HISTOPATHOLOGICAL ANALYSES OF THE LIVER AND GONADS OF VARDAR RIVER FISH *Barbus peloponnesius*

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Abstract. The *Barbus pelopnnesius* is a typical benthophagous fish, feeding on the zoobenthos and plant components and thanks to its sensitivity to the changes in surrounding area is an ideal tool for indication of the health of aquatic ecosystems. The aim of this study was histopathological analysis of the liver and gonad tissue of *Barbus peloponnesius* fish of Vardar River. Liver and gonad of 25 fish individuals collected from two research stations, at the exit point of Veles city during 2013, were excited and proccesed for standart histopathological analysis. Microscopically analysis revealed pathological changes in the liver and gonad tissue including hypertrophy of epithelial cell nuclei in bile ducts and proliferation of their epithelium, presence of hemosiderin, hemorrhage in the hepatocellular parenchyma, bacterial and verminous infection, as well as macrophagous aggregation. Estimation of water quality from two points of vardar River near Veles on the basis of physic-chemical and microbiological indicate that waste water have negative impact to the natural fish population.

Keywords: fish; biomarkers; Barbel; histopathological; lesions; liver; gonads; aquatic ecosystems.

INTRODUCTION

Rivers worldwide serve as the recipients of great quantities of waste discharged by agricultural, industrial and domestic activities. Agriculture seems to be the most considerable source of pollution due to runoff from fertilized land. Industrial processing and solid waste dumps are considered to be the main anthropogenic sources of metal pollution. Shortage of freshwater throughout the world can be attributed to human abuse which most commonly is in the form of pollution [11].

Supplying around 75% of the country's total water resources Vardar River bisects the country from north to south, before flowing into Greece and entering the Aegean Sea near Thessaloniki. After several years of urbanization and unregulated industrial processes in the River's surrounding areas, it has become heavily contaminated by untreated urban and industrial pollution, including heavy metals and chemicals [19]. *Barbus peloponnesius* Valenciennes, 1842 has been described on the basis of specimens fished in the Alphios River (Karytaena), in Peloponnisos. *B. peloponnesius* is widely distributed in the Axios River (Vardar) in the Former Yugoslav Republic of Macedonia [17]. The *Barbus* species is a typical benthophagous fish feeding on the zoobenthos and plant components in the course of the autumn. Every natural community of the bottom, mainly the larvae, is included in the *Barbus* nutrition [13].

In order to evaluate or to predict the pollution impact on the aquatic ecosystems, thereby on aquatic organisms, there is increased need of establishing early-warning signals (biomarkers) using bioindicator organisms (i.e., a biomarker approach) [20]. Histopathological biomarkers can be indicators of the effects on organisms of various anthropogenic pollutants and are a reflection of the overall health of the entire population in the ecosystem. The alterations in cells and tissues in vertebrate fish are recurrently used biomarkers in many studies, but such changes occur in all vertebrates and invertebrates inhabiting aquatic basins. Histopathological biomarkers embody tissue lesions arising as a result of a previous or current exposure of the organism to one or more toxins. Well-documented lesions based on experimental data in liver, ovary, skeleton system and skin have been used as biomarkers to date [6]. We know from earlier on that the development of oxidative stress and biochemical changes can become toxic in fish exposed to pollutants [14].

Also, high concentration of ions (especially nitrogen and potassium) found in sewages of mine works; alter drastically the chemical composition of the aquatic ecosystem water, the composition of endangered species, the structure of nutritive chain and the fish's organism itself. Aside from the low density of fish population, the liver, gill and other organs' histology is an indicator to the unpleasant conditions for fishes living in aquatic ecosystems polluted with mining pollutants [22]. The liver is the main and central organ of the metabolism in aquatic organisms such as the fish and has many anabolic and catabolic functions. The liver plays a key role in the metabolism and biochemical transformations of pollutants from the environment, which inevitably reflects on its integrity by creating lesions and other histopathological alterations of the liver parenchyma or the bile duct [21]. The liver is able to metabolize and degrade toxic components, but its regulating mechanisms can be overpowered by increased concentrations of these components, resulting in structural damage of the parenchyma [2].

