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The comparative enzyme and histological analysis of the *Barbus peleponnesius* from the aquaculture and natural population in the Vardar River in the Republic of Macedonia

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Enzymatic biomarkers are sensitive to environmental changes and they respond by changing their activity. *Barbus peloponnesius* is considered a potential bioindicator to changes that can be caused in an environment by various pollutants. The results from the organs of the experimental group of fish from the aquaculture that were treated with a sublethal dose of insecticides (1 mg/L), showed a significant increase in the kinetic activity of the enzymes EROD and B(a)PMO compared to the group control. Whereas the group of fish treated with the same dose of herbicides did not show a significant increase, there was a kinetic activity alteration in these enzymes. A significant increase in the enzymatic activity (EROD and B(a)PMO) was also seen in the fish from the polluted Vardar River. The fish treated with sublethal concentrations (2 μg/L) of insecticides and herbicides showed haemolysis, coagulation, degeneration and cytoplasmic vacuolization of the hepatocytes, dilatation of the sinusoids, degeneration of the glomeruli and renal tubules, widening of the space between the basal membranes and the epithelial cells of the renal tubules, fracture and degeneration of the ovoplasm and disruption of the follicular epithelium in the hepatic parenchyma, kidney tissue, and the ovaries.

Keywords: EROD, B(a)PMO, CYP 450, CYP1A, Vardar River, *Barbus peloponnesius*

INTRODUCTION

The pollution of water ecosystems is an increasingly serious problem. The rise of the number of industrial, agricultural and commercial chemicals unloaded in aquatic environments has caused negative effects on aquatic organisms (McGlashan, Hughies, 2001). The Vardar River is the largest river in the Republic of Macedonia, which flows from the north to the south into the Aegean Sea near Thessaloniki. Years after urbanization and industrialization in the areas around the river, it was contaminated on a large scale by untreated urban and industrial pollutants, including heavy metals and other chemical substances (Beadini et al., 2015). Since the 1930s, global production of chemicals has increased from 1 million tonnes

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to 400 million tonnes. About 100,000 different substances have been registered in the EU market, of which 30,000–70,000 are used on a daily basis (Schwarzenbach et al., 2006; EC 2001). Most of the chemicals penetrate into aquatic environments through industrial, agricultural, and household waters, including municipal wastewaters (Ohe et al., 2004). In aquatic organisms, including the fish, the pollutants are accumulated directly through the polluted water and indirectly through the food chain. The selection of fish as a model in ecogenotoxicological studies could be made necessary since fish are a very sensitive biomarker indicator of water quality. They can highlight the potential danger of new chemicals introduced in the aquatic environment and also respond to toxicants in a manner similar to that of higher vertebrates (Katsumiti et al., 2013). *Barbus peloponnesius*, *Valenciensi 1842* was described based on the samples caught in the Alphyos (Karytaena) River in Peloponez. *B. peloponnesius* is quite spread in the Vardar River (Panos S, 1989). 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 (Moravec, 1997). Biomarkers represent an early answer or biological changes that are measurable and are caused by the exposure of the organisms to pollutants; they are widely applied in biological monitoring researches (Miranda et al., 2008; Oliveira Ribeiro et al., 2005; Winkaler et al., 2001; Lagadic et al., 2000). Biomarkers have been increasingly accepted all over the world as a tool for assessing the pollution of sea environments, and some of them have already been included in the environment monitoring programmes (Viarengo et al., 2007).

The status of detoxification enzymes in the fish tissues, the histopathological analyses, and the DNA integrity are quite often used as such biomarkers. Enzyme biomarkers are fairly sensitive to environmental changes and are characterized by changes in their activity. In fish, the activity of these enzymes can be inducted or inhibited after the exposure to xenobiotics (Bucheli, Fent, 1995). The enzyme induction represents the intensification of the enzymes activity. For example, the inductivity of two phases of CYP450 has been noticed in the Californian Trout exposed to polychlorinated biphenyls (PCB). In the first phase, the activation of the existing enzymes occured, whereas in the second phase the *de novo* enzyme synthesis took place (Sijm, Opperhuizen, 1989). It is supposed that the *de novo* synthesis of the proteins is the most important process in the enzyme induction process (Stegeman and Hahn, 1994). The liver, kidneys, and gills are adequate organs for performing histological analysis in order to verify the pollution effect (Halis et al., 2010). The liver is one of the most used organs for such purposes, because it contains the mechanisms that play a crucial role in the detoxification process (Ayas et al., 2007; Lemes, Braccini, 2004), biotransformation, and secretion of pollutants (Gernhöfer et al., 2001). The liver of the Teleostei fish was the focus of toxicological studies and proved to be quite sensitive to pollution (Blazer et al., 2007; Pinkney et al., 2004). However, some of the produced metabolites are so reactive and toxic that they affect the structural identity of DNA. the damage to DNA results in the initiation of carcinogenesis, decrease in the reproduction potential, decrease of life expectancy, deceleration of the growth process, etc. All of these can have negative effects on fish populations (Theodorakis et al., 2000). The conditioning factor (CF) is an indicator of the health situation of fish and can be used to assess the pollution level during in-field research. Changes caused by the pollutants are in correlation with the distribution of the energy for the detoxification mechanisms and the expenditure of dedicated reserves, especially for growth (Valdez et al., 2009). The hepatosomatic index (HSI) is an indicator of metabolism in fish. The increase of the HSI is closely related to the acceleration of detoxification processes, as a result of the presence of chemical components (Pereira, Kuch, 2005). On the other hand, the decrease of the HSI shows that fish populations are endangered by chemical substances (Kopecka et al., 2006).

The aim of this study was (1) to determine the activity of EROD and B(a)PMO enzymes in

the liver, kidneys, and gills in experimental fish populations, specifically, in the *Barbus peloponnesius* from aquaculture; (2) to determine the activity of EROD and B(a)PMO enzymes in the same organs in the population of the *Barbus peloponnesius* caught near the source of the Vardar River, in the village of Vrutok, and at the exit of the city of Veles; (3) to determine conditioning factors and the somatic index of the liver, kidneys, and gonads as general indicators of the health of fish and to analyze the possible pathological changes in these organs; (4) to compare the lesions present in *Barbus peloponnesius* from aquaculture and those from the Vardar River, and (5) to process statistically all the data acquired, in order to analyze the histopathological impact of the pollution and the enzyme activity in order to see if there are any differences between the fish from aquaculture (treated with insecticides and herbicides) and those from the Vardar River, compared to the control group of *Barbus peloponnesius*.

