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Histopathology investigation on the Vardar chub (*Squalius vardarensis*) populations captured from the rivers impacted by mining activities

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Abstract

Many natural freshwater ecosystems, especially in the north eastern Macedonia, are polluted with heavy metals, which are released by active mines. Long-term exposure to high levels of dissolved metals might result in increased metal bioaccumulation in organs of aquatic organisms, and consequently might cause various sub-toxic and toxic effects. The aim of this study was to assess the health of Vardar chub (*Squalius vardarensis*) inhabiting mining impacted rivers Zletovska and Kriva, in comparison with chub from the reference Bregalnica River. It was done by use of indicators of tissue damage (histopathology of liver and gonads) and general indicators of exposure to environmental stressors (condition factor, organo-somatic indices and external/internal macroscopic lesions). Histological assessment of gonads revealed good reproductive health in all three rivers, indicating high tolerance of gonads to contaminant exposure. Contrary, several external/internal lesions were more pronounced in chub from severely metal contaminated Zletovska River. Prevalence of hepatic lesions was also higher in mining impacted rivers (in Kriva, 70%; in Zletovska, 59%) compared to Bregalnica River (38%). The spectrum of histological lesions observed in chub liver varied from non-specific minor degenerative conditions, such as lymphocyte infiltration, fibrosis, parasites, granulomas and lipidosis, to extensive and/or more severe changes such as bile duct proliferation, necrosis, megalocytosis, light-dark hepatocytes and hepatocytes regeneration. The results of histopathological investigation for all three rivers showed clear signs of water contamination, especially prominent in mining influenced rivers. More research efforts should be devoted to study of environmental conditions and metal contamination in the mining impacted rivers worldwide, especially of their effects on health of local ichtyofauna.

Keywords

Vardar chub, histopathology, mining, monitoring, liver, gonads, metal contamination

1. Introduction

For hundreds of years, countless thousands of pollutants have been produced and released into the environment (van der Oost et al., 2003). Among them, metals present a serious threat for natural ecosystems, since they are not biodegradable and tend to accumulate in organisms that reside there (Sary and Mohammadi, 2012). For aquatic ecosystems, a special problem is presented in mine drainage, because it contains high metal amount and is highly acidic, which triggers the transformation of metals into ionic forms, as the most dangerous metal forms for living organisms (Wojtkowska, 2013).

High concentrations of metals in water and/or sediments can result with their accumulation in aquatic biota, including fish (Koca et al., 2005, 2008; Filipović Marijić and Raspor, 2007; Podrug et al., 2009; Yildiz et al., 2010; Dragun et al., 2012, 2015; Javed and Usmani, 2013). For several reasons, fish present good bioindicators of environmental contamination with metals. Fish are ubiquitous in the aquatic environment, and represent the species at the top of the aquatic food chain. They accumulate metals in their organs directly from water or via food in concentrations much higher than present in the water and/or sediment. Consequently, they also can suffer negative health effects (Hinton et al., 1987, 1992; Hinton, 1993, 1994; Dragun et al., 2015).

Although metal accumulation depends on fish species, as well as on metal itself, it is generally higher in the liver, as main detoxification organ, compared to 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). Several types of hepatic lesions were proven as reliable biomarkers in assessing anthropogenic stress, and were consistently associated with contamination exposure, including metal contamination (Hinton, 1993; 1994). Consequently, histology presents a successful tool, which is sensitive and selective for monitoring the sub-lethal effects of metals on the aquatic biota (Arellano et al., 1999). However, different pathologies and abnormalities on hepatic tissue caused by metals were studied only sporadically (Arellano et al.,1999; Yacoub and Abdel Satar, 2003, Olojo et al., 2005; Kraemer et al., 2005; Koca et al., 2005, 2008; Giari et al., 2008; Yildiz et al., 2010; Gurcu et al., 2010; Roberts and Rodger, 2012; Hadi and Alwan, 2012; Javed and Usmani, 2013).