To date, there have been no studies investigating the occurrence of histopathological lesions indicative of anthropogenic contaminant exposure in *Barbus peloponnesius* fish species. This investigation provides the first such assessment of lesions occurring in the livers and gonads of barbel fish in Veles area from the

Vardar River, with particular focus on putative toxicological lesions.

MATERIAL AND METHODS

In this study livers and gonads of 25 barbel (*Barbus peloponnesius*) were collected and examined. The collecting was carried out during June and July 2013, from two stations at Veles city exit point: S1 – a station nearby an accessory medical store "Dimko Mitrev" with these geographic coordinates: N 41^0.70'08.42", E 21^0.79'25.59" and S2 – a station nearby an animal food factory "Agria-Agroindustry- Group" with these geographic coordinates: N 41^0.68'77.02", E 21^0.80'74.93" (Fig. 1).

For collecting the "Electrofishing" method has been applied [10], whereas the geographic coordinates have been determined using GPS (Bushnell Back-Track). Each fish was dissected according to the following protocol: measurement of the fish length, dissection by opening the abdominal cavity, determination of the sex, dissection of the digestive and reproductive system and taking the liver and gonads.

Isolated tissue samples were fixed at the 9 % formalin and treated by the standard paraffin procedure. The 5µm paraffin sections, gained after cuting with a microtome (LEICA SR 2000) were stained with the Hemalaun & Eosin method and approximately 2–4 sections of each individual fish were analyzed and photographed by light microscope (Nikon eclipse 80i) equipped with a digital camera. The prevalence (%) of recorded histopathological changes was calculated as a percentage of affected fishes. During the same period there have been collected samples of water from two stations at Veles city exit point S1 and S2.

The analysis of physicochemical and bacteriological parameters was carried out in accordance with world standard methods, applied in analyzing the quality of waters [1] and also refered to the Macedonian Regulation of Water Categorization [15].

RESULTS

The microscopic analysis of the histological preparations of liver and gonad from the investigated specimens indicated presence of many types of tissue lesions. The obtained results revealed structural changes in the liver and gonad tissue including an inflammatory processes, fibrosis and necrosis on a level of hepatocelular parenchyma, presence of hemosiderin, bile duct proliferation and bile duct epithelium necrosis on a level of hepatic bile tract. However, depending on the amount of intracellular lipid and glycogen, individual hepatocytes displayed a significant variation in appearance within H&E stained sections. Frequencies of pathological changes in the liver and gonad are presented as percentage in the following table.

Hepatocytes were generally granular in appearance with varying amounts of vacuolation (due to lipid storage) present and conspicuous regions of relatively basophilic material most likely representing endoplasmic reticulum. Most fish showed hepatocellular and nuclear pleomorphism (Fig. 1B 6 narrow). This condition occurred throughout the liver with significantly enlarged nuclei containing granular and marginated chromatin. The examinated samples of the histological preparations indicated presence of hemorrhaging in the hepatocellular parenchyma (Fig. 1C 7); moreover the pigment hemosiderin was evidenced, probably as a result of internal bleeding in the hepatic tissue of barbel (Fig. 1A 3). In some cases, hepatocytes were associated with the presence of nematode parasites (9%), most probably of the class Nematodes (Fig. 1A 2). Macrophage aggregates (MA) were present in 26% of fish examined and usually

Figure 1. Satellite image of fish collection stations at the exit point of Veles city (S1 – the station nearby the accessory medical store "Dimko Mitrev" and S2 – the station nearby the animal feed factory "Agria-Agroindustry-Group"

Table. 1. Prevalence (%) of lesion types detected in the liver of all examined samples. Note that individual fish may have more than one lesion

	Lesion type				
Prevalence (%)	Non abnormalities detected	Hemorrhage	Hemosiderin	Parasites	Macrophage aggregate
	48.0	25.5	15.5	9	26