MATERIALS AND METHODS

Stations of fish collection from the natural population of the Vardar River

The fish from the natural population of the Vardar River was collected in the periods from May to August 2014 and May to August 2015 from two stations that are considered as potential spots of pollution: station V0 (the blue spot on the map), the Vardar River near the ELEM hydropower plant in the village of Vrutok (41°.77'03.59"N, 21°.84'12.3"E) and station V1 (the red spot on the map), the River Vardar near the Agria-Agroindustry-Group factory producing animal food at the exit of the town of Veles (41°.68'77.02"N, 21°.80'74.93"E) (Fig. 1). Authorization to carry out electrical fishing was

Fig. 1. Stations of fish collection from the natural population of the Vardar River.

Station V0 (the blue spot on the map), the Vardar River near the ELEM hydropower plant in the village of Vrutok, and station V1 (the red spot on the map), the River Vardar near the Agria-Agroindustry-Group animal food factory at the exit of the town of Veles. Geographic coordinates were defined with the help of the GPS tool (Medion GoPal S3867), whereas the selection of fish was done by using the electric inverter (SUSAN-735 MP), according to the protocol for electrofishing (Michael et al., 1998). In total, 50 fish were collected from the natural population in the Vardar River (ten from station V0 and 40 from station V1)

obtained from the management of the "Ribarska Vistina Ohrid" Fishermen's Club, according to the Law of Protection and Welfare of the Animals, Article 13 of Section III (Macedonian Food and Veterinary Agency).

Fish from the aquaculture

Seven aquariums with dimensions 90 × 80 × 40 cm and 100 l capacity were used for keeping the fish in the laboratory. In one of them, the fish from the control group, which was free from toxic substances, were placed (A0). The other six contained the fish from the experimental group that were treated with toxic substances. In three aquariums the fish were treated with different insecticides (A1-1, A1-2, A1-3), whereas in the other three the fish were treated with different herbicides (A2-1, A2-2, A2-3). Each of the aquariums contained 7–10 fish. The water temperature was 16°C, pH 7.4, and the amount of oxygen in the water 6 mg/L. Water ventilation was enabled by ventilation and continual water filtration pumps (Shark SB-1200F); chlorine was removed from the water supply system using the dechclorination filter (So-Safe CTSLW 10D).

The determination of the somatic index

The fish were measured separately. Their length was measured and then dissection took place – the liver, the kidneys and the gonads were isolated and wet weighted were recorded. The determination of the conditioning factor was done by using the formula

CF = [body weight (g)/length (cm)3] × 100.

The somatic index of the organs (the liver, the kidneys and the gonads) was determined by using the formula

OSI = [organs weight (g)/body weight] × 100 (Slooff et al., 1983).

The treatment of the fish from the aquaculture with chemicals

The treatment of the fish with chemicals (insecticides and herbicides) was done in an intraperitoneal way, by injecting a certain dose of the toxicant into the zone of anal fins. The NOEC (no-observed-effect concentrations) grade of toxicant concentration was applied in the analysis of enzyme activity (1 mg/L). The insecticide and herbicide components were diluted in 0.02% acetone. The United States Environmental Protection Agency recommends maximum allowable limits of 0.05% solvent for acute tests and 0.01% for chronic tests. This concentration of acetone was not significant with regard to toxicity (Ma et al., 2007) or distilled water until the final concentration of 1 mg/L was obtained. In order to cause histological changes, a concentration of 2 μg/L of the toxicant, prepared in accordance with the standard technique (APHA, 1985), was applied on days 1, 2, 3, 7, and 14. According to this technique, the toxicants were diluted in 1 ml of absolute ethyl alcohol and distilled water until the derivation of the final concentration of 2 μg/L.

The determination of the activity of the ethoxyresorufin-o-deethylase (EROD) enzyme

The determination of the activity of the ethoxyresorufin-o-deethylase (EROD) enzyme was realized by using the method applied by Monod et al., (1987). The prepared content of the enzyme reaction with 1.01 mL volume contained the following: 0.7 mL phosphate-buffered saline with pH 7.6, 0.1 mL ethoxyresorufin diluted in 0.1 mL methoxyethanol and 0.1 mL post mitochondrial fraction (S9) of the organ. The enzyme reaction was inhibited by adding 2.5 mL methanol to it. The enzyme reaction product was mixed for 30 s with an electric mixer (Vortex Genie-2) and was centrifuged for 60 s in 5,000 g with a centrifuge (SANYO, HARRIER 18/80, Refrigerated).

The fluorescence of the remaining product (resorufin) in the spectrofluorometer (SHIMADZU RF-1501) at an exciting/emitting wavelength of 510 nm/585 nm standardized with 0.1 mM Rodamine B solution in glycol ethylene was determined for the supernatant placed in a quartz cuvette with a 10 mm optical field, containing the product of the enzyme reaction. As a controlling reaction, the content without the cofactor NADP+G-6-P was used. The remaining amount of the fluorescent product was read with the help of a calibration curve adjusted with 0.05–0.5 nM resorufin concentration,

whereas the activity of the EROD enzyme was expressed in pmol resorufin/min/mg protein.