In this study, we have focused on freshwater ecosystems in northeastern Macedonia, which are becoming increasingly contaminated with heavy metals, due to continuous input of mine drainage from Pb/Zn active mines Zletovo and Toranica into the Zletovska and Kriva rivers, respectively. Our study on the influence of mining on these two rivers so far has indicated high concentrations of many metals in the river water (Ramani et al., 2014a), as well as serious histopathological damage to gills of bioindicator Vardar chub (*Squalius vardarensis*) inhabiting those rivers (Barišić et al., 2015). Preliminary data on metal bioaccumulation even indicated that long-term exposure of Vardar chub to high levels of dissolved metals in the Zletovska and Kriva rivers has resulted in increased accumulation of several metals in chub liver (Ramani et al., 2014b), which could have caused various sub-toxic and toxic effects on hepatic tissue. Facing the above, our aims in the present study were to assess the impact of confirmed high metal contamination of river water on the health status of the local ichthyofauna, represented by Vardar chub (*S. vardarensis*), with the special emphasis put on studying toxicopathic changes in hepatic tissue. Gonadal tissue was also investigated in order to determine if some reproductive disorders in Vardar chub have occurred. And, finally, general health indices were determined and analyzed, as well as external/internal macroscopic lesions. In this study, so far unknown consequences of the long term metal contamination impact on the wild autochthonous fish Vardar chub (*S. vardarensis*), collected within an environment affected by mining, were established.

2. Materials and Methods

2.1. Fish sampling and dissection

Selected bioindicator organism for this study was Vardar chub (*S. vardarensis*). Vardar chub belongs to genus *Squalius* of family Ciprinidae (http://www.cabi.org/isc/datasheet/117313). It is closely related to European chub (*Squalius cephalus*), which is a long lived fish that inhabits slow and moderate water flows from a wide range of European waters and has high mobility due to its pelagic conditions (http://www.cabi.org/isc/datasheet/117313). It is omnivorous, and its food sources range from small (i.e. detritus, plants, invertebrates) to large (i.e. tadpoles, small fish) items. In addition it has high fecundity, fast growth rate, and is considered tolerant of anthropogenic pressures (http://www.cabi.org/isc/datasheet/117313). Chub samplings were performed in May, June and October of 2012 in three rivers located in the north-eastern Macedonia: Bregalnica (n=60), as relative referent site contaminated mainly with agricultural drainage and municipal waste waters, and Zletovska River (n=41) and Kriva River (n=56), two rivers which are under direct influence of Pb/Zn mines, and proven as highly metal polluted (Ramani et al., 2014a). For example, the concentrations of Pb in Bregalnica, Zletovska and Kriva rivers were 0.45-0.69, 0.31-0.82 and 0.56-1.85 μg L^{-1}, respectively; of Cd, 0.04, 0.27-2.01 and 0.05-0.27 μg L^{-1}, respectively; and of Zn 16.9-20.6, 197-1427 and 17.0-37.2 μg L^{-1}, respectively (Ramani et al., 2014a). Detailed information on sampling sites and other physico-chemical characteristics of the water of all three rivers in the time of fish sampling was previously described by Ramani et al. (2014a). Fish were collected by electro fishing (electrofisher Samus 725G) according to CEN EN 14011, 2003 standard. Fish capture and their handling complied with the current laws of the Republic of Macedonia. After capture, alive fish were transported from sampling sites in plastic container with aerated river water to the laboratory. Each animal was anaesthetized with Clove Oil, and then the total length (TL) and body mass (BM) were measured. To avoid the influence of the development of gonads on the examined parameters, BM was measured without gonads. Fulton CF was later calculated according to the following formula: CF = BM×100/TL. After measurements and visual assessment for external gross lesions were completed, fish were dissected and macroscopically inspected for abnormalities of visceral organs. Then, the liver and gonads were carefully removed, and their masses (LM and GM, respectively) were measured. Hepatosomatic (HSI) and gonadosomatic (GSI) indices were calculated according to the following formulas: HSI= LM×100/BM and GSI=GM×100/BM.

2.2. Histopathological assessment of chub liver and gonads

Pieces of liver and gonads were immersed in Bouin's fixative for at least 48 hours. 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. To obtain objective analyses, all slides were coded, so researchers 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 already 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).

2.3. Statistical analyses

Statistical analyses were made using the software Statistica 7.0 for Windows. Differences were considered significant at $p<0.05$. To find out if significant differences existed in the lesion prevalence (*i.e.*, percentage of affected fish) between sampling sites, we have used two sided t-test for proportions. To determine the differences of biometric characteristics of examined Vardar chub between the sampling locations, we have used ANOVA, after checking for normality and homogeneity of variances of the data sets. Whenever the ANOVA indicated significant difference, statistical comparisons between pairs were performed using the post-hoc Newman-Keuls test.

