International scientific workshop

INFLUENCE OF ACTIVE MINES ON FRESHWATER ECOSYSTEMS

May 12-16, 2014

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Pau, France

organize

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within activities of the Projects:

1. The assessment of availability and effects of metals on fish in the rivers under the impact of mining activities (project leaders: Dr. Zrinka Dragun and Dr. Maja Jordanova)
2. Bacterial and parasitical communities of chub as indicators of the status of environment exposed to mining activities (project leaders: Dr. Damir Kapetanović and Dr. Rodne Nastova)
3. Intracellular mapping of essential and nonessential trace elements in the organs of indigenous fish by NanoSIMS (project leaders: Dr. Zrinka Dragun and Dr. Dirk Schaumlöffel)

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PROGRAMME

Monday, May 12, 2014

Arrival and registration

Tuesday, May 13, 2014

10:00-10:15 Zrinka Dragun, Damir Kapetanović: Welcome address and introduction to the workshop

10:15-10:35 Zrinka Dragun: Water quality of mining impacted rivers in the north-eastern Macedonia: I. Physico-chemical parameters and concentrations of dissolved metals/metalloids

10:35-10:50 Damir Kapetanović: Water quality of mining impacted rivers in the north-eastern Macedonia: II. Microbiological water quality of rivers Bregalnica, Zletovska and Kriva - Preliminary results

10:50-11:20 Coffee break

11:20-11:40 Katerina Rebok: Morphometric data of Vardar chub (*Squalius vardarensis*) in the rivers under the impact of mining activity

11:40-12:00 Sheriban Ramani: Accumulation of metals and metalloids in the liver and gills of Vardar chub (*Squalius vardarensis*) from three mining impacted rivers in north eastern Macedonia

12:00-14:00 Lunch break

14:00-14:20 Vlatka Filipović Marijić: Evaluation of dietary metal exposure of *Squalius vardarensis* dwelling in mining impacted rivers in the north-eastern Macedonia

14:20-14:40 Nesrete Krasnići: Cytosolic distribution of Cd, Co, Cu, Fe, Pb, V and Zn in liver, gills and intestine of Vardar chub (*Squalius vardarensis*) from mining impacted rivers in Macedonia

14:40-14:50 Irena Vardić Smrzlić: Molecular characterisation of the metazoan parasites of Vardar chub (*Squalius vardariensis*) from three rivers in north eastern Macedonia

14:50-15:20 Coffee break

15:20-15:40 Vlatka Filipović Marijić: Acanthocephalans, fish intestinal parasites, as bioindicators of metal exposure in rivers impacted by mining waste

15:40-16:00 Nesrete Krasnići: Metallothionein and total protein concentrations in gills and liver of Vardar chub (*Squalius vardarensis*) as biomarkers of water contamination in three rivers in Macedonia

Wednesday, May 14, 2014

10:00-10:15 Damir Kapetanović: Bacterial community of Vardar chub (*Squalius vardarensis*): Preliminary results

10:15-10:30 Damir Valić: Hematological assessment of Vardar chub (*Squalius vardarensis*) from three rivers in north-eastern Macedonia

10:30-11:00 Coffee break

11:00-11:20 Josip Barišić: Spatial and seasonal variability of histopathological alterations on the gills of Vardar chub (*Squalius vardarensis*) from mining impacted rivers in the north-eastern Macedonia

11:20-11:40 Maja Jordanova: Toxicopathic changes in Vardar chub (*Squalius vardarensis*) in rivers under the impact of mining activities

12:00-14:00 Lunch break

14:00-14:30 Dirk Schaumlöffel: Potential and challenges of NanoSIMS for element imaging in biological cells

14:30-14:45 Zehra Hajrulai-Musliu: Fatty acid composition in some river fish species in Republic of Macedonia

14:45-15:00 Risto Uzunov: Detection of methyltestosterone with ELISA method in fish

Thursday, May 15, 2014

08:00-21:00 Visit to Research marine station “Martinska” near Šibenik and National Park “Krka”

Friday, May 16, 2014

Departure

L E C T U R E
A B S T R A C T S

(in accordance with the timetable)

Water quality of mining impacted rivers in the north-eastern Macedonia: I. Physico-chemical parameters and concentrations of dissolved metals/metalloids

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The mine waste was recognized as one of the most serious threats for freshwater ecosystems, and it still represents one of the biggest environmental concerns in Macedonia. Some of the reasons for its toxicity are the acidity and high content of trace metals, which could lead to significant loss of biodiversity. Macedonian most significant mineral deposits are Pb and Zn ores, the exploitation of which is carried out in active mines in the north-eastern part of the country, such as Zletovo in Probishtip and Toranica in Kriva Palanka. Two rivers were chosen for this study, the Zletovska River, which receives the waste from the mine Zletovo, and the Kriva River, which receives the waste from the mine Toranica, whereas a location at the Bregalnica River was chosen as non-impacted sampling site.

The impact of mining on the surface water quality was studied by measuring physico-chemical parameters, nutrient concentrations, and the concentrations of dissolved macro and trace elements. The sampling was performed in spring (May/June) and autumn (October) of 2012. A number of physico-chemical parameters (temperature, pH, redox potential, conductivity, and dissolved oxygen) were measured on site with a portable multiparameter instrument, whereas the other parameters (alkalinity, turbidity, total dissolved solids, chemical oxygen demand, total hardness, and concentration of nutrients and other anions) were measured in the river water samples in the laboratory by standardized ISO, APHA and EPA methods. The measurements of both trace and macro elements were performed on high resolution inductively coupled plasma mass spectrometer.

The Bregalnica River was characterized mainly by slightly alkaline pH and weak contamination with As, Ba, Fe, Mo, Ti, U, V, nitrates and phosphates, which could be connected to agricultural activities, whereas the impact of the mines was not observed. Interesting finding for the Bregalnica River was several degrees higher water temperature compared to the other two rivers, in both sampling periods. The cause of that increase was possibly the mixing of the river water with the hot water from the reservoirs of the nearby situated geothermal system Kezhovica-Ldzhi.

Contrary, both Zletovska and Kriva rivers revealed a clear impact of Pb/Zn mines on water quality. The sampling location at the Zletovska River had slightly acidic water, the highest conductivity, total dissolved solids, total hardness, the concentration of $CaCO_3$, the

concentrations of macro elements (Na, K, Ca, Mg), as well as sulphates and chlorides. Both sulphates and conductivity are useful indicators of acid mine drainage contamination, because they remain increased even when pH approaches neutral values due to large dilutions. In the Zletovska River increased concentrations of Cd, Co, Cs, Cu, Li, Mn, Ni, Rb, Sn, Sr, Tl, and Zn were also found, among which Cd and Ni belong to priority toxic substances, and Cd even exceeded the environmental quality standards set by the European Water Framework Directive for inland surface waters. Due to low water level, especially high concentrations of several elements were measured in autumn (e.g. Cd: 2.0 μg L^{-1}; Mn: 2.5 mg L^{-1}; Zn: 1.5 mg L^{-1}).

The Kriva River water had slightly alkaline pH and continuous slight increase of several trace elements characteristic for agricultural activities (Ba, Fe, Mo and V). In the Kriva River increased concentrations of metals which could be associated with the impact of the nearby mine, such as Cd (0.270 μg L^{-1}) and Pb (1.85 μg L^{-1}), were found only in spring, possibly due to sediment resuspension during higher water discharge, indicating probable sediment contamination by mining waste.