The determination of the benzo(a)pyrene monooxygenase enzyme (B(a)PMO)

The determination of the benzo(a)pyrene monooxygenase enzyme (B(a)PMO) was carried out based on Nebert and Gelboini's (1968) original method and the modifications applied by Payne and Penrose (1975). The prepared content of the enzyme reaction with a volume of 1.2 mL contained the following:

0.8 mL Tris-HCL with pH 7.6 (0.05 M Tris-HCL, 0.15 M KCL, 0.25 M sucrose), 0.1 mL NADPH (1.25 mg/mL diluted in puffer TRIS-HCL), 0.02 ml benzo(a)piren (0.002 M diluted in acetone) and 0.1 mL post mitochondrial fraction of the tissue homogenate. Such content was incubated in a water bath (assistant) at a temperature of 29°C. The reaction was stopped by adding 1 mL acetone to it. The product of the enzyme reaction was extracted in 4 mL normal hexane mixed with an electric mixer (Vortex Genie-2) for 30 s and was centrifuged for 3 min in 5,000 g using a centrifuge (SANYO, HARRIER 18/80, Refrigerated). The supernatant of the hexane extract was transferred to another test tube and 4 mL of NaOH 1 N were added to it. It was again mixed for 30 s and centrifuged for 3 min in 5,000 g. Hexane upper layer is removed with great care, meanwhile the liquid phase, which contained the product of the enzymatic reaction was given the remaining fluorescence of the product. The whole reaction was carried out in a polypropylene tube, in complete darkness (under the activity of infrared rays). The controlling substance consisted of the content with the NADPH cofactor and the denaturalized enzyme at 75°C and 5-minute duration. The fluorescence of the remaining product was measured using a spectrofluorometer (SHIMADZU RF-1501) at an exciting/emitting wavelength of 395/520 nm, standardized with 1μg quinine sulfate in 1 mL of H_2SO_4 1 N. The amount of the fluorescent product was compared to the fluorescence of the 3-OH benzopyrene, whereas the activity of the enzyme was expressed in pmol 3-OH benzopyrene/min/mg protein.

The determination of proteins

The determination of the amount of proteins was carried out in 0.1 mL homogenate of the post-mitochondrial fraction based on the method applied by Lowry et al. (1951). The measurement of the amount of proteins was done as follows: 0.1 mL homogenate supernatant was diluted in 0.5 mL of NaOH 0.1 M. 5 mL of the a reagent were added to the 0.5 mL supernatant and were mixed for several seconds with an electric mixer (Vortex-Genie 2). Ten minutes later, 0.5 mL of the B reagent were added to the substance and was left still for 30 min. Later, the amount of proteins present in the homogenate was measured with the help of a polyphenol reagent, through the reading of the absorbance at 710 nm wavelength, using a spectrophotometer (GENESYS 10 uv).

The histopathological analysis

The unrefined organs were cut 5μm-thick with a microtome (Leica 2000R) and coloured with hematoxilin and eosine with an automatic colouring device (Leica EG1140H block-forming unit and Varistain 24–4 K, Thermo Shandon). The photographing of the analyzed tissue fragments was done with a digital photo camera of an optical microscope (Motic) equipped with computer software (Motic Images 2000 v 1.3). The determination of the frequency (%) of histopathological lesions was done based on the analysed tissue fragments, in accordance with the histomorphometric criteria described by other authors (Sonia et al., 2007; Myers et al., 1994; Moore and Mayers, 1994; Hinton, 1993; Hinton et al., 1992).

The statistical analysis

The statistical software package Statistika 8 was used for statistical processing of the results. The obtained duplicated results for the activity of enzymes were expressed as average values (standard deviation). A value with a significant statistical difference was considered the one having $P < 0.005$. The morphometric data of the fish were processed according to the variance analysis method (ANOVA) one-way and were presented as average values (variance coefficient).

RESULTS

Results from this study are presented in tables as well as through photomicrographs of the optical microscopy. Unlike the control group from the aquaculture (A0), the 2.41 and 1.60 of the kinetic activity of EROD and B(a)PMO enzymes were slightly higher in the natural population group of the Vardar River in the village of Vrutok (V0) (Table 1). This difference in the activity of the two enzymes in the natural population group of the Vardar River in the village of Vrutok (V0), compared to the control group from the aquaculture (A0) kept under laboratory controlled conditions, can be a result of permanent oscillation of the abiotic factors of the water in this area.

In the experimental groups of fish treated with insecticides Chlomorel D (A1-1), Karate Zeon (A1-2), and King (A1-3), a significant increase in the EROD and B(a)PMO enzymes was observed compared to the control group of the fish from the aquaculture (A0) and that of the fish from the natural population of the Vardar River in the village of Vrutok. Based on the obtained results, we can see that all three insecticides that were applied caused induction of the kinetic activity of these two enzymes. In the experimental groups treated with herbicides Linurex 50SC (A2-1), Stomp 330 E (A2-2), and Devrinol (A2-3), there was no significant increase in the activity of these two enzymes, compared to the control group, which means that applied herbicides did not cause induction but just a change in the kinetic activity of the enzymes under supervision.

Results showed that there were differences in the kinetic activity of the two concerning enzymes between the group of fish from the aquaculture (A0) and the group of fish from the natural population of the Vardar River (V0) in the village of Vrutok (see Table 2). Differences in the kinetic activity of the EROD enzyme were noticed in the liver and the gills, whereas with regard to the other enzyme, i.e., the B(a)PMO, differences could be noticed in all three examined organs.

For each parameter, CAPITAL letters show the differences between the fish from the control groups A0 and V0, for each enzyme and organ, whereas the lower-case letters show the differences between the fish in natural population groups V0 and V1 for each enzyme and organ separately.

Compared to the control group from the aquaculture (A0), there were differences in the kinetic activity of EROD and B(a)PMO in the three organs in three groups of fish treated with various insecticides (A1-1, A1-2 and A1-3) (Table 3). Differences in the kinetic activity of

Table 1. **Results of the activity of the EROD and B(a)PMO enzymes in organs within all the examined groups**