3. Results

3.1. Biometric data nad organosomatic indices

The basic biometric data of the examined chub, as well as the results of GSI and HSI are displayed in Table 1. Analyses of the data by ANOVA showed that the animals having different health statuses, without (WL) or with lesions (L) in the liver, did not differ in any of the examined parameters. Concerning locality, generally the most pronounced differences referred to fish from the Zletovska River, which had the lowest mass and length. This was consequently reflected in a significantly lower CF of fish from the Zletovska River compared to fish from Bregalnica and the Kriva River.

Table 1. Biometry of Vardar chub (*Squalius vardarensis*) captured during 2012 in reference Bregalnica River, and two rivers impacted by mining waste, Zletovska and Kriva: body mass (BM), total length (TL), condition factor (CF), gonadosomatic (GSI) and hepatosomatic index (HSI) in fish separated in two groups, without liver lesions (WL) and with liver lesions (L) .

Sampling sites	BM (g)		TL(cm)		CF (%)		GSI (%)		HSI (%)	
	WL	*L*	*WL*	*L*	*WL*	*L*	*WL*	*L*	*WL*	*L*
Bregalnica (n = 60)	82.3 (0.47)[a]	70.3 (0.61)[a]	19.3 (0.16)[a]	18.1 (0.21)[a]	1.06 (0.08)[a]	1.06 (008)[a]	4.88 (0.72)	3.22 (0.75)	1.65 (0.34)	1.54 (0.37)
Zletovska (n = 41)	23.0 (0.49)[b]	26.9 (0.46)[b]	13.5 (0.15)[b]	14.1 (0.14)[b]	0.88 (0.10)[b]	0.90 (0.09)[b]	5.64 (0.43)	6.51 (0.59)	1.46 (0.32)	1.48 (0.31)
Kriva (n = 56)	44.2 (0.58)[b]	55.1 (1.05)[a]	15.8 (0.20)[b]	17.2 (0.28)[a]	1.01 (0.06)[a]	1.01 (0,08)[a]	4.89 (1.31)	7.46 (0.75)	1.72 (0.25)	1.56 (0.21)

[a,b]Different lowercase superscript letters (read vertically) represent significant differences between sampling sites according to ANOVA followed by Newman-Keuls test (i.e. the river assigned letter “a” differs significantly from the river assigned the letter “b”).

3.2. Necropsy-based fish health assessment

Prevalences of the most common external and internal pathological findings are presented in Table 2. Externally, high prevalence of skin oedema and absence of scales, mainly on the upper dorsal fin and/or near caudal fin, was detected. These abnormalities were much more pronounced in the Zletovska and the Kriva River compared to Bregalnica. Parasites were also often found mainly in the body cavity, around the digestive tract and the liver or rarely on the kidney tissue of examined fish from the Zletovska and Kriva River. In the fish from the Zletovska River parasites were located in body cavity but more often were attached on the gill filaments. It can be seen that there were significant differences in the occurrence of parasites between these rivers. In comparison to the other two rivers, the lowest amount of parasites was found in fish from the Zletovska River. The percentage of fish with gill damage, which mainly included absence of filaments, pale, very light color of filaments, and in one fish complete absence of first gill arch, were the highest in the Zletovska River. The changes in the kidneys included mainly enlarged or swollen kidneys, and were comparably present in fish from all three rivers. In the Kriva River, one fish was found in which only half of the kidney was present. Some additional lesions were found in small prevalence: hemorrhage in chub from the Bregalnica River, pink or blue color of gallbladder in the Zletovska and the Kriva River, absence of one gonad in a male chub from the Zletovska River and focal discoloration of the liver in the Kriva River. Although for some lesions differences between rivers were noted, prevalence of all lesions together was around 20% and comparable in all three investigated river ecosystems.

Table 2.The prevalence[¥] (%) of external/internal lesions recorded in Vardar chub (*Squalius vardarensis*) captured during 2012 in reference Bregalnica river, and two rivers impacted by mining waste, Zletovska, and Kriva.

