by dissolving a necessary amount of salts in distilled water. The concentrations in acute test were 0.5; 1.0; 2.0; 4.0; 8.0 µg Cd/L. The concentration of 2 µg Cd/L was chosen according to the 96-hour LC50 for rainbow trout larvae (Table 1). Nominal Cd concentrations were checked with an atomic absorption spectrophotometer (SHIMADZU AA-6800, Japan). Mean measured concentrations were within 5% of target [17].

2.6 Micronucleus (MN) and nuclear abnormalities (NA) assays

Induction of micronuclei (MN), nuclear buds (NB), bi-nucleated (BN), fragmented-apoptotic (FA) cells were analysed in erythroblasts of embryos and larvae. Cell smears were prepared from whole embryos and larvae (with removed yolk sac) body (gently nipped with tweezers): directly smeared on glass slides and air-dried. Smears were fixed in methanol for 10 min. and later were stained with 10 % Giemsa solution in phosphate buffer pH = 6.8 for 20 - 40 min. The stained slides were analysed under light microscopes Olympus BX51 at final magnification of 1,000×. Micronuclei and other NA were identified following criteria described by Fenech et al. [18]. The frequencies of abnormalities were recorded in 1,000 erythroblasts per slide using blind scoring by a single observer.

2.7 Statistical analyses

The 96-hour Median-Lethal-Concentration (LC50) values and their 95 % confidence intervals were estimated using the trimmed Spearman-Karber method [19]. Means and standard deviations were calculated for embryos and larvae to each studied parameter. Differences between the evaluated characteristics studied were tested by two-way ANOVA at $p < 0.05$ using Statistica 7.0 software's (USA). Pearson correlation analysis was performed to identify possible relationships between biological parameters of embryos and larvae and the duration of exposure. Micronucleus and nuclear abnormalities assays were evaluated by non-parametric Mann-Whitney *U*-test (GraphPad Prism 5, USA). Differences were accepted as significant at the 95% level of confidence ($p < 0.05$).

ISBN: 978-960-6865-94-7

3. RESULTS AND DISCUSSION

3.1 Acute toxicity

Mortality of embryos/larvae increased with increasing Cd concentration. The highest mortality of embryos was observed at 8 μg Cd/L, where 97.5 % embryos died during exposure period (first test). In the exposure period 96-hour at 4 μg Cd/L 90 % of hatched larvae (second test) and 89 % of larvae (third test) were dead (Table 1). The calculated 96-hour LC50 values of Cd for rainbow trout embryos/larvae were: 3.32, 1.14, and 2.23 μg Cd/L, respectively. The larvae were consistently more sensitive than the embryos. The most sensitive were hatched larvae.

Eaton with co-authors [20] found that concentrations ranging from 4 to 12 μg Cd/L were fatal to the embryos and larvae of freshwater fish. Fish at early stages of development are more sensitive to toxicants [21]. The larvae were consistently more sensitive than the embryos [20]. Jezierska and co-authors [14] indicated that hatching is a particularly sensitive developmental period. The difference in sensitivities is most likely due to a chorion protecting the embryonic fish [21]. During embryonic development, the embryos are protected by the egg shell and so the chance of metals entering into the egg is relatively small [14].

Table 1 Acute toxicity of Cd to rainbow trout in early life stages

Test	Rainbow trout (*Oncorhynchus mykiss*)	96-hour LC50 (μg Cd/L)	0.95 confidence interval (μg Cd/L)
First (start from eyed embryos 96-hour before hatching)	Eye-stage embryos	3.32	2.67-4.13
Second (continuous first test included hatching period)	Hatched larvae	1.14	0.72-1.56
Third (start from 1-day old larvae)	Hatched larvae	2.23	1.78-2.79

3.2 Chronic toxicity

3.2.1 Mortality

The concentration of 2 μg Cd/L induced a significant ($p < 0.05$) increase in mortality of embryos (chronic test 12-day) after 1-, 4-, 8- and 12-day of exposure, ranging from 6.4 ± 1.1 % to 15.5 ± 1.7 % (in control mortality was from 0.0 ± 0.0 % to 5.2 ± 0.6 %). Mortality of embryos significantly increased ($p < 0.05$) with the exposure duration (12-day) compared to the 1-day exposure (Table 2 and Figure 1a).

Treatment with 2 μg Cd/L had profound effects on the overall health conditions of the larvae (chronic test 24-day). After 1-, 4-, 8- and 12-day exposure the mortality of larvae significant increased ($p < 0.05$) to 25.8 ± 1.2; 36.7 ± 4.7; 49.2 ± 13.0; 49.2 ± 13.0 %, respectively, compared to the control (control respective values 0.0 ± 0.0 to 2.5 ± 3.5 %; Table 4 and Figure 1a). Mortality of larvae significantly increased ($p < 0.05$) with the increase in the exposure duration (4-, 8- and 12-day) compared to the 1-day exposure (Table 3 and Figure 1a).

Mortality of embryos and larvae of various fish species due to Cd exposure has been reported before. Mortality was observed in embryos and pro-larvae of *Silurus soldatovi* exposed to various concentrations of Cd [22]. According to Ismail and Yusof [23], exposure of fertilized eggs of *Oryzias javanicus* to 100 μg Cd/L completely inhibited development and resulted in death of all embryos. Cd was also observed to cause higher mortality of newly hatched larvae than embryos [14]

Table 2 Chronic effect of Cd on rainbow trout embryos mortality and HR

13th International Conference on Protection and Restoration of the Environment
Editors: A. Kungolos, C. Christodoulatos, A. Koutsospyros, C. Emmanouil, C. Laspidou, Z. Mallios, D. Dermatas
ISBN: 978-960-6865-94-7

in relation with the duration of exposure (mean ± SD)

Exposure days	Parameters	
	Mortality % (N = 38)	HR counts/min (N = 20)
Cd (2 μg Cd/L)		
1	6.4 ± 1.1*#	77.6 ± 6.5*#
4	6.4 ± 1.1*#	78.8 ± 5.3*#
8	9.1 ± 2.8*#	85.2 ± 6.5*
12	15.5 ± 1.7*	88.0 ± 5.7*
Control		
1	0.0 ± 0.0	95.2 ± 6.5
4	0.0 ± 0.0	94.4 ± 6.3
8	5.2 ± 0.6	95.2 ± 2.5
12	5.2 ± 0.6	96.0± 3.3

* Significantly different from control ($p < 0.05$); # Significantly different between the duration of exposure compared with the 12-day exposure ($p < 0.05$)

Table 3 Chronic effect of Cd on rainbow trout larvae in relation with the duration of exposure (mean ± SD)

Exposure days	Parameters		
	Mortality % (N = 37)	GVF counts/min (N = 20)	HR counts/min (N = 20)
Cd (2 μg Cd/L)			
1	25.8 ± 1.2*	88.0 ± 6.5*	97.6 ± 5.7*
4	36.7 ± 4.7*#	97.2 ± 7.6*#	93.2 ± 8.4*
8	49.2 ± 13.0*#	118.4 ± 4.2*#	97.6 ± 9.6
12	49.2 ± 13.0*#	127.6 ± 6.2*#	116.8 ± 7.3*#
Control			
1	0.0 ± 0.0	104.8 ± 6.5	109.6 ± 5.4
4	0.0 ± 0.0	108.0 ± 6.5	106.0 ± 3.9
8	2.5 ± 3.5	110.0 ± 6.4	104.0 ± 4.1
12	2.5 ± 3.5	113.6 ± 7.4#	109.6 ± 6.3

* Significantly different from control ($p < 0.05$); # Significantly different between the duration of exposure compared with the 1-, 2-, 4-day exposure ($p < 0.05$)

3.2.2 Cardio-respiratory parameters

The concentration of 2 μg Cd/L significantly decreased heart rate (HR) of embryos during the entire test (chronic test 12-day) (Table 2, Figure 1b). HR of embryos during 12-day exposure was within the range 77.6 ± 6.5–88.0 ± 5.7 counts/min (in control 96.0 ± 3.3 counts/min). HR of embryos exposed to only 1- and 4-day Cd was significantly ($p < 0.05$) different from 12-day exposure.

Cd induced a significant ($p < 0.05$) decrease in HR of larvae during 1- and 4-day (chronic test 24-day) and were within the range 93.2 ± 8.4–97.6 ± 5.7 counts/min (Table 3, Figure 1c). Meanwhile, after 12-day exposure HR of larvae significantly increased ($p < 0.05$) to 116.8 ± 7.3 counts/min as compared to control (109.6 ± 6.3 counts/min) (Table 3, Figure 1b). It was found that

ISBN: 978-960-6865-94-7

HR of larvae significantly ($p < 0.05$) increased only on the 12-day of exposure (compared with stable HR during the 1-, 4- and 8-day exposure).

Significantly ($p < 0.05$) decreased gill ventilation frequency (GVF) of larvae after 1- and 4-day of exposure of 88.0 ± 6.5, 97.2 ± 7.6 counts/min, respectively (in control 104.8 ± 6.5; 108.0 ± 6.5 counts/min) (Table 3 and Figure 1d) was observed (chronic test 24-day). After 12-day exposure GVF of larvae significantly increased ($p < 0.05$) to 127.6 ± 6.2 counts/min comparing to control - 113.6 ± 7.4 counts/min. It was found that respiratory parameters of larvae were significantly ($p < 0.05$) related to the duration of exposure. GVF of larvae was significantly induced ($p < 0.05$) with the increase in the duration of exposure (Table 3, Figure 1c).

The Pearson correlation analysis of mortality, HR, GVF and the duration of exposure (1-, 4-, 8- and 12-day) of embryos and larvae showed a positive correlation. Strong correlation between mortality and the duration of exposure of embryos (r = 0.88) and larvae (r = 0.77); between HR and the duration of exposure of embryos (r = 0.60) as well as larvae (r = 0.61); and between GVF and the duration of exposure of larvae (r = 0.92) were found.

According to Reddy and Reddy [24] cadmium chloride caused negative effects on opercular beats/minute of *Catla catla.* The differences between means of opercular beats of exposed and control fish were highly significant at 24, 48, 72 and 96 hours exposure periods. It was pointed that Cd induced gill necrosis in fish, which may also be a cause of decrease in oxygen consumption and increase in ventilation frequency [24]. Jezierska et al. [14] study revealed that Cd exposure caused an increase in HR of embryos and larvae of common carp and grass carp. HR is a reliable indicator of metabolic rate in embryos [14].

3.2.3 Developmental disorders

In our study, 8-day exposure of rainbow trout eyed embryos (test started 12-day before hatching) to 2 μg Cd/L resulted in visible embryonic haemorrhage in the head area ~14 ± 1.4 % that was significantly different ($p < 0.05$) from control (Figure 2a). Meanwhile, even after 4-day exposure of larvae to the same concentration of Cd various developmental disorders were found (Figure 1a, b, c and d). During 4- and 12-day blood clot was the most visible in larval head area and in other parts of the body: around the eyes, in the yolk sac or in the dorsal fin (29 ± 1.4 % larvae, significantly different ($p < 0.05$) from control (Figure 2b). Spine curvature deviations ~5 ± 1.4 % (Figure 2c) and yolk sac edema ~6.5 ± 2.1 % (Figure 2d) were observed in larvae of rainbow trout (in control 2.5 ± 3.5 %).

Malformations of the yolk sac and curvature in the abdominal region were also observed throughout El-Greisy and El-Gamal [25] study. The impact of heavy metals was the most pronounced during the hatching process of common carp. Embryonic deformations in common carp like spine curvature, incomplete eye pigmentation, lack of tail, lack of head were the most commonly observed. The newly hatched larvae showed severe body malformation, and they were not able to swim or feed [25]. Sublethal effects in fish, notably malformation of the spine, have been reported [6]. More specifically, Cd affected genes involved in the blood coagulation cascade, calcium ion binding and apoptosis [7].

3.2.4 Hatching rate

The percentage of egg hatching rate was recorded during the hatching period (1-6-day) (Table 4, Figure 1d). The hatching rate in the larvae was significantly ($p < 0.05$) lower for the treated group when compared with control. Meanwhile, the duration of exposure and larvae hatching period had significantly ($p < 0.001$) correlated (r = 0.95).

The El-Greisy and El-Gamal [25] study indicated that the fertilization and hatching rates as well as the development of the embryos of common carp were adversely affected by heavy metal treatment. The percentage of fertilization and hatching rates and deformed eggs were recorded 96-hour post-hatching. When compared to the control group, it was found that the both rates in the larvae treated with 0.03 and 0.06 mg of $CdCl_2$/L had decreased. Fertilization and hatching rates decreased in

Editors: A. Kungolos, C. Christodoulatos, A. Koutsospyros, C. Emmanouil, C. Laspidou, Z. Mallios, D. Dermatas
ISBN: 978-960-6865-94-7

relation to the increase in the concentration of the heavy metals [25]. Other investigators observed the acceleration of larvae hatching after exposure to a high concentration of Cd (0.2 mg Cd/L or higher) [14, 26]. Thus it is possible that Cd damaged the hatching glands, disturbed the egg envelope hardening, and could act as an additive activator of chorionase (due to the chemical similarity of Cd and calcium) or oxygen deficit in water could cause the acceleration of hatching [26].

Table 4 Effect of Cd on the hatching rate (1-6-day during hatching) in rainbow trout eggs

Experimental	Hatching rate (%)					
trials	**1-day**	**2-day**	**3-day**	**4-day**	**5-day**	**6-day**
Cd	0.0±0.0	3.6±5.1	22.6±8.4*	53.6±5.1*	59.9±6.2*	62.7±2.2*
Control	2.4±3.4	5.2±0.6	63.9±3.9	89.7±1.8	94.8±0.6	94.8±0.6

* Significantly different from control ($p < 0.05$)

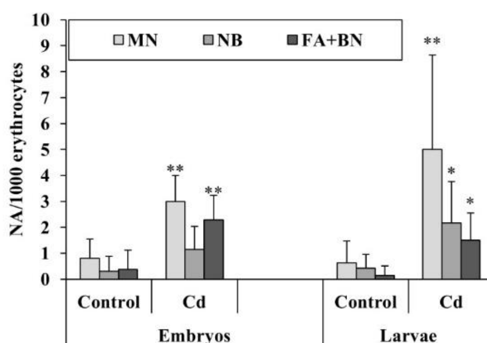


Figure 1. The frequency of micronuclei (MN), nuclear buds (NB) and fragmented-apoptotic (FA) + bi-nucleated (BN) cells in erythroblasts of 4-day embryos and larvae. Differences between control and exposed groups shown: *$p < 0.05$; **$p < 0.005$.

3.2.5 Micronucleus (MN) and nuclear abnormalities (NA) assays

Cd exposure caused both genotoxic and cytotoxic effects during developmental periods of rainbow trout (Figure 1). After 96-hour exposure to Cd, significant elevation of MN and pooled cytotoxicity (bi-nucleated (BN) + fragmented-apoptotic (FA) cells) were detected in the embryo erythroblasts. Micronuclei and NB frequencies increased approximately 4 times, pooled cytotoxicity increased 6 times compared to the control levels. The long-term exposure of larvae to Cd significantly induced the formation of MN, NB and pooled cytotoxicity (Figure 2 g-j). Micronuclei, NB and pooled cytotoxicity frequencies increased approximately 8, 5, 11 times, respectively compared to control level. This study confirms that the MN and NA assays can be used successfully to detect Cd genotoxicity and cytotoxicity in erythroblasts from whole embryos and larvae.

Editors: A. Kungolos, C. Christodoulatos, A. Koutsospyros, C. Emmanouil, C. Laspidou, Z. Mallios, D. Dermatas
ISBN: 978-960-6865-94-7

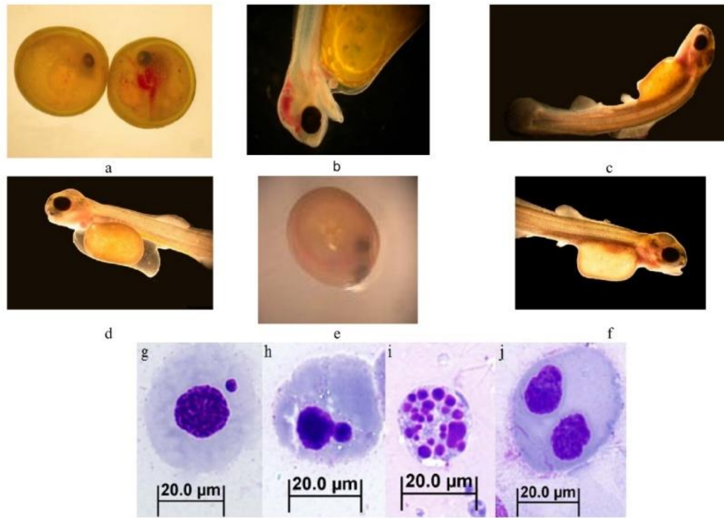


Figure 2. Development disorders induced by Cd: (a) haemorrhage in embryos, (b) blood clot in head, (c) spine curvature, (d) yolk sac edema of embryos and larvae of rainbow trout during exposure of Cd (chronic tests 12-, 24-day), (e) normal embryos, (f) normal larvae; larvae erythroblasts with (g) micronucleus (MN), (h) nuclear bud (NB), (i) fragmented-apoptotic (FA) erythroblast, (j) bi-nucleated (BN) erythroblast.

3.2.5 Micronucleus (MN) and nuclear abnormalities (NA) assays

Cd exposure caused both genotoxic and cytotoxic effects during developmental periods of rainbow trout (Figure 1). After 96-hour exposure to Cd, significant elevation of MN and pooled cytotoxicity (bi-nucleated (BN) + fragmented-apoptotic (FA) cells) were detected in the embryo erythroblasts. Micronuclei and NB frequencies increased approximately 4 times, pooled cytotoxicity increased 6 times compared to the control levels. The long-term exposure of larvae to Cd significantly induced the formation of MN, NB and pooled cytotoxicity (Figure 2 g-j). Micronuclei, NB and pooled cytotoxicity frequencies increased approximately 8, 5, 11 times, respectively compared to control level. This study confirms that the MN and NA assays can be used successfully to detect Cd genotoxicity and cytotoxicity in erythroblasts from whole embryos and larvae.

The micronucleus assay is the most widely used assay for the detection of chromosomal aberrations and other genotoxicological endpoints *in vivo.* Increased frequencies of micronucleated and binucleated cells in fish tissues exposed to Cd were recorded by many authors [11, 27]. Güner and Muranlı [28] reported significant elevation of total nuclear abnormalities (notched, lobed, bud, fragmenting) in erythrocytes of *Gambusia affinis* following exposure to 0.1 ppm and 1 ppm of Cd for one and two weeks. Chronic exposure to $CdCl_2$ (0.37 and 0.62 mg/L) induced elevated formation of micronuclei and other nuclear abnormalities (nuclear bud, binucleates, lobed, notched and vacuolated nuclei) in peripheral blood erythrocytes of fish *Labeo rohita* [27]. Cd induced cell death by apoptosis was determined in rainbow trout hepatocytes [29].

ISBN: 978-960-6865-94-7

Several investigations have demonstrated that embryo or larvae life stage responds to known DNA damaging agents. DNA damage caused by Cd in early fish development was emphasized in several fish species. The genotoxicity potential of Cd in the sediments (from 1.9 μg/g) on Japanese medaka (*Oryzias latipes*) embryos, of $CdCl_2$ (0.112 - 11.2 mg/L) on embryonic zebrafish cells ZF4 and of $CdCl_2$ (0.1 μM) on *O. latipes* larvae was investigated by Barjhoux et al. [30], Pereira et al. [31], Morin et al. [32], respectively. Risso-de Faverney with co-authors [32] determined that Cd induced apoptosis and DNA strand breaks in trout hepatocytes that are partially provoked by the generation of ROS. According to Hsu et al. [33] study, Cd at sublethal levels induced oxidative stress in zebrafish (*Danio rerio*) embryos. Consequently, oxidative stress and DNA repair inhibition are major mechanisms triggering Cd genotoxicity [33].

Summarizing the study results, we demonstrated that laboratory – controlled assessment of toxicity and geno-cytotoxicity effects can provide new information and accurate assessment about Cd exposure impact on fish in early stages of development. Based on the obtained results, we demonstrate that Cd induced geno-cytotoxicity, reduced survival, and disturbed cardio-respiratory system activity, affected development and hatchability of fish in early life stages causing negative consequences for the well-being of the fish populations and communities. Further investigation of the sublethal concentration Cd toxicity to fish in early stages of development could be a useful approach in assessment of ecological risk of heavy metals in aquatic ecosystems.

4. CONCLUSIONS

The calculated 96-hour LC50 values of Cd for rainbow trout embryos/larvae were: 3.32, 1.14, and 2.23 μg Cd/L, respectively (acute toxicity test). The larvae were consistently more sensitive than the embryos. The most sensitive were hatched larvae. Chronic tests (12-, 24-day) on rainbow trout at early stages of development results showed that Cd at sublethal concentration had a negative effect on the biological parameters of embryos and larvae and disturbs the vital functions of organism: significant ($p<0.005$) increased mortality, disturbed the cardio-respiratory system activity, hatching rate and observed various developmental disorders (haemorrhage in embryos; blood clots, yolk sac edema, and spine curvature in larvae). Mortality of larvae significantly increased ($p < 0.05$) with the increase in the duration of exposure. It was found that respiratory parameters of larvae significant ($p < 0.05$) depended on the duration of exposure. GVF of larvae 1-, 4-day significant decreased and 8-, 12-day increased ($p < 0.05$) with the increase on the duration of exposure. The Pearson correlation analysis of mortality, HR, GVF and the duration of exposure (1-, 4-, 8- and 12-day) of embryos and larvae showed a positive correlation. Strong correlation between mortality and the duration of exposure of embryos ($r = 0.88$) and larvae ($r = 0.77$); between HR and the duration of exposure of embryos ($r = 0.60$) as well as larvae ($r = 0.61$); and between GVF and the duration of exposure of larvae ($r = 0.92$). The 96-hour exposure of embryos to Cd induced significantly increased ($p < 0.005$) formation of MN and pooled cytotoxicity [bi-nucleated (BN) + fragmented-apoptotic (FA) cells]. The long-term Cd exposure caused significant ($p < 0.005$) increase of all analysed geno-cytotoxicity endpoints (MN, NB and pooled cytotoxicity) in larvae erythroblasts.

ACKNOWLEDGEMENTS

This research was supported by the Open Access to research infrastructure of the Nature Research Centre and National Cancer Institute under Lithuanian open access network initiative. This work is funded by the Research Council of Lithuania, Project No MIP-108/2015. Geno-cytotoxicity studies were performed under Nature Research Centre program 5 "Investigations of hazardous substances distribution in the ecosystems, regularities and mechanisms of ecotoxicity and biological effects under the impact of anthropogenic stress". We are thankful to Dalia Baršytė Lovejoy (Toronto University) for the language check.

ISBN: 978-960-6865-94-7

References

[1] Raj, V.M., Thirunavukkarasu, A.R., Kailasam, M., Muralidhar, M., Subburaj, R., Stalin, P., 2013. Acute Toxicity Bioassays of Cadmium and Mercury on the Juveniles of Asian seabass *Lates calcarifer* (Bloch). *Indian Journal of Science and Technology*, **6 (4)**, 4330–4335.

[2] El-Said El-Boshy, M., Gadalla, H.A., El-Hamied, F.M.A., 2014. Immunological, hematological and biochemical changes induced by short term exposure to cadmium in catfish (*Clarias gariepinus*). *Journal of Coastal Life Medicine*, **2 (3)**, 175–180.

[3] Rzigalinski, B.A., Strobl, J.S., 2009. Cadmium-containing nanoparticles: Perspectives on pharmacology and toxicology of quantum dots. Toxicology and Applied Pharmacology, **238 (3)**, 280–288.

[4] Leigh, K.L., Bouldin, J.L., Buchanan, R.A., 2014. Measurement of Accumulation of Semiconductor Nanocrystal Quantum Dots by Pimephales Promelas. *Dose-Response: An International Journal*, **10 (3)**, 331–334.

[5] Benjamin, D., Thatheyus, A.J., 2012. Acute Toxicity of Nickel and Cadmium to the Cichlid Fish, *Oreochromis mossambicus* (Peters). *Research in Zoology*, **2 (4)**, 19–22.

[6] Okocha, R.C., Adedeji, O.B., 2011. Overview of Cadmium Toxicity in Fish. *Journal of Applied Sciences Research*, **7 (7)**, 1195–1207.

[7] Bougas, B., Normandeau, E., Pierron, F., Campbell, P.G.C., Bernatchez, L., Couture, P., 2013. How does exposure to nickel and cadmium affect the transcriptome of yellow perch (*Perca flavescens*) – Results from a 1000 candidate-gene microarray. *Aquatic Toxicology*, **142–143**, 355–364.

[8] Heydarnejad, M.S., Khosravian-Hemamai, M., Nematollahi, A., 2013. Effects of cadmium at sub-lethal concentration on growth and biochemical parameters in rainbow trout (*Oncorhynchus mykiss*). *Irish Veterinary Journal*, **66**, 11.

[9] Rani, S., Gupta, R.K., Rani, M., 2015. Heavy Metal Induced Toxicity in Fish with Special Reference to Zinc and Cadmium. *International Journal of Fisheries and Aquatic Studies*, **3 (2)**, 118–123.

[10] *Waalkes, M.P.,* 2000. *Cadmium carcinogenesis in review. Journal of Inorganic Biochemistry*, **79,** 241–*244.*

[11] Cavas, T., Garanko, N.N., Arkhipchuk, V.V., 2005. Induction of micronuclei and binuclei in blood, gill and liver cells of fishes subchronically exposed to cadmium chloride and copper sulphate. *Food and Chemical Toxicology*, **43**, 569–574.

[12] Kensova, R., Blazkova, I., Vaculovicova, M., Milosavljevic, V., Blazkova, L., Hynek, D., Kopel, P., Novotna, M., Zehnalek, J., Pohanka, M., Trnkova, L., Adam, V., Kizek, R., 2015. The Effect of Cadmium Ions and Cadmium Nanoparticles on Chicken Embryos and Evaluation of Organ Accumulation. *International Journal of Electrochemical Science*, **10**, 3623–3634.

[13] Mahrous, K.F., Hassan, A.M., Radwan, H.A., Mahmoud, M.A., 2015. Inhibition of cadmium - induced genotoxicity and histopathological changes in Nile tilapia fish by Egyptian and Tunisian montmorillonite clay. *Ecotoxicology and Environmental Safety*, **119**, 140–147.

[14] Jezierska, B., Ługowska, K., Witeska, M., 2009. The effects of heavy metals on embryonic development of fish (a review). *Fish physiology and biochemistry*, **35**, 625–640.

