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The impact of multiple stressors on the biomarkers response in gills and liver of freshwater breams during different seasons

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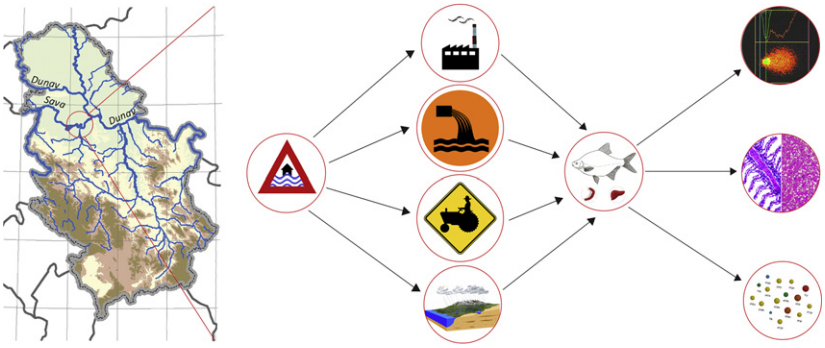
HIGHLIGHTS

- The impact of multiple stressors was studied by biomarkers response in fish.
- DNA damage, histopathology and metal accumulation were studied in gills and liver.
- DNA damage was higher in gills, changes in histopathology were prevalent in liver.
- The variation of the biomarkers response depended on the sampling season.
- Use of multibiomarker approach is essential for confident water quality assessment.

GRAPHICAL ABSTRACT



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ABSTRACT

Biomarkers attract increasing attention in environmental studies, as a tool for detection of exposure and effects of pollution, from both natural and anthropogenic sources. This study aims to assess the impact of multiple stressors during distinctive seasons, covering also extreme hydrological events (extensive flooding in the mid May 2014), on different levels of biological organization in the liver and gills of three closely related freshwater breams. Our previous study on DNA damage in blood cells of these specimens showed increased DNA damage in June 2014, one month after the flooding event. As a continuation of that research, the present study was conducted. As a biomarker of exposure DNA damage was measured by applying the alkaline comet assay, while histopathological alterations were monitored as a biomarker of effect. Additionally, concentrations of metals and metalloids in gills, liver and muscle were assessed. Sampling of fish tissues was performed in 2014, during winter (January and February), spring (March and early June) and summer (late June, July and August). Significant seasonal difference in DNA damage was observed for both tissues. During spring and summer the level of DNA damage in gills was significantly higher when compared to the liver. Histopathological analyses showed higher frequency of alterations in gills during spring, and in liver during summer, but without a significant seasonal difference. Gills had the highest concentration of metals and metalloids during the spring and summer, and liver during

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winter. Muscle was the least affected tissue during all three seasons. This study highlighted the importance of the multiple biomarker approach and the use of different fish tissues in assessment of surface water pollution.

1. Introduction

Monitoring of the surface water quality based solely on the analysis of a limited number of xenobiotics, cannot be considered as reliable due to the presence of a large number of pollutants. Generally, a mixture of low concentrations of different compounds is the main reason for many adverse effects in aquatic animals (Dizer et al., 2002; Navarro-Ortega et al., 2015). Beside toxic, these agents can exert genotoxic effects, inducing damage in the DNA molecule, which, if not repaired, could lead to mutations and alterations in cells, tissues, organism, whole population and the ecosystem. Biomarkers attract increasing attention in environmental studies, as a tool for detection of exposure and effects of pollution (Deutschmann et al., 2016). Assessment of alterations on different levels of biological organisation, such as subcellular (genotoxic effects) and cellular/tissue level (histopathological effects) may lead to significant improvement of knowledge about the overall response of aquatic organisms to pollution (Pacheco and Santos, 2002).

The surface water quality at any given moment reflects the impact of both anthropogenic and natural sources of pollution. Besides, extreme hydrological events which are related to a particular season, such as water scarcity and flooding, may further impair the already vulnerable state of freshwater bodies (Aborgiba et al., 2016). It is well known that many areas in Balkans and South-Eastern Europe are characterized by the seasonality in respect to regular shift of high and low water level. In such conditions, variation of both water quality and quantity, may compromise availability of water for human consumption and energy production. Therefore, monitoring of seasonal changes in surface water quality represents an imperative for the assessment of pollution impact from both natural and anthropogenic sources.

Among the chemicals released into the environment, metals and metalloids take a special place due to their toxicity, genotoxicity, persistence, bioaccumulation and biomagnification in the food chain (Skorić et al., 2012; Štrbac et al., 2015; Sunjog et al., 2016). When found in cells, metals and metalloids have a potential to produce reactive oxygen species (ROS) which may interact with proteins, lipids and the DNA molecule of all exposed organisms, which could be seen as a histopathological change (Fatima et al., 2015). Assessment of metals and metalloids concentration in different fish tissues is extremely important as fish represent an important part of the human diet (Sevcikova et al., 2011).

The integrity of the DNA can be impaired by genotoxic agents. DNA damage in the form of adduct formation, the absence of methylation or chain breaks, has been proposed as a useful parameter for assessing the genotoxic potential of pollutants in the environment (Everaarts et al., 1993). The magnitude of DNA strand breaks can be used as a sensitive indicator of genotoxicity and thus, as a biomarker of exposure. Single cell gel electrophoresis (SCGE) or comet assay, is a relatively simple, versatile, rapid, sensitive and extensively used tool to assess DNA damage on the cellular level (Jha, 2008; Frenzilli et al., 2009).

Histopathological biomarkers are considered as valuable indicators of the status of aquatic organisms and depict the effect of exposure to a series of anthropogenic pollutants (Van der Oost et al., 2003). Histopathological alterations of organs represent endogenous and exogenous time-integrated effects on the organism, derived from changes in the lower levels of biological organisation (Teh et al., 1997). Histopathological methods can be used in conjunction with other ecotoxicological indicators as an early warning system for the survival of species and ecosystem protection (Fatima et al., 2014).

When examining surface water quality the *in situ* approach and the use of feral fish are particularly valuable, since they provide a realistic insight into the consequences of exposure and bioavailability of a number of xenobiotics (Deutschmann et al., 2016). Fish may be exposed to harmful substances through water, sediment and food. Different fish tissues may accumulate metals, metalloids and other xenobiotics at different rates, depending on the biochemical properties of pollutants (Rashed, 2001; Jezierska and Witeska, 2006). In environmental studies, gills are used as they represent the first organ in direct contact with water and waterborne pollutants, while the liver is used due to its role in the intermediary metabolism and as a major organ for metabolic breakdown of xenobiotics (Fasulo et al., 2010).

Common bream, *Abramis brama* (Linnaeus, 1758), white bream, *Blicca bjoerkna* (Linnaeus, 1758) and white-eye bream, *Ballerus sapa* (Pallas, 1814) are three closely related, benthivorous cyprinids in European waters, native for the Sava River. These bream species usually inhabit slow flowing lowland rivers and still waters, near the sandy and silty bottom, feeding on worms, mollusks, and larvae of insects (Simonović, 2001). By feeding on bottom dwelling organisms, benthivorous fish disturb sediment and ingest sediment particles. This process leads to the resuspension of the sediment and the release of substances from the sediment into the water column (Breukelaar et al., 1994). Moreover, in our previous study, we marked freshwater bream as a potentially good bioindicator organism in ecogenotoxicity studies (Kostić et al., 2016). These bream species are chosen as they are rated as stationary (Bartel et al., 2007) and are highly abundant in the Sava River (Jovičić et al., 2014). Regarding the similarity and high relatedness of three bream species, this study was conducted collectively on bream species (formerly Abramis sp.). It is expected that the health status of highly related fish species which are exposed to the same environmental conditions will be affected in the same way (Van Dyk et al., 2009).