[a] EROD (pmol resorufin/min/mg protein)				[a] B(a)PMO (3-OH benzo(a)pyrene/min/mg protein)		
	Organs			Organs		
Groups	Liver	Kidney	Gill	Liver	Kidney	Gill
A0	2.41 (0.23)*^{A}	0.30 (0.013)*^{B}	0.038 (0.006)*^{C}	1.60 (0.22)*^{A}	0.11 (0.008)*^{B}	0.03 (0.005)*^{B}
A1-1	141.71 (7.59)^{A}	0.96 (0.021)^{B}	0.246 (0.009)^{B}	71.76 (4.36)^{A}	0.41 (0.012)^{B}	0.14 (0.010)^{B}
A1-2	132.43 (7.16)^{A}	0.80 (0.035)^{B}	0.207 (0.011)^{B}	39.19 (4.36)^{A}	0.50 (0.013)^{B}	0.13 (0.005)^{B}
A1-3	104.34 (8.86)^{A}	0.60 (0.012)^{B}	0.187 (0.008)^{B}	26.90 (1.74)^{A}	0.31 (0.011)^{B}	0.11 (0.005)^{B}
A2-1	16.18 (1.81)^{A}	0.40 (0.018)^{B}	0.088 (0.006)^{B}	10.23 (1.50)^{A}	0.22 (0.021)^{B}	0.06 (0.006)^{B}
A2-2	14.85 (1.92)^{A}	0.30 (0.017)^{B}	0.085 (0.006)^{B}	8.40 (0.70)^{A}	0.13 (0.009)^{B}	0.05 (0.007)^{B}
A2-3	17.79 (1.14)^{A}	0.22 (0.014)^{B}	0.051 (0.009)^{B}	6.13 (0.53)^{A}	0.10 (0.007)^{B}	0.04 (0.004)^{B}
V0	3.41 (0.53)^{A}	0.32 (0.034)^{B}	0.075 (0.009)^{B}	2.10 (0.33)^{A}	0.18 (0.010)^{B}	0.04 (0.005)^{B}
V1	45.89 (6.00)^{A}	0.47 (0.051)^{B}	0.172 (0.010)^{B}	22.56 (2.46)^{A}	0.27 (0.008)^{B}	0.06 (0.007)^{B}

* Average values (standard deviation).

Table 2. **Results obtained from the activity of the EROD and B(a)PMO enzymes in target organs of the fish from the aquaculture and the fish caught in two locations in the Vardar River**

[a] EROD (pmol resorufin/min/mg protein)				[a] B(a)PMO (3-OH benzo(a)pyrene/min/mg protein)		
Organs				Organs		
Groups	Liver	Kidney	Gill	Liver	Kidney	Gill
A0	2.41 (0.23)^{*A}	0.30 (0.013)*	0.038 (0.006)^{*A}	1.60 (0.22)^{*A}	0.11 (0.008)^{*A}	0.03 (0.005)^{*A}
V0	3.41 (0.53)^{aB}	0.32 (0.034)^{a}	0.075 (0.009)^{aB}	2.10 (0.33)^{aB}	0.18 (0.010)^{aB}	0.04 (0.005)^{aB}
V1	45.89 (6.00)^{b}	0.47 (0.051)^{b}	0.172 (0.010)^{b}	22.56 (2.46)^{b}	0.27 (0.008)^{b}	0.06 (0.007)^{b}

* Average values (standard deviation).

Table 3. **Results of the activity of EROD and B(a)PMO enzymes in the organs from the control group from the aquaculture, the experimental group, and the polluted location group from the Vardar River**

[a] EROD (pmol resorufin/min/mg protein)				[a] B(a)PMO (3-OH benzo(a)pyrene/min/mg protein)		
Organs				Organs		
Groups	Liver	Kidney	Gill	Liver	Kidney	Gill
A0	2.41 (0.23)^{*a}	0.30 (0.013)^{*a}	0.038 (0.006)^{*a}	1.60 (0.22)^{*a}	0.11 (0.008)^{*a}	0.03 (0.005)^{*a}
A1-1	141.71 (7.59)^{bB}	0.96 (0.021)^{bB}	0.246 (0.009)^{bB}	71.76 (4.36)^{bB}	0.41 (0.012)^{bB}	0.14 (0.010)^{bB}
A1-2	132.43 (7.16)^{cC}	0.80 (0.035)^{cC}	0.207 (0.011)^{cC}	39.19 (4.36)^{cC}	0.50 (0.013)^{cC}	0.13 (0.005)^{cC}
A1-3	104.34 (8.86)^{dD}	0.60 (0.012)^{dD}	0.187 (0.008)^{dD}	26.90 (1.74)^{dD}	0.31 (0.011)^{dD}	0.11 (0.005)^{dD}
V1	45.89 (6.00)^{A}	0.47 (0.051)^{A}	0.172 (0.010)^{A}	22.56 (2.46)^{A}	0.27 (0.008)^{A}	0.06 (0.007)^{A}

* Average values (standard deviation).

these enzymes were also noticed in target organs of fish from the polluted location in the Vardar River (V1) and those from the experimental groups treated with different insecticides.

For each parameter, lower-case letters show the differences between fish from the control group A0 and experimental groups A1, A2, and A3, treated with different insecticides, for each enzyme and organ separately, whereas CAPITAL letters show the differences between the fish from the polluted location in the Vardar River and those from the experimental groups A1, A2, and A3 treated with different insecticides, for each enzyme and organ separately.

The data obtained from the determination of EROD and B(a)PMO enzyme activity, between the bodies within the control group from aquaculture A0 and groups from the experiment treated with various herbicides A2-1, A2-2 and A2-3, as well as between fish from polluted locality Vardar River with groups from experiment are shown in Table 4.

For each parameter, lower-case letters show the differences between fish from the control group A0 and experimental groups A1, A2, and A3, treated with different herbicides, for each enzyme and organ separately, whereas CAPITAL letters show the differences between the fish from the polluted location in the Vardar River and those from the experimental groups A1, A2, and A3, treated with different herbicides, for each enzyme and organ separately.

Based on the acquired data as shown in Table 5, it was ascertained that between the control group of fish from the aquaculture (A0) and those caught in the river near the village of Vrutok, there were differences in their body weight (BW), total length (TL), the liver somatic index (LSI) and the kidney somatic index (KSI). There were differences in the liver somatic index (LSI) between the fish caught in the Vardar River, in the village of Vrutok (V0) and those caught in the same river at the exit of the town of Veles (V1).