[15] Annabi, A., Said, K., Messaoudi, I., 2013. Cadmium: Toxic Effects and Physiological Impairments in Fishes. *International Journal of Advanced Research*, **1 (3)**, 372–382.

[16] Batool, M., Abdullah, S., Javed, M., 2014. Behavioral Intoxication of *Channa marulius* and *Wallago attu* during Acute Exposure of Cadmium and Copper. *Pakistan Journal of Life and Social Sciences*, **12 (1)**, 7–15.

[17] Svecevičius, G., 2010. Acute toxicity of nickel to five species of freshwater fish. *Polish Journal of Environmental Studies*, **19 (2)**, 453–456.

[18] Fenech, M., Chang, W.P., Kirsch-Volders, M., Holland, N., Bonassi, S., Zeiger, E., 2003. HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutation Research*, **534**, 65–75.

[19] Hamilton, M. A, Russo, R. C, Thurston, R. V., 1977. Trimmed Spearman-Karben method for estimating median lethal concentration in toxicity bioassays. *Environmental Science & Technology,* **11 (7)**, 714–719.

[20] Eaton, J.G, McKim, J.M, Holcombe, G.W., 1978. Metal toxicity to embryos and larvae of seven freshwater species – I. Cadmium. *Bulletin of Environmental Contamination and Toxicology*, **19**, 95–103.

[21] Kazlauskienė, N., Vosylienė, M.Z., 2008. Characteristic features of the effect of Cu and Zn mixture on rainbow trout *Oncorhynchus mykiss* in ontogenesis. *Polish Journal of Environmental Studies*, **17 (2)**, 291–293.

[22] Zhang, H., Cao, H., Meng, Y., Jin, G., Zhu, M., 2012. The toxicity of cadmium (Cd_2) towards embryos and pro-larva of Soldatov's catfish (*Silurus soldatovi*). *Ecotoxicology and Environmental Safety*, **80**, 258–265.

[23] Ismail, A., Yusof, S., 2011. Effect of mercury and cadmium on early life stages of Java medaka (*Oryzias javanicus*): a potential tropical test. *Marine Pollution Bulletin,* **63**, 347–349.

[24] Reddy, J.S., Reddy, D.C., 2013. Impact of Cadmium toxicity on Behavioural and Haematological Biomarkers of Freshwater Fish, *Catla catla*. *International Journal of Bioassays*, **02 (09)**, 1199–1204.

Editors: A. Kungolos, C. Christodoulatos, A. Koutsospyros,
C. Emmanouil, C. Laspidou, Z. Mallios, D. Dermatas
ISBN: 978-960-6865-94-7

[25] El-Greisy, Z.A., El-Gamal, A.H.A., 2015. Experimental studies on the effect of cadmium chloride, zinc acetate, their mixture and the mitigation with vitamin C supplementation on hatchability, size and quality of newly hatched larvae of common carp, *Cyprinus carpio*. *The Egyptian Journal of Aquatic Research*, **41 (2)**, 219–226.
[26] Szczerbik, P., Mikołajczyk, T., Sokołowska-Mikołajczyk, M., Socha, M., Chyb, J., Epler, P., 2008. The influence of cadmium on Prussian carp oocyte maturation, development of eggs and hatching. *Czech Journal of Animal Science*, **53 (1)**, 36–44.
[27] Jindal, R., Verma, S., 2015. *In vivo* genotoxicity and cytotoxicity assessment of cadmium chloride in peripheral erythrocytes of *Labeo rohita* (Hamilton). *Ecotoxicology and Environmental Safety*, **118**, 1–10.
[28] Güner, U., Muranlı, F.D.G., 2011. Micronucleus test, nuclear abnormalities and accumulation of Cu and Cd on *Gambusia affinis* (Baird & Girard, 1853). *Turkish Journal of Fisheries and Aquatic Sciences*, **11**, 615–622.
[29] Risso-de Faverney, C., Devaux, A., Lafaurie, M., Girard, J.P., Bailly, B., Rahmani, R., 2001. Cadmium induces apoptosis and genotoxicity in rainbow trout hepatocytes through generation of reactive oxygene species. *Aquatic Toxicology*, **53 (1)**, 65–76.
[30] Barjhoux, I˙, Baudrimont, M., Morin, B., Landi, L., Gonzalez, P., Cachot, J., 2012. Effects of copper and cadmium spiked-sediments on embryonic development of Japanese medaka (*Oryzias latipes*). *Ecotoxicology and Environmental Safety*, **79**, 272–282.
[31] Pereira, S., Cavalie, I., Camilleri, V., Gilbin, R., Adam-Guillermin, C., 2013. Comparative genotoxicity of aluminium and cadmium in embryonic zebrafish cells. *Mutation Research,* **750 (1–2)**, 19–26.
[32] Morin, B., Filatreau, J., Vicquelin, L., Barjhoux, I., Guinel, S., Leray-Forget, J., Cachot, J., 2011. Detection of DNA damage in yolk-sac larvae of the Japanese Medaka, Oryzias latipes, by the comet assay. *Analytical and Bioanalytical Chemistry,* **399 (6)**, 2235–2242.
[33] Hsu, T., Huang, K.M., Tsai, H.T., Sung, S.T., Ho, T.N., 2013. Cadmium (Cd)-induced oxidative stress down-regulates the gene expression of DNA mismatch recognition proteins MutS homolog 2 (MSH2) and MSH6 in zebrafish (*Danio rerio*) embryos. *Aquatic Toxicology*, **126**, 9–16.

XI

Long-term toxicity and geno-cytotoxicity of quantum dots to rainbow trout *Oncorhynchus mykiss* embryos

Cibulskaitė Ž, Stankevičiūtė M, Kazlauskienė N, Baršienė J, Kulvietis V, Rotomskis R

Proceedings of the 13th International Conference on Protection and Restoration of the Environment 2016, ISBN: 978-960-6865-94-7. Mykonos island, Greece. p. 460–470

Long-term toxicity and geno-cytotoxicity of quantum dots to rainbow trout *Oncorhynchus mykiss* embryos

Ž. Cibulskaitė[1], M. Stankevičiūtė[1*], N. Kazlauskienė[1], J. Baršienė[1], V. Kulvietis[2], R. Rotomskis[2,3]

[1]Institute of Ecology of Nature Research Centre, Akademijos st. 2, LT-08412 Vilnius-21, Lithuania
[2]Laboratory of Biomedical Physics, National Cancer Institute, Baublio st. 3b, LT-08660 Vilnius, Lithuania
[3]Biophotonics Group of Laser Research Center, Vilnius University, Saulėtekio ave. 9, LT-10222 Vilnius, Lithuania
*Corresponding author: Email: milda.stan@gmail.com

Abstract

The aim of study was to assess the toxicity and geno-cytotoxicity of CdSe/ZnS-COOH quantum dots (QD) to rainbow trout *Oncorhynchus mykiss* at early stages of development ("eye-egg" stage embryos). Bioassay testing was conducted under controlled laboratory conditions; with a goal of exploring relationship between the duration of exposure to QD and biological effects in exposed fish embryos. Long-term (12-day) tests on fish at early developmental stages were performed under static conditions. QD photoluminescence analysis showed that the outer layer of the egg protects embryos from the penetration and accumulation of QD. The present study indicated that biological effects of QD are related to the structure of chorion. Toxicity study demonstrated that QD did not induce a significant decrease in survival, but induced heart rate changes of embryos during the entire test. Geno-cytotoxicity assay revealed that QD induced micronuclei in erythroblasts of embryos.

Keywords: quantum dots; fish; embryos; toxicity;geno-cytotoxicity

1. INTRODUCTION

The application of nanotechnology in various fields, such as biomedical science and electronics, cosmetic and pharmaceutical industries, is likely going to increase the release of nanoparticles (NP) into the environment in the near future [1]. Therefore, the increasing use of nanotechnologies will lead to significant accumulation of engineered NP into the aquatic organisms [2]. The potential ecotoxicological consequence of NP is a growing concern, but their impact is still poorly characterized [3].

Quantum dots (QD) are small semiconductor NP composed of a semiconductor core, which is often encapsulated by a shell or coated with organic molecules [4]. They are only a few nanometers in diameter (2-10 nm) with great optical properties and are commonly used as fluorophores in vital bioimaging of developmental processes [5]. These properties enable the use of QD *in vitro* and *in vivo* experiments and establish QD as promising tools in disease treatment and targeted therapy. Unfortunately, the limitation of their usage is the toxicity. QD are composed of different metals that may have detrimental effects on the environmental and human health [6].

Several recent review articles dealing with NP toxicity to aquatic organisms focus on NP ecotoxicological potential [1]. The nanocrystal core of many types of QD contains the toxic metal cadmium (Cd), possibly resulting in release of Cd from the QD core [7]. However, QD exerted toxic effects in fish not only due to Cd^{2+} release, but also toxicity that might be associated with QD nanoscale properties [8]. Toxicity, cytotoxicity, mutagenicity, genotoxicity and immunotoxicity in fish exposed to ionic form of Cd have been described. Therefore, high heavy metal content in NP

13th International Conference on Protection and Restoration of the Environment
Editors: A. Kungolos, C. Christodoulatos, A. Koutsospyros,
C. Emmanouil, C. Laspidou, Z. Mallios, D. Dermatas
ISBN: 978-960-6865-94-7

makes them potentially harmful to living creatures. In zebrafish, QD induced dose- and age-dependent endpoints of sublethal toxicity, including increased mortality, reduced growth, apparent necrosis, yolk sac malformation, malformed tail and bioaccumulation in body tissues [8-10]. The study by Zhang with co-authors [10] indicated that the mortality increased significantly after zebrafish embryos were exposed to CdTe QD coated with thioglycolic acid. Compared to the CdTe QD, the CdSe/ZnS QD showed lower toxicity in zebrafish embryos and larvae [8, 9]. Blickley with co-authors [11] showed that the chronic QD exposure could cause a negative effect at the population level and maternal transfer of QD or their degradation products to developing progeny may pose a threat to future generations of aquatic organisms. For this reason, QD could be a source of toxicity with fatal impact to fish during the sensitive stages of the embryonic period [11, 9]. Toxicity of different NP (nanosilver, titanium dioxide, silver NPs, single walled carbon nanotubes and QD) on rainbow trout adult was also investigated [12-15, 2]. Knowledge of possible effects in fish embryonic development is still being acquired and could present as valuable information on the QD environmental impact.

The scientific information on the NP toxicity mechanism at early life stages of fish is very limited, especially under varying environmental conditions [3, 7]. Furthermore, there are many factors, which could influence the toxicity of NP in embryos. For example, chemical composition, particle size, shape, surface modification, concentration of particles, crystal form and degree of agglomeration can influence bioaccumulation and the biological effects during embryogenesis [4].

The micronucleus assay is one of the most frequently used tools for the NP genotoxicity assessment [4]. The *in vivo* micronucleus test in fish early life stages were applied in several studies [16-18]. Micronucleus and erythroblast nuclear abnormalities assays previously were used as biomarkers to assess genotoxicity and cytotoxicity of different NP in fish [19, 2]. As emphasized by Rocha with co-authors [20] cytotoxicity and genotoxicity effects of Cd-based QD in aquatic species need to be comprehensively investigated. *In vitro* or *in vivo* chromosome abnormalities, DNA fragmentation induced by CdSe/ZnS core/shell QD have been previously shown in various organisms [21, 22]. Munari with co-authors [2] investigated genotoxicity and cytotoxicity of CdS QD and silver sulphide (Ag_2S) coated with methyl polyethylene glycol in a rainbow trout cell line RTG-2. The results showed that CdS QD was highly cytotoxic at high concentrations, and demonstrated concentration-dependent genotoxicity response in the sub-toxic range after 24 h exposure.

Recently, an expanding number of studies have focused on embryonic stages; however, the comparison between these experiments is difficult as different species of fish, various types of NP, their sizes, concentrations, exposure durations were tested [4]. To the best of our knowledge, there has been no report of using rainbow trout *Oncorhynchus mykiss* embryos as a model for QD long-term toxicity and geno-cytotoxicity studies. It should be noted that this experimental study was done at equal laboratory conditions. Little is known about geno-cytotoxicity effects of QD at early developmental stages of fish in an exposure duration-dependent manner. It is also very important to determine the penetration abilities of QD through the egg envelope that would help to explain QD toxic effects on embryos of various organisms.

The aims of the present study were: (i) to determine the penetration abilities of QD through the egg envelope, (ii) to assess the toxicity and geno-cytotoxicity of QD using the test fish, rainbow trout, in early stages of development – "eye-egg" stage embryos and (iii) to define the relationship between the duration of QD exposure and biological alterations in exposed fish embryos.

2. MATERIALS AND METHODS

Exposure of fish was performed at the Laboratory of Ecology and Physiology of Hydrobionts (Nature Research Centre, Lithuania). Rainbow trout *Oncorhynchus mykiss* eggs ("eye-egg" stage

embryos) were obtained from the Simnas hatchery (Lithuania). Studies have been carried out with non-protected life-stages accordance with EU Directive 2010/63/EU. The laboratory treatment was carried out in climatic camera (Bronson PGC-660, Germany) under static conditions.

Deep well water of high quality was used for storing control embryos [23]. The average hardness of dilution water was approximately 284 mg L^{-1} as $CaCO_3$, alkalinity was 244 mg L^{-1} as HCO_3^-, the mean pH was 8.0, temperature was maintained at 10 ± 0.5°C, and the oxygen concentration ranged from 8 to 10 mg L^{-1}. Dissolved oxygen in the tanks, temperature and pH were measured routinely with a hand-held multi-meter (WTW Multi 340i/SET, Germany).

Long-term (12-day) toxicity test of QD was conducted using "eye-egg" stage embryos (starting from "eye-egg" embryos 12-day before hatching). The effect of QD on the biological parameters: survival (%) (N = 39 in the control group; N = 38 in the QD group) and heart rate (HR, counts/min) of embryos were recorded. HR of embryos was measured during 1-minute periods for each embryo individually, and the mean value for 10 embryos was calculated. The unfertilized eggs were removed from the groups. Survival was recorded at 24-hour intervals. Studies with embryos were performed in two replications.

Toxic effects of CdSe core, ZnS shell and carboxyl group terminated QD, abbr. CdSe/ZnS-COOH (QD ITK 625, Life Technologies) were analyzed at sublethal concentration of 4 x 10^{-9} *M* according to [24]. The volume of 100 µl of stock solution of 8 µM was dissolved in the fish media to achieve final QD concentration of 4 x 10^{-9} *M*. Fluorescence microscopy was used to visualize QD localization in embryonic tissues in order to determine QD distribution. Embryos were prepared using the standard paraffin embedding technique for microscopical examination. They were fixed in 4% formaldehyde solution for 24 hours and were embedded in paraffin blocks for cutting with the microtome. The unstained slices were used for fluorescence microscopy and they were imaged using Nikon C1si confocal microscope, which is equipped argon ion laser (λ=488 nm). The 500-590 nm band pass filter (represented in green color) was used for autofluorescence detection and 620-720 nm band-pass filter (represented in red color) for QD photoluminescence detection.

Geno-cytotoxicity assay was performed on "eye-egg" stage embryos. Genotoxicity [induction of micronuclei (MN) and nuclear buds (NB)] and cytotoxicity [induction of bi-nucleated (BN) and fragmented-apoptotic (FA) cells] endpoints were analysed in erythroblasts. Blood smears were prepared from whole embryonic body (gently nipped with tweezers): directly smeared on glass slides and air-dried. Smears were fixed in methanol for 10 min. and later were stained with 10% Giemsa solution in phosphate buffer pH = 6.8 for 20 - 40 min. The stained slides were analysed under light microscopes Olympus BX51 at final magnification of 1,000×. Micronuclei (MN) were identified according to the following criteria: (1) round and ovoid-shaped non-refractory particles in the cytoplasm, (2) color and structure similar to chromatin of the main nucleus, (3) diameter of 1/3-1/20 of the main nucleus, (4) particles completely separated from the main nucleus [25]. Identification of nuclear buds, fragmented-apoptotic and bi-nucleated cells was done using criteria described by Fenech with co-authors [26]. The frequencies of abnormalities were recorded in 1,000 erythroblasts per slide using blind scoring by a single observer. The 4-day duration test was carried out with embryos (7 specimens in QD exposed and 7 specimens in control groups) for evaluating geno-cytotoxicity.

Differences between the evaluated characteristics studied were tested by two-way ANOVA at $p < 0.05$ using Statistica 7.0 software's (USA). Pearson correlation analysis was performed to identify possible relationships between biological parameters of embryos and the duration of exposure. GraphPad Prism 5 (USA) statistical analysis software was used in geno-cytotoxicity statistical analysis. Non-parametric Mann-Whitney *U*-test was used to compare frequencies of nuclear abnormalities observed in embryos from control and exposed groups. Differences were accepted as significant at the 95% level of confidence ($p < 0.05$).

3. RESULTS

3.1 Incubation of the embryos with QD

Rainbow trout embryos, in early stages of development – "eye-egg" stage were cultivated and exposed to the QD according to the guidelines presented in ISO standard (ISO 7346-1:1996). The formation of aggregates of QD in the incubation water were detected (Figure 1A). However, spectroscopy reveals that QD are stable in the incubation solution. The photoluminescence intensity decay with increasing of the incubation time is related to the aggregation of QD in water and agglomeration on the eggs surface (Figure 1). Confocal fluorescence microscopy was used to visualize QD localization in egg in order to determine QD distribution. QD are covering the envelope of the egg, but they were not observed in the deeper layers. The green color represents the autofluorescence of the embryonic tissues and red color represents the fluorescence of QD (Figure 1B). For rainbow trout embryos, most of the particles were accumulated at the surface of the envelope surrounding the embryos (Figure 1B).

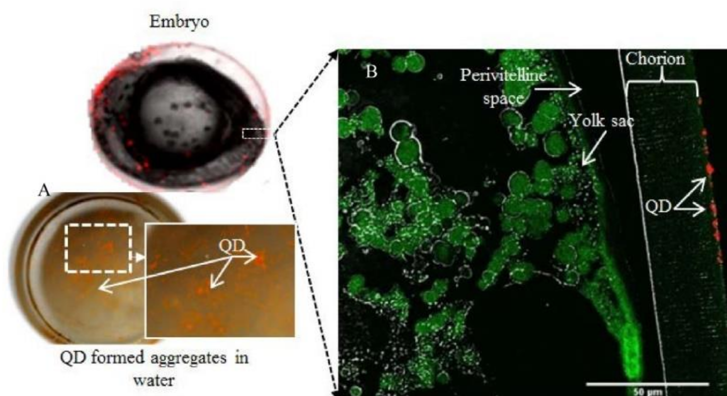


Figure 1. QD formed large aggregates in water (A). Confocal fluorescence microscopy image (B) rainbow trout egg exposed to QD for 8-day. QD (red color) adhere to the envelope but do not penetrate through it to the inner layers of the egg.

3.2 Toxicity

Survival of the embryos exposed to QD during the 1-, 4-, 8-, 12-day of the test did not significantly differ from the survival in control group (Figure 2A). The heart rate (HR) of embryos exposed to QD during the 1-, 4-day was significantly ($p < 0.05$) lower (80.4 ± 4.8; 82.0 ± 4.2 count/min, respectively) as compared to the control (95.2 ± 6.5; 94.8 ± 6.3 count/min, respectively). Meanwhile after 8- and 12-day exposure HR of embryos (89.2 ± 7.4; 90.0± 8.5 counts/ min, respectively) didn't differ significantly from the control (96.0 ± 3.3 counts/min). The alterations in HR of embryos were related to the duration of exposure of QD (Figure 2B). HR of embryos after 1- and 4-day exposure was significantly ($p < 0.05$) different from 12-day exposure. Pearson correlation analysis of HR and the duration of exposure of embryos showed moderate and positive correlations ($r = 0.53$). It shows that HR is decreased at the day of adding QD to the media, but later it recovers to the level of the control group.

Editors: A. Kungolos, C. Christodoulatos, A. Koutsospyros,
C. Emmanouil, C. Laspidou, Z. Mallios, D. Dermatas
ISBN: 978-960-6865-94-7

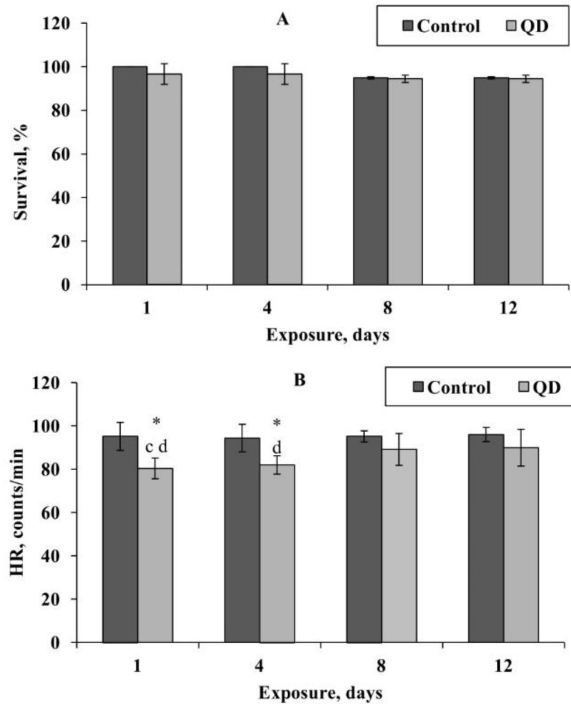


Figure 2. Effect of QD on the survival (A) and the HR (B) of embryos depending on the duration of exposure. Columns and bars represent means ± SD.

* Significantly different from control: $p < 0.05$. Significantly different between the duration of exposure ($p < 0.05$): c- significantly different from the 8-day of exposure; d-significantly different from the 12-day of exposure.

3.3 Geno-cytotoxicity

Exposure (4-day) to QD significantly ($p = 0.044$) increased frequency of micronuclei (MN) in embryos, but did not induced nuclear buds (NB), bi-nucleated and fragmented-apoptotic erythroblasts. However, all analysed geno-cytotoxicity responses were elevated approximately 2 times compared to the control levels (Figure 3). Micronucleated erythroblast, erythroblast with nuclear bud, bi-nucleated and fragmented-apoptotic erythroblast in embryos exposed to QD are presented in Figure 4.

Editors: A. Kungolos, C. Christodoulatos, A. Koutsospyros, C. Emmanouil, C. Laspidou, Z. Mallios, D. Dermatas
ISBN: 978-960-6865-94-7

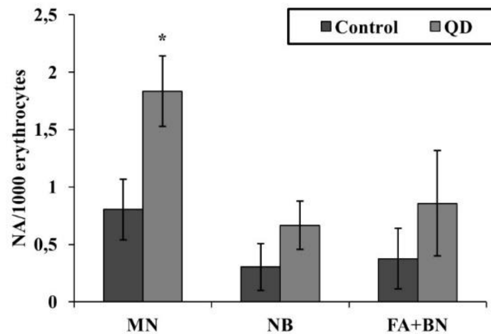


Figure 3. Frequency of micronuclei, nuclear buds and fragmented-apoptotic (FA) + bi-nucleated (BN) erythroblasts of embryos exposed 4-day to QD. Data are reported as mean ± SE. Differences between control and exposure groups shown: * $p \leq 0.05$.

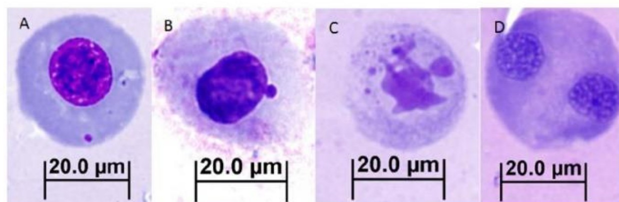


Figure 4. Rainbow trout embryo erythroblast with (A) micronucleus (MN), (B) nuclear bud (NB), (C) fragmented-apoptotic (FA) and (D) bi-nucleated (BN) erythroblast.

4. DISCUSSION

Our results showed that QD formed large aggregates in water (Figure 1 A). QD adhere to the envelope but do not penetrate through in to the inner layers of the rainbow trout egg (Figure 1 B). For this reason, QD did not penetrated the chorion of embryo but QD aggregates affected the ability of larval fish to hatch [8, 11]. These results are also supported by our observations obtained using the confocal microscope. QD adhered to the envelope but did not penetrate through it to the inner layers of the egg [24]. Similar results were observed when photoluminescence SiO_2 nanoparticles (~60 and ~200 nm in diameter) were exposed to zebrafish embryos; the nanoparticles adhered to the surface of the chorion but did not enter the embryo [27]. Exposure of *Fundulus heteroclitus* embryos to aqua-nC60 resulted in very little mortality and aggregates adhered to the chorion, not affecting development of the embryos or their hatching success [11].

Our data showed that survival of rainbow trout embryos exposed to QD in long-term tests remained statistically unchanged (Figure 2A). According to King-Heiden with co-authors [8] coating or size of QD can influence the embryonic toxicity, as it was shown in case of CdSe/ZnS QD coated with PEG or polylysine in zebrafish embryos. QD induced higher mortality than the

Editors: A. Kungolos, C. Christodoulatos, A. Koutsospyros,
C. Emmanouil, C. Laspidou, Z. Mallios, D. Dermatas
ISBN: 978-960-6865-94-7

equivalent amount of Cd^{2+}, probably due to the specific organic coating, QD scale or degree of their agglomeration in solution [8]. But there is another opinion to interpret the toxicity of QD. Because of a lack of protection of the egg membrane, a large amount of water- soluble Cd likely penetrated into the embryos and accumulated around the eggs, especially at high concentrations, and finally led to death [28]. However, interestingly chorion of various fish species may differentially retain Cd. For example, in rainbow trout (*Oncorhynchus mykiss*) 98 % of total Cd was retained by chorion, meanwhile, 61 % in zebrafish (*Danio rerio*) [28]. Annabi with co-authors [28] provided the evidence of the protective effect of the chorion and showed that hatched larvae are more susceptible to Cd than unhatched embryos [28]. Meanwhile, rainbow trout embryos during hatching are more sensitive to external substances at the early stages rather than larval or adult stages [29].

Effects of NP on early life stages of fish are emerging, with reports of crossing the chorion (e.g., Ag-NPs) [29]. The evidence on the protective effect of the chorion (a membrane envelope surrounding the egg) suggested that it acts as a barrier to Cd transfer to the developing embryos [32].