The results obtained in this study revealed severe impact which the active mines Zletovo and Toranica have on the water quality of the rivers receiving their waste (Ramani et al., 2014). The need for the continuous monitoring of those rivers should be emphasized, as well as the necessity to investigate possible detrimental effects of mining waste on the aquatic organisms.

References

Ramani, S., Dragun, Z., Kapetanović, D., Kostov, V., Jordanova, M., Erk, M., Hajrulai-Musliu, Z. 2014. Surface water characterization of three rivers in the lead/zinc mining region of northeastern Macedonia. *Archives of Environmental Contamination and Toxicology* **66**:514-528.

Water quality of mining impacted rivers in the north-eastern Macedonia: II. Microbiological water quality of rivers Bregalnica, Zletovska and Kriva – Preliminary results

Damir Kapetanović[1], Damir Valić[1], Irena Vardić Smrzlić[1], Maja Jordanova[2], Katerina Rebok[2], Sheriban Ramani[3], Zrinka Dragun[4], Vlatka Filipović Marijić[4], Nesrete Krasnići[4], Risto Uzunov[5], Aleksandar Cvetkovikj[5], Zehra Hajrulai-Musliu[5], Stojmir Stojanovski[6], Rodne Nastova[7], Vasil Kostov[7]

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Contamination of surface waters is a world-wide environmental problem. Anthropogenic activities like industry and mining can increase metal concentrations in the surface waters and may result in significant deterioration of water quality. Microorganisms are commonly used as biological indicators of surface water conditions. Microorganisms respond rapidly to changes in their environment due to their short generation time. Metal toxicity, acidic pH and low nutrient levels result in stressed microbial communities. High levels of heavy metals can affect the structure of microbial communities, resulting in decreased metabolic activity and diversity of microorganisms.

Therefore, there is a need to monitor microbial water quality and to provide insight into their interactions with the impact of mining and the subsequent implications on the rivers ecosystem function.

Two rivers were chosen for this study in autumn 2012, the Zletovska River, which receives waste from the Zletovo mine, and the Kriva River, which receives waste from the Toranica mine. The Bregalnica River was chosen as a non-impacted aquatic system.

Microbiological analyses of river water were performed using defined substrate technology. Total coliforms bacteria were identified using Colilert test (IDEXX Laboratories., Westbrook, USA), whereas enterococci were identified using Enterolert-E test (IDEXX Laboratories). Total coliforms and enterococci were enumerated using Quantitray 2000 (IDEXX Laboratories), which used a 97-test-well system and provided the most probable number (MPN) of bacteria/100 mL.

Analysis of the microbial water quality showed higher levels of bacterial indicators in the rivers Kriva and Bregalnica downstream from the big cities. Together with significantly higher number of bacterial indicators an increase in nitrate and phosphate was observed, indicating a particular anthropogenic impact on these rivers. A small number of bacterial

indicators were found in the Zletovska River, indicating less anthropogenic influence, but it is not possible to say is that the effect of pollution from mining activities.

Based on these preliminary results from one sampling season, it can be concluded that the impact of large cities is significant on analyzed rivers from a microbiological point of view.

Morphometric data of Vardar chub (*Squalius vardarensis*) in the rivers under the impact of mining activity

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Disturbed natural balance as a result of significant pollution pressure by mining activities has been already reported for the major waterways in the Republic of Macedonia. An exceeded metal concentration in the aquatic environment has the potential to induce serious harm in wild organisms, to impair their health, condition, reproduction or their habitats. The implementation of the EU Water Framework Directive establishes that the ecological status of the aquatic ecosystems must be determined by biological quality elements, including fish, and has to be supported by hydro-morphological and physico-chemical parameters. Therefore, the aim of this study was to obtain basic data for the effect of mining activities on fish, using Vardar chub (*Squalius vardarensis*) as a bioindicator organism, and using biomarkers that are relatively simple and non-specific, but indicative of cumulative organism-level changes.

For achieving this goal, 158 chub were captured with electro fishing methods from three rivers in northeastern R. Macedonia, two of them under lead/zinc mine activities (Zletovska River and Kriva Rriver) and one control, reference river, free from mine pollution (Bregalnica River). Fish were captured in two seasons – autumn and spring of 2012. Total length (TL), body mass (BM), liver (LM) and gonad mass (GM) were measured. The condition factor (CF), hepatosomatic index (HSI) and gonadosomatic index (GSI) were calculated. With tendency to minimize the effect of the reproductive cycle on these indices, organosomatic indices corrected for the gonad mass were also calculated by subtracting the mass of the gonads from the body mass in the computation of HSI* and GSI*. All obtained data was statistically analysed by ANOVA/MANOVA followed by post-hoc Newman-Keuls' test.

In general, as was expected for this fish species, females in both seasons were heavier and longer than males. Concerning the localities, females in both seasons and males in autumn revealed significantly lower BM, BM* and TL values in the Zletovska River compared with the reference Bregalnica River. Those observations were also reflected on fish condition, expressed by the condition factor which revealed that both sexes in both seasons had disturbed condition (significantly lower CF) in the Zletovska River when compared to Bregalnica and Kriva rivers. Furthermore, seasonal differences were observed in both sexes, with significantly higher CF values in spring in all three locations (except for

males from the Bregalnica River). Strong seasonal influence was also observed for GM, GSI and GSI* with higher values detected in spring compared with autumn and only week effect from localities (only in females in spring with highest values in Kriva River). As for the LM, HSI and HSI*, the lowest values were noticed in the Zletovska River in comparison with the other two rivers, significantly lower for both sexes in autumn. Seasonal differences were observed for LM only in females from Kriva River and for HSI and HSI* in both sexes from Zletovska River. For all parameters spring collected samples revealed higher values than those collected in autumn.

Although all investigated parameters are not specific and not very sensitive, they are widely accepted representative indices of overall organism health and they may serve as an initial screening biomarker to indicate the consequences of exposure to contaminants. Observed lower values for almost all investigated parameters in the Zletovska River suggested disturbed general fish condition and organ disturbance in fish that inhabit waterways that are under pressure by mining activities. However, deeper investigations are needed, using several biomarkers at multiple levels of biological organization, to assess the current pollution impacts on the health status of the local ichthyofauna.

Accumulation of metals and metalloids in the liver and gills of Vardar chub (*Squalius vardarensis*) from three mining impacted rivers in north eastern Macedonia

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Mining activity can seriously affect the quality of surface water, especially regarding the levels of trace elements, which present a serious threat to the aquatic organisms because of their toxicity, persistence and bioaccumulation. The aim of this study was to assess bioavailability of metals and metalloids in the river water based on their accumulation in two target organs (liver and gills) of Vardar chub (*Squalius vardarensis*) from three rivers in north-eastern Macedonia, agriculturally impacted Bregalnica River and two mining impacted rivers, the Zletovska and the Kriva River.

For the assessment of metal accumulation, 12-30 chub specimens were caught per site in spring (May/June) and autumn (October) of 2012. Metal/metalloid concentrations were determined by high resolution inductively coupled plasma mass spectrometer in the soluble cytosolic fractions of gills and liver, obtained after tissue homogenization and centrifugation at 50 000xg.

