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THE ASSESSEMENT OF METAL AVAILABILITY AND THE EFFECTS ON FERAL FISH IN THE RIVERS UNDER THE IMPACT OF MINING ACTIVITIES

DOCTORAL THESIS

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INFORMATION ABOUT THE SUPERVISOR

Dr. Zrinka Dragun was born on June 6^{th}, 1971, in Vinkovci, where she completed her elementary and secondary education in mathematics and computer sciences. She graduated at the Faculty of Pharmacy and Biochemistry at the University of Zagreb in 1996 and earned her B.Sc. in medical biochemistry. She earned her master's degree in 2001 at the Department of Biology, Faculty of Science, University of Zagreb (branch of toxicology), and a doctorate degree in 2006 at the same faculty. She is employed as a senior research associate at the Ruđer Bošković Institute in Zagreb, where she has been working since 2002. In 2018, she has earned the title of scientific advisor in the field of interdisciplinary natural sciences.

She is the author and co-author of numerous scientific and professional papers and communications at scientific conferences, as well as the leader of several scientific projects and supervisor of student theses.

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ABSTRACT

Mine waste is recognized as one of the most serious threats to freshwater ecosystems, and it still represents one of the greatest environmental concerns in North Macedonia. Contamination of freshwater ecosystems with mining waste can result with high concentrations of toxic metals in the water, and their bioaccumulation in the organs of aquatic organisms, especially after long-term exposures, consequently causing various sub-toxic and toxic effects in those organisms. In this study, the impact of active Pb/Zn mines Zletovo and Toranica on the water quality of three rivers (Bregalnica, Zletovska, and Kriva) in the northeastern part of North Macedonia was assessed during spring and autumn 2012, by analysis in the surface water and in two target organs (gills and liver) of selected aquatic bioindicator organism (Vardar chub, *Squalius vardarensis*). The aim of the study was to deepen the understanding of the mining influence on freshwater ecosystems based on the assessment of metal exposure, by determining dissolved metal concentrations in the surface water, on the assessment of metal bioaccumulation, by determining metal concentrations in the cytosols of liver and gills of Vardar chub, and the assessment of final effects of water contamination on Vardar chub, by defining histopathological changes in the fish liver and gonads, as well as fish general health status. The Bregalnica River, near Shtip, was characterized mainly by weak contamination with As, Ba, Fe, Mo, Ti, U, V, nitrates, and phosphates, which all together indicated the impact of agricultural activities; however, an impact of the mines was not observed. Contrary, in both Zletovska and Kriva rivers a clear impact of Pb/Zn mines on water quality was observed. In the Zletovska River water, increased concentrations of Cd, Co, Cs, Cu, Li, Mn, Ni, Pb, Rb, Sn, Sr, Tl, Zn, sulphates, and chlorides were found, and they were especially high in autumn (e.g., Cd 2.0 µg L^{-1}; Mn 2.5 mg L^{-1}; Zn 1.5 mg L^{-1}) during the extremely low water level, confirming the severe impact of the mine waste on the water quality of the Zletovska River. In the Kriva River, increased Cd (0.270 µg L^{-1}) and Pb (1.85 µg L^{-1}) concentrations, indicating the impact of the mining waste, were found only in spring, possibly due to sediment resuspension during greater water discharge. Contrary, in autumn, high increase of nutrients was recorded in the surface water of the Kriva River, revealing the additional impact of the waste from cultivated land. For the assessment of metal bioaccumulation in the organs of Vardar chub, 19 elements were measured in the cytosolic fractions of the gills and liver, to obtain information on metabolically available metal species. The following ranges of cytosolic concentrations of few highly toxic elements were measured in the Vardar chub gills from all three rivers (in µg L^{-1}): Cd, 0.24-59.2; Cs, 0.39-24.4; Tl, 0.01-1.00; and Pb, 0.65-87.2. Their ranges measured in the liver (in µg L^{-1}) were the following: Cd, 1.18-184; Cs, 0.25-25.4; Tl, 0.02-5.80; and Pb, 0.70-61.1. The majority of essential elements (Na, K, Ca, Mg, Co, Cu, Fe, Mn, Mo, and Zn) did not reflect the exposure levels, indicating the strict physiological regulation of their concentrations. The only exception was essential element Se, which was present in the highest concentrations in both seasons and in both organs at the Kriva River, probably due to increased exposure in the river water. With the exception of Ba, all studied nonessential elements (Cd, Cs, Pb, Rb, Sr, Tl, and V) reflected the level of exposure in the river water. Significantly increased gill and hepatic concentrations of Cs, Rb, Sr, and Tl were always detected in Vardar chub from the Zletovska River compared to the other two rivers, of Cd and Pb in the Zletovska and Kriva River compared to Bregalnica, depending on the season, and of V in the Bregalnica River compared to Zletovska and Kriva rivers in both seasons.

For the assessment of the health of Vardar chub, general indicators of exposure to environmental stressors were used, such as condition factor, organo-somatic indices and external/internal macroscopic lesions, as well as histopathological assessment of liver and gonads. In both seasons, the smallest fish, the lowest Fulton condition indices, the lowest hepatosomatic indices and the lowest gill masses were observed for Vardar chub at the Zletovska River, most likely associated to severe metal contamination of surface water. Total prevalence of external/internal lesions was similar in all three rivers and relatively low, around 20%. However, several external/internal lesions were more pronounced in severely metal contaminated Zletovska River. The spectrum of histological lesions observed in Vardar chub liver varied from non-specific minor degenerative conditions, such as lymphocyte infiltration, fibrosis, parasites, granulomas and lipidosis, to extensive and more severe changes such as bile duct proliferation, necrosis, megalocytosis, light-dark hepatocytes and hepatocytes regeneration. Prevalence of hepatic lesions was higher in the mining impacted rivers (in Kriva, 70%; in Zletovska, 59%) compared to agriculturally impacted Bregalnica River (38%). Histological assessment of gonads revealed good reproductive health in all three rivers, indicating higher tolerance of gonads than liver to contaminant exposure. Although the results of histopathological assessment cannot be directly associated to a single contaminant, some lesions observed in this study were probably a consequence of environmental metal contamination. This study has clearly demonstrated detrimental effect that mining pollution has on water quality and health of native freshwater fish. Such information is essential in a process of creating water management plans, with an aim to protect, as well as to improve, quality of freshwater ecosystems worldwide, and especially in areas affected by active mining.

Keywords: agricultural activities, condition factor, cytosolic fraction, essential metals, mine waste, fish, gills, gonads, histopatology, liver, bioaccumulation, nonessential metals, organo-somatic indices, rivers, Vardar chub

CONTENTS

1. INTRODUCTION

The aquatic environment is polluted with a tremendous amount of chemicals derived from human activities (Skouras et al., 2003). In the 20th century, thousands of organic trace pollutants have been produced and in part released into the environment (van der Ost et al., 2003). Additionally, mining activity presents an important source of metal contamination and can seriously affect the quality of surface water. Metals can present a serious threat to the aquatic organisms because of their toxicity, persistence and tendency to bioaccumulate (Eisler, 1993). Some metals are toxic for living organisms even at low concentrations. Others are essential and necessary in trace amounts for the functioning of biological systems, but can also be toxic at higher concentrations (Bajc et al., 2005).

In the north-eastern part of North Macedonia, there are several active mines, which are known to cause degradation of environmental statuses of the rivers flowing through their regions. So far available data obtained in the regular monitoring indicated water contamination with metals in the rivers directly influenced by the waste from those mines, such as, for example, the Kriva River in the vicinity of Zn-Pb carbonate mine Toranica and the Zletovska River affected by the waste from Zn-Pb sulphide mine Zletovo (Midžić and Silajdžić, 2005). It could be presumed with high certainty that long-term exposure to high levels of dissolved metals in such rivers will inevitably result with increased levels of metals bioaccumulated in the tissues of aquatic organisms, and consequently cause the various sub-toxic and toxic effects.

The first step in the assessment of the river water contamination with metals refers to determination of metal concentrations in the surface water, mainly in the dissolved phase, which is considered as bioavailable metal fraction. However, simple approaches of chemical water analyses often fail to detect environmental changes that are harmful for aquatic organisms. The complex mixture of pollutants in natural waters, with their synergistic and antagonistic effects, combined with variable physico-chemical conditions, makes it difficult to predict the impact of toxicants on the ecosystem (Dragun et al., 2009). Therefore, next to the direct determination of trace elements in the water, it is important to gather the data on metal levels accumulated in the tissues of selected aquatic organisms, known as bioindicators, living in the areas affected by mining activity, as well as on their health statuses, to evaluate the impact of their chronic exposure to high metal levels (Dragun et al., 2019).

In the monitoring studies of the long-term metal pollution, the fish proved to be the most appropriate bioindicator organisms, due to their long life span, top position in the aquatic food chain and potential human risk as a consequence of fish consumption (Jordanova et al., 2014). Appropriate fish species for the studies in Macedonian rivers is Vardar chub (*Squalius vardarensis*), because it is a representative freshwater species for those rivers, and it is closely related to European chub (*Squalius cephalus*), a widespread species in the European rivers, which has a wide tolerance to temperature and oxygen changes in the aquatic environment (Andres et al., 2000).

To obtain the information about the metal levels bioaccumulated by the bioindicator species, two organs were selected, namely the liver and the gills. The liver were chosen due to their importance in the uptake, storage, metabolism, redistribution, and excretion of environmental toxicants (Di Giulio and Hinton, 2008), which makes this organ appropriate for detection of chronic exposure to metals. The gills, on the other hand, are in direct contact with the ambient water and are expected to respond quickly to changes in metal exposure (Kraemer et al., 2005; Dragun et al., 2007), and therefore present an appropriate organ for monitoring short term metal exposure, as well as sudden changes in the exposure level (De Boeck et al., 2003; Karadede et al., 2004; Kraemer et al., 2005; Giguère et al., 2006; Tekin-Őzan and Kir, 2006). Recently, the assessment of metal bioaccumulation is performed through determination of metal concentrations in the soluble, cytosolic part of the tissues, instead of the determination of total tissue loads. The benefit of such approach lays in the fact that accumulated metals fractionate into metabolically reactive and detoxified pools, and cytosols refer to fraction available for metabolic requirements and potential toxic effects (McGeer et al., 2012; Dragun et al., 2013a).

Although the exposure of fish to metal contamination is likely to induce a number of lesions in different organs, as first indicators of the fish health status, gross observations for abnormalities are often used, together with condition factor and organosomatic indices, as well as histopathological examination (Blazer et al., 2018). Histopathological changes in the liver have received much attention in assessing the effects of fish environmental stress (Blazer, 2002). Investigation of gonadal tissue histopathology also gives important information about reproductive disorders in studied fish.

The general aim of this study was to deepen the understanding of the mining influence on freshwater systems based on the assessment of (1) metal exposure, by determining dissolved metal concentrations in the surface water, (2) metal bioaccumulation, by determining metal concentrations in the cytosols of liver and gills of Vardar chub, and (3) finite effects of water contamination on Vardar chub, by defining histopathological changes in fish liver and gonads, as well as fish general health status.

In that way, it was possible to establish so far unknown consequences of the long term metal contamination impact on the Vardar chub (*S. vardarensis*), collected within an environment affected by mining. This important information will significantly contribute to more effective protection of freshwater environment and fishery resources, by expanding the knowledge and understanding of possible health effects of metal contamination on native aquatic organisms.

The specific aims of the study were:

- to evaluate levels of water contamination in three rivers in north-eastern part of the North Macedonia, which are impacted by active Pb-Zn mines and agriculture, by measurement of concentrations of macro and trace elements dissolved in the river water, as well as several physico-chemical parameters;
- to assess macro and trace element bioaccumulation based on determination of the cytosolic macro and trace element concentrations in two target organs (liver and gills) of Vardar chub as bioindicator organism;
- to assess the current and contribute to the prediction of future long-term metal pollution impacts on the health of Vardar chub, by studying their general health status and toxicopathic changes and lesions, analyzing the condition factors and organo-somatic indices of liver and gonads, gross pathology in necropsized animals, and presence of the cellular and tissue disorders or lesions in the liver, as well as by studying reproductive health of target fish species, by histopathological examination of the gonads;
- to evaluate variability of the investigated parameters evoked by natural physiological sources, such as influence of fish mass, gender and saeason, versus environmental responses, such as spatial variability due to different levels of metal contamination at selected sampling sites;

- to integrate the obtained results, to perform statistical processing of the results, and to make conclusions about possible consequences of mining activity on the aquatic organisms and river ecosystem health.

2. LITERATURE REVIEW

2.1. TRACE AND MACRO ELEMENTS IN THE RIVER WATER

2.1.1. Essential and nonessential trace and macro elements

Rivers are the most important freshwater resources for humans. Social, economic and political development has been, in the past, largely related to the availability and distribution of freshwater contained in the riverine systems (Meybeck et al., 1996*)*. The river water quality depends on many factors, including:

(i) the proportion of surface run-off and groundwater,
(ii) reactions within the river system governed by internal processes,
(iii) the mixing of water from tributaries of different quality (in the case of heterogeneous river basins), and
(iv) inputs of pollutants, such as metals (Meybeck et al., 1996*)*.

Metals are defined as elements which have a specific density above 5 g cm^{-3} (Järup, 2003). They play a central role in the functioning of aquatic systems, and are required for the normal life processes; all animals, including fish, need these inorganic elements (Chanda et al., 2015). One of the principles of classification of elements is their division into groups, depending on their contents in the organisms of vertebrates.

The first group in this classification refers to "macro elements," and they are: O, C, H, N, Ca, P, K, Na, S, Cl, and Mg. The second group refers to "trace elements," and this group includes, among others: Fe, Zn, F, Sr, Mo, Cu, Br, Si, Cs, I, Mn, Al, Pb, Cd, B, and Rb (Skalnaya and Skalny, 2018; http://www.fao.org). Macro elements, constitute the bulk of cells and tissues, and represent "structural" elements.

Another classification is based on the physiological role of metals in the body, according to which metals can be essential or nonessential.

Essential metals are required to support biological processes (Deb and Fukushima, 1998). They form integral components of proteins involved in all aspects of biological function. Thus, they are required for maintenance of health, and their lack causes pathological damage or alterations in living organisms (Soto et al., 2013).

Metals serve a variety of functions as both intra and extracellular components (Davis and Gatlin, 1996). The general functions of metals can be summarized as follows:

- metals are essential constitutents of skeletal structures such as bones and teeth;
- metals play a key role in the maintenance of osmotic pressure, and thus regulate the exchange of water and solutes within the animal body (http://www.fao.org);
- metals play a vital role in the acid-base equilibrium of the body, and thus regulate the pH of the blood and other body fluids (Chanda et al., 2015);
- metals serve as structural constituents of soft tissues (Davis and Gatlin, 1996);
- metals are essential for the transmission of nerve impulses and muscle contractions; and
- metals serve as essential components of many enzymes, vitamins, hormones, and respiratory pigments, or as cofactors in metabolism, catalysts and enzyme activators (http://www.fao.org).

Considering that they can be toxic when present in excess (Watanabe et al., 1997; Amiard, 2011), organisms must tightly coordinate metal acquisition and excretion to maintain metal homeostasis (Bury et al., 2003).

Non-essential elements have no known biological functions (Deb and Fukushima, 1998). Nonessential metals of particular concern to surface water systems are Cd, Hg, Pb, As, and Sb (Kennish, 1992). They have direct toxic or lethal effects even in very low concentrations (Nordberg et al., 2007). Unlike essential elements, which can be deficient, for non-essential metals only a threshold of toxicity exists (Watanabe et al., 1997; Amiard, 2011).

Some of the essential and nonessential metals are shown in Figure 1, and will be shortly presented below.

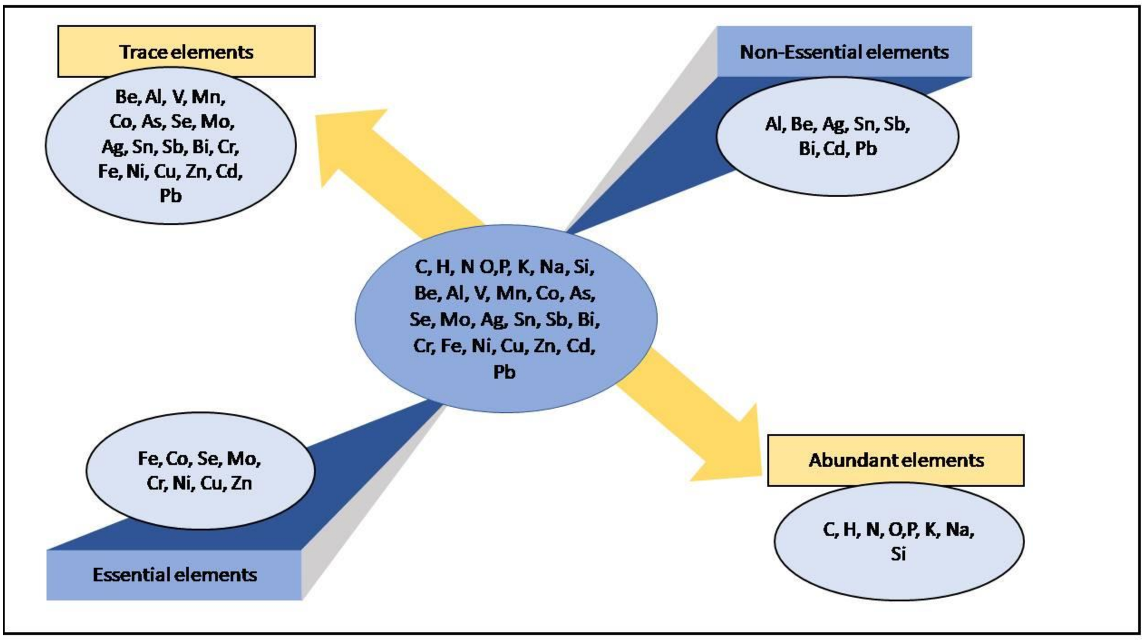


Figure 1. Some essential and non-essential elements, abundant or in traces (modified from Amiard, 2011).

Calcium (Ca) is an essential component of bone and cartilage. It is essential for the normal clotting of blood, by stimulating the release of thromboplastin from the blood platelets (Davis and Gatlin, 1996; http://www.fao.org). Calcium is also an activator for several key enzymes, including pancreatic lipase, acid phosphatase, cholinesterase, ATPases, and succinic dehydrogenase. Through its role in enzyme activation, Ca stimulates muscle contraction (i.e., promotes muscle tone and normal heart beat) and regulates the transmission of nerve impulses from one cell to another through its control over acetylcholine production (http://www.fao.org). In conjunction with phospholipids, Ca plays a key role in the regulation of the permeability of cell membranes and consequently in the uptake of nutrients by the cell, and is believed to be essential for the absorption of vitamin B12 from the gastro-intestinal tract (http://www.fao.org). In the fish, Ca is absorbed through the gastro-intestinal tract (through vitamin D_3 action), gills, skin and fins (http://www.fao.org).

Cobalt (Co) is an integral component of cyanocobalamin (vitamin B12). It has been found to be beneficial for haematology and growth of fish (Watanabe et al., 1997), and as such is essential for red blood cell formation and the maintenance of nerve tissue (http://www.fao.org). Although not confirmed, Co may also function as an activating agent for various enzyme systems (http://www.fao.org). Cobalt is readily absorbed from the gastro-intestinal tract and the surrounding water by fish (http://www.fao.org). Dietary Co availability and absorption is reduced in the presence of high dietary intakes of iodine (http://www.fao.org). Cobalt deprivation can reduce the intestinal synthesis of vitamin B in fish (Watanabe et al., 1997). Although an essential metal, high doses of Co can be toxic to, resulting in haemorrhages in the digestive tract and alterations in white blood cells (Steffens, 1989; Chanda et al., 2015, Terech-Majewska et al., 2016).

Copper (Cu) is an essential component of numerous oxidation-reduction enzyme systems (http://www.fao.org). It is involved in the activity of enzymes such as cytochrome oxidase, superoxide dismutase, lysyl oxidase, dopamine hydroxylase and tyrosinase (Watanabe et al., 1997). As a component of the enzyme ceruloplasmin (ferroxidase), Cu is intimately involved with Fe metabolism, and therefore with haemoglobin synthesis and red blood cell production and maintenance (http://www.fao.org), exhibiting oxidative activity which occurs in blood plasma (Watanabe et al., 1997). Copper also occurs in the formation of the pigment melanin and consequently skin pigmentation, in the formation of bone and connective tissues, and in the maintaining the integrity of the myelin sheath of nerve fibres (http://www.fao.org). The uptake of this metal depends to a great extent on the physiological state of the animal, the Cu content in the water, as well as the levels of Zn, Fe, Cd, Mo, phytates, inorganic sulphates and calcium carbonate in the water (http://www.fao.org), which are metabolic antagonists of Cu (Watanabe et al., 1997). Copper is readily absorbed through the gastro-intestinal tract, gills, fins and skin of fish (http://www.fao.org). High level of Cu inhibits the growth and decreases the food intake, and also can cause gill damage, and the appearance of necrotic foci in the liver and kidneys (Terech-Majewska et al., 2016).

Iron (Fe) has an active part in oxidation/reduction reactions and electron transport associated with cellular respiration. It is found in complexes bound to proteins such as haemoglobin, myoglobin (http://www.fao.org), as well as in enzymes such as microsomal cytochromes, catalase, peroxidases, and the enzymes xanthine and aldehyde oxidase, and succinic dehydrogenase (http://www.fao.org). It is further found in non-haem compounds, such as transferrin, ferritin and flavin iron enzymes. Haemoglobin occurs in erythrocytes, while transferrin is found in plasma; the latter is the principal carrier of iron in blood (Watanabe et al., 1997; Chanda et al., 2015). As a component of the respiratory pigments and enzymes involved in tissue oxidation, Fe is essential for oxygen and electron transport within the body (http://www.fao.org).

Iron is readily absorbed through the gastro-intestinal tract, gills, fins and skin of fish and transported in a protein bound form (Lovell, 1989; Davis and Gatlin, 1996). Dietary iron availability and absorption is usually depressed by high dietary intakes of phosphate, calcium, phytates, Cu and Zn (http://www.fao.org). In general, inorganic sources of Fe are more readily absorbed than organic sources; the ferrous iron (Fe^{2+}) being more available for absorption than ferric iron (Fe^{3+}) (Chanda et al., 2015). Physiological evidence indicates that Fe preferentially crosses the apical membrane of both the gills and intestine in the ferrous state (Bury et al., 2003). Although an essential element, in excess Fe can be toxic. At the cellular level, Fe catalyses the Fenton reaction resulting in the generation of free radical species, including hydroxyl radicals that can potentially cause cellular oxidative damage (Bury et al., 2003). In high concentration Fe can have adverse effects, including retarded growth, high mortatlity, diarrhea and liver damage (Terech-Majewska et al., 2016).

Magnesium (Mg) is an essential component of bone and cartilage, together with Ca. It is an activator of several key enzyme systems, including enzymes kinases (i.e., enzymes that catalyse the transfer of the terminal phosphate of ATP to sugars or other acceptors), mutases (enzymes that catalyse transphosphorylation reactions), muscle ATPases, cholinesterase, alkaline phosphatase, enolase, isocitric dehydrogenase, arginase (Mg is a component of the arginase molecule), deoxyribonuclease, and glutaminase (http://www.fao.org). Through its role in enzyme activation, Mg (like Ca) stimulates muscle and nerve contraction, it is involved in the regulation of intracellular acid-base balance, and plays an important role in carbohydrate, protein and lipid metabolism (http://www.fao.org). Magnesium is essential for the maintenance of intra and extra cellular homeostasis in fish (Moyle and Cech, 1982; Davis and Gatlin, 1996; Terech-Majewska et al., 2016). Magnesium is readily absorbed through the gastro-intestinal tract, gills, skin and fins of fish (http://www.fao.org). At elevated concentration of Ca and Mg, fish have lower gill permeability and osmoregulatory costs. Calcium and Mg retard toxicant uptake and can protect against changes of several water chemistry parameters, such as pH, Na^+, and Cl^- (Wood, 2001). High concentrations of Ca and Mg adversely influence larval survival and growth (da Silva et al., 2005).

Manganese (Mn) functions in the body as activator of enzymes that mediate phosphate group transfer (i.e., phosphate transferases and phosphate dehydrogenases), particularly those involved in the citric acid cycle, including arginase, alkaline phosphatase and hexokinase (http://www.fao.org), glycosyltransferase and non-specific enzymes, such as kinases, transferases, hydrolases, decarboxylases and pyruvate carboxylases (Watanabe et al., 1997). As a cofactor or component of several key enzyme systems, Mn is essential for bone formation (i.e., mucopolysaccharide synthesis), the regeneration of red blood cells, carbohydrate metabolism, and the reproductive cycle (http://www.fao.org). Manganese deficiency causes reduced intake of food, loss of equilibrium in fish, depressed growth and increased mortality (Watanabe et al., 1997), as well as skeletal abnormalities (National Research Council, 1980; Davis and Gatlin, 1996). Manganese is readily absorbed from the gastrointestinal tract and gills. Dietary Mn availability and absorption is reduced in the presence of phytates, and high dietary intakes of Ca (http://www.fao.org). High concentration of Mn can cause liver damage in fish (Javed and Usmani, 2013).

Sodium (Na) and potassium (K) are recognized as essential elements for a number of physiological processes. They occur almost entirely in the fluids and soft tissues of the body. Sodium is the main monovalent ion of extracellular fluids, constituting 93% of the ions (bases) found in the blood stream, and playing a specific role in the absorption of carbohydrates, while K is the major cation of intracellular fluid, and is required for glycogen and protein synthesis (http://www.fao.org). Thirst and total body water are regulated by dietary Na (http://www.nap.edu; http://www.fao.org). Together, Na and K have a vital function in controlling osmotic pressure and acid-base equilibrium, and in stimulating effect on muscle irritability (http://www.fao.org). In high concentrations, Na and K influence the growth and productivity of fish (Al-Saadi, 2017).

Zinc (Zn) is an essential element for normal physiological functions. It has vital structural and catalytic importance in more than 300 proteins involved in growth, reproduction and development processes, as well as visual and immune functions (Watanabe et al., 1997). Thus, it is an important trace element in fish nutrition. This trace element has two roles: first as a cofactor for enzymes which form metal-enzyme complexes (Watanabe et al., 1997), including arginase, enolase, several peptidases, and oxalacetic decarboxylase (http://www.fao.org), and second as an integral part of large number of metalloenzymes, such as alkaline phosphatase, alcohol dehydrogenase, carbonic anhydrase (Watanabe et al., 1997; Chanda et al., 2015), glutamic dehydrogenase, pyridine nucleotide dehydrogenase, superoxide dismutase, pancreatic carboxypeptidase, and tryptophan desmolase (http://www.fao.org). As an active component or cofactor for many important enzyme systems, Zn plays a vital role in lipid, protein, and carbohydrate metabolism; being particularly active in the synthesis and metabolism of nucleic acids (RNA) and proteins (http://www.fao.org). Although not proven, it has been suggested that Zn plays a role in the action of hormones such as insulin, glucagon, corticotrophin, FSH and LH (http://www.fao.org).

Fish can derive Zn from dietary sources, as well as from the water. The gills and gastrointestinal tract are involved in the uptake of this trace element (Watanabe et al., 1997). Deficiency of Zn was reported to lower the digestibility of proteins and carbohydrates, probably due to the reduced carboxypeptidase activity (Ogino and Yang, 1978). Additional signs of Zn deficiency are eye lens cataract and erosion of fins and skin (Ogino and Yang, 1979; Hughes, 1985), as well as fish impaired immunological responses (Kiron et al., 1993; Chanda et al., 2015). In contrast to Cu, it does not form free radicals; it becomes toxic at increased waterborne concentrations due to interference with Ca homeostasis, leading to hypocalcemia (Spry and Wood, 1985; Hogstrand and Wood, 1996). Decreases in weight gains, hemoglobin levels, and hematocrit levels were observed in association to excessive Zn levels (Terech-Majewska et al., 2016).

Cadmium (Cd) is non-essential trace metal, contaminant of surface waters and is extremely toxic to aquatic animals already at low concentrations (USEPA, 2001; CCME, 2002; Franklin et al., 2005), due to its interaction with the metabolism of at least three essential metals: Ca, Zn, and Fe (Komjarova, 2009). Cadmium has a profound capacity of binding to -SH groups, which is higher than that of Zn, and thus has the ability to displace Zn from a number of Zn-containing enzymes (Wittmann, 1979; Moore and Ramamoorthy, 1984). In aquatic organisms, Cd interacts with Ca uptake (Bervoets and Blust, 2003; Reynders et al., 2008) at the gill surface, and disturbe Ca^{2+} balance (Wood, 2001; Franklin et al., 2005), leading to hypocalcemia and, ultimately, death (Verbost et al., 1989; Wood, 2001). The most important sites of Cd absorption in fish are the gills and the gastrointestinal tract (Szebedinszky et al., 2001; Franklin et al., 2005). In fish, Cd accumulates primarily in kidneys, followed by gills, intestine, liver, carcass and muscles (Reynders et al., 2008).

Cesium (Cs) occurs naturally in the environment mainly from erosion and weathering of rocks and minerals. It is also released into the air, water and soil through mining and milling of ores (www.lenntech.com/periodic/elements/cs.htm). Cesium is the most reactive metal and is never found uncombined in nature; it also has high water solubility (www.encyclopedia.com). Cesium is not a common element, and it has few commercial uses. One of its radioactive isotopes, Cs-137, is widely used in a variety of medical and industrial applications (Lalhmunsiama et al., 2018). Stable Cs has a relatively low toxicity to humans, but its radioactive isotope is very hazardous (Phillips and Russo, 1978). It can easily enter human body and cause an internal hazard, such as cancer. Cesium in fish can accumulate from food and water (Phillips and Russo, 1978). Ishak et al. (1977) studied the distribution of ^{134}Cs in Nile catfish, and found its greatest levels in muscle and bone of the fish. Chemically, Cs resembles Na and K. Thus, it can be easily incorporated into terrestrial and aquatic organisms, which makes it a potential toxic contaminant in the water (Lalhmunsiama et al., 2018). However, water with high K levels inhibits Cs uptake. Animals that are exposed to very high doses of Cs show changes in behaviour, such as increased or decreased activity (www.lenntech.com/periodic/elements/cs.htm).

Lead (Pb) is not required for normal physiology in plants or animals and is thus toxic in very low concentrations. The principal toxic effects of chronic Pb exposure to fish are primarily haematological (Hodson et al., 1978), neurological (Davies et al., 1976) and renal impairments (Patel et al., 2006). In fish, waterborne Pb causes the disruption of Na^{+}, Cl^{-} and Ca^{2+} regulation during acute exposure, development of black tails and spinal curvature during chronic exposure, and disruption in hemoglobin synthesis (Sippel et al., 1983; Rogers et al., 2003; 2005). In some polluted areas, dissolved Pb concentrations were reported to vary between 10-80 µg L^{-1} (Bowles et al., 2006), while Pb toxicity has been reported for some freshwater species at Pb concentrations as low as 10 µg L^{-1} (Grosell et al., 2006; Komjarova, 2009).

<u>Thallium (Tl)</u> is a rare trace metal, but its high toxicity, water solubility and tendency of bioaccumulation have made it a United States Environmental Protection Agency (US EPA) priority pollutant (Mijošek et al., 2019). It is more toxic to humans than many other toxic metals, such as Hg, Cd, Pb, Cu or Zn (Zitko, 1975; Peter and Viraraghavan, 2005; Xiao et al., 2004a,b; Couture et al., 2011). It is present in aquatic and terrestrial systems (Nriagu, 1998; Couture et al., 2011; Belzile and Chen, 2017), mainly as Tl^{+} and Tl^{3+}, usually at very low concentrations (Belzile and Chen, 2017), but human activities have greatly increased its presence in nature (Mijošek et al., 2019). The most important anthropogenic sources of Tl in the environment are the combustion of fossil fuels and the smelting of ferrous and non-ferrous ores (Peter and Viraraghavan, 2005). In non-contaminated freshwaters, Tl concentration is generally below 1 µg L^{-1} and rarely exceeds 20 ng L^{-1} in the open ocean (Belzile and Chen, 2017). Its geochemical behaviour and mobility is often compared to that of K, so Tl can enter cells via K^{+} transport systems due to closeness between the K^{+} and Tl^{+} radius (Tl^{+}: 164 p.m., K^{+}: 152 p.m.) (Flegal et al., 1989; Belzile and Chen, 2017). Moreover, thallous ions (Tl^{+}) can inhibit the movement of K^{+} across the mitochondrial membrane and thus inhibit the Na^{+}/K^{+} ATPase pump (Repetto et al., 1998; Belzile and Chen, 2017). Also, this metal binds more tightly than K to N and S ligands (Williams and Frausto da Silva, 1996), being thus very harmful for living organisms even at very low concentrations (Leonard and Gerber, 1997). The more oxidized form of Tl^{3+} has been identified as being ~50,000 times more toxic than Tl^{+} to the common alga *Chlorella* (Ralph and Twiss, 2002), and also much more toxic to *Daphnia magna* (Lan and Lin, 2005), although Tl^{3+} is much less bioavailable (Belzile and Chen, 2017).

2.1.2. Bioavailability of trace and macro elements in the river water

The term bioavailability in metal ecotoxicology has two meanings, one environmental and other toxicological. In the environment, bioavailability corresponds to the metal that is available for capture by a living organism and can be integrated into its metabolic processes (Newman and Jagoe, 1992; Rainbow and Luoma, 2011; Magalhães et al., 2015). Toxicological bioavailability is defined as the fraction of the concentration of the metal that is already absorbed and/or adsorbed on the body (Lopez et al., 2010; Rainbow and Luoma, 2011).

Metals in the aquatic systems occur in different forms, and in order to understand the bioavailability of metals, it is important to characterize the metal species in the water (Gjerde et al., 1993). Speciation according to Templeton et al. (2000) is defined as the distribution of a metal among its possible chemical forms and metal complexes. The bioavailability, and hence toxicity, of metals in the aquatic systems is strongly dependent on the nature of the metal species present (INAP, 2002; Yu et al., 2010; Chi, 2013; Magalhães et al., 2015). For example, the toxicity of As^{3+} is significantly different from the As^{5+} in the aquatic life (Langmuir et al., 2004; Chi, 2013). Therefore, determining the metal species in water is fundamental to predicting metal impact to aquatic biota (INAP, 2002; Magalhães et al., 2015).

In the freshwater environment, according to Nystrand et al. (2012), metals are divided into three fractions: dissolved (<1 nm), colloidal (between 1 nm and 0.45 µm) and particulate (higher than 0.45 µm). Still, the metal fraction obtained after filtration of the river water through 0.45 µm filter is operationally defined as dissolved metal fraction, although it also contains the colloidal fraction. The dissolved form is the most mobile and bioavailable, and is generally the most toxic fraction. It comprises free metal ions, as well as labile inorganic and organic complexes which could be easily introduced in the organs of aquatic organisms, and therefore are considered as bioavailable (INAP, 2002; Dragun et al., 2015). Part of these dissolved metal ions may react with the sediment by adsorption, ion exchange with clay minerals and/or formation of complexes with oxyanions, and thus switch to particulate phase and become less bioavailable (Bjerregaard and Andersen, 2007; Magalhães et al., 2015).

Metal bioavailability and speciation in aquatic environment is influenced by various factors, which can be divided into the following groups: physical, chemical, and biological factors (Newman and Jagoe, 1992; Bonnail et al., 2016). Many of these factors vary seasonally and temporally, and most of the factors are interrelated (John and Leventhal, 1995).

Physical factors include temperature, phase association (solid, liquid or gas), physical adsorption, and sequestration by occlusion within a solid phase or depositional regime as dictated by water movement (Newman and Jagoe, 1992).

Chemical factors include those influencing speciation at thermodynamic equilibrium, complexation kinetics, lipid solubility, and phase transition, such as those associated with precipitation, coprecipitation, or chemical adsorption (e.g., pH, alkalinity, water hardness, total organic content, redox potential; Stumm and Morgan, 1996; Markich et al., 2001).

For example, the pH is one of the most important factors that influence the behavior of the metals (USEPA, 2007). It determines the degree of hydrolysis, polymerization, aggregation and precipitation, and proton competition for available ligands (Magalhães et al., 2015). Low pH induces the dissociation of metals, thus increasing their solubility, and consequently their toxicity, whereas in alkaline pH, metals generally precipitate as oxides and hydroxides, becoming less bioavailable and less toxic (Cornelis and Nordberg, 2007; Magalhães et al., 2015). However, in low pH there is competition between H^+ and metal and this may protect against metal uptake at the gill surface, while in higher pH there is a reduced competition with H^+ and, thus, ionic metal is more available for uptake by the gill (Grosell et al., 2006; Niyogi et al., 2008). The speciation of some metals (Pb, Cu, Hg, and Al) is highly affected by pH, while for some others (Cd and Zn) it is only slightly sensitive to pH changes (Campbell and Stokes, 1985).

Furthermore, in water of higher hardness metal toxicity is lower (Saglam et al., 2013), because carbonate ions of Ca^{2+} and Mg^{2+} compete with the other divalent metal ions for binding to organisms sites (Kozlova et al., 2009), and block metals to enter into the cells.

Also, complexation with organic ligands reduces metal bioavailability, because most organic-metal complexes produce coordination compounds, which are less toxic, have higher molecular weights and are frequently unable to pass through the biological membranes (Richards et al., 1999).

Biological factors that can modify bioavailability include trophic interactions, biochemical or physiological interactions, adaption, microhabitat utilization, animal size and age, feeding strategies, and reproductive stage (Newman and Jagoe, 1992; Bonnail et al., 2016).

2.1.3. The sources of water contamination with trace and macro elements

Metals are natural constituents of the Earth’s crust and since they cannot be degraded or destroyed, they are considered persistent environmental pollutants (Cuong, 2006; De

Lurdes Dinis and Fiúza, 2011). Metals are usually perceived as pollutants due to their toxicity, long persistence, bioaccumulation and biomagnification in the food web (Miracle and Ankley, 2005; Carrasco et al., 2011; Carneiro et al., 2014; Nyeste et al., 2019). There are two sources of metals for the water ecosystems: the natural geological background and anthropogenic activities (Nyeste et al., 2019). Metals are introduced into the aquatic systems as a result of the weathering of the soils and rocks, as well as from volcanic eruptions. Anthropogenic activities often originate from untreated domestic and industrial wastewater discharges, mining and smelting operations and agricultural runoff (Sunjog et al., 2016). For the prevention of ecosystem deterioration and biodiversity loss, it is crucial to evaluate the potential toxic effects of such environmental stressors in non-target organisms, for example in fish (Rodrigues et al., 2016; 2017; 2019; Limbu et al., 2018).

Water contamination originating from metal mining activities can result with significant environmental and ecological degradation and can pose serious risk to human health through contamination of food, as well as of surface waters and groundwaters (Byrne et al., 2012). And it presents a potential barrier to achieving good status of the water bodies, which is required by the EU Water Framework Directive (Gandy et al., 2007).

According to environmental agencies, mining effluents and agricultural runoff can be considered as the most serious threats to freshwater ecosystems (Environment Agency, 2006), the former due to high acidity and high metal concentrations, Fe and its precipitates, and turbidity (Stuhlberger, 2010), and the latter mainly as a source of different types of organic contaminants (Environment Agency, 2006). In mine drainage, the high acidity triggers the transformation of metals into ionic forms, as the most dangerous metal forms for living organisms (Wojtkowska, 2013).

In the active mining areas, the environmental concern is primarily related to spilled mine tailings, emitted dust, and acid mine drainage transported into aquatic ecosystems (Riba et al., 2005; Mayes et al., 2010; Dragun et al., 2019). Mining affects freshwaters through heavy use of water in processing ore, and through water contamination by discharged mine effluents and seepage from tailings and waste rock impoundments (Ugya et al., 2018). The resulting environmental contamination is mainly due to the presence not only of major metals/metalloids mined, but also of the other minor constituents of sulphides which are highly toxic (As, Cd, Hg and Tl) (Dolenec et al., 2005). Increased concentrations of trace elements (Pb, Cu, Ca, Mn, As, Zn, Fe, Ag) can be found around both abandoned and active mines as the result of discharging and dispersion of mine waste materials into nearby soils, food crops, and stream sediments (Davis et al., 2000; Dolenec et al., 2005). In the rivers that receive mine waters with high levels of one or more ecotoxic metals, significant loss of biodiversity can be expected (Stuhlberger, 2010).

Metal mine discharges have resulted in severe degradation of many rivers across the globe (Cerqueira et al., 2011; Silva et al., 2011a, b, c; Byrne et al., 2012). The mining industry has been involved in some of the most widely publicized environmental disasters. Well-known examples of mining-related environmental accidents and long-term deteriorations include Rio Tinto, a river in southern Spain, and the colliery spoil heap failure at Aberfan, Wales, or the Baia Mare cyanide spill in Romania (Stuhlberger, 2010).

2.1.4. Water contamination from Pb/Zn ores in the north-eastern part of the Northern Macedonia

The mine waste still represents one of the biggest environmental concerns in Macedonia (Serafimovski et al., 2016). 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 Probištip and Toranica in Kriva Palanka.

The development of mining and metallurgic industries of Pb and Zn ores in the eastern catchment area of North Macedonia has had a strong influence on the condition of the surface water (Alderton et al., 2005; Midžić and Silajdžić, 2005; Serafimovski et al., 2007; 2016).

Alderton et al. (2005) and Serafimovski et al. (2007) have shown that the waters at Zletovo have a low pH, due to the dissolution of pyrite. High levels of several metals, including Al, Zn, Cd, Fe, and U were reported there, which have exceeded water standards for most of those metals. Also, concentrations of sulphates, Mn, Zn, and Fe were all very high, and there was widespread precipitation of ferric oxyhydroxides downstream from the mine (Alderton et al., 2005). High values of the mentioned metals were maintained 3 km below the mine area, and then dilution reduced the concentrations markedly. In the Zletovo mine the litology is dominated by silicate minerals, so there is little chemical buffering to counteract the acidity. Moreover, Šmuc et al. (2012) have detected increased concetrations of As, Cd, Mo, Pb, Zn, and Cu in the rice and maize samples from the nearby fields, which has been related to the proximity of Pb and Zn mine Zletovo, and their highest concentrations were determined in the rice samples grown in the paddy fields near the Zletovska River. The results indicated that the regular consumption of rice and maize crops containing the highest Cd, Mo, Pb and Zn concentrations could pose a serious threat to human health, because the daily intake exceeded the recommended provisional tolerable daily intake values. Taking into account those results, the area around the Zletovska River is considered as the most anthropogenically impacted part of the Kočani Field (Šmuc et al., 2012).

Toranica mining and processing have had somewhat milder effect on the chemical composition of neighbouring water flows. According to the Serafimovski et al. (2007), waters in the Toranica area have in general a neutral pH and solute contents are fairly low. Elevated values of As, Bi, Co, Fe, Mn, Pb, U and Zn occur in the vicinity of the mine, but rarely exceed reference limits. The high values of some elements in waters upstream of the adit discharges suggest that the mineralization present in bedrock has an impact on the regional geochemical background (Serafimovski et al., 2007). Although the mines at Toranica have exploited similar Pb and Zn rich minerals to those at Zletovo, and the associated river sediments were contaminated, the water contamination at these localities is milder compared to that at Zletovo, and this is due to the lower contents of pyrite in the ore and buffering by the limestone which cause low acidity and low dissolved solids (Serafimovski et al., 2007).

Chronic contamination of riverine systems can be exacerbated by episodic flood events (Bradley, 1984; Hudson-Edwards et al., 1999; Dennis et al., 2009) or by the failure of tailing dams (Byrne et al., 2012). Such event, the collapse/failing of the tailings dam of Zletovo mine, happened in 1976 (JICA, 2008; Stuhlberger, 2010) when material of 150,000 m^3 spilled from the tailings dam to the downstream area and no measures have been taken to prevent soil contamination, flooding villages and agricultural land downstream (JICA, 2008; Stuhlberger, 2010). People in the area use the waters from several tributaries for drinking purposes and household needs. They also use the water for irrigation and agriculture, particularly in the production of many kinds of vegetables (Spasovski and Dambov, 2009).