Table 1 Chorion thickness and total Cd was retained by chorion of various fish species (Literature review)

Fish species	Chorion thickness/ Total Cd was retained by chorion	Authors
Fundulus heteroclitus	13 μm	[31]
Oryzias latipes	12-15 μm/ 94.6%	[32]/[28]
Danio rerio	0.5-0.6 μm/ 61 %	[33]/[28]
Oncorhynchus mykiss	44.2-48.6 μm/ 98 %	[34]/[28]

According to Ninness with co-authors [35], the majority of fish embryos develop surrounded by a fluid layer known as the perivitelline fluid encased in a tough acellular shell or chorion that is composed mainly of protein and glycoprotein. One of the major functions of the chorion is to physically and chemically protect and isolate the embryo from external environmental conditions/impacts. In addition, the chorion is involved in respiration, excretion of metabolic waste and ionic and osmotic balance. In Table 1 the chorion thickness of various fish species are presented. According to Berois with co-authors [36], there are numerous channels cross the entire width of the chorion in a regular pattern. The main focus in Henn's [37] work has been to determine the influence of the chorion on chemical toxicity for zebrafish embryos.

Meanwhile, the exposure to QD induced significant decrease in HR of embryos in relation to the duration of exposure ($r = 0.53$) (Figure 2B). Interestingly the HR of embryos exposed to QD for 1-, 4-day, was significantly ($p < 0.05$) lower as compared to the control. However, at the later time the HR recovers to the normal levels.

This indicates the possibility of acclimation to QD effect. Meanwhile, little is known about acclimation mechanisms in early developmental stages of fish. Possibility of acclimation of larvae of *Salmo trutta* and *Pimephales promelas* by exposure to Cd were observed [38]. According to Jezierska with co-authors [39] the increase in HR during development might have been related to the observed increase in embryonic activity and is an indicator of metabolic rate in embryos. A decrease in HR during hatching period was particularly pronounced in metal-exposed embryos [39]. Wang with co-authors [40] hypothesized that the accumulation of the QD occurs in the zebrafish heart and the substance provokes malformed heart. This is confirmed and by other authors [8, 9].

The clastogenic/aneugenic properties of CdSe/ZnS QD have been observed in some studies [21, 22]. Galeone with co-author [22] suggested classifying CdSe/ZnS QD as significantly toxic in

ISBN: 978-960-6865-94-7

long-term *in vivo* treatments. Nanoparticle-induced oxidative stress is thought to be a key mechanism responsible for genotoxicity effects [41]. Brunetti with co-authors [42] reported Cd^{2+} ions leaching from the CdSe core despite the two-layer ZnS shell. Furthermore, a coating-dependent reactive oxygen species (ROS) generation of CdSe/ZnS core/shell QD was emphasized by Galeone and co-authors [22]. Several studies have pointed to PEG-coating that can protect from Cd^{2+} leak. It was concluded that coating with PEG decreases toxicity, but does not eliminate it [43, 22]. Zhang with co-authors [44] emphasized that PEG-coated silanized QD causes minimal impact to cells. Saez with co-author [45] showed that genotoxicity potential and reactivity of Cd with intracellular targets are influenced by its nano or ionic form. Nano-Cd showed a stronger genotoxic activity compared with ionic-Cd in *Hediste diversicolor*. However, Aye's and co-authors' [21] results showed, that even though QD induced ROS, the mutagenic/clastogenic properties of QD are not completely accounted for. The inherent physico-chemical properties of QD may generate various reactions, which results in genotoxicity/mutagenicity effects in cells or organisms. Other possible mechanisms underlying genotoxicity of QD are inflammation, aberrant signaling responses and direct interaction with DNA and nuclear proteins [42]. Thus, potential for genotoxicity effects and mechanisms of DNA damage of QD still remains unclear [45].

Heavy metal embryonic uptake is known to be blocked by the chorion via binding, most likely via complexation by anionic charged groups, possibly thiol-groups, which are abundantly present in the chorion [37]. Furthermore, it was suspected, that the chorion pores potentially restrict the uptake of compounds depending on their size [37]. In addition, NP cause increased mucus production in fish, may thicken the mucus layer and impair gas exchange [46]. Our results showed significantly increased frequencies of MN, slight increase of other nuclear abnormalities and reduced HR after QD exposure. However, fluorescence microscopy showed that QD accumulation is visible around the outer layer of the egg; indicating the QD did not penetrate the chorion and were not observed in the embryo. More follow-up studies must provide information, which mechanism: Cd leakage or QD adhesion to the envelope of the egg and thus possible disruption of gas exchange is responsible for toxicity and genotoxicity. Therefore, the long-term fish exposure to QD requires further detailed toxicological analysis.

In the present study, the toxicity and geno-cytotoxicity of QD were assessed using rainbow trout embryos as test organism. The toxicity and geno-cytotoxicity of QD and their effect on embryonic survival, physiological parameters such as HR, induction of micronucleus, penetration abilities can provoke developmental defects of fish in larvae or adult stages. The nanocrystal core of CdSe/ZnS-COOH QD contains the toxic metal Cd, thus possible release of Cd from the QD core could contribute to the effects seen. The long-term study of QD toxicity and geno-cytotoxicity effects on embryos should provide new information for understanding QD impact mechanisms. The laboratory-controlled assessment of toxicity and geno-cytotoxicity effects on embryos of rainbow trout reported here provides new information about environmental risk of the QD and opens new avenues for investigating the emerging effects of QD.

5. CONCLUSIONS

CdSe/ZnS-COOH QD does not cause embryonic survival of rainbow trout at the dose of 4×10^{-9} *M* up to 12 days. QD exposure causes the decrease of the HR in the first 4 days of exposure, but this effect is later compensated and restored to a normal level. Exposure to QD causes genotoxicity (increased the frequency of micronuclei in erythroblasts) in embryos. The outer layer of the egg protects embryo from the penetration and accumulation of QD.

ACKNOWLEDGEMENTS
This research was supported by the research infrastructure Open Access Centre for Nature Research and the National Cancer Institute at the initiative of the Open R&D Lithuania network. This work is funded by the Research Council of Lithuania, Project No MIP-108/2015. Geno-cytotoxicity studies were performed under Nature Research Centre programe 5 "Investigations of hazardous substances distribution in the ecosystems, regularities and mechanisms of ecotoxicity and biological effects under the impact of anthropogenic stress". We are thankful to Dalia Baršytė Lovejoy (Toronto University) for the language check.

References

[1] Jovanovic, B., 2011. Immunotoxicology of titanium dioxide and hydroxylated fullerenes engineered nanoparticles in fish models. *Graduate Theses and Dissertations*, 10134.

[2] Munari, M., Sturve, J., Frenzilli, G., Sanders, M. B., Brunelli, A., Marcomini, A., Nigro, M., Lyons, B. P., 2014. Genotoxic effects of CdS quantum dots and Ag_2S nanoparticles in fish cell lines (RTG-2). *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, **16 (775–776)**, 89–93.

[3] Kim, J., Park, Y., Yoon, T. H., Yoon, Ch. S., Choi, K., 2010. Phototoxicity of CdSe/ZnSe quantum dots with surface coatings of 3-mercaptopropionic acid or tri-n-octylphosphine oxide/gum arabic in *Daphnia magna* under environmentally relevant UV-B light. *Aquatic Toxicology*, **97 (2)**, 116–124.

[4] Celá, P., Veselá, B., Matalová, E., Večeřa, Z., Buchtová, M., 2014. Embryonic Toxicity of Nanoparticles. *Cells Tissues Organs*, **199 (1)**, 1–23.

[5] Michalet, X., Pinaud, F.F., Bentolila, L.A., Tsay, J.M., Doose, S., Li, J.J., Sundaresan G.,Wu, A.M., Gambhir, S.S.,Weiss, S., 2005. Quantum dots for live cells, in vivo imaging, and diagnostics. *Science*, **307 (5709)**, 538–544.

[6] Blažková, I., Konečná, M., Kenšová, R., Adama, V., Kizeka, R., 2014. Application of quantum dots into chicken embryos. *Journal of Metallomics and Nanotechnologies*, **3**, 26–28.

[7] Leigh, K.L., Bouldin, J.L., Buchanan, R.A., 2014. Measurement of Accumulation of Semiconductor Nanocrystal Quantum Dots by *Pimephales Promelas*. *Dose-Response: An International Journal*, **10 (3)**, 331–334.

[8] King-Heiden, T. C., Wiecinski, P. N., Mangham, A. N., Met, K. M., Nesbit, D., Pedersen, J.A., Hamers, R. J., Heideman, W., Peterson, R. E., 2009. Quantum dot nanotoxicity assessment using the zebrafish embryo. *Environmental Science & Technology*, **43 (5)**, 1605–1611.

[9] Duan, J., Yu, Y., Li, Y., Yu, Y., Li, Y., Huang, P., Zhou, X., Peng, S., Sun, Z., 2013. Developmental toxicity of CdTe QDs in zebrafish embryos and larvae. *Journal of Nanoparticle Research*, **15**:1700.

[10] Zhang, W., Lin, K., Miao, Y., Dong, Q., Huang, C., Wang, H., Guo, M., Cui, X., 2012. Toxicity assessment of zebrafish following exposure to CdTe QDs. *Journal of Hazardous Materials*, **213–214**, 413–420.

[11] Blickley, T. M., Matson, C., W., Vreeland, W. N., Rittschof, D., Di Giulio, R. T., McClellan-Green, P., D., 2014. Dietary CdSe/ZnS quantum dot exposure in estuarine fish: Bioavailability, oxidative stress responses, reproduction, and maternal transfer. *Aquatic Toxicology,* **148**, 27–39.

[12] Federici, G., Shaw, B.J, Handy, R.D., 2007. Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): gill injury, oxidative stress, and other physiological effects. *Aquatic Toxicology*, **84 (4)**, 415–430.

[13] Smith, C.J., Shaw, B.J., Handy, R.D., 2007. Toxicity of single walled carbon nanotubes to rainbow trout, (*Oncorhynchus mykiss*): respiratory toxicity, organ pathologies, and other physiological effects. *Aquatic Toxicology*, **82 (2)**, 94–109.

[14] Shahbazzadeh, D., Ahari, H., Rahimi, N.M., Dastmalchi, F., Soltani, M., Fotovat, M., Rahmannya, J., Khorasani, N., 2009. The Effects of Nanosilver (Nanocid®) on Survival Percentage of Rainbow Trout (*Oncorhynchus mykiss*). *Pakistan Journal of Nutrition,* **8 (8)**, 1178–1179.

[15] Scown, T.M., Santos, E.M., Johnston, B.D., Gaiser, B., Baalousha, M., Mitov, S., Lead, J.R., Stone, V., Fernandes, T.F., Jepson, M., van Aerle, R., Tyler, C.R., 2010. Effects of aqueous exposure to silver nanoparticles of different sizes in rainbow trout. *Toxicological Sciences*, **115 (2)**, 521–534.

[16] Hayashi, M., Ueda, T., Uyeno K., Wada, K., Kinae, N., Saotome, K., Tanaka, N., Takai, A., Sasaki, Y.F., Asano, N., Sofuni, T., Ojima, Y., 1998. Development of genotoxicity assay systems that use aquatic organisms. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, **399 (2)**, 125–133.

[17] Braunbeck, T., Böttcher, M., Hollert, H., Kosmehl, T., Lammer, E., Leist, E., Rudolf, M., Seitz, N., 2005. Towards an Alternative for the Acute Fish LC50 Test in Chemical Assessment: The Fish Embryo Toxicity Test Goes Multi-species – an Update. *Alternatives to Animal Experiments*, **22 (2)**, 87–102.

[18] Le Bihanic, F., Clérandeau, C., Le Menach, K., Morin, B., Budzinski, H., Cousin, X., Cachot, J., 2014. Developmental toxicity of PAH mixtures in fish early life stages. Part II: adverse effects in Japanese medaka. *Environmental science and pollution research international,* **21 (24)**, 13732–13743.
[19] Vignardi, C.P., Hasue, F.M., Sartório, P.V., Cardoso, C.M., Machado, A.S., Passos, M.J., Santos, T.C., Nucci, J.M., Hewer, T.L., Watanabe, I.S., Gomes, V., Phan, N.V., 2015. Genotoxicity, potential cytotoxicity and cell uptake of titanium dioxide nanoparticles in the marine fish *Trachinotus carolinus* (Linnaeus, 1766). *Aquatic Toxicology*, **158**, 218–229.
[20] Rocha, T.L., Gomes, T., Cardoso, C., Letendre, J., Pinheiro, J.P., Sousa, V.S., Teixeira, M.R., Bebianno, M.J., 2014. Immunocytotoxicity, cytogenotoxicity and genotoxicity of cadmium based quantum dots in the marine mussel *Mytilus galloprovincialis*. *Marine Environmental Research,* **101**, 29–37.
[21] Aye, M., Di Giorgio, C., Berque-Bestel, I., Aime, A., Pichon, B.P., Jammes, Y., Barthélémy, P., De Méo, M.. 2013. Genotoxic and mutagenic effects of lipid-coated CdSe/ZnS quantum dots. *Mutation Research*. **750 (1 – 2)**, 129–138.
[22] Galeone, A., Vecchio, G., Malvindi, M.A., Brunetti, V., Cingolani, R., Pompa, P.P., 2012. In vivo assessment of CdSe-ZnS quantum dots: coating dependent bioaccumulation and genotoxicity. *Nanoscale*, **4**, 6401–6407.
[23] Svecevičius, G., 2010. Acute toxicity of nickel to five species of freshwater fish. *Polish Journal of Environmental Studies*, **19 (2)**, 453–456.
[24] Cibulskaitė, Ž., Kazlauskienė, N., Kulvietis, V., 2015. Sublethal toxicity of quantum dots and heavy metals *to* rainbow trout (*Oncorhynchus mykiss*) in early ontogenesis. Proceedings of the 18th Conference for Junior Researchers „Science – Future of Lithuania", *Environmental protection engineering*. 9 April 2015, Vilnius, Lithuania, 31–37.
[25] Heddle, J.A., Cimino, M.C., Hayashi, M., Romagna, F., Shelby, M.D., Tucker, J.D., Vanparys, P., MacGregor, J.T., 1991. Micronuclei as an index of cytogenetic damage: past, present, and future. *Environmental and Molecular Mutagenesis*, **18 (4)**, 277–291.
[26] Fenech, M., Chang, W.P., Kirsch-Volders, M., Holland, N., Bonassi, S., Zeiger, E., 2003. HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated humanlymphocyte cultures. *Mutation Research*, **534**, 65–75.
[27] Sun, J., Zhang, Q., Wang, Z. and Yan B., 2013. Effects of Nanotoxicity on Female Reproductivity and Fetal Development in Animal Models. *International Journal of Molecular Sciences*, **14**, 9319–9337.
[28] Annabi, A., Said, K., Messaoudi, I., 2013. Cadmium: Toxic Effects and Physiological Impairments in Fishes. *International Journal of Advanced Research*, **1 (3)**, 372–382.
[29] Vosylienė, M. Z., Kazlauskienė, N., Svecevičius, G., 2003. Effect of heavy metal model mixture on biological parameters of rainbow trout *Oncorhynchus mykiss*. *Environmental Science & Pollution Research.* **10**, 103–107.
[30] Shaw, B.J., Handy, R.D., 2011. Physiological effects of nanoparticles on fish: a comparison of nanometals versus metal ions. *Environment International*, **37 (6)**, 1083–1097.
[31] Shanklin, D.R., 1959. Studies on the Fundulus chorion. *Journal of Cellular and Comparative Physiology*, **53**, 1–11.
[32] Hart, N.H., Pietri, R., Donovan, M., 1984. The structure of the chorion and associated surface filaments in Oryzias – evidence for the presence of extracellular tubules. *Journal of Experimental Zoology,* **230**, 273–296.
[33] Joo, K.B., Kim, D.H., 2013. Comparative ultrastructures of the fertilized egg envelopes in *Danio rerio* and *Danio rerio* var. frankei, *Cyprinidae,* Teleostei. *Applied Microbiology*, **43**, 14–20.
[34] Lahnsteiner, F., 2000. Morphological, physiological and biochemical parameters characterizing the over-ripening of rainbow trout eggs. *Fish Physiology and Biochemistry,* **23**, 107–118.
[35] Ninness, M.M., Don Stevens, E., Wright, P. A., 2006. Removal of the chorion before hatching results in increased movement and accelerated growth in rainbow trout (*Oncorhynchus mykiss*) embryos. *The Journal of Experimental Biology*, **209**, 1874–1882.
[36] Berois, N., Arezo, M.J., Papa, N.G., 2011. Gamete interactions in teleost fish: the egg envelope. Basic studies and perspectives as environmental biomonitor. *Biological Research,* **44 (2)**, 119–124.
[37] Henn, K., 2011. Limits of the fish embryo toxicity test with Danio rerio as an alternative to the acute fish toxicity test. Dissertation for the degree of Doctor, Ruperto-Carola University of Heidelberg, Germany.
[38] Witeska M., Sarnowski P., Ługowska K., and Kowal E., 2014. The effects of cadmium and copper on embryonic and larval development of ide *Leuciscus idus* L. *Fish Physiology and Biochemistry.* **40 (1)**, 151–163.
[39] Jezierska, B., £ugowska, K., Witeska, M., 2002. The effect of temperature and heavy metals on heart rate changes in common carp *Cyprinus carpio* L. and grass carp *Ctenopharyngodon idella* (Val.) during embryonic development. *Archives of Polish Fisheries*, **10 (2)**, 153–165.
[40] Wang, Z.G., Zhou, R., Jiang, D., Song, J.E., Xu, Q.,Si, J.,Chen, Y.P., Zhou, X., Gan, L., Li, J.Z., Zhang, H.,Liu, B., 2015. Toxicity of Graphene Quantum Dots in Zebrafish Embryo. *Biomedical and Environmental Sciences*, **28 (5)**, 341–351.

Editors: A. Kungolos, C. Christodoulatos, A. Koutsospyros,
C. Emmanouil, C. Laspidou, Z. Mallios, D. Dermatas
ISBN: 978-960-6865-94-7

[41] Singh, N., Manshian, B., Jenkins, G.J., Griffiths, S.M., Williams, P.M., Maffeis, T.G., Wright, C.J., Doak, S.H., 2009. NanoGenotoxicology: the DNA damaging potential of engineered nanomaterials. *Biomaterials*, **30 (23–24)**, 3891–3914.

[42] Brunetti, V., Chibli, H., Fiammengo, R., Galeone, A., Malvindi, M.A., Vecchio, G., Cingolani, R., Nadeau, J.L., Pompa, P.P., 2013. InP/ZnS as a safer alternative to CdSe/ZnS core/shell quantum dots: *in vitro* and *in vivo* toxicity assessment. *Nanoscale*, **5 (1)**, 307–317.

[43] Ju, Li., Zhang, G., Zhang, C., Sun, Li., Jiang, Y., Yan, C., Duerksen-Hughes, P.J., Zhang, X., Zhu, X., Chen, F.F., Jun, Yang, 2013. Quantum dot-related genotoxicity perturbation can be attenuated by PEG encapsulation. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, **753 (1)**, 54–64.

[44] Zhang, T., Stilwell, J.L., Gerion, D., Ding, L., Elboudwarej, O., Cooke, P.A., Gray, J.W., Alivisatos, A.P., Chen, F.F., 2006. Cellular effect of high doses of silica-coated quantum dot profiled with high throughput gene expression analysis and high content cellomics measurements. *Nano Letters*, **6 (4)**, 800–808.

[45] Saez, G., Aye, M., De Meo, M., Aimé, A., Bestel, I., Barthélémy, P., Di Giorgio, C., 2015. Genotoxic and oxidative responses in coelomocytes of Eisenia fetida and Hediste diversicolor exposed to lipid-coated CdSe/ZnS quantum dots and $CdCl_2$. *Environmental Toxicology*, **30 (8)**, 918–926.

[46] Villarreal, F.D., Das, G.K., Abid, A., Kennedy, I.M., Kültz, D., 2014. Sublethal effects of CuO nanoparticles on Mozambique tilapia (*Oreochromis mossambicus*) are modulated by environmental salinity. *PLoS One*, **9 (2)**, e88723.

Editors: A. Kungolos, C. Christodoulatos, A. Koutsospyros, C. Emmanouil, C. Laspidou, Z. Mallios, D. Dermatas
ISBN: 978-960-6865-94-7

XII

Assessment of heavy metals bioconcentration factor (BCF) and genotoxicity response induced by metal mixture in *Salmo salar* tissues

Sauliutė G, Stankevičiūtė M, Svecevičius G, Baršienė J, Valskienė R

10th International Conference on Environmental Engineering

2017, eISBN 978-609-476-044-0

https://doi.org/10.3846/enviro.2017.043

"Environmental Engineering" 10th International Conference
Vilnius Gediminas Technical University
Lithuania, 27–28 April 2017
http://enviro.vgtu.lt

eISSN 2029-7092 / eISBN 978-609-476-044-0

Article ID: enviro.2017.043
DOI: https://doi.org/10.3846/enviro.2017.043

Assessment of heavy metals bioconcentration factor (BCF) and genotoxicity response induced by metal mixture in *Salmo salar* tissues

Gintarė Sauliutė[1], Milda Stankevičiūtė[2], Gintaras Svecevičius[3], Janina Baršienė[4], Roberta Valskienė[5]

[1, 2, 3, 4, 5]Nature Research Centre, Akademijos 2, 08412 Vilnius, Lithuania
E-mails: [1]gintare.sauliute@gmail.com (corresponding author); [2]milda.stan@gmail.com; [4]janbar@ekoi.lt; [5]roberta.valskiene@gamtostyrimai.lt

Abstract. The aim of this study was to evaluate metals bioconcentration factor (BCF) in gills, liver, kidneys and muscle in relation with genotoxicity effects of metal mixture in peripheral blood, kidneys, gills and liver erythrocytes of the Atlantic salmon (*Salmo salar*). Fish were exposed to maximum-permissible waterborne concentrations of Zn – 0.1, Cu – 0.01, Ni – 0.01, Cr – 0.01, Pb – 0.005 and Cd – 0.005 mg/L, respectively for 7 and 14 days. Genotoxicity was studied using the micronucleus test. In addition, erythrocyte nuclear abnormalities (ENAs) were analysed. Our study indicates that metal BCF in Atlantic salmon is tissue-dependent. Based on the BCF classification scale, the relatively low values of metals bioconcentration were assessed, except for Zn (gills) and Cu (liver) (359.6 and 594.0, respectively). Zn intensively concentrated in fish tissues, while Pb – least of all. Overall, metals were concentrated mostly in the liver, least – in the muscle. Significant differences among BCF values of Pb in gills and muscle and Cd in gills were measured between 7 and 14 d exposure groups. Treatment with metal mixture significantly increased micronucleus frequencies after 7 d of exposure in liver and peripheral blood erythrocytes. Significant genotoxicity response was not observed after 14 d treatment. The erythrocytic nuclei abnormalities determined in *S. salar* blood were nuclear bud on filament (NBf), nuclear bud (NB), blebbed (BL), kidney shaped, vacuolated (VacNuc), 8-shaped nuclei and fragmented-apoptotic (FA) erythrocytes. Significant elevation in total ENAs level was detected in kidneys and liver erythrocytes after 7 d treatment, while after 14 d – in gills and kidneys erythrocytes. No significant differences among analysed responses were measured between 7 and 14 d exposure groups, except total ENAs level in liver erythrocytes.

Keywords: *Salmo salar*, metal mixture, bioconcentration factor (BCF), genotoxicity, nuclear abnormalities, micronuclei.

Conference topic: Environmental protection.

Introduction

Metals are the chemical toxicants that can disturb environmental homogeneity due to their indeterminate persistence, non-degradation, affinity for bioaccumulation and complex interactions (Roy *et al.* 2011). Heavy metals (Zn, Cu, Ni, Cr, Pb, Cd) are assigned to priority hazardous substances (pollutants) in many countries (Directive 2008/105/EC; US EPA 2009). Bioconcentration and biomagnification processes are capable of leading to adverse effects of metals in fish, even at low exposure concentrations as metals integrate into important protein synthesis reactions and as a result perturb vital processes (Valavanidis *et al.* 2006). In the longer time, the pollutants present in the environment at very low levels may accumulate within the body of aquatic organisms by diverse mechanisms to the quantity that they exert noxious effects. Therefore, it is crucial to know the bioaccumulation potential of a pollutant (Palaniappan, Karthikeyan 2009).

Experimental measurement of bioconcentration factor (BCF) is used to assess the potential for a chemical to bioaccumulate (Parkerton *et al.* 2008). Bioconcentration is a situation in which the levels of a toxin in an organism exceed the levels of that toxin in the surrounding environment. This terminology is often used specifically in reference to aquatic environments and aquatic organisms. BCF is used to express bioconcentration levels in a numeric way. The BCF can be calculated as the ratio of a toxin concentration in an organism and the levels in the surrounding environment. The higher the ratio, the more intense the bioconcentration of toxins, in this case, metals in fish. Information on BCF is required for regulatory purposes within the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), which regulates chemicals in the European Union (EU) (Crookes, Brooke 2011).

Due to the ability to accumulate persistent pollutants, fish are excellent bioindicators revealing the relative health of aquatic ecosystems (Lasheen *et al.* 2012). Aquatic organisms can accumulate chemical compounds by two ways: directly from the environment (via skin or respiratory surface) and indirectly (by collecting and concentrating a chemical compound from food). Bioaccumulation (bioconcentration, biomagnification) is a dynamic process, by which chemicals accumulation and excretion occur at the same time (Ivanciuc *et al.* 2006). Due to specific biochemical,

© 2017 Gintarė Sauliutė, Milda Stankevičiūtė, Gintaras Svecevičius, Janina Baršienė, Roberta Valskienė. Published by VGTU Press. This is an open-access article distributed under the terms of the Creative Commons Attribution (CC BY-NC 4.0) License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

physiological properties of the fish body tissues, diverse metal accumulation intensity may occur in different tissues. Therefore, it is important to identify and assess the potential for bioconcentration and to perform comparative analysis.

Aquatic organisms are typically exposed to mixtures of metals. Considerable amount of data show that certain metals affect accumulation of other metals in fish. Interactions among metals are related to their competitive uptake from the surrounding environment and different allocation in fish tissues. Interactions among metals may be different (additive, synergistic or antagonistic), therefore, the effects of their various mixtures on fish survival may also vary (Jezierska, Witeska 2001; Svecevičius *et al.* 2014).