Table 4. **Results of the activity of EROD and B(a)PMO enzymes in the organs from the control group from the aquaculture, the experimental group and the polluted location group from the Vardar River**

[a] EROD (pmol resorufin/min/mg protein)				[a] B(a)PMO (3-OH benzo(a)pyrene/min/mg protein)		
Organs				Organs		
Groups	Liver	Kidney	Gill	Liver	Kidney	Gill
A0	2.41 (0.23)*[a]	0.30 (0.013)*[a]	0.038 (0.006)*[a]	1.60 (0.22)*[a]	0.11 (0.008)*[a]	0.03 (0.005)*[a]
A2-1	16.18 (1.81)[bB]	0.40 (0.018)[bB]	0.088 (0.006)[bB]	10.23 (1.50)[bB]	0.22 (0.021)[bB]	0.06 (0.006)[bA]
A2-2	14.85 (1.92)[bB]	0.30 (0.017)[aC]	0.085 (0.006)[bB]	8.40 (0.70)[cC]	0.13 (0.009)[cC]	0.05 (0.007)[cB]
A2-3	17.79 (1.14)[cB]	0.22 (0.014)[cD]	0.051 (0.009)[cC]	6.13 (0.53)[dD]	0.10 (0.007)[aD]	0.04 (0.004)[cB]
V1	45.89 (6.00)[A]	0.47 (0.051)[A]	0.172 (0.010)[A]	22.56 (2.46)[A]	0.27 (0.008)[A]	0.06 (0.007)[A]

* Average values (standard deviation).

Table 5. **Morphometric data on the fish from the control group from the aquaculture and those from the natural population caught in two locations in the Vardar River**

Morphometric parameters							
Groups	BW (g)	TL (cm)	CF (%)	LSI (%)	SSI (%)	GSI (%)	KSI (%)
A0	76.67 (0.23)[a]	19.35 (0.06)[a]	1.29 (0.07)	0.63 (0.26)	0.06 (0.10)[a]	1.02 (0.63)	0.20 (0.26)[a]
V0	148.60 (0.46)[b]	23.26 (0.13)[b]	1.35 (0.08)	1.22 (0.52)[A]	0.05 (0.09)[b]	1.52 (0.44)	0.37 (0.16)[b]
V1	158.57 (0.54)	23.34 (0.17)	1.44 (0.24)	0.86 (0.25)[B]	0.05 (0.22)	3.13 (0.94)	0.39 (0.32)

* Average values (standard deviation).

For each group, lower-case letters show the differences between the fish from control groups A0 and V0, whereas CAPITAL letters show the differences between the fish from natural population groups V0 and V1.

There were differences in their body weight (BW), total length (TL), and the kidney somatic index (KSI). There were also differences in the liver somatic index (LSI) and the spleen somatic index (SSI) between the fish from the polluted location in the Vardar River and those treated with insecticides and herbicides (Table 6).

For each group, lower-case letters show the differences between the fish from the control group (A0), those from the experimental group treated with insecticides (A1), and those treated with herbicides (A2). CAPITAL letters show the differences between the fish from the polluted location in the Vardar River, the group of fish treated with insecticides (A1), and those treated with herbicides (A2)

The frequency of histological changes in the liver parenchyma in the fish caught in the Vardar River at the exit of the town of Veles (V1) was 54.3%; the frequency of toxicopathologic

Table 6. **Morphometric data on the fish from the control group from the aquaculture, from the groups of fish treated with insecticides and herbicides and those from the polluted location in the Vardar River**

Morphometric parameters							
Groups	BW (g)	TL (cm)	CF (%)	LSI (%)	SSI (%)	GSI (%)	KSI (%)
A0	76.67 (0.23)[a]	19.35 (0.06)[a]	1.29 (0.07)	0.63 (0.26)	0.06 (0.10)	1.02 (0.63)	0.20 (0.26)[a]
A1	136.20 (0.28)[b]	23.32 (0.08)[b]	1.33 (0.11)	0.66 (0.36)[JA]	0.12 (1.50)[B]	2.54 (1.17)	0.53 (0.30)[b]
A2	161.75 (0.19)[b]	24.78 (0.06)[b]	1.30 (0.12)	1.09 (0.49)[B]	0.05 (0.29)[AB]	2.65 (0.34)	0.42 (0.63)[ab]
V1	158.57 (0.54)	23.34 (0.17)	1.44 (0.24)	0.86 (0.25)[AB]	0.05 (0.22)[A]	3.13 (0.94)	0.39 (0.32)

* Average values (standard deviation).

changes in the liver, kidneys, and gonads of the fish treated with insecticides (A1) was 98.5%, whereas in the fish treated with herbicides (A2) it was 95.3% (see Table 7).

During histological analysis of the liver parenchyma of the fish caught in the Vardar River at the exit of the town of Veles, we could notice that most of the examined fragments contained

Table 7. **The frequency (%) of histological and toxic pathologic changes in the fish from the natural population of the Vardar River at the exit of the town of Veles (V1) and the fish from the aquaculture treated with insecticides (A1) and herbicides (A2)**

Frequency (%) of histological and toxicopathologic changes in the examined groups											
Groups	**No changes**	**MA**	**HV**	**HE**	**KG**	**HM**	**HS**	**PI**	**ZS**	**GD**	**FD**
V1	45.7%	20.5%	4%	–	2%	12.5%	9.3%	6	–	–	–
A1	2.5%	22.7%	5%	8%	6.5%	15.6%	11.3%	–	7.5%	10.4%	11.5%
A2	4.7%	21.9%	4%	7.3%	6.2%	15.4%	11.2%	–	7.2%	10.2%	11.9%

Abbreviations: MA – macrophage aggregation; HV – hepatocyte vacuolation; HE– haemolysis; KG – coagulation; HM – bleeding; HS – hemosiderin; PI – parasite infection; ZS – sinusoidal expansion; GD – glomerular degeneration; FD – folicular degeneration.

a large number of aggregated macrophages (MA), cytoplasmic vacuolation of hepatocytes, bleeding, parasite and bacterial infection, coagulation, and hemosiderin (Fig. 2 B, C, D).

In the majority of the analyzed fragments of the liver parenchyma, hepatocyte vacuolation, haemolysis, coagulation, and sinusoidal expansion were noticed (Figure 3E, 3F). A degeneration of glomeruli and extension of the space between the basic membrane and epithelial cells of kidney canals were noticed in most of the fragments taken from kidney tissues (Fig. 3H), whereas a degeneration of follicles, evoplasm fractures, and separation of the follicular epithelium were noticed in the largest part of ovarian tissue fragments (Fig. 3 J).