Lesion type	River		
	Bregalnica	Zletovska	Kriva
Skin oedema and absence of scales N_1=5, N_2=7, N_3=7	8^{a}	17^{b}	12^{b}
Parasites N_1=6, N_2=1, N_3=5	10^{a}	2^{b}	9^{a}
Gill damage N_1=1, N_2=2, N_3=1	2^{a}	5^{b}	2^{a}
Kidney damage N_1=2, N_2=1, N_3=1	3	2	2
Other lesions N_1=2, N_2=2, N_3=1	3^{a}	5^{b}	2^{a}
All lesions N_1=14, N_2=10, N_3=14	23	24	25

[¥]The prevalance (%) was computed as number of a fish with specific lesion in each river divided by total number of fish captured in that river during investigating period.
* N_1, N_2, N_3 – number of fish with specific lesion in the Bregalnica, Zletovska, and Kriva rivers, respectively.
[a,b]Different lowercase superscript letters (read horizontally) represent significant differences between sampling sites according to two sided proportion test (i.e. the river assigned letter "a" differs significantly from the river assigned the letter "b").

3.3 Histopathological assessment of gonads and liver

All examined Vardar chub in this study were mature individuals, with well-developed ovaries and testes tissue. Microscopy analyses did not show any gonad abnormality in male or female specimens. Contrary in the hepatic tissue numerous pathological conditions were noted (Table

3). In general, prevalence of the hepatic lesions was significantly higher in fish from the Zletovska and the Kriva River compared to the reference site, the Bregalnica River.

Table 3.The prevalence[¥] (%) of lesions recorded in the liver of Vardar chub (*Squalius vardarensis*) captured during 2012 in reference Bregalnica River, and two rivers impacted by mining waste, Zletovska, and Kriva.

	River		
Lesion type	Bregalnica	Zletovska	Kriva
Lymphocyte infiltration	17	24	25
$N_1=10$, $N_2=10$, $N_3=14$			
Fibrosis	15	21	29
$N_1=9$, $N_2=9$, $N_3=16$			
Bile duct proliferation	32^{a}	33^{a}	62^{b}
$N_1=19$, $N_2=14$, $N_3=35$			
Parasites and granulomas	3^{a}	2^{a}	7^{b}
$N_1=2$, $N_2=1$, $N_3=3$			
Necrosis	15^{a}	17^{a}	28^{b}
$N_1=9$, $N_2=7$, $N_3=15$			
Megalocitosis	7^{a}	14^{a}	16^{b}
$N_1=4$, $N_2=6$, $N_3=9$			
Light-dark hepatocytes	0^{a}	2^{b}	5^{b}
$N_1=0$, $N_2=1$, $N_3=3$			
Hepatocyte regeneration	0^{a}	5^{b}	7^{b}
$N_1=0$, $N_2=2$, $N_3=4$			
Lipidosis	0^{a}	5^{b}	7^{b}
$N_1=0$, $N_2=2$, $N_3=4$			
All hepatic lesions	38^{a}	59^{b}	70^{b}
$N_1=23$, $N_2=25$, $N_3=39$			

[¥]The prevalance (%) was computed as number of fish with specific hepatic lesions in each river divided by total number of fish captured in that river during investigating period.

* N_1, N_2, N_3 – number of fish with specific lesions in the Bregalnica, Zletovska, and Kriva rivers, respectively.

[a,b]Different lowercase superscript letters (read horizontally) represent significant differences between sampling sites according to the two sided proportion test (i.e. the river assigned letter “a” differs significantly from the river assigned the letter “b”).

Inflammatory changes, namely lymphocyte infiltration was very often present in fish from all three rivers. They could be observed alone, as individual lesions in parenchyma and around the vascular-biliary stromal tracts, or, more often, in association with proliferation of the bile ducts. Normally, chub biliary tract contains thin layer of the connective tissue. If connective tissue increases at least twice, it is diagnosed as fibrosis. Fibrosis was found only in stromal tracts with biliary profiles and was also often seen in examined animals. Fibrosis is also almost always accompanied with bile duct proliferation, which was predominant lesion in collected chub (Fig. 1). Lymphocyte proliferation and fibrosis were found in higher prevalence in animals captured in mining impacted rivers. Prevalence of bile duct proliferation was even significantly higher in the Kriva River compared with the Bregalnica River. Inside bile duct, rare Mixosoan parasites were found (Fig. 1), whereas uncapsulated granulomas in the parenchyma were more common. Both parasites and granulomas occured with the highest prevalence in the Kriva River.