Transition metals play an important role in oxidative reactions and are known for their potential to cause oxidative stress (Valavanidis *et al.* 2006). DNA is particularly susceptible to oxidative damage by reactive oxygen species (ROS). Micronucleus (MN) test together with erythrocyte nuclear abnormalities (ENAs) assay could be used as biomarkers of genotoxicity of a variety of genotoxic agents. As concluded by Luzhna *et al.* (2013), the role of metals in micronuclei formation and DNA damage arise from: binding to DNA and proteins, altered gene expression, mutations, altered cell cycle, chromosome non-disjunction, cytoskeleton dysfunction. Depending on the metal, clastogenic and aneugenic effects could lead to MN formation. Erythrocyte nuclear abnormalities could arise during the DNA replication process (Gomes *et al.* 2015).

Due to their susceptibility to water quality and commercial importance, Salmonids species were selected for experimental study (McCain 1998). The aim of this study was to evaluate metals bioconcentration factor (BCF) at steady-state in fish body tissues (gills, liver, kidneys and muscle) in relation with genotoxicity effects of metal mixture (Zn, Cu, Ni, Cr, Pb and Cd) in peripheral blood, kidneys, gills and liver erythrocytes of the Atlantic salmon (*Salmo salar*).

Material and methods

Experimental set-up

The experimental treatments were conducted on hatchery-reared one-year-old Atlantic salmon (*Salmo salar* Linnaeus, 1758) smolts, average total weight 42.1±4.43 g and average total length 167.4±7.17 mm (mean±SD, $N = 21$, respectively). The fish was obtained from Meškerinė fish hatchery (Švenčionys District, Lithuania) and 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 rate 1 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, the fish were fed in the same manner. Fish were accepted as acclimated to a new medium when their behavior became normal and they fed well. Deep-well water was used as the dilution water.

Reagent grade metal salts («REACHIM» Company, 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.

The tests were conducted under semi-static rotating water-current conditions on 3 groups (2 treatments and one control, each group consisting of seven individuals) using polyethylene (PE) plastic tanks of 35-L total volume filled to a level of 30 L with continuously aerated dilution water. Test fish were exposed for the 7 and 14 days period to a six metal mixture at a concentration corresponding to Lithuanian inland water standards or Maximum-Permissible-Concentrations (MPC) for the receiving water-bodies (Directive 2008/105/EC) (Table 1). Test solutions and clean water were renewed every day, and test fish were transferred into freshly prepared solutions after they were fed.

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

Metal	Source	Concentration	
		Maximum-Permissible-Concentration (MPC) (mg/L)	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

Analytical procedures

The main physico-chemical parameters of the water (temperature, dissolved O_2, pH and conductivity) were measured routinely with a hand-held multi-meter (WTW Multi 340i/SET, Germany). Designed nominal metal concentrations in the tanks were checked during blank tests (without fish) ($N = 4$) with an atomic absorption spectrophotometer (SHIMADZU AA-6800, Japan) by graphite furnace technique using proprietary software. Each water sample was acidified with reagent-grade nitric acid (final concentration 0.5% v/v) and analysed in triplicate. Mean measured concentrations were within 5% – 20% of the target.

Metal bioaccumulation analysis

After the testing was completed, fish (of control and metal-exposed groups) were sacrificed. Fish were measured (total body length (L) and fork-length (l_C), mm) and weighed (total body weight (Q) and body weight without stomach weight (q), g). Later they were used in the removal of needed tissues: muscle without skin (~3 g), gills (whole organ), liver (whole organ) and kidneys (whole organ); organs were weighed to an accuracy of ± 0.001 g. Fish samples were hot air oven-dried at 85 °C for 24 hours until reached constant weight, pre-digested tightly in a concentrated ultrapure HNO_3 (60%) and H_2O_2 (30%) (Lach-Ner, Chempur, respectively) at a ratio of 5:1 v/v for eight hours at a room temperature and then microwave-digested quickly (Jia *et al*. 2005). After that cooling solutions were filtered through a 0.45 μm glass filter and diluted with deionized water. Metal concentrations were measured by atomic absorption spectrophotometry on Varian Spectr AA 55 (USA) with a graphite furnace technique in accordance with standardized procedure ISO 15586:2003 final concentration being expressed as mg/kg of wet weight. Accuracy of analytical procedure was checked using certified reference material fish homogenate (IAEA–407). Recoveries were in acceptable range (within 10%) of the certified values.

Bioconcentration factors (BCF) estimations

Tissues with BCF greater than 1,000 are considered high, and less than 250 low, with those between classified as moderate (Landis *et al.* 2011).

BCF values in this study were calculated as reported by Gobas *et al.* (2009) where bioconcentration factor (BCF) is defined as the ratio of the steady-state metal ions concentrations in the fish vs the concentration in water:

$$BCF = \frac{C_{fish}\left(mg/kg\ wet\ fish\right)}{C_{water}\left(mg/L\right)}. \quad (1)$$

Micronucleus (MN) and erythrocyte nuclear abnormalities (ENAs) analyses

Blood was immediately taken from the caudal vein. A drop of blood was directly smeared on microscopic slides and air-dried. After the sacrifice, small pieces of cephalic kidneys, liver and gills were dissected, softly dragged along clean slide and allowed to dry for 1-2 h (Baršienė *et al.* 2006). Dried smears were fixed in methanol for 10 min. and were stained with 10% Giemsa solution in phosphate buffer pH = 6.8 for 8 min. (Baršienė *et al.* 2004). A light microscope Olympus BX51 (Tokyo, Japan) was used to examine a total of 4,000 cells per sample. Final results were expressed as the mean value (‰) of sums of analysed individual lesions scored in 1000 erythrocytes per fish sampled from every study group. The formation of micronuclei (MN), nuclear buds (NB), nuclear buds on filament (NBf), 8-shaped nuclei, fragmented-apoptotic (FA), kidney-shaped, blebbed (BL), vacuolated (VacNuc) erythrocytes were identified using criteria described by Fenech *et al.* (2003) and Baršienė *et al.* (2014).

Statistical analysis

The statistical analysis was performed using STATISTICA 7.0 (StatSoft Inc., Tulsa, Oklahoma, USA) software package. Significance of differences between the non-exposed and treated groups were tested using one-way analysis of variance ANOVA followed by Bonferroni post hoc test. The results were expressed as mean ± standard error or standard deviation. The level of significance was established at $p < 0.05$.

Results and Discussion

Bioconcentration factor (BCF) assessment

BCF values were calculated in gills, liver, kidneys and muscle tissues after salmon exposure to metal mixture (composed of Zn, Cu, Ni, Cr, Pb and Cd) for 7 and 14 days. According to BCF classification scale, low BCF values of analysed metals were measured in fish tissues after 7 and 14 d exposure period (Fig. 1). However, average values of BCF for Zn [(in gills (356.8–359.6), in kidneys (293.6–294.0)] and Cu [in liver (566.7–594.0)] were recorded. As shown in Fig. 1, metal accumulation intensity in salmon tissues was similar after 7 and 14 d exposure period. Significant differences ($p < 0.05$) among BCF values of Pb in gills and muscle and Cd in gills were measured between 7 d and 14 d exposure groups. BCF shows the potential of particular metals to bioaccumulate in specific tissue. BCF values

of Zn, Ni, Cr and Cd in different tissues followed the same sequence: gills>kidneys>liver>muscle; while Cu – liver>kidneys>gills>muscle; Pb (after 7 d): muscle>kidneys>gills>liver, after 14 d – muscle>gills>kidneys>liver.

Generally, metal levels in fish body tissues usually follow the ranking: liver>gills>kidneys>muscle. The highest metal concentration was detected in the liver of salmon, the least – in muscle. Liver is an important target organ involved in metabolic and detoxification mechanisms (Liebel *et al.* 2013). Based on the data, metals exposure may cause an increase in metallothionein (MT) levels in animals, including fish (Hogstrand, Haux 1991). Reduced levels of proteins, lipids activities in the muscles were measured after fish exposure to metals. According to Allen-Gill and Martynov (1995), low levels of metals accumulated in muscles are due to slower synthesis of proteins in this tissue. BCF values revealed metals accumulation patterns in tissues of Atlantic salmon. Metals accumulation in the tissues showed the following sequences: gills: Zn>Cu>Cd>Cr>Ni>Pb; liver: Cu>Zn>Cd>Cr>Ni>Pb; kidneys: Zn>Cu>Cd>Cr>Ni>Pb and muscle: Cu>Zn>Pb>Cr>Ni>Cd. Zinc and Cu showed the highest levels of accumulation in the tissues. Essential metals (copper, zinc) are vital for the health of fish, involved in all aspects of biological function. However, an excess amount of such metals produces cellular and tissue damage, forming dangerous free radicals. Consequently, there is a fine balance between metal deficiency and surplus and it is crucial for organisms to maintain metal homeostasis via tight regulation by maintaining a balance between uptake and excretion (Bury *et al.* 2003). Bioaccumulation levels of Cd (in gills, liver and kidneys) and Pb (in muscle) take third place in the sequences. Cd and Pb have no known biological functions and are considered as non-essential toxic metals, which tend to accumulate in carnivorous fish tissues (Yousafzai *et al.* 2010). Pb and Cd disrupt calcium uptake in gills and may affect the metabolism of essential trace element by having an effect on normal tissue distribution of Zn and Cu (Komjarova, Blust 2009; Birceanu *et al.* 2008).

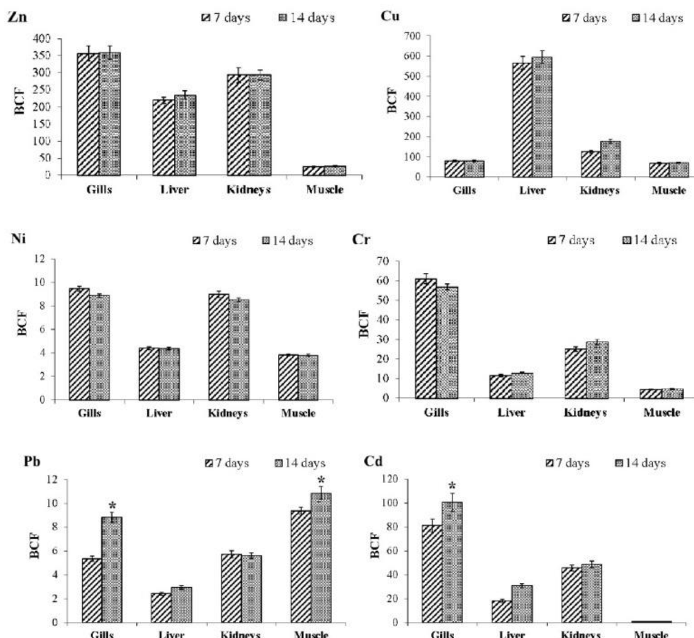


Fig. 1. Bioconcentration factor (BCF) in the selected organ tissues exposed to metal mixture for 7 and 14 d (mean ± SEM, $N = 7$). Asterisks (*) denote significant differences among exposure groups ($p < 0.05$)

According to Sauliutė and Svecevičius (2014) study result, salmon exposure to metal mixture (Zn, Cu, Ni, Cr, Pb, Cd) and single metal (Ni, Cr, Pb) at standing water-current conditions for 14 days showed different BCF values of

metals in fish tissues. BCF values of analysed metals were lower in comparison to this study. However, higher BCF level was measured for Zn in muscle (BCF = 283). Significantly higher BCF values of metals after fish exposure to metal mixture than to single metal were reported in that study. This could be influenced by synergistic interaction within metal mixture. Contrary to our result (during rotating water-current conditions), Sauliutė and Svecevičius (2014) study showed the highest BCF values in salmon muscle, the least in kidneys. The reasons why the Atlantic salmon accumulated the highest amounts of metals in the muscle are due to the experimental design and fish behavior (fish activity was low – lying on the bottom of the tank). Atlantic salmon is a very active rheophilous species which actively searches for food and performs distant and long-term anadromous and catadromous migrations. As confirmed by field studies, fish activity can promote the release of metals from the tissues (Svecevičius *et al.* 2014; Mohammadnabizadeh *et al.* 2014; Jezierska, Witeska 2001; Ray 1978). Therefore, in this study a new experimental system using rotating water-current condition was developed, which particularly reflect fish natural living conditions.

Micronucleus (MN) and erythrocyte nuclear abnormalities (ENAs) analyses

Fig. 2 shows MN frequencies in *S. salar* gills, kidneys, liver and peripheral blood erythrocytes. Significant increases occurred at 7 d of exposure in liver and blood erythrocytes. Significant genotoxicity response was not observed after 14 d treatment. The presence of micronuclei is an irreversible change and reflects genotoxic damage (Javed *et al.* 2016). Exposure to single metals such as Cd, Pb, Cr, Cu, Zn and Ni at high concentrations is known to induce MN in peripheral blood erythrocytes in different fish species (Gomes *et al.* 2015; Ahmed *et al.* 2013; Çavaş 2008; Bagdonas, Vosylienė 2006). There are experimental studies showing capacity of single metal (such as Cr) to induce MN even at low (environmentally relevant) concentrations (Zhu *et al.* 2004). Micronuclei induction after fish exposure to environmentally relevant metal mixture concentrations is scantily discussed. Significant MN induction was observed in blood after treatment with Cd, Cu, Pb, Zn and Cu, Zn metal mixtures using higher concentrations in several fish species (Harabawy, Mosleh 2014; Obiakor *et al.* 2010).

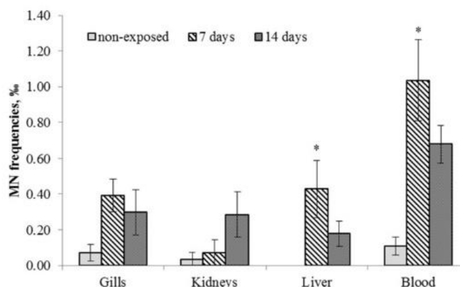


Fig. 2. Genotoxicity responses in *Salmo salar* gills, kidneys, liver and peripheral blood erythrocytes (mean ± SEM, $N = 21$). Asterisks (*) denote significant differences from control during exposure time ($p < 0.05$)

The erythrocytic nuclei abnormalities determined in *S. salar* blood were nuclear bud on filament (NBf), nuclear bud (NB), blebbed (BL), kidney-shaped, vacuolated (VacNuc), 8-shaped nuclei and fragmented-apoptotic (FA) erythrocytes. Levels of total ENAs are shown in Fig. 3. Significant ENAs induction was found in *S. salar* kidneys and liver erythrocytes after 7 days of exposure, while after 14 d of exposure – in gills and kidneys erythrocytes. No significant differences among analysed responses were measured between 7 d and 14 d exposure groups, with the exception of total ENAs level in liver erythrocytes. ENAs induction in different tissues erythrocytes is scantily discussed. Erythrocyte nuclear abnormalities are markers of genetic instability. It is suggested that ENAs could arise from the DNA replication process (Gomes *et al.* 2015). Induction of various ENAs such as lobed, blebbed, notched, bud, vacuolated and condensed nuclei after treatment with single Cd was reported (Gomes *et al.* 2015). Binucleated, kidney-shaped nuclei, blebbed nuclei, lobed nuclei, bilobed nuclei, notched nuclei, hook-shaped nuclei and vacuolated nuclei induction in Nile tilapia, *Oreochromis niloticus* was reported after treatment with Cd, Cu, Pb and Zn (1.25 mgL^{-1} of each) metal mixture (Harabawy, Mosleh 2014). As Gomes *et al.* (2015) study shows, MN induction exhibited the lowest frequency in most of Cd treatments, while the specific ENAs showed the highest frequencies in all treatments. As shown in Fig. 2, significant ENAs induction was noticed in *S. salar* gills and kidneys erythrocytes, while MN frequencies in these tissues were statistically insignificant during all exposure time. The results of this study suggest using MN test in combination with ENAs assay, which together bring better results considering genotoxicity evaluation after treatment with metal mixture at low exposure concentrations.

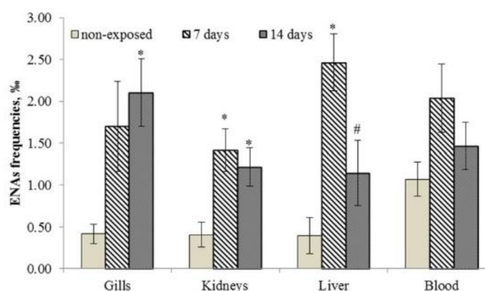


Fig. 3. Erythrocyte nuclear abnormalities level in *Salmo salar* gills, kidneys, liver and peripheral blood erythrocytes (mean ± SEM, $N = 21$). Asterisks (*) denote significant differences from control during exposure time, while # – differences among exposure groups ($p < 0.05$)

Conclusions

This study attempted to investigate the genotoxic potential and bioconcentration factor of complex metal mixture at environmentally relevant (maximum-permissible waterborne concentrations) concentrations, whose effects on aquatic organisms are still poorly investigated. The obtained results showed that used metal mixture caused genotoxic damage in *S. salar* erythrocytes. The results of this study suggest using MN test in combination with erythrocyte nuclear abnormalities assay, which together brings better results considering genotoxicity evaluation after metal treatment. Our study indicates that metal BCF in Atlantic salmon is tissue-dependent. However, measured BCF values of analysed metals were low in fish tissues after 7 and 14 d exposure period. The highest metal concentration was detected in the liver of salmon, the least – in muscle. Significant differences among BCF values of Pb (gills, muscle) and Cd (gills) were measured between 7 and 14 d exposure groups.

Acknowledgements

We are thankful to Irena Žalakevičienė for the language check.

Funding

This research was supported by the research infrastructure Open Access Centre for Nature Research at the initiative of the Open R&D Lithuania network. This work is funded by the Research Council of Lithuania [Project No MIP-108/2015]. Geno-cytotoxicity studies were performed under Nature Research Centre programme 5 "Investigations of hazardous substances distribution in the ecosystems, regularities and mechanisms of ecotoxicity and biological effects under the impact of anthropogenic stress".

Disclosure statement

The authors declare that have no conflict of interest.

References

Ahmed, M.; Kundu, G.; Al-Mamun, M.; Sarkar, S.; Akter, M.; Khan, M. 2013. Chromium (VI) induced acute toxicity and genotoxicity in freshwater stinging catfish, Heteropneustes fossilis, *Ecotoxicology and Environmental Safety* 92: 64–70. https://doi.org/10.1016/j.ecoenv.2013.02.008

Allen-Gill, S. M.; Martynov, V. G. 1995. Trace metals burdens in nine species of freshwater and anadromous fish from the Pechora River, Northern Russia, *Science of the Total Environment* 161: 653–659. https://doi.org/10.1016/0048-9697(95)93634-T

Bagdonas, E.; Vosylienė, M. Z. 2006. A study of toxicity and genotoxicity of copper, zinc and their mixture to rainbow trout (*Oncorhynchus mykiss*), *Biologija* 1: 8–13.

Baršienė, J.; Lazutka, J.; Syvokienė, J.; Dedonytė, V.; Rybakovas, A.; Bagdonas, E.; Bjornstad, A.; Andersen, OK. 2004. Analysis of micronuclei in blue mussels and fish from the Baltic and the North Seas, *Environmental Toxicology* 19(4): 365–371. https://doi.org/10.1002/tox.20031

Baršienė, J.; Dedonytė, V.; Rybakovas, A.; Andreikenaitė, L.; Andersen, OK. 2006. Investigation of micronuclei and other nuclear abnormalities in peripheral blood and kidney of marine fish treated with crude oil, *Aquatic Toxicology* 78S: S99–S104. https://doi.org/10.1016/j.aquatox.2006.02.022

Baršienė, J.; Butrimavičienė, L.; Grygiel, W.; Lang, T.; Michailovas, A.; Jackūnas, T. 2014. Environmental genotoxicity and cytotoxicity in flounder (*Platichthys flesus*), herring (*Clupea harengus*) and Atlantic cod (*Gadus morhua*) from chemical munitions dumping zones in the southern Baltic Sea, *Marine Environmental Research* 96: 56–67. https://doi.org/10.1016/j.marenvres.2013.08.012

Birceanu, O.; Chowdhury, M. J.; Gillis, P.; McGeer, J.; Wood, C. M.; Wilkie, M. P. 2008. Modes of metal toxicity, and impaired branchial ionoregulation in rainbow trout exposed to mixtures of Pb and Cd in soft water, *Aquatic Toxicology* 89: 222–231. https://doi.org/10.1016/j.aquatox.2008.07.007

Bury, N. R.; Walker, P. A.; Glover, C. N. 2003. Nutritive metal uptake in Teleost fish, *Journal of Experimental Biology* 206: 11–23. https://doi.org/10.1242/jeb.00068

Çavaş, T. 2008. In vivo genotoxicity of mercury chloride and lead acetate: micronucleus test on acridine orange stained fish cells, *Food and Chemical Toxicology* 46: 352–358. https://doi.org/10.1016/j.fct.2007.08.015

Crookes, M.; Brooke, D. 2011. *Introduction. In Estimation of fish bioconcentration factor (BCF) from depuration data*. Environment Agency. Environment Agency, Evidence. Horizon House, Deanery Road, Bristol, BS1 5AH. 11–12.

Directive 2008/105/EC of the European Parliament and of the Council of 496 16 December 2008 on environmental quality standards in the field of water 497 policy, amending and subsequently repealing Council Directives 82/176/EEC, 498 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 499 2000/60/EC of the European Parliament and of the Council. Official Journal of the European Communities L 500 348, 24/12/2008 p. 0084-0097.

Fenech, M.; Chang, W. P.; Kirsch-Volders, M.; Holland, N.; Bonassi, S.; Zeiger, E. 2003. Human MicronNucleus project. HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures, *Mutation Research* 534: 65–75. https://doi.org/10.1016/S1383-5718(02)00249-8

Gomes, J. M. M.; Ribeiro H. J.; Procópio, M. S.; Alvarenga, B. M.; Castro, A. C. S.; Dutra, W. O., *et al.* 2015. What the erythrocytic nuclear alteration frequencies could tell us about genotoxicity and macrophage iron storage?, *PLoS ONE* 10(11): e0143029. https://doi.org/10.1371/journal.pone.0143029

Gobas, F. A. P. C.; Wolf, D.; Burkhard, W.; Verbruggen, L. P.; Plotzke, K., *et al.* 2009. Revisiting bioaccumulation criteria for POPs and PBT assessments, *Integrated Environmental Assessment and Management* 5: 624–637. https://doi.org/10.1897/IEAM_2008-089.1

Harabawy, A. S. A.; Mosleh, Y. Y. I. 2014. The role of vitamins A, C, E and selenium as antioxidants against genotoxicity and cytotoxicity of cadmium, copper, lead and zinc on erythrocytes of Nile tilapia, *Oreochromis niloticus, Ecotoxicology and Environmental Safety* 104: 28–35. https://doi.org/10.1016/j.ecoenv.2014.02.015

Hogstrand, C.; Haux, C. 1991. Mini review. Binding and detoxification of trace metals in lower invertebrates with references to metallothionin, *Comparative Biochemistry and Physiology* 100: 383–390.

ISO 15586:2003. Water quality – determination of trace elements using atomic absorption spectrometry with graphite furnace. Geneva, International Organization for Standardization.

Ivanciuc, T.; Ivanciuc, O.; Douglas, K. D. 2006. Modeling the bioconcentration factors and bioaccumulation factors of polychlorinated biphenyls with posetic quantitative superstructure/activity relationships (QSSAR), *Molecular Diversity* 10: 133–145. https://doi.org/10.1007/s11030-005-9003-3

Javed, M.; Ahmad, I.; Ahmad, A.; Usmani, N.; Ahmad, M. 2016. Studies on the alterations in haematological indices, micronuclei induction and pathological marker enzyme activities in *Channa punctatus* (spotted snakehead) perciformes, channidae exposed to thermal power plant effluent, *Springerplus* 5(1): 761. https://doi.org/10.1186/s40064-016-2478-9

Jezierska, B.; Witeska, M. 2001. *Metal toxicity to fish*. Siedlce, Poland: Wydawnictwo AP.

Jia, H.; Ren, H.; Satoh, S.; Endo, H.; Hayashi, T. 2005. Comparison of pretreatment condition of cadmium in fish sample and diet by microwave digestion method for ICP-AES, *Journal of the Tokyo University Marine Science and Technology* 1: 41–46.

Komjarova, I.; Blust, R. 2009. Multimetal Interactions between Cd, Cu, Ni, Pb, and Zn Uptake from Water in the Zebrafish *Danio rerio*, *Environmental Science & Technology* 43(19): 7225–7229. https://doi.org/10.1021/es900587r

Landis, W.; Sofield, R.; Yu, M. H.; Landis, W. G.; Sofield, R. M. 2011. *Introduction to environmental toxicology: molecular substructures to ecological landscapes.* 4th ed. Boca Raton, Florida: CRC Press. ISBN 9781439804100.

Lasheen, M. R.; Abdel-Gawad, F. Kh.; Alaneny, A. A.; Abd El bary, H. M. H. 2012. Fish as bio indicators in aquatic environmental pollution assessment: a case study in Abu-Rawash area, Egypt, *World Applied Sciences Journal* 19(2): 265–275.

Liebel, S.; Tomokake, M. E. M.; Oliveira Ribeiro, C. A. 2013. Fish histopathology as biomarker to evaluate water quality, *Ecotoxicology and Environmental Contamination* 8(2): 9–15. https://doi.org/10.5132/eec.2013.02.002

Luzhna, L.; Kathiria, P.; Kovalchuk, O. 2013. Micronuclei in genotoxicity assessment: from genetics to epigenetics and beyond, *Frontiers in Genetics* 4: 131. https://doi.org/10.3389/fgene.2013.00131

McCain, B. B. 1998. Uptake of aromatic chlorinated hydrocarbons by juvenile chinook salmon (*Oncorhynchus tshawytscha*) in an urban estuary, *Archives of Environmental Contamination and Toxicology* 19: 10–16. https://doi.org/10.1007/BF01059807

Mohammadnabizadeh, S.; Pourkhabbaz, A.; Afshari, R. 2014. Analysis and determination of trace metals (Nickel, Cadmium, Chromium, and Lead) in tissues of *Pampus argenteus* and *Platycephalus indicus* in the Hara Reserve, Iran, *Hindawi Publishing Corporation Journal of Toxicology*. https://doi.org/10.1155/2014/576496

Obiakor, M. O.; Okonkwo, J. C.; Ezeonyejiaku, C. D.; Ezenwelu, C. O. 2010. Genotoxicology: single and joint action of copper and zinc to *Synodontis clarias* and *Tilapia nilotica, Journal of Applied Sciences and Environmental Management* 14(3): 59–64. https://doi.org/10.4314/jasem.v14i3.61468

Palaniappan, P. L. R. M.; Karthikeyan, S. 2009. Bioaccumulation and depuration of chromium in the selected organs and whole body tissues of freshwater fish *Cirrhinus mrigala* individually and in binary solutions with nickel, *Journal of Environmental Sciences* 21: 229–236. https://doi.org/10.1016/S1001-0742(08)62256-1

Parkerton, T. F.; Arnot, J. A.; Weisbrod, A. V.; Russom, C.; Hoke, R. A; Woodburn, K.; Traas, T.; Bonnell, M.; Burkhard, L. P.; Lampi, M. A. 2008. Guidance for evaluating in vivo fish bioaccumulation data, *Integrated Environmental Assessment and Management* 4(2): 139–155. https://doi.org/10.1897/IEAM_2007-057.1

Ray, S. 1978. Bioaccumulation of lead in Atlantic salmon (*Salmo salar*), *Bulletin of Environmental Contamination and Toxicology* 19: 631–636. https://doi.org/10.1007/BF01685850

Roy, U. S.; Chattopadhyay, B.; Datta, S.; Mukhopadhyay, S. K. 2011. Metallothionein as a biomarker to assess the effects of pollution on Indian major carp species from wastewater-fed fishponds of East Calcutta wetlands (a Ramsar site), *Environmental Research, Engineering and Management* 4(58): 10–17.