Under conditions of low metal exposure, following ranges of metal/metalloid concentrations were measured in the chub liver: <1 μg L^{-1} for Tl; 1-10 μg L^{-1} for As, Cd, Co, Cs, Pb, Sr, and V; 10-100 μg L^{-1} for Ba and Mo; 100-500 μg L^{-1} for Mn, Rb, and Se; and >1000 μg L^{-1} for Cu, Fe, and Zn. In chub gills, concentration ranges slightly varied, as follows: <1 μg L^{-1} for Cd, Tl, and V; 1-10 μg L^{-1} for As, Co, Cs, Mo, and Pb; 10-100 μg L^{-1} for Ba, Cu, Mn, and Se; 100-500 μg L^{-1} for Rb and Sr; and >1000 μg L^{-1} for Fe and Zn. The comparison between two organs indicated higher basal concentrations of several elements in the liver: As, Cu, Mn, Mo, Se, Tl, and V were higher 2, 30-50, 3-7, 8, 2-3.5, 2, and 9 times, respectively, in both seasons, whereas Fe was 9 times higher only in autumn. Higher concentrations in the gills were found for Sr (13 times) in both seasons and for Fe and Zn (2 times) only in one season (in spring and autumn, respectively).

As a result of increased exposure to metals in the river water, increased bioaccumulation was observed for several elements in both liver and gills of Vardar chub in both seasons, specifically for V in the Bregalnica River, and for Cs, Rb, Sr, and Tl in the Zletovska River. Increased exposure to Cd was evident in both seasons in the Zletovska River, but only in spring in the Kriva River, and accompanied by adequate increase of

cytosolic Cd concentrations in both organs. In the Kriva River, dissolved Cd concentrations decreased in autumn and resulted with decrease of cytosolic Cd concentrations in both organs. Increased exposure to Pb in the river water was observed in the Kriva River in spring and in the Zletovska River in autumn, and followed by increase of cytosolic Pb concentrations in both organs. Decrease of Pb concentrations in the river water resulted with partial decrease of cytosolic Pb concentration in both organs. Among listed elements, more pronounced accumulation of Cd, Rb, Tl and V was observed in the liver, of Pb and Sr in the gills, while accumulation of Cs was comparable in both organs.

Contrary to above listed elements, several elements did not exhibit signs of bioaccumulation, even under conditions of extremely high exposure in the river water, indicating existence of regulation mechanisms for those elements in chub organism. For example, despite increased concentrations of dissolved Fe in the Bregalnica River and dissolved Mn and Zn in the Zletovska River in both seasons, as well as dissolved Fe in the Kriva River in autumn, increased cytosolic concentrations of these elements were generally not found in the cytosols of chub liver and gills. Only in the Zletovska River, cytosolic concentrations of Mn in spring and Zn in autumn were found increased in the gills (3 times and 30%, respectively) compared with other two rivers, at exposure level of 350 μg L^{-1} of dissolved Mn and 1.5 mg L^{-1} of dissolved Zn. However, at much higher exposure level of dissolved Mn in autumn (2.4 mg L^{-1}), increased bioaccumulation of Mn was not observed.

In order to exclude chub physiology as a cause of differences in metal/metalloid concentrations between three rivers, we have analyzed their association with the fish size (mass range: 13.86-307.89 g), sex (43% females, 57% males) and season, using the data obtained at less impacted Bregalnica River. The significant associations with chub mass and sex were determined for several elements, but only for cytosolic Ba concentrations in the liver and for cytosolic Mn concentrations in the gills they were determined in both seasons. Negative correlations with chub mass and higher concentrations in males were obtained for Ba, whereas positive correlations with the chub mass and higher concentrations in females were obtained for Mn. Seasonal differences were also observed, with 2-3 times higher concentrations of Cu and Sr in both organs in spring and Fe and Zn in the gills in spring and autumn, respectively. Elements which have been found to accumulate in chub liver and gills due to increased exposure to metals in water (i.e. Cs, Rb, Tl, Cd, Pb, and V) generally showed no association with the chub size, sex or season.

Integrated approach to estimation of metal pollution of river water requires linking of data on concentrations of dissolved metals in water with data on metal accumulation in target organs of indicator organisms. In the cytosol of liver and gills of Vardar chub (*Squalius vardarensis*), increased concentrations of Cd, Cs, Pb, Rb, Sr, Tl, and V were determined in association with increased concentrations of these elements in the river water, whereas, at the same time, their dependence on fish physiology was not established. This indicates the possibility of applying cytosolic concentrations of these metals in chub liver and gills in investigative monitoring as indicators of increased biological availability of metals in the river water.

Evaluation of dietary metal exposure of *Squalius vardarensis* dwelling in mining impacted rivers in the north-eastern Macedonia

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Fish are frequently selected as the organisms of choice in aquatic biomonitoring due to their top position in the biotic communities and their sensitivity to low concentrations of environmental pollutants. Among other pollutants, metals represent a serious threat to the aquatic environment, since they are not biodegradable and can accumulate in the sediments and biota, including fish. For normal cellular metabolism, fish require essential metals which may be taken up from water, food and/or sediments in varying degrees. Metals are taken up to fish through skin, gills and gastrointestinal tract. In the last decade there is a growing concern that dietborne metal uptake may be of equal or greater importance than the waterborne for native fish. Accordingly, in the present field study gastrointestinal tissue was selected as an indicator organ for evaluating dietary metal exposure of freshwater fish Vardar chub (*Squalius vardarensis*) in two mining impacted rivers (Zletovska and Kriva) and in less contaminated river (Bregalnica).

High contamination of the aquatic systems by the waste from active mines can seriously affect the quality of surface water, with the regard to metal/metalloid levels, and consequently influence the aquatic life. The exposure of Vardar chub to dietborne metal levels in three differently impacted rivers by Pb/Zn active mines Toranica and Zletovo, situated in the north-eastern Macedonia, was assessed by measuring 22 trace and 4 macroelements in sub-cellular gastrointestinal tissue fraction, cytosol, in order to determine the levels of bioavailable metal form. Cell cytosol was isolated from posterior part of gastrointestinal tissue by homogenisation at 4°C in 5 volumes of homogenising buffer (100 mM Tris/Base, pH=8.1) containing 1 mM DTT as a reducing agent and 0.5 mM PMSF and 0.006 mM leupeptin as inhibitors of proteolitic activity. The homogenates were centrifuged at 50000xg for 2 hours (4°C), resulting in water soluble cytosolic fraction (S50), which was 10 times diluted for determination of trace and 100 times for determination of macroelements. Metal measurements were carried out by high resolution inductively coupled plasma-mass spectrometer (HR ICP-MS). The sampling campaigns were performed in spring (May/June) and in autumn season (October), which coincided with chub spawning and post-spawning period.

