Evaluation of histopathological alterations in the gills of Vardar chub (*Squalius vardarensis* Karaman) as an indicator of river pollution

Josip Barišić[a]*, Zrinka Dragun[b], Sheriban Ramani[c], Vlatka Filipović Marijić[b], Nesrete Krasnići[b], Rozelindra Čož-Rakovac[a], Vasil Kostov[d], Katerina Rebok[e], Maja Jordanova[e]

[a]Ruđer Bošković Institute, Division of Materials Chemistry, Laboratory for Ichtyopathology – Biological Materials, Bijenička c. 54, Zagreb, Croatia; jbarisic@irb.hr; rrakovac@irb.hr

[b]Ruđer Bošković Institute, Division for Marine and Environmental Research, Laboratory for Biological Effects of Metals, Bijenička c. 54, Zagreb, Croatia; zdragun@irb.hr; vfilip@irb.hr; nkrasnic@irb.hr;

[c]Ministry of Agriculture, Forestry and Water Economy, Hydrometeorological Administration, Division for Water, Air and Soil Quality Monitoring and Laboratory Analysis, Hydrology and Ecology Department, Skupi b.b., Skopje, Macedonia; sheriban_ramani@hotmail.com

[d]Institute of Animal Sciences, Ile Ilievski 92-a, Skopje, Macedonia; vasilkostov@yahoo.com

[e]Ss. Cyril and Methodius University in Skopje, Faculty of Natural Sciences and Mathematics, Gazi baba b.b., Skopje, Macedonia; katerinarebok@yahoo.com; majaj@pmf.ukim.mk

*Corresponding author:
Josip Barišić
Laboratory for Ichtyopathology – Biological Materials
Division of Materials Chemistry
Ruđer Bošković Institute
Bijenička c. 54, 10000 Zagreb, Croatia
Phone/Fax: xx385-1-4571232;
E-mail: jbarisic@irb.hr

Abstract

Quantification of histopathological alterations in the gills of Vardar chub (*Squalius vardarensis* Karaman) was performed in 2012 in rivers of north-eastern Macedonia, with the aim to examine the effects of water quality in the rivers (Zletovska and Kriva River - impacted by active Pb/Zn mines; Bregalnica River - contaminated by agricultural waste). The biological alterations in chub were classified as: circulatory disturbances, regressive and progressive changes, but their severity differed. Altogether the mildest changes were observed in the gills of chub from the Bregalnica River, a less polluted river, whereas mining impacted rivers were characterized by more severe alterations. In the gills of chub from the Zletovska River, which is highly contaminated with numerous metals, sulphates and chlorides, the highest lesion indices were found for the regressive changes of both epithelium and supporting tissue, with typical lesions referring to atrophy, thinning and lifting of epithelial cells, necrosis of epithelium and chloride cells, as well as deformations of lamellar cartilaginous base. Gill damages of chub from the Kriva River were overall milder compared to the Zletovska River, in accordance with pollution status. In the gills of chub from that river, progressive changes were more pronounced, specifically severe hyperplasia of mucous cells and epithelium in the interlammellar space, leading to fusion of lamellae, as well as hypertrophy of chloride cells. The comparison between seasons indicated higher intensity of progressive changes in all three rivers in autumn, when water level was very low, and consequently, water contamination was more pronounced due to concentration effect. The pattern and severity of histopathological alterations in the chub gills reflected differences in contamination levels and type of contaminants in different rivers and sampling periods, and thus have been proven as a valuable indicator of water quality.

Keywords: agriculture, freshwater fish, gills, histopathology, mining

1. Introduction

Nowadays, aquatic environments are exposed to extreme pressure, as final recipients of numerous contaminants (e.g. inorganic, organic, microbiological, and pharmaceutical) from various sources (e.g. industry, mining, municipal wastewaters, and agricultural runoff). 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 and the latter mainly as a source of different types of organic contaminants. In Macedonia, mining is still one of the most important industries, with lead and zinc ores in the north-eastern part of the country being the most significant mineral deposits for exploitation (Midžić and Silajdžić 2005). The influence of two currently active lead/zinc mines (Zletovo and Toranica) on the water quality of rivers in that region (Zletovska and Kriva) was detected, referring to contamination with number of metals and changed physico-chemical characteristics (Ramani et al. 2014). However, the sole information on water contamination is not sufficient for evaluation of the risks for health and for survival of aquatic organisms inhabiting such environments. Supplementary indicators should be analysed simultaneously, to obtain the information on effects which have arisen as a consequence of exposure of aquatic organisms to identified contaminants. Such approach to monitoring of natural waters, which links water contamination with its effects on aquatic life, is indispensable for prevention of further deterioration of affected ecosystems and for preservation of biodiversity.

Usually, the effect of contaminants is evaluated by analyses of numerous biochemical and molecular biomarkers. However, histopathology should not be overlooked in monitoring of contaminant effects, since it provides an opportunity for rapid detection of consequences of exposure to contaminants by localisation, description and even quantification of lesions that occur on selected organs of bioindicator organism (Bernet et al. 1999; Reddy and Rawat 2013). It is, however, essential to choose wisely both bioindicator and the organ which will be examined. For this study, fish species Vardar chub (*Squalius vardarensis* Karaman) was selected for two reasons: first, fish are at the top of the aquatic food chain, and therefore mirror the combination of the biotic and abiotic conditions in the particular aquatic environment (Chovanec et al. 2003; Dragun et al. 2007), and second, this specific species as a member of genus *Squalius*, which is wide spread in European rivers, provides the possibility for comparison among distant geographical regions. Selection of appropriate organ for histopathological analyses, on the other hand, was governed by the wish to recognize the consequences of contamination events promptly after they occur. Although, the exposure of fish to chemical and microbiological contaminants could induce a number of lesions in different organs, we have chosen gills, which have been described as good indicator of current environmental conditions (Amiri et al. 2011), due to their direct and permanent contact with contaminants in the water (Bernet et al. 1999; Amiri et al. 2011), their fast response and high sensitivity even to low concentrations of pollutants (Monteiro et al. 2008).