The Sava River is one of the most important water courses in Serbia in terms of water supply for the Serbian capital Belgrade, but also the cities of Šabac, Obrenovac, Sremska Mitrovica, etc. On the other hand the degree of sewage treatment is very low or absent, both for urban and industrial wastewater (Kolarević et al., 2016). Besides, the majority of agricultural activities are settled near the river bank. This may lead to both microbiological pollution originating from untreated urban wastewater and agricultural runoff (Kirschner et al., 2009), and chemical pollution originating from untreated urban wastewater, agricultural runoff and industrial wastewater (Loos et al., 2009). In our previous study, we have characterized anthropogenic waste as the main source of faecal pollution in the Sava River, indicating the presence of untreated urban wastewaters (Vrzel et al., 2016).

This research was conducted in January 2014, with the aim to monitor seasonal variations in water quality on the site exposed to multiple pollution sources. In the middle of May 2014, extensive flooding (100-year flood) occurred at the examined site. In our previous study, in which DNA damage in blood cells of these specimens was monitored, we observed an increased DNA damage in June 2014, one month after the flooding event (Aborgiba et al., 2016). Having that in mind, this research aims to assess the impact of multiple stressors during different seasons on different levels of biological organization, subcellular (genotoxic effect) and cellular/tissue level (histopathological effects) in the liver and gills of these bream species. Additionally, concentration of metals and metalloids in gills, liver and muscle were assessed. Data obtained at the level of each individual were combined to obtain seasonal data about the effect of multiple stressors on freshwater breams.

2. Material and methods

2.1. Sampling site

The sampling site Duboko is situated on the Sava River, 23 km upstream from the Belgrade city center and from the confluence with the Danube River (Fig. 1). The site is exposed to untreated wastewater from the town of Obrenovac (circa 70,000 inhabitants), intensive agricultural activity, but also the cumulative pollution of different type from upstream sections. The largest thermal power plant in Serbia "Nikola Tesla - TENT A" (producing 1650 MW power) with the belonging ash field is situated approximately 15 km upstream of the sampling site, which additionally provides potential stress to aquatic ecosystems within investigated stretch of the Sava River.

2.2. Physical, chemical and microbiological analyses of water

Basic physical (pH, temperature, oxygen concentration, electrical conductivity) and chemical (NO_2, NO_3^-, NH_4^+, PO_4^{3-}) parameters were measured on site by using portable instruments (HI 9126 pH/ORP Meter, HI 9146 Dissolved Oxygen Meter, WTW-Photolab spectrophotometer, Hanna Instruments, USA; Digital Einstich-Thermometer, TFA Dostmann GmbH & Co, Germany; EC59/EC60, EC/TDS/TEMP meter, Martini Instruments, USA). Microbiological indicators of faecal pollution, total coliforms (TC) and *Escherichia coli* (EC), were assessed by using a most probable number approach (MPN) by Colilert 18 (IDEXX, Germany). Enterococci (EF) concentrations were also determined by the MPN using MUD/SF microtiter plates (BIORAD, Austria) and standard method according to ISO 7899-1:1998. Presumptive *Clostridium perfringens* (CP) numbers were determined by using membrane filtration and incubation on TSC (Tryptose Sulphite Cycloserine) media, according to the standard ISO 14189:2013.

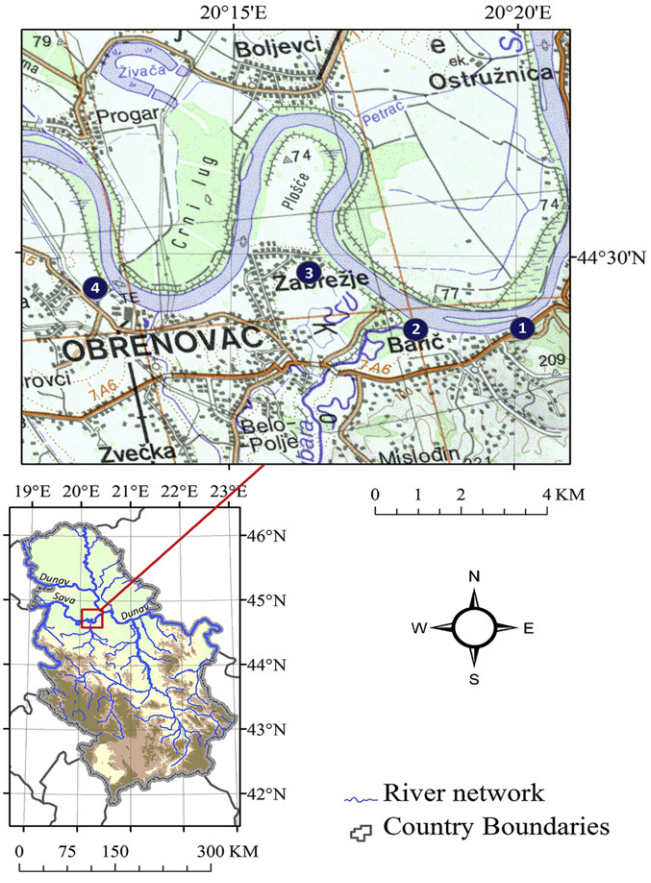

Fig. 1. Sampling locality Duboko: 1. Sampling site, 2. The mouth of the Kolubara River, 3. Town of Obrenovac, 4. TENT A with belonging ash field.

Data on the water level variation during sampling months was provided by the Republic Hydrometeorological Service of Serbia and given as a Supplementary material (Fig. S1).

2.3. Sampling of fish tissue

Sampling of breams for comet assay and histopathological analyses, was done during winter (January and February- 10 specimens), spring (March and early June- 10 specimens), and summer (late June, July, and August- 12 specimens), for a total of 32 specimens. Analysis of metals and metalloids was performed only on fish sampled in February (for winter- 5 specimens), early June (for spring- 5 specimens) and in July and August (for summer- 6 specimens), in total 16 specimens. Experiment was approved by the Animal Ethics Committee of the Institute for Multidisciplinary Research, University of Belgrade. Fish were caught by angling with a rod. Prior to dissection specimens were anesthetized using clove oil (50 μL/L) and measured for total length (TL in cm) and weight (W in g). The condition factor, which is often used to express the overall state of the fish, was calculated based on the length-weight relationship, according to the formula (Bagenal and Tesch, 1978):

$$CF = W \times L^{-3}.$$

2.4. Comet assay analysis

Cell suspensions of liver and gills were prepared based on the protocol used in our previous study (Kostić et al., 2016). Briefly, tissue samples were cut into small pieces in Hank's Balanced Saline Solution (HBSS) and exposed to trypsin (final concentration 0.05%, 10 min, room temperature). Suspensions were then centrifuged and diluted in HBSS to obtain approximately 50,000 cells/mL. Cell viability was assessed by differential acridine orange/ethidium bromide (AO/EB) staining (Squier and Cohen, 2001). Samples showing >70% cell viability were further processed for the comet assay.