Significantly higher gastrointestinal metal concentrations in the mining impacted Kriva River compared to the Bregalnica River in both seasons were found for Se, in the mining impacted Zletovska River for Li, Rb, Mo, Cs, Tl, U, Co, Ni, Cu, Sr, As, Ca, while the highest

V, Mn, Zn and Ba concentrations were found in the agriculturally contaminated Bregalnica River. Although dietborne metal levels usually do not directly reflect total dissolved metal concentrations in water, spatial variability of gastrointestinal cytosolic metal levels were in accordance with higher Ba and V total dissolved concentrations in the Bregalnica water and Co, Cs, Cu, Li, Ni, Rb, Sr, Tl and Ca in the Zletovska water. Moreover, seasonal differences of metal concentrations in water were followed by gastrointestinal metal variability, which was evident as higher Pb levels in Bregalnica in spring, higher Tl and Sr in Zletovska in autumn, and higher Cd and Pb levels in the Kriva River in spring. In addition, significant correlation of cytosolic Pb and chub total length and body mass ($r=0.5$, $p<0.01$) from the Kriva River in spring indicated size dependence of Pb accumulation in chub. Cytosolic gastrointestinal metal levels of Vardar chub evidently reflected metal concentrations in the river water and, therefore, reflected significant impact of the nearby mine on the Zletovska River and occasional contaminations of the Kriva River.

Cytosolic distribution of Cd, Co, Cu, Fe, Pb, V and Zn in liver, gills and intestine of Vardar chub (*Squalius vardarensis*) from mining impacted rivers in Macedonia

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Several metals, such as Co, Cu, Fe, and Zn, are essential micronutrients required for wide selection of physiological processes in living organisms. However, both essential metals and nonessential metals, such as Cd, Pb, and V, can be toxic, depending on their concentrations. As a response to increased metal exposure, binding of metals to various cytosolic biomolecules as ligands can be expected. Therefore, the assessment of potential metal toxicity requires the knowledge on metal speciation and distribution within cells. In this study, cytosolic distributions of seven metals associated with biomolecules of different molecular masses were analysed in the liver, gills and intestine of Vardar chub (*Squalius vardarensis*) caught in three differently contaminated rivers in north-eastern Macedonia in spring (May/June) of 2012. Comparison was made between metal distribution profiles obtained for two mining impacted rivers (Zletovska and Kriva), and one less contaminated river (Bregalnica), which was considered as a reference site.

Cytosolic fractions were isolated from liver, gills and intestine of three chub specimens, one from each river, by homogenisation and subsequent centrifugation at 50000xg. Separation of cytosols in the fractions, which contained proteins of different molecular masses, was carried out by size exclusion high performance liquid chromatography, using Superdex™ 200 10/300 GL column (molecular mass range 10-600 kDa). Metal concentrations in obtained fractions were determined by high resolution inductively coupled plasma mass spectrometry.

The distribution profiles of essential metals differed more between the organs than between the rivers, due to comparable cytosolic metal concentrations in organs of chub from all three rivers. Distribution profile of Co was characterized by several peaks. The first one occurred only in hepatic cytosol, within high molecular mass protein category (HMM; >100 kDa). The other peaks occurred within medium (MMM; 30-100 kDa), low (LMM; 10-30 kDa) and very low molecular mass protein category (VLMM; <10 kDa). The association of Co with VLMM protein fraction (maximum at 1.4 kDa) in all organs could be presumably explained as binding to known Co-containing compound, cobalamin (1.3 kDa). The elution times of Co peaks were almost identical in all tissues, but peak heights differed in accordance to cytosolic Co concentrations. Therefore, peaks in all hepatic cytosols and intestinal cytosol from the Bregalnica River were higher, narrower and sharper compared to gills and the other two intestinal samples. Observed increase of Co concentrations in hepatic and intestinal cytosols resulted with its increase predominantly within HMM and MMMregions.

Copper distribution profile was characterized by a peak with maximum at elution time of 30 minutes (12.2 kDa), which coincided with elution time of metallothioneins (MTs). That

peak was the highest in the liver, followed by intestine and then gills, which corresponded well with Cu cytosolic concentrations in those organs.

Iron distribution profile in hepatic and intestinal tissues was characterized by two clear peaks. The maximum of the first peak corresponded to proteins of molecular mass around 400 kDa, such as ferritin (450 kDa), which serves as Fe storage protein, indicating probable participation of liver and intestine in Fe storage. The second Fe-peak appeared in all three tissues and covered the range of molecular masses from 10-60 kDa, which could possibly involve binding to known Fe-containing proteins, such as catalase (60 kDa) or transport protein myoglobin (17 kDa). Unlike other essential metals, the binding of Fe to ferritin differed between rivers, and was especially pronounced in liver in mining impacted rivers (Zletovska and Kriva), whereas in the gills the highest MMM peak was observed in the Kriva River, indicating higher Fe transport through gills, but not its storage.

Interesting finding for Zn were different distribution profiles in three organs, not just regarding the peak heights, but also their elution times. In liver, three peaks of the following ranges of molecular masses were evident: 1) >300kDa, 2) 237-9.3 kDa, 3) 20.9-7.11 kDa. The last one, with elution time at 30 minutes, was the highest and coincided with MT elution time. In gills, the most of Zn was eluted in HMM and MMM regions, whereas MT peak was not observed. Contrary, in the intestine the highest Zn peak was found at elution time of 38 minutes (1.8 kDa) indicating Zn binding to small compounds.

Distribution profiles of nonessential metals varied both between the organs and between the rivers, due to different exposure levels in the river water and their consequent accumulation in chub organs. In all organs, the most prominent Cd peak was found in the LMM protein region, with maximum at elution time of MTs (30 minutes, 12.2 kDa), same as previously described for Cu and hepatic Zn. The height of this peak corresponded well with cytosolic Cd concentrations, and was the highest in the liver in the Zletovska and Kriva River, followed by intestine and gills in the Zletovska River. In the gill cytosol, a small portion of cytosolic Cd was also found associated with HMM fractions, whereas in the intestinal cytosols, LMM peak did not only increased in height with increasing cytosolic Cd concentrations, but also expanded its width in direction of lower molecular masses.

Distribution profiles of Pb from the Kriva River had the best resolved peaks, due to the highest cytosolic Pb concentrations in all three organs of chub from that river. The highest Pb concentrations and the highest peaks were found in the intestine, than liver, and they were the lowest in the gills. In liver, two sharp peaks were observed within HMM and MMM regions, with maxima at elution time of 23 minutes (105.7kDa), and 30 minutes, respectively. The second peak coincided with elution time of MTs. In gills, several peaks in HMM and MMM regions were found, but were rather small. In the intestine, Pb was mainly eluted in MMM protein region with clear, sharp peak with maximum at 27 minutes (35.9 kDa).

Distribution profiles of V could be defined only for liver and intestine of chub from the Bregalnica River, because in all the other cases V concentrations were too low. In liver, sharp, narrow peak was obtained in HMM region (~500 kDa), whereas wide and much lower peak was observed in MMM region (181.3-12.2 kDa). A similar pattern was found in the intestine, but the peaks were much lower, due to lower cytosolic V concentrations.

Presented cytosolic metal distribution profiles for three organs of Vardar chub, living under different conditions of metal exposure,were the first step towards recognition of specific metal-binding proteins, which could be further on used as biomarkers of metal pollution.