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.

Svecevičius, G.; Sauliutė, G.; Idzelis, R. L.; Grigelevičiūtė, J. 2014. Accumulation of heavy metals in different body tissues of Atlantic Salmon, *Salmo salar* L., exposed to a model mixture (Cu, Zn, Ni, Cr, Pb, Cd) and singly to nickel, chromium, and lead, *Bulletin of Environmental Contamination and Toxicology* 92: 440–445. https://doi.org/10.1007/s00128-014-1237-2

US EPA. 2009. National recommended water quality criteria. Office of Water, Office of Science and Technology (4304T) [online], [cited 10 October 2016]. Available from Internet: http://water.epa.gov/scitech/swguidance/standards/criteria/current/

Valavanidis, A.; Vlahogianni, T.; Dassenakis, M.; Scoullos, M. 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants, *Ecotoxicology and Environmental Safety* 64: 178–189. https://doi.org/10.1016/j.ecoenv.2005.03.013

Yousafzai, A.; M.; Chivers, D. P.; Khan, A. R.; Ahmad, I.; Siraj, M. 2010. Comparison of heavy metals burden in two freshwater fishes *Wallago attu* and *Labeo dyocheilus* with regard to their feeding habits in natural ecosystem, *Pakistan journal of zoology* 42(5): 537–544.

Zhu, Y.; Wang, J.; Bai, Y.; Zhang, R. 2004. Cadmium, chromium, and copper induce polychromatocyte micronuclei in carp (*Cyprinus carpio* L.), *Bulletin of Environmental Contamination and Toxicology* 72(1): 78–86. https://doi.org/10.1007/s00128-003-0243-6

View publication stats

XIII

Erythrocytic nuclear abnormalities, DNA damage, bioconcentration factor and haematological changes induced by metal mixture at environmentally relevant concentrations in *Rutilus rutilus*

Stankevičiūtė M, Sauliutė G, Markuckas M, Virbickas T, Baršienė J

Proceedings of the 14th International Conference on Protection and Restoration of the Environment 2018, ISBN: 978-960-99922-4-4. Thessaloniki, Greece. p. 785–794.

ERYTHROCYTIC NUCLEAR ABNORMALITIES, DNA DAMAGE, BIOCONCENTRATION FACTOR AND HEMATOLOGICAL CHANGES INDUCED BY METAL MIXTURE AT ENVIRONMENTALLY RELEVANT CONCENTRATIONS IN *RUTILUS RUTILUS*

M. Stankevičiūtė*[1], G. Sauliutė[1], A. Markuckas[2], T. Virbickas[1], J. Baršienė[1]

[1] Nature Research Centre, Akademijos st. 2, LT-08412 Vilnius, Lithuania
[2]Vilnius University, Life Sciences Center, Department of Biochemistry and Molecular Biology, Saulėtekio av. 7, 10223 Vilnius, Lithuania

*Corresponding author: e-mail: milda.stan@gmail.com, tel: +370 60716809

Abstract
The aim of this study was to assess bioconcentration factor (BCF), metallothioneins (MT), genotoxicity, cytotoxicity and changes of haematological parameters in roach *Rutilus rutilus* after 14 days treatment with a six metals mixture (MIX) at environmentally relevant concentrations (Zn – 0.1, Cu – 0.01, Ni – 0.01, Cr – 0.01, Pb – 0.005 and Cd – 0.005 mg/L) and with 6 variants (reduced concentration of single metal while other metals concentration remain constant) of the MIX. Most frequently the highest accumulated amount of metals in tissues (gills, liver, kidneys, muscle) was detected after treatment with variants of MIX. Significantly reduced concentration of accumulated Ni was measured after Cu↓, Cr↓, Pb↓ and Cd↓ treatments (10 times reduced Cu^{2+}, Cr^{6+}, $Pb^{2+}$and Cd^{2+} concentration, respectively) in all tissues (except in liver after Cu↓, Cr↓ and Cd↓ treatments) compared with MIX. Significant *induction* of *MT in liver and kidneys* was *not detected. However, positive correlation* ($r = 0.83$; $p = 0.022$) *was measured between MT and Zn amount in liver.* DNA damage in erythrocytes of roach was examined by comet assay. Additionally, erythrocytic nuclear abnormalities were assessed in erythrocytes of peripheral blood, liver, kidneys and gills. Significant DNA damage was measured after Cr↓, Pb↓ and Zn↓ treatments. Significant elevations in total ENAs were measured after Cr↓ and Ni↓, MIX or Ni↓ treatments in peripheral blood, gills and kidneys erythrocytes, respectively. The frequencies of *separate ENAs* such as micronuclei, enucleus were significantly elevated after Cr↓, Ni↓ treatments in peripheral blood, respectively; apoptotic cells – after MIX treatment in gills and enucleus after Ni↓ treatment in liver compared to control level. Decreased number of *red blood cells*, *haematocrit level*, haemoglobin concentration and increased number of white blood cells in peripheral blood was measured after MIX treatment. However, only decrease in haemoglobin concentration was statistically significant.

Keywords: Genotoxicity; comet assay; cytotoxicity; bioconcentration factor (BCF); *Rutilus rutilus;* metallothioneins

1. INTRODUCTION

Metals in the environment continue to create serious global health concerns, because metals cannot be degraded into non-toxic forms and are persistent pollutants in the ecosystems (Ayangbenro and Babalola, 2017). Metals at certain concentrations are toxic to all life forms. However, contamination of the ecosystems with metals continues to increase and exceed the recommended limit in the environment (Dixit et al, 2015). Several studies showed that metals are toxic to fish even at low

concentrations and are capable of inducing genotoxicity, cytotoxicity, DNA fragmentation and other toxicity endpoints (Zhu et al, 2004; Cavas et al, 2005). Genotoxicity and cytotoxicity of metal mixture at Maximum-Permissible-Concentrations (MPC) previously were evaluated in rainbow trout (*Ochorhyncus mykiss*) (Valskienė et al, 2015) and Atlantic salmon (*Salmo salar*) (Stankevičiūtė et al, 2017). Significant accumulation of metals in *S. salar* tissues also was reported after treatment with metal mixture at MPC (Stankevičiūtė et al, 2017). However, most of the studies evaluating joint metal toxicity are dealing with binary metal mixtures toxicity at high concentrations (Driessnack et at., 2016, 2017; Duran et al, 2015; Winter et al, 2012). Notwithstanding, fish in the environment encounters with complex metal mixtures. Such exposure may lead to higher toxicity and bioaccumulation levels due to interactions of compounds in the metal mixture (Heys et al, 2016; Cedergreen, 2014).

This study was designed to evaluate metal mixture induced genotoxicity, cytotoxicity, changes in haematological parameters, bioaccumulation and metallothioneins content in *R. rutilus* tissues using whole mixture approach. Whole mixture testing is *more similar* to the current *environment exposure, because chemicals in the environment exist in mixtures* and at low concentrations (Heys et al, 2016).

The main objectives of the present study were: 1) to assess bioconcentration factor (BCF) of metals in different tissues (gills, liver, kidneys and muscle) of *Rutilus rutilus* after exposure to metal mixture at a concentration corresponding to Maximum-Permissible-Concentrations (MPC) accepted for the inland waters in EU, 2) to assess DNA damage and nuclear abnormalities in erythrocytes of roach after treatment with metal mixture and variants of this mixture, 3) to evaluate metallothioneins content in liver and kidneys tissue and 4) to assess haematological changes after fish exposure to metal mixture.

2. MATERIALS AND METHODS

2.1 Experimental set-up

The test was conducted on hatchery-reared 3–4 years old juveniles roach (*Rutilus rutilus* Linnaeus, 1758), average total weight 50.9 ± 12.4 g and average total length 160.6 ± 12.2 mm (mean ± SD, *N* = 56 respectively). The fish was obtained from fish hatchery (Elektrėnai District, Lithuania) and kept for acclimation in holding tanks (1000-L volume) supplied with flow-through aerated deep-well water at least two weeks prior to testing. Fish were kept under a *natural light* cycle and fed commercial fish 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 supply and diet were kept as during the acclimation period. Deep-well water was used as the dilution water. Its chemical and physical characteristics have been presented in our previous research (Stankevičiūtė et al, 2017). Reagent grade metal salts («REACHIM» Company, Russia) were used as the toxicants. Stock solution was prepared by dissolving the necessary amount of the salt in distilled water, the final concentration being recalculated according to the amount of metal ion. The experiment was conducted under semi-static rotating water-current conditions on 8 groups of fish (treatment and control, *N* = 56). Seven *R. rutilus* were put in each *polyethylene* (PE) tank of 35-L total volume filled to a level of 30 L with continuously aerated dilution water (7 treatments), a total of 49 fish in treatment and 7 in control groups. Test fish were exposed for 14 days period to a six metal (Zn, Cu, Ni, Cr, Pb and Cd) mixture (hereinafter referred to as MIX) at a concentration corresponding to Maximum-Permissible-Concentrations (MPC) accepted for the inland waters in EU (Directive 2008/105/EC) (Table 1). Other treatments were performed by reducing MPC of single metal in the mixture (MIX) made of 6 metals by 10-times, while other 5 metals concentrations remain constant (e.g. Zn↓ (metal with reduced concentration in MIX), while Cu, Ni, Cr, Pb, Cd concentrations remain constant (hereinafter referred to as Zn↓) and etc.). Test solutions and clean water were renewed every day, and test fish were transferred into freshly prepared solutions after they were fed.

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

Metal	Source	Concentration (mg/L)			
		MIX (MPC) nominal	MIX Measured (mean ± SD)	Metal↓ nominal	Metal↓ Measured (mean ± SD)
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

The main physico-chemical parameters of the water were measured routinely with a hand-held multi-meter (WTW Multi 340i/SET, Germany). Designed nominal metal concentrations in the tanks were checked during blank tests (without fish) (N = 4) with an atomic absorption spectrophotometer (SHIMADZU AA-6800, Japan) by graphite furnace technique using proprietary software. Each water sample was acidified with reagent-grade nitric acid (final concentration 0.5% v/v) and analysed in triplicate. Mean measured concentrations are presented in Table 1.

2.2 Metal bioaccumulation analysis

After the testing was completed, fish (of control and metal-exposed groups) were sacrificed. 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 (~3 g), gills (whole organ), liver (whole organ) and kidneys (whole organ); organs were weighed to an accuracy of ±0.001 g. Fish samples were hot air oven-dried at 85 °C for 24 hours until reached constant weight, pre-digested tightly in a concentrated ultrapure HNO_3 (60%) and H_2O_2 (30%) (Lach-Ner, Chempur, respectively) at a ratio of 5:1 v/v for eight hours at a room temperature and then microwave-digested quickly (Jia et al 2005). After cooling solutions were filtered through a 0.45 μm glass filter and diluted with deionized water. Metal concentrations were measured by atomic absorption spectrophotometry on Varian Spectr AA 55 (USA) with a graphite furnace technique in accordance with standardized procedure ISO 15586:2003 final concentration being expressed as mg/kg of wet weight. Accuracy of analytical procedure was checked using certified reference material fish homogenate (IAEA–407). Recoveries were in acceptable range (within 10%) of the certified values.

2.3 Bioconcentration factors (BCF) estimations

Tissues with BCF greater than 1,000 are considered high, and less than 250 is low bioaccumulation potential, with those between classified as moderate (Landis et al, 2011). BCF values in this study were calculated as reported by Gobas et al (2009) where bioconcentration factor (BCF) is defined as the ratio of the steady-state metal ions concentrations in the fish vs the concentration in water:

$$BCF = \frac{C_{fish}\,(mg/kg\ wet\ fish)}{C_{water}\,(mg/L)}, \quad (1)$$

2.4 Metallothioneins determination

Metallothionein content determination was assayed according to the method of Peixoto et al (2003). For metallothionein level assays, the liver and kidney were removed, weighted and frozen (-80 °C). The organs were homogenized with Potter-Elvehjem homogenizer in 4 volumes of 20 mM Tris-HCl buffer, pH 8.6, containing 0.5 mM PMSF and 0.01% β-mercaptoethanol. The homogenate was then centrifuged at 17,000 × g for 30 min at 4 °C. Aliquots of 1 ml of supernatant containing metallothioneins were added with 1.05 ml of cold (-20 °C) absolute ethanol and 80 μl chloroform.

The samples were centrifuged at 6000 × *g* for 10 min at 4 °C. The collected supernatant was combined with three volumes of cold ethanol (-20 °C), maintained at -20 °C for 1 h and centrifuged at 6000 × *g* for 10 min at 4 °C. The metallothionein-containing pellets were then rinsed with 1 ml of 87% ethanol and 1% chloroform mix and centrifuged at 6000 × *g* for 10 min at 4 °C. The metallothionein content in the pellet was evaluated using the colorimetric method with DTNB reagent. The pellet was suspended in 150 µl 0.25 M NaCl and subsequently 150 µl 1 N HCl containing 4 mM EDTA was added to the sample. 4.2 ml 2 M NaCl containing 0.43 mM DTNB buffered with 0.2 M Na-phosphate, pH 8.0 was then added to the sample at room temperature. The sample was centrifuged at 3000 × *g* for 5 min at room temperature. The supernatant absorbance was evaluated at 412 nm. Metallothionein concentration was estimated using molar absorption coefficient at 412 nm 14140 $M^{-1}cm^{-1}$ (Eyer et al, 2003) and expressed as micrograms of SH groups per gram of wet weight.

2.5 Erythrocytic nuclear abnormalities (ENAs) analysis in in vivo assay

ENAs analysis was performed in peripheral blood, gills, kidneys and liver erythrocytes. Blood was immediately taken from the caudal vein. A drop of blood was directly smeared on microscopic slides and air-dried. After the sacrifice, small pieces of cephalic kidneys, liver and gills were dissected, softly dragged along clean slide and allowed to dry for 1-2 h. Dried smears were fixed in methanol for 10 min. and were stained with 10% Giemsa solution in phosphate buffer pH = 6.8 for 8 min. (Baršienė et al, 2004). The stained slides were analysed under bright-field microscopes Olympus BX51 (Tokyo, *Japan*) using an immersion objective (1000×) and the photos were taken *with* an Olympus U-CMAD3 (Tokyo, *Japan*) camera. 4,000 erythrocytes with intact cellular and nuclear membrane per fish were evaluated using blind scoring by a single observer. Final results were expressed as the mean value (‰) of sums of analysed individual lesions scored in 1000 erythrocytes per fish sampled from every study group. The formation of micronuclei (MN), binucleated erythrocyte with nucleoplasmic bridge (BNb), nuclear buds (NB), nuclear buds on filament (NBf), 8-shaped nuclei, fragmented (Fr), apoptotic (Ap), binucleated (BN) erythrocytes were identified using criteria described by Fenech et al (2003) and Baršienė et al (2014). Additionally, kidney-shaped, blebbed (BL), vacuolated nuclei (VacNuc), enucleus (EN) erythrocytes were identified (Harabawy and Mosleh 2014).

2.6 Cell isolation and Comet assay

Peripheral blood samples were collected from the caudal vein using an insulin syringe (30G needle, 3.8% *sodium citrate*). Blood was placed in a 15 mL glass bottles containing 10 mL of chilled phosphate buffered saline (PBS). The viability of the erythrocytes was assessed through the Trypan Blue exclusion method (Anderson and Wild, 1994). Only cell suspensions with viability >90% were used. Alkaline comet assay version technique was used as described by Singh et al (1988) with slight modifications (Fatima et al, 2014). The slides were stained with ethidium bromide, placed under a glass cover and analysed by fluorescence microscopy (Olympus BX51, *Olympus U-RFL-T*, Tokyo, *Japan*); the photos were taken *with* an Olympus U-CMAD3 (Tokyo, *Japan*) camera. 50 nuclei of each individual were scored randomly and captured at 40× magnification. Images were analysed using Comet assay IV version 4.2 software and percentage of DNA in the tail (% Tail DNA) was assessed.

2.7 Haematological analysis

Blood was sampled from the caudal vein of fish using an insulin syringe (30G needle, 3.8% *sodium citrate*). Following indices of blood parameters were assessed: erythrocytes (RBC, $10^6 \times mm^{-3}$), haemoglobin concentration (Hb, g/l), haematocrit level (Hct, l/l), leukocyte count (WBC, $10^3 \times mm^{-3}$) were determined using routine methods (Svobodova et al, 1991).

2.8 Data analysis and statistics

Geno- and cytotoxicity data do not follow a normal distribution (Kolmogorov-Smirnov and Shapiro-Wilk normality test). Geno-cytotoxicity data were analysed by the nonparametric Kruskal-Wallis test

followed by Dunns post hoc test (using GraphPad Prism® 5.01 (GraphPad Software Inc., San Diego, CA, USA)). BCF and MT content data follow a normal distribution. Data for BCF in tissues were evaluated by two-way factorial ANOVA followed by Bonferroni post hoc test, MT levels was analysed by a one-way ANOVA followed by Bonferroni post hoc test through STATISTICA 7.0 (StatSoft Inc., Tulsa, Oklahoma, USA) software. Spearman correlation was used to assess the relationship between MT content and metal accumulation in liver and kidneys tissues. The results were expressed as mean ± standard error or standard deviation. The level of significance was established at $p<0.05$.

3. RESULTS

3.1 Bioconcentration factor

According to BCF classification scale (Landis et al 2011), low BCF values of analysed metals were measured in fish tissues, except for Zn [in gills (393.1–613.1)), in liver (170.5–318.8), in kidneys (249.4–476.7)] and Cu (in liver (971.7–1789.8), in kidneys (139.1–561.7)] (Fig. 1). BCF values varied depending on metal, metal mixture treatment and specific tissue. *BCF values* for Zn and Cd in *different tissues* after treatment with metal mixtures followed *the sequence*: gills>kidneys>liver>muscle; Cu – liver>kidneys>gills>muscle; Ni – kidneys>muscle>gills>liver, Cr – liver>gills>muscle>kidneys; Pb – gills>liver>kidneys>muscle.

The highest BCF value for Zn was detected in gills tissue after treatment with Pb↓ mixture, in liver – after Ni↓ and in kidneys – Cu↓ treatment (Fig. 1). Treatments with metal mixtures resulted in the highest Cu BCF values measured in liver tissue. The highest BCF value of Cu was detected in liver after Pb↓ treatment, while in kidneys – after Ni↓ treatment. The lowest BCF values for all analysed metals mostly were detected in muscle tissue after metal mixtures treatment.

BCF of Ni was highly affected by reduction of concentration of all metals (Fig. 1). BCF values for Ni significantly differ after all treatments (with reduced concentrations of single metal) performed in comparison to MIX treatment. The highest Ni amount accumulated in gills and muscle tissues was measured after MIX treatment, in liver – after Cu↓, in kidneys – after Zn↓ treatment. BCF values for Cr in gills and muscle tissues were significantly higher after Ni↓ treatment compared to MIX treatment. While MIX treatment resulted in the highest Cr BCF value in liver tissue, and Cu↓ treatment – in the highest Cr BCF value in kidneys compared to MIX treatment. Significant differences between BCF values for Pb were not detected in kidneys and muscle tissues after all treatments performed. However, the highest Pb accumulation was measured after MIX treatment in gills and liver tissues, followed by Pb↓ treatment in gills tissue. Significant differences between BCF values for Cd were not detected after all treatments performed and in any analysed tissues in comparison to MIX treatment. The highest Cd BCF was measured after Cr↓ treatment in gills and kidneys tissues, while after Zn↓ and Cu↓ treatments in liver tissue.

In summary, 10 times reduction of MPC of certain metal, was not always associated with a significant decrease in the same metal amount accumulated in *R. rutilus* tissues compared to MIX treatment. Furthermore, the highest BCF values for metals were measured mostly after treatments with metal mixtures with reduced metal concentration in comparison to BCF values after MIX treatment.

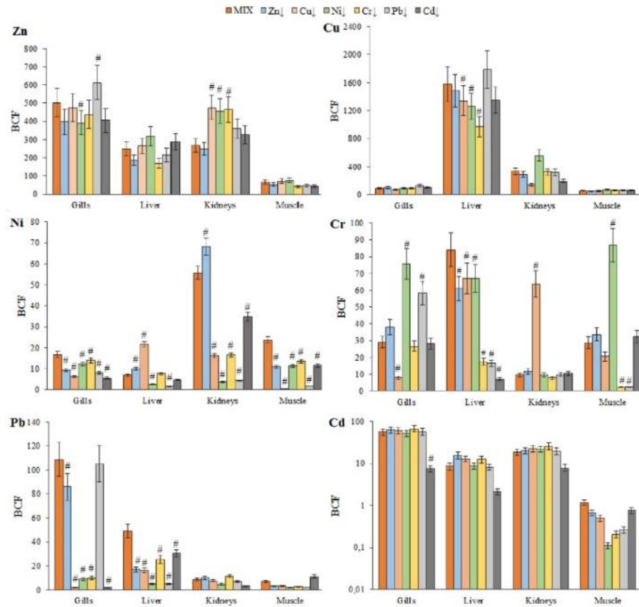


Figure 1. Bioconcentration factor (BCF) in the selected organ tissues exposed to different metal mixture (mean±SD, *N*=7). Grades (#) denote significant differences from MIX treatment groups (p<0.05).

3.2 Erythrocytic nuclear abnormalities

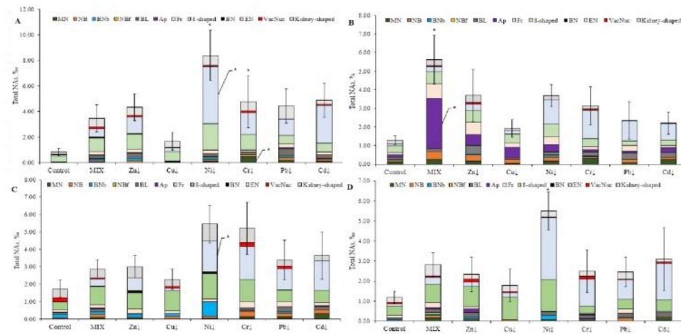


Figure 2. Erythrocyte nuclear abnormalities (ENAs) in (A) peripheral blood, (B) gills, (C) liver and (D) kidneys erythrocytes in control fish and fish treated with metal mixtures (mean±SE, N=7). Asterisks (*) denote significant differences from control group (p<0.05).

14 days treatment with metal mixtures significantly affected micronuclei, enucleus and apoptotic erythrocytes frequencies. The frequencies of *separate ENAs* such as MN, EN were significantly elevated after Cr↓, Ni↓ treatments in peripheral blood erythrocytes, respectively; apoptotic (Ap) cells – after MIX treatment in gills and enucleus after Ni↓ treatment in liver erythrocytes compared to control level. Significant elevations in total ENAs were measured after Cr↓, MIX or Ni↓ treatments in peripheral blood, gills or kidneys erythrocytes (Fig. 2).

3.3 Comet assay

The exposure of fish to Zn↓, Cr↓ and Pb↓ metal mixtures resulted in significant DNA damage compared to those from the control group (Fig. 3). The highest percentage of DNA in the tail (22.59 %) was observed after Cr↓ treatment followed by Pb↓ (16.55 %) and Zn↓ (16.08 %) treatments.

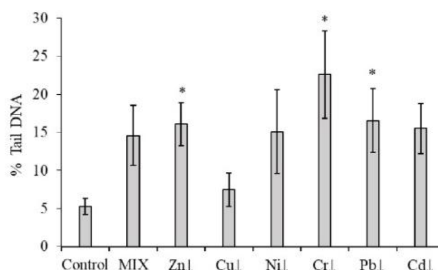


Figure 3. DNA damage (*percentage* of *DNA* in the *tail*) in control fish and fish treated with metal mixtures (mean±SD, *N* = 7). Asterisks (*) denote significant differences from control group (p<0.05).

3.4 Haematological parameters and metallothioneins content

Decreased number of *red blood cells*, *haematocrit* level, haemoglobin concentration and increased number of white blood cells in peripheral blood was measured after MIX treatment (Table 2). However, only decrease in haemoglobin concentration was statistically significant. Metallothionein content in liver and kidneys is presented in Table 2. Liver and kidneys MT level increased 1.22 and 1.25-fold after MIX treatment, respectively, nevertheless, significant differences were not detected. Positive correlation ($r = 0.83$; $p = 0.022$) was measured between MT and Zn amount in liver.

Table 2. Effects of metal mixture (MIX) on haematological parameters and metallothioneins (MT) content (mean±SD, *N* = 7) in *R. rutilus* liver and kidney.

Treatment	Hb, g/l	Hct, l/l	RBC count, $10^6 \times mm^{-3}$	WBC count, $10^3 \times mm^{-3}$	MT	
					Liver	Kidneys
Control	84.83±11.07	0.328±0.07	1.33±0.12	23.42±9.05	41.7±15.3	12.5±0.969
MIX	64.33±14.88 *	0.233±0.06	1.03±0.33	28.75±11.86	50.8±8.84	15.5±4.31

Asterisks (*) denote significant differences from control group ($p < 0.05$)

4. DISCUSSION AND CONCLUSIONS

The results demonstrated significant DNA damage, elevation in micronucleus, enucleus and apoptotic erythrocytes frequencies depending on analysed tissue and performed treatment. Moreover, the findings of this study revealed that *R. rutilus* exposure to metal mixture and its variants induced

variation in metals BCF values, indicating possible interactions between components of primary metal mixture (MIX) and its variants with 10-times reduced concentration of a single metal. Significant effect of MIX on MT content was not detected. Nevertheless, significant decrease in haemoglobin concentration was noted after MIX treatment.