The alkaline comet assay was performed as described in our previous study (Kostić et al., 2016). Briefly, slides precoated with two layers of the 1% NMP (normal melting point) agarose were covered with 70 μL of 1% LMP (low melting point) agarose mixed with 30 μL of cell suspensions. For each tissue, per specimen, one slide was prepared. Slides were immersed into freshly made, ice-cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 10) and held on 4 °C for 16-18 h. After lysis, slides were covered with cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) to enable denaturation (20 min, 4 °C) and electrophoresis (0.75 V/cm, 300 mA, 20 min, 4 °C). The slides were neutralized (0.4 M Tris, pH 7.5, 15 min, 4 °C) and fixed in cold methanol (15 min, 4 °C). Slides were stained using acridine orange and examined under a fluorescence microscope. A total of 250 comets for each tissue were randomly scored and analyzed by using the Comet IV computer software (Perceptive Instruments, UK). Tail intensity, % of DNA in the comet tail (TI) was used to express the DNA damage level.

2.5. Histopathological analysis of liver and gill tissue

After fixation in 4% formaldehyde, tissues were processed in an automatic tissue processor Leica TP 1020 (Leica, Germany), dehydrated in a graded ethanol series, cleared with xylene and embedded in paraffin. Paraffin blocks were serially sectioned at 5 μm thickness on a microtome Leica SM 2000R (Leica, Germany), dewaxed and stained using a combination of haematoxylin and eosin (HE). All slides were blinded and assessed by two histopathologists (B.R. and V.P.). Micrographs were taken by using a Leica DM LS microscope equipped with a Leica DFC 310 FX camera (Leica, Germany).

The type and the extent of histological alterations were described by using a method developed by Bernet et al. (1999). Histopathological

Table 1
Overview of the number of sampled fish during three seasons, with their total length (TL) and weight (W), and condition factor (CF).

	Winter				Spring				Summer					
	01/21/2014		02/19/2014		03/20/2014		06/09/2014		06/27/2014		07/16/2014		08/20/2014	
	TL [cm]	W [g]	TL [cm]	W [g]	TL [cm]	W [g]	TL [cm]	W [g]	TL [cm]	W [g]	TL [cm]	W [g]	TL [cm]	W [g]
1	21.5	133	22	139	20.5	69	21	90	16	57	22.5	97	22.5	103
2	21.5	83	21.5	124	18	72	24	97	18	61	16	41	22.2	98
3	21	98	21	107	18.5	63	20	76	15	41	19.5	73	18	61
4	20.5	83	19.9	89	20	65	18	63			18.5	58	14.5	31
5	21	76	19.5	91	19	60	18	76					15	38
Total number of sampled specimens per season	10				10				12					
CF [Mean ± SD]	1.11 ± 0.19				0.97 ± 0.19				1.03 ± 0.17					

(HP) alterations are classified into four reaction patterns: circulatory, regressive, progressive, and inflammatory. Pathological conditions of blood and tissue fluid flow are classified within circulatory disturbances. Regressive alterations are defined as processes which lead to reduction in function or loss of an organ, while progressive alterations are defined as increased activity of cells or tissues. Inflammatory changes are often coupled with processes belonging to other reaction patterns and it is difficult to attribute them to one single reaction pattern. Since two inflammatory changes (leukocyte infiltration and presence of granulomas) were found in the present study, we decided to introduce `Inflammatory alterations` category as suggested in Bernet et al. (1999), and not incorporate those changes to other patterns. Each alteration was evaluated by using the importance factor ranging from 1 (alteration is of minimal importance) to 3 (alteration is of marked importance) in order to point out the relevance and pathological significance of a lesion. Importance factor of each HP alteration is showed in the Table 2. A score value ranging from 0 (absence of alteration) to 6 (severe occurrence of alteration) is specified in accordance to the degree and extent of a specific alteration. To obtain value of the organ index, importance factor and score value were multiplied, according to the following formulas (Bernet et al., 1999):

(a) Histopathological index of the organ:

$$\mathbf{I_{org}} = \Sigma_{rp}\Sigma_{alt}\left(a_{orgrpalt} \times w_{org\ rp\ alt}\right)$$

(b) Reaction index of the organ ($I_{org\ rp}$):

$$\mathbf{I_{org\ rp}} = \Sigma_{alt}\left(a_{org\ rp\ alt} \times w_{org\ rp\ alt}\right)$$

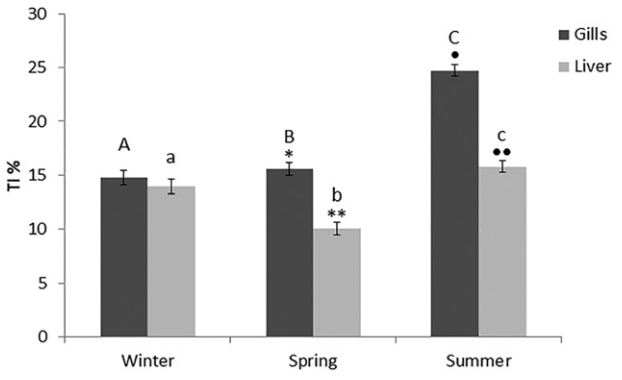

Fig. 2. DNA damage in gills and liver cells expressed using Tail Intensity - TI (%) (mean ± SE) during winter (10 specimens), spring (10 specimens) and summer (12 specimens). *, **; ●, ●● - observed differences in DNA damage between gills and liver within a particular season; ^{A,B,C}- values marked with different letters are significantly different between seasons in gill tissue; ^{a,b,c}- values marked with different letters are significantly different between different seasons in liver tissue; statistical significance for three seasons was tested using Mann-Whitney *U* test with corrected p level ($p < 0.017$).

(c) The sum of the indices for both organs-Total index for the individual fish:

$$\mathbf{I_T} = \Sigma_{org}\Sigma_{rp}\Sigma_{alt}\left(a_{org\ rp\ alt} \times w_{org\ rp\ alt}\right)$$

where *org* represents the organ (I_L-liver; I_G-gills); *rp*- reaction pattern, *alt*- the alteration, *a*- score value, and *w*- importance factor.

Frequency of lesions (FQ) was expressed by dividing the number of fish on which the change was found with the number of fish analyzed:

$$\mathbf{FQ}(\%) = \mathbf{N_{lesion}} \times N_{total}{}^{-1} \times 100.$$

2.6. Analysis of metals and metalloids in liver and gill tissue

For analysis of metals and metalloids samples of liver, gills and muscle were immediately washed with distilled water and stored on −18 °C until analysis. All samples were dried by Freeze Dryers Rotational-Vacuum-Concentrator (GAMMA 1–16 LSC, Germany). Sample portions between 0.2 and 0.5 g (dry weight) were digested in a microwave digester (Speedwave™ MWS-3+; Berg of Products + Instruments GmbH, Germany) using 6 mL of 65% HNO_3 and 4 mL of 30% H_2O_2 (Merck suprapure, USA) at a food temperature program (100–170 °C). After cooling to room temperature, digested samples were diluted with distilled water to a total volume of 25 mL. Analysis was performed by inductively-coupled plasma optical emission spectrometry (ICP-OES, Spectro Genesis EOP II, Spectro Analytical Instruments DmbH, Germany), and included assessment of concentrations of 16 elements (Al, As, B, Ba, Cd, Co, Cr, Cu, Fe, Li, Mn, Mo, Ni, Pb, Sr and Zn). The following wavelength lines of the ICP-OES analysis were used: Al 396.152 nm, As 234.948 nm, B 249.773 nm, Ba 230.424 nm, Cd 226.502 nm, Co 228.616 nm, Cr 267.716 nm, Cu 324.754 nm, Fe 259.941 nm, Li 460.289 nm, Mn 257.611 nm, Mo 202.095 nm, Ni 231.604 nm, Pb 220.353 nm, Sr 460.733 nm, and Zn 213.856 nm. Concentrations of elements in all tissues are expressed as μg/g dry weight (dw).