2.2. BIOINDICATORS FOR THE STUDY OF TRACE AND MACRO ELEMENT BIOACCUMULATION

2.2.1. Fish as bioindicators

Bioindicators are defined as a species or a group of species that readily reflects the abiotic or biotic state of an environment, reflecting the impact of the environmental change on a habitat, community or ecosystem, and can be indicative of the diversity of a subset of taxa or the whole diversity within an area (Gerhardt, 1999). The use of bioindicators (Fig. 2) should help to describe the natural environment, to detect and assess human impacts on the environment and to evaluate restoration or remediation measures; in all these cases, fish are intensively used for indication purposes (Chovanec et al., 2003).

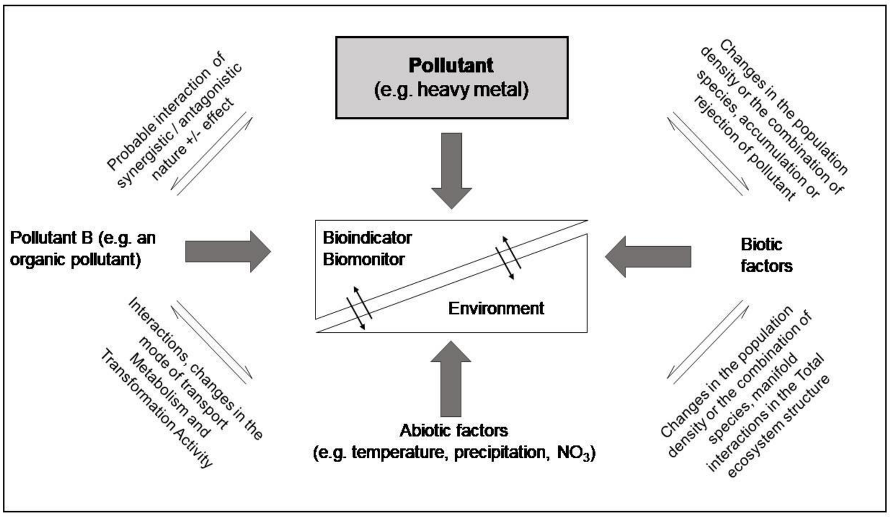


Figure 2. Simplified representation of complex ecosystem interrelations with regard to a pollutant, and consequences for bioindication and biomonitoring (from Markert et al., 2003).

In the aquatic environment, fish are an important component of the ecosystem: fish are distributed in virtually all aquatic systems, are involved in the flow of energy through different trophic levels, have varied habits, relatively long life cycles, different reproductive and feeding strategies, a relatively high mobility in the water column (van der Oost et al., 2003; Linde-Arias et al., 2008), and are relatively easy to capture, manage and identify; in addition, they are able to show biological responses to contaminants exposure at different organizational levels (suborganismal, individual, population, and community); they can be of different sizes and ages and, in comparison with invertebrates, they are also more sensitive to many toxicants; hence, they are commonly chosen as sentinel organisms (Powers, 1989; van der Oost et al., 2003; Yancheva et al., 2015).

Furthermore, fish respond to environmental toxic changes by adapting their metabolite functions (Yancheva et al., 2015). Exposure of feral populations of animals to environmental contaminants is a global problem, and fish populations offer appropriate models for examining the effects of contamination (Jenner et al., 1990). More importantly, fish species are at the top position in the aquatic food chain and may directly affect the health of humans, which makes the use of fish highly significant for the biomonitoring programs (Zhou et al., 2008).

Fish have, moreover, proved to be of significance as bioindicators of the so-called ecological integrity (Karr, 1991; Schiemer, 2000) because during their life cycle, the various fish integrate a wide range of riverine conditions, including the properties of bed sediments relevant for egg development and the longitudinal integrity for spawning migrations (Copp, 1989; Schiemer et al., 1991; 2001; Persat et al., 1995; Gaudin, 2001; Chovanec et al., 2003).

So fish communities are widely used in monitoring the impacts of aquatic contaminations (Hermenean et al., 2015; Jia et al., 2016; Liu et al., 2016; Nyeste et al., 2019), whereas concentrations of chemicals in fish organs present excellent indicator of their bioavailability in the environment (Chovanec et al., 2003).

2.2.2. Characteristics of Vardar chub (*Squalius vardarensis*) as a selected bioindicator

Vardar chub (*S. vardarensis*) (Fig. 3a) was selected as an abundant and representative freshwater species in the Macedonian rivers, which is closely related to European chub (*Squalius cephalus*) (Fig. 3b), a fish of the same genus and of wide tolerance to the temperature and oxygen changes in the aquatic environment (Andres et al., 2000; Podrug et al., 2009). Chub are some of the most widespread cyprinid species in Europe and are a very popular game fish (Kottelat and Freyhof, 2007). They are the most abundant in small rivers and large streams, but also occur in slow-flowing lowland rivers and very small mountain streams (Kottelat and Freyhof, 2007), undertaking spawning migration to inflowing streams. The preferred substrate of chub is coarse gravel (Lelek and Lusk, 1965; Cowx and Welcomme, 1998; Caffrey et al., 2008). Young fish form groups in shallow water, while larger specimens tend to be solitary, often residing under overhanging trees or roots (Wheeler, 1998; Caffrey et al., 2008).

Figure 3a. Vardar chub (*Squalius vardarensis*)

Figure 3b. European chub (*Squalius cephalus*)

Several studies showed that there are three different types of diet of chub, depending on their age (Balestrieri et al., 2006; Marković, 2007; Caffrey et al., 2008). Chub juveniles (age 0+) have a plankto-phytophage diet; they feed mainly on diatoms, green algae, and zooplankton (Marković, 2007). The older, sexually immature chub (age 1+, 2+) have a plankto-phyto-zoophage diet, feeding on diatoms, green algae, and macrophytes, and also on animals, such as larvae and imagines of Odonata, Trichoptera, Diptera and Ephemerophtera insects (Marković, 2007). The sexually mature adult chub (age 3+, and thereafter) have mainly carnivorous diet. They prey predominantly on fish, but also feed on insects, molluscs, and amphibians (Ünver and Erkakan, 2011). These significant shifts in diet can lead to different bioaccumulation and biomagnification features in the different age groups of chub (Merciai et al., 2014). European chub lives up to 15 years, and females live longer than males. Males reproduce for the first time at 2-4 years of age, while females at 4-6 years. Maturity is influenced by environmental factors and individuals may mature much later. Spawning occurs in fast-flowing water above gravel bottom, rarely among submerged vegetation. Spawning occurs in May to August, when temperature rises above 12°C. Female spawn more than once during a season (www.fws.gov./fisheries/).

Chub is a generalist fish species; it also survives in more polluted water (Machala et al., 2001; Dragun et al., 2012, 2016). Therefore, chub is increasingly used to assess the contamination levels of water bodies and give relevant information regarding the vulnerability of aquatic organisms (e.g., Dragun et al., 2007; Triebskorn et al., 2007; Yılmaz et al., 2007; Krasnići et al., 2013; Nyeste et al., 2019).

2.2.3. Uptake, distribution, accumulation, toxicity and excretion of trace and macro elements in the fish

Tissue concentrations of chemicals are a function of uptake, storage, and excretion (Chovanec et al., 2003). The uptake of dissolved metals by aquatic organisms into the cell include passive diffusion, active transport, and endocytosis (Fig. 4) (Puckett et al., 2010).

According to Puckett et al. (2010), passive diffusion involves the movement of molecules directly through the lipid bilayer down their concentration gradient. Active transport (using proteins) moves a particular class of cargo, and its expression varies by tissue type. It can be energy-independent, using channels and passive carriers, or be energy-dependent, using ATP-powered pumps. Endocytosis is the uptake of macromolecules and solutes by vesicles derived from the plasma membrane. One clear dividing line between different mechanisms is whether uptake requires energy, as for endocytosis and active transport proteins, or is energy-independent, as is the case for passive diffusion through the membrane and diffusion facilitated by channels and carriers (Puckett et al., 2010).

Such metal uptake can occur through several pathways in fish (Dragun, 2006), specifically through gills, skin or the digestive system by feeding (Yılmaz et al., 2007; Lenhardt et al., 2012; Nyeste et al., 2019). However, the most important pathways for uptake in fish are water filtration through the gills and food consummation.

Due to the low solubility of oxygen in the water (www.nzqa.govt.nz), fish gills are built of filaments covered in lamellae which are only a few cells thick and contain blood capillaries, and they pass large volumes of water in a unit of time. During the countercurrent flow of the water over the filaments and lamellae, oxygen by diffusion pass from water to blood, whilst CO_2 goes in the opposite direction, by diffusion from blood to water. Such structure gives a large surface area and a short distance for gas exchange (www.biologymad.com), facilitating uptake of metals from the water.

Uptake of metals across gill membranes is thus a function of water flow along the gills (McKim and Erickson, 1991). In addition, rise of temperature, oxygen depletion or metabolic stimulation (e.g., during reproduction or stress) accelerate gill ventilation and thus also the uptake of toxicants (Chovanec et al., 2003). Metals are then carried via blood to organs, bound to carrier proteins, and can reach high tissue concentrations through association with metal binding proteins in specific tissues (Sönmez et al., 2016; Elbeshti et al., 2018).

The described uptake of trace elements may lead to their accumulation in the cells of aquatic organisms (Jezierska and Witezka, 2006) and can cause toxic effects. Once in the cells, metals can interact with cytosolic molecules and interfere with cell functions, leading to altered physiological processes and reducing the normal functioning of the organism (Grobler et al., 1989; Wepener et al., 2001), and eventually leading to deleterious effects (Wang, 2013; Urien et al., 2018).

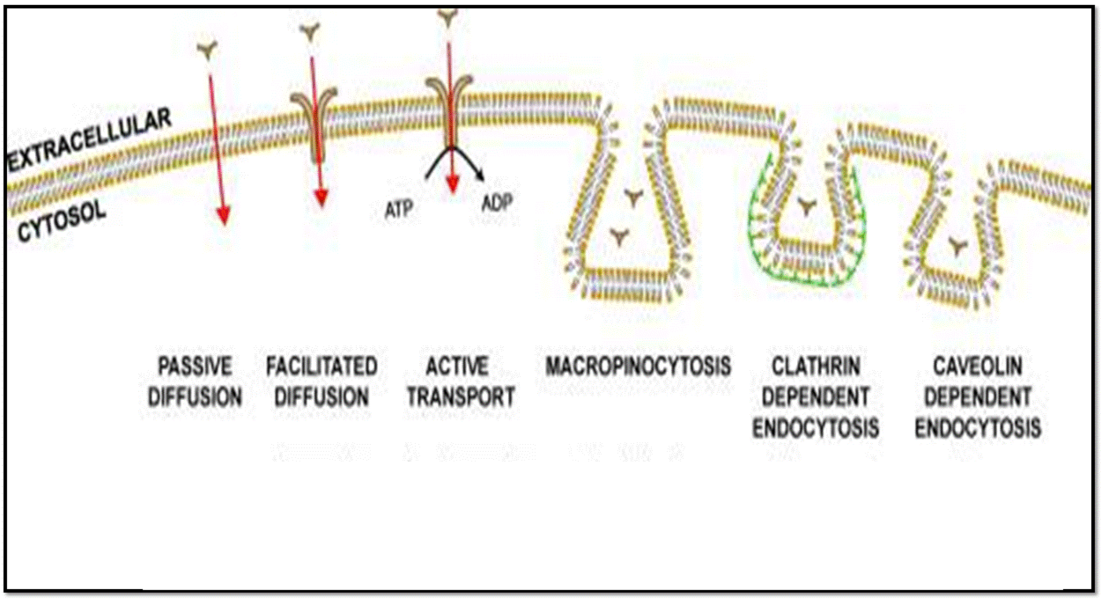


Figure 4. Routes into the cell. Passive diffusion, facilitated diffusion, active protein transporters, and several endocytic pathways are illustrated (from Puckett et al., 2010).

Metal toxicity depends upon the absorbed dose, the route of exposure and duration of exposure, causing various disorders, and can also result in excessive damage of various organs, such as kidney, nervous system, respiratory system, endocrine, and reproductive systems, for example due to oxidative stress induced by free radical formation (Hollenberg, 2010; Jaishankar et al., 2014). In addition, metals can produce toxicity through several other mechanisms, including ionoregulatory disturbance, respiratory disturbance (Playle, 1997) changes in enzyme activity (Weis and Weis, 1992), displacement of essential metals from biologically important molecules and cellular damage (Mason and Jenkins, 1995), disruption of the structure and/or function of subcellular organelles, as well as interaction with DNA leading to mutagenesis or carcinogenesis (Hollenberg, 2010).

Metal accumulation in fish depends on both the chemical properties of the elements and different factors such as ecological needs, and the physiological state, size and age of individuals, gender, as well as their life cycle, life history and feeding habits (Newman and Doubet, 1989; Canli and Atlı, 2003; Canpolat and Çalta, 2003; Jezierska and Witeska, 2006; Zhang and Wong, 2007; Lenhardt et al., 2015; Nyeste et al., 2019). It also depends on metal uptake routes, deposition and excretion rates (Chovanec et al., 2003). The proportion of accumulated toxicants between different organs of the fish largely depends on dynamic processes between uptake, storage, and elimination. After short term exposure, gills and the digestive tract usually show a high load of toxicants, whereas concentrations in liver, kidney, bones (Pb, Zn), and muscles (especially for lipophilic substances) increase more slowly after a time-lag, but remain more persistent compared to the other organs (Olson et al., 1978; Kock et al., 1996). Metal accumulation is generally the highest in the liver, as main detoxification organ, compared to the other fish organs, which makes fish liver widely used target organ for monitoring of long term metal pollution of water ecosystems (Arellano et al., 1999; Yacoub and Abdel Satar, 2003; Filipović Marijić and Raspor, 2007; Koca et al., 2008; Podrug et al., 2009; Jovanović et al., 2011; Dragun et al., 2012, 2015). Due to active regulation, tissue accumulation of essential metals (e.g., Cu, Zn) is saturated at low levels, and thus presents a relatively weak indicator of environmental contamination (McGeer et al., 2000).

Fish have different routes for possible excretion of harmful chemicals, such as metals, including the excretion through the gills, liver and bile, kidney and skin (Heath, 1995; Chovanec et al., 2003; Azevedo et al., 2012). For example, several metals have been found in elevated concentration in the bile of the fish during or following the ingestion of metals or waterborne exposure to metals (Felts and Heath, 1984). Excretion of metals via skin and gills involve the mucus, which is constantly secreted and sloughed off by these tissues (Heath, 1995). According to Hardy et al. (1987), gills are the major excretory route for dietary Zn.

2.2.4. Fish gills and liver as target organs for the study of trace and macro element bioaccumulation

Numerous studies have quantified contaminants in different fish organs to evaluate environmental quality, seeking causal relationships with fish health, and, based on these, the liver is likely the best choice, followed by the gills (Begum et al., 2004; Pokorska et al., 2012; Majnoni et al., 2013). The liver and the gills are among the most sensitive organs frequently evaluated as indicators in assessing the health status of the fish (Bernet et al., 1999; Rašković et al., 2010; Yancheva et al., 2015).

The liver is a detoxification organ and it is essential for both the metabolism and the excretion of toxic substances in the fish body (Hinton and Laurén, 1990; Van der Oost et al., 2003; Salamat and Zarie, 2012; Yancheva et al., 2015). It has several physiological functions, including the biotransformation of organic xenobiotics, excretion of harmful metal traces, storing of energy sources (such as glycogen), and protein synthesis; this organ also has high enzymatic activity (Ruiz-Picos et al., 2015). Additionally, the liver of fish is sensitive to environmental contaminants because some contaminants tend to highly accumulate in this organ. Therefore, the responses observed in the liver are indicative of the overall health of the fish upon exposure to toxic xenobiotics, carcinogenic materials, and urban pollution in general, although this organ has no direct contact with pollutants dissolved in water as do gills (Olsson et al., 1998; Au, 2004; Gul et al., 2004; Wolf and Wolfe, 2005; Fernandes et al., 2008; Nunes et al., 2008; Zhu et al., 2008; Capkin and Altinok, 2013; Nunes et al., 2013; Valon et al., 2013; Ruiz-Picos et al., 2015).

Fish gills are multifunctional organ involved in ion transport, gas exchange, acid–base regulation and waste excretion (Dang et al., 2001; Gernhöfer et al., 2001; de La Torre et al., 2005; Evans et al., 2005; Oliveira Ribeiro et al., 2006; Nigro et al., 2006; Vigliano et al., 2006; Salamat and Zarie, 2012; Singh, 2014). According to several authors (Carpene and Vašak, 1989; Perry and Laurent, 1993; Pourang, 1995; Tkacheva et al., 2004; and Rosseland et al., 2007) the gills are the major route of toxicant penetration into the fish organism, thus they are the first organs which come in contact with environmental pollutants and are sensitive subjects for identifying the effects of water toxicants on the fish organism. Thus, gills are the first organ to respond to environmental changes (Evans et al., 2005; Rašković et al., 2010; Rodrigues et al., 2017). In addition, the toxicant concentrations in the gills reflect the toxicant concentrations in the water where the fish live; whereas, the concentrations in the other organs, such as liver and kidney, which represent storage of toxicants (Kroglund et al., 2008; Podrug et al., 2009; Dragun et al., 2013a; Yancheva et al., 2015) can indicate the exposure level at the site where the fish have previously resided. Furthermore, Heier et al. (2009) stated that, as the fish gills can accumulate bioavailable pollutants, the measurement in the gills can reflect the speciation of pollutants, and in particular metals in water, which make them a useful tool for assessing metal bioavailability in the water. In addition, the toxicants could induce gill histological alterations such as epithelium degeneration and necrosis (Camargo and Martinez, 2007; Ayandiran et al., 2009; Vigário and Sabóia-Morais, 2014), but also can induce numerous defense mechanisms, which could prevent their negative effects. These mechanisms are expressed in different morphological changes of gills, including edema, proliferation of epithelium and fusion (Velmurugan et al., 2009). Many studies revealed that interstitial edema is one of more frequent lesions observed in the gill epithelium of fish exposed to pollutants such as metals (Karlsson-Norggren et al., 1986; Reid and McDonald, 2011; Hwang and Tsai, 1993; Sola et al., 1995; Bury et al., 1998; Karan et al., 1998; Cengiz and Ünlü, 2002; Pane et al., 2004; Cengiz, 2006; Nero et al., 2006; Velmurugan et al., 2009). In gills during the increased exposure to metals mucus secretion occur which cause uptake and binding of metals to gill surface (Shephard, 1994; Authman et al., 2015). This may explain a part of the elevated metal concentrations observed in the fish gills (Felts and Heath, 1984; Chovanec et al., 2003).

2.3. EFFECTS OF WATER CONTAMINATION WITH TRACE ELEMENTS ON FISH HEALTH CONDITION

2.3.1. Assessment of biometric parameters and organosomatic indices

The effects of environmental stressors on fish are hard to interpret because of the complexity of the environment itself (Adams and Ryon, 1994). In stressed ecosystems, growth and condition are expected to decline because energy is diverted into acclimation and compensation (Cairns and Niederlehner, 1993). Exposure of fish to sublethal concentrations of contaminants may also impose considerable physiological stress, resulting in a number of manifestations such as reduced growth, impaired reproduction, predisposition to disease, reduced locomotory and predatory performance, or reduced capacity to tolerate subsequent stress (Adams et al., 1989; Dragun et al., 2013b).

For the assessment of the fish health, standard fisheries indices consisting of fish mass, length, condition factor and organosomatic indices are generally used. The latter two are used as indicators of fish well being, and may vary in response to different kind of pollutants in the river water, including metals (Schmitt and Dethloff, 2000; Jovanović et al., 2011; Dragun et al., 2013b). Health indices of fish indicate stress in aquatic ecosystems, they are therefore often examined in relation to pollution (Hinck et al., 2007b; Roussel et al., 2007; Felipe-Sotelo et al., 2008; Szlinder-Richert et al., 2009; Güngördü et al., 2012; Kerambrun et al., 2012).

Variation of length and mass of fish of a particular age over time can display changes in growth condition. The condition factor is commonly used as a simple general indicator of physical and physiological status of fish, in the sense of a relative measure of body composition, fatness, feeding, growth, reproductive stage, and body energy content of fish (Encina and Granado-Lorencio, 1997; Dragun et al., 2013b). The condition factor would remain unaffected, if length and weight increased proportionally to each other over time. Only if weight increases stronger than length, the condition factor would increase (Bolger and Connoly, 1989; Cone, 1989). The larger the factor is, the better is the condition (Ricker, 1975).

The hepatosomatic index which is the ratio of liver mass to body mass is also used to evaluate the well-being or fitness (Bolger and Connoly, 1989), as well as the energy reserves of the fish. Hepatosomatic index (HSI), moreover, was confirmed as a useful biomarker of aquatic pollution (van Dyk et al., 2012). Hall et al. (2012) have shown that hypertrophy or an increase in liver size occurs due to exposure to various contaminants metabolized by the liver. In this case a higher index would be indicative of exposure to certain chemical classes.

However, when evaluating hepatosomatic index (HSI), some factors other than pollution should be considered, such as the age of the fish (van Dyk et al., 2012; Dragun et al., 2013b).

As indicator of the reproductive health and status of fish populations, gonadosomatic indices (GSI) and gonad histopathology have been used. The sex and reproductive status are the most important factors that affect GSI of fish species, and this index is also influenced by the seasonal changes of the abiotic parameters, such as temperature and photoperiod (Blazer, 2000).

2.3.2. Necropsy-based fish health assessment

Organisms that inhabit contaminated water bodies are faced with continuous exposure to mixtures of xenobiotics, which can cause disruption in the health of the biota (van der Oost et al., 2003). In most aquatic systems it is difficult to establish a clear link between environmental pollutants and the health of organisms due to the wide variety of environmental and ecological factors that influence the response of aquatic biota (Adams et al., 1989; Ruiz-Picos et al., 2015).

According to Blazer (2000), two types of assessments have been directed toward whole fish or gross (visible to the naked eye) observations: the incidence of gross external pathological disorders and a more comprehensive necropsy-based fish health assessment (internal and external). The necropsy-based fish health assessment provides data on visible abnormalities and lesions, parasites, condition and organosomatic indices. It includes external and internal observations and allows for the measurement of additional condition indices. The necropsy-based assessment supplemented by age analysis and histopathology of various organs allows for the calculation and comparison of various condition indices, prevalence of visible abnormalities, as well as microscopic tissue changes, by sex, age, site and sampling period. Additional tissue collections can be made for many other analyses including electron microscopy, bacteriology, virology, parasitology and chemical concentrations. These methods can also be part of more in-depth analyses used to diagnose the cause of fish kills or mortalities of captive fishes (Blazer et al., 2018). The prevalence or percentage of fish with visible pathological disorders has been used for many years as a convenient and relatively easy indicator of environmental quality by fisheries managers and field personnel (Blazer, 2000). Visible lesions generally include fin erosion, skin ulcers, eye disorders, visible tumors and skeletal deformities. The index of biotic integrity, which was designed to evaluate quality or condition of an aquatic ecosystem, includes external abnormalities in its calculation (Oliveira and Cortes, 2006). Three categories of fish community metrics, species abundance, trophic composition, and health and abundance of fishes, are used to reflect the condition of the fish community and the environment in which it is found (Blazer, 2000). Health is determined by the proportion of fish with disease or anomalies (Karr, 1981). Goede (1989) developed a systematic fish health/condition or necropsy-based system for use by fisheries personnel at the field level.

As an example, a study by Environmental Monitoring and Assessment Program can be presented, during which fish were collected from estuarine sites in the Virginian province and the Louisianian province (Gulf Coast), and a total of 24,291 fish representing 143 species were examined. Skin lesions were the most prevalent gross abnormalities in both provinces, followed by ocular abnormalities in the Virginian province, and by branchial chamber abnormalities (gill parasites, gill arch deformities) in the Louisianian province. Prevalence, in general, was three-fold higher for demersal fishes than pelagic and eight-fold higher at sites with high sediment contaminant concentrations (Macauley et al., 1999).

2.3.3. Histopathological assessment of the liver

Histopathological data are frequently applied in ecotoxicological studies to assess the effects of different classes of environmental stressors in fish, including heavy metals and metalloids.

Histopathology is a discipline which can provide an indication of fish health by determining early injury to cells. It involves the microscopic examination of cells and tissues of an organism and the semi-quantitative determination of histological abnormalities (Paolini et al., 2005; Teh et al., 2005; Yancheva et al., 2015).

Histological analyses observe cell structures and assess lesions that are not always visible to the naked-eye. Histological changes are medium-term responses to sub-lethal stressors, and histology represents a rapid method to detect effects of toxicants in various organs (Bernet et al., 1999; Au, 2004; Rebok, 2013).

Histopathology is a suitable ecotoxicological method, since it provides a valid and rapid detection of consequences of exposure to contaminants; it allows to locate, to describe and even to quantify lesions that occur on selected key organs of exposed fish (Bernet et al., 1999; Nero et al., 2006; Barišić et al., 2015; Jordanova et al., 2017). The link between water pollution and its effects on aquatic life is useful for prevention of further deterioration of affected ecosystems and for preservation of biodiversity (Barišić et al., 2015). By doing so, it is possible to prevent adverse effects in aquatic organisms, namely in fish health (Gil and Pla, 2001). The use of histopathological indicators is a frequent practice in fish health research, as they are able to provide information on chronic and sub-lethal effects of xenobiotics on organs and for the assessment of fish stress. They are usually applied in research of polluted aquatic ecosystems, since they are good indicators of altered or polluted environments.

Liver are suitable organ for histological examination in order to determine the effects of pollution, since they easily respond to xenobiotic exposure (Oliveira Ribeiro et al., 2006; Rašković et al., 2013; Nunes et al., 2015). Furthermore, tissue alterations in liver are an indicative parameter of the general health condition of fish (Nunes et al., 2013).

Thus, according to Lang (2002) and Feist et al. (2004), in the recent years the fish diseases and liver histopathological alterations have been used as indicators of pollution effects and have been implemented in monitoring programs. Triebskorn et al. (1997) and Schramm et al. (1998) described methods to study the liver ultrastructure using quantitative and semi-quantitative electron microscopy. The liver is organ that is highly susceptible to damage by virtue of its detoxifying role, active metabolic functions in the organism, glycogen storage and excretion (Rebok, 2013). Stentiford et al. (2003) stated that numerous categories of liver pathology are present as reliable biomarkers of toxic damage. The presence of inflammatory lesions, hepatocellular fibrillar inclusions, and preneoplastic and neoplastic lesions is higher in fish captured in polluted environments than in the fish from the reference sites. However, according to Pinto et al. (2009), the sort of histological alterations observed depends on individual exposition time to pollutants, as well as on pollutant type and concentration. Exposure to heavy metals and different organic pollutants, for example, may cause histological changes in the liver and a histological investigation of exposed specimens may therefore produce meaningful results (van Dyk et al., 2007; Triebeskorn et al., 2008; Marchand et al., 2009; Hued et al., 2012; Georgieva et al., 2014). Figueiredo-Fernandes et al. (2007) suggested that high metal deposition in the fish liver leads to the abnormalities in the hepatic structure and hepatocytes, which can lead to subsequent cell death (Yancheva et al., 2015).

2.3.4. Histopathological assessment of the gonads

Histology offers a powerful tool in the study of reproductive health of fishes. It is routinely used for sex verification, identifying stages of development, documenting presence of intersex, tumors, parasites and other abnormalities and quantifying atresia. The recognition that many environmental chemicals may act as endocrine disruptors and hence influence reproduction has lead to interest in assessing reproductive health of wild aquatic animals (Sumpter, 1995; Goodbred et al., 1997; Rolland et al., 1997; Jobling et al., 1998; Kendall et al., 1998; Kime, 1998). The histopathological assessment of fish reproductive organs can be divided into two separate components: the evaluation of gonads for abnormal findings and for gonad staging (Wolf et al., 2004). Since fish reproduction is controlled by environmental factors, such as temperature and photoperiod (Blazer, 2002), there are many histological changes, both normal and abnormal, that can be observed in fish gonadal tissue throughout their development and reproductive cycle (Blazer, 2002).

The most investigated reproductive disruptions on fish exposed to various contaminants are the presence of increased rates of atretic oocytes and of intersex, i.e. the simultaneous presence of male and female gonad tissue in the same gonad of individual gonochoristic fish (Blazer, 2002; Rebok, 2013). Most fish species currently used in monitoring programs are gonochoristic, i.e. existing as one sex, either male or female (Blazer, 2002). Atretic oocytes (which have also been named corpora atretica) represent a common, normal ovarian structural component in wild individuals (Rebok, 2013).

Reproductive strategies of fish are extremely varied and so hermaphroditism and sex reversal are normal condition in some fish species (Blazer, 2002). The most common intersex condition observed in fish species is ovo-testis, the presence of ovarian tissue in the testes of male fish (Blazer, 2002). For example, wild male roach with female-like reproductive ducts were observed as e results of exposure to treated sewage effluents during early-life (Rodgers-Gray et al., 2001; Rebok, 2013). Based on these findings, reproductive indices in association with other biomarkers could be powerful tool for assessing fish health status.

3. MATERIALS AND METHODS

3.1. CHEMICALS

3.1.1. Milli-Q water (conductivity 0.057 μS cm^{-1}) was used for all solutions.
3.1.2. pH calibration solutions:
3.1.2.1. pH 4.01 buffer solution (±0.01 pH) for HI 7004 HANNA instruments (Padova, Italy)
3.1.2.2. pH 7.01 buffer solution (±0.01 pH) for HI 7007 HANNA instruments (Padova, Italy)
3.1.3. Conductivity calibration solutions (1413 µS cm^{-1}) for HI 7031 HANNA instruments (Padova, Italy)
3.1.4. Anesthetic Clove oil (Sigma Aldrich, Chemie Gmbh, Switzerland)
3.1.5. Trizma®/Hydrochloride (Tris(hydroxymethyl)aminomethane hydrochloride), $C_4H_{11}NO_3 \cdot HCl$, (Sigma Chemical Co., USA)
3.1.6. Trizma®/Base (Tris(hydroxymethyl)aminomethane), $C_4H_{11}NO_3$ (Sigma Chemical Co., USA)
3.1.7. DTT, dithiothreitol, $C_4H_{10}O_2S_2$ (Sigma Chemical Co., USA)
3.1.8. Standard solutions for high resolution inductively coupled plasma mass spectrometer (HR ICP-MS):
3.1.8.1. Multielement standard solution for trace elements (100 mg L^{-1}, Analitika, Czech Republic)
3.1.8.2. Single element standard solutions of Sb and Sn (1 g L^{-1}; Analytika, Czech Republic), Rb and U (1 g L^{-1}; Sigma-Aldrich, Germany), and Cs (1 g L^{-1}; Fluka, Germany)
3.1.8.3. Macro elements standard solution (Ca 2.0 g L^{-1}; Mg 0.4 g L^{-1}; Na 1.0 g L^{-1}; K 2.0 g L^{-1}; Fluka, Germany)
3.1.9. Indium (1 μg L^{-1}; Indium Atomic Spectroscopy Standard Solution, Fluka, Germany)
3.1.10. Quality control sample for trace metals (QC Trace Metals, Catalog number 8072, Lot Number 146142-146143, UNEP GEMS, Burlington, Canada)
3.1.11. Quality control sample for macro elements (QC Minerals, Catalog number 8052, Lot Number 146138-146139, UNEP GEMS, Burlington, Canada)
3.1.12. Ammonium test 1.14752 Spectroquant® (Merck, Germany)
3.1.13. Phosphate test 1.14848 Spectroquant® (Merck, Germany)
3.1.14. AQUANAL-plus Nitrate 37409 (Fluka Analytical, Sigma-Aldrich, Germany)
3.1.15. HNO_3, 65%, Suprapur (Merck, Germany)
3.1.16. HNO_3, 65%, p.a. (Merck, Germany)
3.1.17. HCl, 37%, p.a. (Merck, Germany)
3.1.18. H_2SO_4, 95-97%, p.a. (Merck, Germany)
3.1.19. Oxalic acid, 0.05 mol L^{-1}(Fluka Analytical, Sigma-Aldrich, Germany)
3.1.20. Titrival® Kompleksal® III, 0.1 mol L^{-1}, (Kemika, Croatia)
3.1.21. Potassium permaganate solution, 0.05 mol L^{-1}, (Merck, Germany)
3.1.22. Sodium hydroxide solution (Merck, Germany)
3.1.23. Sodium thiosulphate solution 0.1 mol L^{-1} (Merck, Germany)
3.1.24. Buffered 4% formalin (Sigma-Aldrich, Germany)
3.1.25. Bouin's fixative (Merck, Germany)
3.1.26. Etanol 96%
3.1.27. $Hg(SCN)_2$, p.a. (Sigma-Aldrich, Germany)
3.1.28. $Fe(NO_3)_3 \cdot 9H_2O$, ACS reagent, (Sigma-Aldrich, Germany)
3.1.29. Argon, 99.999%.

3.2. INSTRUMENTS

3.2.1. Field instruments

3.2.1.1. WTW Multi 340i/SET portable (Germany)
3.2.1.2. Electrofisher Samus 725G, voltage of inverter 550-600 V, 650 W

3.2.2. Laboratory instruments

3.2.2.1. Freezer -80 ºC, Polar 370H (Angelantoni Industrie s.p.a., Italy)
3.2.2.2. Refrigerator +4 ºC with freezer at -20 ºC (Gorenje, Slovenia)
3.2.2.3. Avanti J-E centrifuge (Beckman Coulter, USA)
3.2.2.4. CARY 100 Scan UV-visible Spectrophotometer (Varian, Australia)
3.2.2.5. Photometer SQ 118 (Merck, Germany)
3.2.2.6. Analitycal balance (Sartorius CP 224S, Germany)
3.2.2.7. Digital balance (Mettler Toledo BB300, Switzerland)
3.2.2.8. pH meter pHM210 (Radiometer, Copenhagen)
3.2.2.9. Conductivity meter CDM210 (Radiometer, Copenhagen)
3.2.2.10. Centrifuge Sorvall RC28S (Kendro, USA) with cooling (maximum 100,000×g)
3.2.2.11. High resolution inductively coupled plasma mass spectrometer (HR ICP-MS, Element 2, Thermo Finnigan, Germany), equipped with an autosampler ASX 510 (CETAC Technologies, USA)
3.2.2.12. Potter-Elverhjem homogenizer (Glas-Col, USA)
3.2.2.13. Precise and transportable balance Viper SW 3 (Mettler-Toledo, Switzerland)
3.2.2.14. Ice maker (Eurfrigor, Italy)
3.2.2.15. Rotation microtome RAZOR, manual (Croatia)
3.2.2.16. Microscope (Nicon, USA)
3.2.2.17. Water bath Julabo (Germany)

3.3. METHODS

3.3.1. Study area

North Macedonia is a landlocked country located in southern part of the Balkan Peninsula, surrounded by Albania, Greece, Bulgaria, Kosovo and Serbia. Its territory spreads over 25,713 km^2 (JICA, 2008; Milevski et al., 2017). It is a mountainous country (mean elevation: 829 m), where hills and mountains cover 79%, plains and valleys 19.1%, and water surfaces 1.9% of the territory (JICA, 2008), comprising of 35 rivers, 3 natural lakes (Ohrid Lake, Prespa Lake and Dojran Lake), and 50 artificial lakes. With large areas of erodible soils, crystalline rocks (gneiss, mica-schists, other schists), sandstones, lacustrine and fluvial deposits, steep slopes (39.5 % of the area is above 15°), semi-arid climate and sparse vegetation, landslides are very common in North Macedonia (Milevski et al., 2017).

The country is under the influence of several climate zones, which result in cold winters and long and warm summers with much sunshine. Nearly half of the total area of the country is used by agriculture, split equally between cultivated areas and pastures. About 37% of the total territory of North Macedonia is classified as forest lands, which is high in comparison to the other countries in Europe. Forest cover plays an important ecological function in terms of watershed protection and soil conservation (JICA, 2008).

North Macedonia is relatively rich in mineral resources. The main metallic resources of North Macedonia are iron, lead, zinc, copper, nickel, chromium, antimony, arsenic and manganese, while main non-metallic resources are coal, clay, diatomite, gypsum, quartz and marble. The north-eastern part of North Macedonia encompasses several ore districts, among which there are Kratovo-Zletovo and Sasa-Toranica districts (Fig. 5) (JICA, 2008). Lead and zinc are the most important non-ferrous metals in North Macedonia and the total reserves of contained Pb and Zn at three mines of Sasa, Zletovo and Toranica, located in the northeastern part of North Macedonia, are reported to be, respectively, 385,000 t and 310,000 t. The most important Macedonian metal deposits are linked to regional magmatic activity that occurred in the southern parts of the Carpatho-Balkanides from the Eocene to the Pliocene (Serafimovski and Aleksandrov, 1995; Alderton et al., 2005).

This study was done to investigate the influence of Pb-Zn mineralization and mining on the river water quality and on fish species Vardar chub (*Squalius vardarensis*) near Zletovo and Toranica mines. Three rivers were included in this study: Bregalnica, Zletovska River and Kriva River (Fig. 5) (Ramani et al., 2014), wich flow through the north-eastern part of North Macedonia. The map of the study area with the sampling sites is shown in Figure 5, while the list of the sampling sites with coordinates, known pollution sources, and the dates of the samplings is presented in Table 1.

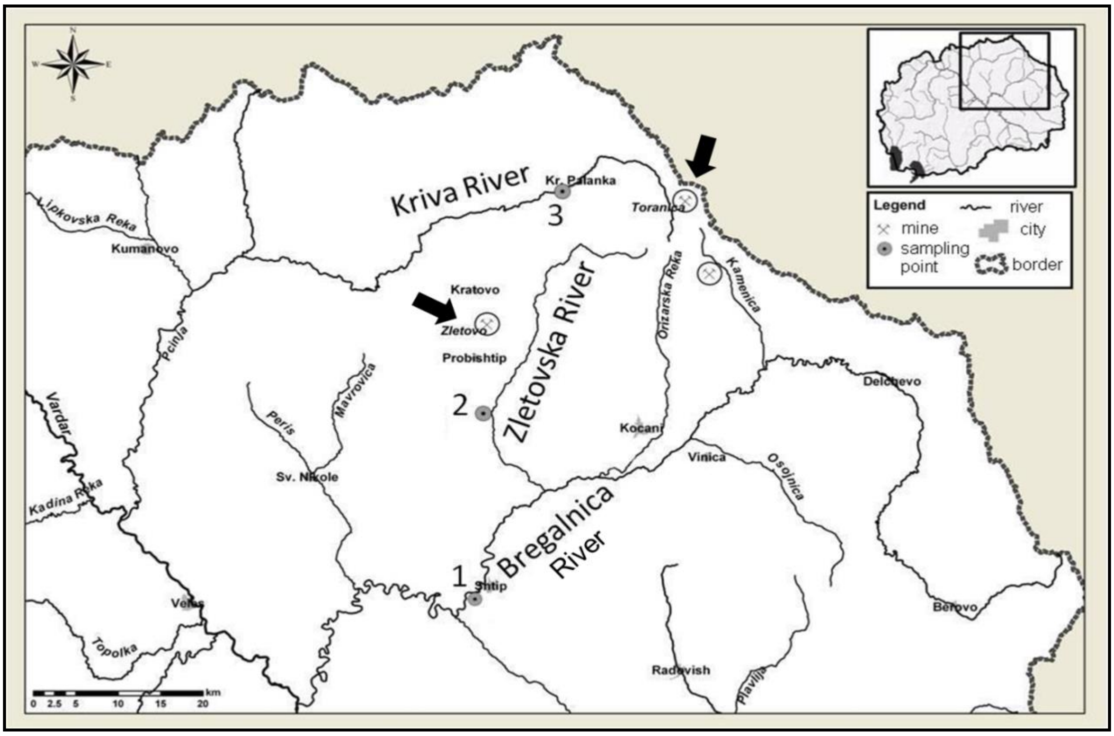


Figure 5. Map of the sampling area with marked sampling sites. Black arrows point to studied Zletovo and Toranica Pb/Zn mines.

3.3.1.1. The Zletovo Mine

The Zletovo Mine (Pb and Zn mine), located close to Dobrevo Village, approximately 3 km to the north-east of the town of Probištip, started operating in 1940s actively until today, with several interruptions. Dacite ignimbrite is the most characteristic volcanic rock that is found in this ore deposit (Serafimovski and Aleksandrov, 1995; Serafimovski and Boev, 1996; JICA, 2008). The main ore minerals in the Zletovo Mine are galena (PbS) and sphalerite (ZnS), followed by pyrite (FeS_2) and chalcopyrite ($CuFeS_2$), and number of other minerals which occur only sporadically (Alderton et al., 2005; Serafimovski et al., 2006). Ores from the Zletovo Mine, with grades higher than 9% of Pb and 2% of Zn, and with significant presence of Ag, Bi, Cd, and Cu (Alderton et al., 2005), are concentrated by flotation at Probištip, while tailings are disposed of in two impoundments situated in the adjacent valleys. Tailings are mixtures of waste ground rock, spent processing water and reagents produced at mine processing plant through ore extraction and manufacturing. The properties of tailings are dependent on the ore body being mined, the grinding and processing circuits, the reagent properties and the thickening process prior to disposal.

Since the mine workings are located at the upper stream of the Koritnica River, and processing plant and tailings dams are located in the Kiselica River area, in and near Probištip, the environmental impact caused by mining activities are more serious in the latter area than in the former. Both rivers flow into the Zletovska River.

Table 1. The list of the sampling sites with coordinates, known pollution sources, and the dates of the samplings.

Sampling site	Coordinates	Pollution sources	Sampling dates
Bregalnica River (1)	N 41°43.57’ E 22°10.27’	municipal wastewater from the city of Štip, agricultural waste	Spring: May 11, 2012 Autumn: October 17, 2012
Zletovska River (2)	N 40°58.54’ E 21°39.45’	waste from Pb/Zn mine Zletovo and from Pb-battery factory	Spring: May 11, 2012 Autumn: October 16, 2012
*Kriva River (3)	N 42°11.39’ E 22°18.34’	waste from Pb/Zn mine Toranica, municipal wastewater from Kriva Palanka	Spring: June 13, 2012 Autumn: October 18, 2012

*In the autumn sampling, the water was additionally collected at an upstream location closer to the mine, near Zhidilovo (N 42°13.50' E 22°22.18').

3.3.1.2. The Toranica Mine

The Toranica Mine (Pb and Zn mine) is situated in the north-eastern part of the North Macedonia, approximately 18 km south-east of the town of Kriva Palanka and 2 km west of the Bulgarian border (JICA, 2008), close to the Sasa deposit, but in a separate watershed. The production of Pb and Zn from the Toranica Mine have been lasting from 1987, with a few year interruptions (Alderton et al., 2005). The ore from Toranica Mine consists predominantly of galena, sphalerite, chalcopyrite and pyrite (Fidancev et al., 2011). Ore grades are about 6.5% of Pb+Zn with additional elevated concentrations of Ag, As, Bi, Cd, Cu, Mn, and Sb (Serafimovski et al., 1997; 2007). Milling and flotation occur at the mine and there is a tailings dam below the mine site with a culvert directing the Toranica River beneath the dam.

There are a number of environmental concerns associated with this site. In particular, there is extensive metal contamination (by Cd, Pb and Zn) in the river water downstream of the mine (Toranica and Kriva rivers) (Stuhlberger, 2010).