Necrosis was observed in a form of individual necrotic cells, with destroyed nuclei, or in a form of necrotic areas located mainly around stromal tracts with biliary profiles. Its prevalence, as well as prevalence of megalocytosis (Fig. 2a), enormous enlargements of cells and nuclei, was significantly higher in the Kriva River compared to the other two rivers, Bregalnica and the Zletovska River. Light-dark hepatocytes and hepatocyte regenerations were found in the Zletovska and the Kriva River, but not in Bregalnica. The light dark hepatocytes were seen as the fields consisting of dark and light hepatocytes, side by side (Fig.2b). Regenerating hepatocytes also appeared as fields, with smaller cells containing darker cytoplasm in comparison to normal hepatocytes (Fig. 3a).Lipidosis was also a type of lesion which was found only in the Zletovska and the Kriva River. Lipidosis, presence of vacuoles inside hepatocytes, had mainly diffuse distribution within parenchyma and sometimes occupying large areas of parenchymal tissue (Fig. 3b).

4. Discussion

The present study was primarily designed to investigate effects of rivers contaminated by mining waste, Zletovska and Kriva, on Vardar chub health. For the assessment of fish health, we have used standard fisheries indices consisting of fish mass, length, condition factor and organosomatic indices. The latter two were used as indicators of fish well being, and may vary in response to different kind of pollutants in the river water, including heavy metals (Schmitt and Dethloff, 2000; Jovanović et al., 2011; Liebel et al., 2013; Dragun et al., 2013). In our study, we have classified the fish within each locality in two groups according to liver lesion status (with and without lesions), but no significant differences were noted in examined indices between two groups. Contrary, when differences between localities were considered, the fish captured in the Zletovska and the Kriva River, contaminated with mining waste, had lower mass, length and consequently CF compared to the fish sampled in Bregalnica, as a reference site. Especially small fish and low condition factor observed in the Zletovska River could be associated with high metal exposure (Filipović Marijić and Raspor, 2007), which is consistent with extremely high concentrations of several metals found in the Zletovska River water in the time of chub sampling (Ramani et al., 2014a). Conditon factor of fish from the Kriva River did not differ significantly from the reference site. It is consistent with the fact that the Kriva River, although being mining impacted river, was less contaminated with metals compared to the Zletovska River (Ramani et al., 2014a). In addition to severe water contamination, smaller CF of the fish from the Zletovska River could be also the result of insufficient nutrition (Munkittrick and Dixon, 1988).

Both HSI and GSI are considered as useful indicators in monitoring studies, with HSI being commonly associated with contaminant exposure. 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). Our results indicated that a significant difference in HSI and GSI did not exist either within each sampling point between fish with and without lesions or between sampling points. Furthermore, obtained HSI and GSI values did not indicate an impaired condition of the fish in either of three examined aquatic ecosystems. This is in accordance with previous studies, which reported that presence of toxicants in the environment was not always associated with fluctuations in HSI. For example, exposure to paper mill effluent (Oikari and Niitylä, 1985), benzo(a)pyrene (Grady et al., 1992) or chromium picolinate (Mehrim, 2012) have not shown any effect on fish HSI. Similarly, our results for GSI, as well as microscopic investigations of chub gonads indicated good reproductive health. We have noted only small, but insignificant increase in GSI in chub with lesioned liver from mining impacted rivers, which is in accordance with investigations of Billiard and Khan (2003) who found GSI increase in various fishes from the locations contaminated with pulp and paper mill effluent. However, absence of pronounced changes in HSI and GSI does not necessarily mean that we deal with an unpolluted environment and/or healthy organisms. For example, investigation performed on the liver of the *Astyanax aff. fasciatus* and *Oreochromis niloticus* living in a contaminated environment showed that liver lesions (necrosis and leukocyte infiltration) have occurred even though no changes in HSI were recorded (Liebel et al., 2013).