To compare the total metal content in different tissues and through different seasons metal pollution index (MPI) was calculated according to equation MPI = (cf1 x cf2 x cf3 x…cfn) $^{1/n}$, where cfn = concentration of the metal n in the sample (Usero et al., 1997).

2.7. Statistical analyses

Statistical analyses of the results were performed by using the Statistica 7.0 Software (StatSoft, Inc.) and SPSS 20.0 (Inc., Chicago, IL, USA). Normality of data distribution was tested by using the Kolmogorov-Smirnov test. In cases where data followed normal distribution, homogeneity of variance was tested by Levene's test, significant differences were tested using One-way ANOVA and the post-hoc Tamhane's T2 test and Dunett's T3 test, since these tests tolerate the lack of homogeneity. For all tests significance was tested for $p < 0.05$. In cases where the data did not follow normal distribution, differences

Table 2
Histopathological alterations (with importance factor in brackets) in gills and liver during examined period, with presented Frequency of lesions (FQ), NF- no frequency. Values are presented as mean ± SE for each season (winter- 10 specimens, spring- 10 specimens, summer- 12 specimens). [a, b] - values marked with different letters are significantly different during different seasons (One-way ANOVA with post-hoc Tamhane's T2 test, $p < 0.05$; only significant differences are shown).

	Histopathological changes	Winter		Spring		Summer	
	Gills	HP indices	FQ	HP indices	FQ	HP indices	FQ
Circulatory alterations	Hyperaemia [1]	2.8 ± 0.6	80%	3.6 ± 0.6	100%	4.4 ± 0.6	83%
	Telangiectasia [1]	0.0 ± 0.0	NF	0.1 ± 0.1	20%	0.1 ± 0.1	17%
	Aneurism [1]	0.0 ± 0.0	NF	0.4 ± 0.4	10%	0.2 ± 0.2	8%
	Intercellular edema [1]	0.2 ± 0.2	10%	0.8 ± 0.4	30%	1.2 ± 0.4	42%
Progressive alterations	Hypertrophy of epithelial cells [1]	0.2 ± 0.2	10%	0.8 ± 0.3	40%	0.2 ± 0.2	8%
	Hyperplasia of epithelial cells [2]	0.5 ± 0.2	60%	0.9 ± 0.3	70%	0.4 ± 0.2	42%
	Hyperplasia of goblet cells [1]	1.4 ± 0.4	60%	0.8 ± 0.4	30%	0.6 ± 0.4	17%
	Complete lamellar fusions [2]	0.0 ± 0.0	NF	0.3 ± 0.2	20%	0.0 ± 0.0	NF
Regressive alterations	Epithelial lifting [1]	1.8 ± 0.4	80%	2.0 ± 0.5	70%	2.4 ± 0.7	58%
	Architectural and structural alterations [1]	1.2 ± 0.3	60%	2.4 ± 0.7	70%	1.8 ± 0.5	58%
	Presence of goblet cells in secondary lamellae [1]	0.0 ± 0.0	NF	0.6 ± 0.3	30%	2.0 ± 0.8	33%
	Necrosis [3]	0.4 ± 0.3	30%	0.6 ± 0.4	30%	0.4 ± 0.1	50%
	Liver						
Circulatory alterations	Sinusoidal congestion [1]	2.2 ± 0.6[a]	70%	2.8 ± 0.7[ab]	70%	5.0 ± 0.5[b]	100%
	Stasis [1]	2.2 ± 0.6[a]	70%	2.0 ± 0.7[a]	60%	4.7 ± 0.4[b]	100%
Regressive alterations	Vacuolation of hepatocytes [2]	1.8 ± 0.8	40%	1.4 ± 0.7	40%	0.2 ± 0.2	8%
	Necrosis [3]	1.0 ± 0.4	40%	0.6 ± 0.4	20%	1.0 ± 0.3	50%
	Fibrosis of periportal and portal areas [2]	0.4 ± 0.3	20%	0.8 ± 0.3	40%	1.2 ± 0.5	42%
Inflammatory alterations	Leukocyte infiltration [2]	1.8 ± 0.5[a]	70%	1.4 ± 0.4[a]	60%	4.2 ± 0.6[b]	92%
	Presence of granuloma [2]	0.8 ± 0.3	40%	0.4 ± 0.3	20%	0.5 ± 0.3	25%
Progressive alterations	Haemorrhage [1]	0.2 ± 0.2	10%	0.2 ± 0.2	10%	0.0 ± 0.0	NF

were tested using Mann-Whitney *U* test, and significance level was corrected in accordance with the number of overall comparisons. Correlation analysis between groups of histological alterations in gills was performed by using the Pearson correlation with significance level of $p < 0.05$.

2.8. Basal level of DNA damage

Since it was impossible to find reference (unpolluted) site, with low anthropogenic impact in the Serbian part of the Sava River we provided basal DNA damage level assessed in our laboratory for chub *Squalius cephalus* obtained on the Uvac River "Zlatar" reservoir (protected natural area) (Sunjog et al., 2014). This information is added so we could get insight on the variation of DNA damage assessed in breams during different seasons and their sensitivity as experimental organisms. Values expressed using TI % were 4.20 ± 1.07 for liver and 4.44 ± 1.59 for gills. Additionally breams belong to the same subfamily Leuciscinae as chub.

3. Results

3.1. Condition of fish

The condition of the fish was worst during spring, followed by summer and winter, however, no significant seasonal difference in condition was observed.