3.3.1.3. The Bregalnica River

Based on the information gathered during existing monitoring programmes (Milevski et al., 2004), in this study Bregalnica was selected as the river nonimpacted by mining. It is the longest left tributary of the Vardar River, the principal river in North Macedonia. Bregalnica has a length of 225 km and its river basin covers an area of ~4,300 km^2 (Jovanovska et al., 2018). Its water discharge in 2012, when the sampling was performed, was in the range from 1.24-66.30 m^3 s^{-1} (Table 2). To avoid the influence of the contamination from the Zletovska River, a location situated approximately 35 km downstream from the mouth of the Zletovska River into the Bregalnica River was chosen as the sampling point at Bregalnica (N 41º43.570 E 22º10.270). However, this site is located downstream from the city of Štip (Table 1, Fig. 6), the largest town in the eastern part of Macedonia, and therefore it is influenced by sewage and household water discharges, as well as waste from industrial facilities and farms, which are partly released in the collection system, and partly directly into the river (Spasovski, 2011; Rebok, 2013). Furthermore, Bregalnica is also impacted by rice field runoff (Andreevska et al., 2013). According to the State Statistical Office (2012a), Bregalnica River Basin administratively encompasses 19 municipalities and sustains a population of about 180,000 (State statistical office, 2002). The region is characterized by poor economic growth, considerable rural-urban migration and high rate of emigration (State statistical office, 2012a; 2012b). Human presence is most prominent along the rivers, as about 40% of the settlements and villages situated in the area are adjacent to rivers and streams. The main economic activity, especially in the lowland areas is agriculture (Jovanovska et al., 2018).

Figure 6. Sampling site at the Bregalnica River

Figure 7. Sampling site at the Zletovska River

3.3.1.4. The Zletovska River

The Zletovska River is one of the most polluted tributaries of the Bregalnica River (Dolenec et al., 2005). It is 56 km long, with the catchment area of 460 km^2. Water discharge of the Zletovska River in 2012 was in the range from 0.167 to 26.55 m^3 s^{-1} (Table 2). This river drains the central part of the Kratovo-Zletovo volcanic complex, the abandoned old mining sites and bare tailings, as well as the effluents from the Pb/Zn mine Zletovo and its ore processing facilities located near the town of Probištip in the northeastern Macedonia (Alderton et al., 2005; Dolenec et al., 2005). In the town of Probištip, there is also a battery factory, as a potential source of contamination (Spasovski and Dambov, 2009; Ramani et al., 2014). As the sampling point at the Zletovska River (N 40º58.540 E 21º39.450), a site was chosen 5-6 km downstream from the tailings impoundment of the Zletovo mine (Table 1, Fig. 7). That site was additionally impacted by municipal and industrial wastewaters of Probištip town. Two tributaries of the Zletovska River, the Kiselica River and the Koritnica River, drain the flotation plant at Probištip and the main excavation points of the Zletovo mine (Alderton et al., 2005).

3.3.1.5. The Kriva River

The Kriva River is the longest tributary of the River Pčinja, which is the left tributary of the Vardar River. The length of the Kriva River is 78.7 km and the catchment area is 1,001.8 km^2 (Vasilevski and Radevski, 2014). Water discharge of the Kriva River in 2012 was in the range from 0.08-8.42 $m^3 s^{-1}$ (Table 2). As the sampling point at the Kriva River (N 42º11.390 E 22º18.34') the site was chosen 15-20 km downstream from the Toranica mine (Table 1, Fig. 8). The valley of the Kriva River is predominantly enclosed in crystalline rocks (gneisses, mycashites, chlorite, muscovite shale), whereas the area downstream of the Belakovce is mainly embedded in tertiary volcanoes: andesite, ignimbrite, tuff, breach, etc. (Milevski et al., 2017). From the village Belakovce until the very inflow of Pčinja, the valley of the Kriva River is encased in eocene (marine) sediments: sandstones and flat limestones.

Figure 8. Sampling site at the Kriva River

Figure 9. Sampling site at the Kriva River (Zhidilovo)

The western part of the river basin of the Kriva River is built in volcanic (magmatic) rocks, which are the result of tertiary volcanism within the Kratovo-Zletovska area. These are predominantly andesites, dacites, laties, ignimbrates of a dacic composition, then tuffs, breccias, etc. In fact, most of the catchment area of Kriva River is built of rocks with low waterproofness, which is why the surface leakage is strong, and the infiltration of atmospheric waters is low (www.igeografija.mk). Ore milling and flotation occur at the mine, and there is a tailing dam below the mine with a culvert directing the Toranica River, the Kriva River tributary, beneath the dam (Alderton et al., 2005). During the autumn sampling, the water of the Kriva River was additionally collected at an upstream location closer to the mine, near Zhidilovo (N 42°13.50' E 22°22.18') (Fig. 9). The Kriva River also flows through land used for gardens and orchards, so it was exposed to the influence of agricultural runoff (Ramani et al., 2014; Dragun et al., 2017).

3.3.2. Study period

The study of the influence of Pb-Zn mineralization and mining on the river water quality and on metal bioaccumulation in two organs (liver and gills) of freshwater fish Vardar chub near Zletovo and Toranica mines was carried out in two campaigns, spring (May/June) and autumn campaign (October) in 2012 in three rivers in north-eastern part of North Macedonia (Table 1, Fig. 5), mentioned in the section 3.3.1. Study area.

Table 2. Water discharge ($m^3 s^{-1}$) for the rivers Bregalnica, Zletovska and Kriva in the time of sampling, and during the year 2012 (the exact date of the water discharge measurement is shown in Table 1).

Date	Bregalnica	Zletovska	Kriva
May 11th/ June 13th	2.90	9.51	0.933
October	1.60	0.424	0.099
Annual minimum	1.24	0.167	0.080
Annual maximum	66.30	26.55	8.42
Annual average	6.36	2.65	0.705

Hydrological information for the rivers Bregalnica, Zletovska, and Kriva at the time of each water sampling and for the whole year 2012 are shown in Table 2 and in Figs. 10, 11, and 12 (data were obtained by the courtesy of the Hydrometeorological Service of the Republic of North Macedonia). During the spring period the water discharge was higher than during the autumn period, when water discharge was close to annual minimum (Table 2).

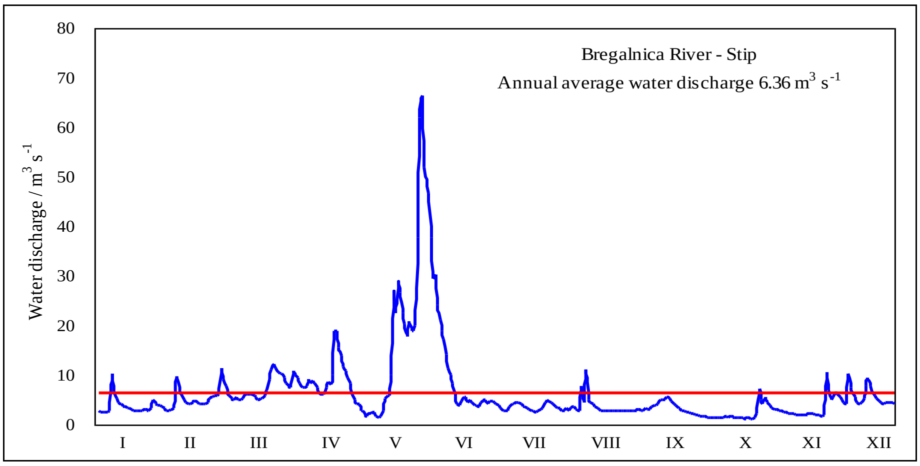


Figure 10. The Bregalnica River water discharges ($m^3\ s^{-1}$) for the period from January 1st to December 31st 2012 at hidrological station Štip.

3.3.3. Water sampling

The river water was sampled for analyses of dissolved metals and physico-chemical parameters in each river according to the norm ISO 5667-6:2005, once in the spring period and once in the autumn period. On each occasion, the river water samples were collected by grab water sampling in the polyethylene plastic bottles (three bottles of 0.25 L for metal analyses and one bottle of 1.0 L for physico-chemical parameters) which were, prior to sampling, rinsed with acid (for metal analyses: v/v 10% HNO_3, p.a.,Merck, Germany; for physico-chemical parameters: v/v 5% HCl, p.a., Merck, Germany) and with Milli-Q water. The samples for metal analyses were stored at +4°C for at most 24 hours before filtration through a cellulose nitrate filter (0.45 μm pore diameter, Sartorius, Germany). The filtrates were acidified with nitric acid (acid concentration in the samples: 0.65%; Suprapur, Merck, Germany), and stored at +4°C.

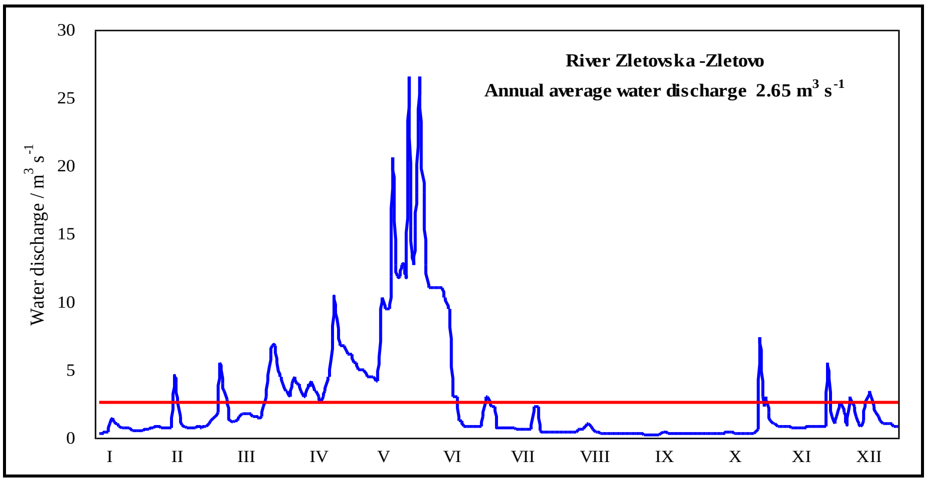


Figure 11. The Zletovska River water discharges ($m^3\ s^{-1}$) for the period from January 1st to December 31st 2012 at hidrological station Zletovo.

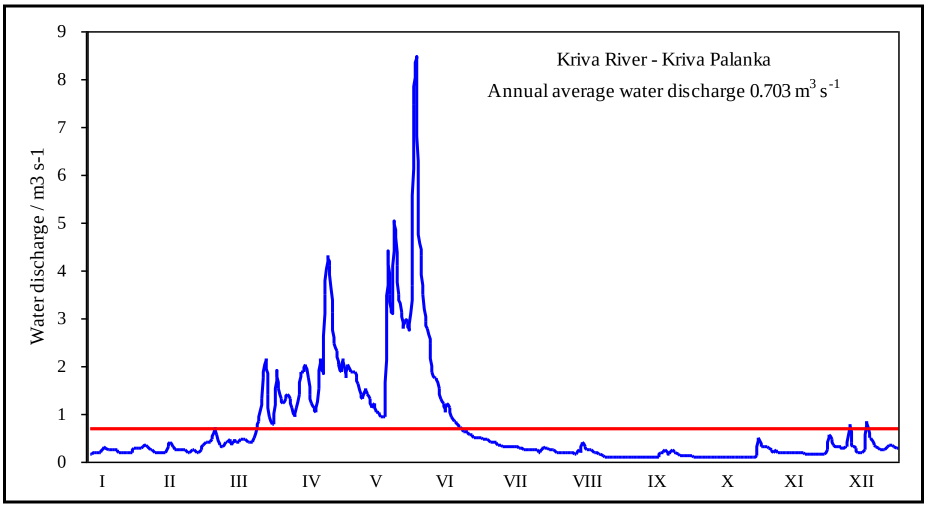


Figure 12. The Kriva River water discharges ($m^3\ s^{-1}$) for the period from January 1st to December 31st 2012 at hidrological station Kriva Palanka.

3.3.4. Determination of physico-chemical parameters

For the determination of physico-chemical parameters, standardized ISO, American Public Health Association (APHA), and United States Environmental Protection Agency (USEPA) methods were used.

Figure 13. On site measurements of physico-chemichal parameters.

Several physicochemical parameters, such as temperature, pH, conductivity, and dissolved oxygen (DO), were measured on site with a portable meter (WTW Multi 340i/SET; Germany) (Fig. 13) according to the following methods:

- temperature was determined according to APHA AWWA 2550 B;
- pH was determined according to ISO 10523:1994;
- DO was determined according to APHA 4500-O G;
- alkalinity was determined with titration method using acid (HCl) according to ISO 9963-1:1994; and
- conductivity was determined according to ISO 7888:1985.

Several other physicochemical parameters were measured subsequently in the river water samples in the laboratory by the following methods:

- turbidity was determined with nephelometric method (APHA 2130 B);
- total dissolved solids (TDS) were determined with gravimetric method according to APHA2540 C;
- COD_{KMnO4} (mg O_2 L^{-1}) was determined with titrimetric method according to ISO 8467:1993;
- total hardness (°dH) was determined with EDTA titrimetric method (APHA 2340 C);
- NO_3^- (mg N L^{-1}) was determined with spectrophotometric method (AQUANAL®-plus test);
- NO_2^- (mg N L^{-1}) was determined with spectrophotometric method (APHA 4500-NO_2^- B);
- NH_4^+ (mg N L^{-1}) was determined with spectrophotometric method (APHA 4500-NH_3 D; ISO 7150/1);
- total N (TN; mg N L^{-1}) was determined with spectrophotometric method (DIN EN ISO 11905-1);
- PO_4^{3-} (mg PO_4^{3-} L^{-1}) was determined with ascorbic acid method (APHA 4500-P E);
- total P (TP; mg P L^{-1}) was determined with ascorbic acid method (APHA 4500-P E);
- SO_4^{2-} (mg L^{-1}) was determined with $Ba(ClO_4)_2$ titration method (APHA 4500-SO_4^{2-});
- Cl^- (mg L^{-1}) was determined with ferricyanide colorimetric method (EPA 325.2 Cl^-).

3.3.5. Dissolved metals in the river water

Dissolved trace elements were measured directly in the filtered and acidified river water samples, whereas macro elements (Na, K, Ca, Mg) were measured in 10 times diluted filtered and acidified samples, due to their higher concentrations. The measurements of both trace and macro elements were performed on high resolution inductively coupled plasma mass spectrometer (HR ICP-MS, Element 2, Thermo Finnigan, Germany), equipped with an autosampler ASX 510 (CETAC Technologies, USA). Measurements of ^{7}Li, ^{85}Rb, ^{98}Mo, ^{111}Cd, ^{120}Sn, ^{121}Sb, ^{133}Cs, ^{205}Tl, ^{208}Pb, and ^{238}U were operated in low resolution mode, ^{23}Na, ^{24}Mg, ^{42}Ca, ^{47}Ti, ^{51}V, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{86}Sr, and ^{138}Ba in medium resolution mode, whereas ^{39}K and ^{75}As were measured in high resolution mode. Indium (1 µg L^{-1}; indium atomic spectroscopy standard solution, Fluka, Germany) was added to the samples as an internal standard (Dautović, 2006; Fiket et al., 2007). The external calibration was performed using adequately diluted standard solutions prepared from multielement stock standard solution for trace elements (100 mg L^{-1}, Analitika, Czech Republic) in which single element standard solutions of Sb and Sn (1 g L^{-1}; Analytika, Czech Republic), Rb and U (1 g L^{-1}; Sigma-Aldrich, Germany), and Cs (1 g L^{-1}; Fluka, Germany) were added. Separate external calibration was made using standard solution for macro elements (Ca 2.0 g L^{-1}; Mg 0.4 g L^{-1}; Na 1.0 g L^{-1}; K 2.0 g L^{-1}; Fluka, Germany). All standard solutions were prepared in 1.3% HNO_3 (Suprapur, Merck, Germany). Measurements were also performed in filtration blanks (Milli-Q water filtered and acidified in the same way as the samples of the river water). If necessary, the blank corrections of measured trace element concentrations in the river water were made. The accuracy of metal determination was controlled with 100 times diluted quality control sample for trace metals (QC Trace Metals, Catalog number 8072, Lot Number 146142-146143, UNEP GEMS, Burlington, Canada) and with 10 times diluted quality control sample for macro elements (QC Minerals, Catalog number 8052, Lot Number 146138-146139, UNEP GEMS, Burlington, Canada). The limits of detection for trace element measurement in the filtered river water were the following (in µg L^{-1}): As (0.005), Ba (0.020), Cd (0.001), Co (0.001), Cs (0.001), Cu (0.010), Fe (0.100), Li (0.010), Mn (0.010), Mo (0.020), Ni (0.020), Pb (0.020), Rb (0.010), Sb (0.001), Sn (0.001), Sr (0.020), Ti (0.020), Tl (0.002), U (0.001), V (0.002), and Zn (0.100) (Dautović, 2006; Roje, 2008).

3.3.6. **Fish sampling and organ dissection**

Fish were sampled by electro fishing, applying the electrofisher Samus 725G and following the guidelines stated in the standard MKC EN 14011:2009. Opaque plastic reservoir (Fig. 14) was used for transport of sampled fish to the laboratory and for keeping them alive in aerated river water, which was taken from each respective sampling point.

Figure 14. Transportation of the sampled fish.

For the purposes of this study, 158 individuals of Vardar chub (*S. vardarensis* Karaman) (Fig. 15a,b) were caught. In the spring campaign 90 individuals were caught, 30 from each river, whereas in the autumn campaign 68 individuals were caught, 30 from the Bregalnica River, 26 from the Kriva River, and 12 from the Zletovska River.

Fish were anesthetized with Clove Oil (Sigma Aldrich, USA) prior to killing and dissection. Then followed measurements of fish total lengths (TL) (cm) and body masses (BM) (g), with and without gonads, to avoid the influence of the gonad development on the measured parameters, based on which Fulton condition indices (FCI) were calculated using the formula from Rätz and Lloret (2003), FCI = BM×100/TL.

After these measurements and visual assessment of external gross lesions were completed, fish were killed and opened (Fig. 15b), and then macroscopically inspected for abnormalities of visceral organs. Liver, gills and gonads were then dissected, the organ masses were measured, and organosomatic indices calculated. Gonadosomatic indices (GSI) were calculated according to Şaşi (2004), as ratios of gonad masses to total body masses, multiplied with 100 (GSI=GM×100/BM). Hepatosomatic indices (HSI) were calculated according to the following formula: HSI= LM×100/BM, where LM refers to liver mass.

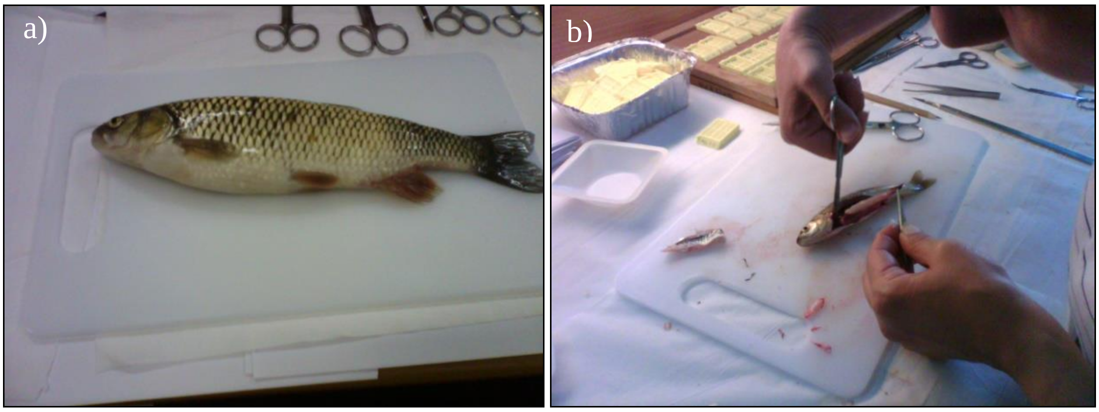


Figure 15. a) Vardar chub (*Squalius vardarensis* Karaman), and b) dissection of Vardar chub organs.

Fish sex was determined histologically using samples of gonad tissues which were put in Bouin's fixative (Merck, Germany). The liver and the gills were stored at -80ºC for later trace and macro element analyses, whereas liver and gonad tissues for histological analyses were cut into ~3 mm thick slabs.

3.3.7. Homogenization of Vardar chub livers and gills and isolation of soluble cytosolic fractions

Isolation of soluble cytosolic fractions from liver and gills of Vardar chub was performed according to Standard Operational Procedure (1999), which was developed in the framework of the Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM) (Dragun et al., 2009). Frozen samples of Vardar chub liver and gills were chopped up inside cooled glass dishes, and then cooled homogenization buffer [100 mM Tris-HCl/Base (Sigma, pH 8.1 at 4 ºC) containing the reducing agent (1 mM dithiothreitol, Sigma)] was added into the dishes (w/v 1:5). Obtained suspensions were homogenized in glass tube, which was cooled on ice, applying 10 strokes of PTFE-coated pestle at 6,000 rpm (Potter-Elvehjem homogenizer, Glas-Col, USA). The homogenates were afterwards centrifuged for 2 h in the Avanti J-E centrifuge (Beckman Coulter, USA) at 50,000×g and +4 ºC. Supernatants obtained after centrifugation represented soluble cytosolic fractions of Vardar chub liver and gills, which contained cytosolic biomolecules, as well as microsomes and lysosomes, whereas cell membranes, nuclei, mitochondria and granules were excluded from the samples (Bonneris et al., 2005; Podrug et al., 2009; Dragun et al., 2013a). Cytosolic fractions of Vardar chub liver and gills were then stored at -80 ºC for latter macro and trace element analyses.

3.3.8. Macro and trace element analyses in the cytosolic fractions of Vardar chub liver and gills

For macro and trace element analyses in the cytosolic fractions of Vardar chub liver and gills the method was the same as described for dissolved elements in the river water (section 3.3.5.). The differences referred to preparation of the samples for macro and trace element analyses in the cytosolic fractions of Vardar chub organs, as well as to analysed elements. Simple sample preparation for macro and trace element analyses was applied, consisting of dilution of cytosolic tissue fractions with Milli-Q water and acidification with HNO_3 (Suprapur®, Merck, Germany) (Dragun et al., 2013a). Dilution factors for Na, K, Mg, and Ca were 100, whereas for all the remaining elements they were 10. Final concentration of HNO_3 in the samples was 0.65%. The samples were prepared for measurement in duplicate.

Measured elements were ^{82}Se, ^{85}Rb, ^{98}Mo, ^{111}Cd, ^{133}Cs, ^{205}Tl, and ^{208}Pb (low resolution mode), ^{23}Na, ^{24}Mg, ^{42}Ca, ^{51}V, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{63}Cu, ^{66}Zn, ^{86}Sr, and ^{138}Ba (medium resolution mode), and ^{39}K (high resolution mode).

Same as in the case of the river water analyses, accuracy control of macro and trace element measurements was performed using quality control samples acquired from UNEP/GEMS (QC trace metals, catalogue no. 8072, lot no. 146142-146143; QC minerals, catalogue no. 8052, lot no. 146138-146139; Burlington, Canada). Ten measurements in control samples were carried out and the following recoveries (%) were obtained: Ba (97.2 ± 3.0), Ca (100.2 ± 2.5), Cd (97.5 ± 1.8), Co (98.4 ± 2.2), Cu (98.2 ± 1.8), Fe (104.1 ± 6.3), K (96.7 ± 11.5), Mg (97.1 ± 2.3), Mn (97.6 ± 1.4), Mo (96.4 ± 2.9), Na (100.1 ± 2.5), Pb (97.5 ± 3.3); Se (100.9 ± 6.7), Sr (99.9 ± 2.9), Tl (100.4 ± 2.3), and V(99.3 ± 1.8). Limits of detection (LOD) were calculated as three standard deviations of ten successive measurements of macro and trace element concentrations in the blank samples (adequately diluted 100 mM Tris-HCl/Base supplemented with 1 mM dithiotreitol). The following LODs (mg L^{-1}) were determined for macro elements in the hepatic and gill cytosolic fractions: Na, 1.23; K, 0.216; Mg, 0.047; and Ca, 1.05. The following LODs (μg L^{-1}) were determined for trace elements in the hepatic and gill cytosolic fractions: Ba, 0.471; Cd, 0.013; Co, 0.001; Cs, 0.009; Cu, 0.438; Fe, 1.46; Mn, 0.088; Mo, 0.044; Pb, 0.120; Rb, 0.179; Se, 0.378; Sr, 0.451; Tl, 0.020; V, 0.015; and Zn, 95.0 (Dragun et al., 2019). All concentrations measured in Vardar chub liver and gills in this study were presented as mg L^{-1}or μg L^{-1} of cytosolic tissue fractions.

3.3.9. Histopathological assessment of Vardar chub liver and gonads

Pieces of liver and gonads were immersed in Bouin's fixative for at least 48 h. After fixation, tissues were routinely processed to paraffin wax blocks, cut in 5 μm thick serial sections and stained with haematoxylin and eosin. Five sections randomly taken at various locations throughout the liver were examined applying light microscopy (Nicon microscope). To obtain objective analyses, all slides were coded, so the analyst did not have previous knowledge of the capture location for each specific fish that was being analyzed. Toxicopathic hepatic lesions were diagnosed according to the histopathology criteria previously described for the other fish species (Myers et al., 1987, 1992; Hinton et al., 1992; Hinton, 1993; Wolf and Wolfe, 2005; Blazer et al., 2006; Rebok 2013).

Gonads were analyzed for determination of the sex and breeding stage, and also for detection of eventual reproductive disorders, such as presence of intersex.

The histopatplogical evaluation of the gonads was primarily intended to monitor the presence of intersex. Fish could be identified as having intersex when individual small foci of undeveloped oocytes were observed within the testis or when germinal male cells were observed within the ovary.

3.3.10. Data processing and statistical analyses

Statistical program SigmaPlot 11.0 for Windows was applied for graph creation and statistical analyses. In the case of water analyses, due to the small number of data several nonparametric statistical tests were applied. Kruskal-Wallis one way analysis of variance on ranks with post hoc Dunn’s test was used for comparison of trace and macro element concentrations measured in three rivers, separately for each sampling. Mann-Whitney rank sum test was used for comparison of the results obtained during spring and autumn, separately for each site. The differences were considered statistically significant when p was lower than 0.05.

The data analyses for bioaccumulation of metals in the cytosolic fractions of Vardar chub were performed using SAS Studio from SAS University Edition® software. In order to explore the time-spatial distribution patterns and compare response group means among representative rivers and different sampling periods, analysis of covariance (ANCOVA) was performed after adjusting for the covariate - fish mass. As a data pre-treatment, natural log transformation (ln(x)) was used to meet the fundamental assumptions required by the model, namely normality and variance homogeneity, but also this transformation improves data linearity. The continuous covariate variable was grand-mean centred (Hofmann and Gavin, 1998; Enders and Tofighi, 2007) in order to ensure numerical stability of the model. The ANCOVA model was utilized using the PROC GLM procedure with REML estimation method and unstructured covariance (option type = UN in random statement) at the 0.05 level of significance. Mann-Whitney rank sum test was used for comparison of bioaccumulated trace and macro element concentrations during spring and autumn, separately for each river. The differences were considered statistically significant when p was lower than 0.05.

To assess the impact of metal pollution of the river water on the health status of Vardar chub, the software Statistica 7 for Windows was used. Differences were significant at $p<0.05$. Two sided t-test for proportions was used to find out if significant diffrences existed in the lesion prevalence (i.e., percentage of the affected fish) between the sampling sites. ANOVA was used to determine differences between the sampling points, after cheking for normality and homogeneity of variances of the data sets. Whenever the ANOVA indicated significant differences, statistical comparisons between pairs were performed using the post-hoc Newman Keuls test. For biometric parameters, differences between the groups regarding the non parametric data derived from semi-quantitative analyses were first evaluated by the Kruskal-Wallis' ANOVA, and followed by using the Mann-Whitney's test whenever the ANOVA revealed significant differences.

4. RESULTS

4.1. SURFACE WATER CHARACTERIZATION OF THE SELECTED RIVERS

4.1.1. Spatial variations

4.1.1.1. Physico-chemical and hydrological parameters

The complete overview of general physico-chemical and hydrological parameters for three rivers in two sampling periods is given in Table 3, but several changes characteristic for particular watercourses should be especially emphasized. The water temperature of the Bregalnica River, for example, was few degrees higher (2-6°C) compared to the other two rivers in both sampling periods. In the spring period, the difference between Bregalnica and the Kriva River was less pronounced, but this was due to later sampling date at the Kriva River. Except for the increase of the water temperature at Bregalnica, all the other deviations of the physico-chemical parameters from the recommendable values (Table 3) were mainly observed at the Zletovska River. The river water of the Zletovska River was characterized with slightly acidic pH, positive redox potential (pE), very high conductivity and total dissolved solids (TDS), as well as the highest alkalinity and total water hardness in both sampling periods. The Bregalnica River and the Kriva River were characterized by slightly alkaline pH and negative redox potential (pE), TDS were rather low in the spring samples, but they somewhat increased in autumn, and the levels of other parameters were much lower compared to the Zletovska River, in both periods (Table 3). The concentrations of dissolved oxygen were around the saturation levels, whereas turbidity was higher than recommendable (Table 3) in all three rivers, in both sampling periods. During the spring the water discharge of the Zletovska River was higher (3-10 times) compared to the other two rivers, while in autumn the water discharge of all three rivers was generally very low.

Table 3. General physico-chemical and hydrological parameters determined in the river water of the Bregalnica River, the Zletovska River and the Kriva River in the spring and autumn periods of 2012.

	Bregalnica River		**Zletovska River**		**Kriva River**	
	Spring	**Autumn**	**Spring**	**Autumn**	**Spring**	**Autumn**
T (ºC)	19.8	17.1	13.7	13.1	17.7	12.4
pH	8.12	8.11	6.88	6.52	7.90	8.02
Alkalinity (mg $CaCO_3$ L^{-1})	140.1	255.2	365.3	410.4	94.2	144.0
pE (mV)	-68	-63	5	28	-54	-57
Turbidity (NTU)	16	6	27	3	7	10
Conductivity (μS cm^{-1})	390	595	1490	2020	211	347
TDS (mg L^{-1})	316	510	987	1568	153	261
DO (mg O_2 L^{-1})	10.38	9.40	9.17	8.23	8.87	8.97
COD_{KMnO4} (mg O_2 L^{-1})	2.96	0.89	6.08	0.40	2.24	4.84
Total hardness (ºdH)	10.43	14.27	37.60	70.00	5.92	8.62
Water discharge (m^3 s^{-1})	2.90	1.60	9.51	0.424	0.933	0.099

4.1.1.2. Nutrients and anions

The increase of total nitrogen and nitrogen based nutrients was generally found more pronounced at the Bregalnica and Kriva rivers compared to the Zletovska River (Table 4). The increase of NH_4^+ and total nitrogen was especially pronounced in the water of the Kriva River in the autumn sampling. Similar finding was made for phosphates and total phosphorus, with slight increase at both Bregalnica and the Kriva River, and marked increase only at the Kriva River in October. Contrary, both sulphates and chlorides were much higher at the Zletovska River compared to Bregalnica and the Kriva River, in both sampling periods, and especially in autumn.

Table 4. The concentrations of nutrients and other anions determined in the river water of the Bregalnica River, the Zletovska River and the Kriva River in the spring and autumn periods of 2012.

	Bregalnica River		**Zletovska River**		**Kriva River**	
	Spring	**Autumn**	**Spring**	**Autumn**	**Spring**	**Autumn**
NO_3^- (mg N L^{-1})	0.639	1.131	0.364	0.033	0.596	0.744
NO_2^- (mg N L^{-1})	0.014	0.037	0.0001	0.0013	0.008	0.097
NH_4^+ (mg N L^{-1})	0.053	0.096	0.019	0.123	0.360	2.18
TN (mg N L^{-1})	0.90	1.90	1.50	0.50	0.95	5.10
PO_4^{3-} (mg PO_4^{3-} L^{-1})	0.230	0.188	0.028	0.044	0.175	0.916
TP (mg P L^{-1})*	<0.5	0.089	<0.5	0.032	<0.5	0.325
SO_4^{2-} (mg L^{-1})	53.78	44.47	453.2	883.9	29.75	25.02
Cl^- (mg L^{-1})	10.18	18.72	20.62	43.83	4.89	12.13

*For total phosphorus determination, Spectroquant phosphate cell test 114729 was used during the spring period, whereas Spectroquant phosphate test 114848 was used in the autum period; these two tests have different detection limits, which is the reason for the different manner of expressing the results for the two seasons.

4.1.1.3. Macro and trace elements

The complete overview of the concentrations of four dissolved macro elements and 21 dissolved trace element in the river water of three rivers in the north-eastern part of North Macedonia in two sampling periods is presented in Table 5 and Table 6, respectively. In both sampling periods, the most pronounced increase of four macro elements (Na, K, Ca, and Mg) was observed in the Zletovska River, whereas the lowest values were obtained in the Kriva River (Table 5, Fig. 16).

Table 5. The concentrations (mg L^{-1}) of dissolved macro elements (Na, K, Ca, and Mg) (mean ± standard deviation) determined in the river water of the Bregalnica River, the Zletovska River and the Kriva River in the spring and autumn periods of 2012.

	Bregalnica River		**Zletovska River**		**Kriva River**	
	Spring	**Autumn**	**Spring**	**Autumn**	**Spring**	**Autumn**
Na (mg L^{-1})	18.37±0.08	39.29±1.18	79.11±0.45	117.35±1.98	4.76±0.07	10.67±0.05
K (mg L^{-1})	5.88±0.119	5.93±0.11	17.08±0.45	18.09±0.36	1.88±0.16	3.56±0.14
Ca (mg L^{-1})	47.33±0.36	73.09±1.42	197.45±0.58	332.20±5.99	27.99±0.29	41.82±1.26
Mg (mg L^{-1})	11.49±0.17	21.89±0.42	43.49±0.45	70.36±1.97	5.91±0.02	9.59±0.06

Table 6. The concentartions (μg L^{-1}) of dissolved trace elements (mean ± standard deviation) determined in the river water of the Bregalnica River, the Zletovska River and the Kriva River in the spring and autumn periods of 2012.

	Bregalnica River		**Zletovska River**		**Kriva River**	
	Spring	**Autumn**	**Spring**	**Autumn**	**Spring**	**Autumn**
As (μg L^{-1})	2.69±0.02	2.60±0.10	0.674±0.012	0.398±0.041	0.855±0.015	0.758±0.028
Ba (μg L^{-1})	55.97±0.96	79.05±7.12	5.16±0.88	8.07±0.35	28.89±0.12	37.29±0.99
Cd (μg L^{-1})	0.039±0.008	0.021±0.019	0.272±0.002	2.012±0.036	0.270±0.010	0.029±0.011
Co (μg L^{-1})	0.095±0.001	0.078±0.007	1.51±0.01	0.841±0.033	0.053±0.005	0.074±0.003
Cs (μg L^{-1})	0.067±0.002	0.091±0.002	1.300±0.008	1.03±0.018	0.026±0.001	0.015±0.000
Cu (μg L^{-1})	2.16±0.36	1.07±0.26	3.38±0.07	3.16±0.23	1.21±0.21	1.37±0.195
Fe (μg L^{-1})	61.28±3.37	37.59±2.58	14.18±5.06	5.90±0.54	11.57±0.69	25.67±0.384
Li (μg L^{-1})	5.46±0.10	14.50±0.06	47.58±0.41	67.23±2.79	1.26±0.014	2.60±0.014
Mn (μg L^{-1})	13.27±0.79	4.40±0.58	351.9±6.5	2527.5±219.01	9.90±1.88	9.65±0.957
Mo (μg L^{-1})	0.950±0.010	1.071±0.026	0.303±0.058	0.136±0.079	0.442±0.016	0.476±0.033
Ni (μg L^{-1})	0.561±0.034	0.462±0.031	1.33±0.13	4.57±0.21	0.318±0.022	0.765±0.627
Pb (μg L^{-1})	0.692±0.096	0.307±0.216	0.313±0.044	0.748±0.10	1.84±0.22	0.420±0.031
Rb (μg L^{-1})	2.03±0.01	3.20±0.03	22.75±0.20	25.87±0.55	0.844±0.008	1.616±0.023
Sb (μg L^{-1})	0.284±0.001	0.082±0.003	0.103±0.003	0.141±0.002	0.525±0.007	0.584±0.013
Sn (μg L^{-1})	0.029±0.020	0.017±0.004	0.023±0.003	0.032±0.002	0.004±0.001	0.014±0.002
Sr (μg L^{-1})	348.7±2.1	514.8±28.14	2796.2±11.6	4307.40±542.4	121.3±1.0	182.17±6.18
Ti (μg L^{-1})	0.513±0.059	0.065±0.035	0.046±0.004	0.026±0.021	0.052±0.003	0.153±0.168
Tl (μg L^{-1})	0.014±0.000	0.008±0.001	0.043±0.001	0.146±0.002	0.006±0.000	0.003±0.001
U (μg L^{-1})	2.05±0.01	2.39±0.08	0.411±0.010	0.074±0.002	0.575±0.004	0.496±0.008
V (μg L^{-1})	1.13±0.03	0.838±0.046	0.059±0.005	0.029±0.001	0.220±0.006	0.386±0.011
Zn (μg L^{-1})	4.97±1.84	6.14±8.51	197.0±2.95	1506.6±138.09	22.07±6.86	3.81±2.84

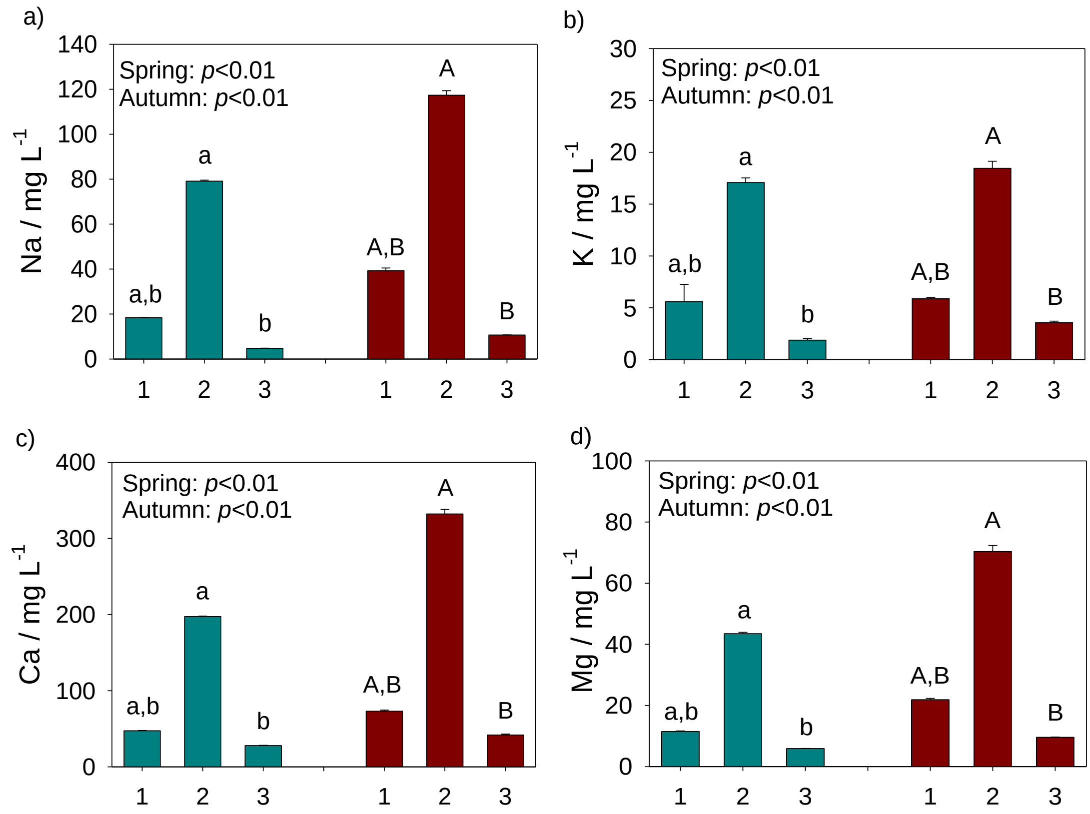


Figure 16. The concentrations (mg L^{-1}) of dissolved macro elements (a) Na, b) K, c) Ca, and d) Mg) in the river water of three rivers in the north-eastern part of North Macedonia (1 – Bregalnica River; 2 – Zletovska River; 3 – Kriva River). The results are presented as error bars (bar – mean value; error – standard deviation; based on three samples). The statistically significant differences between sites according to Kruskal-Wallis one way analysis of variance on ranks (levels of significance indicated in the figure) followed by *post-hoc* Dunn's test (level of significance set at $p<0.05$) are indicated by different letters (lower case letters for spring, and upper case letters for autumn). Legend: green bars – spring; red bars – autumn.

Several trace elements were found in higher concentrations even in the river water of the reference Bregalnica River compared to other two rivers in both periods, namely As, Ba, Fe, Mo, Ti, U, and V (Table 6, Fig. 17). However, the majority of elements were found in exceptionally high concentrations in the water of the Zletovska River (Cd, Co, Cs, Cu, Li, Mn, Ni, Pb, Rb, Sn, Sr, Tl, and Zn; Table 6, Fig. 18 and 19a), also in both periods. The Kriva River was characterized by the increase of the same elements as Bregalnica (As, Ba, Fe, Mo, Ti, U, and V), but less pronounced. Except for Fe and Ti, which were increased only in autumn, the other elements were present in increased concentrations in both periods (Table 6, Fig. 17). In addition, prominent increase of Cd and Pb in the Kriva River was also observed, but only in the spring period (Table 6, Fig. 18a and Fig. 19a), whereas slightly increased concentrations of Sb were measured in both periods (Table 6, Fig. 19b).

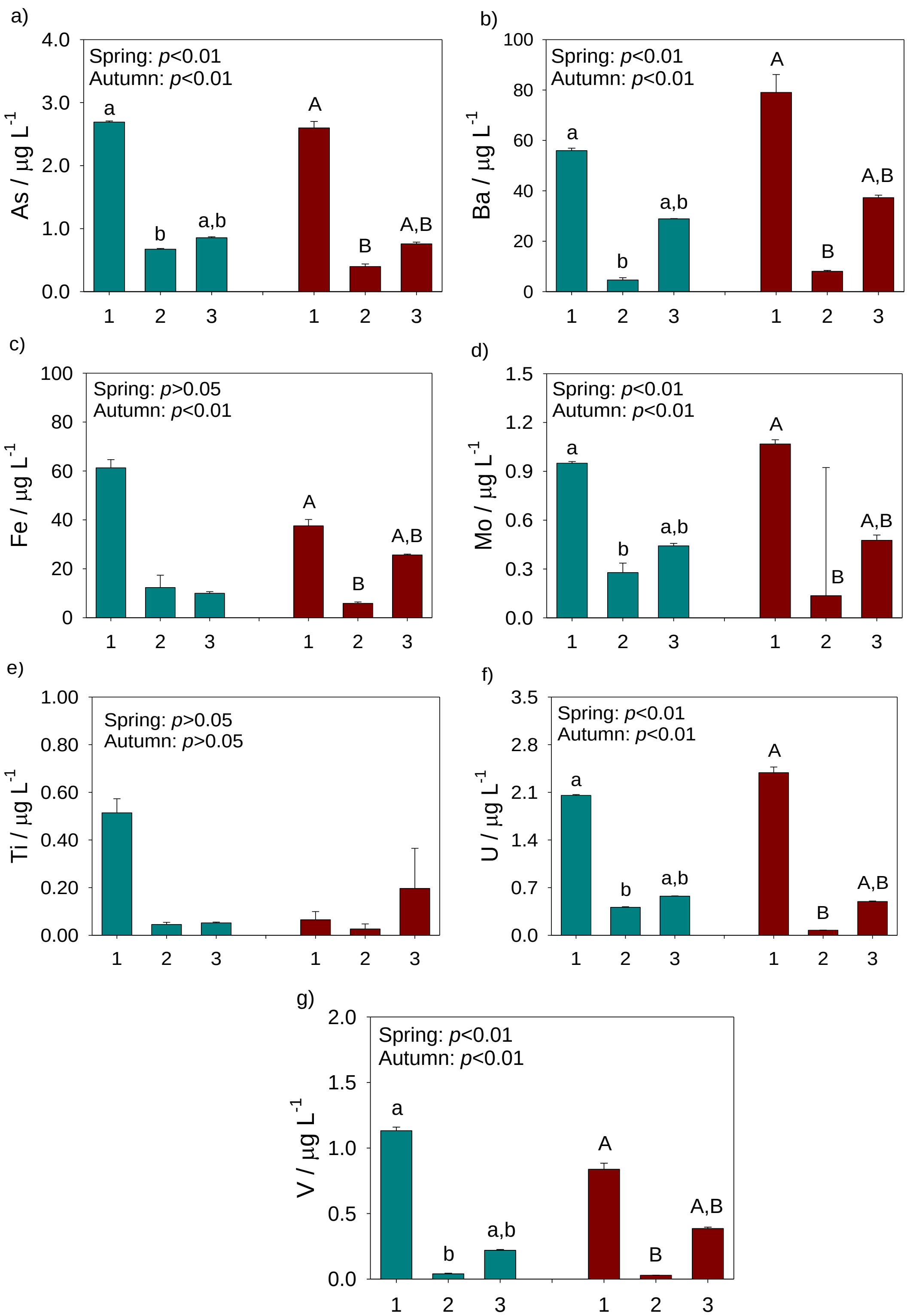


Figure 17. The concentrations (µg L^{-1}) of dissolved trace elements in the river water of three rivers in the north-eastern part of North Macedonia (1 – Bregalnica River; 2 – Zletovska River; 3 – Kriva River). This figure presents trace elements which are generally increased in the Bregalnica River (a) As, b) Ba, c) Fe, d) Mo, e) Ti, f) U, and g) V). The results are presented as described in the caption of Fig. 16.