The main aim of this study was to evaluate the possibility to use combination of qualitative and quantitative histopathological examination of Vardar chub gills as a supplementary indicator of water pollution in mining impacted rivers applying the procedure according to Bernet et al. (1999). That procedure has been widely accepted and applied for histopathology-based evaluation of water pollution (Liebel et al. 2013; Lujić et al. 2015; Rašković et al. 2015). Since chemical and microbiological water quality of three rivers in north-eastern Macedonia which were selected for this study, Bregalnica, Zletovska and Kriva, was determined simultaneously with chub sampling (Ramani et al. 2014), we were able to associate observed histopathological alterations to specific degrees and types of water contamination. Additional benefit of this study, being conducted in the field, is that it provided the possibility to assess the effect of complex mixture of contaminants on the fish showing adapted behaviours (e.g. avoidance, feeding) and physiology whilst living under realistic, natural conditions in a contaminated ecosystem (Flores-Lopes and Thomaz 2011; Bernet et al. 2004). The importance of such approach is supported by the fact that recently several other field surveys of contamination of aquatic ecosystems, which were based on gill histopathology, have been performed in different regions in the world: in Egypt, using Nile catfish (*Clarias gariepinus*; Authman et al. 2013), in Pakistan, using Mozambique tilapia (*Oreochromis mossambicus*; Jabeen and Chaudhry 2013), in Brasil, using fish species *Astyanax aff. fasciatus* and Nile tilapia (*Oreochromis niloticus*;

Liebel et al. 2013), and in Serbia, using pike (*Esox lucius*), zander (*Sander lucioperca*) and Wels catfish (*Silurus glanis*; Lujić et al. 2015), as well as barbel (*Barbus barbus*) and sterlet (*Acipenser ruthenus*; Rašković et al. 2015). Since our study was conducted in two periods, spring and autumn of 2012, which differed concerning fish physiology and physico-chemical characteristic of the river water, seasonal variations of histopathological alterations on Vardar chub gills were also assessed.

2. Materials and methods

2.1 Fish sampling

Vardar chub (158 specimens) were sampled in spring (May/June) and autumn (October) of 2012 in three rivers in north-eastern Macedonia: the Bregalnica River as the least contaminated freshwater system or a reference site, and the Zletovska and Kriva rivers as the rivers under direct influence of active Pb/Zn mines Zletovo and Toranica, respectively. The map, detailed description of the sampling area, as well as detailed physico-chemical and microbiological characterization of the river water of these three rivers in the time of chub sampling were already published by Ramani et al. (2014). Fish sampling was performed by electro fishing (electrofisher Samus 725G) according to relevant standards (CEN EN 14011:2003). It was previously shown that capture of fish by electrofishing produces gill damage in form of a bleeding in very small percentage of sampled fish (Hawkins 2002). Thus, electrofishing can be considered as acceptable sampling method for histopathological study of fish gills. After capture, fish were kept alive in an opaque plastic tank with aerated river water taken from the sampling site where they were caught. It lasted for approximately 4-5 hours, until further processing in the laboratory. Application of electrofishing, aeration of the river water, as well as short time from sampling to dissection were the precaution measures which should have prevented or at least minimized the appearance of ante mortem artefacts. Individual fish were anesthetized with Clove Oil (Sigma Aldrich). Total length and total mass were measured and Fulton condition indices (FCI) were calculated in accordance with Rätz and Lloret (2003). After fish sacrifice by a strong blow to the head with a blunt object, the gills and gonads were dissected, their mass measured and then they were stored in buffered 4% formalin (Sigma Aldrich) and Bouin's fixative (Merck, Germany), respectively, until further analyses. Gonads were used for histological determination of fish sex. Gonadosomatic indices (GSI, %) were calculated as ratios between gonad mass and total body mass (g), multiplied with 100 (Şaşi 2004). The biometric characteristics and number of analysed specimens of Vardar chub per site and season are given in Table 1.

2.2 Histological examination of chub gills

Routine histology was used to produce tissue sections for histological analyses of chub gills (Suvarna et al. 2013). In brief, chub gills were preserved in 4% buffered formalin for 24-72 hours, after which the samples were transferred to 50% ethanol for indefinite time. For embedding preparation, gill tissues were dehydrated further through 70%, 96% and absolute ethanol and cleared in xylene, and soaked in paraffin wax (Paraplast, Fluka, Germany) using Leica-TP 1020 (Leica, Germany) processor. Sections were cut at 2 μm using a microtome Leica RM 2255 (Leica, Germany) and dewaxed overnight in an oven at 60°C. Dried sections were stained with Mayer's haematoxylin and Young's eosin stains (Biognost, Croatia) and with periodic acid Schiff kit (PAS; Biognost, Croatia), following the methods described by Suvarna et al. (2013). After staining, sections were dehydrated in increasing concentrations of ethanol (70-100%), cleared in xylene, and mounted in Biomount DPX (Biognost, Croatia). Tissue sections were examined microscopically using BX51 light binocular microscope (Olympus, Japan). Microphotographs were taken using digital camera DP 70 (Olympus, Japan) connected to microscope. The screening protocol was qualitative and conducted in a subjective manner.