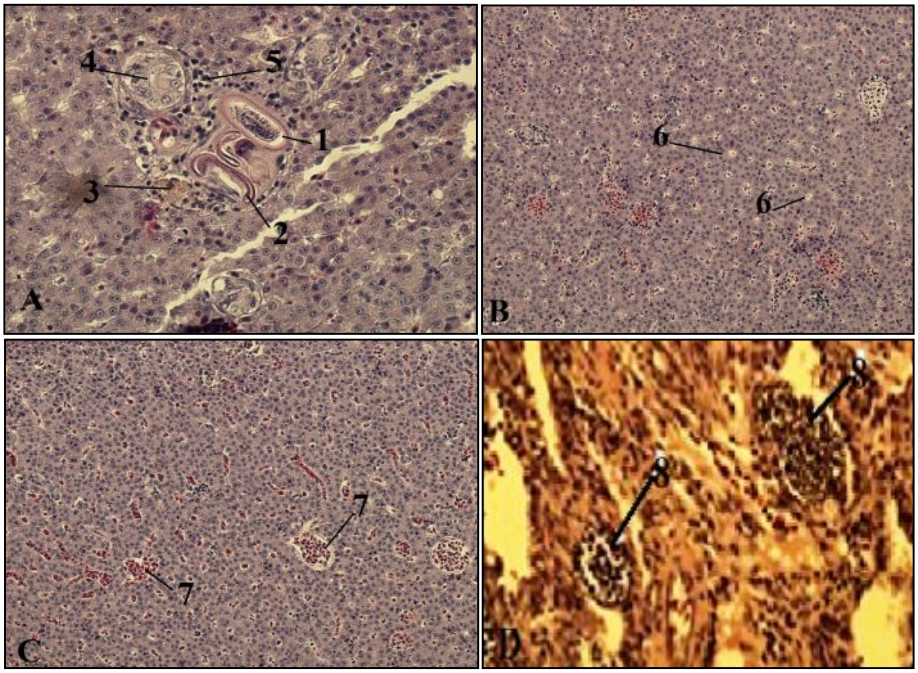

Figure 3. A – Histological changes of a fish liver and gonad sections. All sections stained with H&E. 1– parasite cyst; 2 – parasites, most likely, Nematodes; 3 – pigment hemosiderin in hepatocellular parenchyma ; 4 – hypertrophy of epithelial cell nuclei of biliary duct; 5 – bacterial infection X 600; **B** – section showing the vacuolization of hepatocytes X 400; **C** – 7 hemorrhage in the liver tissue associated with parenchyma cell necrosis X 200; **D** –8 a fish spawn section showing macrophagous aggregates 200X.

Table 2. Categorization of Vardar River water at Veles city exit point according to total coliform and heterotrophic bacteria (The Regulation on Water Categorization, Official Gazette of the Republic of Macedonia No. 18/99) [18]

Stations	Bacteriological parameters of Vardar River water		
	Coliform bacteria (bact./100mL)	Heterotrophic bacteria (bact./1mL)	Categorization (by class)
S1	24.000	84.6	III
S1	24.000	151.3	III

Table 3. Categorization of Vardar River water at Veles city exit point based on obtained values of physiochemical parameters (The Regulation on Water Categorization, Official Gazette of the Republic of Macedonia No. 18/99)

Stations	Physiochemical parameters of Vardar River water					
	Dissolved O_2 (mg/l)	BOD_5 (mg/l O_2)	Organic matters – Consumption of $KMnO_4$ (mg/l)	$N\text{-}NH_3$ (mg/l)	Total phosphorus (mg/l)	Categorization (by class)
S1	12.110	8.700	7.850	1.130	2.437	IV
S2	12.330	8.715	7.867	1.142	2.440	IV

occurred with even distribution throughout the liver, sometimes associated with blood vessels (Fig. 1D 8). Affected hepatocytes showed loss of cellular integrity and separation from adjacent cells and the presence of strongly basophilic pyknotic nuclei.

The obtained results of the conducted analysis during the summer period of 2013, at the exit point of Veles indicate to a substantially decreased quality of the water, that is specially increased development of the investigated bacteria [9], as well as increased values for all related physicochemical parameters [15], (Table. 2 and 3).