In the present study, muscle tissue exhibited the lowest and less significant variations in metals BCF values after treatment with MIX variants with 10-time reduced concentration of a single metal, as compared with MIX (except Ni and Cr BCF). The highest values of BCF were detected in metabolic body tissues of *R. rutilus* – gills, liver, kidneys, the least – in muscle. In accordance, Sauliutė et al (2017) study showed similar results of metals BCF values in *S. salar* tissues (Sauliutė et al, 2017). Pb↓ treatment highly affected accumulated amount of Zn and Cr in gill (increased 1.2 and 2.0–fold, respectively) compared to MIX treatment. Zn↓ treatment 1.2-fold increased Ni accumulation in kidneys, meanwhile Ni↓ highly affected accumulated amount of Cr in gill and muscle (increased 2.6 and 3.0–fold, respectively) compared to MIX treatment. The highest amount of Zn, Cr and Ni accumulated was measured after Cu↓ treatment in kidneys and liver, respectively. However, treatments with reduced concentration of a single metal showed the lowest variation in the amount of accumulated Cd in tissues, compared to accumulated amount changes of other metals.

In this study, metal mixtures at MPC induced significant formation of MN, EN and Ap in Cr↓, Ni↓ or MIX treatments depending on analysed tissue. The total level of ENAs was also significantly elevated after Cr↓, Ni↓ or MIX treatments. Gills erythrocytes, considering ENAs induction, were mostly affected by MIX treatment. In peripheral blood erythrocytes, significant changes in single endpoints or total ENAs frequencies were detected after Cr↓ or Ni↓ treatments, in liver and kidneys erythrocytes – after Ni↓ treatment. The potential of metal induced damage to the genetic material using environmentally relevant concentrations has scarcely been investigated. Prior studies, that have evaluated toxicity responses in salmonids after treatment with metal mixture at MCP, also reported significant elevation in geno- and cytotoxicity endpoints after 14 days treatment (Stankevičiūtė et al, 2017, Valskienė et al, 2015). Stankevičiūtė et al (2017) study reported significant genotoxicity induction in kidneys erythrocytes, while significant cytotoxicity was detected in gills erythrocytes of *Salmo salar* after 14-day treatment. Rainbow trout 14 days exposure to metal mixture at MPC also resulted in elevation of genotoxicity endpoints in blood and kidneys erythrocytes, while significant cytotoxicity was detected in all analysed tissues (Valskienė et al, 2015).

In the present study, treatment with MIX resulted in decrease of all analysed haematological parameters, except leukocyte count (WBC). Nevertheless, only decrease in haemoglobin concentration was significant. The results are in accordance with Vosylienė et al (2006) findings, which showed decrease in erythrocyte count, haematocrit level and increase in leukocyte count after rainbow trout exposure to complex metal mixture at various concentrations.

Acknowledgments
This work was funded by the Research Council of Lithuania, Project No. S-MIP-17-10. Metallothioneins determination was funded by the Research Council of Lithuania, Project No. MIP-108/2015.

References

1. Anderson S.L. and G.C. Wild (1994) 'Linking genotoxic responses and reproductive success in ecotoxicology', **Environmental Health Perspectives,** Vol. 102(9-12).
2. Ayangbenro A.S. and O.O. Babalola (2017) 'A New Strategy for Heavy Metal Polluted Environments: A Review of Microbial Biosorbents', **International Journal of Environmental Research and Public Health**, Vol. 14(94).
3. Baršienė J., J. Lazutka, J. Syvokienė, V. Dedonytė, A. Rybakovas, E. Bagdonas, A. Bjornstad, O.K. Andersen (2004) 'Analysis of micronuclei in blue mussels and fish from the Baltic and the North Seas', **Environmental Toxicology,** Vol. 19(4), pp. 365-371.

4. Baršienė J., L. Butrimavičienė, W. Grygiel, T. Lang, A. Michailovas, T. Jackūnas (2014) 'Environmental genotoxicity and cytotoxicity in flounder (*Platichthys flesus*), herring (*Clupea harengus*) and Atlantic cod (*Gadus morhua*) from chemical munitions dumping zones in the southern Baltic Sea', **Marine Environmental Research,** Vol. 96(56-67).

5. Cavas T., N.N. Garanko, V.V. Arkhipchuk (2005) 'Induction of micronuclei and binuclei in blood, gill and liver cells of fishes subchronically exposed to cadmium chloride and copper sulphate', **Food and Chemical Toxicology**, Vol. 43(4), pp. 569-74.

6. Cedergreen N. (2014) 'Quantifying Synergy: A Systematic Review of Mixture Toxicity Studies within Environmental Toxicology', **PLoS ONE,** Vol. 9(5): e96580.

7. Directive 2008/105/EC of the European Parliament and of the Council of 496 16 December 2008 on environmental quality standards in the field of water 497 policy, amending and subsequently repealing Council Directives 82/176/EEC, 498 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 499 2000/60/EC of the European Parliament and of the Council. Official Journal of the European Communities L 500 348, 24/12/2008 p. 0084-0097.

8. Dixit R., D. Malaviya, K. Pandiyan, U.B. Singh, A. Sahu, R. Shukla, B.P. Singh, J.P. Rai, P.K. Sharma, H. Lade (2015) 'Bioremediation of heavy metals from soil and aquatic environment: An overview of principles and criteria of fundamental processes', **Sustainability**, Vol 7, pp. 2189-2212.

9. Driessnack M.K., A.L. Matthews, J.C. Raine, S. Niyogi (2016) 'Interactive effects of chronic waterborne copper and cadmium exposure on tissue-specific metal accumulation and reproduction in fathead minnow (*Pimephales promelas*)', **Comparative Biochemistry and Physiology** – Part C 179 (165-173).

10. Driessnack M.K., A. Jamwal, S. Niyogi (2017) 'Effects of chronic waterborne cadmium and zinc interactions on tissue specific metal accumulation and reproduction in fathead minnow (*Pimephales promelas*)', **Ecotoxicology and Environmental Safety,** Vol. 140, pp. 65-75.

11. Duran S., M. Tunçsoy, B. Yeşilbudak, Ö. Ay, B. Cicik, C. Erdem (2015) 'Metal Accumulation In Various Tissues Of *Clarias Gariepinus* Exposed To Copper, Zinc, Cadmium And Lead Singly And In Mixture', **Fresenius Environmental Bulletin,** Vol. 24(12c).

12. Eyer P., F. Worek, D. Kiderlen, G. Sinko, A. Stuglin, V. Simeon-Rudolf, E. Reiner (2003) 'Molar absorption coefficients for the reduced Ellman reagent: reassessment', **Analytical Biochemistry,** Vol. 312, pp. 224-227.

13. Fatima M., N. Usmani, M.M. Hossain, M.F. Siddiqui, M.F. Zafeer, F. Firdaus, S. Ahmad (2014) 'Assessment of genotoxic induction and deterioration of fish quality in commercial species due to heavy-metal exposure in an urban reservoir', **Archives of Environmental Contamination and Toxicology,** Vol. 67(2), pp. 203-2013.

14. Fenech M., W.P. Chang, M. Kirsch-Volders, N. Holland, S. Bonassi, E. Zeiger (2003) 'HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures', **Mutation Research,** Vol. 534, pp. 65-75.

15. Gobas F.A.P.C, D. Wolf, W. Burkhard, L.P. Verbruggen, K. Plotzkeet (eds) (2009) 'Revisiting bioaccumulation criteria for POPs and PBT assessments', **Integrated Environmental Assessment and Management,** Vol. 5, pp. 624-637.

16. Harabawy A.S.A. and Y.Y.I Mosleh (2014) 'The role of vitamins A, C, E and selenium as antioxidants against genotoxicity and cytotoxicity of cadmium, copper, lead and zinc on erythrocytes of Nile tilapia, *Oreochromis niloticus*', **Ecotoxicology and Environmental Safety**, Vol. 104, pp. 28-35.

17. Heys K.A., R.F. Shore, M.G. Pereira, K.C. Jones, F.L. Martin (2016) 'Risk assessment of environmental mixture effects', **RSC Advances,** Vol. 6, pp. 47844-47857.

18. ISO 15586:2003. Water quality. Determination of trace elements using atomic absorption spectrometry with graphite furnace. Geneva, International Organization for Standardization.

19. Jia H., H. Ren, S. Satoh, H. Endo, T. Hayashi (2005) 'Comparison of pre-treatment condition of cadmium in fish sample and diet by microwave digestion method for ICP-AES', **Journal of the Tokyo University Marine Science and Technology**, Vol. 1, pp. 41-46.

20. Landis W., R. Sofield; M.H Yu, W.G. Landis, R.M. Sofield (2011) 'Introduction to environmental toxicology: molecular substructures to ecological landscapes', 4th ed. Boca Raton, Florida: CRC Press.

21. Peixoto N.C., T. Roza, E.M.M. Flores, M.E. Pereira (2003) 'Effects of zinc and cadmium on $HgCl_2$-ALA-D inhibition and Hg levels in tissues of suckling rats', **Toxicology Letters**, Vol. 146, pp. 17-25.

22. Sauliutė G., M. Stankevičiūtė, G. Svecevičius, J. Baršienė, R. Valskienė (2017) 'Assessment of heavy metals bioconcentration factor (BCF) and genotoxicity response induced by metal mixture in *Salmo salar* tissues', **"Environmental Engineering" 10th International Conference.**

23. Singh N.P., M.T. McCoy, R.R. Tice, E.L. Schneider (1988) 'A simple technique for quantitationof low levels of DNA damage in individual cells', **Experimental Cell Research**, Vol. 175, pp. 184-191.

24. Stankevičiūtė M., G. Sauliutė, G. Svecevičius, N. Kazlauskienė, J. Baršienė (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**, Vol. 26(8), pp. 1051-1064.

25. Svobodova Z. and B. Vykusova (Eds.) (1991) 'Diagnostics, Prevention and Therapy of Fish Diseases and Intoxications' Vodnany: Czechoslovakia.

26. Valskienė R, Stankevičiūtė M, Butrimavičienė L, Greiciūnaitė J, Svecevičius G (2015) 'Induction of nuclear abnormalities in rainbow trout (*Oncorhynchus mykiss*) after exposure to a model mixture of heavy metals (Zn, Cu, Ni, Cr, Cd, Pb) at maximum permissible concentration', **Proceedings of the 18th Conference for Junior Researchers "Science – Future of Lithuania"**, Vol. 15, pp. 100–105.

27. Vosylienė M.Z. and A. Jankaitė (2006) 'Effect of heavy metal model mixture on rainbow trout biological parameters', **Ekologija,** Vol. 4, pp. 12-17.

28. Winter A.R., R.C. Playle, D.G. Dixon, U.W.E. Borgmann, M.P. Wilkie (2012) 'Interactions of Pb and Cd mixtures in the presence or absence of natural organic matter with the fish gill', **Ecotoxicology and Environmental Safety**, Vol. 83, pp. 16-24.

29. Zhu Y., J. Wang, Y. Bai, R. Zhang (2004) 'Cadmium, chromium, and copper induce polychromatocyte micronuclei in carp (*Cyprinus carpio* L.)', **Bulletin of Environmental Contamination and Toxicology,** Vol. 72(1), pp. 78-86.

XIV

Geno-, cytotoxicity and toxicity induced by *Saprolegnia parasitica* and cadmium alone and in combination to *Oncorhynchus mykiss*

Stankevičiūtė M, Jurgelėnė Ž, Greiciūnaitė J, Markovskaja S, Kazlauskienė N, Baršienė J

Proceedings of the 14th International Conference on Protection and Restoration of the Environment 2018, ISBN: 978-960-99922-4-4. Thessaloniki, Greece. p. 795–804

GENO-, CYTOTOXICITY AND TOXICITY INDUCED BY *SAPROLEGNIA PARASITICA* AND CADMIUM ALONE AND IN COMBINATION TO *ONCORHYNCHUS MYKISS*

M. Stankevičiūtė*[1], Ž. Jurgelėnė[1], J. Greiciūnaitė[1], S. Markovskaja[1], N. Kazlauskienė[1], J. Baršienė[1]

[1] Nature Research Centre, Akademijos st. 2, LT-08412 Vilnius, Lithuania

*Corresponding author: e-mail: milda.stan@gmail.com, tel: +370 60716809

Abstract

The aims of present study were to determine genotoxicity, cytotoxicity and toxicity induced by *Saprolegnia parasitica* at concentrations 92000, 22400 and 5500 *colony-forming units per milliliter* (cfu/mL) and Cd (2 μg Cd/L as $CdCl_2{\cdot}H_2O$) alone and in combination to rainbow trout *Oncorhynchus mykiss* larvae after 8-day treatment. The formations of micronuclei (MN) and nuclear buds (NB) were assessed as genotoxicity, while 8-shaped nuclei and fragmented-apoptotic (FA) erythroblasts were assessed as cytotoxicity endpoints. Significant induction of MN frequency was detected after treatment with the lowest concentration of *S. parasitica* and after co-exposure. In contrast, significant elevation of NB was measured exceptionally after exposure to the highest *S. parasitica* concentration. Total level of genotoxicity endpoints showed significant elevation after the highest, the lowest *S. parasitica* concentrations and co-exposure treatments. Significant changes in cytotoxicity endpoints were not detected after all treatments performed. Surprisingly, exposure to Cd did not induce any significant changes of selected biomarkers. During the treatment, biological parameters such as heart rate (HR, counts/min) and gill ventilation frequency (GVF, counts/min) were assessed. Toxicity study demonstrated that HR of larvae exposed to *S. parasitica* at concentrations 22400 and 5500 cfu/mL, and 5500 cfu/mL+Cd after 8 days was significantly ($p<0.05$) lower as compared to the control. Additionally, *S. parasitica* at 5500 cfu/mL, and 5500 cfu/mL+Cd induced a significant decrease in GVF in larvae at the end of the test.

Keywords: fish; genotoxicity; cytotoxicity; toxicity; *Saprolegnia parasitica*; cadmium

1. INTRODUCTION

The aquatic fungus-like heterotrophs or straminipilous fungi referred also as "water moulds" (traditionally oomycetes) of the order *Saprolegniales* is common and widespread in freshwater environment (Rietmüller, 2000; Dick, 2001). Most of them are saprotrophs decomposing dead organic material, but some species are known to be pathogens and have the ability to infect various aquatic organisms including fish or crustaceans and induce a number of economically important diseases (Willoughby, 1994; Wicker et al, 2001). Fungal disease such as saprolegniosis is known as one of the common salmonids disease (Thoen et al, 2011). Naturally, *Saprolegnia* species are found in all lotic and lentic freshwater basins (Rietmüller, 2000; Markovskaja, 2006). In aquaculture, *Saprolegnia* infection causes severe problem in incubating eggs and newly hatched fry (Hussein et al, 2001; Thoen et al, 2011; Van Den Berg et al, 2013). The lethal impact of saprolegniosis could cause major financial loss in an industry of the global fish industry production (Phillips et al, 2008). According to Bruno et al (2011), over 10% of salmonid eggs become infected with oomycetes in hatcheries each year. Since 2002, when the use of malachite green, an organic dye very efficient at killing the pathogen and previously widely used, was banned due to its toxicity, *Saprolegnia* infection

has reemerged in aquaculture. There are no chemicals now available that provide sufficient protection against the saprolegniosis after hatching (Fornerisa et al, 2003). In order to mitigate *Saprolegnia* infection in aquaculture, the development and testing of general or specific *antifungal agents* has increased (Ali et al, 2014).

Songe et al (2016) emphasized, that *Saprolegnia* infection in salmonids eggs has been scarcely investigated and the role of such infection in fish eggs remains unclear. Moreover, *Saprolegnia parasitica* is thought to be most frequent species of *Saprolegnia* genus infecting fish egg (van West, 2006; Shahbazian et al, 2010). *S. parasitica* causes rapid death of eggs, because of hyphae penetration into the chorion, and consequently failure of osmosis regulation (Songe et al, 2016). In fish, disease is characterized by visible white or grey patches of filamentous mycelium on the body or fins of fish, hyphae penetrate epidermal tissues causing dermal, epidermal damage and cellular necrosis. Furthermore, lethargic behaviour, loss of equilibrium and death are the results of severe infection (Pickering et al, 1982). The parasitic lifecycle of *S. parasitica* has been well described by Andersson and Cerenius (2002), Dieguez-Uribeondo et al (1994), Torto-Alalibo et al (2005), Robertson et al (2009), and van West (2006). The zoospores of this pathogenic oomycete may be transmitted by fish eggs, wild fish, water sources, and equipment (Saha et al, 2016).

It is important to note, that toxins are very important virulence factors for many fungal diseases. Oomycetes are known to secrete toxins, proteinaceous substances or hydrolytic enzymes (Soanes et al, 2007). Torto-Alalibo et al (2005) have exuded and isolated several proteins of *S. parasitica* (CBD proteins, CBEL-like proteins, glycosyl hydrolases, proteases, protease inhibitors) and emphasized that these proteins can have a range of impacts on health. Moreover, *Saprolegnia* infection induce a strong inflammatory response in fish. As concluded by Belmonte et al (2014) *S. parasitica* produces the metabolite prostaglanding E_2 (PGE_2), which increases the inflammatory response in fish leukocytes. Consequently, inflammatory responses may trigger the genotoxicity. Furthermore, joint effects of parasitism and pollution may lead to unexpected toxicity endpoints. Parasites exposed to environmental contaminants is a phenomenon, which is not well understood and, which deserves further investigation (Sures et al, 2017). Additionally, no studies have been conducted to understand the combined impact of the *S. parasitica* infection and sublethal concentration of toxic metal Cd and their geno-, cytotoxicity and toxicity to developing fish. For this reason, the present study has the *following objectives*: a) to identify possible genotoxicity and cytotoxicity potential of *S. parasitica* infection using rainbow trout larvae, b) to assess combined effects of S. *parasitica* infection and Cd exposure on geno- and cytotoxicity endpoints, c) to determine biological effects of *S. parasitica* and Cd alone and in combination.

2. MATERIALS AND METHODS

2.1 Experimental set-up

Rainbow trout *Oncorhynchus mykiss* eggs (at 20 stages, eyed-egg stage embryos (Ballard, 1973)) were obtained from the Simnas hatchery (Lithuania) and risen in bare-bottom tanks supplied with flow-through aerated deep-well water. Studies have been carried out with non-protected life-stages accordance with EU Directive 2010/63/EU. The laboratory treatment was carried out in an environmental chamber (Bronson PGC-660, Zaltbommel, The Netherlands) with continuous aeration under static conditions (static non-renewal experiment) according to ISO 7346-1:1996, without the water being changed. According to the OECD 210 (OECD, 1992), the experiments were carried out in the dark and the larvae were not fed (ISO 10229:1994).

The fungus-like organism *Saprolegnia parasitica* Coker was isolated from naturally infected perch (*Perca fluviatilis*). The identification of *Saprolegnia* isolate was performed at species level, by taxonomic analysis of the sexual structures combined with morphological characterization of its asexual stage under light microscope Nikon eclipse Ci with phases contrast at magnifications x 400

(up to x 1000). The nomenclature of identified species follows Seymour, 1970; Rietmüller, 2000; Dick, 2001; Markovskaja, 2006.

The pure living cultures of *Saprolegnia parasitica* were isolated by the baiting technique (Seymour, 1970). Hemp seeds were used as baits, placed into the vessels with 100 mL of distilled water and hyphae scraped from naturally infected fish. After 5-7 days white hyphae appeared on the hemp seeds with developing asexual and later sexual organs. For the experiments a suspension of *S. parasitica* colony-forming units (cfu - zoospores, oospores, hyphae), prepared from pure living culture with concentration levels of 92000, 22400 and 5500 cfu/mL was used. Additionally, cadmium (2 µg Cd/L) induced geno-, cytotoxicity and toxicity alone and in combination with *S. parasitica* at concentration 5500 cfu/mL were assessed.

Reagent grade cadmium chloride ($CdCl_2 \cdot H_2O$) («REACHIM» Company, Russia) was used as the toxicant and stock solutions were prepared by dissolving a necessary amount of salts in distilled water. The concentration of 2 µg Cd/L was chosen according to the 96 h LC50 for rainbow trout larvae (Cibulskaitė et al, 2015). Nominal metal concentrations in the tanks were checked during blank tests (without larvae) (N = 3) with an atomic absorption spectrophotometer (SHIMADZU AA-6800, Japan). Mean measured concentrations were within 10 – 15% of target.

Experiments were conducted on *O. mykiss* larvae 4 days post hatching. All treatments were carried out with 3 replications and control groups (using glass tanks of 1-L total volume filled to a level of 500 mL with continuously aerated dilution water, a total of 35 larvae per treatment tank and 35 larvae in control tank were used).

2.2 Analytical procedures

The main physico-chemical parameters of the water (temperature, dissolved O_2, pH and conductivity) were measured routinely with a hand-held multi-meter (WTW Multi 340i/SET, Germany). Physico-chemical parameters of the laboratory water (deep-well) were as follows: dissolved oxygen 10±1 mg/L, temperature 10±0.5°C, pH 8.1±0.1. Chemical characteristics of the deep-well water have been presented in our previous research (Stankevičiūtė et al, 2017).

2.3 Nuclear abnormalities (NAs) analysis

Nuclear abnormalities (NAs) analysis was performed in erythroblasts of *O. mykiss* larvae. Blood smears were prepared from larvae body (gently nipped with tweezers): directly smeared on glass slides and air-dried. Smears were fixed in methanol for 10 min. and later were stained with 10% Giemsa solution in phosphate buffer pH = 6.8 for 20 - 40 min. The stained slides were analyzed under light microscope Olympus BX51 (Tokyo, *Japan*) at final magnification of 1,000× and the photos were taken *with* an Olympus U-CMAD3 (Tokyo, *Japan*) camera. Identification of micronuclei, nuclear buds, fragmented-apoptotic and bi-nucleated cells was done using criteria described by Heddle et al (1991) and Fenech et al (2003). The frequencies of abnormalities were recorded in 1,000 erythroblasts per slide using blind scoring. The test was carried out with 10 specimens of larvae in each treatment and control groups for evaluating genotoxicity and cytotoxicity. Genotoxicity [induction of micronuclei (MN) and nuclear buds (NB)] and cytotoxicity [induction of fragmented-apoptotic (FA) and 8-shaped nuclei cells] endpoints were analysed. Considering low frequencies of separate cytotoxicity endpoints, total cytotoxicity level (FA+8-shaped) was assessed as the sum of the frequencies of cytotoxicity endpoints.

2.4 Toxicity assay

Heart rate (HR, counts/min) and gill ventilation frequency (GVF, counts/min) were investigated. Samples of larvae were taken upon days 8 after exposure start. HR and GVF of larvae was measured for each larvae individually, and the mean value for 10 larvae was calculated.

2.5 Data analysis and statistics

The geno- and cytotoxicity data follow a normal distribution (Kolmogorov-Smirnov and Shapiro-Wilk normality test). Geno-cytotoxicity data were analysed by the one-way ANOVA followed by Bonferroni post hoc test (using GraphPad Prism® 5.01 (GraphPad Software Inc., San Diego, CA, USA)) for comparison of differences between groups. Toxicity data (HR and GVF) do not follow a normal distribution (Kolmogorov-Smirnov and Shapiro-Wilk normality test). Differences between the evaluated characteristics studied were tested by nonparametric Kruskal-Wallis test using STATISTICA 7.0 (StatSoft Inc., Tulsa, Oklahoma, USA) software. The results were expressed as mean ± standard error or standard deviation. The level of significance was established at $p<0.05$.

3. RESULTS

Results of micronucleus tests with *O. mykiss* larvae are given in Figure 1. Treatment with the lowest *S. parasitica* concentration (Sap3 – 5500 cfu/mL) significantly increased MN frequencies in erythroblasts of larvae. Cadmium alone did not induce significant MN formation. However, Cd in combination with *S. parasitica* at concentration 5500 cfu/mL significantly increased MN frequencies. Notwithstanding, MN frequencies induced by Cd in combination with the lowest *S. parasitica* concentration (Sap3) did not significantly differ from exposure to *S. parasitica* (Sap3) alone.

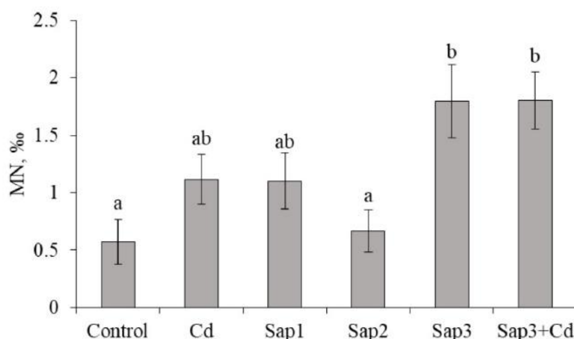


Figure 1. Mean values (mean ± SEM, *N* = 10) of micronuclei (MN) frequencies in erythroblasts of *O. mykiss* larvae treated with cadmium (Cd, 2µg/L), three concentrations of *Saprolegnia parasitica* (Sap1 – 92000, Sap2 – 22400, Sap3 –5500 (cfu/mL)) and Cd in combination with *S. parasitica* (2 µg Cd/L + 5500 cfu/mL). Letters denote significant differences between groups

Analysis of nuclear bud (NB) revealed a significant increase after treatment with the highest *S. parasitica* concentration (Sap1). Exposure to Cd, other *S. parasitica* concentrations and co-exposure did not significantly affect NB responses in erythroblasts of larvae (Figure 2).

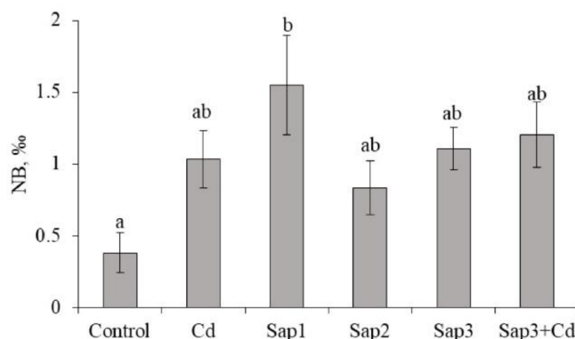


Figure 2. Mean values (mean ± SEM, *N* = 10) of nuclear bud (NB) frequencies in erythroblasts of *O. mykiss* larvae treated with cadmium (Cd, 2µg/L), three concentrations of *Saprolegnia parasitica* (Sap1 – 92000, Sap2 – 22400, Sap3 – 5500 (cfu/mL)) and Cd in combination with *S. parasitica* (2 µg Cd/L + 5500 cfu/mL). Letters denote significant differences between groups

Treatment with all *S. parasitica* concentrations and co-exposure treatment significantly increased total genotoxicity level in larvae erythroblasts, except for the 22400 cfu/mL (Sap2) concentration level.