2.3 Determination of lesion indices and prevalence of histopathological alterations on chub gills

For the quantification of gill damage, the standardized assessment method according to Bernet et al. (1999) was applied. This method classifies pathological changes into five reaction patterns: circulatory disturbances, regressive changes, progressive changes, inflammation and tumours (Table 2). Circulatory disturbances result from a pathological condition of blood and tissue fluid flow, regressive changes are processes which terminate in a functional reduction or loss of an organ, progressive changes are processes which lead to an increased activity of cells or tissues, inflammatory changes are often associated with processes belonging to other reaction patterns, and therefore the term inflammation is used in very strict sense, as presented in Table 2, whereas tumours are uncontrolled cell and tissue proliferations (Bernet et al. 1999). To each of these alterations, an importance factor and a score value were assigned. The value of importance factor (1-3) reflects the relevance of a lesion, which depends on its effect on organ function and on the ability of the fish to survive. Score value (0-6), on the other hand, depends on the degree and extent of the alteration. The sum of the multiplied importance factors and score values of all alterations found within examined organ is called total lesion index, and it represents the degree of overall damage to gills (Bernet et al. 1999). In Fig. 1, we have presented the lesion indices for each reaction pattern, as well as total lesion indices. The prevalence of each alteration was also calculated as the percentage of occurrence of an alteration within all analysed chub specimens (Table 2).

2.4 Data processing and statistical analyses

Histological microphotographs were edited using Microsoft® AnalySIS Soft Imaging System. The calculations of the indices and prevalence were performed in Microsoft Office Excel, whereas graph plotting and statistical analyses were performed using the statistical program SigmaPlot 11.0 for Windows. The comparison of biometric parameters of chub caught in three rivers in two seasons, as well as comparison of lesion indices obtained for three rivers in each season was done by Kruskal-Wallis one way analysis of variance on ranks, with *post-hoc* pair wise multiple comparison (Dunn's method). The comparison of indices obtained in two seasons was done for each river by Mann-Whitney U rank sum test.

3. Results and discussion

Histopathological characteristics of an organ can be influenced by several other factors along with pollution (for example, fish age and size; Bernet et al. 1999). Therefore, histological examinations in this study were performed on chub of comparable size, thus including approximately one third of sampled fish (total of 52 specimens), whereas very small and big specimens were excluded (Table 1).

Still, the chub from the Zletovska River were somewhat smaller and had lower condition indices compared to chub from the other two rivers (Table 1). Condition indices primarily reflect fish nutritional status, i.e. the food availability, which could be associated to somewhat higher FCI in all three rivers in the spring than autumn season. However, observed differences in FCI between rivers were probably the result of different water quality, since animals collected in the field often display low FCI values at sites contaminated with organic contaminants, metals and urban sewages (Liebel et al. 2013). The lowest FCI of chub from the Zletovska River was in accordance with the worst water quality in the both sampling periods established for that river (Ramani et al. 2014). In addition, in all three rivers, in both seasons, the percentage of females in analysed subsamples was comparable, ~30% or less, except for the Zletovska River in autumn, when percentage of males (33%) was lower than percentage of females (67%) (Table 1). Since in general differences in analysed parameters between sexes were not observed, females and males from selected groups were analysed together. Evident seasonal differences between analysed specimens referred only to values of GSI, which were mainly significantly higher in spring than autumn (Table 1). GSI for mature chub (*Squalius cephalus*) during the reproductive phase was reported to be above 3% for females and above 1.5% for males (Şaşi, 2004). In our study in spring sampling GSI was above 6% in all three rivers indicating pre-spawning period. Contrary, in the autumn GSI was notably lower and amounted to approximately 1.5% at two rivers, Bregalnica and Kriva, which together with personal observation during dissection indicated the period of fish spawning. Only for chub caught in the Zletovska River, GSI was above 3% even in autumn, as a sign of shift in reproduction cycle compared to the other two rivers, possibly in association with rather unfavourable conditions for living in that highly contaminated river.

Histological impairments belonging to three reaction patterns, i.e. circulatory disturbances, regressive and progressive changes (Table 2) were observed on the gills of chub from all three studied rivers, even from the reference Bregalnica River, indicating low water quality and evident pollution of freshwater ecosystems in north-eastern Macedonia. The specific signs of inflammation and tumours were not found (Table 2). However, the lowest prevalence of histopathological alterations (Table 2) and especially the lowest values of lesion indices were still found in the gills of chub caught in the reference river (Fig. 1). Accordingly, although the gills of all chub were damaged - only to a different degree, non-impaired surfaces with general structure of normal gills (Fig. SI1), typical for teleostean fish (Genten et al. 2009), were most frequently found in Vardar chub from the Bregalnica River. The gills consisted of primary and secondary gill lamellae. The secondary lamellae were lined by pavement cells (squamous epithelial cells), the blood cells were visible inside the capillary, and the pillar cells in the basis, giving them support. Between secondary lamellae, the primary lamellae were lined by stratified epithelium and mucous cells, while the chloride cells (faintly stained acidophilic cells) were scattered in the base of lamellae and in the interlamellar region (Fig. SI1).