DISCUSSION

Histology offers a powerful tool in the study of detailed structures of different tissues in fish. It is used for sex verification, identification of development stages, presence of parasites, tumour diagnosis, and other abnormalities and changes in the arrangement of tissue layers, including the digestive tract (Monsefil et al., 2010). 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 (Doherty, 2010). Activity of the enzymes included in the biotransformation of the xenobiotics is determined in order to assess the levels of exposure of vertebrates against xenobiotics. The determination of mixed-function oxidase (MFO) activity in fish was suggested as a quantitative measure for the demonstration of the presence of xenobiotics in aquatic ecosystems a long time ago (Kurelec et al., 1977).

Measurement of MFO activity and other parameters in fish ascertains once again the great importance of fish as bioindicators (Reichenbach-Klinke, 1974).

The power of the EROD inductiveness differs in different fish species. The fish from the Cyprinidae family are quite often used as a model for exploring the activity of the EROD enzyme (Hongyan et al., 2003). In our study, the applied concentration of 1 mg/L of insecticides such as Chlomorel D, Karate Zeon and King in *Barbus peloponnesius* resulted in CYP450 inductiveness.

It has been proven that the applied concentrations of herbicides Sencor 70 WG (0.25 and 2.5 mg/l) and Successor 600 (0.06, 0.22 and

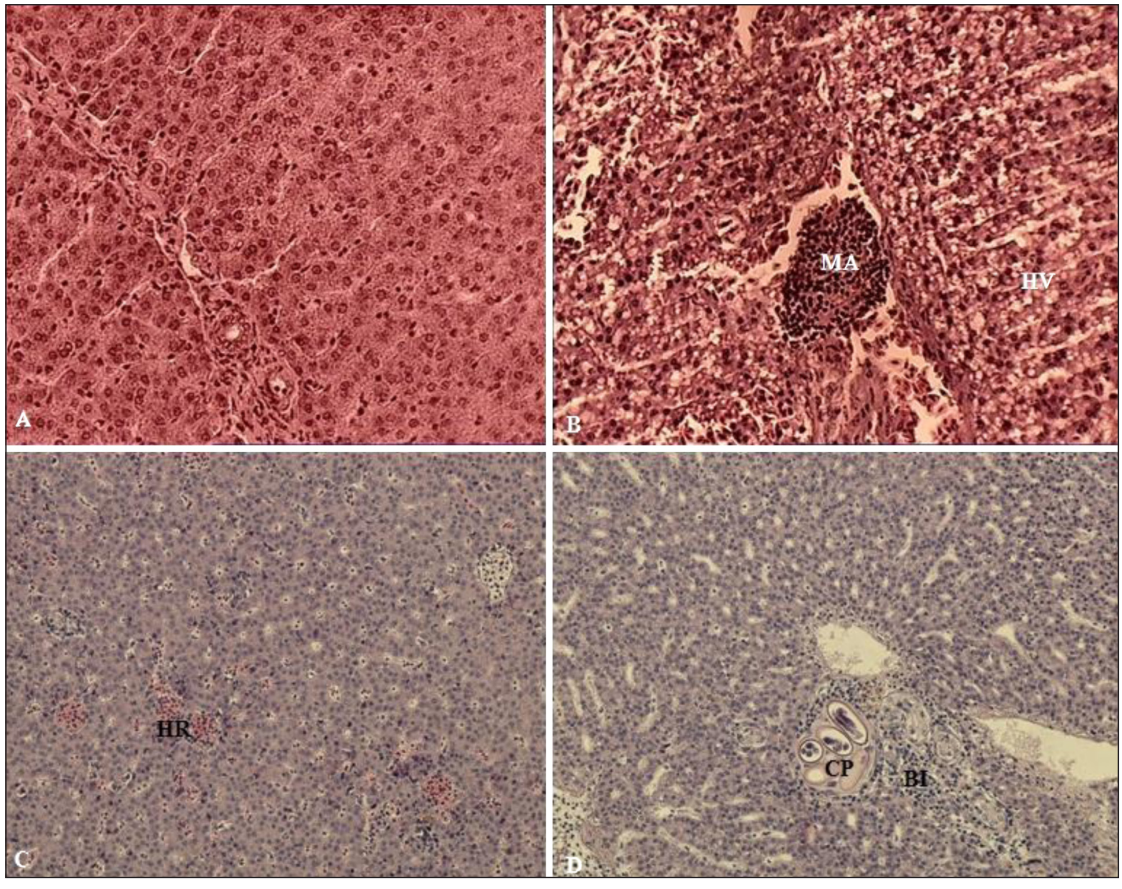

Fig. 2. Photomicrography of fragments taken from the liver of *Barbus peloponnesius* obtained by an optic microscope photo camera. Figure 2A – the liver of the fish from the control group from the natural population of the Vardar River, near the village of Vrutok (V0), H&E colouring, 400X magnification. Figures 2B, 2C, 2D – the liver of the fish from the polluted location in the Vardar River (V1), H&E colouring, magnification: B-400X, C&D-100X (MA – macrophage aggregation; HV – hepatocyte vacuolation, HR – bleeding, CP – parasite cysts, BI – bacterial infection)

0.60 mg/l) did not cause any effects on CYP450 (Haluzovà et al., 2011). Our results are in line with this finding. It was found that the application of 1 mg/L herbicides Linurex 50 SC, Stomp 330 E and Devrinol, did not cause any effects on CYP450. Results from our detoxification tests reveal that applied insecticides in the experimental fish caused an increased activity of the EROD and B(a)PMO enzymes compared to the control fish, unlike the herbicides, which did not cause any significant increase in the activity of these two enzymes. Results obtained from the measurement of the activity of EROD and B(a)PMO enzymes in fish treated with herbicides (A2-1, A2-2 and A2-3) prove the current results obtained by other authors having examined other fish species. It means that the inductiveness of EROD and B(a)PMO enzymes is not caused by herbicides, most probably because of the presence of inhibitors and suppressors in the synthesis of proteins P-450 in these pollutants.