According to external/internal evaluation of Vardar chub, on average 20% of fish from all three rivers had macroscopically visible disorders, with chub from the Zletovska River being somewhat more intensely affected. Changes on that level reflect an advanced stage of toxicant impact. In other words, when high incidence of gross abnormalities occurs, it can be regarded as a consequence of significant presence and effect of toxicants in the water (Schmitt and Dethloff, 2000; Noga, 2000; Blazer et al., 2010). However, gross observations, as well as condition and organosomatic indices 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 (Hinck et al., 2006; Hinton et al., 1992; Hinton, 1993; Wolf and Wolfe, 2005).

According to our histological examination of the gonadal tissue, no signs of disease (presence of atretic oocytes, intersex, disorganization of the lobules in testes) were found in either male or female fish. All examined fish were obviously in good reproductive condition, indicating that gonads were probably more tolerant to metal exposure. 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).

Contrary to gonads, numerous non-neoplastic and neoplastic lesions were found in the chub liver, with lymphocyte infiltration, fibrosis and bile duct proliferation being the most frequently observed.

Lymphocyte infiltration and connective tissue proliferation, which were non-neoplastic in nature, were found in chub at all three locations and seemed unrelated to differences in environmental contamination. Accumulation of lymphocytes was previously detected in fish exposed to heavy metals (Sorensen et al., 1984; Schmidt et al., 1999; Liebel et al., 2013; Javed and Usmani, 2013), but also to pesticides and to other contaminants in the environment (Rousseaux et al., 1995; Myers et al., 1992; Schmidth-Posthamus et al., 2001; Hinck et al., 2007; 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 Wolke, 2005; Roberts and Rodger, 2012). 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, often in association with fibrosis. Its prevalence was the highest in metal contaminated rivers, especially in the Kriva River where it has been registered in as much as 62% of fish. The bile duct proliferation was previously recognized as being closely associated to pollution (Murchelano and Wolke, 1991; Schmit et al., 1999; Rousseaux et al., 1995, Stentiford et al., 2003), such as heavy metal pollution (Roberts and Rodger, 2012) and was suggested as promising toxicopathological indicator (Blazer et al., 2006). .

Compared with three above mentioned lesions, parasites and granulomas had relatively low prevalence, which was still significantly higher in the chub from the Kriva River compared to chub from the other two rivers. 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). Negative relationship between abundance of intestinal parasites and metal contamination was often recorded (Dragun et al., 2013; Vardić Smrzlić et al., 2015). In addition, during this research, complete absence of intestinal parasites in chub 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 chub from the Zletovska River, which was highly metal contaminated (Ramani et al., 2014a), corroborated the assumption on negative association between parasite prevalence and water contamination. Interesting finding, however, was higher number of parasites and granulomas in the chub liver at mining impacted Kriva River, which was similar to 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 2012 (Ramani et al., 2014a).

Furthermore, necrosis and megalocytosis (enlargement of hepatocyte cytoplasm and nucleus) were also present in fish from all three locations. Again, prevalence for both lesions was higher in both mining impacted rivers compared to reference Bregalnica River, but significantly higher only in the Kriva River. 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 in fish liver in response to metal exposure (Bernet et al., 2004; Figueiredo-Fernandes et al., 2007; Koca et al., 2005; 2008; Triebskorn et al., 2008; Yidiz et al., 2010, Gurcu et al., 2010; Hadi and Alawan, 2012), but also after exposure to some other contaminants (Boorman et al., 1997; Figueiredo-Fernandes et al., 2007; Liebel et al., 2013; Javed and Usmani, 2013). In fish from the River Elbe, the observed hepatocytomegaly was also due to high level of metal pollution (Peterse et al., 1987). It has even been suggested that megalocytoses can be an indicator of hepatocarcinogen-hepatotoxin exposure in the environment (Myers et al., 1987; 1990).

Remaining three types of more neoplastic lesions, light-dark hepatocytes, hepatocyte regeneration, and lipidosis, were observed in both metal polluted rivers, the Zletovska and the Kriva River, but have not been found at the reference location, the Bregalnica River. These types of lesions were also observed in the fish from the lower Elbe River, which is 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 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). Hepatocytes undergo regeneration to replace necrotic foci, after hepatocellular necrosis occurs (de Melo et al., 2008). The occurrence of lipidosis 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; Koca et al., 2005; 2008; Gurcu et al., 2010; Hadi and Alwan, 2012; Yacoub and Abdel Satar, 2013; Chavan and Muley, 2014; Javed and Usmani, 2013). Similar to other lesions, next to its association with exposure to toxicants, lypidosis can also be a normal feature of fish liver (Roberts, 1978; Wolf and Wolfe 2005). In our case, as we have not found lipidosis in fish from the reference river, we suppose that lipidosis in the investigated chub was probably a pathological condition.