The analyzed samples of water from all investigated localities indicate to a relatively high level of organic load, which in accordance to the obtained values for the presence of the organic matter (represented as consumption of KMnO4) indicate that the water is mainly with quality of III and IV class. These obtained results indicate to an increased nutrient loading of the water at the respective points.

DISCUSSION

Many industrial, agricultural and urbanization processes cause environmental pollution and contribute to the contamination of water ecosystems, thereby threatening the health of aquatic biota and humans. The health of all living organisms in an aquatic ecosystem is also affected as a result of the deterioration of water quality [4]. The biological integrity of an ecosystem can often be assessed based on the health of its fauna [22]. Considering the pollution as a kind of general threat to fish [10], only 48.0 % of total analyzed samples of this study showed no structural abnormalities (Table 1), while 52.0 % of the samples revealed tissue changes as a result of certain organic pollution. These changes occurred probably from the communal sewage and wastewater from food factory that flows directly into the Vardar River. In the present study as expected in all analyzed samples of water contamination were recorded at the sampling sites

during the summer months. The values of the coliform bacteria are similar at the two studied stations, whereas the heterotrophic bacteria shown higher values at the S2 station. Histology offers a detailed study of the structure of different fish tissues. It can also verify gender, identify growth stages and the presence of parasites, diagnose tumors and other abnormalities as well as changes caused in tissue layer set-up, including the digestive tract [12]. Histopathological biomarkers are closely related to other stress biomarkers because the majority of pollutants need to go through metabolic activations so that they can be able to cause changes in the attacked organism. Acting mechanism of some xenobiotics could trigger the creation of a specific enzyme which causes metabolic changes, leading towards intoxication and death at cellular level, manifested as necrosis and representing histopathological biomarkers at tissue level. Chemical matters provoke necrotic and degrading changes accompanied by inflammations and protective reactions [3, 23]. Aside from chemical components, histopathological lesions could appear as a result of infectious diseases and parasites, triggering necrotic and degrading changes, making the body to respond defensively with an inflammation [23, 25]. A large number of macrophagous aggregates which are present in gonad (Fig.1 D 8) can be found in the liver, kidneys, spleen and testicles of fish, under the influence of chemical pollutants, bacteria, fungi or parasites [20]. The results of histopathological changes of our study allow us to speculate that these changes are caused by numerous chemical pollutants and bacteria. The liver deposits carbohydrates in a shape of glycogen and especially fats, before the breeding period. Hepatocytes that normally have a compact shape could make us believe that they are more or less vacuolated during their observation with an optical microscope, depending on the level of fat depositions [16]. The phenomenon of a fatty liver or better defined as large fat deposits in fish liver is a normal occurrence at certain periods, including periods of sexual maturity, when fish get to deposit considerable amounts of fats in their hepatocytes [8]. Eating disorders can often time cause fats to deposit in fish liver [3]. But when fish are fed properly and unseasonable for breeding, then a fatty liver should undoubtedly be considered as a pathological occurrence [21]. Pacheco and Santos [16] described increased vacuolisation of the hepatocytes as a signal of degenerative process that suggests metabolic damage, possibly related to exposure to polluted water. The results suggested also that the vacuolization need not be related to a degenerative process. The presence of hemosiderin (in this study 15.5 %) in the liver comes as a result of hemoglobin degradation which gets infiltrated through the lymphatic system of macrophages [7].

In the present study 9% of the examinated samples showed rupture of the hepatocytes which caused hemorrhage, like aneurysm, this lesion can be interpreted as a reflection of the direct action of toxic agents on the tissue [24]. The histological changes observed in the liver and gonads of the *B. peloponnesius* indicate that the fish were responding to the direct effects of the contaminants as much as to the secondary effects caused by stress.

Histological changes of liver and gonad may serve as a useful marker of polluted water. The differences of the histological abnormalities between two stations of our study were not significant. In 52% of all affected fish both liver and gonad tissues showed at least 9% abnormalities with high correlation in accordance to the level of water pollution. The liver was more affected by histological changes according to the total coliform and heterotrophic bacteria.

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