Unlike the progressive and regressive changes, which had higher prevalence (Table 2) and were more severe in the chub from the mining impacted rivers (Fig. 1c-f), the circulatory disturbances in spring period were comparably intensive in all chub (Fig. 1a), whereas in autumn, those disturbances were milder in Bregalnica compared to the Zletovska River, but still comparable with the Kriva River (Fig. 1b). Disturbances of blood flow included vascular congestion, telangiectasis and oedema in the interlamellar space. However, only circulatory alteration found in all analysed fish from all three rivers was congestion (Table 2), as seen in primary and secondary gill lamellae of chub from the Bregalnica River (Fig. 2a). The other two circulatory alterations, telangiectasis and intercellular oedema which usually appear immediately after acute exposure to pollutants (Monteiro et al. 2008), were found in the highest prevalence in chub from the Bregalnica River (Table 2). Various stages of telangiectasis in capillaries could be seen throughout the entire lamella of chub from Bregalnica River, with occasional occurrence of thrombotic clusters of erythrocytes and severe oedema in the intralamellar space (Fig. 2b-c). Observed circulatory disturbances are the alterations of minimal pathological importance (Bernet et al. 1999), which are easily reversible as exposure to contaminants ends (Bernet et al. 1999; Altinok and Capkin 2007). Therefore, they indicate only weak contamination of the river water, which is in accordance with simultaneously established weak contamination of Bregalnica River with several trace elements (As, Ba, Fe, Mo, Ti, U, and V), nitrates and phosphates, as well as critical faecal pollution, which altogether pointed to agricultural water contamination, and absence of mining impact (Ramani et al. 2014). The association of agricultural runoff and histopathological changes, especially on fish gills, was already reported, probably as a consequence of water contamination with bacteria and pesticides (Altinok and Capkin 2007; Kurtović et al. 2009; Devi and Mishra 2013). For example, much more pronounced histological alterations on gills than liver and intestine were found for European chub (*Squalius cephalus*) from the Sava River section highly contaminated with heterotrophic and coliform bacteria (Kurtović et al. 2009), whereas oedema in the filamentary epithelium and intense lamellar vasodilatation in the gills of *Channa punctatus* resulted from exposure to sublethal concentrations of pesticide chlorpyrifos (Devi and Mishra 2013). Furthermore, exposure to pesticide methiocarb has affected the gills of rainbow trout (*Oncorhynchus mykiss*) while alterations on the other trout organs, such as liver, spleen, kidney and brain, were still not observed (Altinok and Capkin 2007). This can be explained by the fact that gills are exceptionally sensitive to water contamination, due to direct contact with contaminated medium, since they consist of a network of capillaries, where blood is basically separated from the surrounding water by only one or two layers of cells (Thophon et al. 2003). Therefore, the histopathological alterations on gills, unlike on other fish internal organs, could arise even as a consequence of exposure to less toxic substances, moderate contamination level or shorter exposure periods, and could be even detected in controls, as reported for brown trout (*Salmo trutta f. fario*) (Schwaiger et al. 1997). The changes on gills could also occur as a consequence of the stress caused by sampling, because of too long fish keeping in a trawl net, or a delay before fish are placed in holding tanks (Speare and Ferguson 1989). However, in this study all necessary precaution measures were taken to prevent sampling induced histological changes. Despite of the observed mild changes on the gills, total lesion indices were the lowest in the chub from the Bregalnica River in both seasons (Fig. 1g-h), confirming it as a least polluted among three studied watercourses.

It was expected to find more severe damages in the gills of chub from two mining impacted rivers, the Zletovska and Kriva River, since different types and degrees of contamination were demonstrated in the water of these rivers at the time of chub samplings (Ramani et al. 2014). These expectations were indeed confirmed by higher total lesion indices in both seasons in chub from both mining impacted rivers compared to the reference Bregalnica River (Fig. 1g-h). The influence of mining effluents on histological structure of freshwater fish gills was previously reported for the rivers of north-western Russia (Tkatcheva et al. 2004) and Romania (Triebskorn et al. 2008). One of the main characteristics of mining impacted rivers is high content of metals in water, which could be included among prime causes of pathological alterations on fish gills dwelling in those rivers. Occurrence of various histological changes (e.g. degenerative and circulatory changes, hyperplasia advancing to fusion of secondary lamellae, and focal necrosis) in the gills of several freshwater fish (e.g. zebra fish, *Danio rerio*; Gibelio carp, *Carassius gibelio*; African catfish, *Clarias batrachus* and *C. gariepinus*; Indian flying barb, *Esomus danricus*; and Indian major carp, *Labeo rohita*) was reported after exposure to different metals, like Cd, Cu, Pb, and Zn (Campagna et al. 2008; Velcheva et al. 2010; Khan et al. 2011; Wani et al. 2011; Das and Gupta 2012; Sheriff et al. 2012).

Specifically, at the time of both chub samplings, the Zletovska River was highly contaminated by a number of trace elements (Cd, Co, Cs, Cu, Li, Mn, Ni, Rb, Sn, Sr, Tl, and Zn), sulphates and chlorides, and had disrupted physico-chemical characteristics, indicating the clear and continuous impact of the waste from the Zletovo mine (Ramani et al. 2014). Accordingly, the highest prevalence and the highest lesion indices of the most severe regressive alterations, encompassing both the alterations on epithelium and supporting tissue, were found in the chub from the Zletovska River (Table 2, Figs. 1c-d). Such alterations are generally irreversible, lead to partial or total loss of the organ function (Bernet et al. 1999), and are considered as direct toxic effect of the pollutants (Mallatt 1985; Mohamed 2009). Typical regressive lesions on the gills of chub from the Zletovska River included atrophy causing shortening and curling of secondary lamellae, as well as thinning and lifting of epithelial cells (Fig. 3a). Such separation of respiratory epithelium from the pillar cells usually occurs due to fluid accumulation in the subepithelial space (Mallatt 1985; Roberts 2012), i.e. it is a result of circulatory disturbances, such as congestion and oedema, which were observed in the gills in parallel with described regressive changes (Fig. 3a). In addition, necrosis of the epithelium and chloride cells as the most severe form of regressive changes, as well as cartilage deformity as an example of the regressive change of supporting tissue, were also found (Fig. 3b). Furthermore, atrophy and necrosis of supporting tissue were registered in all analysed specimens from the Zletovska River, which was not the case in the chub from the other two rivers (Table 2). Occasional incidence of rupture of pillar cells, associated with swollen tips of secondary lamellae, was also noticed in the chub from the Zletovska River in the spring period (Table 2, Fig. 3c). Such alteration can cause shortening of lamellar length and hence a decrease in the blood-water diffusion area (Garcia-Santos et al. 2006), and it was previously reported for catfishes (*Clarias macrocephalus* and *C. gariepinus*) exposed to Cd (Pantung et al. 2008). The alterations observed in the gills of chub from the Zletovska River were probably caused by chronic fish exposure to mining effluents, since it has been reported that severe gill damages, such as necrosis, occur following the chronic exposure to toxicants, and are more frequently observed after exposure to heavy metals or complex mixtures of environmental pollutants than after exposure to organic pollutants (Mallatt 1985; Poleksić and Mitrović-Tutundžić 1994; Takashima and Hibiya 1995; Triebskorn et al. 2008; Javed and Usmani 2013).