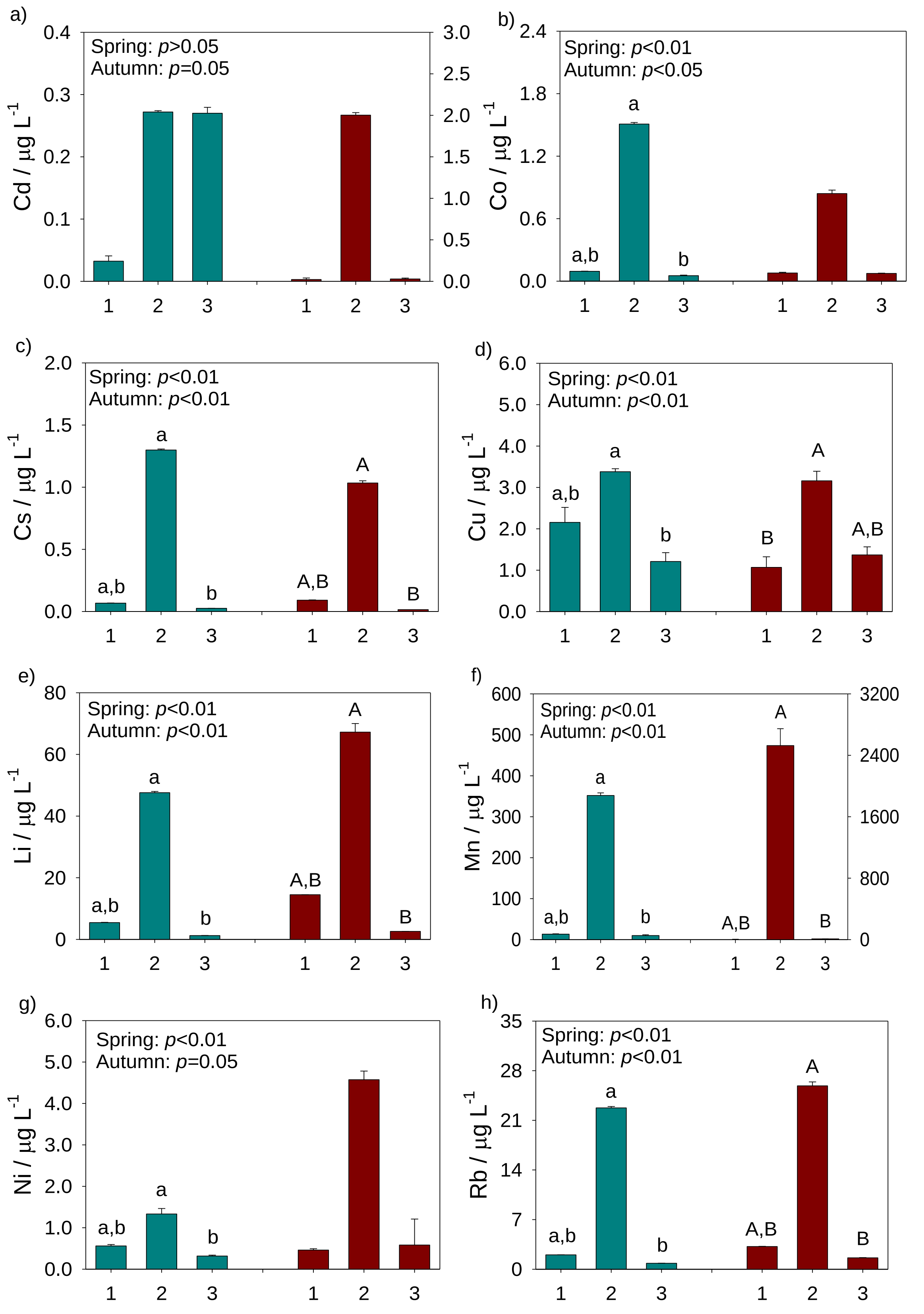


Figure 18. The concentrations (µg L^{-1}) of dissolved trace elements in the river water of three rivers in the north-eastern part of North Macedonia (1 – Bregalnica River; 2 – Zletovska River; 3 – Kriva River). This figure presents trace elements which are generally increased in the Zletovska River (a) Cd, b) Co, c) Cs, d) Cu, e) Li, f) Mn, g) Ni, h) Rb), (i) Sn, j) Sr, k) Tl, and l) Zn). The results are presented as described in the caption of Fig. 16.

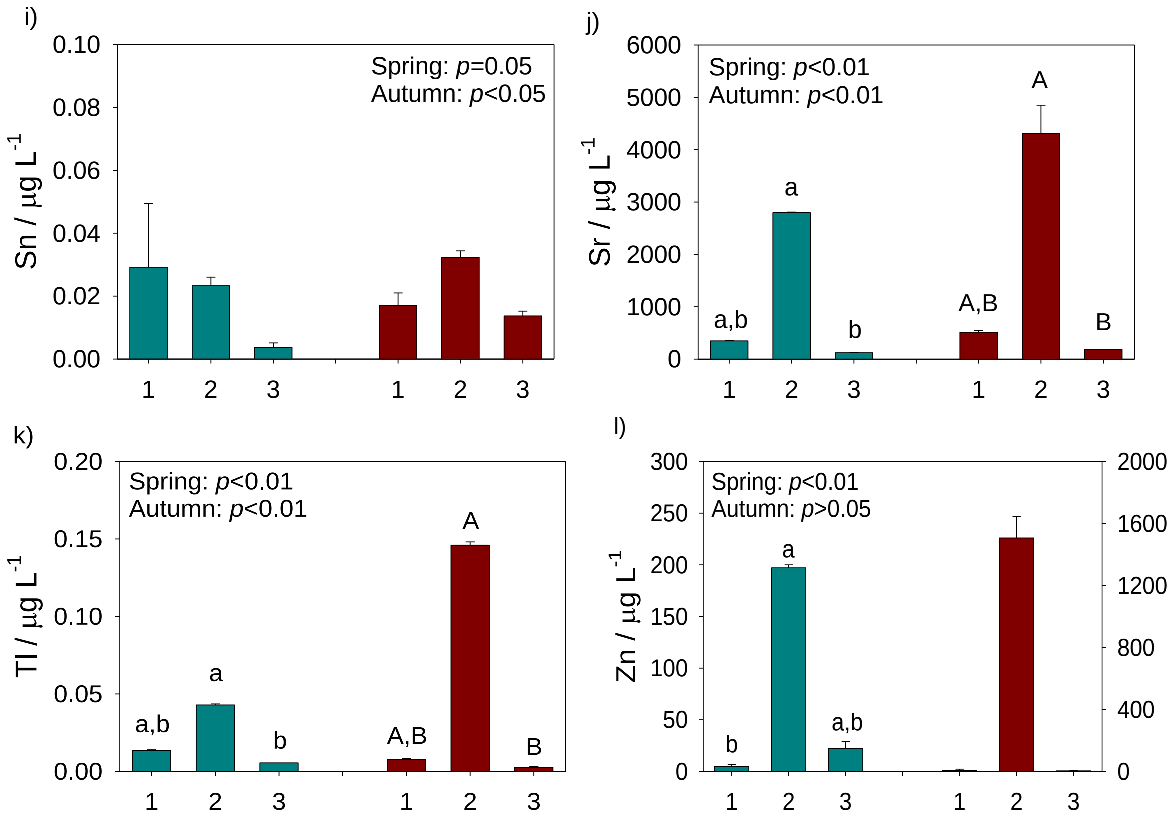


Figure 18. – continued. The concentrations (µg L^{-1}) of dissolved trace elements in the river water of three rivers in the north-eastern part of North Macedonia (1 – Bregalnica River; 2 – Zletovska River; 3 – Kriva River). This figure presents trace elements which are generally increased in the Zletovska River, (a) Cd, b) Co, c) Cs, d) Cu, e) Li, f) Mn, g) Ni, h) Rb), (i) Sn, j) Sr, k) Tl, and l) Zn). The results are presented as described in the caption of Fig. 16.

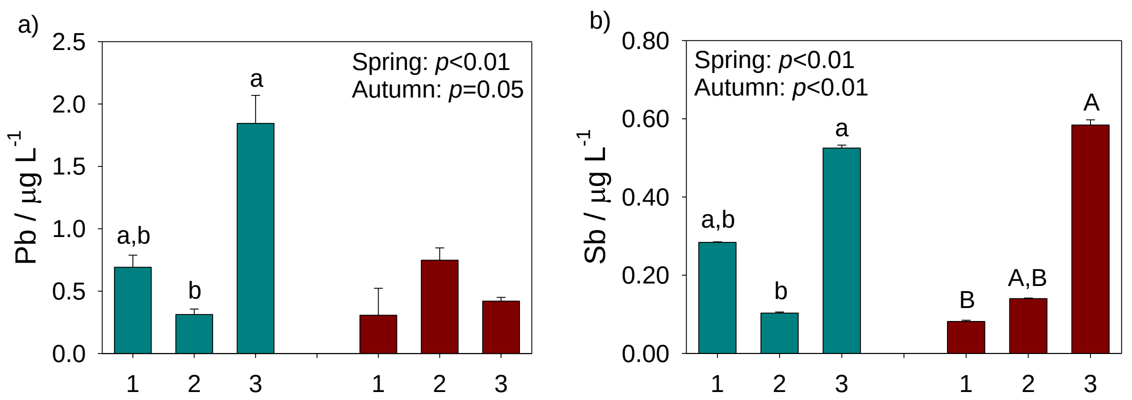


Figure 19. The concentrations (µg L^{-1}) of dissolved trace elements in the river water of three rivers in the north-eastern part of North Macedonia (1 – Bregalnica River; 2 – Zletovska River; 3 – Kriva River). This figure presents trace elements which are generally increased in the Kriva River (a) Pb and b) Sb). The results are presented as described in the caption of Fig. 16.

4.1.2. Seasonal variability

4.1.2.1. Physico-chemical parameters, nutrients and anions

The water discharge differed between sampling periods. In the spring sampling the water discharge of all three rivers was approximately 2-20 times higher compared to autumn, when it was close to the annual minimum (Table 3). Although this study encompassed two distinct sampling periods, both sampling periods were characterized by similar, moderate, temperature range. Therefore, the water temperature was mainly comparable, and differences between sites were more pronounced than the differences between sampling periods (Table 3). Comparable values in both seasons were also found for pH, pE and DO. The other physico-chemical parameters differed between sampling periods. Higher values in the autumn period were observed for conductivity, alkalinity, TDS and total hardness. Contrary, COD_{KMnO4} and turbidity were higher in the spring period, but only in the Bregalnica and Zletovska River, whereas the values measured in the Kriva River were higher in the autumn (Table 3). Nutrient and anion concentrations were also found generally increased during the autumn season (Table 4). Especially increased autumn concentrations were found for nitrogen and phosphorus based nutrients in the Bregalnica and Kriva River, and for sulphates in the Zletovska River.

4.1.2.2. Macro and trace elements

Higher concentrations in the spring period were observed for Cu, Mn, Ti and Pb in the Bregalnica River (2-8 times higher compared to autumn) (Table 6, Fig. 18d, f, Fig. 17e and Fig. 19a). Similar situation was seen in the Zletovska River, but only for Fe and U (2 and 5.5 times higher spring than autumn concentrations, respectively) (Table 6, Fig. 17c, f). In the Kriva River, the concentrations of Cd, Pb and Zn were also prominently higher in the spring than autumn (4.5-9.5 times) (Table 6, Fig. 18a, Fig. 19a and Fig. 18l). In the autumn period, the concentration increase of Cd, Tl, Pb, Mn, Ni, Zn (2-7.5 times higher autumn than spring concentrations) was evident in the Zletovska River (Table 6, Fig. 18a, k, f, g, l and Fig. 19a). The observed differences between the spring and the autumn sampling were not statistically significant ($p>0.05$), due to small number of data per site within each period (n=3).

4.2. BIOMETRIC DATA OF VARDAR CHUB AND ORGANOSOMATIC INDICES

Vardar chub (*Squalius vardarensis*) was selected as bioindicator organism for the study of metal/metalloid bioaccumulation after the exposure in the mining impacted rivers. Ten biometric parameters were determined as indicators of biological status of Vardar chub caught in spring (May/June) and autumn (October) of 2012 in three rivers in the northeastern part of North Macedonia: the Bregalnica River, the Zletovska River and the Kriva River. Determined parameters included sex, total length, total mass and masses of several organs (liver, gills and gonads), as well as Fulton condition index (FCI) and organosomatic indices (hepatosomatic (HSI), gonadosomatic (GSI) and gill index).

In Table 7, the distribution of sex among three sampling sites is shown for two seasons. The ratio of females to males (F:M) in spring in the Bregalnica River was 1:1.1; while in autumn it was 1.3:1; in the Zletovska River in spring it was 1.7:1, while in autumn it was 1.4:1; in the Kriva River in both seasons the ratio was approximately 1:2. In total, in the spring season 43 chub were female and 47 males, while in autumn 33 chub were females and 35 chub were males, so the total number of females and males in each season was almost identical.

Table 7. The number of the collected Vardar chub regarding the sampling site, season and sex in the Bregalnica River, the Zletovska River and the Kriva River in the spring and autumn periods of 2012.

Season	Spring			Autumn		
Sex	F+M	F	M	F+M	F	M
	n	n (%)	n (%)	n	n (%)	n (%)
Bregalnica River	30	14 (46.7)	16 (53.3)	30	17 (56.7)	13 (43.3)
Zletovska River	30	19 (63.3)	11 (36.7)	12	7 (58.3)	5 (41.7)
Kriva River	30	10 (33.3)	20 (66.7)	26	9 (34.6)	17 (65.4)

F-female, M-male, n-number of Vardar chub specimens, % - percentage of Vardar chub specimens

The obtained results for all biometric parameters are presented in Table 8 for each studied river in each season, for females and males together, shown as medians, minimums and maximums**.** In Table 9, a summary is presented of the influences of selected natural factors (locality, season, sex), and of their interactions, on all biometric parameters, based on the ANOVA analyses. In the following five tables (10 to 14) the biometric data are presented separately for females and males, for each river in each season.

To minimize the effects of the reproductive cycle on the biometric parameters, total fish masses and organosomatic indices corrected for the gonad masses were also calculated, and the statistical analyses were performed on both crude and corrected data.

In general, females of Vardar chub were heavier than males. Specifically, females from the Bregalnica and Kriva rivers in spring were statistically significantly heavier than males, while in autumn this trend was observed only in the Bregalnica River (Table 10; Figs. 20 and 21). Concerning the localities, it was observed that Vardar chub varied significantly in mass among three rivers. The chub from the Zletovska River were smaller compared to the other two rivers in spring, both female and male, but the differences were statistically significant only for females. On the other hand, in the autumn season chub from both Zletovska and Kriva rivers, both female and male, were smaller compared to chub from Bregalnica, but the differences were again statistically significant only for females (Table 10;

Figs. 20 and 21). Seasonal differences were observed only in female Vardar chub from the Kriva River, with higher values recorded in spring.

In accordance with masses, females from the Bregalnica and Kriva rivers were statistically significantly longer than males in spring, while in autumn this trend was observed at the Bregalnica and Zletovska rivers (Table 11; Fig. 22). Concerning the localities, it was observed that Vardar chub varied significantly in length among three rivers. In spring, both female and male chub from the Zletovska River were shorter compared to chub from the other two rivers, but the differences were statistically significant only for females. In autumn, male chub from the Zletovska River were statistically significantly shorter compared to both other rivers, while the female chub from both Zletovska and Kriva rivers were shorter compared to Bregalnica (Table 11; Fig. 22). Significant seasonal differences were observed only for females from the Kriva River and males from the Zletovska River, with higher values observed in spring.

The differences between females and males were generally not statistically significant at any sampling site in any season for FCI and were rather small, amounting from 2.4 to 10% (Table 11; Fig. 23). The exception was the Kriva River in spring, where females had statistically significantly higher FCI than males (Table 11; Fig. 23). FCI varied notably among three rivers, and both females and males in spring have shown lower values of FCI in the Zletovska River compared to both Bregalnica and Kriva River. For females, differences were statistically significant only in comparison to Kriva River, while for males compared to both Bregalnica and Kriva rivers. In autumn, both females and males again had the lowest FCI values at the Zletovska River. However, statistically significantly lower values compared to both Bregalnica and Kriva rivers were obtained only for females, while for males FCI was statistically significantly lower compared to Bregalnica not only at the Zletovska, but also at the Kriva River (Table 11; Fig. 23). Seasonal differences were observed in both females and males of Vardar chub at all sampling sites, with higher values recorded during the spring season. The only exceptions were the males from the Bregalnica River, for which the differences were not significant (Table 11; Fig. 23).

Regarding the gonad masses (GM) and gonadosomatic indices, it was observed that female Vardar chub from the Bregalnica and Kriva rivers in spring had statistically significantly higher GM than males, while only females from the Kriva River had statistically significantly higher GSI and GSI* than males (Table 12; Figs. 24, 25 and 26). In autumn, there were no observable differences between females and males in either of three parameters. All three parameters, GM, GSI and GSI,* varied significantly among three rivers only for females, and only in the spring, and the highest values were recorded at the Kriva River. Statistically significant seasonal differences were observed for GM, GSI and GSI* for female Vardar chub at the Bregalnica and Kriva rivers, and only for GSI and GSI* for males at all three sampling sites (Table 12; Figs. 24, 25 and 26), with higher values recorded in the spring.

Liver masses (LM) followed the trend of total masses of Vardar chub. Females had generally higher liver masses than males, and the differences were statistically significant at the Bregalnica and Kriva rivers in spring, and at the Bregalnica River in autumn (Table 13; Fig. 27). Liver masses varied significantly among sampling sites only in females. In spring, the lowest values were noticed at the Zletovska River, and the differences were statistically significant compared to both Kriva and Bregalnica rivers. In the autumn season, LM of female chub from both Zletovska and Kriva rivers were significantly lower compared to chub from the Bregalnica River. Seasonal differences were observed only in females of Vardar chub from the Kriva River, with significantly higher values recorded in spring (Table 13; Fig. 27).

Differences in HSI and HSI* between female and male Vardar chub were observed only in the spring season, with higher values in females at all three sites (Figs. 28 and 29), but the difference was statistically significant only at the Kriva River for HSI* (Table 13). Contrary, for both HSI and HSI,* statistically significant differences among sampling sites were observed only in autumn for both sexes, with the lowest values noticed in the Zletovska River (Table 13; Figs. 28 and 29) in comparison with the other two rivers. Significant seasonal differences for both HSI and HSI* were observed for both females and males only at the Zletovska River, with higher values recorded in spring (Table 13; Figs. 28 and 29).

In general, females of Vardar chub had higher gill masses than males. The differences were statistically significant for chub from the Bregalnica and Kriva rivers in spring, and the Bregalnica River in autumn (Table 14; Fig. 30). Gill masses of females in both seasons, and males in autumn, varied significantly among sampling sites. Significantly lower values were observed at the Zletovska River in spring compared to the other two rivers for females. In the autumn season, gill masses at both Zletovska and Kriva rivers were statistically significantly smaller compared to the Bregalnica River for females, while for males only the gill masses at the Zletovska River were significantly smaller compared to the Bregalnica River. Seasonal differences were observed only for gill masses of females at the Kriva River, with significantly higher values recorded in spring (Table 14; Fig. 30).

Concerning the gill index, the differences between females and males were generally not significant. The only statistically significant difference referred to higher gill indices of females than males in autumn at the Zletovska River (Table 14; Fig. 31). Furthermore, only the gill indices of males in autumn varied significantly among sampling sites, with lower values observed at the Zletovska River compared to the Bregalnica and Kriva rivers. Significant seasonal differences in gill indices were observed for females at the Bregalnica River, with higher values recorded in autumn, and for males at the Zletovska River with higher values recorded in spring (Table 14; Fig. 31).

The coefficients of variation (CVs) for several biometric parameters were very high, as follows: TM (33-88%), TM*(33-88%), GM (27-157%), GSI (22-64%), GSI* (25-67), LM (25-95%), HSI (13-59%) and HSI* (13-52%). For TL, CVs were much lower (12-29%), while for FCI they were extremely low (6-15%).

Table 8. The biometric parameters (median in the first row, minimum and maximum in the second row) determined for Vardar chub caught in the Bregalnica River, the Zletovska River and the Kriva River in the spring and autumn periods of 2012.

		Total mass / g	**Total length / cm**	**FCI / %**	**Gonad mass / g**	**GSI / %**	**Liver mass / g**	**HSI / %**	**Gill mass / g**	**Gill index / %**
Bregalnica River	**S**	86.82 13.86-307.89	18.59 10.90-29.90	1.16 0.95-1.39	5.898 0.247-26.82	6.16 1.14-11.97	1.231 0.302-5.50	1.38 0.85-2.96	0.773 0.142-1.60	0.957 0.95-1.39
	A	89.33 39.72-281.85	19.79 15.70-29.40	1.05 0.91-1.20	1.816 0.400-6.58	1.87 0.73-3.97	1.603 0.405-7.21	1.75 0.71-3.48	1.090 0.399-3.04	1.18 0.91-1.20
Zletovska River	**S**	29.16 12.99-106.66	13.91 10.90-21.30	0.99 0.87-1.17	2.224 0.260-13.81	6.58 1.80-12.95	0.424 0.054-1.63	1.45 0.38-2.24	0.276 0.058-0.79	1.003 0.87-1.17
	A	27.15 6.07-107.19	13.36 8.70-22.70	0.85 0.72-0.97	1.308 0.073-4.89	3.48 0.79-6.30	0.267 0.029-1.12	0.85 0.48-1.34	0.333 0.088-0.96	0.935 0.72-0.97
Kriva River	**S**	90.43 32.70-350.00	18.84 14.20-30.20	1.15 0.96-1.30	9.364 2.224-25.24	9.82 5.17-18.25	1.330 0.371-5.45	1.37 0.96-2.06	0.902 0.286-2.31	1.04 0.96-1.30
	A	33.45 10.87-95.33	14.33 10.50-21.60	1.00 0.90-1.15	0.621 0.069-5.03	1.49 0.56-5.27	0.525 0.177-1.10	1.68 1.15-2.44	0.377 0.119-0.97	1.04 0.90-1.15

S-spring, A-autumn, HSI- hepatosomatic indices, GSI- gonadosomatic indices, FCI- Fulton condition indices

Table 9. Summary of the ANOVA showing the effects of locality, season, and sex, as well as their interactions, on all investigated biometric parameters (crude and corrected for gonad masses*), which are presented in Table 8 for each site in each season, as well as in Tables 10 to 14, additionaly separated by sex. The results of the ANOVA are presented in the form of *p* values.

	Locality	Season	Sex	Locality × Season	Locality × sex	Season × Sex	Locality × Season × Sex
TM	<0.001	<0.001	<0.001	<0.001	<0.05	<0.01	<0.01
TM *	<0.001	<0.01	<0.001	<0.001	<0.05	<0.01	<0.01
TL	<0.001	<0.001	<0.001	<0.001	n.s.	n.s.	<0.001
FCI	<0.001	<0.001	<0.01	n.s.	n.s.	<0.01	n.s.
Gonad mass	<0.001	<0.001	<0.001	<0.001	<0.01	<0.001	<0.001
GSI	<0.001	<0.001	<0.05	<0.001	n.s.	n.s.	<0.001
GSI*	<0.001	<0.001	<0.01	<0.001	<0.05	n.s.	<0.001
LM	<0.001	<0.01	<0.001	<0.001	<0.05	<0.01	<0.01
HSI	<0.001	n.s.	<0.01	<0.001	n.s.	<0.05	n.s.
HSI*	<0.001	n.s.	<0.001	<0.001	n.s.	<0.01	n.s.
Gill mass	<0.001	n.s.	<0.001	<0.001	n.s.	n.s.	<0.001
Gill index	<0.001	n.s.	n.s.	<0.01	n.s.	<0.01	n.s.

n**.s.** - not significant (*p*>0.05), TM - total mass, TM* - total mass corrected for gonad mass, TL - total length, FCI - Fulton condition indices, GSI - gonadosomatic indices, GSI* - gonadosomatic indices corrected for gonad mass, LM - liver mass, HSI- hepatosomatic indices, HSI* - hepatosomatic indices corrected for gonad mass

Table 10. Vardar chub total masses[1] (crude and corrected for gonad masses*) from the rivers Bregalnica, Zletovska and Kriva in the north-eastern part of North Macedonia, in two sampling seasons, spring and autumn 2012. The data are shown for two sexes separately. Statistically significant differences (p<0.05) reported in the table refer to the results of ANOVA followed by Newman-Keuls test.

	Total mass / g				**Total mass* / g**			
Season	**Spring**		**Autumn**		**Spring**		**Autumn**	
Sex	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**
Bregalnica	130.97 (51)^{Aa}	48.19 (33) ^{B}	110.82 (53)^{Aa}	61.22 (42) ^{B}	121.94 (49)^{Aa}	45.03 (33)^{B}	108.33 (52)^{Aa}	60.29 (42)^{Ba}
Zletovska	32.36 (69) ^{b}	23.63 (43)	39.16 (88) ^{b}	10.33 (42)	29.87 (65)^{b}	21.87 (42)	37.26 (88)^{b}	10.11 (42)^{b}
Kriva	158.42(48)$^{\circ Aa}$	56.44 (68) ^{B}	30.30 (73)$^{\circ b}$	35.11 (66)	139.97(54)$^{\circ Aa}$	51.62 (67)^{B}	29.88 (73)$^{\circ b}$	34.38 (64)^{ab}

[1]values are expressed as mean (coefficient of variation (%))
A,B - different uppercase superscript letters represent differences between the sexes within each season and within the same sampling site (read horizontal)
a,b - different lowercase superscript letters represent differences between the sampling sites within each sex and within the same season (read vertical)
° - represent differences among fish of the same sex between two different seasons and within the same sampling locality

Table 11. Vardar chub total lengths[1] and condition factors[1] from the rivers Bregalnica, Zletovska and Kriva in the north-eastern part of North Macedonia, in two sampling seasons, spring and autumn 2012. The data are shown for two sexes separately. Statistically significant differences ($p<0.05$) reported in the table refer to the results of ANOVA followed by Newman-Keuls test.

	Total length /cm				**Fulton condition factor (%)**			
Season	**Spring**		**Autumn**		**Spring**		**Autumn**	
Sex	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**
Bregalnica	21.52 (19)^{Aa}	16.03 (12)^{B}	21.47 (16)^{Aa}	17.60 (12)^{Ba}	1.20(8) $^{\circ ab}$	1.13 (9)^{a}	1.04 (6)$^{a\circ}$	1.07(5)^{a}
Zletovska	14.27 (18)^{b}	13.29 (14)°	15.33 (29)^{Ab}	10.60 (18)$^{\circ Bb}$	1.00(8)$^{\circ b}$	0.96 (5)$^{\circ b}$	0.86 (8)$^{\circ b}$	0.84 (10)$^{\circ b}$
Kriva	23.08 (14)$^{\circ Aa}$	16.73 (15)^{B}	13.76 (24)$^{\circ b}$	14.64 (23)^{a}	1.22 (5)°A^{a}	1.11 (6)°B^{a}	1.02(9) $^{\circ a}$	0.99 (6)$^{\circ b}$

[1]values are expressed as mean (coefficient of variation (%))
A,B - different uppercase superscript letters represent differences between the sexes within each season and within the same sampling site (read horizontal)
a,b - different lowercase superscript letters represent differences between the sampling sites within each sex and within the same season (read vertical)
° - represent differences among fish of the same sex between two different seasons and within the same sampling locality

Table 12. Vardar chub gonad masses[1] and gonadosomatic indices[1] (crude (GSI) and corrected for gonad masses (GSI*), respectively), from the rivers Bregalnica, Zletovska and Kriva in the north-eastern part of North Macedonia, in two sampling seasons, spring and autumn 2012. The data are shown for two sexes separately. Statistically significant differences ($p<0.05$) reported in the table refer to the results of ANOVA followed by Newman-Keuls test.

	Gonad mass / g				**GSI / %**				**GSI* / %**			
Season	**Spring**		**Autumn**		**Spring**		**Autumn**		**Spring**		**Autumn**	
Sex	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**
Bregalnica	9.03(87)$^{\circ Aa}$	3.16(49)^{B}	2.49 (71)°	0.93(37)	6.04(56)$^{\circ a}$	6.27 (31)°	2.08(42)°	1.59(28)°	6.54(59)$^{\circ a}$	6.73(33)°	2.14(44)°	1.62(29)°
Zletovska	2.49(125)^{b}	1.76(66)	2.22 (76)	0.21(75)	6.35(59)^{a}	6.96(44)°	4.10 (48)	1.93(44)$^{\circ}$	6.95(62)a	7.59(46)°	4.32(58)	1.96(45)°
Kriva	18.45(27)$^{\circ Ac}$	4.82(85)^{B}	0.41(0.90)°	0.73(157)	12.94(35)$^{\circ Ab}$	8.25(22)$^{\circ B}$	1.28(31)°	1.59(64)$^{\circ}$	15.13(39)$^{\circ Ab}$	9.04(25)$^{\circ B}$	1.30(31)°	1.63(67)°

[1]values are expressed as mean (coefficient of variation (%))
A,B - different uppercase superscript letters represent differences between the sexes within each season and within the same sampling site (read horizontal)
a,b - different lowercase superscript letters represent differences between the sampling sites within each sex and within the same season (read vertical)
° - represent differences among fish of the same sex between two different seasons and within the same sampling locality

Table 13. Vardar chub liver masses[1] and hepatosomatic indices[1] (crude (HSI) and corrected for gonad masses (HSI*), respectively), from the rivers Bregalnica, Zletovska and Kriva in the north-eastern part of North Macedonia, in two sampling seasons, spring and autumn 2012. The data are shown for two sexes separately. Statistically significant differences ($p<0.05$) reported in the table refer to the results of ANOVA followed by Newman-Keuls test.

	Liver mass / g				**HSI / %**				**HSI* / %**			
Season	**Spring**		**Autumn**		**Spring**		**Autumn**		**Spring**		**Autumn**	
Sex	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**
Bregalnica	2.01(59)^{Aa}	0.55(24)^{B}	2.05(75)^{Aa}	1.03(60)^{B}	1.54(17)	1.25(41)	1.80(36)^{a}	1.68(37)^{a}	1.64(15)	1.32 (39)	1.84(37)^{a}	1.71(37)^{a}
Zletovska	0.51 (70)^{b}	0.27 (45)	0.43 (95)^{b}	0.07 (46)	1.60(20)°	1.18(39)°	0.84(50)$^{\circ b}$	0.69(25)$^{\circ b}$	1.71(20)°	1.26(37)°	0.88(52)$^{\circ b}$	0.70(25)$^{\circ b}$
Kriva	2.64(41)$^{\circ Aa}$	0.68(67)^{B}	0.47(66)$^{\circ b}$	0.56 (53)	1.72 (13)	1.20 (13)	1.62 (21)^{a}	1.71 (19)^{a}	1.97(15)^{A}	1.31(13)^{B}	1.64(21)^{a}	1.74(18)^{a}

[1]values are expressed as mean (coefficient of variation (%))
A,B - different uppercase superscript letters represent differences between the sexes within each season and within the same sampling site (read horizontal)
a,b - different lowercase superscript letters represent differences between the sampling sites within each sex and within the same season (read vertical)
° - represent differences among fish of the same sex between two different seasons and within the same sampling locality

Table 14. Vardar chub gill masses[1] and gill indices[1] from the rivers Bregalnica, Zletovska and Kriva in the north-eastern part of North Macedonia, in two sampling seasons, spring and autumn 2012. The data are shown for two sexes separately. Statistically significant differences ($p<0.05$) reported in the table refer to the results of ANOVA followed by Newman-Keuls test.

	Gill mass / g				**Gill index / %**			
Season	**Spring**		**Autumn**		**Spring**		**Autumn**	
Sex	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**
Bregalnica	1.07 (40)^{Aa}	0.52 (37)^{B}	1.37 (51)^{Aa}	0.73 (50)^{Ba}	0.85 (20)°	1.12 (34)	1.23 (13)°	1.17 (14)^{a}
Zletovska	0.30 (56)^{b}	0.23 (53)	0.43 (71)^{b}	0.13 (30)^{b}	0.98 (15)	0.95 (28)°	0.90 (68)^{A}	0.61 (93)$^{\circ Bb}$
Kriva	1.50 (30)$^{\circ Ac}$	0.60 (72)^{B}	0.33 (70)$^{\circ b}$	0.41 (71)^{ab}	1.00 (16)	1.06 (13)	1.09 (15)	1.00 (32)^{a}

[1]values are expressed as mean (coefficient of variation (%))
A,B - different uppercase superscript letters represent differences between the sexes within each season and within the same sampling site (read horizontal)
a,b - different lowercase superscript letters represent differences between the sampling sites within each sex and within the same season (read vertical)
° - represent differences among fish of the same sex between two different seasons and within the same sampling locality

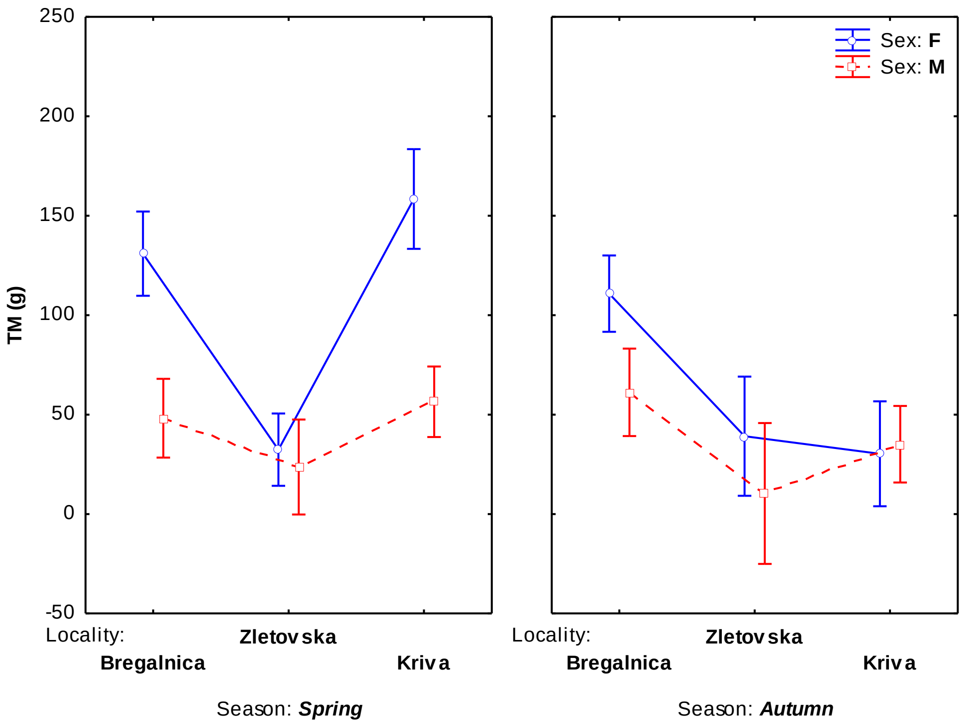


Figure 20. Total masses (TM) of female and male specimens of Vardar chub sampled at the selected sites during the spring and autumn 2012. The data are presented as mean, and vertical lines denote a 0.95 confidence interval**.**

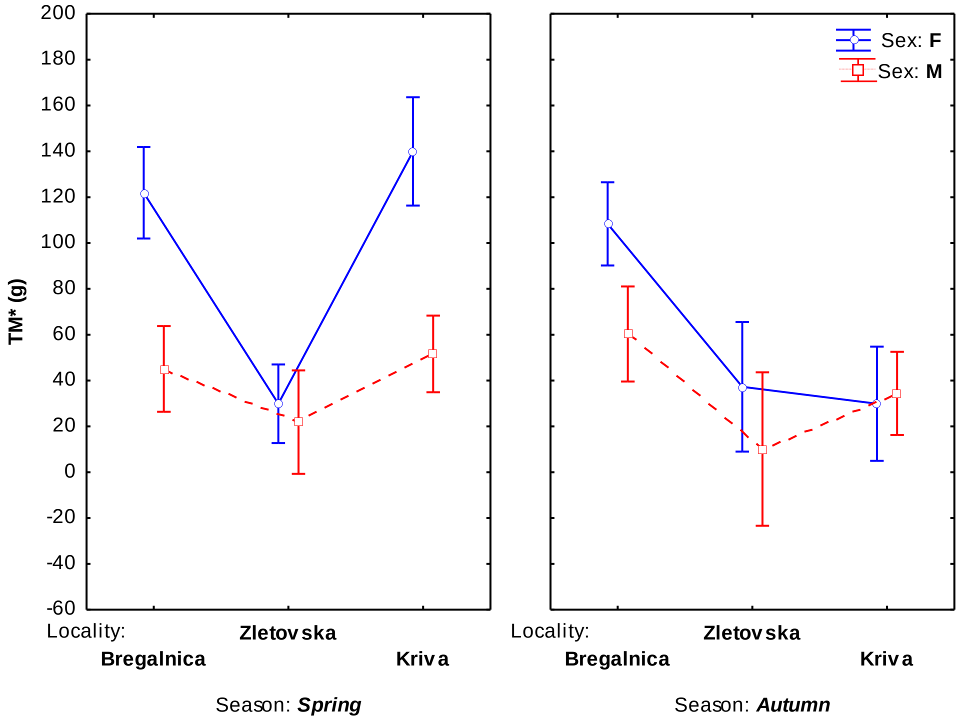


Figure 21. Total masses corrected for gonad masses (TM*) of female and male specimens of Vardar chub sampled at the selected sites during the spring and autumn 2012. The data are presented as mean, and vertical lines denote a 0.95 confidence interval.

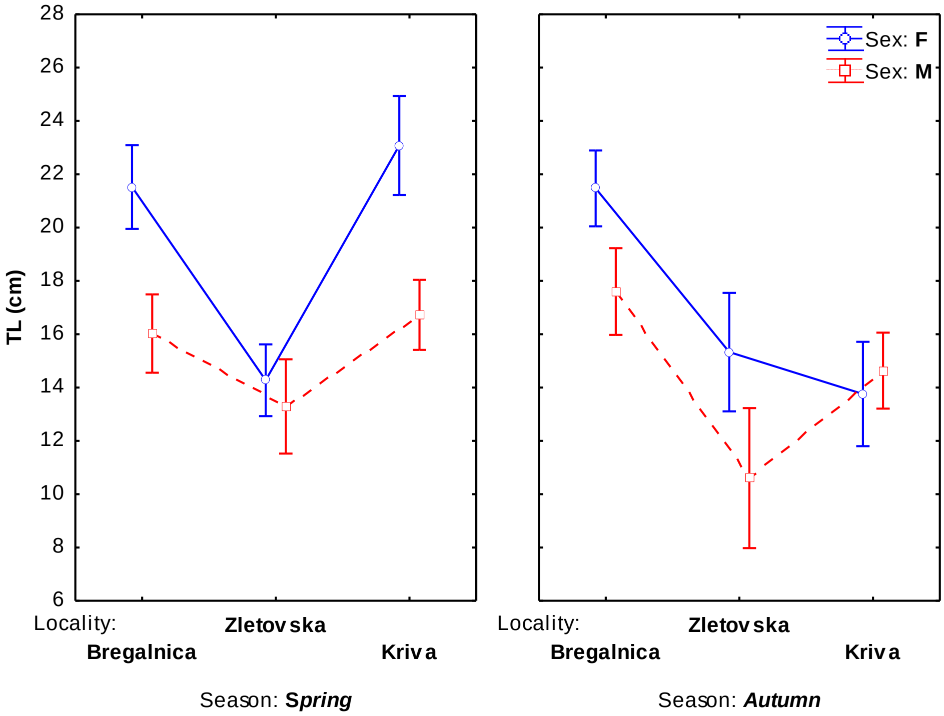


Figure 22. Total lengths (TL) of female and male specimens of Vardar chub sampled at the selected sites during the spring and autumn 2012. The data are presented as mean, and vertical lines denote a 0.95 confidence interval.

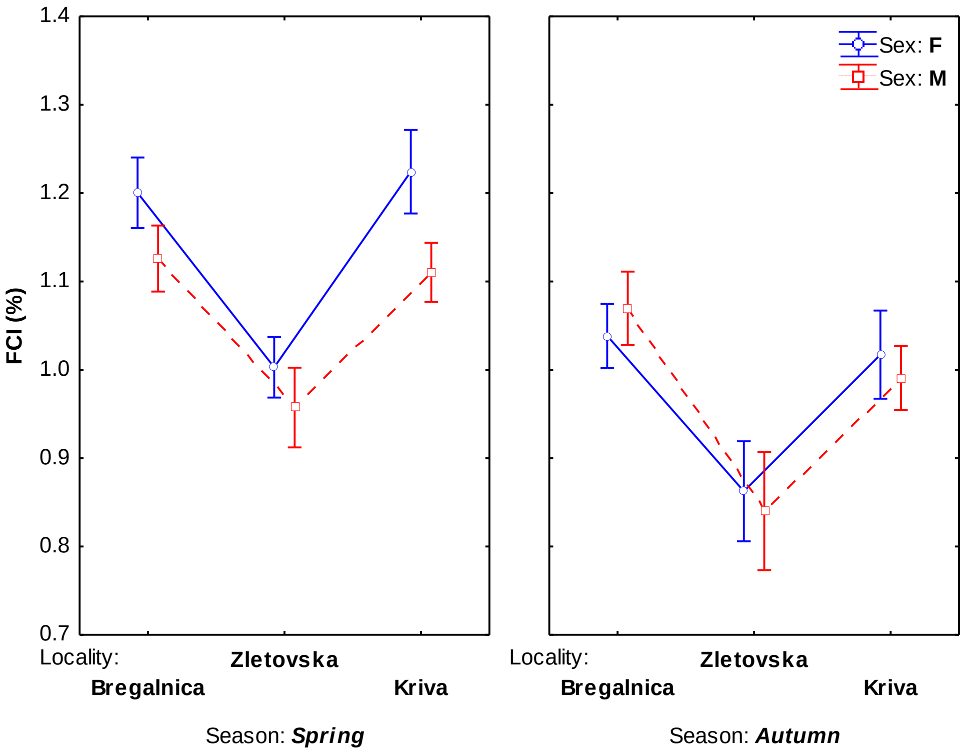


Figure 23. Fulton condition indices (FCI) of female and male specimens of Vardar chub sampled from the selected sites during the spring and autumn 2012. The data are presented as mean, and vertical lines denote a 0.95 confidence interval.