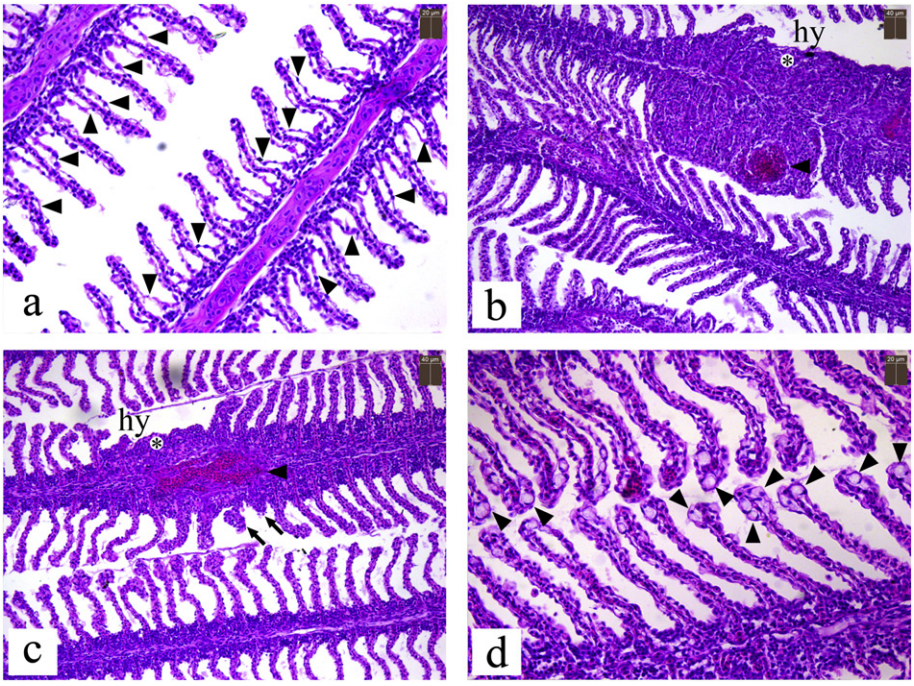

Fig. 3. Histopathological alterations in gills: a. epithelial lifting (arrowheads) (HE ×200); b. hyperplasia (hy) of epithelial cells leading to complete lamellar fusions, with rupture of blood vessel forming hematoma (arrowhead) (HE ×200); c. hyperplasia of epithelial cells (hy), shortening of secondary lamellae (arrows), stasis in the central venous sinus (arrowhead) (HE ×200); d. presence of goblet cells in secondary lamellae (arrowheads) (HE ×400).

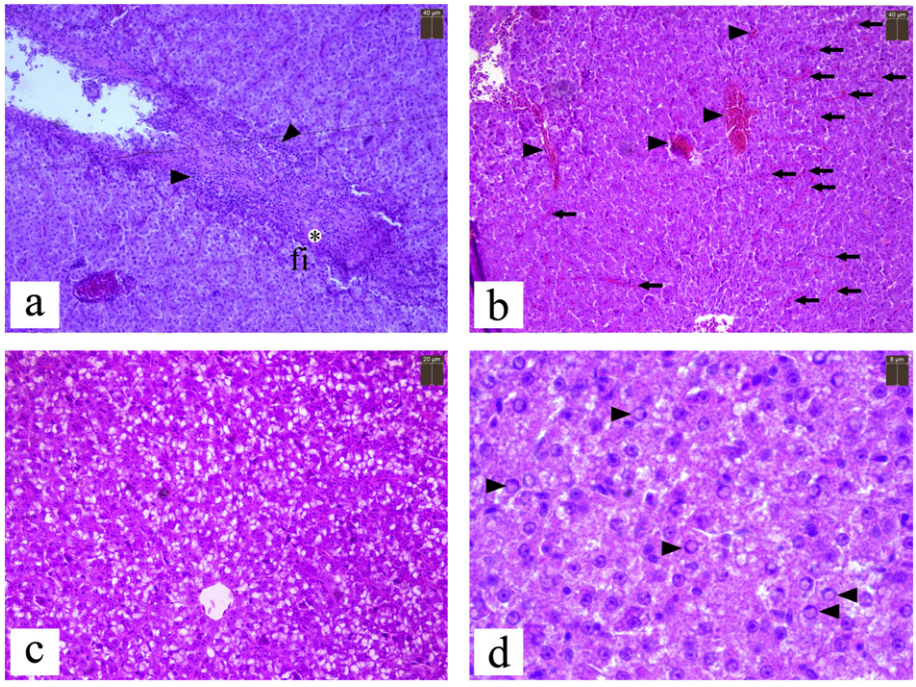

Fig. 4. Histopathological alterations in the liver: a. leukocyte infiltration into liver parenchyma and especially around blood vessels (arrowheads); note extensive fibrosis (fi) of blood vessels (HE ×200); b. congestion of sinusoids (arrows) and presence of stasis inside the blood vessels (arrowheads) (HE ×200); c. vacuolation of hepatocytes (HE ×400); d. vacuolation of nuclei in hepatocytes (arrowheads) (HE ×1000).

An overview of the number of specimens, their TL, W and condition are shown in Table 1.

3.2. Physical, chemical and microbiological analyses of water

Results on basic physical, chemical and microbiological parameters are given in the Supplementary Table S1. For physical and chemical parameters, both monthly and derived average values for each season are given in this table. For microbiological data classification of the water quality based on faecal indicators was provided according to Kirschner et al. (2009).

3.3. Comet assay analysis

The DNA damage level in gills and liver cells was expressed by using the Tail Intensity- TI (%) as a mean ± SE (Fig. 2). A significant seasonal difference in DNA damage level of both gills and liver was observed for all three seasons. Both tissues had the highest level of DNA damage during summer. Gills had the lowest level of DNA damage in winter and liver in spring. The level of DNA damage in gills was significantly higher than in liver, during spring and summer, while during winter difference in DNA damage between gills and liver was not observed.

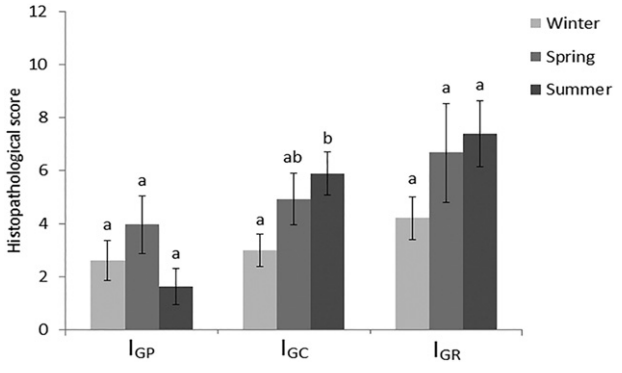

Fig. 5. Extent of progressive (I_{GP}), circulatory (I_{GC}), and regressive (I_{GR}) changes in gills presented as mean ± SE, during winter (10 specimens), spring (10 specimens) and summer (12 specimens). [a,b] -values marked with different letters are significantly different between seasons (One-way ANOVA with post-hoc Tamhane's T2 test, $p < 0.05$).

3.4. Histopathological analysis of gills and liver tissue

3.4.1. Specific histopathological alterations

Detailed overview of specific histopathological alterations assessed in gills and liver tissue during the study period, together with marked seasonal differences for each alteration is given in the Table 2. Additionally, frequency of each lesion- FQ (%) is presented in the Table 2.

In general, branchial apparatus was not extensively damaged in fish caught at the study location. The most frequent alterations in gills were hyperaemia and epithelial lifting, both belonging to the group of easily reversible alterations with mean scores above 2 (out of a maximum 6). Architectural and structural alterations of epithelium and supportive tissue, and presence of goblet cells in secondary gill lamellae had histopathological scores between 0 and 2. Other alterations were scarce,

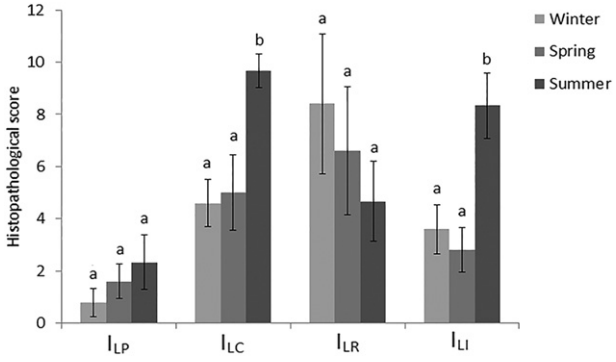

Fig. 6. Extent of progressive (I_{LP}), circulatory (I_{LC}), regressive (I_{LR}) and inflammatory (I_{LI}) changes in liver presented as mean ± SE, during winter (10 specimens), spring (10 specimens) and summer (12 specimens). [a,b] -values marked with different letters are significantly different between seasons (One-way ANOVA with post-hoc Tamhane's T2 test, $p < 0.05$).