Molecular characterisation of the metazoan parasites of Vardar chub (*Squalius vardarensis*) from three rivers in north eastern Macedonia

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Fish parasites with a complex life cycle, including intermediate and paratenic hosts, can be used as indicators of aquatic environmental stress. Their identification is however often difficult, due to the high level of intraspecific variability. The aim of the present study was to identify metazoan parasites found in Vardar chub (*Squalius vardarensis*) from three rivers in northeastern Macedonia under different minning impact.

Parasite specimens were collected from the dissected Vardar chub (*S. vardarensis*) sampled in spring and autumn from three rivers in northeastern Macedonia: Zletovska River and Kriva River under mining activity impact and Bregalnica River which is non-impacted. Abdominal cavity cysts were observed in the fish from Bregalnica (spring and autumn) and Kriva River (spring) and cysts from two fish specimens were used for the identification. Intestinal parasites were observed in the fish from Bregalnica (spring and autumn) and Zletovska River (spring) and specimens from four different fish were used for identification. Morphological analysis of the parasitic cysts content was done by light microscope, while molecular identification was performed by sequence analysis of 18S rRNA region. Molecular identification of intestinal parasites was done by sequence analyses of three different DNA regions: 18S rRNA, ITS and COI gene. For the phylogenetic analysis of intestinal parasites based on partial COI sequence data, maximum likelihood as well as maximum parsimony method was applied by MEGA 6 software.

Members of two different parasitic phyla were determined from the examined Vardar chub: Myxozoa (Cnidaria) in the abdominal cavity and Acanthocephala in the intestine. Morphological analysis of myxozoan cysts indicated presence of *Myxobolus* sp., while molecular analysis based on 18S rRNA analysis confirmed this genus. Molecular analysis of acanthocephalans based on the 18S rRNA and ITS region confirmed two different species:

Pomphorhynchus laevis and *Acanthocephalus* sp. most closely related to the *A. anguillae* (99.8% identity to the sequence from the GenBank). Phylogenetic analysis of *P. laevis* based on COI sequence data indicated separated clustering of Macedonian specimens in relation to the other European specimens available from the GenBank.

Identification of *Myxobolus* sp. in Vardar chub is important as members of this genus are potentially dangerous to their fish host. Determination of acanthocephalans is important not just for their further phylogeographic studies, but also for the water contamination studies, as these parasites were suggested as a sensitive biological indicator of metal bioavailability in the river water.

Acanthocephalans, fish intestinal parasites, as bioindicators of metal exposure in rivers impacted by mining waste

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In the past decades, the interrelation between parasites and contaminants has gained increasing interest, especially in aquatic ecotoxicology. Fish intestinal parasites, acanthocephalans, were reported to effectively accumulate metals in concentrations that are orders of magnitude higher than those in the commonly used indicator organisms like fishes, bivalves or crustaceans. Acanthocephalans lack a gastrointestinal system and consequently, depend on host micronutrients and highly absorb essential metals from the fish intestine, as elements of physiological importance. Due to their competition for essential elements, non-essential elements are also absorbed by the parasites, even more effectively than essential metals. Accordingly, attempts have been made to utilize acanthocephalans as biological indicators of metal exposure in environmental risk assessment studies.

Application of acanthocephalans as bioindicators in metal exposure assessment of mining impacted rivers in the north-eastern Macedonia involved parasites hosted by Vardar chub (*Squalius vardarensis*) dwelling in the Zletovska and Kriva River impacted by Pb/Zn active mines, and in the less contaminated Bregalnica River. Sample preparation involved acid digestion of acanthocephalans in a drying oven at 85°C for 3.5 hours using 1.5 ml of concentrated HNO_3 and 0.5 ml H_2O_2, followed by measurement of 24 trace and 4 macroelements by high resolution inductively coupled plasma-mass spectrometer (HR ICP-MS). For the purposes of calculation of bioconcentration factors (BCFs), cytosolic gastrointestinal metal levels were multiplied with tissue dilution (6 fold) and BCFs were determined as the ratio of the element concentration in the parasites to that in host gastrointestinal tissue ($BCF = C_{[parasite]}/C_{[host\ intestine]}$).

Two acanthocephalan species, *Pomphorhynchus laevis* and *Acanthocephalus sp.,* were found in Vardar chub, but only from Bregalnica River and not from two mining affected rivers. Such occurrence of acanthocephalans only in moderately contaminated river disables their application as bioindicators of environmental pollution in mining impacted rivers. It follows that absence of acanthocephalans might be considered as a first sign of highly polluted aquatic environment.

In the Bregalnica River, prevalence in both sampling periods, spring (May/June) and autumn (October), was comparable, i.e. around 70% of infected chub specimens. In spring, all measured metal concentrations were higher in *P.laevis* than *Acanthocephalus sp.*, statistically significantly for 14 trace elements, while in autumn only for 7 trace elements. The

data on BCF confirmed higher metal accumulation in *P. laevis* than *Acanthocephalus sp.*, which was evident in both seasons for all measured metals, except for Sr, Na, Mg and Ca. Estimation of metal accumulation based on BCF indicated in both acanthocephalan species and in both seasons 10-45 times higher Al, Pb, Sr, Tl, Li, and Ba, 3-20 times higher Cr, Ti, Ca, Cu, and Mn, and 1-6 times higher Cd, Ni, V, U, Fe, Mg, As, and Na concentrations in acanthocephalans than in fish gastrointestinal tissue. In contrast, Se, Rb, Mo, Cs, Co, Zn and K were not accumulated by parasites. Although involvement of acanthocephalans in metal exposure assessment gives valuable data, which might serve as a sensitive bioindicator of bioavailable metal levels in the freshwater ecosystem, acanthocephalan-chub system unfortunately cannot be applied as an indicator of acute or chronic metal exposure in mining impacted rivers.

Evaluation of possible protective role of acanthocephalans against metal accumulation in chub, regarding high metal uptake capacity of acanthocephalans, pointed to significantly higher Rb, Cs, Tl, Pb, Mn and Cu levels in uninfected compared to chub infected with acanthocephalans in spring, while in autumn only for Cd and Tl. It follows that acanthocephalans can reduce levels of some metals in the gastrointestinal tissue of their host, related to the uptake of bile-bound metals by parasites and reduction of metal levels available for reabsorption by the host. Our data suggest that in metal exposure assessment acanthocephalans should be considered as confounding factors, due to their ability to modify metal concentrations in fish gastrointestinal tissue.

Metallothionein and total protein concentrations in gills and liver of Vardar chub (*Squalius vardarensis*) as biomarkers of water contamination in three rivers in Macedonia

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Metallothionein (MT) and total cytosolic protein (TP) concentrations were determined as biomarkers of water contamination in gill and hepatic cytosols of Vardar chub (*Squalius vardarensis*) caught in three rivers in north-eastern Macedonia, one agriculturally impacted (Bregalnica) and two mining impacted (Zletovska and Kriva), in spring (May/June) of 2012. MT concentrations were determined in heat-treated (10 min at 85˚C) cytosols by differential pulse voltammetry on 797 VA Computrace (Metrohm). TP concentrations were determined on the spectrophotometer/fluorometer Infinite M200 (Tecan) at 750 nm wavelength using the method according to Lowry. Comparison of the results obtained for two organs indicated generally higher concentrations of analyzed proteins in the liver. However, for TPs differences were statistically significant only at the Bregalnica River ($p<0.01$), and for MTs at Bregalnica and Zletovska River ($p<0.001$).