5. Conclusion

Hepatic lesions were observed in all three examined rivers: reference Bregalnica, and mining impacted Zletovska and Kriva rivers. However, higher total prevalence of hepatic lesions, as well as occurrence of certain type of lesions, such as neoplastic lesions, were generally registered in mining polluted rivers. Although external/internal malformations and hepatic lesions are not specific indicators of certain individual contaminants, our results have indicated that presence of high concentrations of heavy metals in the river body without a doubt has had a significant toxic influence on the examined Vardar chub health and vitality. Chub from both Zletovska and Kriva River had more severe hepatic lesions compared to the reference river. At least some of the observed lesions, which were found in both mining impacted rivers, were probably the result of metal pollution of the water, such as bile duct proliferation, megalocitosis, light-dark hepatocytes, hepatocyte regeneration and lipidosis. On the other hand, several pathological findings were present more frequently in the Kriva River, which is less metal contaminated among two mining impacted rivers. However, the Kriva River is also an agriculturally impacted river, as seen from high fecal contamination, as well as high concentrations of ammonium in the water. Therefore, high presence of several severe hepatic lesions in chub from the Kriva River, such as bile duct proliferation, granulomas and necrosis, can possibly be associated to synergistic effect of metal and organic pollution of the river water. This study has clearly demonstrated detrimental effect that mining pollution has on native freshwater fish. It is information, which is essential in a process of creating water management plans with an aim to protect, as well as improve, quality of freshwater ecosystems worldwide, especially in areas affected by active mining.

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Fig. 1. Light micrograph of the chub (*Squalius vardarensis*) 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). Note myxosporean parasites within biliary profile lumen (asterisk). Haematoxilin and eosin. Scale bar = 0.1 mm.