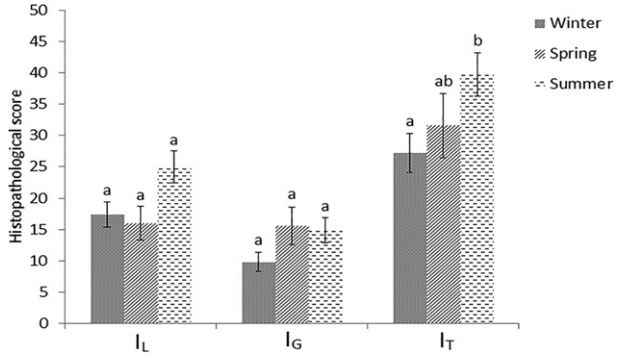

Fig. 7. Liver (I_L), gills (I_G) and total histopathological index (I_T) presented as mean ± SE, during winter (10 specimens), spring (10 specimens) and summer (12 specimens). ^{a,b} -values marked with different letters are significantly different between seasons (One-way ANOVA with post-hoc Tamhane's T2 test, $p < 0.05$).

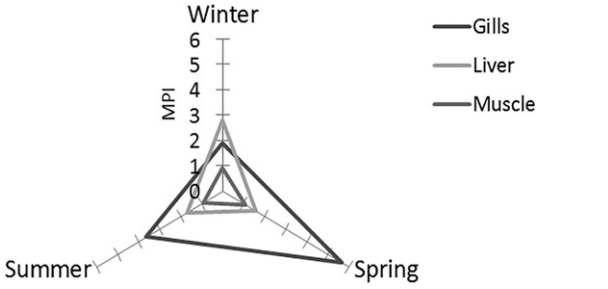

Fig. 8. Metal pollution index of gills, liver and muscle tissue during three examined seasons (5 specimens per season were analyzed).

especially irreversible changes, such as complete lamellar fusions or necrosis of the tissue, which had low scores (below 1). None of the individual alterations in gills did show significant seasonal variation. Some of the gill alterations observed in this study are presented in the Fig. 3.

In liver, alteration with the highest mean histopathological score was sinusoidal congestion, with higher scores in the summer compared to the winter ($p < 0.05$). This change was frequently accompanied with stasis of blood and infiltration of leukocytes. Two latter alterations also had significantly higher scores in summer comparing to both winter and spring ($p < 0.05$). Other alterations had lower scores, but necrosis in liver, during winter and summer periods, had a mean value of 1, which is more than focal extent in the liver. Some of the observed liver alterations in this study are presented in the Fig. 4.

3.4.2. Categorisation of histopathological alterations in gills and liver

In gill tissue most pronounced alterations in all three seasons belonged to the category of regressive changes (I_{GR}), following circulatory (I_{GC}) and progressive disturbances (I_{GP}). Interestingly, significant correlation was observed between regressive and circulatory alterations in gills ($r = 0.5472$, $p = 0.0018$). A significant seasonal difference within the alteration category was observed only within circulatory alterations between winter and summer (Fig. 5).

In liver tissue, circulatory (I_{LC}) and inflammatory (I_{LI}) disturbances dominated during the summer with significant differences in comparison to both winter and spring. During winter and spring the most prevalent in the liver were the regressive changes (I_{LR}) without significant seasonal differences. Progressive alterations were the least frequent hepatic lesions, without significant seasonal variations (Fig. 6).

Overall, greater presence of alterations in gills was visible during spring and in liver during summer, without significant seasonal differences, as shown by tissue histopathological (I_G and I_L) index. A total

Table 3
Concentration of metals and metalloids in muscle, liver and gills; data are presented as mean ± SD (16 specimens were analyzed in total). ^{a,b} -values marked with different letters are significantly different between seasons in a particular tissue (One-way ANOVA with post-hoc Tamhane's T2 test, $p < 0.05$). ^{A,B,C} -values marked with different letters are significantly different between tissues in a particular season (One-way ANOVA with post-hoc Dunett's T3 test, $p < 0.05$); ND- non detected; $^{*/**}$ Value obtained in one specimen/Value obtained in two specimens.