The analysis of spatial variability indicated comparable concentrations of TPs in chub gills from Bregalnica, Zletovska and Kriva rivers (20.2, 20.8, and 21.3 mg mL^{-1}, respectively), whereas in the liver statistically significant ($p<0.001$) concentration decrease was observed at the Zletovska River (19.3 mg mL^{-1}) compared to other two rivers (Bregalnica: 22.2 mg mL^{-1}; Kriva: 21.8 mg mL^{-1}). TPs are regarded as nonspecific biomarkers of pollution, and their synthesis could be induced or supressed due to exposure to pollutants. Therefore, TP concentration decrease in chub liver at the Zletovska River was possibly a consequence of low river water quality, characterized by increased concentrations of number of trace elements, sulphates, chlorides, and other changes of physico-chemical water properties (Ramani et al., 2014).

Contrary to TPs, MT concentrations were comparable in chub liver from all three rivers (317.8, 312.0, and 332.8 µg mL^{-1}, respectively), and differed in the gills, with statistically significantly higher concentrations in the gills of chub from the Kriva River (310.8 µg mL^{-1}) compared to other two rivers (Bregalnica: 215.1 µg mL^{-1}; Zletovska: 202.5 µg mL^{-1}). Since MTs represent inducible biomarkers of metal exposure, the association of their concentrations with three known MT inducers, Cd, Zn and Cu (measured in gill and hepatic cytosols by high resolution inductively coupled plasma mass spectrometry) was also analyzed. In the gills, in all three rivers, positive correlations were obtained with Cd (r ranged from 0.264 to 0.777) and Zn (r ranged from 0.690 to 0.873), whereas in the liver the association between MTs and metal concentrations was much weaker, and referred only to

essential elements Zn and Cu. Based on these results, apparently gill MTs were more sensitive to changes in metal exposure than hepatic MTs, which were maintained constant despite increased exposure to Cd (Ramani et al., 2014), as well as increased hepatic Cd concentrations (Bregalnica: 4.0 ng mL^{-1}; Zletovska: 34.4 ng mL^{-1}; Kriva: 34.5 ng mL^{-1}). However, gill MTs were increased only at the Kriva River, whereas Cd concentrations in the gills were increased in both Zletovska and Kriva rivers (19.3 and 17.8 ng mL^{-1}, respectively), compared to the Bregalnica River (0.5 ng mL^{-1}). Therefore, it could be hypothesized that possibly accumulation of other metals, in addition to Cd, in chub gills could have caused increase of MT concentrations at the Kriva River. For example, cytosolic Pb concentrations were much higher in the gills of chub from the Kriva River (34.0 ng mL^{-1}), compared to both Bregalnica and Zletovska rivers (3.7 and 8.2 ng mL^{-1}, respectively). In addition, some other factor could have influenced MT concentrations in the chub gills from the Kriva River, such as a month later sampling campaign (in June) compared to the other two rivers (in May).

Analysis of association with biometric parameters (total chub mass, condition index (CI), and hepatosomatic index (HSI)), indicated only week negative correlations of TPs in liver with chub mass (r ranged from -0.229 to -0.440), CI (r ranged from -0.086 to -0.404) and HSI (r ranged from -0.237 to -0.494), in some cases even statistically significant. Since TPs were the lowest in the liver of chub from the Zletovska River, where also the smallest chub specimens were caught, this association cannot explain the differences between sites. On the other hand, MT concentrations in gills showed positive correlation only with chub mass (r ranged from 0.137 to 0.678), statistically significant at Zletovska River. The chub specimens from the Kriva River were indeed bigger than from the Zletovska River, but of comparable size as from the Bregalnica River, and thus, the fish size solely could not be the cause of gill MT increase at the Kriva River. TPs in gills and MTs in liver, which did not exhibit spatial variability, also did not exhibit association with biometric parameters.

Analysis of differences between chub sexes, indicated higher concentrations of TPs in both organs in males than females, whereas MTs showed opposite trend with higher concentrations in females. For both parameters, differences were more pronounced in the gills, and in some cases were even statistically significant. However, at the Kriva River, characterized by the highest gill MTs, sampled chub comprised only 33% of females, which was much lower percentage compared to other two rivers (Bregalnica: 47%; Zletovska 60%), and thus chub sex could not be the reason for observed spatial differences in gill MTs.

In a conclusion, in spring period, TP concentrations in the chub liver possibly could be used as a nonspecific indicator of water pollution, whereas MT concentrations in the chub gills possibly could be applied as indicator of increased metal accumulation in that organ. However, further investigation is needed to discern all the factors and metals which could influence MT concentrations.

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Bacterial community of Vardar chub (*Squalius vardarensis*): Preliminary results

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Anthropogenic activities increase discharge of different pollutants in the aquatic environment which cause deterioration of the environment. Mining activities release in the environment different inorganic species and metals, which has negative effects on the water quality, aquatic organisms and humans. Aquatic organisms are affected by metal concentrations not only in their surrounding water but also in their food. Microorganisms are also affected by changes in the environment and can also provide biological responses to the environmental disturbances. Microorganisms associated with aquatic organisms can thus be used to monitor the impact of mining activities on the aquatic environment because of the ubiquity of microorganisms in aquatic environments.

The objectives of the present study were (i) to determine abundance of fish bacterial population and species diversity of bacterial community of Vardar chub (*Squalius vardarensis*) from three Macedonian rivers, (ii) to compare obtained bacterial communities, and (iii) to isolate and characterize the isolates using different methods of bacterial identification (MALDI and VITEK 2 Systems), and (iv) to evaluate whether they provide a good assessment of the potential detrimental effects of pollution on aquatic organisms. For that purpose, we sampled 68 fish from rivers: Zletovska (12), Bregalnica (30) and Kriva (26), and obtained samples for bacteriological analysis from the gills and internal organs (spleen, liver and kidney).

According to bacterial analysis Kriva and Bregalnica Rivers are aquatic environments under high anthropogenic influence. In contrast, Zletovska River constitutes a less microbial polluted ecosystem.

Bacterial analyses were negative for internal organs, whereas samples from the gills were only positive. Bacterial analysis of gills indicated that size of the bacterial population were higher in fish from the Bregalnica River (10.8 CFU/fish) in relation to Kriva (7.2 CFU/fish) and Zletovska River (7 CFU/fish).

Contribution of bacterial genera to the bacterial population assemblage was higher in Zletovska River, with three genera: *Pseudomonas* (52%), *Aeromonas* (24%) and

Acinetobacter (24%), more than in relation to Kriva River, two genera: *Pseudomonas* and *Morganella*, as well as to Bregalnica River with one genus *Acinetobacter*.

This study reveals the influence of microbial water quality on diversity of fish bacterial populations in the rivers. In general, dominant genera *Pseudomonas* and *Acinetobacter*, which are known as genera that can remove metals and have bioaccumulation potentials for metals from polluted aquatic environments, can provide a good assessment for the determination of the potential effects of metal pollution on the aquatic organisms.

Hematological assessment of Vardar chub (*Squalius vardarensis*) from three rivers in the north-eastern Macedonia

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Pollution of surface water presents the major ecological problem in developed industrial world. Various human activities lead to the deterioration of water quality and mining presents one of those impacts. This need for resources has been proven to have extremely negative influence on all biological as well as physico-chemical characteristics of water. Animals that inhabit waters under severe mining activity diversely respond to environmental changes. The changes of hematological parameters in fish can present the consequences of mining influence on the river water.