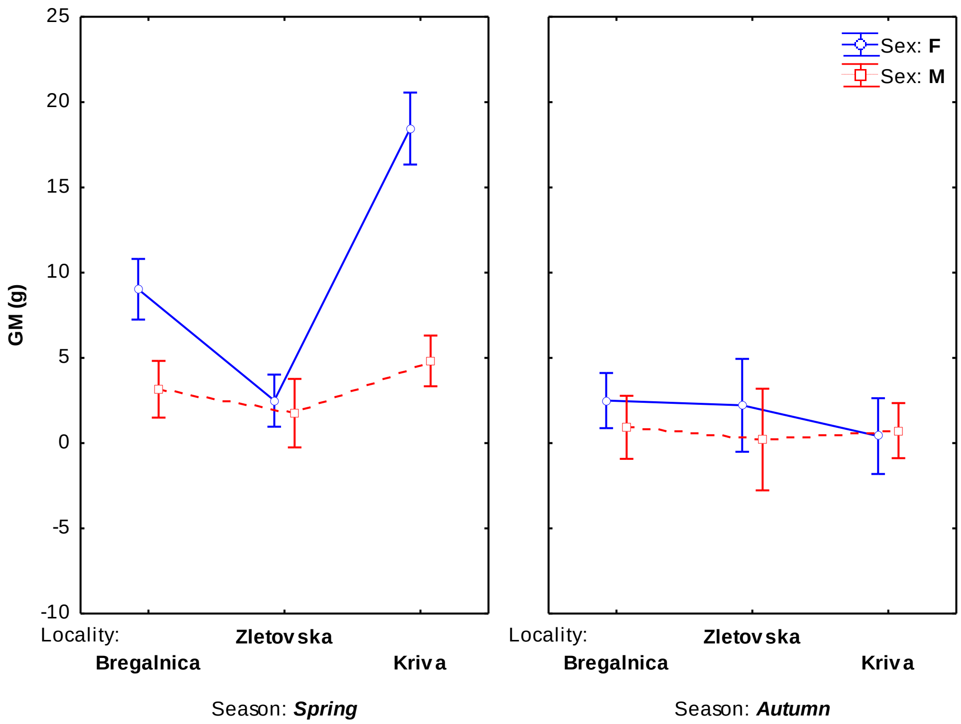


Figure 24. Gonad masses (GM) of female and male specimens of Vardar chub sampled from the selected sites during the spring and autumn 2012. The data are presented as mean, and vertical lines denote a 0.95 confidence interval.

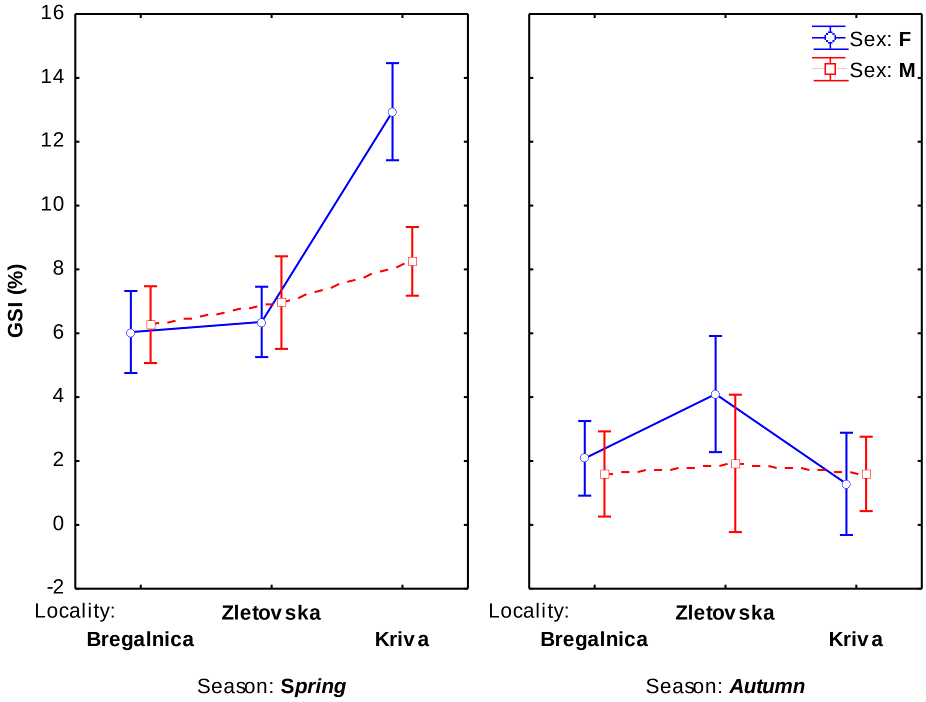


Figure 25. Gonadosomatic indices (GSI) of female and male specimens of Vardar chub sampled from the selected sites during the spring and autumn 2012. The data are presented as mean, and vertical lines denote a 0.95 confidence interval.

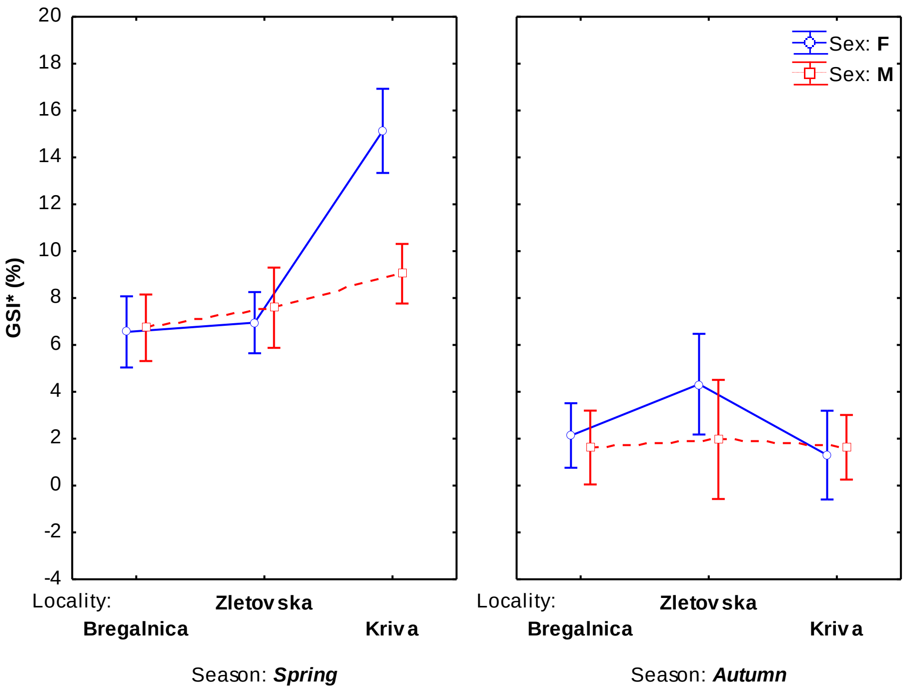


Figure 26. Gonadosomatic indices corrected for gonad masses (GSI*) of female and male specimens of Vardar chub sampled from the selected sites during the spring and autumn 2012. The data are presented as mean, and vertical lines denote a 0.95 confidence interval.

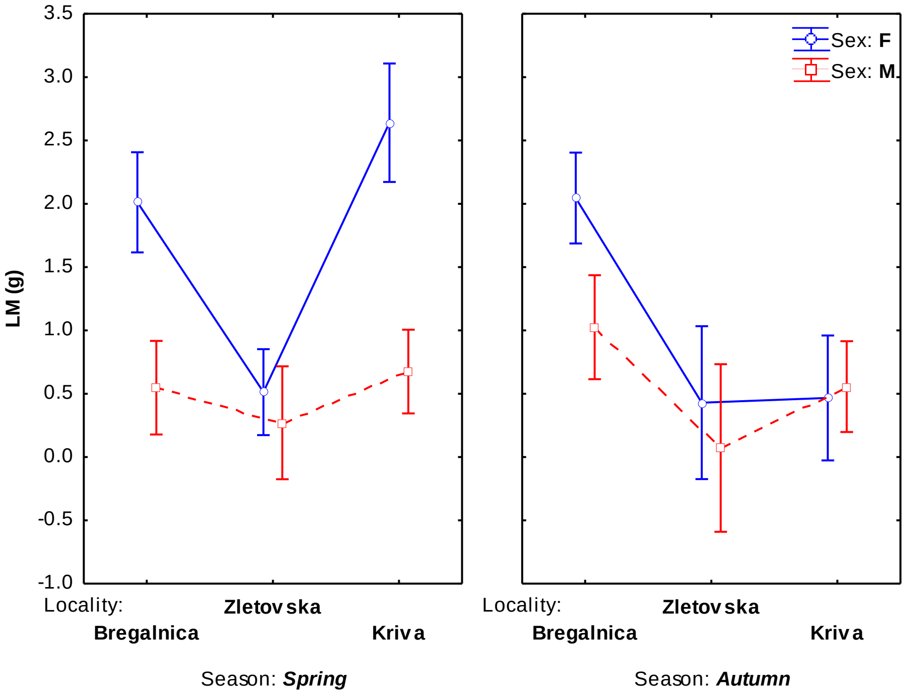


Figure 27. Liver masses (LM) of female and male specimens of Vardar chub sampled from the selected sites during the spring and autumn 2012. The data are presented as mean, and vertical lines denote a 0.95 confidence interval.

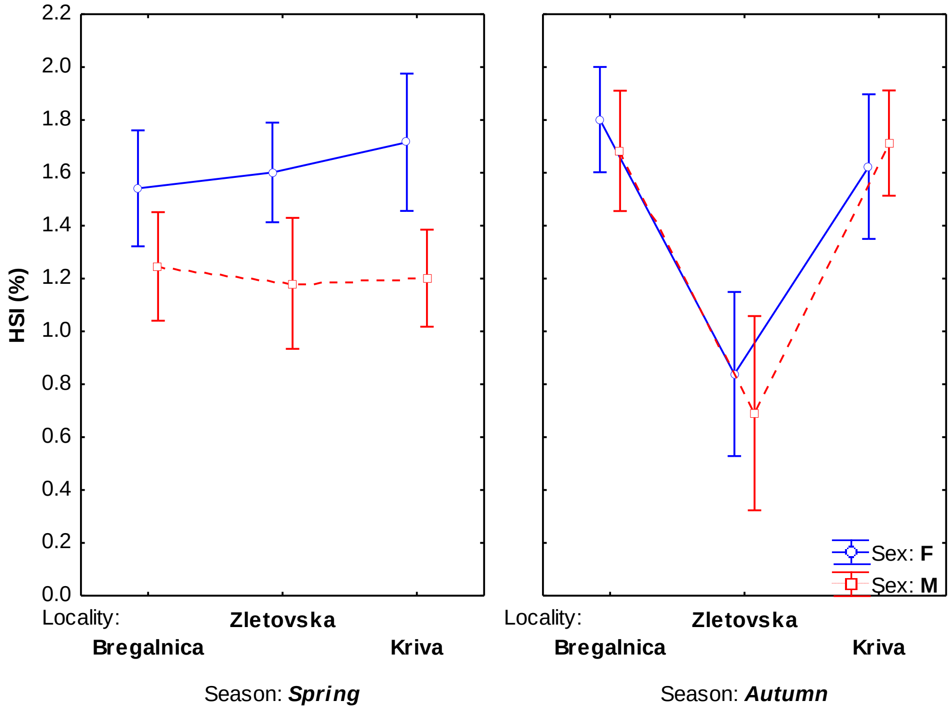


Figure 28. Hepatosomatic indices (HSI) of female and male specimens of Vardar chub sampled from the selected sites during the spring and autumn 2012. The data are presented as mean, and vertical lines denote a 0.95 confidence interval.

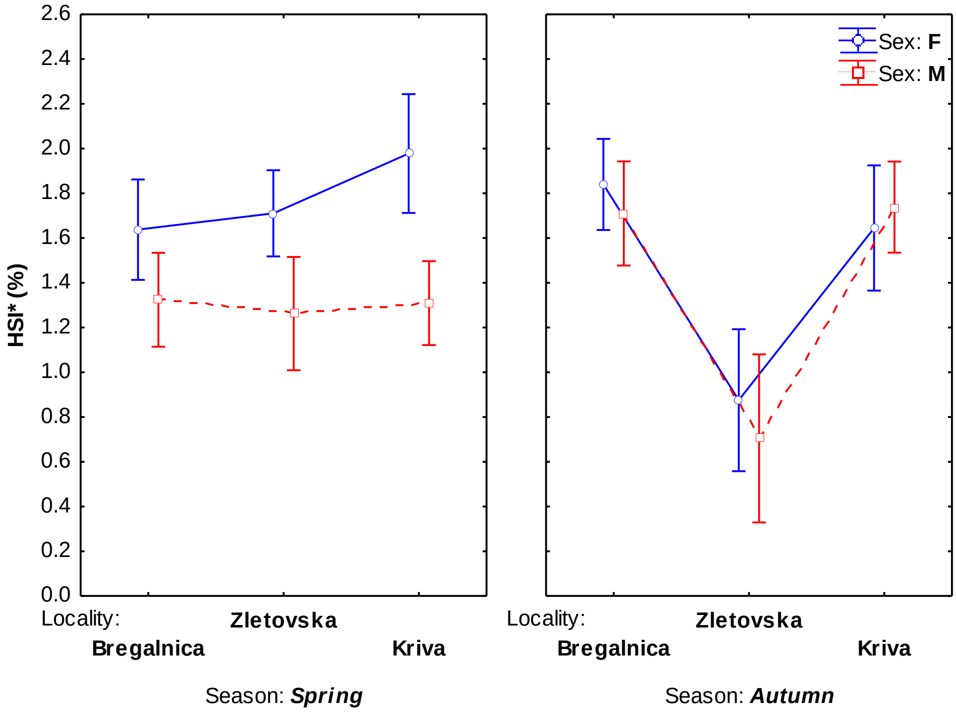


Figure 29. Hepatosomatic indices corrected for gonad masses (HSI*) of female and male specimens of Vardar chub sampled from the selected sites during the spring and autumn 2012. The data are presented as mean, and vertical lines denote a 0.95 confidence interval.

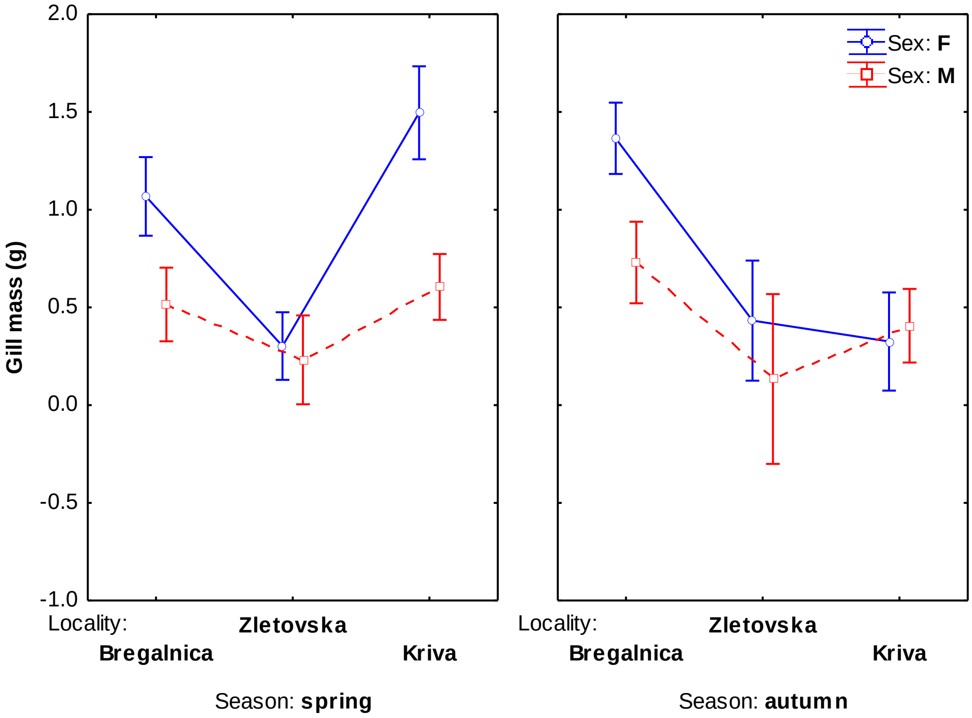


Figure 30. Gill masses of female and male specimens of Vardar chub sampled from the selected sites during the spring and autumn 2012. The data are presented as mean, and vertical lines denote a 0.95 confidence interval.

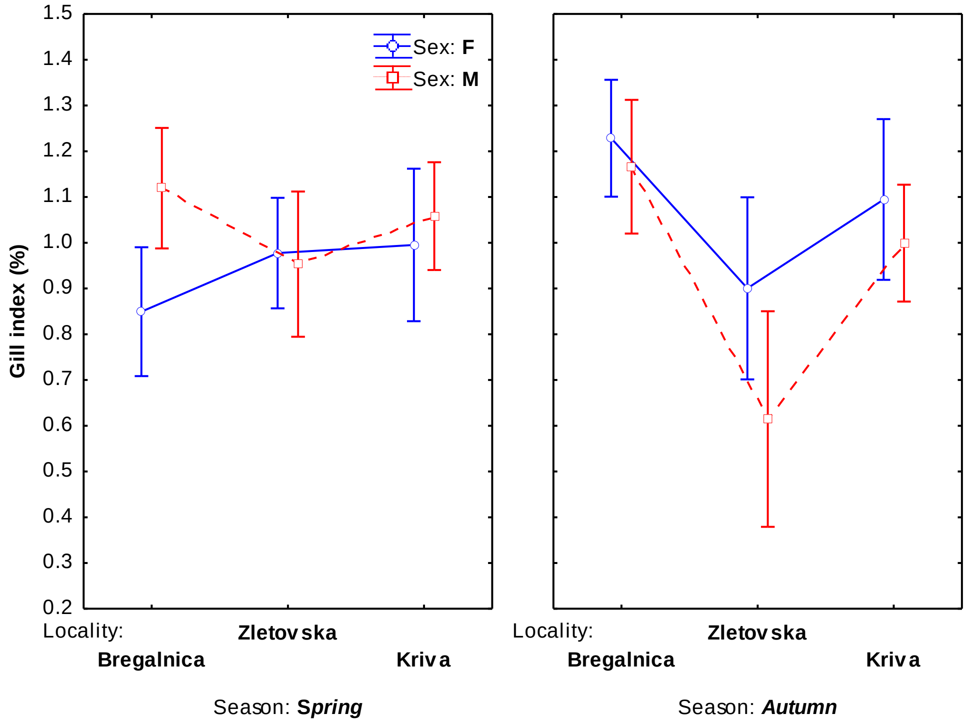


Figure 31. Gill indices of female and male specimens of Vardar chub sampled from the selected sites during the spring and autumn 2012. The data are presented as mean, and vertical lines denote a 0.95 confidence interval.

4.3. CYTOSOLIC CONCENTRATIONS OF MACRO AND TRACE ELEMENTS ACCUMULATED IN THE GILLS OF VARDAR CHUB

4.3.1. Spatial differences of cytosolic concentrations of macro and trace elements in the gills of Vardar chub from three North Macedonian rivers

The complete overview of the cytosolic concentrations of four macro elements and 15 trace elements in the gills of Vardar chub caught in three rivers in the north-eastern part of North Macedonia in two sampling periods, spring and autumn 2012, is presented in Figures 32 to 34 and Table 15.

Since the detailed analysis of biometric parameters of the sampled fish, which was presented in the previous chapter, indicated significant variations in fish size among three rivers, macro and trace element concentrations in the gills obtained at three sites were statistically compared using analysis of covariance (ANCOVA), with fish masses as a covariance, to annul the impact of the fish size. Some of the differences, therefore, are not immediately obvious from the figures, since they are calculated taking in consideration the differences in the size of Vardar chub at three sites. Differences are, thus, indicated in figures by different letters, and described in the following text. For the purpose of simpler data presentation, we have categorized the elements in three groups.

The first groups refers to four essential macro elements, Na, K, Ca, and Mg. Although several statistically significant differences among sites were obtained for cytosolic concentrations of those elements in the gills of Vardar chub, they generally varied rather slightly among three rivers (Fig. 32; Table 15), with the exception of somewhat more pronounced between-site differences observed for Na in autumn (Fig. 32a). Furthermore, all statistically significant differences were limited to the autumn season, while the concentrations of macro elements in the spring were comparable at all three sites.

Specifically, the highest gill cytosolic Na concentrations were obtained for Vardar chub from the Zletovska River in both seasons, but they were statistically significantly higher than Na concentrations in the fish from two other rivers only in autumn (Fig. 32a). The highest gill cytosolic concentrations of both K and Mg in autumn were obtained for Vardar chub from the Kriva River, while their lowest concentrations were measured at the Zletovska River, and the differences between these two rivers were statistically significant (Fig. 32b and d). And, finally, the gill cytosolic Ca concentrations in autumn were statistically significantly higher in Vardar chub from both Zletovska and Kriva rivers compared to the Bregalnica River (Fig. 32c).

The second group of the studied elements refers to seven trace elements that are essential for functioning of fish metabolism, namely Co, Cu, Fe, Mn, Mo, Se and Zn. The concentrations of these elements in the gills of Vardar chub showed the similar behaviour as the concentrations of macro elements. Although some statistically significant differences were observed between sites in both seasons, the concentrations generally exhibited only limited spatial variability (Fig. 33; Table 15), which will be presented in detail below.

In the spring period, gill Co concentrations did not vary significantly between sites, while in autumn statistically significantly higher cytosolic Co concentrations in gills were obtained for Vardar chub from the Zletovska River compared to both other rivers (Fig. 33a). Same as Co, Cu in gills also did not differ significantly between sites in spring, but only in autumn, with the highest gill cytosolic concentrations of Cu obtained for Vardar chub from the Zletovska River, even statistically significantly higher compared to the Bregalnica River (Fig. 33b). Iron concentrations varied significantly between sites in both seasons, with the highest cytosolic concentrations in gills observed in Vardar chub from the Kriva River in spring and from the Zletovska River in autumn; in spring, the difference was significant compared to the Zletovska River and in autumn compared to Bregalnica (Fig. 33c). Like Fe, Mn also showed statistically significant differences between sites in both seasons, with significantly higher gill cytosolic concentrations in spring in Vardar chub from the Zletovska River compared to both Kriva and Bregalnica rivers (Fig. 33d).

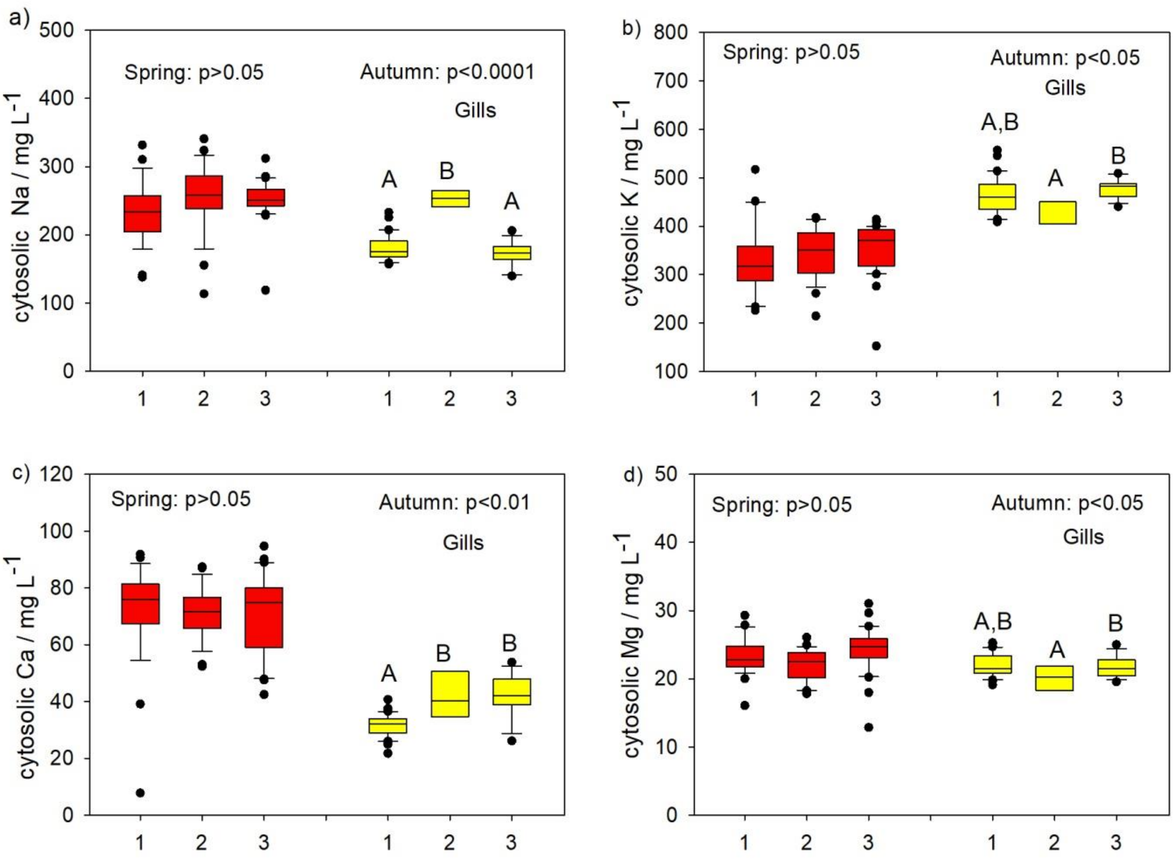


Figure 32. The cytosolic concentrations (mg L^{-1}) of four essential macroelements in the gills of Vardar chub caught in three Macedonian rivers (1 – Bregalnica River; 2 – Zletovska River; 3 – Kriva River) in two sampling campaigns (spring and autumn 2012): a) Na, b) K, c) Ca, and d) Mg. The results are presented as box-plots. The boundaries of box-plot indicate 25^{th} and 75^{th} percentiles; a line within the box marks the median value; whiskers above and below the box indicate 10^{th} and 90^{th} percentiles, whereas the black dots indicate each outlier. Statistically significant differences between sites ($p<0.05$) according to ANCOVA within each season are indicated with different letters (a, b, c), lowercase in spring and uppercase in autumn.

In autumn, higher gill cytosolic Mn concentrations were obtained for Vardar chub from the Zletovska and Bregalnica rivers compared to the Kriva River, and the difference between Bregalnica and the Kriva River was statistically significant (Fig. 33d). For Mo, gill cytosolic concentrations did not differ significantly between sites in either season (Fig. 33e).

Contrary, gill Se concentrations differ notably between sites in both seasons, and they were significantly higher in Vardar chub from the Kriva River, in both spring and autumn, compared to the fish from both other rivers (Fig. 33f). And, finally, Zn significantly differed between sites only in spring, with significantly higher gill cytosolic concentrations obtained for Vardar chub from the Zletovska River compared to Bregalnica (Fig. 33g).

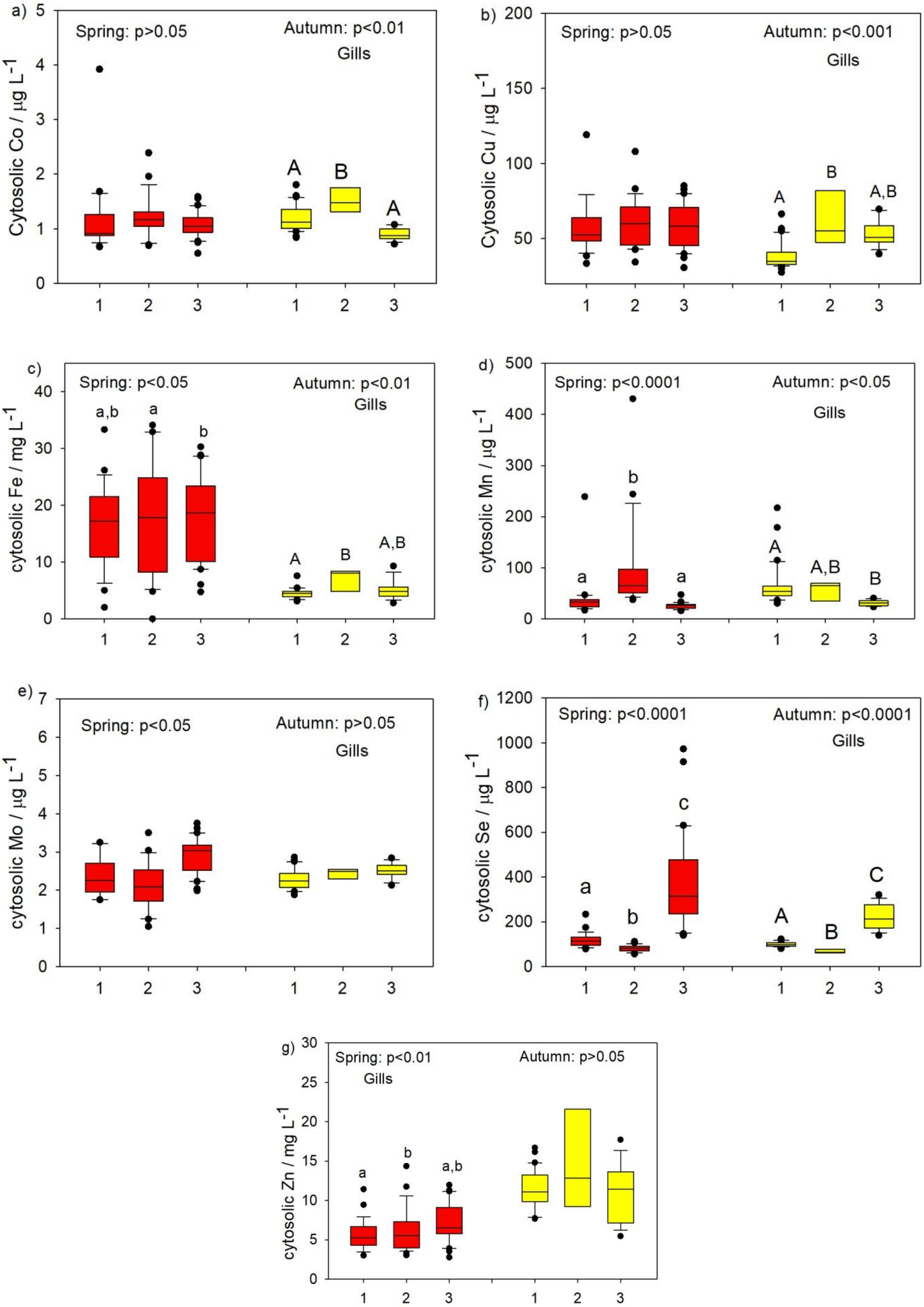


Figure 33. The cytosolic concentrations (µg L^{-1} or mg L^{-1}) of seven essential trace elements in the gills of Vardar chub caught in three Macedonian rivers (1 – Bregalnica River; 2 – Zletovska River; 3 – Kriva River) in two sampling campaigns (spring and autumn 2012): a) Co, b) Cu, c) Fe, d) Mn e) Mo, f) Se, and g) Zn. The results are presented as described in the caption of Fig. 32.

The third group of the studied elements refers to eight trace elements that have no known functions in fish metabolism and that are mostly higly toxic, namely Ba, Cd, Cs, Pb, Rb, Sr, Tl and V. These elements generally exhibited pronounced spatial variability, as will be presented below (Fig. 34; Table 15).

Among all nonessential elements, Ba had the most unclear spatial distribution. Specifically, it did not vary significantly between sites in spring, although its cytosolic concentrations in gills were the lowest at the Zletovska River. Contrary, in the autumn, the highest Ba concentrations were measured precisely in the gills of Vardar chub from the Zletovska River, being even significantly higher compared to Bregalnica (Fig. 34a). All the other nonessential elements had very characteristic spatial distributions. In spring period, statistically significantly higher gill cytosolic concentrations of Cd were obtained for Vardar chub from the Zletovska and Kriva rivers compared to the Bregalnica River (Fig. 34b). However, in autumn, significantly higher cytosolic Cd concentrations were obtained for Vardar chub from the Zletovska River compared to both Bregalnica and Kriva rivers (Fig. 34b). For Pb, the highest gill cytosolic concentrations in spring were obtained for Vardar chub from the Kriva River, followed by the Zletovska River, while the lowest concentrations were measured in fish from the Bregalnica River, and all the differences were statistically significant (Fig. 34d). In autumn, the highest gill Pb concentrations were obtained for Vardar chub from the Zletovska River, followed by the Kriva River, while the lowest were recorded again at the Bregalnica River, and all the differences were statistically significant (Fig. 34d).

The cytosolic concentrations of Cs, Rb, Sr and Tl in Vardar chub gills in both seasons were notably higher at the Zletovska River compared to the other two rivers. Furthermore, concentrations at the Bregalnica River were higher in comparison to the Kriva River in both seasons, and almost all observed differences were statistically significant (Fig. 34c, e, f, and g). The highest gill cytosolic V concentrations were obtained for Vardar chub from the Bregalnica River in both seasons, and they were statistically significantly higher than V concentrations in the fish from two other rivers (Fig. 34h).

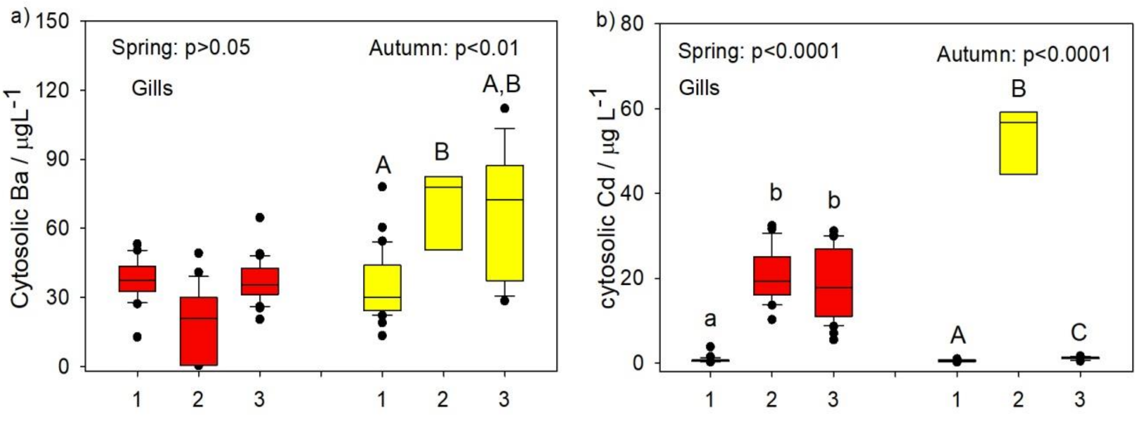


Figure 34. The cytosolic concentrations (µg L^{-1}) of eight nonessential trace elements in the gills of Vardar chub caught in three Macedonian rivers (1 – Bregalnica River; 2 – Zletovska River; 3 – Kriva River) in two sampling campaigns (spring and autumn 2012): a) Ba, b) Cd, c) Cs, d) Pb, e) Rb, f) Sr, g) Tl, and h) V. The results are presented as described in the caption of Fig. 32.

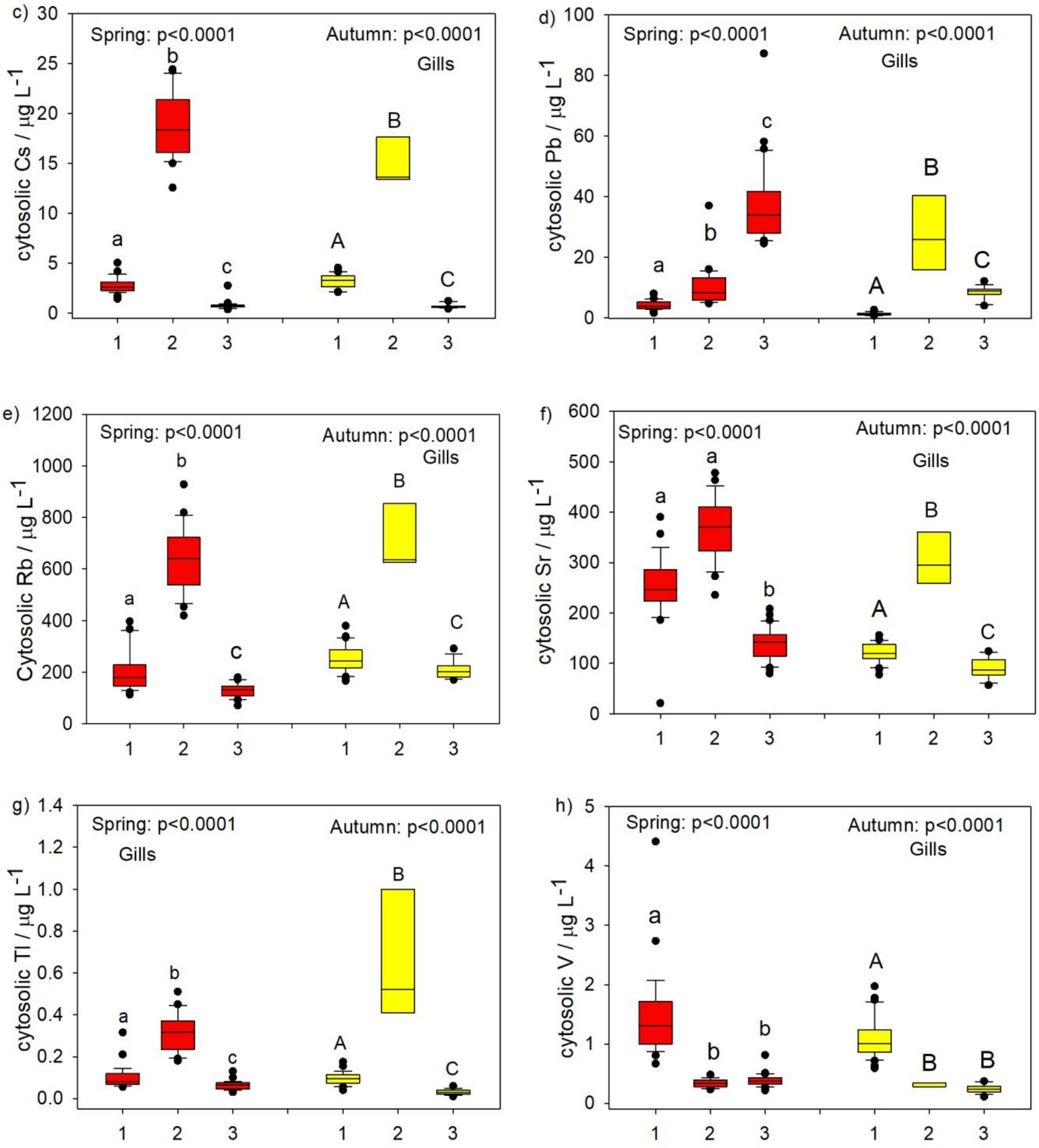


Figure 34. – continued. The cytosolic concentrations (µg L^{-1}) of seven essential trace elements in the gills of Vardar chub caught in three Macedonian rivers (1 – Bregalnica River; 2 – Zletovska River; 3 – Kriva River) in two sampling campaigns (spring and autumn 2012): a) Ba, b) Cd, c) Cs, d) Pb, e) Rb, f) Sr, g) Tl, and h) V. The results are presented as described in the caption of Fig. 32.

4.3.2. Seasonal variability of cytosolic concentrations of macro and trace elements in the gills of Vardar chub from three North Macedonian rivers

To determine the presence of seasonal differences of cytosolic concentrations of macro and trace elements in the gills of Vardar chub, the comparisons between the spring and autumn values were performed separately for each metal at each sampling site by Mann-Whitney rank sum test. The results of these analyses are presented in Table 15. Several seasonal differences were observed, but their intensity and significance depended on the metal and the studied river. Higher concentrations in the gills of Vardar chub from all three rivers in the spring were observed for Na, Ca, Mg, Fe, Se, Sr, and V, and the differences were significant at all three rivers only for Ca, at two rivers for Na, Mg, Fe, and Se, and at only one or none for V and Sr, respectively. Higher gill concentrations in spring limited to only one river were recorded for Cu at the Bregalnica River and for Mo at the Kriva River. In autumn, higher concentrations in Vardar chub gills were observed at all three rivers for K, Mn, Rb and Zn, and the differences were statistically significant at all three rivers for K and Zn, and at two rivers for Mn and Rb.

Unlike essential elements, which were generally higher either in one or the other season at all rivers, the seasonal variability of the majority of studied nonessential elements varied among sites, i.e. the concentrations were higher in spring at one river, and in the autumn at the other river. For example, both Cd and Tl at the Zletovska River were significantly higher in autumn than spring, while they were significantly higher in spring than autumn at the Kriva River. Gill Ba in Vardar chub from the Bregalnica River was significantly higher in spring, while at both other rivers it was significantly higher in autumn. Gill Pb concentrations in Vardar chub from the Bregalnica and Kriva rivers were significantly higher in spring, while at the Zletovska River they were significantly higher in autumn. Significant seasonal variability for gill Cs was observed only at the Bregalnica River, with higher values in autumn. Gill Co was the only element for which no observable seasonal differences were recorded.

Table 15. Macro and trace element cytosolic concentrations (mean±standard deviation) in the gills of Vardar chub from the rivers Bregalnica, Zletovska and Kriva, in the north-eastern part of North Macedonia, in two sampling seasons, spring and autumn 2012 – analysis of seasonal differences.

	Bregalnica River		**Zletovska River**		**Kriva River**	
	Spring	**Autumn**	**Spring**	**Autumn**	**Spring**	**Autumn**
Essential macroelements						
Na / mg L^{-1}	233.9±44.5°	180.7±19.3°	255.9±49.8	253.1±12.1	251.1±31.4°	173.4±17.9°
K / mg L^{-1}	329.9±66.5°	463.8±38.4°	345.3±51.3°	420.0±26.4°	353.3±53.4°	479.6±19.2°
Ca / mg L^{-1}	72.1±17.0°	31.5±3.99°	71.5±8.89°	41.9±8.15°	70.7±14.0°	41.7±7.56°
Mg / mg L^{-1}	23.4±2.67°	22.0±1.69°	22.1±2.27	20.1±1.77	24.3±3.37°	21.7±1.58°
Essential trace elements						
Co / µg L^{-1}	1.12±0.60	1.18±0.23	1.22±0.39	1.51±0.22	1.06±0.23	0.90±0.11
Cu / µg L^{-1}	52.7±270.5°	38.4±8.55°	61.6±16.4	61.5±18.1	58.6±14.5	53.0±8.84
Fe / mg L^{-1}	16.4±7.11°	4.51±0.84°	17.5±9.32	7.10±1.96	18.0±7.36°	5.09±1.60°
Mn / mg L^{-1}	33.3±39.4°	53.6±40.3°	64.7±89.5	65.1±18.7	26.2±6.31°	31.0±5.53°
Mo / µg L^{-1}	3.04±3.87	2.29±0.26	2.12±0.60	2.44±0.13	2.91±0.46°	2.52±0.20°
Se / µg L^{-1}	117.1±32.1°	98.7±10.6°	81.6±14.5	66.8±8.02	384.6±210.6°	221.9±55.1°
Zn / mg L^{-1}	5.64±1.87°	11.5±2.36°	6.23±2.67°	14.5±6.37°	7.13±2.45°	10.9±3.68°
Nonessential trace elements						
Ba / µg L^{-1}	37.9±8.79°	34.1±14.1°	18.5±15.7°	70.3±17.3°	37.0±8.98°	65.9±25.6°
Cd / µg L^{-1}	0.71±0.67	0.56±0.21	20.7±5.93°	53.5±7.90°	18.8±8.10°	1.14±0.30°
Cs / µg L^{-1}	2.77±0.77°	3.22±0.69°	18.9±3.25	14.9±2.38	0.77±0.40	0.72±022
Pb / µg L^{-1}	4.19±1.44°	1.30±0.46°	10.0±6.77°	27.3±12.3°	37.1±13.1°	8.41±2.05°
Rb / µg L^{-1}	198.2±74.8°	252.5±51.2°	636.1±124.7	705.0±128.5	130.2±26.6°	208.9±34.1°
Sr / µg L^{-1}	248.1±65.3	121.1±19.1	368.6±60.0	304.8±51.2	138.4±31.3	91.0±20.7
Tl / µg L^{-1}	0.10±0.05	0.10±0.03	0.31±0.09°	0.64±0.31°	0.06±0.02°	0.03±0.01°
V / µg L^{-1}	1.47±0.72°	1.09±0.33°	0.34±0.07	0.30±0.03	0.38±0.11	0.24±0.07

° - represent statistically significant differences ($p<0.05$) of the cytosolic macro and trace element concentrations between two seasons, within the same sampling locality, according to Mann-Whitney rank sum test

4.4. CYTOSOLIC CONCENTRATIONS OF MACRO AND TRACE ELEMENTS ACCUMULATED IN THE LIVER OF VARDAR CHUB

4.4.1. Spatial differences of cytosolic concentrations of macro and trace elements in the liver of Vardar chub from three North Macedonian rivers

The complete overview of the cytosolic concentrations of four macro elements and 15 trace elements in the liver of Vardar chub caught in three rivers in the north-eastern part of North Macedonia in two sampling periods, spring and autumn 2012, is presented in Figures 35 to 37 and Table 16.