		Muscle	Liver	Gills
Al	Winter	$14.26 \pm 14.94^{A\,a}$	$9.09 \pm 10.61^{A\,a}$	$11.52 \pm 7.06^{A\,a}$
μg/g	Spring	$19.62 \pm 18.88^{A\,a}$	$42.32 \pm 77.3\ 1^{A\,a}$	$233.95 \pm 171.8\ 7^{A\,a}$
	Summer	$7.20 \pm 4.27^{A\,a}$	$36.79 \pm 69.91^{A\,a}$	$47.67 \pm 64.40^{A\,a}$
As	Winter	0.35 ± 0.32	2.32 ± 2.43^{a}	0.52^{*}
μg/g	Spring	0.42^{*}	1.21 ± 1.73^{a}	1.54^{*}
	Summer	0.04^{*}	1.10 ± 0.48^{a}	0.03^{*}
Cr	Winter	$0.20 \pm 0.16^{A\,a}$	$0.22 \pm 0.11^{A\,a}$	$0.64 \pm 0.24^{B\,a}$
μg/g	Spring	$1.72 \pm 3.34^{AB\,a}$	$0.23 \pm 0.18^{A\,a}$	$1.44 \pm 0.3\ 4^{B\,b}$
	Summer	$0.29 \pm 0.23^{A\,a}$	$0.22 \pm 0.11^{A\,a}$	$0.97 \pm 0.16^{B\,ab}$
Cu	Winter	$1.05 \pm 0.67^{A\,a}$	$19.18 \pm 15.83^{A\,a}$	$0.31 \pm 0.44^{A\,a}$
μg/g	Spring	$0.83 \pm 0.31^{A\,a}$	$17.63 \pm 3.39^{B\,a}$	$15.12 \pm 32.78^{AB\,ab}$
	Summer	$0.55 \pm 0.23^{A\,a}$	$19.38 \pm 3.61^{B\,a}$	$1.22 \pm 0.52^{A\,b}$
Fe	Winter	$13.64 \pm 4.20^{A\,a}$	$225.89 \pm 198.85^{AB\,a}$	$148.42 \pm 54.63^{B\,a}$
μg/g	Spring	$16.57 \pm 12.95^{A\,a}$	$223.23 \pm 151.48^{A\,a}$	$331.41 \pm 215.19^{A\,a}$
	Summer	$14.74 \pm 10.13^{A\,a}$	$231.03 \pm 95.03^{B\,a}$	$204.50 \pm 175.42^{AB\,a}$
Mn	Winter	$0.80 \pm 0.21^{A\,a}$	$4.90 \pm 1.11^{B\,a}$	$13.63 \pm 6.30^{B\,a}$
μg/g	Spring	$2.70 \pm 1.15^{A\,ab}$	$6.34 \pm 2.04^{B\,a}$	$81.22 \pm 35.18^{C\,b}$
	Summer	$4.29 \pm 0.80^{A\,b}$	$6.64 \pm 1.30^{B\,a}$	$92.27 \pm 13.67^{C\,b}$
Mo	Winter	$0.21 \pm 0.19^{A\,a}$	0.28 ± 0.15	$1.65 \pm 1.49^{A\,a}$
μg/g	Spring	$0.33 \pm 0.13^{A\,a}$	$0.56 \pm 0.57^{**}$	$2.91 \pm 0.85^{B\,a}$
	Summer	$0.40 \pm 0.18^{A\,a}$	0.60^{*}	$2.57 \pm 0.43^{B\,a}$
Pb	Winter	0.11^{*}	0.53 ± 0.25^{a}	ND
μg/g	Spring	0.06 ± 0.05^{a}	0.30 ± 0.21^{a}	1.07^{*}
	Summer	0.07 ± 0.04^{a}	0.36 ± 0.23^{a}	0.28^{*}
Sr	Winter	$1.52 \pm 0.50^{A\,a}$	$0.38 \pm 0.14^{B\,a}$	$63.17 \pm 31.57^{C\,a}$
μg/g	Spring	$2.19 \pm 1.06^{A\,ab}$	$0.30 \pm 0.11^{B\,a}$	$86.05 \pm 38.57^{C\,a}$
	Summer	$3.04 \pm 1.03^{A\,b}$	$0.49 \pm 0.18^{B\,a}$	$75.41 \pm 5.53^{C\,a}$
Zn	Winter	$31.09 \pm 6.92^{A\,a}$	$55.20 \pm 24.52^{A\,a}$	$48.12 \pm 21.33^{A\,a}$
μg/g	Spring	$20.20 \pm 4.91^{A\,a}$	$42.83 \pm 8.50^{B\,a}$	$59.38 \pm 7.98^{C\,a}$
	Summer	$22.17 \pm 6.21^{A\,a}$	$58.08 \pm 14.52^{B\,a}$	$69.04 \pm 5.08^{B\,a}$
Ba	Winter	$2.01 \pm 0.71^{A\,a}$	$0.28 \pm 0.22^{**}$	$21.05 \pm 7.91^{B\,a}$
μg/g	Spring	$1.39 \pm 0.49^{A\,a}$	2.56^{*}	$37.26 \pm 9.81^{B\,ab}$
	Summer	$1.73 \pm 0.49^{A\,a}$	0.33 ± 0.21	$40.70 \pm 5.71^{B\,b}$

histopathological index (I_T) showed gradual increase from winter to summer, with significantly higher level of alterations in summer in comparison to winter (Fig. 7). Although I_L did not show significant seasonal variation it should be taken into consideration that increase of alterations in liver during summer probably contributed in a large degree to significant increase of total histopathological index I_T during summer.

3.5. Analysis of metals and metalloids in gills and liver tissue

Concentration of metals and metalloids during winter, spring and summer in muscle, liver and gill tissue of examined bream specimens is shown in Table 3, together with significant differences between seasons and between different tissues during the same season.

B was below the detection limit during all seasons in all examined tissues. Cd was only detected in liver during summer, in two specimens during spring, and one specimen in winter. Co was only detected in liver, in one specimen in winter and one specimen in spring. Li was only detected in liver in one specimen during spring. Ni was detected in liver and muscle in one specimen during spring, and one specimen in summer. In gills, Ni was detected in one specimen during spring and all specimens during summer. These elements were not taken into consideration for statistical analyses, due to their irregular detection during sampling period.

Regarding Al concentration no significant difference during different seasons and tissues was observed. As dominated in liver, with no significant seasonal variation. Cr concentration was the highest in gills, during winter and summer in comparison to both muscle and liver, and during spring in comparison to liver. In gills significantly higher concentration of Cr was observed in spring in comparison to winter. Cu concentration was significantly higher in liver, during summer in comparison to muscle and gills, and during spring in comparison to muscle. In gills Cu concentration during summer was significantly higher in comparison to winter. Fe concentration in gills was significantly higher during winter, and in liver during summer in comparison to muscle. Significant seasonal variation in Fe concentration was not observed in either tissue. Mn concentration was the highest in gills during spring and summer in comparison to both liver and muscle, while during winter it was higher in comparison to muscle. In gills Mn concentration was the highest during spring and summer in comparison to winter. In muscle Mn concentration was the highest during summer in comparison to winter, while in liver no significant seasonal variation was observed. Mo concentration was the highest in gills in comparison to muscle. Significant seasonal variation of Mo concentration was not observed in either tissue. Pb was dominant in liver, without significant seasonal variation. Sr concentrations were the highest in gills during all three seasons, in comparison to both liver and muscle. In muscle, Sr concentration was significantly higher during summer in comparison to winter. Zn concentration was the highest in gills during spring in comparison to both muscle and liver, and during summer in comparison to muscle. Zn concentration did not vary significantly during different seasons in either tissue. Ba concentration was the highest in gills in comparison to muscle, during all three seasons. In gills, Ba concentration was significantly higher during summer in comparison to winter.

Metal pollution index was calculated in order to normalize and compare the whole metal contamination of different tissues during different seasons at examined site. According to the MPI, gills were under the highest pressure of metal pollution during spring and summer. Liver was under the highest pressure of metal pollution during winter, while the muscle was the least affected tissue during all three seasons (Fig. 8.).

4. Discussion

This study investigate the seasonal variation of DNA damage level, histopathological alterations and concentrations of metals and metalloids in the liver and gills of freshwater breams on the locality exposed to multiple pollution sources. During the entire period of research the electrical conductivity was above 100 μS/cm which is according to Camargo and Martinez (2007) associated with impaired water quality. Also, pH value was above 8 in all sampling seasons, which could be stressful for fish in terms of the increased O_2 diffusion distance (Marchand et al., 2009).

4.1. Analysis of metals and metalloids in gill and liver tissue

The study of Salem et al. (2014) concluded that due to the benthic way of life, gills of the bottom feeding fish are constantly exposed to the metal-containing sediment and therefore it is not surprising that in our study gills were the tissue under the highest pressure of metals. The highest concentration of elements in gills suggests that water could be the main source of pollution (Kraal et al., 1995). Gills showed specificity for the accumulation of Cr, Mn, Mo, Sr and Ba. Cr had significantly higher levels during spring, Ba during summer, while Mn and Mo had significantly higher levels during both spring and summer, in comparison to winter. This increase in spring and summer concentrations could be related to the flooding event, due to the introduction of pollutants from surrounding area and disturbed sediment, but also due to the metabolism changes. According to Regoli (1998) it is expected that metal concentrations in tissues vary depending on the season, due to the change in both environmental input and changes in metabolism. The study of Pereira et al. (2010) associated seasonal variation in metal concentrations in gills with temperature effect on the metal uptake, which is enhanced during summer. Many studies observed similar affinity of gills towards aforementioned elements (Višnjić-Jeftić et al., 2010; Sunjog et al., 2012; Subotić et al., 2013). Interestingly, in these studies liver is marked as a target organ for Mo accumulation, while in our study we observed the highest concentrations of Mo in gills. It is possible that Mo formed a complex with the mucus on the gills surface which prevented its entrance into the body and other organs, which is reported for some metals by Dural et al. (2006). This is supported by the fact that hyperplasia of goblet cells was the highest during winter and gradually decreased towards the summer, while Mo concentration was the lowest during winter (high mucus production) and increased towards summer (low mucus production). In the study of Reid (2002) Mo in conjunction with histopathological alterations in gills such as increased mucus production, increased gas diffusion distance and decreased gill surface area, which could have result in an increase of ventilatory frequency. This respiratory stress may further increase the level of oxidative damage in gills and be the reason of higher DNA damage level in gills observed in this study.