The objective of this study was to determine how mining activity influence hematological parameters in Vardar chub (*Squalius vardarensis*). Samples from three rivers in the north-eastern Macedonia were obtained: two under mining influence, Zletovska and Kriva River and Bregalnica River as a non-impacted one.

Comparison of the values of hematological parameters (hematocrit (HCT), total serum protein (TSP), refractive index (RI) and serum specific gravity (SG)) of Vardar chub captured in three differently polluted rivers showed significant differences. All four parameters had the lowest values in Zletovska River (HCT 30%, TSP 3.6 g/100 mL, RI 1345, SG 1028), which is characterized by the most prominent water contamination with metals. Hematocrit was also reduced in Kriva River (HCT 35%), where metal contamination and poor microbiological quality of the water was found. Hematocrit has the highest value in Bregalnica River (HCT 39.5%), which was selected for sampling as a reference station. Serum proteins, refractive index and specific gravity were elevated in Vardar chub from the Kriva River (TSP 6.75 g/100 mL, RI 1348, SG 1045).

Except of the degree of water pollution with metals in three investigated rivers, there were physiological and health differences between examined Vardar chubs. Consequently correlation of hematological parameters and several physiological parameters was analyzed (total weight, condition factor and sex). None of hematological parameters showed a clear correlation with the weight and condition factor. In all three investigated rivers hematocrit

values were observed to be somewhat higher in males than in females (about 5% higher median), but the differences were not statistically significant. The other three parameters had higher values in males only in Kriva River, and the differences were statistically significant only for serum proteins ($p<0.05$).

The association between hematological parameters and parasitic infections of Vardar chub was also investigated. Intestinal parasites were found only in Vardar chub from Bregalnica River and comparison between infected (70%) and uninfected (30%) specimens was made. Although the differences were not significant, there was an indication of higher values of serum proteins, the refractive index and specific gravity in uninfected specimens, while hematocrit showed no association with parasitic infection.

Although connections between hematological parameters and some physiological and health indicators of Vardar chub were observed, the differences between the investigated rivers were much more pronounced and suggested the possibility of using hematological parameters as indicators of pollution of river water.

Spatial and seasonal variability of histopathological alterations on the gills of Vardar chub (*Squalius vardarensis*) from mining impacted rivers in the north-eastern Macedonia

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Histopathological alterations on the gills of Vardar chub (*Squalius vardarensis*) were studied in three rivers in Pb/Zn mining region in north-eastern Macedonia. The Zletovska and Kriva River are directly impacted by active Pb/Zn mines Zletovo and Toranica, respectively, whereas the Bregalnica River is moderately contaminated mainly by agricultural waste, and considered in this study as nonimpacted site. The analyses were performed for two seasons of 2012, spring (May/June) and autumn (October), and the gills of 6-10 chub of similar size and age were examined for each river in each season. Tissue samples were fixed in 4% neutral formaldehyde, and processed using a standard histological technique: dehydration in the ethanol series, embedding in paraffin, and serially sectioning at 2 μm. Sections were stained with haematoxylin and eosin. In all three rivers, the circulatory disturbances, regressive and progressive changes were found, but their severity differed. The signs of inflammation and neoplasm were not observed. The circulatory disturbances and regressive changes had the highest scores according to Bernet *et al*. (1999) at the Zletovska River, progressive changes were more pronounced at the Kriva River, whereas all changes were the mildest at Bregalnica. If importance of the changes was considered, the alterations described with importance factors 1-2 had higher scores at Zletovska and Kriva River than Bregalnica, but changes described with importance factor 3, e.g. necrosis, were especially pronounced only at the Zletovska River.

The characteristic alteration on the gills of the chub from the Bregalnica River was hyperplasia and erythrocyte congestion throughout the entire lamella (aneurism). This progressive changes were observed in both seasons, with higher score in autumn. More severe lesions were observed in the gills of the chub from two mining impacted rivers. Typical lesions on the Zletovska River included thinning and lifting of epithelial cells, congestion and oedema, fusion of several secondary lamellae usually on the tips of secondary lamellae, acute necrosis of epithelium and calcified cartilage, as well as oedema and deformations of the lamellar cartilaginous base. In some areas the secondary lamellae had atrophied. On the Kriva River, moderate to marked gill damage was found, such as degeneration and necrosis, exfoliation and delaminating of respiratory epithelium. Extreme

telangiectasia was also found. In both seasons hyperplasia and hypertrophy of mucous and epithelial cells appeared severe.

The comparison between seasons indicated higher intensity of progressive changes in all three rivers in autumn, when water level was very low, and consequently, water contamination more pronounced due to preconcentration effect. All these findings were in agreement with observed water contamination by mining waste, which was continuously the worst in the Zletovska River, occassionaly at Kriva River, whereas the best water quality was found in the Bregalnica River (Ramani et al., 2014). Therefore, the histopathological alterations on the chub gills could be used as a reliable, although nonspecific, indicator of water quality.

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Toxicopathic changes in Vardar chub (*Squalius vardarensis*) in rivers under the impact of mining activities

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The natural environment of many river ecosystems, especially in the north eastern part of Macedonia, is more and more polluted with heavy metals, most of which were released into the environment by the active mines in this region. It could be presumed with high certainty that long-term exposure to high levels of dissolved metals will result in increased levels of metals bioaccumulated in the tissues of aquatic organisms, and consequently cause various sub-toxic and toxic effects. In monitoring studies on long-term metal pollution, fish proved to be the most appropriate indicator organisms due to their long life span and top position in the aquatic food chain, as well as potential risk of human consumption of "metal-loaded" fish.

Histopathology is one approach used to monitor toxicopathic tissue changes in fish. Several liver lesions are reliable biomarkers in assessing anthropogenic stress, being consistently associated with contamination exposure including metal contamination. Facing the above, our aim was to assess the current metal pollution impact on the health status of the local ichthyofauna, namely by studying toxicopathic changes in gonad and liver tissue.

In this vein, we studied the gonads and liver of 158 chubs collected from three rivers: the Bregalnica River, as the site nonimpacted by mines and contaminated mainly with agricultural and domestic waste waters, as well as the Kriva River and the Zletovska River, two rivers suspected to be under high degree of metal pollution from active Pb/Zn mines Toranica and Zletovo, respectively. Fish were collected in two seasons, spring and autumn of 2012. Gonad and liver tissues were routinely dehydrated through ascending series of ethanol, cleared in xylene, infiltrated, and embedded in paraffin. The obtained 5 μm thin sections were stained with hematoxylin and eosin.