In the same way as for the gills, macro and trace element concentrations in the liver obtained at three sites were statistically compared using analysis of covariance (ANCOVA), with fish masses as a covariance, to annul the impact of the fish size. Again, comparable to the gills, some of the differences are not evident from the figures, since they are calculated taking in consideration the differences in the size of Vardar chub at three rivers. In all figures, differences are indicated by different letters, and will be described in the following text.

For the cytosolic concentrations of four essential macro elements, Na, K, Ca, and Mg, in the liver of Vardar chub, several statistically significant differences among sites were obtained, but they generally varied rather slightly among three rivers (Fig. 35; Table 16).

Specifically, in both seasons the highest cytosolic Na concentrations in the liver were obtained for Vardar chub from the Zletovska River. The differences were statistically significant compared to both Bregalnica and Kriva rivers in spring, and only compared to the Kriva River in autumn (Fig. 35a). The remaining three macro elements showed comparable spatial distributions, with the highest cytosolic concentrations of K, Ca and Mg in spring in the liver of Vardar chub from the Bregalnica River, even significantly higher compared to the Kriva River for K and Ca (Fig. 35b, c, d). In autumn, the highest cytosolic concentrations of all three elements were found in the liver of Vardar chub from the Zletovska River (Fig. 35b, c, d), significantly higher compared to the Bregalnica River for Ca and Mg (Fig. 35c, d), while differences were not statistically significant between sites for K (Fig. 35b).

The cytosolic concentrations of seven essential trace elements, namely Co, Cu, Fe, Mn, Mo, Se and Zn, in the liver of Vardar chub from three rivers also varied only slightly (Fig. 36, Table 16). For example, for Fe and Mn in the liver of Vardar chub, there were no statistically significant differences between sites in either season (Fig. 36c, d), while the remaining five elements exhibited some statistically significant spatial variability.

For hepatic Co, higher cytosolic concentrations were obtained in both periods in fish from the Bregalnica River and the Kriva River compared to the Zletovska River, but the differences were statistically significant only in spring (Fig. 36a). For hepatic Cu, the highest cytosolic concentrations were obtained for Vardar chub from the Kriva River in both periods, and they were statistically significantly higher compared to the Bregalnica River (Fig. 36b).

Hepatic Mo also showed differences between sites, with the highest cytosolic concentrations in the Vardar chub from the Kriva River and the lowest in the fish from the Zletovska River in both periods, but the differences between these two rivers were statistically significant only in spring (Fig. 36e). Contrary to all the other essential elements, hepatic Se concentrations differed notably between sites in both seasons, with the highest concentrations observed in Vardar chub at the Kriva River, followed by the Bregalnica River and the lowest concentrations at the Zletovska River, and all the differences were statistically significant (Fig. 36f).

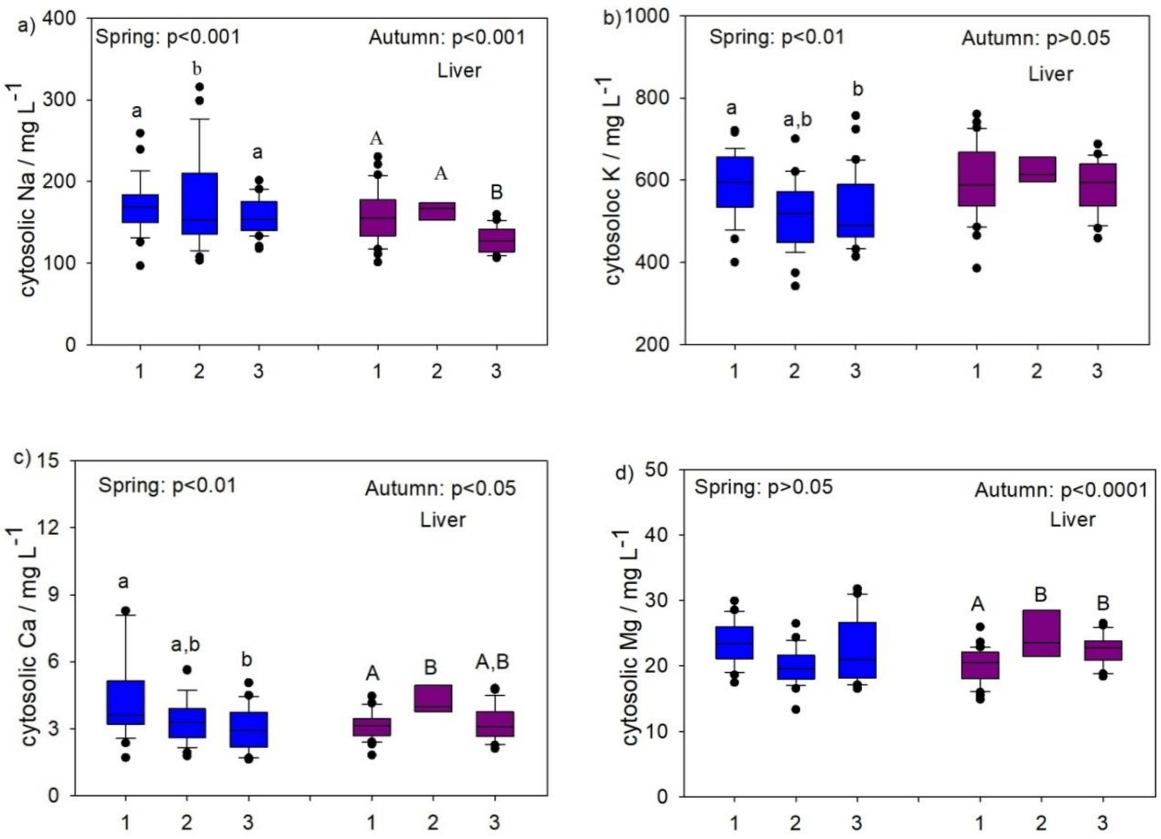


Figure 35. The cytosolic concentrations (mg L^{-1}) of four essential macroelements in the liver of Vardar chub caught in three Macedonian rivers (1 – Bregalnica River; 2 – Zletovska River; 3 – Kriva River) in two sampling campaigns (spring and autumn 2012): a) Na, b) K, c) Ca, and d) Mg. The results are presented as box-plots. The boundaries of box-plot indicate 25th and 75th percentiles; a line within the box marks the median value; whiskers above and below the box indicate 10th and 90th percentiles, whereas the black dots indicate each outlier. Statistically significant differences between sites ($p<0.05$) according to ANCOVA within each season are indicated with different letters (a, b, c), lowercase in spring and uppercase in autumn.

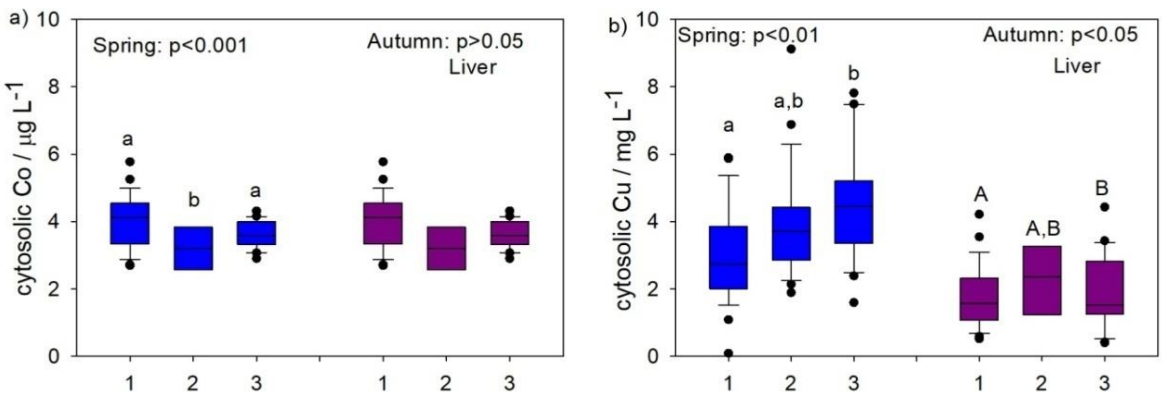


Figure 36. The cytosolic concentrations (µg L^{-1} or mg L^{-1}) of seven essential trace elements in the liver of Vardar chub caught in three Macedonian rivers (1 – Bregalnica River; 2 – Zletovska River; 3 – Kriva River) in two sampling campaigns (spring and autumn 2012): a) Co, b) Cu, c) Fe, d) Mn e) Mo, f) Se, and g) Zn. The results are presented as described in the caption of Fig. 35.

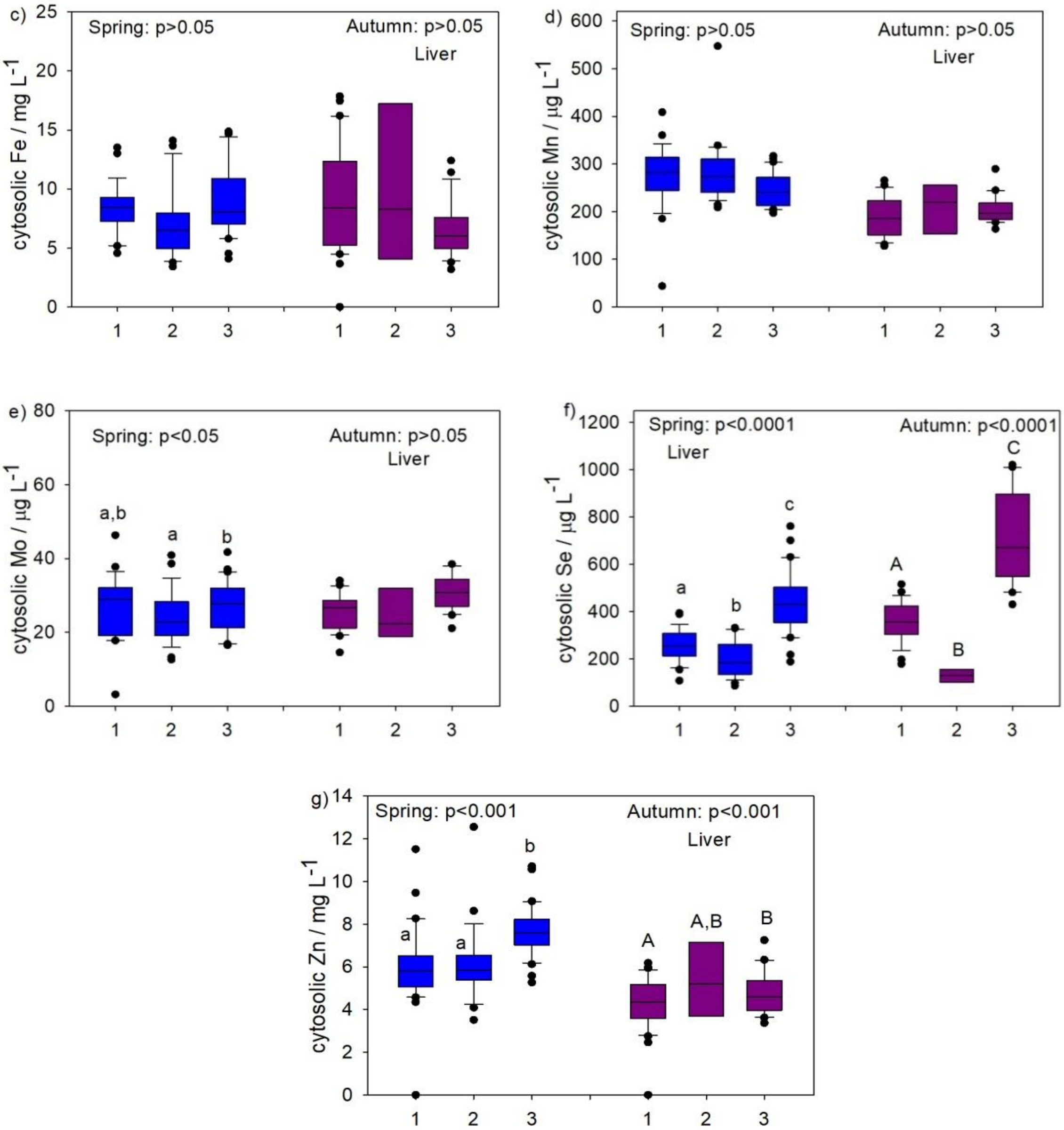


Figure 36. – continued. The cytosolic concentrations (µg L^{-1} or mg L^{-1}) of seven essential trace elements in the liver of Vardar chub caught in three Macedonian rivers (1 – Bregalnica River; 2 – Zletovska River; 3 – Kriva River) in two sampling campaigns (spring and autumn 2012): a) Co, b) Cu, c) Fe, d) Mn e) Mo, f) Se, and g) Zn. The results are presented as described in the caption of Fig. 35.

The last of the essential elements, hepatic Zn, significantly differed between sites in both spring and autumn, with the highest cytosolic concentrations obtained for Vardar chub from the Kriva River in both seasons. The differences were statistically significant compared to both Bregalnica and Zletovska rivers in spring, and only compared to the Bregalnica River in autumn (Fig. 36g).

The same as was observed for the gills, nonessential and higly toxic elements, namely Ba, Cd, Cs, Pb, Rb, Sr, Tl and V, generally exhibited more pronounced spatial variability compared to all essential elements (Fig. 37; Table 16).

For hepatic Ba, statistically significant differences between sites were obtained only in spring, with the highest cytosolic concentrations measured in Vardar chub at the Bregalnica

River, which were significantly higher compared to the Kriva River. Although hepatic Ba concentrations did not vary significantly between sites in autumn, opposite spatial distribution was then obtained, with higher hepatic Ba concentrations at the Zletovska River and the Kriva River compared to the Bregalnica River (Fig. 37a).

Again, comparable to the gills, all the other nonessential elements had very characteristic spatial distributions. For hepatic Cd, in the spring period statistically significantly higher cytosolic concentrations were obtained for Vardar chub from the Zletovska and Kriva rivers compared to the Bregalnica River (Fig. 37b). And, in autumn, the highest cytosolic Cd concentrations were obtained for Vardar chub from the Zletovska River, followed by the Kriva River, while the lowest concentrations were measured at the Bregalnica River, and all the differences were statistically significant (Fig. 37b). For hepatic Pb, the highest cytosolic concentrations in spring were obtained for Vardar chub from the Kriva River, and they were statistically significantly higher compared to both Zletovska and Bregalnica rivers (Fig. 37d). In autumn, however, the highest hepatic Pb concentrations were obtained for Vardar chub from the Zletovska River, followed by the Kriva River, while the lowest concentrations were recorded at the Bregalnica River, and the differences between the Bregalnica River and both other rivers were statistically significant (Fig. 37d).

Comparable to the gills, the cytosolic concentrations of four nonessential elements, namely Cs, Rb, Sr and Tl, in Vardar chub liver in both seasons were notably higher at the Zletovska River compared to both other rivers. Moreover, their concentrations at the Bregalnica River were higher compared to the Kriva River, also in both seasons, and most of the observed differences were statistically significant (Fig. 37c, e, f, and g). For hepatic V, statistically significantly higher cytosolic concentrations were obtained for Vardar chub from the Bregalnica River compared to both other rivers in both seasons (Fig. 37h). In autumn, the additional statistically significant difference was observed between the Zletovska and Kriva rivers, with the lowest hepatic V concentrations recorded at the Kriva River (Fig. 37h).

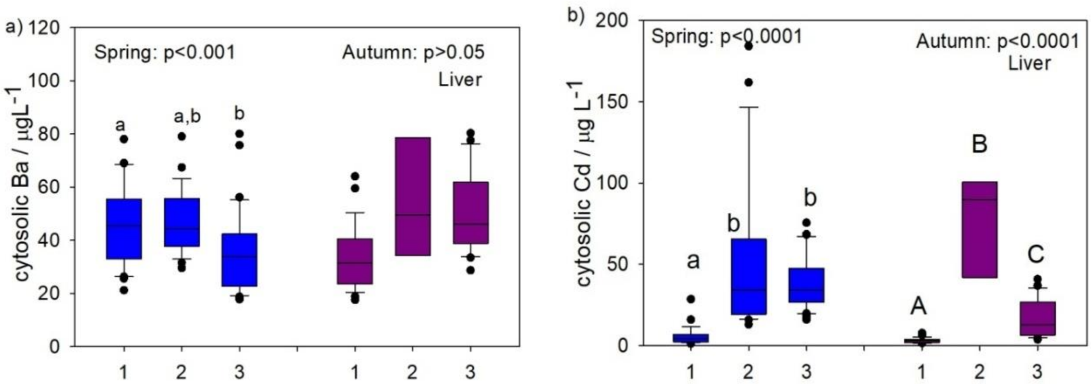


Figure 37. The cytosolic concentrations (μg L^{-1}) of eight nonessential trace elements in the liver of Vardar chub caught in three Macedonian rivers (1 – Bregalnica River; 2 – Zletovska River; 3 – Kriva River) in two sampling campaigns (spring and autumn 2012): a) Ba, b) Cd, c) Cs, d) Pb, e) Rb, f) Sr, g) Tl, and h) V. The results are presented as described in the caption of Fig. 35.

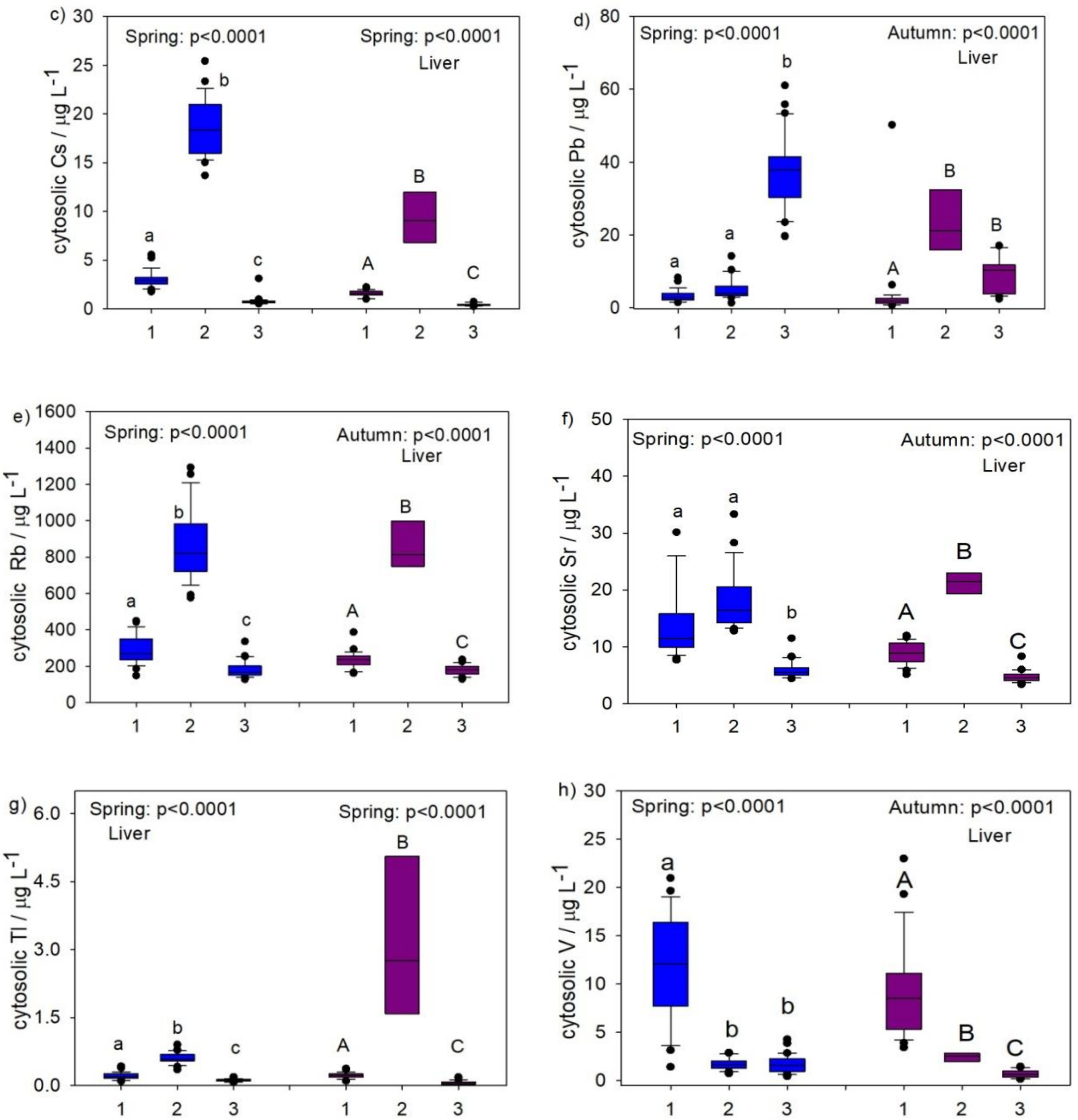


Figure 37. – continued. The cytosolic concentrations (µg L^{-1}) of eight nonessential trace elements in the liver of Vardar chub caught in three Macedonian rivers (1 – Bregalnica River; 2 – Zletovska River; 3 – Kriva River) in two sampling campaigns (spring and autumn 2012): a) Ba, b) Cd, c) Cs, d) Pb, e) Rb, f) Sr, g) Tl, and h) V. The results are presented as described in the caption of Fig. 35.

4.4.2. Seasonal variability of cytosolic concentrations of macro and trace elements in the liver of Vardar chub from three North Macedonian rivers

The comparison between the spring and autumn values was performed in the same way as for the gills, separately for each metal at each sampling site, by Mann-Whitney rank sum test, to determine the presence of seasonal differences of cytosolic concentrations of macro and trace elements in the liver of Vardar chub. The results of these analyses are presented in Table 16.

Comparable to the gills, several seasonal differences were observed, with variable intensities and significances, depending on the studied metal and the river. Higher concentrations in the liver of Vardar chub from all three rivers in the spring were observed for

Na, Co, Cu, Mn, Zn, and Cs, and the differences were significant at all three rivers for Cu and Cs, at two rivers for Co, Mn, and Zn, and at only one for Na. Statistically significantly higher hepatic concentrations in spring limited to only one or two rivers were recorded for Ca and Rb at the Bregalnica River, Fe and Cd at the Kriva River, and Sr at the Bregalnica and Kriva rivers. In autumn, higher concentrations in Vardar chub liver compared to spring were observed at all three rivers only for K, and the differences were statistically significant at two rivers. Higher hepatic concentrations in autumn limited to only one river were observed for Mo at the Kriva River. For all the other elements, the seasonal variability varied among sites, with significantly higher concentrations in one season at one site and in the other at another site. For example, hepatic Mg at the Bregalnica River was higher in spring, while it was higher in autumn at the Zletovska River. For hepatic Se, higher concentrations in spring were recorded at the Zletovska River, while at the Bregalnica and Kriva rivers higher values were measured in autumn. Hepatic Ba was higher in spring at the Bregalnica River, while it was higher in autumn at the Kriva River. Hepatic Pb concentrations in Vardar chub from the Bregalnica and Zletovska rivers were significantly higher in autumn than in spring, while at the Kriva River they were significantly higher in spring. For hepatic Tl, statistically significant seasonal difference was observed at the Zletovska River, with higher values in autumn, while at the Kriva River statistically significantly higher Tl concentrations were recorded in spring. And lastly, for hepatic V, at two rivers, the Bregalnica and the Kriva River, higher concentrations were obtained in spring, while at the Zletovska River they were higher in autumn.

Table 16. Macro and trace element concentrations (mean±standard deviation) in the hepatic cytosols of Vardar chub from the rivers Bregalnica, Zletovska and Kriva, in the north-eastern part of North Macedonia, in two sampling seasons, spring and autumn 2012 – analysis of seasonal differences.

	Bregalnica River		**Zletovska River**		**Kriva River**	
	Spring	**Autumn**	**Spring**	**Autumn**	**Spring**	**Autumn**
Essential macroelements						
Na / mg L^{-1}	169.5±32.4	156.8±31.9	174.2±57.8	164.6±11.4	158.1±23.1°	129.8±15.6°
K / mg L^{-1}	591.9±78.1	600.3±90.7	517.5±81.0°	622.5±33.3°	524.1±87.9°	586.0±64.0°
Ca / mg L^{-1}	5.99 ±10.3°	3.13±0.60°	3.36±0.96	4.23±0.69	3.00±0.97	3.21±0.73
Mg / mg L^{-1}	23.5±3.09°	20.2±2.62°	19.9±2.74°	24.5±3.84°	22.5±5.05	22.5±2.23
Essential trace elements						
Co / µg L^{-1}	5.25±0.88°	4.01±0.78°	3.62±0.68	3.20±0.69	4.84±0.91°	3.66±0.40°
Cu / mg L^{-1}	3.02±1.41°	1.76±0.91°	3.99±1.59°	2.29±1.07°	4.57±1.60°	1.92±1.03°
Fe / mg L^{-1}	8.46±2.04	9.16±4.21	6.93±2.84	9.86±7.13	8.79±2.85°	6.50±2.27°
Mn / µg L^{-1}	272.9±66.7°	189.1±41.3°	281.4±65.5	209.7±71.3	245.0±35.6°	203.1±28.7°
Mo / µg L^{-1}	26.8±8.56	25.7±4.78	24.0±6.79	24.4±7.18	27.2±6.76°	34.8±19.8°
Se / µg L^{-1}	258.4±68.6°	362.3±87.0°	201.5±73.0°	129.0±29.1°	440.4±130.5°	711.6±192.7°
Zn / mg L^{-1}	6.18±1.54°	4.39±0.97°	6.09±1.73	5.35±1.89	7.71±1.16°	4.78±1.02°
Nonessential trace elements						
Ba / µg L^{-1}	44.8±13.5°	33.6±12.3°	46.0±10.5	54.2±23.7	33.9±13.2°	51.3±14.9°
Cd / µg L^{-1}	5.64±5.49	3.27±1.49	52.5±48.6	77.4±34.5	38.6±15.9°	16.0±11.3°
Cs / µg L^{-1}	3.03±0.83°	1.59±0.32°	18.6±2.94°	9.26±2.85°	0.80±0.45°	0.42±0.12°
Pb / µg L^{-1}	3.26±1.65°	3.68±9.03°	5.03±2.79°	23.2±8.80°	37.1±10.1°	9.03±4.60°
Rb / µg L^{-1}	292.0±79.9°	234.5±45.6°	872.6±199.0	853.3±137.4	182.6±46.3	179.5±28.4
Sr / µg L^{-1}	20.2±37.9°	8.85±1.84°	18.1±5.27	21.2±1.92	5.91±1.49°	4.77±1.09°
Tl / µg L^{-1}	0.21±0.08	0.22±0.06	0.60±0.12°	3.21±1.99°	0.12±0.03°	0.06±0.04°
V / µg L^{-1}	11.8±5.54°	9.06±4.78°	1.69±0.58°	2.45±0.45°	1.74±0.96°	0.68±0.39°

° - represent statistically significant differences ($p<0.05$) of the cytosolic macro and trace element concentrations between two seasons, within the same sampling locality, according to Mann-Whitney rank sum test

4.5. VARDAR CHUB HEALTH STATUS

4.5.1. Necropsy-based fish health assessment

To assess the current metal contamination impacts on the health status of the Vardar chub from the Bregalnica, Zletovska, and Kriva rivers in the north-eastern part of North Macedonia, external/internal macroscopic lesions of the Vardar chub were investigated first. The results of the external and internal lesions observed in Vardar chub are presented in Table 17 in the form of prevalences (%) and number of affected Vardar chub specimens in both seasons together, whereas the data for each season separately are given in Table 18. The prevalances (%) were computed as numbers of a fish with specific lesions in each river divided by total number of fish captured in that river during the investigating period.

Table 17. The prevalences (%) of external/internal lesions in Vardar chub (*Squalius vardarensis*) from the rivers Bregalnica, Zletovska and Kriva in the north-eastern part of North Macedonia, presented together for two sampling seasons, spring and autumn 2012.

Locality / Lesion type	Bregalnica River (n_t=60) % (n)	Zletovska River (n_t=42) % (n)	Kriva River (n_t=56) % (n)
Skin oedema and absence of scales	8 (5) ^{a}	17 (7) ^{b}	12 (7) ^{b}
Parasites	10 (6) ^{a}	2 (1) ^{b}	9 (5) ^{a}
Gill damage	2 (1) ^{a}	5 (2) ^{b}	2 (1) ^{a}
Kidney damage	3 (2)	2 (1)	2 (1)
Other lesions	3 (2) ^{a}	5 (2) ^{b}	2 (1) ^{a}
Total number of fish with lesions	23 (14)	24 (10)	25 (14)

a,b - different lowercase superscript letters represent statistically significant differences between the sampling sites according to the binomial proportion test
n_t – total number of analyzed fish

As external pathological signs, skin oedema and absence of scales mainly on the upper dorsal fin and/or near caudal fin were detected with high prevalence. From the results presented in Tables 17 and 18, it can be noted that those abnormalities were much more pronounced at the Zletovska and the Kriva River and were significantly higher compared to the Bregalnica River. In the examined fish, parasites were found mainly in the body cavity, around the digestive tract and on the liver, or rarely on the kidney tissue, at the Bregalnica and the Kriva River. Parasites at the Zletovska River were located in body cavity, but more often they were attached on the gill filaments. The parasite prevalences at the Bregalnica and the Kriva River were significantly higher compared to the Zletovska River, where the prevalence of parasites was much lower (Table 17). Gill damages were present in Vardar chub mainly as absence of filaments or as pale and very light color of filaments, while in one fish complete absence of first gill arch was observed. The prevalence of gill damages was significantly higher at the Zletovska River compared to the Bregalnica and Kriva rivers (Table 17). Enlarged or swollen kidneys were observed in Vardar chub from all three rivers, and one fish from the Kriva River was even found with only half of the kidney. The prevalence of kidney damage was almost equally present in the fish from all three rivers (Table 17). Additional lesions in Vardar chub were found in low prevalences, including the following: pink or blue color of gallbladder at the Zletovska and the Kriva River, absence of one gonad in a male Vardar chub from the Zletovska River, hemorrhage in Vardar chub from the Bregalnica River, and focal discoloration of the liver at the Kriva River. Regarding the prevalence of these lesions, statistically significantly higher value was observed at the Zletovska River compared to the Bregalnica and Kriva rivers (Table 17). The prevalence of all lesions together amounted to 23% to 25% for three sampling sites, showing no differeneces among three rivers (Table 17). Clear seasonal trend was not observed, but somewhat higher prevalence of several lesions was observed in spring at the Kriva River, concurrently with higher metal exposure level, and in autumn at the Zletovska River, during the drought period and extremely high concentrations of several metals in the water (Table 18).

Table 18. The prevalences (%) of external/internal lesions in Vardar chub (*Squalius vardarensis*) from the rivers Bregalnica, Zletovska and Kriva in the north-eastern part of North Macedonia, presented separately for two sampling seasons, spring and autumn 2012.

Locality	**Bregalnica % (n)**		**Zletovska % (n)**		**Kriva % (n)**	
Season / **Lesion type**	**Spring** n_t**=30**	**Autumn** n_t**=30**	**Spring** n_t**=30**	**Autumn** n_t**=12**	**Spring** n_t**=30**	**Autumn** n_t**=26**
Skin oedema and absence of scales	7 (2)	10 (3)	13 (4)	25 (3)	17 (5)	8 (2)
Parasites	7 (2)	13 (4)	3 (1)	0 (0)	10 (3)	8 (2)
Gill damage	3 (1)	0 (0)	3 (1)	8 (1)	0 (0)	4 (1)
Kidney damage	7 (2)	0 (0)	0 (0)	8 (1)	3 (1)	0 (0)
Other lesions	3 (1)	3 (1)	7 (2)	0 (0)	0 (0)	4 (1)
Total number of fish with lesions	23 (7)	23 (7)	23 (7)	25 (3)	27 (8)	23 (6)

n_t – total number of analyzed fish

4.5.2. Histopathological assessment of Vardar chub liver

During the study period, numerous lesions were observed in the liver of Vardar chub. The results of the prevalances of various lesions observed in the liver of Vardar chub captured during spring and autumn 2012 in three rivers, Bregalnica, Zletovska, and Kriva, in the north-eastern part of North Macedonia are presented in Table 19 for both seasons together, whereas the data for each season separately are given in Table 20.

In general, prevalence of all hepatic lesions was significantly higher in Vardar chub from the Kriva and Zletovska rivers compared to the Bregalnica River (Table 19).

Lymfocite infiltration was sometimes observed as a single lesion in the form of aggregations of lymfocytes distributed in the parenchyma or associated with the conective tissue around the vascular-biliary stromal tracts, but more often it was observed in association with proliferation of the bile ducts (Fig. 38). Lymfocite infiltration did not vary significantly among sampling sites, but the prevalence was higher at two rivers impacted by mining activity (Table 19).

Table 19. The prevalence (%) of lesions observed in the liver of Vardar chub (*Squalius vardarensis*) from the rivers Bregalnica, Zletovska and Kriva in the north-eastern part of North Macedonia, presented together for two sampling seasons, spring and autumn 2012.

Locality / Lesion type	Bregalnica River (n_t=60) % (n)	Zletovska River (n_t=42) % (n)	Kriva River (n_t=26) % (n)
Lymphocyte infiltration	17 (10)	24 (10)	25 (14)
Fibrosis	15 (9)	21 (9)	29 (16)
Bile duct proliferation	32 (19)^a	33 (14)^a	63 (35)^b
Parasites and granuloma	3 (2)^a	2 (1)^a	5 (3)^b
Necrosis	15 (9)^a	17 (7)^a	27 (15)^b
Megalocitosis	7 (4)^a	14 (6)^a	16 (9)^b
Light-dark hepatocyte	0 (0)^a	2 (1)^b	5 (3)^b
Hepatocyte regeneration	0 (0)^a	5 (2)^b	7 (4)^b
Lipidosis	0 0)^a	5 (2)^b	7 (4)^b
Total number of fish with lesions	38 (23)^a	59 (25)^b	70 (39)^b

a,b - different lowercase superscript letters represent statistically significant differences between the sampling sites according to the binominal proportion test
n_t – total number of analyzed fish

At least twofold increase of connective tissue around the biliary profiles compared to normal was diagnosed as fibrosis. The fibrosis was often observed in examined Vardar chub, and was found ony in stromal tracts with biliary profiles. The prevalence of fibrosis was higher at the rivers impacted by mining activity, and especially at the Kriva River, but did not vary significantly among sampling sites (Table 19).

Increased number of variably shaped bile ducts compared to the normal appearence was diagnosed as the bile duct proliferation (Fig. 38). Fibrosis is often connected with bile duct proliferation. The bile duct proliferation was observed as dominant lesion at all three studied sites (Table 19). The prevalence of the bile duct proliferation was significantly higher at the Kriva River compared to the Zletovska and Bregalnica rivers (Table 19).

Rare Mixosoan parasites were observed inside the bile ducts, whereas uncapsulated granulomas in the parenchyma were observed more commonly. Significantly higher prevalence of both parasites and granulomas were noticed at the Kriva River compared to the Zletovska and Bregalnica rivers (Table 19).

Another lesion found in the Vardar chub was necrosis, which was observed in a form of the individual necrotic cells with destroyed nuclei or in a form of necrotic areas located around stromal tracts with biliary profiles. Megalocytosis was also noted, which was typically characterized by development of enlarged hepatocyte cytoplasm and enlarged nucleus (Fig. 39a). The prevalence of necrosis and megalocytosis differed notably among sampling sites and the higher prevalence was observed at the Kriva River compared to the other rivers, Zletovska and Bregalnica (Table 19).

Table 20. The prevalence (%) of lesions observed in the liver of Vardar chub (*Squalius vardarensis*) from the rivers Bregalnica, Zletovska and Kriva in the north-eastern part of North Macedonia, presented separately for two sampling seasons, spring and autumn 2012.

Locality	**Bregalnica % (n)**		**Zletovska % (n)**		**Kriva % (n)**	
Season **Lesion type**	**Spring (n_t=30)**	**Autumn (n_t=30)**	**Spring (n_t=30)**	**Autumn (n_t=12)**	**Spring (n_t=30)**	**Autumn (n_t=26)**
Lymphocyte infiltration	7 (2)	27 (8)	13 (4)	50 (6)	20 (6)	31 (8)
Fybrosis	20 (6)	10 (3)	20 (6)	25 (3)	37 (11)	19 (5)
Bile duct proliferation	33 (10)	30 (9)	40 (12)	17 (2)	77 (23)	46 (12)
Granuloma	3 (1)	3 (1)	0 (0)	8 (1)	0 (0)	12 (3)
Necrosis	20 (6)	10 (3)	13 (4)	25 (3)	43 (13)	8 (2)
Megalocitosis	10 (3)	3 (1)	13 (4)	25 (3)	27 (8)	4 (1)
Light-Dark hepatocyte	0 (0)	0 (0)	3 (1)	0 (0)	10 (3)	0 (0)
Hepatocyte regeneration	0 (0)	0 (0)	0 (0)	17 (2)	13 (4)	0 (0)
Lipidosis	0 (0)	0 (0)	7 (2)	0 (0)	7 (2)	8 (2)
Total number of lesions	33 (10)	43 (13)	60 (18)	58 (7)	57 (17)	85 (22)

n_t – total number of analyzed fish

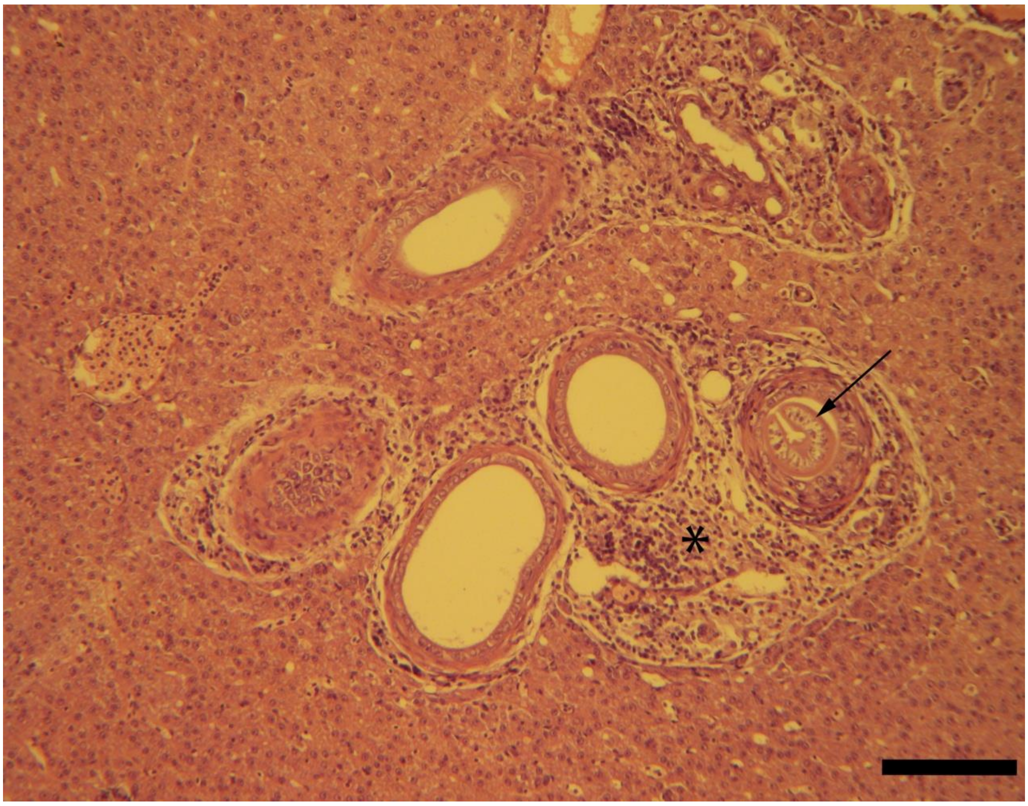


Figure 38. The example of light micrograph of the Vardar chub liver from the Kriva River, showing bile duct proliferation in form of the nests of biliary ducts, around which the lymphocyte infiltration can be seen (arrows). The presence of myxosporean parasites within biliary profile lumen is noted with asterisk. Haematoxylin and eosin. Scale bar = 0.1 mm.

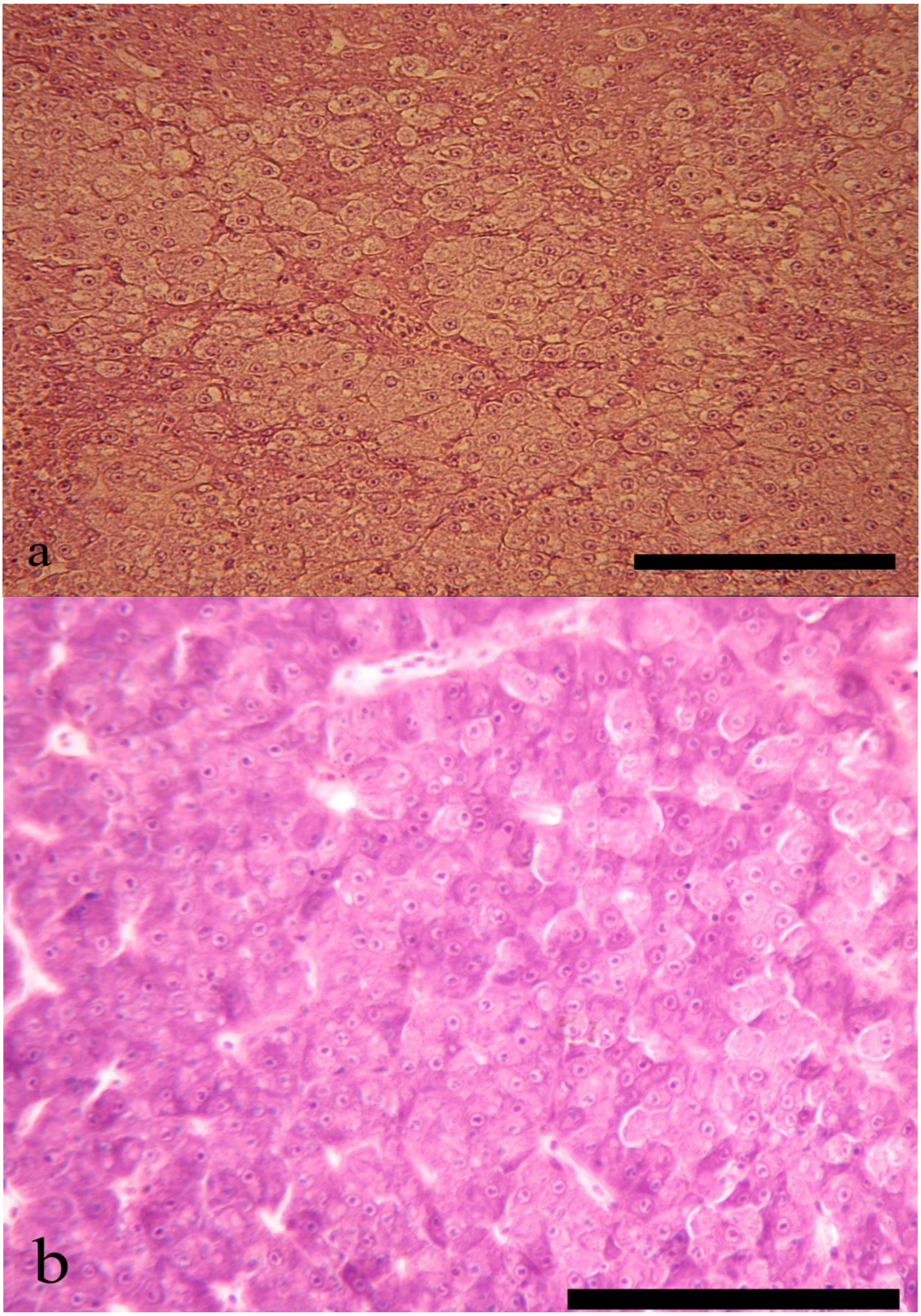
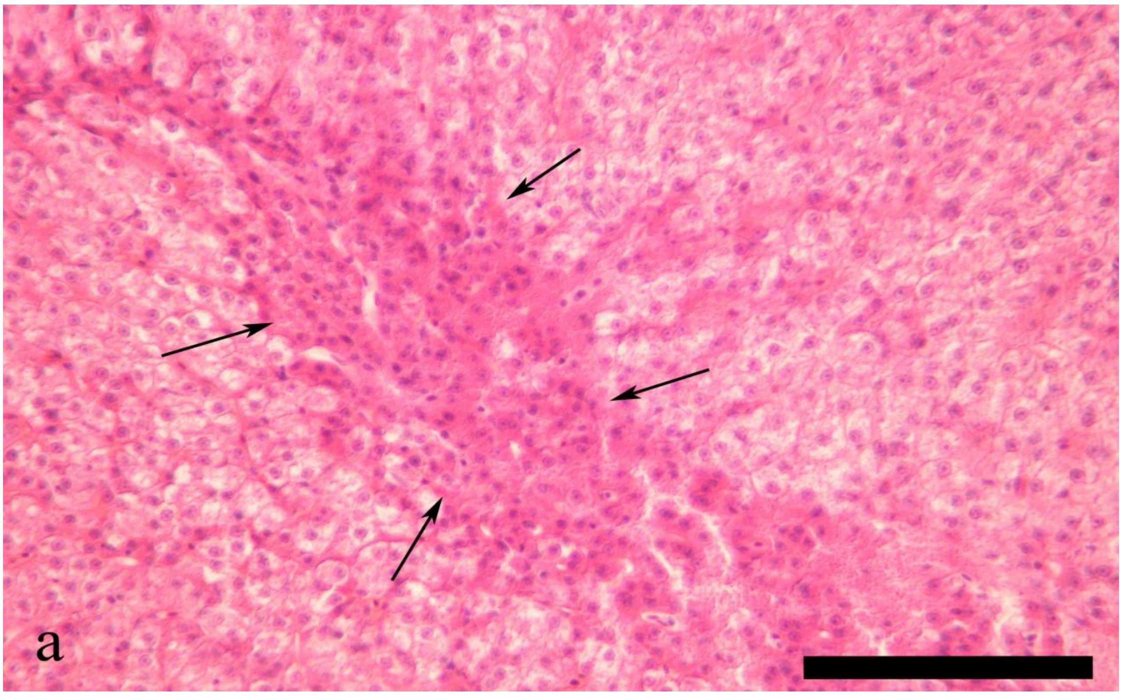


Figure 39. The example of light micrograph of the Vardar chub liver from the Zletovska River illustrating: **a)** megalocitosis, enlarged cells; and **b)** light-dark hepatocytes with prominent nucleoli. Haematoxylin and eosin. Scale bar = 0.05 mm.