Based on in-field research, we found that there was a significant increase in the activity of EROD and B(a)PMO enzymes in the microsomal liver fraction of the *Cyprinus carpio* caught in the Vardar River at the exit of the city of Skopje (Beadini et al., 2001). The same happened with *Barbus peloponnesius* caught in the Vardar River at the exit of the town of Veles (Sejfullai L,

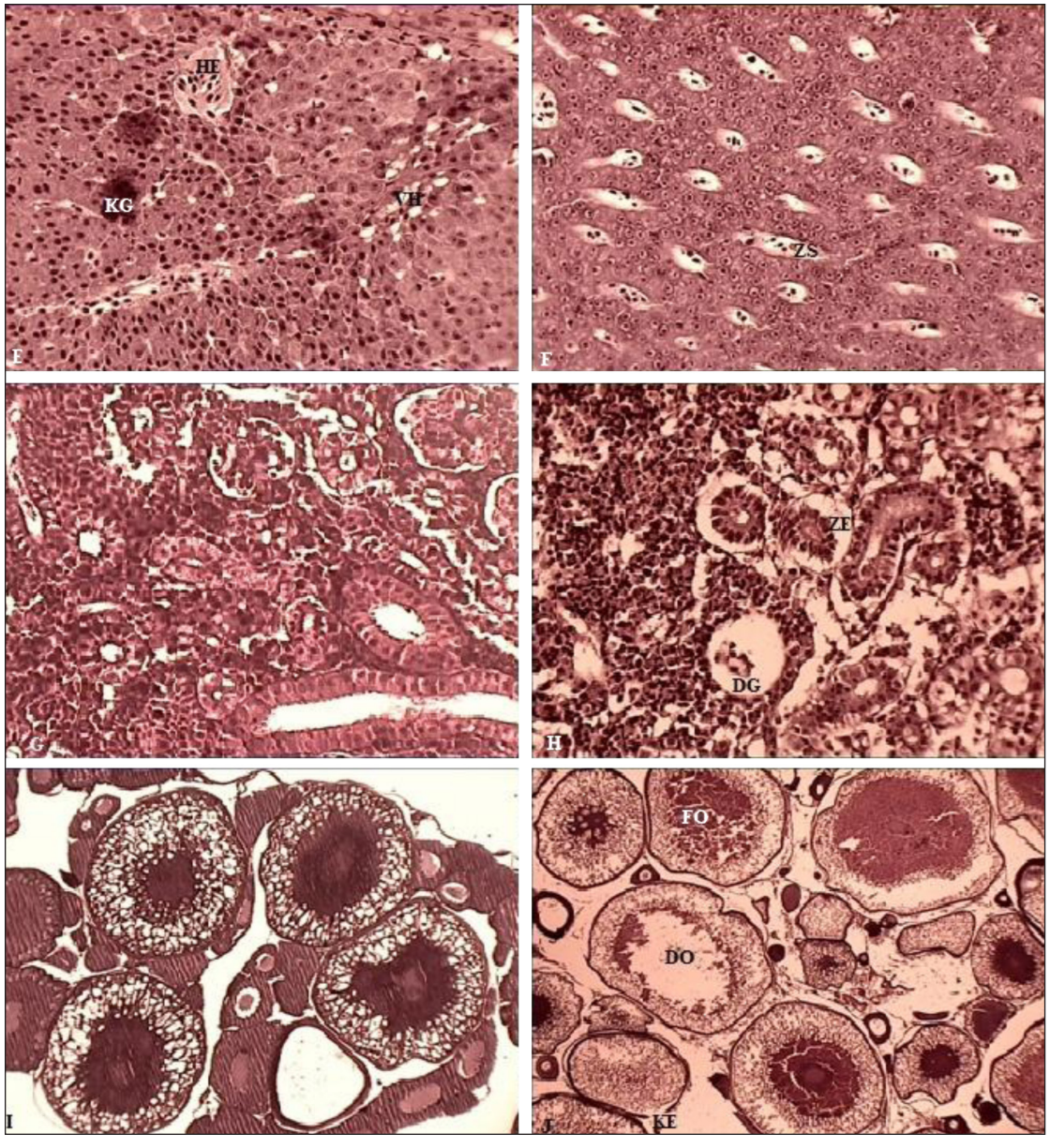

Fig. 3. Photomicrography of *Barbus peloponnesius* with toxicopathic changes caused by the intraperitoneal application of insecticides and herbicides. Figure 3E – the liver with toxicopathic changes caused by the application of the insecticide Chlomorel D (KG – coagulation, HE – haemolysis, VH – hepatocyte vacuolation). Figure 3F – the liver with toxicopathic changes caused by the application of the herbicide Stomp 330 E (ZS – sinusoide expansion). G – a kidney from the control group from the aquaculture (A0). Figure 3H – a kidney with toxicopathic changes caused by the application of the insecticide Karate Zeon (DG – degeneration of glomeruli, ZE – extension of the space between the basic membrane and epithelial cells of kidney canals). Figure 3I – the ovary from the control group from the aquaculture (A0). Figure 3J – the ovary with toxicopathic changes caused by the application of the herbicide Linurex 50 SC (KE – separation of the follicular epithelium, DO – ooplasm degeneration, FO – ooplasm fractures)

2003). The results we obtained from the measurement of the activity of the EROD and B(a) PMO enzymes in fish caught in the Vardar River at the exit of the town of Veles are in line with the above-mentioned findings. They revealed a significant increase in the kinetic activity of

the EROD and B(a)PMO enzymes. Histology deals with the detailed examination of the different tissue structures in fish. It also enables the definition of the sex, identification of development phases and the presence of parasites as well as the diagnose of tumours, other anomalies and changes caused in the organization of tissue layers, including the digestive tract (Monsefil et al., 2010).

Based on our research, it was found that during the intoxication with this insecticide in liver hepatocytes induced necrosis and apoptosis. In kidneys, the renal tube disintegration occurred, along with glomeruli reduction, bleeding, necrosis, and karyopyknosis. In the ovaries of this fish, damages in eggs, cytoplasm traction, destruction of follicles, and contraction of the caryoplasm were noticed.

Our results are in correlation with other authors' results in terms of the toxicopathic changes in fish tissues caused by the treatment with insecticides and herbicides. It was found that the applied concentration (2 μg/L) of the insecticides and herbicides in *Barbus pelopponeius* for a given period caused a series of changes in the liver, kidneys and gonads. Hemolysis, coagulation, cytoplasmic vacuolation of the hepatocytes, and expansion of sinusoids were among the most emphatic changes in the liver parenchyma (Fig. 3 E and F). As regards the kidney tissue of this particular fish, the following were recorded: degeneration of glomeruli and expansion of the space between the basic membrane and the epithelial layer of kidney canals (Fig. 3 H). Separation of the follicular epithelium, ooplasm fractures and degeneration of follicles were noticed in the ovarian tissues (Fig. 3 J). The frequency of toxic pathologic lesions in the fish treated with insecticides was 98.5%, whereas in those treated with herbicides, it was 95.3% (Table 7).