The water contamination of the Kriva River at the time of chub sampling, on the other hand, was less pronounced and not permanent, despite the fact that the sampling site was located downstream from the Pb/Zn mine Toranica. Only occasional high water contaminations, which could resulted in the shorter exposure to pollutants, were observed at that river (with Cd and Pb in the spring sampling, and with faecal bacteria in the autumn sampling) (Ramani et al. 2014). Accordingly, the gill damages of chub from the Kriva River were overall milder compared to the Zletovska River, but more pronounced compared to the reference Bregalnica River (Fig. 1). A characteristic finding for the chub from the Kriva River was the highest prevalence and the highest indices for progressive changes in both sampling periods (Table 2, Fig. 1e-f, Fig. 4a-b), which were accompanied by circulatory disturbances such as oedema (Fig. 4a), and regressive changes such as desquamation (Fig. 4c). Especially frequent progressive lesion in chub from this river was hyperplasia of cells in the interlammellar space, leading to fusion of lamellae (Fig. 4a). By use of PAS staining, it became obvious that hyperplasia also affected mucous cells (5b). Severe hypertrophy of chloride cells (Fig. 4a) was also observed in high percentage of chub specimens from the Kriva River, which was not the case in the other two rivers (Table 2). The progressive changes, such as hyperplasia and hypertrophy of epithelial cells, can be considered as general safety measures against toxicants (Das and Gupta 2012), which increase the distance between external environment and the blood and thus serve as a barrier to the entrance of xenobiotics (Mallatt 1985; Mohamed 2009). Additional protective mechanism is hyperplasia of mucous cells, which secrete mucus, a layer of glycoproteins and glycolipids, to establish a protective barrier over gill epithelium (Breseghelo et al. 2004; Pereira et al. 2012). Although gill alterations, like epithelial hyperplasia accompanied by lamellar swelling and telangiectasis, were previously described for the gills of chub (*S. cephalus*) from the Sava River, living under similar conditions of high faecal contamination (Kurtović et al. 2009), such as found in the Kriva River (Ramani et al. 2014), such changes could not be considered as a specific response to one type of water contamination, i.e. they can occur under various conditions of exposure to different contaminants (e.g., heavy metals, pulp and paper mill effluents, crude oil, excessive ammonium concentrations, detergents; Mallatt 1985; Belicheva and Sharova 2011; Pereira et al. 2012). However, hyperplasia of the epithelial cells has been repeatedly associated with ammonium (El-Shebly and Gad 2011). For example, epithelial hyperplasia in brown trout (*Salmo trutta*) was associated with ammonium concentrations from 0.032-0.177 mg L^{-1} (Bernet et al. 2004), whereas in carp (*Cyprinus carpio*) it was observed whenever ammonium level increased above 0.5 mg L^{-1} for a longer period (Rašković et al. 2010). In the Kriva River, ammonium concentrations were 0.36 and 2.18 mg L^{-1}, in spring and autumn sampling, respectively (Ramani et al. 2014), which is considerably higher than acceptable threshold value for cyprinid waters set at 0.2 mg L^{-1} (EPCEU 2006) or recommended ammonium concentrations for fish ponds which should not surpass 0.1 mg L^{-1} (El-Shebly and Gad 2011).

Seasonal differences were generally less pronounced than spatial differences. Main seasonal effect observed in this study referred to higher prevalence and statistically significant increase ($p<0.05$) of indices calculated for progressive changes in gills of chub sampled in the autumn compared to spring in all three rivers (Table 2, Fig. 1e-f). Contrary, statistically significant autumn decrease of indices for circulatory disturbances of chub from the Bregalnica ($p<0.001$) and Kriva River ($p<0.05$) was recorded (Table 2, Fig 1a-b). Total indices had the same spatial pattern and comparable values in both seasons. As mentioned before, chub in this study were sampled in two physiologically distinct periods, pre-spawning and spawning, and it is well known that seasonality of pathological conditions of fish can be explained by the role of hormonal variations in disease susceptibility or even by the influence of temperature on fish (Bernet et al. 1999). However, there is a possibility, even more probable, that the observed higher severity of progressive changes in autumn was the consequence of seasonal change of exposure to contaminants. Lower water discharge in October compared to May/June of 2012 has lead to temporarily higher contamination of the river water in all three rivers: strong faecal contamination of the Bregalnica and Kriva River, and exceptionally high contamination with several metals (e.g. Cd, Mn, and Zn) in the Zletovska River (Ramani et al. 2014). Since the gills are very sensitive to changes in the quality of the water (Amiri et al. 2011), more prominent progressive changes on gills in autumn than in spring were probably found as a result of transient increase in exposure to pollutants, which then caused the activation of defence mechanisms. Seasonal variability in histopathological alterations on gills of several fish from El-Salam canal in Egypt was also attributed to seasonal differences in type and degree of water contamination (Mohamed 2003).