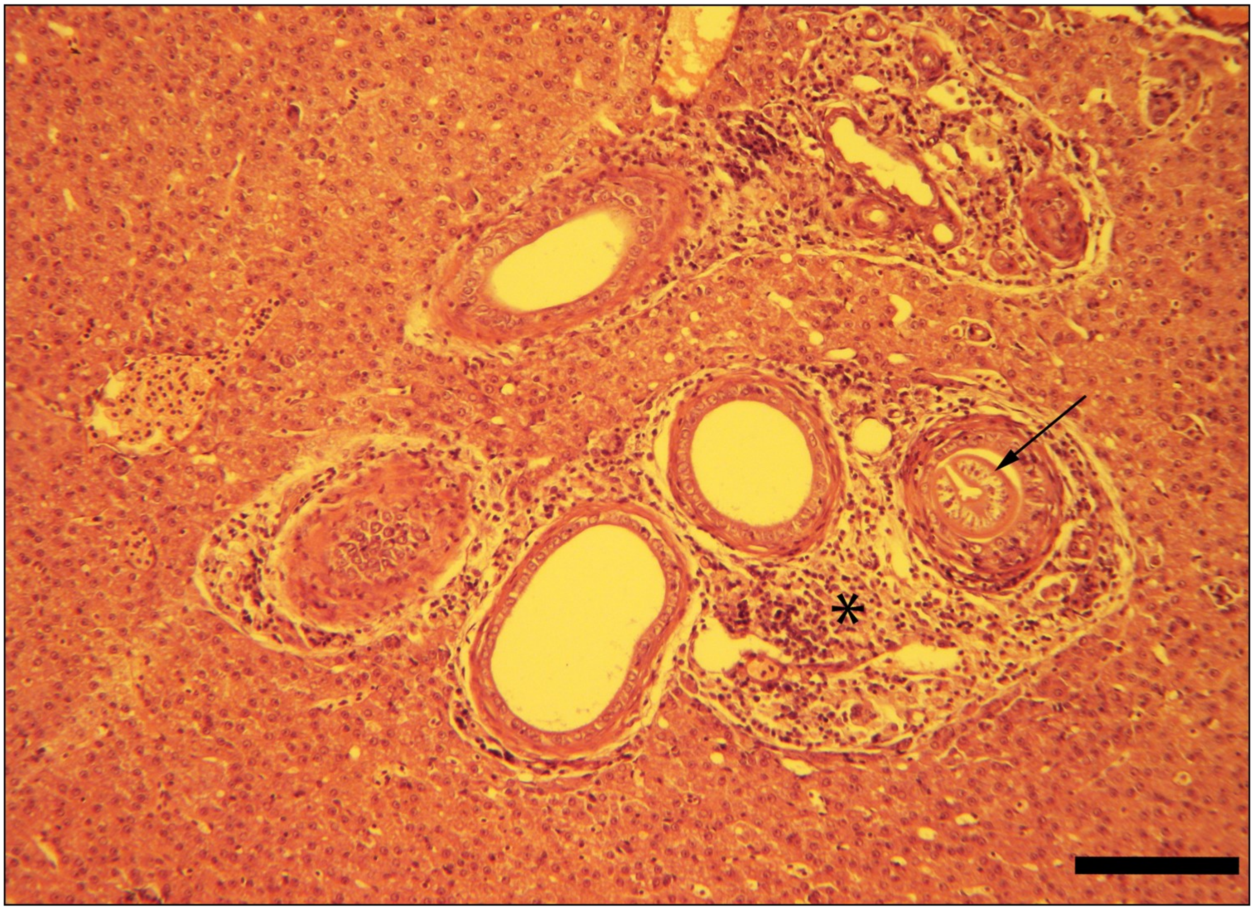

Fig. 2. Light micrograph of the chub (*Squalius vardarensis*) liver from the Zletovska River illustrating: **a)** megalocitosis, enlarged cells; and **b)** light-dark hepatocytes with prominent nucleoli. Haematoxilin and eosin. Scale bar = 0.05 mm.

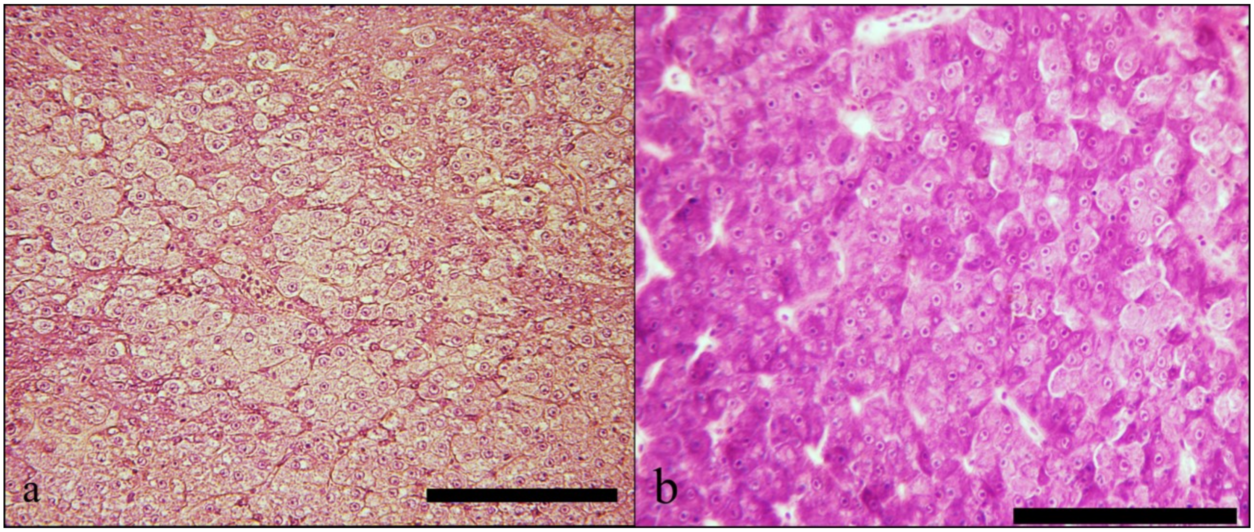

Fig. 3. Light micrograph of the chub (*Squalius vardarensis*) liver from the Kriva River showing: **a)** hepatocytes regeneration (arrows); note the relative increase in cytoplasmic eosinophilia; **b)** lipidosis (on right site) characterized by large introcytoplasmic vacuoles within hepatocytes. Haematoxilin and eosin. Scale bar a = 0.05 mm, b = 0.1 mm.