During our in-field research, in a large number of analyzed fragments from the liver parenchyma of the fish caught in the Vardar River, at the exit of the town of Veles, a great number of aggregated macrophages (MA), cytoplasmic vacuolation of hepatocytes, presence of hemosiderine, bleeding, parasite and bacterial infections were noticed (Beadini et al., 2015). The results we obtained speak in favor of these data; namely, based on the histological analysis of the fragments of the liver parenchyma of the fish caught in the same location of the Vardar River, we could notice a large number of aggregated macrophags, cytoplasmic vacuolation of hepatocytes, bleeding, parasite infection (cysts and nematode larvae) and bacterial infections (Fig. 2 B, C and D). The frequency of histological changes in the parenchyma of the fish liver from the polluted location in the Vardar River was 54.3%.

The ascertained concentrations of terbutryn or symazine in the environment affected the biometric parameters of the zebrafish (*Danio rerio*). The symazine concentrations of 2 or 4 μg/L caused an increase of HIS in the experimental fish compared to the controlling ones. Our results are in correlation with the ones obtained by other authors as regards fish treated with chemical substances. The treatment of fish with insecticides and herbicides resulted with changes in biometric parameters, compared to the fish from the control group from the aquaculture (A0). The treatment for a given period of time with sublethal dose of 2 μg/L of the toxicant, resulted in increase of HIS and KSI in the fish of the A1 group treated with insecticides and A2 group treated with herbicides (Table 6). In addition, there was an increase of HIS and KSI in the fish from the polluted location in the Vardar River at the exit of the town of Veles (V1), compared to the control group (Table 5).

As regards the biomonitoring of the pollutants, it would be better if it were applied intraperitoneally, because in this way we could avoid the influence of those pollutants on the reduction of the activity of the EROD and B(a)PMO enzymes; as a consequence, the results obtained from the biomonitoring of the xenobiotics could be unrealistically low. The measurement of the activity of the EROD and B(a)PMO enzymes in microsomal fractions of the organs of *Barbus peloponnesius* and histological changes showed that biochemical and histologic parameters are potential biomarkers for the detection of xenobiotics in aquatic ecosystems, and especially rivers, whereas *Barbus peloponnesius* proved to be a very good bio-indicator. It was also found that

examined organs, and especially the liver, have a potential for the transformation of xenobiotics and inductiveness of CYP1A and can be used as biomarkers for the monitoring of xenobiotics. This was also verified by measuring the inductiveness of EROD and B(a)PMO enzymes in the fish treated with chemical substances.

On the basis of the information presented in different studies, there is no doubt that the application of histopathological changes as a biomarker of organism exposure to contaminated sites offers important information that can contribute to environmental monitoring programs designed for surveillance, hazard assessment, or regulatory compliance. One of the most important benefits of the use of histopathological biomarkers in environmental screening is the possibility of examining specific target organs including liver, kidney, and gills.

During this study we came to ascertain that the exposure of *Barbus peloponnesius* against applied chemicals resulted in causing stress to the organism, followed by changes in the kinetic activity of the two examined enzymes and histological and histopathologic changes in the liver, kidneys and gonads. All of the applied insecticides caused significant changes in the kinetic activity of the EROD and B(a)PMO enzymes, though they did not cause inductiveness.

The results obtained from the detoxification tests show that these substances can be considered as having great amounts of toxicity with regard to fish and a long-term exposure to them could affect their general condition as well as biochemical and histological biomarkers.

Both enzymes (EROD and B(a)PMO) in the target organs of *Barbus peloponnesius* of the Vadar River can serve as potential biochemical indicators (biomarkers) for monitoring the level of xenobiotics in water, which could be toxic for fish and other organisms in this aquatic ecosystem. Further research and exploration are needed in order to explain that the inductiveness of enzymes caused by pesticides is a result of molecular mechanisms. High concentrations of pollutants and the presence of specific pollutants inhibit cytochrome P450 by hindering the inductiveness of CYP1A.

Therefore, we consider that future studies should focus not only on the effects caused by pesticides themselves, but rather on the interaction of pesticides with other pollutants, because the aquatic environment can be polluted by different toxic substances.

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BARBUS PELEPONNESIUS AKVAKULTŪROS IR NATŪRALIOS VARDARO UPĖS (MAKEDONIJOS RESPUBLIKA) POPULIACIJOS FERMENTO LYGINAMOJI IR HISTOLOGINĖ ANALIZĖ

Santrauka

Fermentiniai biologiniai žymenys jautriai reaguoja į aplinkos pokyčius – kinta jų aktyvumas. *Barbus peloponnesius* yra potencialus biologinis rodiklis pokyčių, kuriuos gali sukelti įvairūs aplinkos teršalai. Akvakultūros žuvų grupių organų, paveiktų pusiau mirtina insekticidų doze (1 mg/l), rezultatai rodo reikšmingą EROD fermento, taip pat B(a)PMO kinetinio aktyvumo padidėjimą, palyginti su kontroline grupe. Užterštos Vardaro upės žuvyse taip pat pastebėtas didesnis fermentinis aktyvumas (EROD ir B(a)PMO). Insekticidų ir herbicidų subletalinėmis koncentracijomis (2 μg/L) paveiktų žuvų kepenų parenchimoje, inkstų audinyje ir kiaušidėse nustatyta hemolizė, sutrikęs krešėjimas ir citoplazminė hepatocitų vakuolizacija, išsiplėtę sinusoidai, glomerulų degeneracija, inkstų kanalėlių, erdvės tarp bazinių membranų ir inkstų kanalėlių epitelio ląstelių išsiplėtimas, ikrų įtrūkimas ir degeneracija, folikulinio epitelio pažeidimai.

Raktažodžiai: EROD, B(a)PMO, CYP 450, CYP1A, Vardaro upė, *Barbus peloponnesius*