4. Conclusions

Histopathological alterations on the gills of Vardar chub from all three studied rivers indicated in general low water quality status of all selected freshwater ecosystems in north-eastern Macedonia. However, the reference, agriculturally impacted, Bregalnica River was characterized with the mildest changes on chub gills, among which only reversible circulatory disturbances were comparably severe as in the other two more heavily contaminated rivers. The impairments on the gills of chub from two mining impacted rivers were more severe. In chub from the Kriva River, which was characterized by occasional high water contamination with metals and faecal bacteria, as well as by constantly high level of ammonium, the highest indices were observed for progressive changes. In chub from the Zletovska River, which was characterized by high and continuous metal contamination, frequent occurrence of severe regressive changes of both epithelium and supporting tissue was observed, resulting with the highest total lesion indices. Seasonal assessment indicated higher indices of progressive changes in the autumn compared to spring at all three rivers, probably as a result of sudden increase in level of exposure to different contaminants, due to their preconcentration during the period of very low water discharge. Our results implied that, although histopathological alterations on fish gills do not constitute specific response to exposure to isolated contaminants, they can indicate different types and intensities of contamination of freshwater ecosystems. Therefore, based on this study, as well as other previously published researches (Jabeen and Chaudhry 2013; Paulino et al. 2014), in which histological changes on the fish gills were demonstrated as useful biomarkers of environmental contamination, histopathological alterations on chub gills could be suggested as very valuable supplementary biomarker of water pollution for the monitoring studies.

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Figure legends

Figure 1. The indices of histopathological alterations in the gills of Vardar chub (*Squalius vardarensis* Karaman) from three rivers in the north-eastern Macedonia (Bregalnica River, Zletovska River, and Kriva River) in two seasons, classified in three reaction patterns: a) circulatory disturbances in spring; b) circulatory disturbances in autumn; c) regressive changes in spring; d) regressive changes in autumn; e) progressive changes in spring; f) progressive changes in autumn; g) total indices in spring; h) total indices in autumn. 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 dots indicate outliers. Differences among sites are indicated with different letters (a, b), based on Kruskal-Wallis One Way Analysis of Variance on Ranks (*p* values indicated in figures) and *post hoc* Dunn's test ($p<0.05$). Number of samples per each site in each season is given in Table 1.

Figure 2. Typical histopathological alterations on the gills of Vardar chub (*Squalius vardarensis* Karaman) from the Bregalnica River, referring to disturbances of blood flow, with black arrows pointing to specific changes: a) vascular congestion in primary (1) and secondary gill lamellae (2); b) various stages of telangiectasis throughout the entire lamella (1), with thrombotic clusters of erythrocytes in the intralamellar space (2); c) severe oedema in the interlamellar space (1).

Figure 3. Typical histopathological alterations on the gills of Vardar chub (*Squalius vardarensis* Karaman) from the Zletovska River, referring to regressive changes, with black arrows pointing to specific changes: a) atrophy resulting in shortening of secondary lamellae (1), thinning and lifting of epithelial cells (2), accompanied by congestion and oedema (3), as well as lamellar curling (4); b) necrosis of the epithelium and chloride cells (1), as well as regressive change of supporting tissue in a form of cartilage deformity (2); c) rupture of pillar cells, associated with swollen tips of secondary lamellae (1).

Figure 4. Typical histopathological alterations on the gills of Vardar chub (*Squalius vardarensis* Karaman) from the Kriva River, referring to progressive changes, with black arrows pointing to specific changes: a) hyperplasia of cells in the interlamellar space (1) and severe hypertrophy of chloride cells (2), leading to fusion of lamellae (1), and accompanied by oedema (3); b) mucous cell proliferation and excess mucous production visualized by use of PAS staining (1); c) epithelial layer desquamation (1).

Figure SI1. Non-impaired gill surface with general structure of normal gills in Vardar chub (*Squalius vardarensis* Karaman) from the Bregalnica River.

Table 1. Biometric characteristics of Vardar chub (*Squalius vardarensis*Karaman) specimens analyzed in this study, caught in three rivers in north-eastern Macedonia in spring and autumn of 2012.