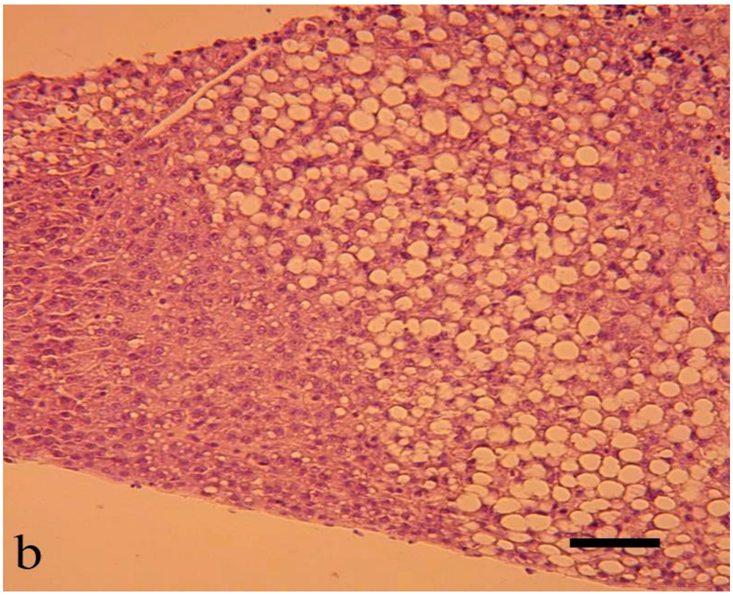


Figure 40. The axample of light micrograph of the Vardar chub liver from the Kriva River showing: **a)** hepatocytes regeneration (arrows), with the relative increase in cytoplasmic eosinophilia; **b)** lipidosis (on right site) characterized by large introcytoplasmic vacuoles within hepatocytes. Haematoxylin and eosin. Scale bar: a = 0.05 mm, b = 0.1 mm.

Another lesion recorded in Vardar chub liver was occurence of dark and light hepatocytes, side by side in a tissue section, seen as fields (Fig. 39b). Furthermore, hepatocyte regeneration was also observed, and included degenerating, dying or dead hepatocytes. They appeared as fields with smaller cells containing darker cytoplasm compared to normal hepatocytes (Fig. 40a). In addition, hepatocellular vacuolization diagnosed as lipidosis was present as diffuse distribution within parenchyma, sometimes occupying large areas of parenchymal tissue (Fig. 40b). Light-dark hepatocytes, hepatocyte degeneration and lipidosis were observed only at the rivers afected by mining activity, namely at the Zletovska and Kriva rivers, and were not found at the Bregalnica River. Thus, the prevalence was significantly higher at the Zletovska and Kriva rivers compared to the Bregalnica River (Table 19).

Similar to external/internal lesions, clear seasonal trend was also not observed for hepatic lesions, but somewhat higher prevalences of more severe lesions, such as granulomas, necrosis and megalocitosis, were observed in spring at the Kriva River, and in autumn at the Zletovska River (Table 20).

4.5.3. Histopathological assessment of Vardar chub gonads

Examination of the ovary and testes of the collected Vardar chub showed that all chub used in this study were mature individuals with well developed ovaries and testes tissues. In spring and autumn season, studied specimens of Vardar chub were in prespawning/spawning and postspawning stage, respectively.

In the prespawning/spawning stage in males, in the spring period, testicular tissue showing lobular nature of seminiferous tubules and heads of free spermatozoa filled the lumen of the tubules (Fig. 41a). In females, in the prespawning/spawning period, body cavity was full of mature eggs.

In autumn, in the postspawning season, male gonads (testes) contained tubules or lobules that contained mainly spermatogonia (Fig. 41b). Female gonads typically, in the postspawning stage in autumn, contained oocytes, more precisely pre-vitellogenetic oocytes with light red cytoplasm and nucleus located in the center of the cells (Fig. 42).

Microscopy analyses applied to observe histopatological changes in the gonads did not show any gonad abnormality either in male or female specimens of Vardar chub from any river in either season.

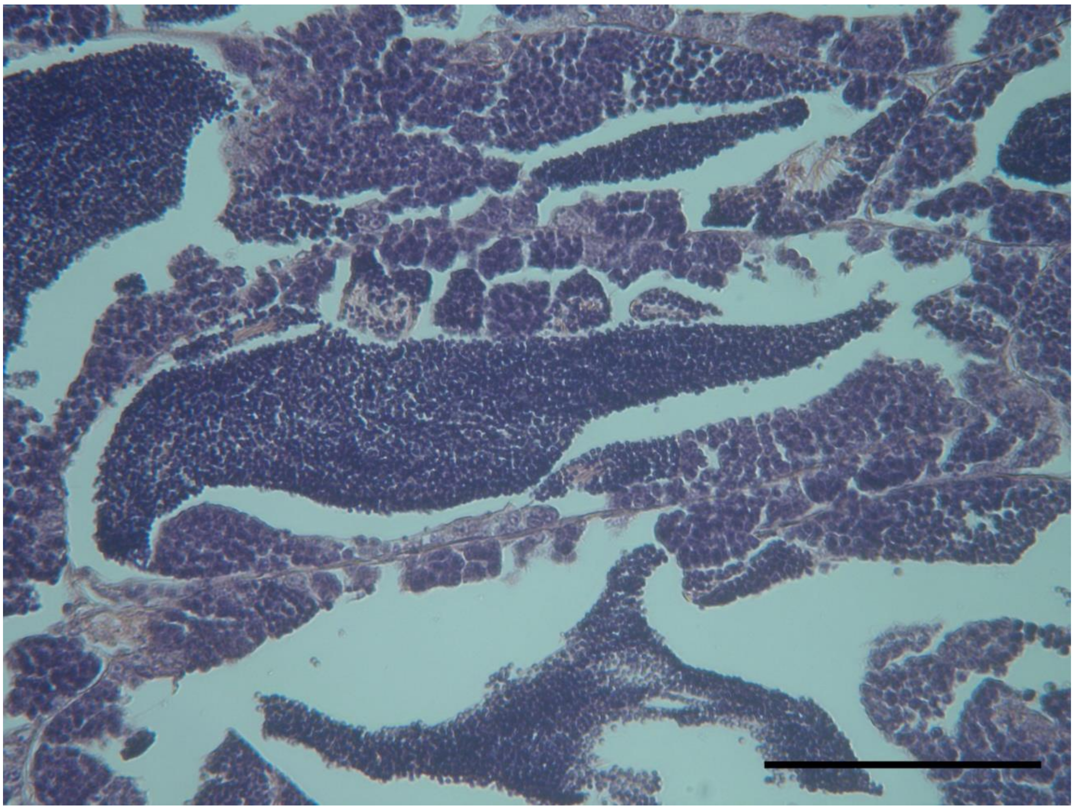


Figure 41a. Gonads of male in the spring season. Haematoxylin and eosin. Scale bar = 10 µm

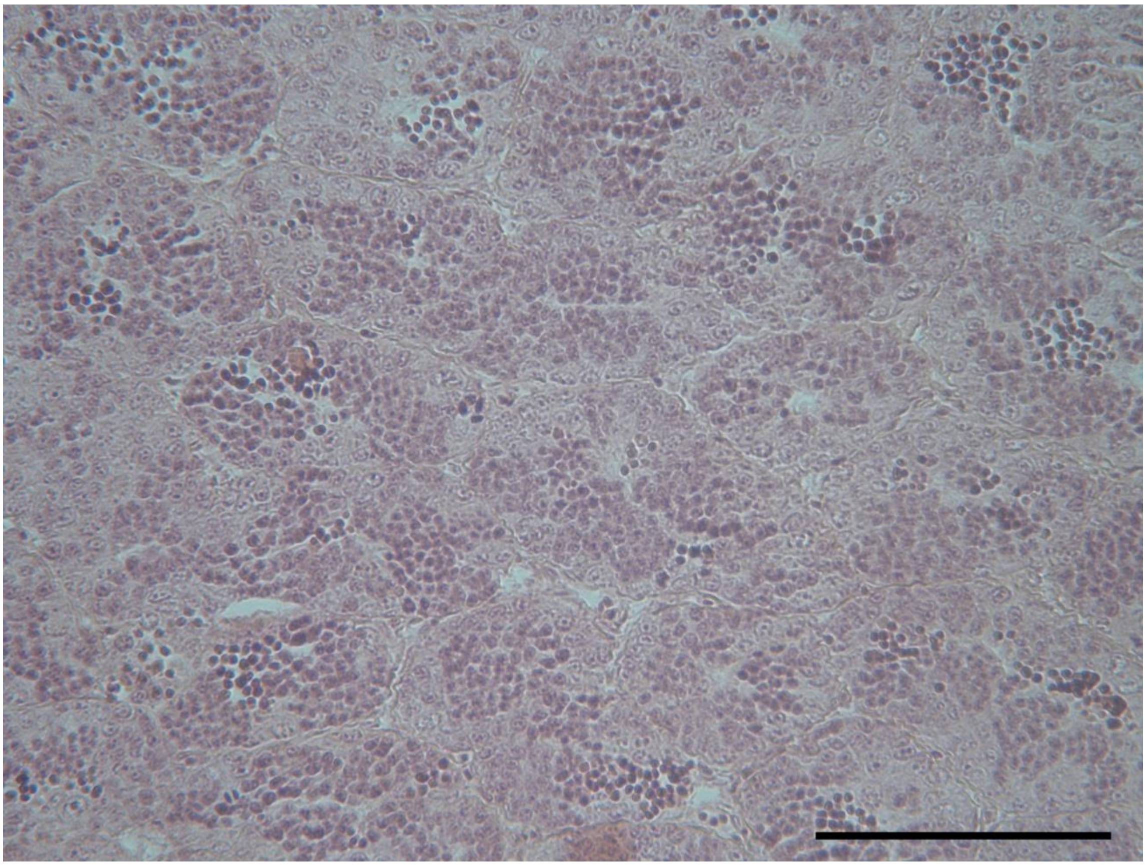


Figure 41b. Gonads of male in the autumn season. Haematoxylin and eosin. Scale bar = 10 µm

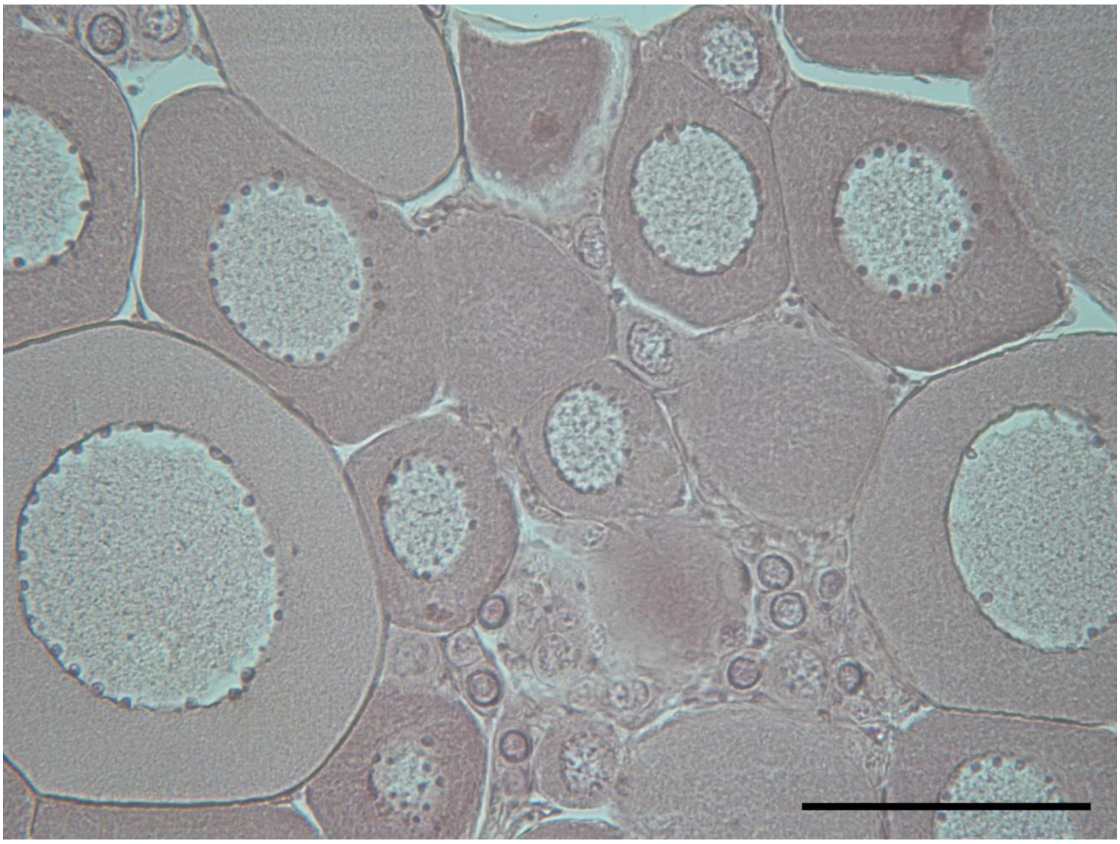


Figure 42. Gonads of female in the autumn season. Haematoxylin and eosin. Scale bar = 10 µm

5. DISCUSSION

The aquatic environment for hundreds of years has been disturbed by human activities and thousands of pollutants have been produced and released into the water ecosystems, including metals. Metals could present a hazard for functions of natural ecosystems due to their toxic effects, long persistence, and bioaccumulative properties, as well as their biomagnification in the food chain (Gheorghe et al., 2017). However, despite being the pollutants, metals also present an important component of aquatic environment, and the biodiversity of aquatic ecosystems often depends on their presence (Krasnići et al., 2019). They have high importance in aquatic systems, and are required for the normal functions of all animals, including the fish.

Metals like Co, Cu, Fe, Mn, Mo, and Zn are considered as essential, since they are required for the growth and life cycles of organisms, but still can be toxic at high concentrations, while nonessential elements, which have no known functions in the organism (e.g., Cd, Hg, and Pb), are toxic even at very low concentrations (Jakimska et al., 2011). Already at low exposure to nonessential metals or excessive bioaccumulation of essential metals, the maintenance of metal homeostasis can be impaired (Krasnići et al., 2019). So, the metal concentrations in the aquatic environment, which surpass the recommended levels, have the potential to induce serious harm in the wild organisms, to impair their health, condition, reproduction or their habitats (Rebok et al., 2014). They also present potential risk for humans, which consume those aquatic organisms, including fish (Jordanova et al., 2014).

One of the most important sources of metal contamination in the environment is the mining activity, which can seriously affect the quality of surface waters, first by large volumes of wastes from mines, and second due to the acidity and high contents of metals (Fashola et al., 2016). In northeastern part of North Macedonia, mine waste still represents one of the biggest environmental concerns (Dragun et al., 2014; Serafimovski et al., 2016), and the most important mineral deposits are Pb and Zn ores, such as found in the Zletovo mine near Probištip and in the Toranica mine near Kriva Palanka. Their waste has strongly influenced the condition of the surface waters running through that area (Alderton et al., 2005; Midžić and Silajdžić, 2005; Serafimovski et al., 2007; Serafimovski et al., 2016). For these reasons, it is important to monitor the water quality of the rivers influenced by mine activity, as well as the consequences of the water contamination on aquatic life.

Having that in mind, fish species Vardar chub (*Squalius vardarensis*) was chosen in this study as bioindicator organism, because it is representative freshwater species in the studied Macedonian rivers. It is also closely related to fish species European chub (*Squalius cephalus*), which is widespread throughout the European freshwaters, and frequently used as bioindicator organism in the monitoring studies. In many environmental investigations, European chub was used to assess the consequences of metal contamination of water bodies and to give relevant information about the vulnerability of aquatic organisms (e.g., Dragun et al., 2007; Triebskorn et al., 2007; Yılmaz et al., 2007; Krasnići et al., 2013; Rašković et al., 2018; Nyeste et al., 2019).

As target organs, the liver and the gills were selected, to assess both chronic and acute metal bioaccumulation. Specifically, the assessment of the metal bioaccumulation was performed in the soluble cytosolic tissue fractions of these two organs, to obtain the information on the metal portions in the cells which are available for metabolic requirements and possible toxic effects (Dragun et al., 2013a, b).

So the general aim of this study was to assess the metal bioavailability and effects in the rivers under the impact of active mining, applying comparative determination of (1) dissolved trace and macro element levels in the river water, (2) their bioaccumulated levels in two organs of Vardar chub, and (3) their impacts on the health status of the Vardar chub. Each of these, as well as their interdependences, will be discussed further on.

5.1. WATER CONTAMINATION OF THREE RIVERS IN THE Pb/Zn MINING REGION IN THE NORTHEASTERN PART OF NORTH MACEDONIA (published in Ramani et al., 2014)

Within this study, sampling sites were chosen at each of three studied rivers (Table 1; Fig. 5). One of the basic criteria for the sampling site selection was that some essential characteristics of the river water, such as pH and water oxygenation, should be compatible with possibility for fish survival. Accordingly, the sampling sites at two mining-impacted rivers were selected at locations being far enough from the mines to assure the presence of the aquatic life, namely of Vardar chub.

As a result of the targeted selection of the sampling locations, both pH and dissolved oxygen (DO) levels in all three rivers in this study (Table 3) were categorized as the first class of surface waters (Table 21; GRM, 1999). However, as expected, a number of other measured parameters did not comply with the requirements for the first class water. For example, turbidity in all samples of the studied rivers was high (Table 3), and characteristic for fourth-class of water (Table 21; GRM, 1999). Increased turbidity in the vicinity of mines can be the result of Fe precipitates. It decreases the incidence of light in a water body, thus impeding photosynthesis and causing a breakdown of food chains, which finally results in decreased biodiversity in the affected areas (Stuhlberger, 2010).

The Bregalnica River receives water from the Zletovska River, which is directly influenced by the active mine Zletovo (Table 1; Fig. 5). However, a location was selected at Bregalnica, which is presumably far enough from the source of contamination to be considered a mining nonimpacted site. Downstream and further away from the mining regions, decrease in dissolved metal concentrations can be expected as a result of dilution in noncontaminated river water as well as a result of a removal from solution due to precipitation of the oxide, hydroxide, and sulphate phases and coprecipitation or adsorption of metals onto those phases in sediments (Hudson-Edwards et al., 1999; Alderton et al., 2005; Ribeiro et al., 2013a,b; Silva et al., 2013). Furthermore, the contamination also generally decreases in river sediments, not only in water, downstream from the contaminant source (Byrne et al., 2012) due to hydraulic sorting (Wolfenden and Lewin, 1978), dilution by uncontaminated sediments (Marcus, 1987), hydrogeochemical reactions (Hudson-Edwards et al., 1996), and biological uptake (Lewin and Macklin, 1987).

In the Bregalnica River, the pH of the water was slightly alkaline (Table 3) and comparable to pH values characteristic for the sites with agricultural activity of high intensity, which were reported to be approximately 8 (Cooper and Fortin, 2010). In addition, total dissolved solids (TDS) (Table 3) were rather low in the spring samples, but they somewhat increased in the period of low water level during autumn (Table 2). The levels of nitrates and phosphates were slightly increased compared to the Zletovska River (Table 4). Somewhat disrupted physical and organoleptic properties of the water, accompanied by an increased level of suspended substances have already been reported for the Bregalnica River under the town of Štip as a result of drainage of sewage from households, the collection system, and industry (Spasovski, 2011; Rebok, 2013). An interesting finding for the Bregalnica River was the several degrees higher water temperature compared to the other two rivers during both sampling periods (Table 3), which can have a profound influence on the aquatic ecology (Huet, 1986), e.g., by affecting the solubility of gases (Cokgor et al., 2009). The cause of this increase could be found in the geothermal system Kezhovica-Ldzhi, which is situated nearby on the right bank of the Bregalnica River and approximately 2 km to the southwest from the center of Štip. The temperature increase of the river water was possibly the result of the mixing of the river water with hot water from the deep springs of cracked granites, which are the reservoirs of this geothermal system (Spasovski, 2012).

The concentrations of several dissolved trace elements, namely As, Ba, Fe, Mo, Ti, U, and V, were moderately increased in the surface water of this river (Table 6; Fig. 17). This increase was mostly notable when a comparison was made with the Zletovska River with concentrations being approximately 4-30 times higher. However, the concentrations of metals defined as priority toxic substances by the European Water Framework Directive (EU WFD), such as Cd, Ni, and Pb (EPCEU, 2008), were not increased in this river. Among seven elements present in the increased concentrations in this river, only Ba concentration surpassed the fresh water screening benchmark as defined by the USEPA (Fig. 17b, Table 22). The concentrations of other six elements did not exceed either USEPA benchmarks (http://www.epa.gov) or the strict limits defined by the Canadian guidelines for the protection of aquatic life (Table 22), which are derived based on a goal of no long-term observable adverse effects on aquatic ecosystems (http://st-ts.ccme.ca/).

The specificity of these moderate changes of the river water quality observed at the Bregalnica River was that they could be usually seen in the rivers flowing through agriculturally developed regions. In the Sutla River in Croatia, for example, a mild increase of the same elements as in the Bregalnica River (As, Ba, Fe, U, V, and Ti) was observed at the agriculturally impacted river section (Dragun et al., 2011). Both synthetic and natural fertilizers, herbicides, and insecticides, which were used in agriculture, were reported as sources of metals (e.g., As, Bednar et al., 2002; Ba, Senesi et al., 1983; Ti, Anke and Seifert, 2004; and V, Vachirapatama et al., 2002) that might contaminate nearby rivers (Nash et al., 2003; Bolan et al., 2004; Cooper and Fortin, 2010). Increased concentrations of As, Cu, and Zn have been previously associated with the poultry litter (Jackson and Bertsch, 2001; Jackson et al., 2003); therefore, an increase of As concentration could be also associated to the presence of a poultry farm in the town of Štip (Spasovski, 2011). In addition, increased U concentrations at agriculturally impacted sites can occur due to its complexation with humic substances (Sachs et al., 2007) abundantly present in the river water because of their use as additives to fertilizers (Peňa-Měndez et al., 2005). Finally, increased levels of phosphorus and nitrogen in aquatic ecosystems were also reported in association with agricultural activities (Carpenter et al., 1998; Dragun et al., 2011). The concentrations of several elements (Cu, Mn, Pb, and Ti; Fig. 18d, f, Fig.19a and Fig.17e), otherwise present in rather low concentrations, were somewhat increased in spring compared with autumn samples (by 2-8 times), which could be also connected to leaching from agriculturally used soils during rainy periods. Previous analyses of the sediments indicated that the effects of the Zletovo mine extended all the way to the Bregalnica River because even at the confluence between the Zletovska and Bregalnica rivers, sediment still contained Zn in a concentration of 990 mg/kg (Alderton et al., 2005). However, the effect of mining was not visible in the river water downstream from the confluence of these two rivers even during the spring period of greater water discharge when sediment resuspension and a consequent increase in metal concentrations could be anticipated (Neal et al., 2000; Dragun et al., 2009).

The Zletovska River sampling location had slightly acidic water (Table 3), probably due to the influence of drainage waters from the tailings impoundment of the Zletovo mine. Furthermore, characteristic findings for the Zletovska River in both sampling periods were positive pE, the highest water hardness, and the highest conductivity and TDS (Table 3), classified as third- and fifth-class of water, respectively, during spring and autumn (Table 21;

GRM, 1999). Total hardness, the concentration of $CaCO_3$ (Table 3), and the concentrations of macroelements (Na, K, Ca, and Mg; Table 5; Fig. 16) were also the highest in that river, with Ca concentration being even higher than USEPA benchmark. Because conductivity is especially sensitive to sulphate ions (Jiměnez et al., 2009), concentrations of SO_4^{2-} were accordingly higher in this river compared to the Bregalnica and Kriva rivers by 8-15 times in the spring and 20-35 times in the autumn (Table 3). An observed increase of Cl^- concentrations in the Zletovska River was somewhat less pronounced (Table 3). Sulphate concentrations were approximately 2-4 times higher than the recommended limit for drinking water (250 mg L^{-1}; Stuhlberger, 2010; Salem and Alshergawi, 2013; https://water-research.net). Both sulphate and conductivity are useful indicators of acid mine drainage contamination because they remain increased even when pH approaches neutral values due to large dilutions (Jiměnez et al., 2009), as observed at the sampling site at the Zletovska River. This occurs because sulphur is not easily adsorbed and thus can migrate further than heavy metals; its migration can represent the largest range of mine-tailing impact (Gray, 1996). Similar changes as in the Zletovska River were observed in the Kocacay River, near the Balya mine in Turkey, where metallurgic wastes were composed mainly of metal sulphides, pyrite, galena, sphalerite, which was similar to the ore composition in the Zletovo mine (Aykol et al., 2003).

Table 21. The permitted ranges and upper limits of several physico-chemical parameters (GRM, 1999) defined for five classes of surface water quality, as well as classification of three rivers in two sampling periods according to each specific parameter.

		pH	**Turbidity NTU**	**TDS mg L^{-1}**	**DO mg O_2 L^{-1}**
1st class surface water		6.5-8.5	<0.5	350	>8.0
2nd class surface water		6.3-6.5	0.5-1.0	500	6.00-7.99
3rd class surface water		6.0-6.3	1.1-3.0	1,000	4.00-5.99
4th class surface water		5.3-6.0	>3.0	1,500	2.00-3.99
5th class surface water		<5.3	>3.0	>1,500	<2.00
Classification of each river					
Bregalnica	**Spring**	1st	4th-5th	1st	1st
	Autumn	1st	4th-5th	3rd	1st
Zletovska	**Spring**	1st	4th-5th	3rd	1st
	Autumn	1st	4th-5th	5th	1st
Kriva	**Spring**	1st	4th-5th	1st	1st
	Autumn	1st	4th-5th	1st	1st

The majority of acid production by mine wastes occurs precisely due to the oxidation of iron sulphide minerals, such as pyrite and pyrrhotite (Stumm and Morgan, 1981; Lapakko, 2002; Belzile et al., 2004), which is the reason why an increase in pyrite content, such as characteristic for the Zletovo mine, commonly results in greater acidity (Alderton et al., 2005), as well as in the formation of soluble metal sulphates in waste dumps (Aykol et al., 2003). Consequently, the first indicator of sulphide mineral oxidation is the presence of sulphates as the dominant anions and H^+ in mine-drainage waters (Nordstrom and Alpers, 1997; Lapakko, 2002; Aykol et al., 2003). Next to sulphates and low pH, mine drainage is commonly characterized by high levels of dissolved toxic metals (Robb and Robinson, 1995; Braungardt et al., 2003). Such acidic, metal-rich waters, which flow from abandoned or active mines, can contaminate streams and rivers far downstream from the drainage source and consequently can have toxic effects on biota (Aykol et al., 2003). With increasing distance from the contamination source, the acidity of mine water is generally buffered to a greater or lower degree by dilution (Aykol et al., 2003). Relatively high pH of the river water (>6.1) favours the incorporation of metals into the particulate phase through the processes of sorption (Bird et al., 2010). However, even then river water can contain considerable amounts of metals, which can seriously endanger the aquatic ecosystem as seen in the Kocacay River, in which several elements (As, Cd, Cr, Cu, Fe, Mn, Pb, and Zn) were reported to be an important environmental concern (Aykol et al., 2003). Similarly, the analysis of trace elements in the Zletovska River, at pH of 6.52-6.88 (Table 3), indicated serious contamination of the river water with a number of metals (Cd, Co, Cs, Cu, Li, Mn, Ni, Pb, Rb, Sn, Sr, Tl, and Zn; Table 6; Figs. 18 and 19a), among which Cd and Ni belong to priority toxic substances and Cd even exceeded the environmental quality standards (EQS) set by the EU WFD for inland surface waters (EPCEU, 2008). Comparison with the Canadian guidelines and USEPA benchmarks indicated a possible troublesome increase of several elements above their recommended limits, namely Cd, Cu, Li, Mn, Sr and Zn. The increase of Zn concentrations as a result of the activities in the Pb/Zn mine Zletovo has already been reported in the water of the Zletovska River and its tributary, the Kalnistanska River, accompanied by increase of Cd concentrations, because Cd geochemically follows Zn-containing minerals (Spasovski and Dambov, 2009). The increase of Cu and Mn concentrations in the water of the Kalnistanska River was also observed, where the Cu increase was a result of a chalcopyrite presence associated with Pb and Zn minerals (Spasovski and Dambov, 2009).

Concentrations of a few metals (Cd, Mn, Ni, Pb, Tl, and Zn; Table 6) were especially high in October during low water discharge (Table 2). Specifically, dissolved Cd was approximately 10 times higher than its EQS and 20 times higher than Canadian recommendations (Table 22), whereas dissolved Mn and Zn were as much as 12 and 50 times higher than Canadian limits, respectively (Table 22; Figs. 18a, f, and l). From our results, it was obvious that contamination of the river water with Zn was much more pronounced than with Pb, even though ZnS and PbS are present in equal amounts in Pb/Zn mines (Barnes, 1979), such as the Zletovo mine. This could be explained by greater solubility of ZnS compared with PbS (Barnes, 1979), as well as by the fact that Pb is readily adsorbed by aluminum and Fe oxide phases in the sediment (Lee et al., 2002), thus resulting in a much quicker decrease of dissolved Pb than Zn concentrations (Zhang et al., 2004). Therefore, Pb is to a lesser degree present in the water in the dissolved phase, and therefore Zn is a better indicator of the effects of mining (Alderton et al., 2005).

Previously, Zn and Cd, bound to Fe and Mn oxides/hydroxides, as well as several other metals (Cs, Cu, and Tl), were also found in highly increased concentrations in paddy soil samples in the vicinity of the Zletovska River (Dolenec et al., 2005). This was undoubtedly the consequence of the discharge of untreated acid mine water and effluents from tailings, rich in Zn and Cd (39 and 176 mg L^{-1}, respectively), into the river water that was used for the irrigation of the paddy fields on the western side of the Kočani field (Dolenec et al., 2005). However, not only acid mine drainage, but also a high regional geochemical background, must be considered as a source of high trace element concentrations in the Zletovska River water, because previous studies have also reported high metal levels upstream of the mine (e.g., Zn 330 and Mn 900 µg L^{-1}; Alderton et al., 2005).

The level of nutrients was lower compared with the other two rivers (Table 4), and low inputs of inorganic nutrients and organic matter, next to high pyrite availability, could be an additional cause of the poor water quality regarding trace elements in the Zletovska River (Aykol et al., 2003).

The Kriva River water had slightly alkaline pH and negative redox potential similar to the Bregalnica River (Table 3). However, conductivity, TDS, and alkalinity were lower (Table 3), indicating lower concentrations of various salts in this river, which was also confirmed by low levels of macroelements (Table 5; Fig. 16). Accordingly, although both rivers had higher concentrations of the same trace elements compared to the Zletovska River, being characteristic for agricultural type of contamination, such as Ba, Fe, Mo, and V (Senesi et al., 1983; Vachirapatama et al., 2002; Dragun et al., 2011), their concentrations were much lower in the Kriva River water (Figs. 17b, c, d, and g). Furthermore, it would be expected to find a similar type of contamination in the Kriva River as in the Zletovska River because the sampling location was situated downstream from the Toranica mine, which exploits similar Pb- and Zn-rich minerals as those at Zletovo and exhibits similar sediment contamination with Pb, Zn, Cd, and other ore-related metals (Alderton et al., 2005). However, the water at this locality was much less metal contaminated, as has been shown in previous studies (Alderton et al., 2005). As explained by Alderton et al. (2005), this was probably caused by lack of pyrite in the ore compared with Zletovo, as well as by buffering of acid waters by carbonate host lithologies (limestone), which kept metal concentrations low. A similar finding was reported for parts of the English Peak District with predominating carbonate lithology (Carboniferous limestone), which were characterized by neutral to basic mine discharges and significantly lower concentrations of dissolved toxic metals (Smith et al., 2003). In the carbonate areas, acid formed in the oxidation of sulphides can be neutralized by carbonate rock, such as limestone and dolomite, thus resulting in slightly alkaline surface water and retarding the migration of heavy metals (Holmstrom et al., 1998; Zhang et al., 2004). This could explain the low dissolved metal concentrations in the surface water of the Kriva River, as well as the alkaline pH, which was even higher at an upstream location closer to the mine, near Zhidilovo (autumn pH = 8.16). Characteristic findings for the Kriva River, however, were severe temporary water contaminations with different contaminants. For example, high concentrations of Cd and Pb (Fig. 18a and Fig.19a) were found in the river water in the spring, which could be associated with the impact of the nearby mine. The concentration of Cd in the spring was higher than its EQS (Table 22), whereas Pb concentration, although increased, was still lower than its EQS by approximately four times (Table 22). In the autumn sampling, however, Cd and Pb concentrations decreased at the selected sampling site, but closer to the mine they were still rather high and comparable with spring values (at Zhidilovo: Cd, 210 ng L^{-1} and Pb, 1.95 µg L^{-1}).

The contamination event observed in the autumn referred to increased levels of NH_4^+, PO_4^{3-}, total nitrogen, and total phosphorus (Table 4). The sampling site on the Kriva River is also surrounded by gardens and cultivated land, which could be associated with high level of nutrients in the river water. However, with exception of the continuous slight increase of several trace elements characteristic for agricultural use (Ba, Fe, Mo, and V; Fig. 17b, c, d, and g), it seems that these occasional contamination events were not the consequence of continuous leaching, either from the mine tailings or from the agricultural soil, considering that they were rather extreme, but of short duration. Therefore, it could be hypothesized that contamination of the Kriva River was caused by periodic waste input directly into the river water, either from the mine or manure from the farms.

In the spring sampling, it is even more probable that sediment resuspension, as a consequence of greater water discharge, resulted in the concentration increase of elements sequestered in sediment, such as Cd and Pb, as has often been reported for trace elements in the river water (Neal et al., 2000; Dragun et al., 2009). The contamination of the river sediments has been reported in most metal mining regions of the world with metal concentrations usually being several orders of magnitude higher than those in the water column (Macklin et al., 2006). Sediments of the Toranica River, tributary of the Kriva River, were reported as being highly contaminated with Pb, Zn, and sulfur; although the metal concentrations decreased after the confluence with the Kriva River, they were still significantly increased (Alderton et al., 2005). Similarly, in the Twymyn River in the United Kingdom, in the vicinity of a former Pb/Zn mine, Pb concentrations in sediment were ≤100 times higher than levels reported to have deleterious impacts on aquatic ecology, and they were especially high in the acid-soluble phases (Byrne et al., 2010). It is characteristic for metals introduced into sediments through human activities, such as mining, that they often exist in weakly bound chemical forms (Jain, 2004) and therefore potentially pose serious threats for the aquatic systems (Byrne et al., 2010). Because of that, sediments are not a permanent sink for metals, and sulphide-/organic-bound metals may be released into the water column whenever suitable conditions for dissolution occur, such as, for example, the disturbance and oxidation of sediments during flood flows (Byrne et al., 2010). Therefore, even when concentrations of toxic elements in water decrease due to dilution or precipitation, and pH is not very low, the river sediments still can present the source of high pollution level and high toxicity (Sarmiento et al., 2011).

Table 22. The environmental quality standards (EQS) proposed by the EU WFD, the Canadian water quality guidelines for the protection of aquatic life or protection of agriculture (for irrigation) for dissolved trace elements in the surface waters, and freshwater screening benchmarks defined by US EPA for dissolved trace and macro elements.

	EQS[a]	**Canadian guidelines[d]**	**US EPA[g]**
As / µg L^{-1}	-	5	-
Ba / µg L^{-1}	-	-	4
Cd / µg L^{-1}	0.08-0.25[b]	0.031-0.112[e]	0.25[h]
Co / µg L^{-1}	-	50[f]	23
Cu / µg L^{-1}	8.2[c]	2.25-4[e]	9[h]
Fe / µg L^{-1}	-	300	-
Li / µg L^{-1}	-	2500[f]	14
Mn / µg L^{-1}	-	200[f]	120
Mo / µg L^{-1}	-	73	-
Ni / µg L^{-1}	20.0	91.3-150[e]	52[h]
Pb / µg L^{-1}	7.2	2.95-7.00[e]	2.5[h]
Sb / µg L^{-1}	-	-	30
Sn / µg L^{-1}	-	-	73
Sr / µg L^{-1}	-	-	1500
U / µg L^{-1}	-	15	2.6[h]
V / µg L^{-1}	-	100[f]	20
Zn / µg L^{-1}	-	30	120[h]
Na / mg L^{-1}	-	-	680
K / mg L^{-1}	-	-	53
Ca / mg L^{-1}	-	-	116
Mg / mg L^{-1}	-	-	82

a - European Parliament and the Council of the European Union (EPCEU) (2008); b - EQS for Cd depends on the concentration of $CaCO_3$ in the river water; for the Kriva River, EQS for Cd is 0.150 µg L^{-1}, and for the Zletovska and Bregalnica rivers, 0.250 µg L^{-1}; c - Crane et al. (2007); d - http://st-ts.ccme.ca/; e - for some elements, Canadian guidelines for the protection of aquatic life are based on the concentration of $CaCO_3$ in the water: the lower limit given in the table is based on the lowest $CaCO_3$ level in the Kriva River, whereas the upper limit is based on the highest $CaCO_3$ level measured in the Zletovska River; f - for several elements, Canadian guidelines for the protection of aquatic life are not defined, and the values presented in the table refer to the guidelines for the protection of agriculture; g - http://www.epa.gov/reg3hscd/risk/eco/btag/sbv/fw/screenbench.htm; h - defined for hardness of 100.

5.2. THE BIOMETRY AND ORGANOSOMATIC INDECIES OF VARDAR CHUB (published in Jordanova et al., 2016 and Dragun et al., 2019)

To assess the impact of the described surface water contamination of three rivers in northeastern part of North Macedonia, the fish species Vardar chub (*S. vardarensis*) was used, as already mentioned earlier. Vardar chub sex, mass, length, condition factor and organosomatic indices were used as indicators of fish well being, which may vary in response to different kinds of pollutants in the river water, including heavy metals (Schmitt and Dethloff, 2000; Jovanović et al., 2011; Dragun et al., 2013b; Liebel et al., 2013).

As a starting point, to understand the relationship between the individuals, the environment and the state of the population, it is important to analyze the sex ratio of the sampled fish (Oliveira et al., 2012). The sex ratio (female to male) is expected to be 1:1, but it may vary, species to species, or even within the same population at different times, being influenced by several factors, such as adaptation of the population, reproductive behavior, food availability and environmental conditions (Oliveira et al., 2012).

In this study, in the Bregalnica River ideal ratio of females to males was observed in the spring period, while in autumn in that river, and in both seasons in the Zletovska River, the female dominated (30-70% higher number of females than males) (Table 7). Dragun et al. (2009, 2013b) have used in their investigation the European chub as bioindicator fish species and have observed domination of females (59-80% of females in the studied populations), while Raikova-Petrova et al. (2012), who have investigated European chub in the Iskar River in Bulgaria, showed 30% higher number of females than males. Moreover, Van et al. (2019) showed disproportion of females to males during the investigation of fish species from Kizilirmak-Yesilirmak shelf area, reporting female domination for three fish species (sex ratio (F:M) - 1.3:1 for *Merlangius merlangus*, 1.45:1 for *Pomatomus saltatrix*, 1.80:1 for *Mullus barbatus*) and male domination for only one fish species (sex ratio (F:M) - 1:1.24 for *Trachurus mediterraneus*). Unlike the Bregalnica and the Zletovska rivers, in the Kriva River in both seasons the male domination was observed, with approximately twice higher number of males compared to females (Table 7). The domination of males was also reported by Oliveira et al. (2012) in their study on several marine fish from Brazilian coastal waters, namely *Chloroscombrus chrysurus* (F:M ratio - 1:2), *Opisthonema oglinum* (F:M ratio - 1:1.4) and *Lutjanus synagris* (F:M ratio - 1:4). Raikova-Petrova et al. (2012) have established three periods with different sex ratios: 1) the youngest age classes with male domination; 2) the following period of balanced sex ratio; 3) and the last period of female domination. Such grouping was also reported by Rebok (2013), who have observed that in the early life stages, the number of collected males was higher than the number of females, while in the later life stages the collected number of females was predominant. Rebok (2013), in her study, further explained that for freshwater fish in lower age classes the ability of hatching out the male fish is higher compared to females, while in the upper age classes it is opposite, the rate of hatched males is lower and as a consequence the females become dominant in a population. Accordingly, it can be presumed that the male domination in the Kriva River was the consequence of the younger fish compared to the other two rivers. There is also possibility that differences in the water quality at three rivers have influenced the sex composition at each river, which would be in agreement with the statements of Oliveria et al. (2012).

It was also observed that female chub were longer and also heavier than male chub (Table 10-11; Figs. 20-22), and, according to Rebok (2013), smaller body size in males than in females is common in many fish species. Furthermore, the smallest fish, both concerning the length and mass, were found in the Zletovska River in spring, and in both mining impacted rivers (Zletovska and Kriva) in autumn (Table 10-11; Figs. 20-22), most likely due to severe water pollution, with metals and organic matter, respectively. Reduction in growth at polluted sites compared to unpolluted was observed in many previous studies (e.g., Dragun et al., 2013b; Rebok, 2013).