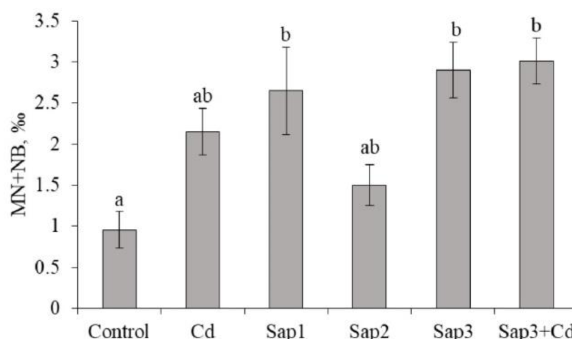


Figure 3. Total genotoxicity (MN+NB) level (mean ± SEM, *N* = 10) in erythroblasts of *O. mykiss* larvae treated with cadmium (Cd, 2µg/L), three concentrations of *Saprolegnia parasitica* (Sap1 – 92000, Sap2 – 22400, Sap3 – 5 500 (cfu/mL)) and Cd in combination with *S. parasitica* (2 µg Cd/L + 5500 cfu/mL). Letters denote significant differences between groups

Significant elevation of total cytotoxicity level was not found after all treatments performed. However, the highest total cytotoxicity level was measured after treatment with the lowest *S. parasitica* concentration (Sap3), followed by Sap3+Cd and Sap2 treatments.

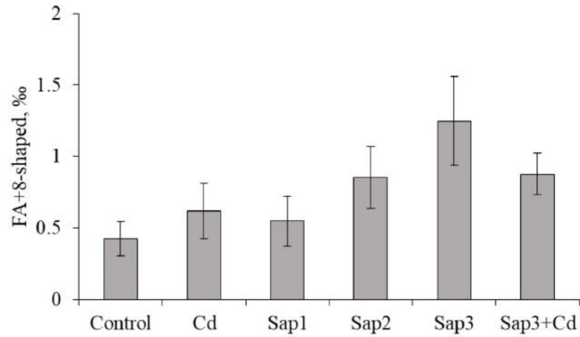
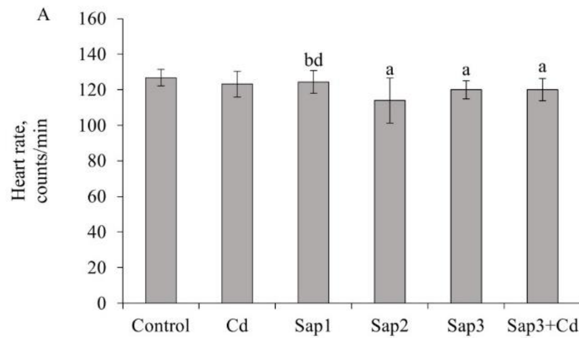


Figure 4. Total cytotoxicity (FA+8-shaped) level (mean ± SEM, *N* = 10) in erythroblasts of *O. mykiss* larvae treated with cadmium (Cd, 2µg/L), three concentrations of *Saprolegnia parasitica* (Sap1 – 92000, Sap2 – 22400, Sap3 –5500 (cfu/mL)) and Cd in combination with *S. parasitica* (2 µg Cd/L + 5500 cfu/mL). Letters denote significant differences between groups

In this investigation, significantly ($p<0.05$) decreased HR of larvae as compared to the control after 8 days of exposure to Sap2 – 22400, Sap3 – 5500 (cfu/mL) and Cd in combination with *S. parasitica* (2 µg Cd/L + 5500 cfu/mL). Meanwhile, in the highest *S. parasitica* treatment (Sap1) HR of larvae did not differ significantly from the control. HR of larvae in the highest *S. parasitica* treatment (Sap1) was significantly ($p<0.05$) different from Sap2 and Sap3+Cd treatments.

The lowest *S. parasitica* concentration (Sap3 – 5500 cfu/mL) and Cd in combination with *S. parasitica* (2 µg Cd/L + 5500 cfu/mL) induced a significant ($p<0.05$) decrease in GVF of larvae. Additionally, GVF of larvae in Cd treatment were significantly ($p<0.05$) different from Sap3 and Sap3+Cd treatment.



800

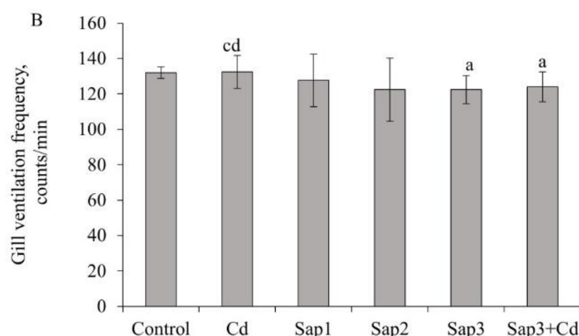


Figure 5. Sub-chronic (8 days of exposure) effect of cadmium (Cd, 2µg/L), three concentrations of *Saprolegnia parasitica* (Sap1 – 92000, Sap2 – 22400, Sap3 –5500 (cfu/mL)) and Cd in combination with *S. parasitica* (2 µg Cd/L + 5500 cfu/mL) on biological parameters of *O. mykiss* larvae: gill ventilation frequency (counts/min) and heart rate (counts/min) (mean ± SD).

[a] Significant difference from the control ($p<0.05$). Significant difference between treatments ($p<0.05$): [b] significant difference from Sap2 treatment; [c] significant difference from Sap3 treatment; [d] significant difference from Sap3+Cd treatment.

4. DISCUSSION AND CONCLUSIONS

This study was designed to identify possible geno- and cytotoxicity potential and to assess biological effects of egg-pathogenic *S. parasitica* infection in rainbow trout larvae. Moreover, the exacerbation of toxicity endpoints of joint parasitism and Cd exposure was assessed. The findings of this study indicated a significant increase of separate genotoxicity endpoints and total genotoxicity depending on exposure concentration of *S. parasitica*. However, genotoxicity endpoints did not show a clear tendency to increase with increasing *S. parasitica* exposure concentration. Belmonte and co-authors (2014) detected the immune suppression in Atlantic salmon before the pathogen infection (establishment) or after early stages of interaction. Moreover, 12 days exposure of fish to *S. parasitica (*10^4 zoospores/cysts liter^{-1}*)* did not cause evidence of infection and no suppression of the antigen, and no induction of proinflammatory genes were detected. These responses might indicate a protection against the oomycetes. In this study, exposure to the highest concentrations of *S. parasitica* did not induce the highest frequencies of all analyzed geno- and cytotoxicity endpoints. These results might indicate the threshold for inhibition of certain geno- and cytotoxicity responses. Further analyses using more frequent sampling and various concentrations of *S. parasitica* are therefore suggested. Scientific literature data related to direct or indirect genotoxic effects induced by *Saprolegnia* do not exist. The genotoxic potential of *Saprolegnia parasitica* in fish has not been investigated at all. This study provides *first* toxicity data that show significantly increased genotoxic activity in rainbow trout after *S. parasitica* exposure. Azimzadeh and Amniattalab (2017) indicated oxidative stress, haematological and histopathological changes in rainbow trout infected with *S. parasitica*. Moreover, parasitic *Saprolegnia* species produces metabolites, which may induce a strong inflammatory response in fish (Belmonte et al, 2014). However, one of the limitations of these findings is that it does not explain which mechanisms (direct or indirect) are responsible for such genotoxicity outcome.

Marcogliese et al (2005) concluded that parasitism in the presence of pollution may further compromise the health by reducing the immunocompetence of the host. Furthermore, exacerbation of toxicity effects may be noted even parasites infestation occurs at low intensities. In agreement with that, the findings of this study, showed the highest total genotoxicity level after joint treatment with the lowest *S. parasitica* concentration and Cd in comparison to other treatments.

In the present study, significant cytotoxicity was not induced by any *S. parasitica* concentration tested, as well as after co-exposure treatment. As emphasized by Schaumburg et al (2006), parasites can induce anti-apoptotic activities in the host.

In addition, during a sub-chronic test, *S. parasitica* induced negative effects on biological parameters (decreased heart rate and gill ventilation frequency) of rainbow trout larvae. Moreover, these effects did not relate to the concentration of *S. parasitica*. However, significant difference between *S. parasitica* treatments was observed only in heart rate measurement. In fish gills serve as a principal organ for respiration, osmoregulation, and excretion (Evans et al, 2005), they also become a potentially important site of penetration by parasites (Mikheev et al, 2014). Therefore, in this study, saprolegniosis seems to have damaged the gill, then gill ventilation frequency decreased in larvae and the deficiency of oxygen induced bradycardia. Furthermore, saprolegniosis-induced hypoxia may be responsible for the significant genotoxicity responses measured in this study. In fish, infection begins on the head, gills, or fins and spreads over the entire surface of the body, for this reason, often osmoregulatory failure results in the death of fish (van West, 2006). On the other hand, in contrast to our study, Mikheev et al (2014) demonstrated that rainbow trout reacted to low oxygen concentration with wider expansion of parasites (*Diplostomum spathaceum*), leading to an increase in gill ventilation frequency. Additionally, physiological or social stressors could produce similar effects on the transmission success of the parasites penetrating fish hosts using the gills.

This study result showed the negative effects of combined parasites and pollutant exposure. Similar results of negative effects were found by Gheorgiu et al (2006), where significantly increased mortality of guppies (*Poecilia reticulata*) exposed to Zn and infected with the monogenean *Gyrodactylus turnbulli* were observed.

Marcogliese et al (2005) noted that cumulative effects of multiple stressors are becoming a major problem in ecotoxicology and many other fields. In conclusion, this study highlights the potential to advance our current understanding of the significance of a biological stressor (pathogen) on geno-, cytotoxicity and toxicity endpoints. Furthermore, the potential to exacerbate toxicity endpoints after fish exposure to multiple environmental stressors (pathogen infection and pollution) is emphasized.

Acknowledgments
This work was funded by the Research Council of Lithuania, Project No. S-MIP-17-10.

References

1. Ali S.E., E. Thoen, Evensen Ø and I. Skaar (2014) 'Boric acid inhibits germination and colonization of Saprolegnia spores in vitro and in vivo', PLoS One, Vol 9, pp. e91878.
2. Andersson M.G. and L. Cerenius (2002) 'Pumilio homologue from *Saprolegnia parasitica* specifically expressed in undifferentiated spore cysts', **Eukaryotic Cell**, Vol 1(1), pp. 105-111.
3. Azimzadeh K. and Amniattalab A. (2017) 'Total Sialic Acid, Oxidative Stress and Histopathological Changes in Rainbow Trout Saprolegniasis (*Oncorhynchus mykiss*)', **Kafkas Universitesi Veteriner Fakultesi Dergisi**, Vol. 23(1), pp. 55-62.
4. Belmonte R., T. Wang, G.J. Duncan, I. Skaar, H. Mélida, V. Bulone, P. van West and C.J. Secombes (2014) 'Role of Pathogen-Derived Cell Wall Carbohydrates and Prostaglandin E_2 in Immune Response and Suppression of Fish Immunity by the Oomycete *Saprolegnia parasitica*', **Infection and Immunity**, Vol 82(11), pp. 4518-4529.

5. Bruno D.W., P. van West and G.W. Beakes (2011) 'Saprolegnia and other oomycetes in: Fish Diseases and Disorders: Viral, Bacterial and Fungal Infections', **CABI International,** Vol 3(2), pp. 669-720.

6. Cibulskaitė Ž., N. Kazlauskienė and V. Kulvietis (2015) 'Sublethal toxicity of quantum dots and heavy metals to rainbow trout (*Oncorhynchus mykiss*) in early ontogenesis. Proceedings of the 18th Conference for Junior Researchers „Science – Future of Lithuania"', **Environmental protection engineering**, pp. 31–37.

7. Dick M.W. (2001) 'Straminipilous Fungi: systematics of the peronosporomycetes, including accounts of the marine straminipilous protists, the plasmodiophorids, and similar organisms', **Kluwer Academic Publishers, Dordrecht.**

8. Dieguez-Uribeondo J., L. Cerenius and K. Soderhall (1994) 'Repeated zoospore emergence in *Saprolegnia parasitica*', **Mycological Research**, Vol 98(7), pp. 810-815.

9. Evans D.H., P.M. Piermarini and K.P. Choe (2005) 'The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid–base regulation, and excretion of nitrogenous waste', **Physiological Reviews**, Vol. 85(1), pp. 97–177.

10. Fornerisa G., S. Bellardib, G.B. Palmegianoc, M. Sarogliad, B. Sicuroa, L. Gascoe and I. Zoccarato (2003) 'The use of ozone in trout hatchery to reduce saprolegniasis incidence', **Aquaculture**, Vol 221(1-4), pp. 157-166.

11. Gheorgiu C., D.J. Marcogliese and M. Scott (2006) 'Concentration-dependent effects of waterborne zinc on population dynamics of *Gyrodactylus turnbulli* (Monogenea) on isolated guppies (*Poecilia reticulata*)', **Parasitology**, Vol 132(2), pp. 225-32.

12. Hussein M.A., K. Hatai and T. Nomura (2001) 'Saprolegniasis in salmonids and their eggs in Japan', **Journal of Wildlife Diseases,** Vol 37(1), pp. 204-207.

13. ISO (International Organization for Standardization) 10229 (1994) 'Water Quality-Determination of the Prolonged Toxicity of Substances to Freshwater Fish -- Method for Evaluating the Effects of Substances on the Growth Rate of Rainbow Trout (*Oncorhynchus mykiss* Walbaum (Teleostei, Salmonidae))'.

14. ISO (International Organization for Standardization) 7346-1 (1996) 'Water Quality – Determination of the Acute Lethal Toxicity of Substances to a Freshwater Fish [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae)] -Part 1: Static Method'.

15. Marcogliese D.J., L.G. Brambilla, F. Gagné and A.D. Gendron (2005) 'Joint effects of parasitism and pollution on oxidative stress biomarkers in yellow perch *Perca flavescens*', **Diseases of Aquatic Organisms**, Vol. 63(1), pp. 77-84.

16. Markovskaja S. (2006) 'Saprolegniaceae (Peronosporomycetes) in Lithuania. II. The genus Saprolegnia', **Botanica Lithuanica**, Vol. 12(2), pp. 97-112.

17. Mikheev V.N., A.F. Pasternak, E.T. Valtonen and J.Taskinen (2014) 'Increased ventilation by fish leads to a higher risk of parasitism', **Parasites & Vectors**, Vol 7, pp. 281.

18. OECD (Organization for Economic Cooperation and Development) (1992) 'Guideline for the Testing of Chemicals: Fish, Early-life Stage Toxicity Test', Part 210.

19. Phillips A.J., V.L. Anderson, E.J. Robertson, C.J. Secombes and P. van West (2008) 'New insights into animal pathogenic oomycetes', **Trends in Microbiology**, Vol 16(1), pp. 13–19.

20. Pickering A.D. and L.G. Willoughby (1982) 'Saprolegnia infections of salmonid fish. In: Roberts R.J., editor. Microbial Diseases of Fish', Academic Press, pp. 271-297.

21. Rietmüller A. (2000) 'Morphologie, Ökologie und Phylogenie aquatischer Oomyceten', Bibliotheca Mycologica, Vol.185: 1–344.

22. Robertson E.J., V.L. Anderson, A.J. Phillips, C.J. Secombes, J. Dieguez-Uribeondo and P. van West (2009) 'Saprolegnia – fish interactions. In: Lamour K, Kamoun S, editors. Oomycete genetics and genomics, diversity, interactions and research tools', **Hoboken, NJ, USA: Wiley-Blackwell**, pp. 407-424.
23. Saha H., A.K. Pal, N.P. Sahu and R.K. Saha (2016) 'Feeding pyridoxine prevents Saprolegnia parasitica infection in fish *Labeo rohita*', **Fish and Shellfish Immunology**, Vol 59, pp. 382-388.
24. Seymour R.L. (1970) 'The genus *Saprolegnia*', **Nova Hedwigia**, Vol. 19, pp. 1–124.
25. Schaumburg F., D. Hippe, P. Vutova and C.G. Lüder (2006) 'Pro- and anti-apoptotic activities of protozoan parasites', **Parasitology**, Vol 132(S1), pp. S69–S85.
26. Shahbazian N., H.A. Ebrahimzadeh Mousavi, M. Soltani, A.R. Khosravi, S. Mirzargar and I. Sharifpour (2010) 'Fungal contamination in rainbow trout eggs in Kermanshah province propagations with emphasis on Saprolegniaceae', **Iranian Journal of Fisheries Sciences**, Vol 9(1), pp. 151–160.
27. Soanes D.M., T.A. Richards and N.J. Talbot (2007) 'Insights from sequencing fungal and oomycete genomes: what can we learn about plant disease and the evolution of pathogenicity?', **Plant Cell,** Vol 19(11), pp. 3318-3326.
28. Songe M.M., A. Willems, J. Wiik-Nielsen, E. Thoen, Ø. Evensen, P. van West and I. Skaar (2016) '*Saprolegnia diclina IIIA* and *S. parasitica* employ different infection strategies when colonizing eggs of Atlantic salmon, *Salmo salar* L.', **Journal of Fish Diseases**, Vol. 39(3), pp. 343-352.
29. Sures B., M. Nachev, C. Selbach and D.J. Marcogliese (2017) 'Parasite responses to pollution: what we know and where we go in 'Environmental Parasitology'', **Parasites & Vectors**, Vol 10(1), pp. 65.
30. Thoen E., O. Evensen and I. Skaar (2011) 'Pathogenicity of *Saprolegnia* spp. to Atlantic salmon, *Salmo salar* L., eggs', **Journal of Fish Diseases**, Vol 34(8), pp. 601-608.
31. Torto-Alalibo T., M. Tian, K. Gajendran, M.E. Waugh, P. van West and S. Kamoun (2005) 'Expressed sequence tags from the oomycete fish pathogen *Saprolegnia parasitica* reveal putative virulence factors', **BioMed Central Microbiology**, Vol 5, pp. 46.
32. Van Den Berg A.H., D. McLaggan, J. Dieguez-Uribeondo and P. van West (2013) 'The impact of the water moulds *Saprolegnia diclina* and *Saprolegnia parasitica* on natural ecosystems and the aquaculture industry', **Fungal Biology Reviews**, Vol 27(9), pp. 33-42.
33. Van West P. (2006) '*Saprolegnia parasitica*, an oomycete pathogen with a fishy appetite: New challenges for an old problem', **Mycologist**, Vol 20(3), pp. 99-104.
34. Wicker E., M. Hulle, F. Rouxel (2001) 'Pathogenic characteristics of isolates of *Aphanomyces eusteiches* from pea in France', **Plant Pathology,** Vol 50(4), pp. 433-442.
35. Willoughby L.G. (1994) 'Fungi and fish diseases', **Pisces Press**.

XV

Investigation of quantum dots toxicity, genotoxicity, cytotoxicity, and uptake in rainbow trout *Oncorhynchus mykiss* larvae

Jurgelėnė Ž, Stankevičiūtė M, Kazlauskienė N, Montvydienė D, Baršienė J, Jokšas K, Markuckas A

Proceedings of the 14th International Conference on Protection and Restoration of the Environment ISBN: 978-960-99922-4-4. Thessaloniki, Greece. p. 775–806.

INVESTIGATION OF QUANTUM DOTS TOXICITY, GENOTOXICITY, CYTOTOXICITY, AND UPTAKE IN RAINBOW TROUT *ONCORHYNCHUS MYKISS* LARVAE

Ž. Jurgelėnė*[1], M. Stankevičiūtė[1], N. Kazlauskienė[1], D. Montvydienė[1], J. Baršienė[1], K. Jokšas[2, 3], A. Markuckas[4]

[1] Institute of Ecology of Nature Research Centre, Akademijos st. 2, LT-08412 Vilnius, Lithuania,
[2]Vilnius University, Faculty of Chemistry and Geosciences, Naugarduko st. 24, LT-03225 Vilnius, Lithuania
[3]Geology and Geography Institute of Nature Research Centre, Akademijos st. 2, LT-08412 Vilnius, Lithuania
[4]Vilnius University, Life Sciences Center, Department of Biochemistry and Molecular Biology, Saulėtekio av. 7, 10223 Vilnius, Lithuania

*Corresponding author: e-mail: zivile.jurgelene@gmail.com, tel.: +370 63385183

Abstract
Nanoparticles may be released into the environment and induce harmful effects to the aquatic ecosystem. The aims of the present study were to determine: (1) toxicity, genotoxicity and cytotoxicity to larvae of rainbow trout *Oncorhynchus mykiss* exposed to 4 nmol/L CdSe/ZnS quantum dots (QDs); (2) Cd accumulation; (3) the concentration of metallothionein (MT) in larvae after exposure to QDs; and (4) explain the possible impact mechanism of the QDs to fish larvae. QDs at sublethal concentration was used during the tests. Our findings revealed that heart rate (HR, counts/min) of larvae didn't differ significantly ($p < 0.05$) from the control; gill ventilation frequency (GVF, counts/min) significantly ($p < 0.05$) increased only after 10 days of exposure to QDs compared to the control. Total genotoxicity level (erythroblasts with micronuclei and nuclear buds) in larvae significantly ($p < 0.05$) increased after 4 days of exposure. However, 4 nmol/L QDs did not induce significant cytotoxicity over the concentration applied. QDs induced a significant increase in Cd accumulation in larvae after 4-10 days of exposure in comparison with the control. MT was used as a marker of internal Cd exposure, thus providing indirect information on *in vivo* QDs degradation. The concentration of MT did not change in larvae during treatment. Therefore, QDs were stable during 10 days of exposure. QDs absorption was not found to take place in larvae. Possibly, the effects of QDs to larvae are related to mechanical impact of QDs.

Keywords: quantum dots; fish; accumulation; toxicity, genotoxicity and cytotoxicity; metallothioneins

1. INTRODUCTION

The rapid growth in the nanotechnology industry leads to use of novel nanomaterials like quantum dots (QDs) for biomedical applications, such as diagnostics, drug delivery and nanotherapy. The knowledge regarding the uptake mechanisms of nanoparticles (NPs) and toxicity to organism is not well understood (Murugan et al, 2015). QDs are semiconductor crystals of nanometer dimensions (2–10 nm), containing 200–10000 atoms, and can consist of a cadmium/selenide (CdSe) core with a zinc sulfide (ZnS) shell with some type of surface coating. To their strong fluorescence intensity, their stability, water solubility, small size and flexible surface charge enable QDs suitable agents to study uptake with *in vitro* and *in vivo* studies (Zhang et al, 2011). Nevertheless, little is known about the

environmental risks of exposure to these NPs. Due to their possible large scale of production in future and NPs release in the environment has led to alarming on their potential long-term toxicity related to the fact that these materials contain heavy metals such as Cd, As, Zn, Pb (Libralato et al, 2017).

The question is open about the safety of using QDs for treating patients, as there are not enough reliable studies on their toxicological effects. Oh et al (2016) study showed the need for more in-depth analysis for major QDs types, because QDs toxicity is closely correlated with many specific parameters of QDs, such as surface properties (including shell, ligand and surface modifications), diameter, assay type and exposure time. Two major mechanisms are involved in the toxicity effects of QDs: Cd^{2+} in the structure that could cause interference in DNA repair or increase of oxidative stress and free radical formation. The release of Cd^{2+} from the core of QDs influences toxicity and causing ROS generation (Ji et al, 2015).

Studies undertaken to investigate QDs effect have used a broad range of QDs types and included classical cytotoxicity assays, and have examined the effects of QDs on cellular organelles and gene/protein expression, as well as their behavior and fate in vertebrate and invertebrate models (Oh et al, 2016; Rocha et al, 2017). Fishes or mice are usually employed to evaluate *in vivo* effects of contaminants, but studies with mice are time consuming, present ethical issues and are expensive (Yong et al, 2013). In recent years, the use of fish as an established animal model system for NPs toxicity assay is growing exponentially (Chakraborty et al, 2016; Rocha et al, 2017). Different types of parameters are used to evaluate NPs toxicity such as hatching achievement rate, developmental malformation of organs, damage in gill and skin, abnormal behavior (movement impairment), immunotoxicity, genotoxicity or gene expression, neurotoxicity, endocrine system disruption, reproduction toxicity and finally mortality (Chakraborty et al, 2016).

QDs may be released into the environment and induce harmful effects to humans and the ecosystem (Demir and Castranova 2017). QDs can be transferred from prey to predator in a microbial food chain (Werlin et al, 2011). Lee et al (2015) showed a three-level (from *Astasia longa* (protozoa) to *Moina macrocopa* (cladoceran), and to *Danio rerio* (fish)) transfer of QDs in the aquatic environment. In addition, NPs with certain physicochemical characteristics can readily enter biological membranes (Murugan et al, 2015). NPs may reach the embryo from somatic tissues, in case of their capability to cross species-specific barriers, spanning from embryo protective layers (i.e., chorion membrane for zebrafish and Drosophila) up to the highly structured mammalian placenta (Tortiglione 2011).

Many QDs produced characteristic signs of Cd toxicity that were weakly correlated with metallothionein (MT) expression, indicating that QDs were slightly degraded *in vivo* (King-Heiden et al, 2009). Using MT gene induction as an indicator of Cd^{2+} release, these studies could detect breakdown of QDs after absorption by the organism (King-Heiden et al, 2009). Additionally, the fluorescence emission shifts from red to blue and the excitation fluorescence peak become broader during QDs biodegradation (Alaraby et al, 2015). Furthermore, the QDs degradation could be due to low pH or oxidation of QDs surface (Khalil et al, 2011). The low pH conditions of the gastric tract can contribute to QDs degradation in Drosophila larvae and fish (Alaraby et al, 2015; Duan et al, 2013).

The current knowledge is yet too limited to drawing conclusions about risks of QDs to early development stages. Further investigations are needed for clarify toxic mechanisms of QDs, particularly in early development of organisms. The aims of present study were to assess toxicity, genotoxicity and cytotoxicity to larvae of rainbow trout *Oncorhynchus mykiss* exposed to 4 nmol/L CdSe/ZnS quantum dots (QDs), to determine accumulation of Cd and the concentration of metallothionein (MT) in the whole body of larvae exposed to QDs, and to explain the possible impact mechanism of the QDs to fish larvae.

2. MATERIALS AND METHODS

Exposure of fish was performed at the Laboratory of Ecology and Physiology of Hydrobionts (Nature Research Centre, Lithuania). Embryos of *O. mykiss* in the eyed-egg stage were obtained from Simnas Experimental Hatchery (Alytus District, Lithuania). All studies have been carried out with non-protected life-stages in accordance with Directive 2010/63/EU.