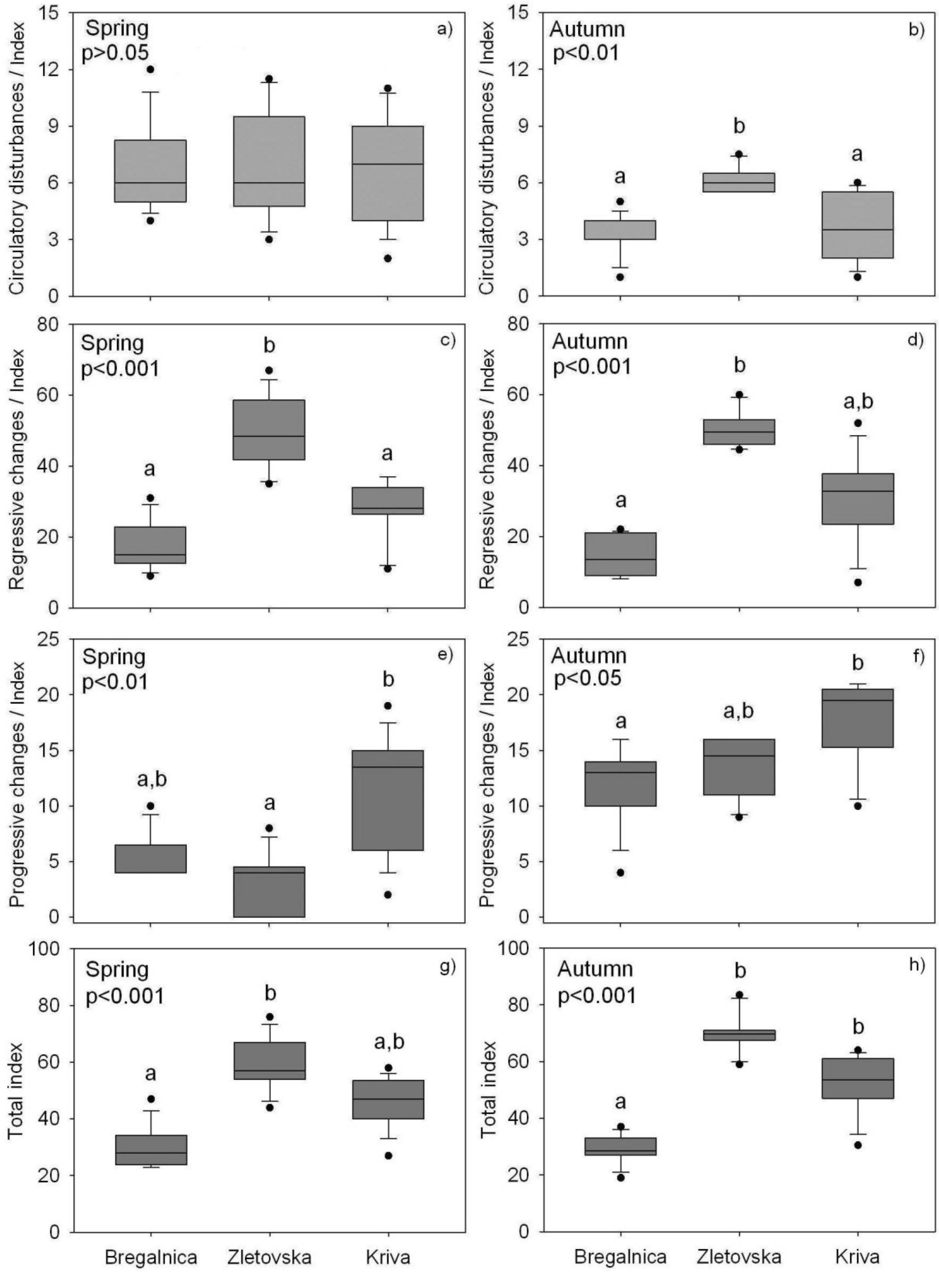
	Bregalnica River		Zletovska River		Kriva River	
	Spring	**Autumn**	**Spring**	**Autumn**	**Spring**	**Autumn**
n	9	10	9	6	10	8
***Total length (cm)**	16.58±0.92	16.50±0.52	[b]15.01±1.02	14.77±2.49	15.84±0.80	[a]16.81±1.07
****Total mass (g)**	[a]51.32±9.84	47.18±6.05	[b]33.84±8.65	29.56±16.77	43.58±6.50	47.22±9.36
****Gill mass (g)**	0.52±0.19	0.55±0.13	0.32±0.11	0.31±0.17	0.45±0.08	0.54±0.13
*****FCI (g cm^{-3})**	[a]1.12±0.11	1.05±0.05	0.98±0.07	[b]0.84±0.08	[a]1.09±0.08	0.98±0.05
*****GSI (%)**	[a]6.75±1.45	[b]1.54±0.43	[a]8.97±1.94	4.61±1.67	[a]8.26±2.14	[b]1.58±0.27
Females/Males (n)	1/8	3/7	3/6	4/2	0/10	2/6

* p<0.05; ** p<0.01; *** p<0.001
[a,b]the values assigned with different letters (a or b) statistically significantly differ from one another according to Dunn's test (p<0.05); the other values do not differ either from one another, or from the assigned values

Table 2. Prevalence of occurrence (%) of histopathological alterations observed in gills classified in five reaction patterns, according to Bernet et al. (1999).

		Bregalnica		Zletovska River		Kriva River	
		Spring	**Autumn**	**Spring**	**Autumn**	**Spring**	**Autumn**
Circulatory disturbances	- telangiectasis	78	40	56	0	80	0
	- congestion (hyperaemia)	100	100	100	100	100	100
	- intercellular oedema	67	80	22	50	60	13
Regressive changes	*Epithelium*						
	- structural alterations	100	100	100	100	100	100
	- plasma alterations	0	0	0	0	0	0
	- intercellular deposits	0	0	0	0	0	0
	- nuclear alterations	0	0	0	0	0	0
	- atrophy	100	100	100	100	100	88
	- necrosis	100	90	100	100	100	88
	- rupture of pilar cells	0	20	33	0	0	0
	Supporting tissue						
	- structural alterations	89	50	100	100	90	100
	- plasma alterations	0	0	0	0	0	0
	- intercellular deposits	0	0	0	0	0	0
	- nuclear alterations	0	0	0	0	0	0
	- atrophy	89	60	100	100	100	100
	- necrosis	56	10	100	100	50	75
Progressice changes	*Epithelium*						
	- hypertrophy	0	10	0	17	60	75
	- hyperplasia	100	100	56	100	100	100
	Mucous cells						
	- hypertrophy	0	10	0	0	10	0
	- hyperplasia	44	90	44	100	90	100
Inflammation	- exudate	0	0	0	0	0	0
	- activation of the reticuloendothelial system (RES)	0	0	0	0	0	0
	- leucocyte infiltration	0	0	0	0	0	0
Tumour	- benign	0	0	0	0	0	0
	- malignant	0	0	0	0	0	0

Figure 1.



Spring
p>0.05
a)
Autumn
p<0.01
b)
Spring
p<0.001
c)
Autumn
p<0.001
d)
Spring
p<0.01
e)
Autumn
p<0.05
f)
Spring
p<0.001
g)
Autumn
p<0.001
h)
Circulatory disturbances / Index
Regressive changes / Index
Progressive changes / Index
Total index
a
b
a,b
Bregalnica
Zletovska
Kriva

Figure 2.