In both seasons, the lowest Fulton condition indices (FCI) were also observed at the Zletovska River, which can be associated with the worst water quality of the Zletovska River, whereas FCI was mildly decreased only in autumn at the Kriva River compared to the Bregalnica River (Table 11; Fig. 23). FCI gives information about the physiological state of the fish in relation to the their general welfare and nutritional status (Barišić et al., 2015), and, thus, it is associated with biological factors such as age, sex, gonadal development, suitability of the environment and fish fatness (Van et al., 2019). The decrease of FCI might reflect metabolic impact of trace metals on fish, as was reported for *Perca flavescens* (Giguère et al., 2004), for *M. barbatus* (Filipović Marijić and Raspor, 2007), and for *S. cephalus* (Dragun et al., 2013b). Schmitt and Dethloff (2000) also reported decreased condition factors of white suckers at sites with elevated concentrations of metal mixtures. Thus, especially small fish and low condition factors observed for Vardar chub at the Zletovska River could be associated with high metal exposure, as was previously reported by Filipović Marijić and Raspor (2007), and which is consistent with extremely high concentrations of several metals found in the surface water of the Zletovska River in the time of chub sampling. In addition to severe water contamination, smaller FCI of Vardar chub from the Zletovska River could be also the result of insufficient nutrition of fish living in that river (Munkittrick and Dixon, 1988). Milder decrease of condition factors of Vardar chub from the Kriva River is consistent with the fact that the Kriva River, although being mining impacted river, was less contaminated with metals compared to the Zletovska River, and the lower condition indices only in the autumn season could be associated to the occurrence of organic contamination of surface water.

Gonad mass and gonadosomatic indices (GSI) showed differences between the seasons with generally higher values in the spring (Table 12; Figs. 24-26). Since GSI reflects the cycling changes in gonad mass in relation to total fish mass (Rebok, 2013), it can be used to determine fish spawning period. Increase of GSI suggests approaching spawning season, while decrease suggests that spawning has already occurred (Rebok, 2013). Accordingly, based on the results of this study, the preparation for the spawning period, i.e. gonad development of Vardar chub occurs in spring. Gonad development is related to increments in the daylight period, water temperature, and food supply (Encina and Granado-Lorencio, 1997). The exact period of spawning of *S. cephalus*, fish species related to Vardar chub, although it depends on the climate, mostly occurs from April to June (e.g., Berg 1964; Öztaş, 1989; Şaşi, 2004). Similarly, Raikova-Petrova et al. (2012) in their study have indicated periods from March to September as the spawning periods of *S. cephalus*, depending on the water bodies which those fish inhabited. Moreover, in this study, the highest GSIs in the spring time were observed in the Kriva River (Table 12; Figs. 24-26), possibly indicating the impact of combined metal and organic pollution. Water contamination was previously indicated as the possible cause of the changes of FCI and GSI indices, specifically of FCI decrease and GSI increase (Dragun et al., 2013b). Billiard and Khan (2003) also found GSI increase in various fishes from the locations contaminated with pulp and paper mill effluent.

Generally the lowest liver mass and hepatosomatic indices (HSI) were also observed for Vardar chub at the Zletovska River (Table 13; Figs. 27-29), probably as a consequence of high metal pollution. According to Dekić et al. (2016), the HSI is a good indicator of the energy status, and, accordingly, the fish in poor habitat conditions have lower values of this index than fish species that inhabit the food rich habitats. HSI values also vary with season (Schmitt and Dethloff, 2000), as well as depending on the gender and stage of gonad development (Fabacher and Baumann, 1985; Förlin and Haux, 1990; Dekić et al., 2016). However, the changes in the size of liver can also be related to the stressors that come from the environment. Many investigators have suggested that HSI increase or decrease in fishes indicates exposure to numerous environmental toxic chemicals (Schmitt and Dethloff, 2000; Blazer et al., 2006), including heavy metals (Figueiredo-Fernandes et al., 2007; Jovanović et al., 2011) and can be linked to hystopathological changes in the liver (Ram and Singh, 1988). For example, HSI was found considerably lower in the fish exposed to toxic substances, such as Cd and Zn (Dekić et al., 2016), which is consistent with the results of this study. Moreover, Larsson et al. (1984) and Schmitt and Dethloff (2000) confirmed that liver size decreased due to exposure of perch (*Perca fluviatilis*) to a mixture of metals.

Gill masses and gill indices were generally lower at the Zletovska River (Table 14; Figs. 30-31), which could be associated with the high contamination of its river water. On the gills of the same specimens of Vardar chub as used within this study, the histopathological assessment was also performed. The fish from the Zletovska River were characterized by frequent occurrence of severe regressive changes of both epithelium and supporting tissue, resulting with higher total lesion indices compared to the other two rivers (Barišić et al., 2015), which could present the changes underlying the decrease of the gill masses of the fish from the Zletovska River.

5.3. METAL BIOACCUMULATION IN THE LIVER AND GILLS OF VARDAR CHUB FROM THREE RIVERS IN THE Pb/Zn MINING REGION IN THE NORTHEASTERN PART OF NORTH MACEDONIA (published in Dragun et al., 2019)

The cytosolic concentrations of four macro and 15 trace elements in two target organs of Vardar chub, gills and liver, were further analyzed as the indicators of their bioaccumulation in differently contaminated environments. Gills were chosen due to their direct contact with the ambient water and are expected to respond quickly to changes in metal exposure (Kraemer et al., 2005; Dragun et al., 2007), while liver were chosen for detection of chronic exposure to metals (Di Giulio and Hinton, 2008). Cytosolic concentrations of those selected 19 elements in the gills and liver of Vardar chub measured in spring and autumn of 2012 (Tables 15 and 16) present the first data of that kind for Vardar chub. Cytosolic concentrations of several of these elements were previously published for the gills and the liver of related fish species European chub (*S. cephalus*) from moderately contaminated rivers in Croatia, and can serve as a basis for comparison with the results of this study. Average cytosolic concentrations of five metals in the gills of *S. cephalus* from the Sava River were as follows: Cd 2.9-3.6 µg L^{-1}, Cu 68.4-79.0 µg L^{-1}, Fe 3.9-9.6 mg L^{-1}, Mn 55.0-63.5 µg L^{-1}, and Zn 6.3-10.3 mg L^{-1} (Dragun et al., 2007), whereas their average cytosolic concentrations in the liver of the same fish were: Cd 8.0 µg L^{-1}, Cu 1.5 mg L^{-1}, Fe 5.0 mg L^{-1}, Mn 150.0 µg L^{-1}, and Zn 5.0 mg L^{-1} (Podrug et al., 2009). Cytosolic concentration ranges of several other hepatic metals in *S. cephalus* from the Sava River were: Co 4.1-5.1 µg L^{-1}, Mo 22.8-30.6 µg L^{-1}, Pb 1.0-5.9 µg L^{-1}, Sr 5.5-10.5 µg L^{-1}, and V 2.9-11.5 µg L^{-1} (Dragun et al., 2013a). Average cytosolic concentrations of four metals in the gills of *S. cephalus* from the Sutla River were as follows: Pb 0.85-17.3 µg L^{-1} (Dragun et al., 2012), and Cd 0.68 µg L^{-1}, Cu 42.6 µg L^{-1}, and Zn 14.3 mg L^{-1} (Dragun et al., 2013b); whereas their average cytosolic concentrations in the liver of those same fish from the Sutla River were: Pb 1.2-18.1 µg L^{-1} (Dragun et al., 2012), and Cd 19.4 µg L^{-1}, Cu 1.5 mg L^{-1}, and Zn 6.6 mg L^{-1} (Dragun et al., 2013b). Cytosolic concentration ranges of several other elements in the gills of *S. cephalus* from the Sutla River were the following: Ba 13-67 μg L^{-1}, Ca 19-62 mg L^{-1}, Co 0.7-2.7 μg L^{-1}, Cs 0.2-1.9 μg L^{-1}, Fe 1.6-6.4 mg L^{-1}, K 225-895 mg L^{-1}, Mg 13-47 mg L^{-1}, Mn 16-69 μg L^{-1}, Mo 1.3-16 μg L^{-1}, Na 78-366 mg L^{-1}, Rb 164-1762 μg L^{-1}, Sr 24-81 μg L^{-1}, and V 0.1-1.8 μg L^{-1} (Dragun et al., 2016). If we compare those numbers with the results of the present study, we can see that cytosolic concentrations of highly toxic elements (Cd, Cs, Pb and Sr) in the gills and the liver of Vardar chub reached higher levels compared to European chub from moderately contaminated Sava and Sutla rivers in Croatia, indicating that increased metal exposure caused by mining contamination of the North Macedonian rivers resulted with an observable impact on metal bioaccumulation in fish organs.

In the present study, bioaccumulated concentrations of four analyzed macro elements, Na, K, Ca and Mg, in the gills and the liver of Vardar chub generally did not reflect the increased exposure from the river water. Although in both sampling periods their highest dissolved concentrations in the river water were measured in the mining impacted Zletovska River (Table 5; Fig. 16) (10-15 times higher compared to the other rivers for dissolved Na, and 3-9 times for dissolved K, Ca and Mg), the highest cytosolic concentrations of these elements were not always found at the Zletovska River, either in Vardar chub gills (Fig. 32) or liver (Fig. 35). Although statistically significant differences between the sites were sometimes observed, the differences were generally rather small and the concentrations of each of these four elements were placed within the narrow ranges indicating strong intracellular regulation.

The bioaccumulated concentrations of seven essential trace elements (Co, Cu, Fe, Mn, Mo, Se and Zn) in the gills and liver of Vardar chub showed the similar behaviour as the macro elements, despite the enormous differences in the exposure levels among three rivers. Four of these elements, dissolved Co, Cu, Mn and Zn, were increased in the river water of the mining impacted Zletovska River (Fig. 18b, d, f, and l), whereas two of them, dissolved Fe and Mo, were increased in the river water of the agriculturally impacted Bregalnica River (Fig. 17c and d). As for Se, there are no available information on its dissolved concentrations in the river water of the studied rivers at the time of the fish sampling. The concentrations of the dissolved Fe and Mo in the Bregalnica River, as well as Cu in the Zletovska River, were only moderately higher compared to the other rivers (up to 8 times). Contrary, the concentrations of dissolved Co, Mn and Zn in the Zletovska River were markedly higher compared to the other two rivers (28-40 times in the spring period, depending on the metal). In the autumn period, characterized by extremely low water level, this differences were even more obvious for dissolved Mn and Zn, with maximally 570 and 400 times higher concentrations in the Zletovska River, respectively. However, although some statistically significant differences of bioaccumulated essential trace element concentrations were observed between the sites for both organs and in both seasons (Figs. 33 and 36), the observed trends mainly could not be associated to the exposure from the water, again indicating probable strong regulation of essential elements in the fish organism, already reported in numerous studies (Olsvik et al., 2000; Moiseenko and Kudryavtseva, 2001; Monna et al., 2011). Only indication of response to increased exposure in the water was recorded in the gills of Vardar chub, in the autumn for cytosolic Co (Fig. 33a) and Cu (Fig. 33b) and in the spring for cytosolic Mn (Fig. 33d) and Zn (Fig. 33g), with significantly higher gill concentrations at the Zletovska River. However, it could not be claimed without a doubt, even for those mild differences observed for those four essential metals, that they were the results of higher exposure in the water. Dissolved Mn and Zn concentrations were, as already mentioned, much higher in the water in the autumn sampling, and thus multiple increase of gill accumulation of Mn and Zn at the Zletovska River would be expected in the autumn sampling compared to spring, which was not the case. Differences, such were observed in the gills, were not observed in the liver; contrary, hepatic Co (Fig. 36a) was even the lowest in the Vardar chub from the site with the highest exposure, i.e. from the Zletovska River. Brumbaugh et al. (2005) also reported that Zn in the liver of common carp (*Cyprinus carpio*) did not reflect water contamination near the former site of Pb/Zn mining. Casiot et al. (2009) reported absence of Cu, Mn and Zn bioaccumulation in the liver of chub (*Leuciscus cephalus*) downstream of the point of contamination by acid mine drainage originating from a former Pb/Zn mine, whereas Zhuang et al. (2013) reported that Zn bioaccumulation factors in the various fish tissues from mining influenced ponds showed a small range (8-29 %), suggesting that Zn was regulated to maintain a homeostatic status.

Furthermore, bioaccumulated Fe (Figs. 33c and 36c) and Mo (Figs. 33e and 36e) concentrations did not reflect the exposure level in either of the target organs of Vardar chub. Their highest bioaccumulation would be expected in Vardar chub from the Bregalnica River which had the highest exposure level (Fig. 17c and d), but that was not confirmed by the obtained results. The differences between sites were actually very small for Fe and Mo in both organs and in both seasons. This was opposite to previous study on related fish species, European chub (*S. cephalus*), when evident Fe bioaccumulation in the gills was reported as a consequence of exposure in the river water (Dragun et al., 2016).

The only element that stands out from this group of essential elements is Se, which was markedly increased in both seasons and in both organs in fish from the mining and agriculturally impacted Kriva River (Figs. 33f and 36f); however, due to the lack of information about the Se levels in the river water, it can only be hypothesized that the cause was higher exposure level at the Kriva River. This contrast between Se and the most of the other essential elements was already described in the literature. Although Se is an essential micronutrient that is vital to biological systems in small amounts, it has a narrow range between essentiality and toxicity in fish (Wilber, 1980). Even at low aqueous concentrations (≤ 1 µg L^{-1}) in freshwater ecosystems, Se can bioaccumulate in aquatic food chains and cause deleterious effects at higher trophic levels in species such as fish (Lemly, 1985, 1993; Bowie et al., 1996; Hamilton, 2004). Thus, whole-body Se concentrations of lake chub (*Couesius plumbeus*) significantly increased after exposure to uranium mill effluent (Phibbs et al., 2011). In the study by Bubach et al. (2015), Se concentrations in the liver of rainbow trout (*Oncorhynchus mykiss*) were doubled at certain areas, whereas at the same time K, Zn, and Fe did not show significant differences among differently contaminated areas. Increased Se accumulation was also previously reported in several studies in the liver of metal exposed fathead minnows (*Pimephales promelas*) (Rozon-Ramilo et al., 2011; Ouellet et al., 2013). Comparable to our study, Ouellet et al. (2013) did not observe simultaneous Cu accumulation in the liver of fathead minnows (*P. promelas*), despite the considerable exposure level in the water (total Cu: ~70 µg L^{-1}), whereas Rozon-Ramilo et al. (2011) reported the increasing trend of both hepatic Se and Cu concentrations after the exposure of fathead minnows to mine effluent (total Cu: ~50 µg L^{-1}). Increased Cu and Zn bioaccumulation was also reported in both liver and gills of grass carp (*Ctenopharyngodon idellus*) in the vicinity of copper mine in China (Liu et al., 2012). Since Cu, like majority of essential elements, is a tightly regulated micronutrient in fish, which ensures the protection from possible toxicity, Cu levels in the water or diet have to be at a relatively high level to cause such noteable Cu bioaccumulation in fish tissues (Schlenk and Benson, 2001). Considering that various authors have reported different bioaccumulation trends for essential metals at comparable exposure levels (Rozon-Ramilo et al., 2011; Liu et al., 2012; Ouellet et al., 2013), some other factors, and not only the exposure level, probably can influence bioavailability and accumulation of essential metals in fish organs, such as fish species and metal speciation in the water. Accordingly, another explanation for differences in bioaccumulation between Se and other essential elements can be found in water chemistry. Water chemistry (pH, Ca content, etc.) tends to make metals more or less bioavailable (and thus more or less toxic) for animals (Monna et al., 2011). Selenium availability is most favored at basic pH, whereas the availabilities of the other elements are in the most cases higher in waters with acidic pH (Eisler, 1993; Ezoe et al., 2001). In our study, all rivers had pH that was either close to neutral (6.52-6.88 in the Zletovska River; Table 3) or slightly basic (7.90-8.12 in the Bregalnica and the Kriva River; Table 3), which was evidently favourable for Se bioaccumulation.

All studied nonessential elements (Cd, Cs, Pb, Rb, Sr, Tl and V), except Ba, clearly reflected the exposure from the water, in both seasons and in both organs of Vardar chub (Figs. 34 and 37). This was consistent with the general knowledge that the levels of non-essential elements, such as Cd and Pb, in various fish species generally follow the peaks of environmental metal contamination more closely than the levels of essential elements (Monroy et al., 2014). Two of the studied nonessential elements had higher dissolved concentrations in the river water of the agriculturally impacted Bregalnica River compared to the other two rivers, namely Ba (Fig. 17b; up to 12 times) and V (Fig. 17g; up to 29 times) . The other elements were mainly higher in the river water of the mining impacted Zletovska River compared to the other two rivers. In the spring period, dissolved Cd (Fig. 18a) and Tl (Fig. 18k) were higher in the Zletovska River compared to one or the other remaining river up to 8 times, dissolved Rb (Fig. 18h) and Sr (Fig. 18j) up to 27 times and dissolved Cs (Fig. 18c) as much as 50 times. In the autumn period, higher concentrations of several of those elements in the Zletovska River compared to the other two rivers were even more notable, especially of dissolved Cd, Cs and Tl, amounting to 50-95 times (Figs. 18a, c, and k). Even dissolved Pb (Fig. 19a), which was rather low in the Zletovska River in the spring, has somewhat increased in the autumn season, probably due to extremely low water level of that river (Table 2). In the Kriva River, which was also impacted by the mining waste, higher concentrations of dissolved Cd (Fig. 18a) and Pb (Fig. 19a) compared to the other two rivers were found only in the spring period. At that time, concentration of dissolved Cd in the Kriva River was comparable to the Zletovska River, whereas dissolved Pb concentration was up to 6 times higher compared to the other two rivers.

As mentioned above, the only nonessential element that have not reflected the exposure in the water was Ba (Fig. 34a and 37a). The highest hepatic and gill concentrations of that metal were not found as expected in the fish from the Bregalnica River. Contrary to our findings, Rašković et al. (2018) found increased Ba bioaccumulation in the liver and gills of chub (*S. cephalus*) after accident at abandoned mining site. However, gill Ba concentrations were shown to exhibit much higher concentrations in smaller than in bigger fish in the study on European chub (*S. cephalus*) from the Sutla River (Dragun et al., 2016). Therefore, the absence of association between water exposure and consequent bioaccumulation which was observed for Ba in this study possibly occurred due to combined effect of too low exposure level in North Macedonian rivers and strong association of Ba with fish physiology.

As for the remaining nonessential elements, the highest hepatic and gill V concentrations (Fig. 34h and 37h) were always found in the Vardar chub from the Bregalnica River, whereas the highest hepatic and gill Cs (Fig. 34c and 37c), Rb (Fig. 34e and 37e), Sr (Fig. 34f and 37f), and Tl (Fig. 34g and 37g) concentrations were always found in the Vardar chub from the Zletovska River, closely reflecting the exposure in the water. Association between Cs bioaccumulation and level of water exposure was already reported for the gills of European chub (*S. cephalus*) (Dragun et al., 2016). Similar finding was reported for Rb and Cs concentrations in the liver of rainbow trout (*O. mykiss*) as a consequence of fish exposure to water contaminated by a volcanic source, with strong association between hepatic Rb and Cs concentrations and the distance to the volcano (Bubach et al., 2015). Ouellet et al. (2013) reported increased accumulation of Rb in the liver and Tl in the gills of fathead minnows (*P. promelas*) during chronic exposure to a metal mine effluent. Campbell et al. (2005) even reported tendency of Rb and Cs to consistently biomagnify in diverse food webs.

Furthermore, hepatic and gill Cd (Fig. 34b and 37b) and Pb (Fig. 34d and 37d) concentrations in Vardar chub followed the pattern of changeable water contamination. Higher hepatic and gill Cd concentrations were found in Vardar chub from both Zletovska and Kriva rivers compared to Bregalnica in the spring period, whereas in the autumn period the highest Cd concentrations were found in the liver and the gills of Vardar chub from the Zletovska River (Fig. 34b and 37b), which was in accordance with the levels of exposure recorded in the river water (Fig. 18a). Hepatic and gill Pb concentrations were the highest in Vardar chub from the Kriva River in the spring period, and from the Zletovska River in the autumn (Fig. 34d and 37d), again in accordance with the current river water contamination with Pb (Fig. 19a). Similar thing was reported by several authors and for various fish species. For example, Biuki et al. (2011) observed increase of Cd and Pb in the liver of juvenile milkfish (*Chanos chanos*) following the increase of exposure to these pollutants; Casiot et al. (2009) reported twofold increase of Cd, threefold increase of Tl and sixfold increase of Pb in the liver of chub (*L. cephalus*) exposed to acid mine drainage of former Pb/Zn mine, whereas Monna et al. (2011) reported three orders of magnitude higher hepatic Cd and Pb concentrations in brown trout (*Salmo trutta fario*) from mining influenced sites compared to those of commercial trout produced by fish farming. Increased Cd was also reported in the liver of *S. cephalus* from the contaminated site of the Sava River (Podrug et al., 2009), whereas increased Pb concentrations were found in both liver and gills of grass carp (*C. idellus*) from freshwater ecosystem influenced by copper mine (Liu et al., 2012) and of European chub (*S. cephalus*) from Pb contaminated site of the Sutla River (Dragun et al., 2012). Moreover, Ebrahimi and Taherianfard (2010) reported increased hepatic Pb concentrations in two cyprinid fish species (*C. carpio* and *Capoeta* sp.) from Pb contaminated section of the Kor River in Iran.

The trends of seasonal variability of elements bioaccumulated in the gills and liver of Vardar chub were not quite clear and striking. However, seasonal changes of few metals, especially nonessential, could be associated to the changes of dissolved metal concentrations in the river water. For example, the concentrations of nonessential elements Cd, Pb and Tl bioaccumulated in the gills and liver of Vardar chub directly followed the changes of exposure levels. Gill and hepatic Cd, Pb and Tl were increased in autumn, during the drought period, compared to spring at the Zletovska River, while they were increased in spring compared to autumn at the Kriva River (Tables 15 and 16), which was consistent with the trends of dissolved concentrations of those metals in the water of two rivers (Table 6). Contrary, concentrations of several essential metals in the gills and the liver exhibited seasonal variability which was probably the result of physiological processes, with their concentrations being higher predominantly in the spring period of higher feeding and growth rates (Farkas et al., 2002), as well as of higher metabolic activity (Andres et al., 2000).

5.4. FISH HEALTH STATUS (published in Jordanova et al., 2016)

Final objective of this study was to determine how established river water contamination with mining waste, as well as consequent metal bioaccumulation in Vardar chub gills and liver, has impacted the fish health status. A combination of analytical and biological methods provides a powerful tool to determine relationships between cause and effects (Lenhardt et al., 2015; Fonseca et al., 2017; Gerber et al., 2017; Giltrap et al., 2017; Rašković et al., 2018). Accordingly, fish health has been used worldwide as indicator of the health of ecosystem (Blazer et al., 2018). And, for the evaluation of fish health, the assessment of external/internal lesions has been often used, as well as the analysis of the histopathological changes on the fish tissues (Rebok, 2013). According to Carrola et al. (2009) and Rašković et al. (2018), histopathological alterations are often related to fish living in water where mining activities occur, and the changes on that level reflect an advanced stage of toxicant impact on fish population (Blazer, 2000). With that in mind, Vardar chub were inspected for external/internal lesions, and the study of toxicopathic changes on liver and gonads was performed. Liver were chosen for the assessment, as mentioned before, due to their vital role in fish metabolism, while gonads were chosen due to their importance in the reproductive health of the fish.

According to external/internal evaluation of Vardar chub, approximately 20% of fish from all three rivers had macroscopically visible disorders, with chub from the Zletovska River being somewhat more intensely affected (Table 17), which is not surprising considering observed high bioaccumulation of highly toxic elements (Cd, Cs, Pb, Rb, Sr, and Tl) in organs of Vardar chub from that river (Figs. 34b, c, d, e, f, and g, and 37b, c, d, e, f, and g). When high incidence of gross abnormalities occurs, it can be regarded as a consequence of significant presence and effect of toxicants in the water (Noga, 2000; Schmitt and Dethloff, 2000; Blazer et al., 2010).

Skin oedema and the absence of scales were the most frequent external disorders in Vardar chub, observed at all three rivers, but with highest prevalence at the Zletovska River, followed by parasites, and gill and kidney damages (Table 17). According to Rebok (2013), high prevalence of skin odema and absence of scales can, for example, occur due to the more aggressive behavior of local predator fish. Moreover, for the development of the gross lesions a set of interacting conditions is required, and factors that play a role in their development include genetics, species, food habits, contaminants and other water quality parameters, weather, handling or capture methods, or traumas of other types (Blazer, 2000). Schmitt and Dethloff (2000) stated that many investigators use different protocols and diagnostic criteria for determining the frequencies of external lesions, making their comparison difficult. And due to the lack of data about external gross lesions on Vardar chub from other investigations, it is difficult to further compare and interpret data on gross pathology of Vardar chub from mining impacted rivers in North Macedonia.

Thus, gross observations, although useful for uncovering previously unknown pathological conditions in fish from contaminated sites (Rebok, 2013), cannot be used for definitive evaluation of fish health, and should be followed by histopathological evaluation (Vethaak et al., 1992). Histopathology can provide very early warning indications of effect of contaminants in the environment (Schmitt and Dethloff, 2000), since, for example, metal exposure of fish can result in adverse biological effects, such as increase in lesion formation, tumors, cancers, and impaired reproductive success (Hinton et al., 1992; Hinton, 1993; Wolf and Wolfe, 2005; Hinck et al., 2006).

In Vardar chub liver, numerous non-neoplastic and neoplastic lesions were found, with lymphocyte infiltration, fibrosis and bile duct proliferation being the most frequently observed (Table 19). Lymphocyte infiltration (Fig. 38) and connective tissue proliferation, which were non-neoplastic in nature, were found in chub at all three locations, thus seemingly unrelated to differences in environmental contamination. Accumulation of lymphocytes was previously detected in the fish exposed to heavy metals (Sorensen et al., 1984; Schmidt et al., 1999; Javed and Usmani, 2013; Liebel et al., 2013), but also to pesticides and to other contaminants in the environment (Myers et al., 1992; Rousseaux et al., 1995; Schmidt-Posthamus et al., 2001; Hinck et al., 2007a; Liebel et al., 2013). This could serve as an explanation why lymphocyte infiltration was found both in agriculturally and mining impacted rivers, although it was somewhat more intensely associated to metal exposure. As for fibrosis, it is not unusual finding for the fish liver around the bile duct; it can have parasitic, inflammatory or a toxic cause or it could be idiosyncratic (Wolf and Wolfe, 2005; Roberts and Rodger, 2012), and it can lead to increased size of blood vessels (Rašković et al., 2018). Fibrosis was also previously described in association with metal exposure (Mallatt, 1985; Triebskorn et al., 2008). But, the most common lesion observed in Vardar chub liver was increased number of bile duct profiles, i.e. bile duct proliferation (Fig. 38), often in association with fibrosis. Its prevalence was the highest in metal contaminated rivers, especially in the Kriva River where it has been found in as much as 63% of fish (Table 19). The bile duct proliferation was previously recognized as being closely associated to pollution (Murchelano and Wolke, 1991; Rousseaux et al., 1995; Schmit et al., 1999; Stentiford et al., 2003), such as metal pollution (Roberts and Rodger, 2012), and was suggested as promising toxicopathological indicator (Blazer et al., 2006).

Parasites and granulomas had relatively low prevalence, which was still significantly higher in the Vardar chub from the Kriva River compared to Vardar chub from the other two rivers (Table 19). Observed granulomas on tissue sections were generally positively associated with occurrence of parasites within the body cavity, whereas number of parasites in fish is generally negatively associated with the presence of toxic pollutants (Lafferty, 1997). Rebok (2013) stated that absence of parasites, together with low condition factor of fish, is indicative of exposure to xenobiotics. Negative relationship between abundance of intestinal parasites in fish and metal contamination was often recorded (Dragun et al., 2013b; Vardić Smrzlić et al., 2015). In addition, in Vardar chub analyzed in this study, complete absence of intestinal parasites at both Zletovska and Kriva River was observed (Filipović Marijić et al., 2014). Accordingly, the lowest prevalence of parasites and granulomas in the liver, as well as of parasites in fish body cavity and on the other organs of Vardar chub from the Zletovska River (Table 19), which was highly metal contaminated, confirmed the negative association between parasite prevalence and water contamination. However, higher numbers of parasites and granulomas were found in the Vardar chub liver at mining impacted Kriva River (Table 19), which was consistent with previous report about commonly found granulomas in the liver of brown trout from the river subjected to mine drainage (Carrola et al., 2009). But, it is possible even for rivers impacted by mining activities to have only moderately increased concentrations of dissolved metals in the water, due to specific geological characteristics of the areas (dominating carbonate lithology), as observed for the Kriva River in this study, thus creating the favorable conditions for infestation with parasites.

Histopatological alterations which were also present in the fish from all three locations, were necrosis and megalocytosis (enlargement of hepatocyte cytoplasm and nucleus) (Fig. 39a). Necrosis occur when cellular homeostasis is disturbed, usually by the presence of free radicals in the cytoplasm, and it presents an irreversible histopathological alteration which is often induced by high levels of metals in the environment (Ribeiro et al., 2005; Farombi et al., 2007; Rašković et al., 2018). Accordingly, prevalence of both lesions was higher at both mining impacted rivers compared to agriculturally impacted Bregalnica River, although significantly higher only at the Kriva River (Table 19). According to Wolf and Wolfe (2005), occurrence of necrosis in fish liver is common and clear pathological response after exposure to toxicants (Ayas et al., 2007). More specifically, necrosis and/or hepatocelular degenerations were often found precisely in the fish liver in response to metal exposure (Bernet et al., 2004; Koca et al., 2005; 2008; Figueiredo-Fernandes et al., 2007; Triebskorn et al., 2008; Gurcu et al., 2010; Yildiz et al., 2010; Hadi and Alwan, 2012), but also after exposure to some other contaminants (Boorman et al., 1997; Figueiredo-Fernandes et al., 2007; Javed and Usmani, 2013; Liebel et al., 2013). In fish from the River Elbe, the observed hepatocytomegaly was also due to high level of metal pollution (Peters et al., 1987). It has also been suggested that megalocytoses can be an indicator of hepatocarcinogen-hepatotoxin exposure in the environment (Myers et al., 1987; 1990).

Light-dark hepatocytes (Fig. 39b), hepatocyte regeneration, and lipidosis, as neoplastic lesions, were observed in both metal polluted rivers, the Zletovska and the Kriva River, but have not been found at the agriculturally impacted Bregalnica River (Table 19). These types of lesions were also observed in the fish from the lower Elbe River, which is also contaminated with metals (Peters et al., 1987). Light-dark hepatocytes were previously observed in barbel collected from the Bregalnica River (Rebok, 2013), and it was suggested that this condition may be connected to toxicant induced injury. Hepatocytes regeneration (Fig. 40a) was also reported as a result of metal contamination of the aquatic environment (Gernhöfer et al., 2001) or even due to contamination by other toxicants (Wolf and Wolfe, 2005). Toxicants cause hepatocyte death after entering in the liver, directly or after metabolic transformation, and cell death is considered the first sign of toxicant effect in fish (Rebok, 2013). After hepatocellular necrosis occurs, hepatocytes undergo regeneration to replace necrotic foci (de Melo et al., 2008). The occurrence of lipidosis (Fig. 40b)) observed in the present study is in agreement with previous reports on fatty acid degeneration found in various fish species after metal exposure (Arellano et al., 1999; Yacoub and Abdel Satar, 2003; Koca et al., 2005; 2008; Gurcu et al., 2010; Hadi and Alwan, 2012). Costa et al. (2011) reported that lipidosis was observed in the liver of fish exposed to sediments contaminated with metals and organic compounds. This is in agreement with the results of this study, because lipidosis was found in the highest prevalence at the Kriva River (Table 19), where resuspension of sediments was presumed to be the cause of the increase of dissolved Cd and Pb in the river water in the spring period (Fig. 18a and Fig. 19a). Similar to other lesions, next to its association with exposure to toxicants, lipidosis can also be a normal feature of fish liver (Roberts, 1978; Wolf and Wolfe 2005). However, as lipidosis have not been found in fish from less contaminated Bregalnica river, but only in two mining impacted rivers, it can be supposed that lipidosis in the investigated Vardar chub was probably a pathological condition caused by high metal exposure.

High prevalence of external and internal lesions on Vardar chub from the Zletovska River, as well as presence of numerous hepatic lesions, is consistent with additional findings on the same Vardar chub, i.e. the highest relative volumes of immune cells in the kidney (Jordanova et al., 2017) and higher frequency and intensity of histopathological alterations in the gills of Vardar chub from the Zletovska River compared to the other two rivers (Barišić et al., 2015).

Many biomarkers and processes in fish, such as metal bioaccumulation, vary seasonally with the reproductive cycle. Thus, to be able to interpret the results obtained studying the fish, it is essential to know their reproductive status, which can be achieved by determining histologically their gonadal stage or condition (Blazer, 2000). Histological examination of gonads is a valuable technique that has been used to evaluate not only the reproductive status, but also to determine the fish sex, and to assess the gonadal health of male and female fish, and it falls into a category of analyses that provide structural, rather than functional, information about gonadal health and maturational stage (Blazer, 2000). Different xenobiotics can alter ovarian development/maturation and cause reproductive abnormalities (Rebok, 2013). Oocyte atresia, defined as an involution or resorption of unfertilized eggs by the ovaries, is a normal physiological event in all fish, but it also occurs as a pathological condition in the enhanced degree in fish after exposure to certain environmental contaminants, and is, thus, used as a biomarker of reproductive impairment (Blazer, 2000).

Many researchers have reported abnormalities of the gonads (e.g., enhanced oocyte atresia) due to the impact of agricultural activities or exposure to organic compounds (Kime, 1995; Johnson et al., 1999). Moreover, Kumar and Pant (1988) have reported atretic oocytes in *Puntius conchonius* caused by exposure to several metals (Cu, Pb, and Zn). Also, Rebok (2013) has reported oocyte atresia and intersex condition in barbell captured in the Bregalnica River. The presence of intersex in fish captured at the contaminated sites was reported by many researchers (van Aerele at al., 2001; Peňaz et al, 2005; Hinck et al., 2008; Tetreault et al., 2011).

Contrary to these findings, histological examination of the gonadal tissue of Vardar chub in this study did not reveal any signs of disease (presence of atretic oocytes, intersex, or disorganization of the lobules in testes), in either male or female fish. All examined fish were evidently in good reproductive condition (Figs. 41 and 42), indicating that gonads were probably more tolerant to metal exposure than liver. It is in accordance with the reports that lower levels of many metals/metalloids (Hg, Pb, Cd, As, Cu, Zn and Cr) were always detected in gonads compared to fish muscle, liver and kidney (Has-Schön et al., 2008).

6. CONCLUSIONS

The study of metal contamination of surface water caused by mining activities in three rivers in northeastern part of North Macedonia, Bregalnica, Zletovska and Kriva, in two seasons (spring and autumn) in 2012, combined with the study of metal bioaccumulation in two organs (gills and liver) of the representative fish species from those rivers, Vardar chub (*Squalius vardarensis*), and with the study of the effects of metal exposure on Vardar chub health, lead to the following conclusions:

1. The Bregalnica River, despite of the inflow of mining impacted Zletovska River into its course, did not show the water contamination characteristic for mining impacted rivers, but only weak contamination by several dissolved trace elements (As, Ba, Fe, Mo, Ti, U, V), as well as by nitrates and phosphates, which is alltogether characteristic for agriculturally developed regions. The pH of the Bregalnica River water was slightly alkaline (8.1) and comparable to pH values characteristic for the sites with high intensity agricultural activity. Moreover, the Bregalnica River had several degrees higher water temperature compared with the other two rivers during both sampling periods, possibly associated with nearby geothermal system Kezhovica-Ldzhi.
2. The Zletovska River exhibited clear signs of water contamination as a result of mining activities of the Pb/Zn mine Zletovo, with high concentrations of several dissolved trace elements in the surface water (namely, Cd, Co, Cs, Cu, Li, Mn, Ni, Pb, Rb, Sn, Sr, Tl, and Zn), among which Cd, Ni and Pb should be pointed out as priority toxic substances. Especially high concentrations of few metals were measured in autumn (e.g., Cd 2.0 µg L^{-1}; Mn 2.5 mg L^{-1}; Zn 1.5 mg L^{-1}), during the drought period. Furthermore, the Zletovska River water was slightly acidic (6.5-6.9), with high concentrations of sulphates and chlorides, positive pE, high total hardness, high conductivity and total dissolved solids, high concentration of $CaCO_3$, as well as high concentrations of four macroelements (Na, K, Ca, and Mg). The Zletovska River water was classified, according to several physico-chemical parameters, as third class of water during spring, and fifth class of water during autumn.
3. In the Kriva River, the impact of mining activities of Pb/Zn mine Toranica was less pronounced, and evident only in the spring period. It was manifested as increased dissolved Cd (0.270 µg L^{-1}) and Pb (1.85 µg L^{-1}) concentrations in the surface water. Increased concentrations of these metals only in the spring period were a possible sign of sediment contamination and its resuspension during higher water discharge. Generally low trace metal concentrations in the surface water of the Kriva River were probably caused by lack of pyrite in the ore compared to Zletovo, as well as by buffering of acid waters by carbonate host lithologies (limestone). Moreover, the Kriva River water had slightly alkaline pH (7.9-8.0) and negative redox potential, similar to the Bregalnica River, but lower conductivity, alkalinity and lower levels of macroelements, indicating lower concentrations of various salts in this river. In autumn, temporary increase of nutrients (NH_4^+, PO_4^{3-}, total nitrogen, and total phosphorus) was observed, which could be the result of the direct periodic input of wastes from gardens and cultivated land nearby the river, and a confirmation of the impact of the agricultural activity on the Kriva River water quality.
4. The impact of water contamination by mining waste on Vardar chub condition and health was observed in the changes of several biometric parameters. In both seasons, the smallest fish, both concerning the length and mass, the lowest Fulton condition indices (FCI), the lowest hepatosomatic indices and the lowest gill masses were

observed for Vardar chub at the Zletovska River, most likely due to severe metal contamination of surface water. At the other mining impacted river, the Kriva River, small fish and mildly decreased FCI were found only in autumn, thus probably associated to organic matter pollution of the Kriva River water.

5. Gonad mass and gonadosomatic indices were higher in the spring than autumn, indicating spring as the period of Vardar chub gonad development, i.e. preparation for spawning.
6. The bioaccumulation of ten studied essential elements (Na, K, Ca, Mg, Co, Cu, Fe, Mn, Mo, and Zn) in the gills and liver of Vardar chub did not clearly reflect the exposure levels in the surface water. Their concentrations were kept in narrow ranges in both organs of selected bioindicator species, even in the conditions of extremely high water exposure. That was especially evident for Mn and Zn at the Zletovska River during the autumn period of extremely low water level, and indicated the physiological regulation of the concentrations of essential elements.
7. The only essential element whose gill and hepatic concentrations probably reflected the level of water exposure was Se, which was markedly increased in Vardar chub from the mining and agriculturally impacted Kriva River, thus indicating its strong tendency to bioaccumulate. This finding is consistent with previous knowledge on Se, which even at low aqueous concentrations (≤ 1 µg L^{-1}) can bioaccumulate in fish and cause them harmful effects.
8. The bioaccumulation of seven nonessential and highly toxic elements (Cd, Cs, Pb, Rb, Sr, Tl, and V) clearly reflected the level of exposure in the water. Significantly higher gill and hepatic concentrations of Cs, Rb, Sr, and Tl were detected in Vardar chub from the Zletovska River compared to the other two rivers in both seasons, of Cd in Vardar chub from the Zletovska and Kriva rivers in spring and from the Zletovska River in autumn in comparison to Bregalnica, of Pb in Vardar chub from the Kriva River in spring and from the Zletovska River in autumn in comparison to the other two rivers, and of V in Vardar chub from the Bregalnica River compared to the Zletovska and Kriva rivers in both seasons.
9. The only nonessential element whose bioaccumulation did not follow the pattern of water contamination was Ba, either due to rather low exposure level or due to previously described pronounced dependence of its concentrations on fish physiology, with much higher Ba concentrations usually found in smaller than in bigger fish.
10. Vardar chub from the Zletovska River were more intensely affected with macroscopically visible external/internal disorders compared to the fish from the other two rivers, which reflected an advanced stage of toxicant impact and was consistent with high bioaccumulation of highly toxic elements (Cd, Cs, Pb, Rb, Sr, and Tl) in Vardar chub organs.
11. Histological assessment of liver revealed hepatic lesions in all three examined rivers: agriculturally impacted Bregalnica River, and two mining impacted rivers, Zletovska and Kriva. However, higher total prevalence of hepatic lesions, as well as occurrence of more severe hepatic lesions, such as neoplastic lesions, were generally registered in mining polluted rivers (Kriva River, 70%; Zletovska River, 59%; Bregalnica, 38%). The lesions which were found in both mining impacted rivers were probably at least partly the result of water contamination with metals, including bile duct proliferation, megalocitosis, light-dark hepatocytes, hepatocyte regeneration and lipidosis. Several

severe hepatic lesions (e.g., granulomas and necrosis) were observed more frequently in Vardar chub from the Kriva River, which is less metal contaminated of two mining impacted rivers, but which is also affected by agricultural contamination, thus indicating possible synergistic effect of metal and organic pollution of the river water on Vardar chub health.

12. Histological assessment of gonads revealed good reproductive health of Vardar chub from all three rivers. No signs of disease, i.e. presence of atretic oocytes, intersex, or disorganization of the lobules in testes, were found in either male or female fish, indicating high tolerance of gonads to contaminant exposure.
13. Although external/internal malformations and hepatic lesions are not specific indicators of certain individual contaminants, the results of this study have indicated that presence of high concentrations of metals in the river body, and especially in combination with organic water pollution, without a doubt has a significant toxic influence on the fish health and vitality.
14. This study has clearly demonstrated detrimental effect that mining pollution has on water quality and health of native freshwater fish. Such information is essential in a process of creating water management plans with an aim to protect, as well as to improve, quality of freshwater ecosystems worldwide, and especially in areas affected by active mining.

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8. BIOGRAPHY

Sheriban Ramani was born on August 5th, 1976 in Skopje, where she completed her primary and secondary education. In 2000 she graduated from the Faculty of Natural Sciences and Mathematics at the State University of Tetovo, in Chemistry. From 2000-2004 she worked as an assistant professor of General inorganic chemistry at the Faculty of Natural Sciences and Mathematics at the State University of Tetovo. In 2006 she obtained her Masters Degree in Natural Sciences and Mathematics from the Department of Chemistry at the University of Priština, with the topic "Electrochemical study of complexation of cadmium with cysteine and cystine in electrolyte solutions" under the supervision of Dr. Biserka Raspor, a senior scientist, and her master's thesis was conducted at the Laboratory for Biological Effects of Metals, Divison for Marine and Environmental Research at the “Ruđer Bošković” Institute, Zagreb, Croatia.

Since 2006, she has been working in the Water Quality Department of the Hydrometeorological Service as an advisor for atomic absorption spectrophotometry for determination of metals in surface water. Later, in 2018, she started working as Head of the Water Quality Laboratory. During that period, she attended several trainings on water quality assessment, including The Water Framework Directive held in Rotterdam, the Netherlands in 2008, and The precipitation chemistry training held in Germany in 2011.

She has participated in several projects, including:

- Monitoring the quantitative and qualitative characteristics of surface waters in the Lepenec River Basin;
- Monitoring the quantitative and qualitative characteristics of surface water reservoirs in Zhaden and the Rasce Source;
- Monitoring the quantitative and qualitative characteristics of surface waters in the Skopje Valley;
- Strumica River Watershed Monitoring Program;
- Bilateral project between Croatia and Macedonia, titled „The assessment of metal availability and effects on feral fish in the rivers under the impact of mining activities,“ the results of which are included in the doctoral thesis.

She is the author or coauthor of several papers, including:

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She is the author or coauthor of several abstracts in conference proceedings, including:

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