Water-soluble, red emitting semiconductor QDs (Qdot® ITK™, Life Technologies, CA, USA) a size of about 5 - 7 nm as determined by transmission electron microscopy were used at a concentration of 4 x 10^{-9} mol/L. The concentration of QDs was chosen according to the study of Yong et al (2013) who showed that LC_{50} values of CdSe–ZnS to zebrafish are in the range of 0.7 - 4.2 x 10^{-7} mol/L. A volume of 100 μL of a stock dispersion of 8 μmol/L QDs was diluted with deep-well water to achieve final concentrations of 4 x 10^{-9} mol/L in the incubation media. Continuous aeration was used to keep the particles suspended.

The toxicity test was performed in a climate cabinet (Bronson PGC-660, Zaltbommel, the Netherlands) with continuous aeration under static conditions according to ISO 7346-1:1996, without water changing. According to the OECD 210 (OECD 1992), the experiments were carried out in the dark and the larvae were not fed (ISO 10229:1994). The studies were performed in three replicates. Deep-well water used for dilution and as control water had a mean pH of 8.0; the temperature was maintained at 10 ± 0.5 °C, and the oxygen concentration was 10 mg/L. Dissolved oxygen in the tanks, temperature and pH were measured routinely with a hand-held multi-meter (Multi 340i/SET, WTW, Weilheim, Germany). Heart rate (HR, counts/min) and gill ventilation frequency (GVF, counts/min) of larvae were evaluated using stereomicroscope (RZ Series, Meiji Techno, Saitama, Japan). Samples of larvae were taken upon days 4, 7 and 10 after exposure start.

Induction of micronuclei (MN), nuclear buds (NB), bi-nucleated (BN), fragmented-apoptotic (FA) cells were analysed in erythroblasts of larvae. Total genotoxicity level was assessed as the sum of MN and NB, as well as total cytotoxicity level – as the sum of BN and FA frequencies. Cell smears were prepared from whole larvae (with removed yolk sac) body (gently nipped with tweezers): directly smeared on glass slides and air-dried. Smears were fixed in methanol for 10 min. and later were stained with 10 % Giemsa solution in phosphate buffer pH = 6.8 for 20 - 40 min. The stained slides were analysed under light microscopes Olympus BX51 at final magnification of 1,000×. Micronuclei and other nuclear abnormalities (NAs) were identified following criteria described by Fenech et al (2003). The frequencies of abnormalities were recorded in 1,000 erythroblasts per slide using blind scoring by a single observer.

Experiment of the accumulation of QDs in larvae lasted 10 days: starting from 1-day-old larvae under static conditions according to ISO 7346-1:1996. The Cd accumulation was measured in the whole body of larvae (10 individuals per 3 replicate). Sampled organisms were dried up on absorbent paper, weighted and then stored at −18 °C until Cd analysis. For Cd analysis in larvae, the digestion method was used (Thomas and Mohaideen 2015). The content of Cd in the experimental water and in the whole body of the fish larvae was analyzed by an atomic absorption spectrophotometer SHIMADZU AA-7000 (Japan) with a graphite furnace atomizer GFA-7000 and auto-sampler ASC-7000 (measured wavelength 185 to 900 ± 0.3 nm, high-speed deuterium lamp 185 to 430 nm, heating temperature range 50 to 3000 0C, repeatability 2.5%) according to the analysis method LST EN ISO 15586: 2004. The concentration of Cd standard (Sigma-Aldrich Chemie GmbH, Germany) for atomic absorption spectrophotometer is 1000 mg/L and Cd detection limit is 0.3 μg/L.

MT content determination was assayed according to the method of Peixoto et al (2003). For MT level assays, 7 and 10 days old larvae of rainbow trout were weighted and frozen (-80 °C). The larvae were homogenized with Potter-Elvehjem homogenizer in 4 volumes of 20 mM tris (hydroxymethyl) aminomethane HCl buffer, pH 8.6, containing 0.5 mM phenylmethylsulphonyl fluoride and 0.01% β-mercaptoethanol. The homogenate was then centrifuged at 17,000 × *g* for 30 min at 4 °C. Aliquots of 1 ml of supernatant containing MT were added with 1.05 ml of cold (-20 °C) absolute ethanol and

80 μl chloroform. The samlpes were centrifuged at 6000 × *g* for 10 min at 4 °C. The collected supernatant was combined with three volumes of cold ethanol (-20 °C), maintained at -20 °C for 1 h and centrifuged at 6000 × *g* for 10 min at 4 °C. The metallothionein-containing pellets were then rinsed with 1 ml of 87% ethanol and 1% chloroform mix and centrifuged at 6000 × *g* for 10 min at 4 °C. The MT content in the pellet was evaluated using the colorimetric method with 5,5'-dithio-*bis*(2-nitrobenzoic acid) reagent. The pellet was suspended in 150 μl 0.25 M NaCl and subsequently 150 μl 1 N HCl containing 4 mM ethylendiamintetraacetic acid calcium disodium salt were added to the sample. 4.2 ml 2 M NaCl containing 0.43 mM 5,5'-dithio-*bis*(2-nitrobenzoic acid) buffered with 0.2 M Na-phosphate, pH 8.0 was then added to the sample at room temperature. The sample was centrifuged at 3000 × *g* for 5 min at room temperature. The supernatant absorbance was evaluated at 412 nm. MT concentration was estimated using molar absorption coefficient at 412 nm 14140 $M^{-1}cm^{-1}$ and expressed as micrograms of SH groups per gram of wet weight.

Means and standard deviations or standard errors for each studied parameter were calculated. Differences between the evaluated characteristics studied were tested by two-way ANOVA using Statistica 7.0 software (StatSoft Inc., Tulsa, Oklahoma, USA). Results of nuclear abnormalities assay were analyzed by non-parametric Mann-Whitney test (GraphPad Software Inc., San Diego, CA, USA). Differences were accepted as significant at the 95 % level of confidence ($p < 0.05$).

3. RESULTS

In this investigation, GVF of larvae after 10 days of exposure to QDs significantly ($p < 0.05$) increased as compared to the control (Figure 1 A). Meanwhile, after 4 and 7 days of exposure to QDs GVF of larvae did not differ significantly from the control. Also, HR of larvae throughout the exposure period did not differ significantly from the control, ranging from 101.60 ± 6.73 to 103.73 ± 8.48 counts/min (in control HR was from 101.33 ± 3.90 to 103.20 ± 5.06 counts/min) (Figure 1 B).

Results of total cytotoxicity and total genotoxicity levels in erythroblasts of *O. mykiss* larvae are given in Figure 2. Significant elevation of the total cytotoxicity level was not found after QDs treatment. However, total cytotoxicity level after 4 days of exposure was approximately 5-fold higher compared to the control level. Treatment with QDs significantly increased total genotoxicity level in larvae erythroblasts, which was 2.5-fold higher than the control level.

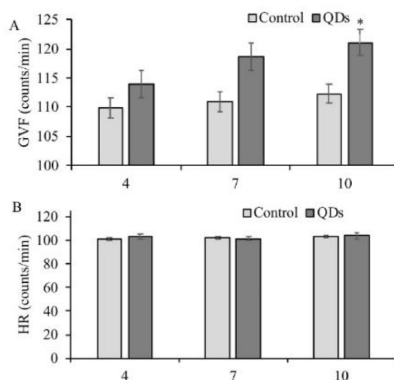


Figure 1. Toxic effect of QDs on biological parameters of *O. mykiss* larvae: (A) GVF (counts/min) and (B) HR (counts/min) (mean ± SEM, N = 15).* Significant difference from the control ($p < 0.05$).

778

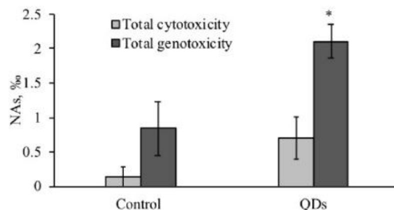


Figure 2. Total cytotoxicity (bi-nucleated (BN)+ fragmented-apoptotic (FA)) and total genotoxicity (micronuclei (MN)+ nuclear buds (NB)) levels (mean ± SEM, N = 7) in erythroblasts of *O. mykiss* larvae in control and QDs exposed groups. Asterisks (*) denote significant differences from control group ($p < 0.05$)

Changes of Cd concentrations in larvae during experiment are shown in Figure 3. Samples of larvae were taken upon days 4, 7 and 10 after exposure start to determine the accumulation of QDs to rainbow trout larvae depending on the duration of exposure. Cd accumulation in larvae after 4, 7 and 10 days exposures to QDs were significantly ($p < 0.05$) different from the control (Figure 3). The maximum value of accumulated Cd was found in larvae exposed to QDs after 10 days of exposure (1.302 ± 0.272 μg/g). However, accumulation of Cd in larvae did not depend on the duration of exposure.

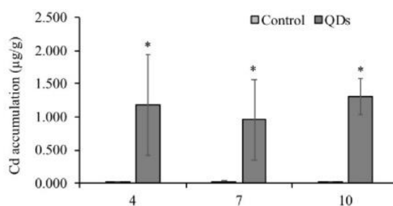


Figure 3. Cd accumulation (wet weight, μg/g) in *O. mykiss* larvae after 4, 7 and 10 days of exposure to QDs.

Measured MT content was used as a marker of internal QDs exposure. However, the MT contents in larvae showed no significant changes after treatment QDs for 7 and 10 days compared to the control (Figure 4).

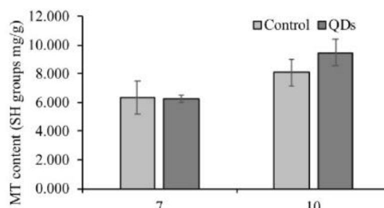


Figure 4. Metallothionein content (SH groups, mg/g) in *O. mykiss* larvae after 7 and 10 days of exposure to QDs.

4. DISCUSSION AND CONCLUSIONS

The toxicity study showed significantly ($p < 0.05$) increased GVF of larvae as compared to the control after 10 days of exposure to QDs, however GVF in larvae showed no significant changes after 4 and 7 days of exposure, and HR in larvae showed no significant changes after 4, and 10 days of exposure to QDs (Figure 1 A and B). One possible explanation for these results is that QDs interfered with breathing due to QDs adhesion in gill. Gill is an important organ for respiration, osmoregulation, acid-base balance and nitrogenous waste excretion (Mansouri et al, 2016). Gills are most important targets of waterborne objects such as NP (Chakraborty et al, 2016). For instance, Cu NPs may damage gills lamellae of zebrafish (Griffitt et al, 2007). It was noticed that gill of larvae forms mucus complex with QDs. Mucus secretion were also observed in the present study and were reported by other authors (Federici et al, 2007; Smith et al, 2007) for rainbow trout exposed to single walled carbon nanotubes and titanium dioxide nanoparticles. In addition, Ag NPs can induce excessive mucus secretion and hyperplasia in gill tissue of zebrafish (Mansouri and Johari 2016). The larvae gradually developed unique systems to protect themselves from the toxicity of chemicals. The number of mucous cells may be an indicator of exposure to stressors (Ostaszewska et al, 2016). An increase in the number of mucous cells secreting sulfated and carboxylated mucins is associated to the increase in mucus viscosity, which improves its protective properties (Kumari et al, 2009). Mucus consists of immunoglobulin, lysosome, and lectin that protect fish against infections. According to Poleksic et al (2010) reduction of mucous cell abundance at the highest AgNPs concentration and a decrease in the number and area of mucous cells in fish exposed to CuNPs show exhaustion of proliferative ability of mucous cells.

The most obvious finding to emerge from the nuclear abnormalities analysis in larvae is that significant increase of total genotoxicity level (as sum of MN and NB frequencies) in QDs exposed larvae eryhtroblasts was determined. Xiao et al (2016) study results revealed that carbon QDs exposure causes significant DNA damage in embryonic cells of Rare Minnow (*Gobiocypris rarus*). Oxidative stress induced DNA damage and the inflammatory response are considered to be the main mechanisms causing toxicity of the NPs (Xiao et al, 2016; Schins and Hei 2006; Schins and Knaapen 2007). Further studies focusing on QDs-induced genotoxicity should be performed using fish erythroblasts/erythrocytes, which, as indicated by the present study, are important targets for *in vivo* QDs toxicity.

As shown in Figure 3, the Cd amount in larvae was significantly ($p < 0.05$) different from the control during the exposure period. QDs potential risk could be caused by the nanomaterial itself or by their free metallic components (Hardman 2006). The determination of chemical concentration in larvae is a challenge since it requires highly sensitive analytical techniques owing to the low sample amount (1 larvae ~ 0.1 g). In this study, larvae do not feed yet, suggesting that one possible way to pass QDs in the larvae are skin-absorption. It is well known that biological barriers play a significant function to determine QDs biodistribution (Chu et al, 2010). A small size of QDs (between 1 and 100 nanometers) permits these NPs to get into the body through cellular barriers and can reach organs and tissues and interact with biological structures, thus impact normal functions in different ways (Maldiney et al, 2011). NPs could accumulate selectively in the head, yolk sac and the tail after NPs enter into the larvae body through swallowing and skin-absorption (Kang et al, 2015). However, QDs could be eliminated in the urine of larvae or degraded into particles and could be removed by lysosome-like vesicles, and then accumulate in the kidney and liver (Lei et al, 2011). In contrast, during normal metabolism, the primary accumulation tissues of heavy metal Cd are the liver and kidneys (Haouem et al, 2007). Lei et al (2011) noted that MAA-QDs were unable to diffuse into the yolk of larvae because of the high content of lipids in the yolk cell.

MT content (an indicator of metal ion exposure) were used to detect toxicity due to Cd^{2+}, however MT contents in larvae did not significantly increase after 7 and 10 days of exposure (Figure 4). Our research data coincides with Fischer et al (2006) data that ZnS shell and surface ligands protect QDs

from degradation *in vivo*. Therefore, QDs were stable during 10 days of exposure and QDs absorption was not in larvae. In contrast with our finding, King-Heiden et al (2009) noticed that QDs degraded at least partially *in vivo*, MT expression correlated with $CdCl_2$ and QDs exposure concentrations.

In summary, this study demonstrates that QDs induced significantly ($p < 0.05$) increased GVF of larvae as compared to the control only after 10 days of exposure to QDs, however QDs did not cause GVF changes in larvae after 4 and 7 days of exposure and HR changes in larvae after 4, 7 and 10 days of exposure (Figure 1 A and B). Furthermore, total genotoxicity level was found to increase significantly after 4 days exposure to QDs (Figure 2). The Cd amount in larvae was significantly ($p < 0.05$) different from the control throughout the exposure period (Figure 3). Our study further demonstrated that the MT levels of larvae were unchanged (Figure 4), which might explain that QDs were stable and QDs absorption was not in larvae. Thus, our findings suggest that exposure to QDs could be due to QDs adhesion in gill, which induced toxicity to larvae. Results of toxicity and genotoxicity studies allow to assuming that the mechanical impact of QDs could be one of the factors induced the changes of physiologic function in fish larvae. However, further investigation must be undertaken to confirm this presumption.

Acknowledgment
Toxicity, accumulation of QDs and MT content assessment was funded by the Research Council of Lithuania, Project No. MIP-108/2015. Genotoxicity and cytotoxicity studies were funded by the Research Council of Lithuania, Project No. S-MIP-17-10.

References

1. Alaraby M., E. Demir, A. Hernández and R. Marcos (2015) 'Assessing potential harmful effects of CdSe quantum dots by using Drosophila melanogaster as *in vivo* model', **Science of the Total Environment,** Vol. 530-531, pp. 66-75.
2. Chakraborty C., A.R. Sharma, G. Sharma and S.-S. Lee (2016) 'Zebrafish: A complete animal model to enumerate the nanoparticle toxicity', **Journal of nanobiotechnology,** Vol. 14: 65.
3. Chu M., Q. Wu, H. Yang, R. Yuan, S. Hou, Y. Yang, Y. Zou, S. Xu, K. Xu, A. Ji and L. Sheng (2010) 'Transfer of quantum dots from pregnant mice to pups across the placental barrier', **Small**, Vol. 6(5), pp. 670-8.
4. Demir E. and V Castranova (2017) 'Evaluation of the Potential Genotoxicity of Quantum Dots. A Review', **Scientific Pages Nanotechnology**, Vol. 1(1), pp. 1-19.
5. Directive 2010/63/EU, of the European Parliament and of the Council of the European Union. On the Protection of Animals Used for Scientific Purposes. Official Journal of the European Union L276, 233-279, 2010.
6. Duan J., Y. Yongbo, L. Yang, Y. Yang, L. Yanbo, H. P, Z. Xianqing, P. Shuangqing and S. Zhiwei (2013) 'Developmental toxicity of CdTe QDs in zebrafish embryos and larvae', **Journal of Nanoparticle Research**, Vol. 15, 1700.
7. Federici G., B.J. Shaw and R.H. Handy (2007) 'Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): gill injury, oxidative stress, and other physiological effects', **Aquatic Toxicology**, Vol. 84, pp. 415-430.
8. Fenech M., W.P. Chang, M. Kirsch-Volders, N. Holland, S. Bonassi and E. Zeiger (2003) 'HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated humanlymphocyte cultures', **Mutation research**, Vol. 534, pp. 65-75.
9. Fischer H.C., L. Liu, K.S. Pang and W.C.W. Chan (2006) 'Pharmacokinetics of nanoscale quantum dots: *In vivo* distribution, sequesteration, and clearance in the rat. **Advanced Functional Materials**, Vol. 16, pp. 1299-1305.

10. Griffitt R.J., R. Weil, K.A. Hyndman, N.D. Denslow, K. Powers, D. Taylor and D.S. Barber (2007) 'Exposure to copper nanoparticles causes gill injury and acute lethality in zebrafish (*Danio rerio*)', **Environmental Science & Technology**, Vol. 41, pp. 8178-8186.

11. Haouem S., N. Hmad, M.F. Najjar, A. El Hani and R. Sakly (2007) 'Accumulation of cadmium and its effects on liver and kidney functions in rats given diet containing cadmium polluted radish bulb', **Experimental and Toxicologic Pathology**, Vol. 59, pp. 77-80.

12. Hardman R. (2006) 'Review A Toxicologic Review of Quantum Dots: Toxicity Depends on Physicochemical and Environmental Factors', **Environmental health perspectives**, Vol. 114, pp. 165-173.

13. ISO 10229: 1994. "Water Quality -- Determination of the Prolonged Toxicity of Substances to Freshwater Fish -- Method for Evaluating the Effects of Substances on the Growth Rate of Rainbow Trout (*Oncorhynchus mykiss* Walbaum (Teleostei, Salmonidae))"

14. ISO 7346-1: 1996. "Water Quality -- Determination of the Acute Lethal Toxicity of Substances to a Freshwater Fish [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae)]"-- Part 1: Static Method.

15. Ji X., F. Peng, Y. Zhong, Y. Su and Y. He (2014) 'Fluorescent quantum dots: Synthesis, biomedical optical imaging, and biosafety assessment', **Colloids Surf. B Biointerf**, Vol. 124, pp. 132-139.

16. Kang Y.F., Y.H. Li, Y.W. Fang, Y. Xu, X.-M. Wei and X.-B. Yin (2015) 'Carbon Quantum Dots for Zebrafish Fluorescence Imaging', **Scientific Reports**, Vol. 5, 11835, DOI: 10.1038/srep11835.

17. Khalil W.K., E. Girgis, A.N. Emam, M.B. Mohamed and K.V. Rao (2011) 'Genotoxicity evaluation of nanomaterials: DNA damage, micronuclei, and 8-hyroxy-2deoxyguanosine induced by magnetic doped CdSe quantum dots in male mice', **Chemical Research in Toxicology**, Vol. 24, pp. 640-650.

18. King-Heiden T.C., P.N. Wiecinski, A.N. Mangham, K.M. Met, D. Nesbit, J.A. Pedersen, R.J. Hamers, W. Heideman and R.E. Peterson (2009) 'Quantum dot nanotoxicity assessment using the zebrafish embryo', **Environmental Science & Technology**, Vol. 43(5), pp. 1605-1611.

19. Kumari U., M. Yashpal, S. Mittal and A.K. Mitta (2009) 'Histochemical analysis of glycoproteins in the secretory cells in the gill epithelium of a catfish *Rita rita* (*Siluriformes*, *Bagridae*)', **Tissue Cell**, Vol. 41, pp. 271-280.

20. Lee W.M and Y.J. An (2015) 'Evidence of three-level trophic transfer of quantum dots in an aquatic food chain by using bioimaging', **Nanotoxicology**, Vol. 9, pp. 407-412.

21. Lei Y., Q. Xiao, S. Huang, W. Xu, Z. Zhang, Z. He, Y. Liu and F. Deng (2011) 'Impact of CdSe/ZnS quantum dots on the development of zebrafish embryos', **The Journal of Nanoparticle Research**, Vol. 13, pp. 6895-6906.

22. Libralato G., E. Galdiero, A. Falanga, R. Carotenuto, E. de Alteriis and M. Guida (2017) 'Toxicity Effects of Functionalized Quantum Dots, Gold and Polystyrene Nanoparticles on Target Aquatic Biological Models: A Review', **Molecules**, Vol. 22, 1439; doi:10.3390/molecules22091439.

23. LST EN ISO 15586:2004. Vandens kokybė. Mikroelementu nustatymas atominės absorbcijos spektrometrija, naudojant grafitine krosni (ISO 15586:2003).

24. Maldiney T., C. Richard, J. Seguin, N. Wattier, M. Bessodes and D. Scherman (2011) 'Effect of core diameter, surface coating, and PEG chain length on the biodistribution of persistent luminescence nanoparticles in mice', **ACS Nano**, Vol. 5(2), pp. 854-62. doi:10.1021/ nn101937h

25. Mansouri B. and S.A. Johari (2016) 'Effects of short-term exposure to sublethal concentrations of silver nanoparticles on histopathology and electron microscope ultrastructure of zebrafish (*Danio rerio*) gills', **Iranian Journal of Toxicology**, Vol. 10, pp. 15-20.

26. Mansouri B., R. Rahmani, N.A. Azadi and S.A. Johari (2016) 'Combined effects of silver nanoparticles and mercury on gill histopathology of zebrafish (*Danio rerio*)', **Journal of Coastal Life Medicine**, Vol. 4(6), pp. 421-425.

27. Murugan K., Y.E Choonara, P. Kumar, D. Bijukumar, L.C du Toit and V. Pillay (2015) 'Parameters and characteristics governing cellular internalization and trans-barrier trafficking of nanostructures', **International journal of nanomedicine**, Vol. 10, pp. 2191-2206.

28. OECD (Organization for Economic Cooperation and Development). 1992. Guideline for the Testing of Chemicals: Fish, Early-life Stage Toxicity Test, Part 210.

29. Oh E., R. Liu, A. Nel, K.B. Gemill, M. Bilal, Y. Cohen and I.L. Medintz (2016) 'Meta-analysis of cellular toxicity for cadmium-containing quantum dots', **Nature Nanotechnology**, Vol. 11, pp. 479-486.

30. Ostaszewska T., M. Chojnacki, M. Kamaszewski and E. Sawosz-Chwalibóg (2016) 'Histopathological effects of silver and copper nanoparticles on the epidermis, gills, and liver of Siberian sturgeon', **Environmental science and pollution research international**, Vol. 23, pp. 1621-1633.

31. Peixoto N.C., T. Roza, E.M. Flores and M.E. Pereira (2003) 'Effects of zinc and cadmium on HgCl2-delta-ALA-D inhibition and Hg levels in tissues of suckling rats', **Toxicology Letters**, Vol. 46, pp.17-25.

32. Poleksic V., M. Lenhardt, I. Jaric, D. Djordjevic, Z. Gacic, G. Cvijanovic and B. Raskovic (2010) 'Liver, gills, and skin histopathology and heavy metal content of the Danube sterlet (*Acipenser ruthenus* Linnaeus, 1758)', **Environmental toxicology and chemistry**, Vol. 29, pp. 515-521.

33. Rocha T.L., N.C. Mestre, S.M.T. Saboia-Morais and M.J. Bebianno (2017) 'Environmental behaviour and ecotoxicity of quantum dots at various trophic levels: A review', **Environment International**, Vol. 98, pp. 1-17.

34. Schins R.P. and A.M. Knaapen (2007) 'Genotoxicity of poorly soluble particles', **Inhalation Toxicology**, Vol. 19, pp. 189-198.

35. Schins R.P. and T.K. Hei (2006) 'Genotoxic Effects of Particles', In: Donaldson K and Borm P, Particle toxicology. **CRC Press/Taylor & Francis Group**, USA, pp. 285-298.

36. Smith C.J., B.J. Shaw and R.D Handy (2007) 'Toxicity of single walled carbon nanotubes to rainbow trout, (*Oncorhynchus mykiss*): respiratory toxicity, organ pathologies, and other physiological effects', **Aquatic Toxicology**, Vol. 82, pp. 94-109.

37. Thomas S. and J. A. Mohaideen (2015) 'Determination of some heavy metals in fish, water and sediments from Bay of Bengal', **International Journal of Chemical Sciences**, Vol. 13(1), pp. 53-62.

38. Tortiglione C. (2011) 'The heritable effects of nanotoxicity. Review', **Nanomedicine (Lond.)**, Vol. 9(18), pp. 2829-2841.

39. Werlin R., J.H. Priester, R.E. Meilke, S. Krämer, S. Jackson, P.K. Stoimenov, G.D. Stucky, G.N. Cherr, E. Orias and P.A. Holden (2011) 'Biomagnification of cadmium selenide quantum dots in a simple experimental microbial food chain', **Nature Nanotechnology**, Vol. 6, pp. 65-71.

40. Xiao Y., L. Liu, Y. Chen, Y. Zeng, M. Liu and L. Jin (2016) 'Developmental Toxicity of Carbon Quantum Dots to the Embryos/Larvae of Rare Minnow (*Gobiocypris rarus*)', **BioMed Research International**, 11 pages.

41. Yong K.T., W.C. Law, R. Hu, L. Ye, L.W. Liu, M.T. Swihart and P.N. Prasad (2013) 'Nanotoxicity assessment of quantum dots: from cellular to primate studies', **Chemical Society Reviews**,42(3), pp. 1236-1250.
42. Zhang L.W, W. Bäumer and N.A Monteiro-Riviere (2011) 'Cellular uptake mechanisms and toxicity of quantum dots in dendritic cells', **Nanomedicine (Lond)**, 6(5), pp. 777-791.

NOTES

Vilniaus universiteto leidykla
Universiteto g. 1, LT-01513 Vilnius
El. p. info@leidykla.vu.lt,
www.leidykla.vu.lt
Tiražas 15 egz.