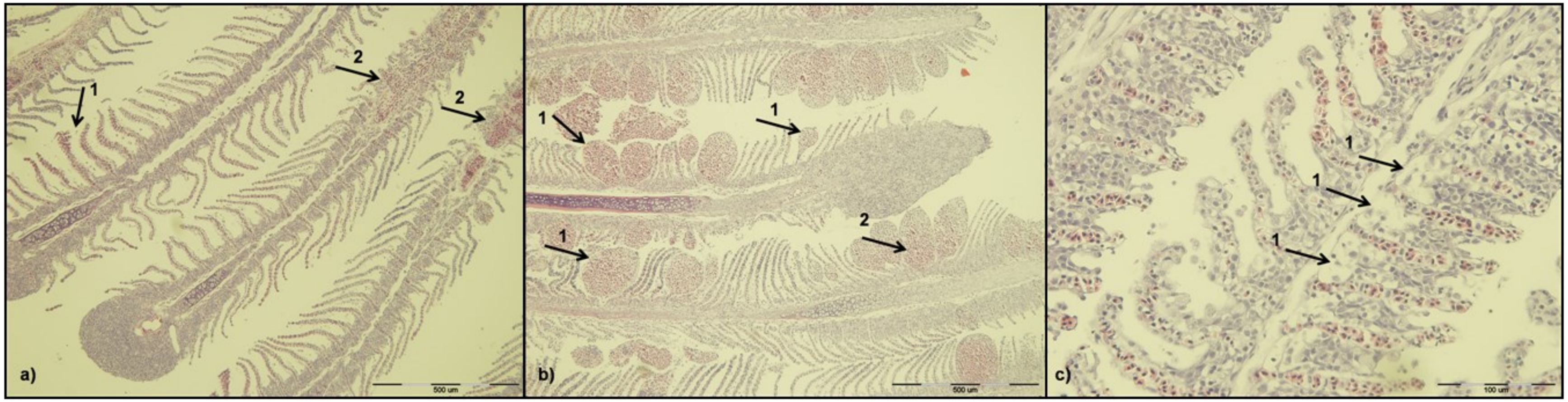


Figure 3.

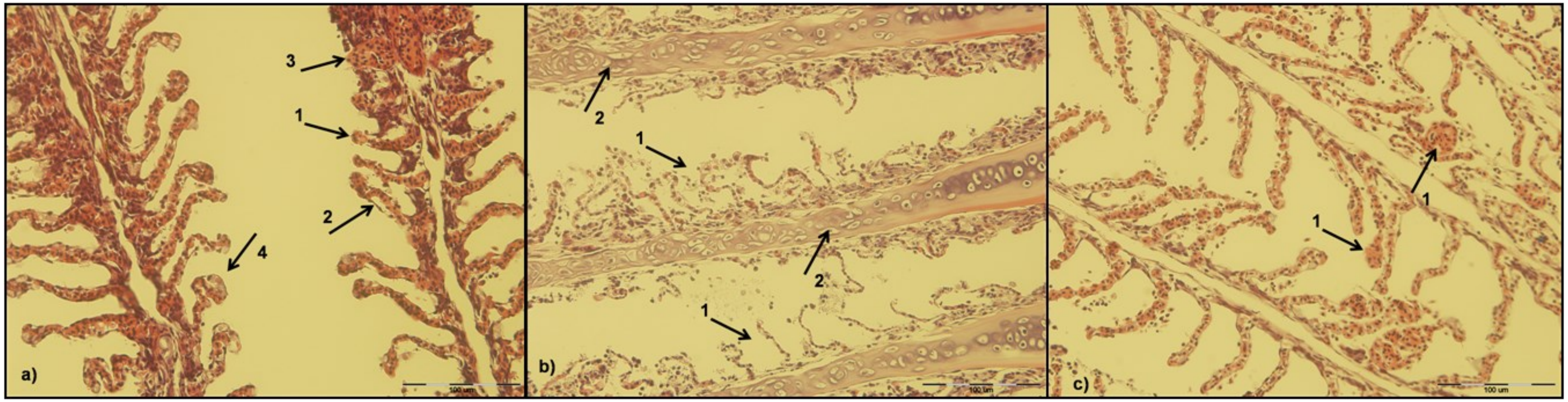


Figure 4.

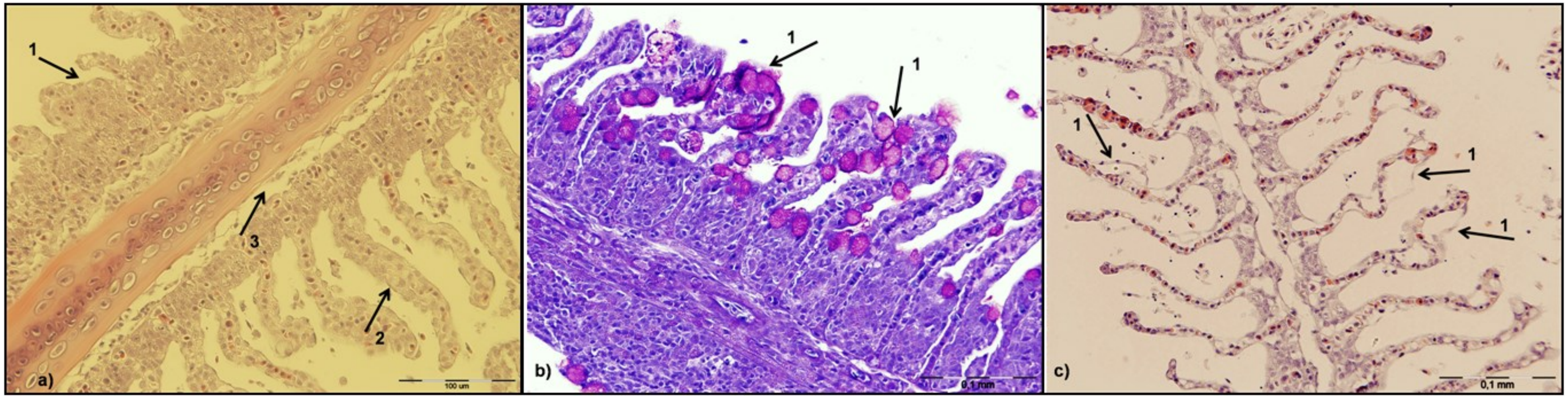


Figure SI-1.