Histological alterations in gonad tissues, in both males and females, were not detected in examined chub. Contrary, if all sites were considered, the spectrum of observed histological lesions in the liver (and prevalence of affected fish) varied from non-specific minor degenerative conditions, such as lymphocyte infiltration (19%), focal necrosis (12%), parasitic granuloma (2%), lipidosis (3%) to extensive and/or more severe changes such as: cholangiofibrosis (19%), bile duct proliferation (21%), megalocytosis (25%), light-dark hepatocytes (6%) and hepatocytes regeneration (3%). Although prevalence of light-dark hepatocytes and hepatocytes regeneration was low, these lesions, as well as megalocytosis, were previously established as histopathological biomarkers of contaminant exposure in both field and laboratory studies, in a variety of species. In general, if all lesions were considered,

no differences in prevalence were found between locations and it varied from 45% in Bregalnica, 43% in the Kriva River and 41% in the Zletovska River. Significant differences were found only for lymphocyte infiltration between Bregalnica (13%) and the Kriva River (29%) and for hepatocytes regeneration between Bregalnica (0%) and the Zletovska River (4%).

The results of histopathological investigation showed clear effects of water contamination on fish health in all three rivers, and not only in two mining impacted rivers. Some lesions observed in this study suggested environmental contamination as the most likely etiology. Although only a long-term monitor programme can provide definitive answers, the obtained data indicated that more research efforts should be devoted to the environmental conditions and metal contamination in the rivers in Macedonia, especially on their effects on local ichtyofauna health.

Potential and challenges of NanoSIMS for element imaging in biological cells

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Probing the distribution of macronutrient and trace elements at the sub-cellular level is one of the major challenges in cell biology. In order to investigate biosorption and bioaccumulation processes of trace elements at the cellular level, element specific imaging techniques have a great potential. Highest spatial resolution down to 30-50 nm combined with high sensitivity can be achieved with nano Secondary Ion Mass Spectrometry (NanoSIMS) for imaging of chemical elements in cells. NanoSIMS imaging is perfectly suited to measure, visualize and quantify the distribution of almost all elements in the periodic table (from hydrogen to uranium) and their stable isotopes (allowing the design of tracer experiments) at the sub-cellular level in biological material. The parallel detection of 7 masses for an ion source configuration is possible. For some elements it can detect concentrations at the low ppm level. Element specific imaging techniques have been successfully applied to biological cells; however, a difficult challenge is sample preparation because physiological important metal ions are often highly diffusible. NanoSIMS operates under high vacuum, thus water has to be removed from the cells and the cellular structure has to be stabilized by embedding in a resin. Therefore, the critical point is the preservation of the *in vivo* distribution of elements in this way that the analysis reveals the chemical and structural integrity of the living cell.

This talk highlights the potential and challenges of NanoSIMS for element imaging in biological cells including sample preparation techniques. This will be illustrated by examples from the literature and data from our laboratory. A prototype of a new O^- ion source has been applied providing high sensitivity and high spatial resolution (50 nm and less). This allows for the first time a parallel sensitive detection of major and trace elements such as Na, Ca, Cu, Fe and Cd at subcellular level. We are working on Cd toxicity in green algae using *Chlamydomonas reinhardtii* as model organism. These data are completed by total metal analysis (ICP MS) in order to understand adaptive metal stress responses to environmental changes and metal tolerance processes.

Fatty acid composition in some river fish species in Republic of Macedonia

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Fatty acids play an important role in human health, especially omega 3 fatty acids such as eicosapentanoic fatty acid (EPA) and docosahexanoic fatty acid (DHA). Presence of omega 3 fatty acid is very important in human diet because of their effect on reduction of the incidence of coronary heart disease, their positive effect on brain and nervous tissue development in infants etc. The human body is not able to synthesize EPA and DHA, so it is very important to recompense them through food. Beside in nuts, olive oil, chia, flaxseed, sunflower seed, broccoli etc., omega 3 fatty acid can be found in fish. The aim of our study is determination of fatty acid composition in some species of river fish.

Three species of river fish were included in our study: *Barbus barbus* (11 samples), *Chondrosotoma nasus* (10 samples) and *Salmo truta fario* (11 samples).The fish was taken from river Vardar in a period of five months (August – December, 2013). Extraction of the lipids and methylation of the samples were performed according to AOAC Official Method 996.06. Determination of fatty acids were carried out on a GC-FID, (GC Agilent Technologies 7890 GC System) with HP88 column (J&W 112 -8867; 250°C; 60m x 250mm x 0,2 mm, Agilent, USA). The injector and detector were kept at 250ºC and 300ºC, respectively. The gas flows which we used were: 1.4 mL/min carrier gas (He), 23 mL/min make up gas (N_2), 30 mL/min H_2 and 400 mL/min flame synthetic air. The split used was 200:1. Injections of 1 µL sample were carried out in duplicate.

The most common fatty acid in the fish was monounsaturated fatty acid: 43.07% in *Chondrosotoma nasus*, 37.80% in *Barbus barbus* and 42.86% in *Salmo truta fario*. Moreover, the content of polyunsaturated fatty acid is very good. *Chondrosotoma nasus* contains 30.84%, *Barbus barbus* 27.93% and *Salmo truta fario* contains 22.85%. The highest content of DHA was detected in *Barbus barbus* (10.07%), while minimum content of these fatty acids was detected in *Salmo truta fario* (5.49%). The content of the EPA was from 0.2 to 0.44%.

The results of the present study indicate that species of river fish are excellent dietary sources of highly unsaturated and polyunsaturated fatty acids as well, especially the omega-3 fatty acids.

Detection of methyltestosterone with ELISA method in fish

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Synthetic hormone like substances anabolic steroids (trenbolone, 19 nortestosterone, methyltestosterone etc.) and stilbenes (diethylstilbestrol, hexestrol etc.) are often used for therapy in farm animals (Shaoa et al., 2005). Moreover, in livestock farming they have been used as growth promoters (Regal et al., 2010). In *Cyprinus carpio*, methyltestosterone induces faster growth in three different ways: activation of secretion of other androgenic anabolic hormones, increased food conversion and direct effect of methyltestosterone on the gene expression in the muscle cells (Felix, 1989). Due to potential carcinogenic effect and human health risk of their residues hormonal growth promoter are prohibited in the European Union since 1988 (Shaoa et al., 2005; Regal et al., 2010; Uzunov et al., 2013). The aim of this study is detection of methyltestosterone in fish with ELISA method.

Forty five fish samples, *Salmo truta fario,* which are included in Macedonian monitoring plan, were analysed for detection of methyltestosterone in fish. Fish samples were taken from the Macedonian fish farm. Analyses were performed with ELISA kit (R-Biopharm, Germany, Art. no. R3603). Prior the analyses of the fish samples, ELISA method were validated according to European Commission Decision 2002/657/EC. During the validation procedure linearity, limit of detection (LOD), detection capability (CCβ), precision and recovery were investigated.

Validation procedure showed that the ELISA kit for detection of methyltestosterone has a good linearity (r^2 = 0.9927), good precision (coefficient of variation was from 0.2 – 11.5%) and good recovery from 82.4 to 97.4%. The limit of detection of the method was 0.14 µg/kg and the CCβ was found to be 0.56 µg/kg. Determined CCβ was less than minimum required performance limit (MRPL) for the methyltestosterone (1.0 µg/kg). Because of these performances the method is applicable as a screening method. All fish samples were analysed in period of two years and the results were lower than the established CCβ.

Fish and fish products play an important role in human nutrition, for this reason they should not contain any factors or substances harmful for human health. In our study fish samples did not contain residue of methyltestosterone. In the future, detection of anabolic steroids in the fish will be continued in order to prevent abuses of these substances.

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