In our study, liver was the main organ for the accumulation of Cu, As and Pb. Cu concentrations in liver did not vary significantly during different seasons. In the study of Farkas et al. (2000) Cu had the highest accumulation rate in livers of three fish species in comparison to gills and muscle, without observed seasonal variation in concentrations. The study of Lenhardt et al. (2012) observed the highest concentration of Cu in the livers of freshwater bream and white bream in comparison to gills, muscle and gonads. Many studies reported that fish liver is one of the major target organs for As accumulation (Sorensen et al., 1979; Farombi et al., 2007; Jarić et al., 2011). According to Bhattacharya and Bhattacharya (2007) As have the potential to induce oxidative stress in the fish liver and alter hematological parameters. Sorensen et al. (1980) showed positive correlation between the levels of arsenic accumulated in the liver of green sunfish and the level of histological damage in that tissue. Pb is marked as a common heavy metal found in the environment, emerging from sources such are urban wastewaters, industrial discharges and agricultural runoff (Olojo et al., 2005). The study of Vinodhini and Narayanan (2008) also reported about Pb preference for accumulation in the fish liver.

Muscle tissue was the least affected during the study period. In this study, Mn and Sr concentrations in muscle were significantly higher during summer in comparison to winter. In environmental studies of

metal accumulation muscle is the tissue of the greatest interest since it is used for human consumption. To compare data obtained in this study to prescribed maximum acceptable concentrations (MAC) according to National Regulation of the Republic of Serbia (Official Gazette of FRY, No 28/2011, 2011), all concentrations obtained in muscle were transformed from dry weight to wet weight (ww). Prescribed MAC values in the fish meat are for Pb: **1.0**, As: **2.0**, Cu: **30.0**, Fe: **30.0** and Zn: **100** mg/kg or μg/g ww. For Pb the following values were obtained: non-detected (ND) during winter, 0.01 μg/g ww during spring and ND during summer; for As: 0.09 μg/g ww during winter, and ND during both spring and summer; for Cu: 0.28 μg/g ww during winter, 0.21 μg/g ww during spring, and 0.09 μg/g ww during summer; for Fe: 3.51 μg/g ww during winter, 4.06 μg/g ww during spring, and 3.35 μg/g ww during summer; and for Zn: 8.08 μg/g ww during winter, 5.05 μg/g ww during spring, and 4.89 μg/g ww during summer. Thus, in our study none of the aforementioned elements did not exceed prescribed MAC values for the fish meat.

Beside monitoring of mercury in fish tissue, that is regulated by the Directive on Priority Substances in the field of Water Policy (EU, 2013), our results imply that it is important to monitor the concentration of other metals and metalloids in tissues of freshwater organisms. Moreover, mentioned regulation (EU, 2013) does not standardize the monitoring of Hg in a sufficient way, thus it is hard to provide comparable datasets over the European continent. In practice, this means that the European regulative on the field of water policy should be constantly developed.

4.2. Comet assay

In this study, we observed significant seasonal differences in DNA damage of both liver and gill cells, with both tissues having the highest DNA damage level during summer. The metabolic rate and physiology of all ectothermic organisms is highly dependent on temperature (Clarke and Johnston, 1999). The study of De Andrade et al. (2004) showed that increased temperature caused an increase in the DNA damage level of blood cells of two fish species, both baseline levels and levels obtained *in vitro* under the treatment with MMS. The same group observed high sensitivity of the comet assay applied *in situ* and seasonal variation in DNA damage of blood cells, with higher levels during warmer seasons. In our previous study on the Danube River, DNA damage in blood, liver and gill cells of freshwater bream was also the highest during summer (Kostić et al., 2016). In the present study, the level of DNA damage in gills was significantly higher in comparison to liver, during spring and summer. Statistical analysis of data from the individual months showed the highest DNA damage in gill cells during early June, and it was significantly higher in comparison to all other sampling months. A possible cause of this incidence could be a withdrawal of water which took place in June, after the flooding event that occurred in the middle of May. It is possible that the water that withdrew from the surrounding agricultural land and thermal power plant ash dump introduced a large amount of the genotoxic substances into the Sava River, which exhibited their genotoxic action in direct contact with gills. According to Polard et al. (2011), the highest concentrations of pesticides in streams near the agricultural area occur during floods, through the surface water runoff. Additionally, floods can have a major impact on sediment disturbance and resuspension of pollutants adsorbed on sediments, promoting the release of pollutants retained in the sediment (Hollert et al., 2000). The study of Brinkmann et al. (2010) showed that genotoxic effects occur after relatively short exposure to resuspended sediments in a simulated flood event. Also, Cr levels were significantly higher in gills during spring (early June), almost a month after flooding occurred. The study of Kumar et al. (2013) pointed out the ability of Cr to increase the levels of reactive oxygen species and induce DNA damage in both peripheral blood and gill cells of common carp (*Cyprinus carpio*). In the study of Velma and Tchounwou (2010), exposure of goldfish (*Carassius auratus*) to Cr resulted in oxidative stress, increased DNA damage and histopathological effects in liver and kidney. Additionally, Mn and Ba concentrations in gills were significantly higher during both spring and summer. Dissolved Mn may reach very high concentrations in natural waters as a result of human activities such as metal mining and industrialization (Morillo and Usero, 2008). In the study of Vieira et al. (2012) Mn caused generalized oxidative stress in different fish organs, with gills being the most sensitive organ. Treatment of rats with Ba via drinking water increased total chromosomal aberrations and micronucleus frequencies in bone-marrow cells (Elwej et al., 2016). This, together with increased temperature could be the main reason of increased DNA damage in gills during the summer season. On the other hand, liver had the highest DNA damage during August, and it was significantly higher compared to other months. This could be explained by a higher metabolic activity of fish during summer, under increased temperature, which was the highest in August. Also it is possible that DNA damage in liver increased in August as a consequence of processing a large amounts of genotoxic substances introduced in water, sediment and biota after flooding event (Pailler et al., 2009).

4.3. Histopathological analysis of gill and liver tissue

Gill filaments and lamellae provide a large surface area which is in direct and constant contact with waterborne pollutants, making gills extremely sensitive to chemicals from water (Bernet et al., 1999; Au, 2004). However, gills respond non-specifically to pollutants and various pollutants may cause similar reactions of the gill tissue. In this study, gill histopathological index (I_G) did not show significant seasonal variations, however it was the lowest during winter, the highest in spring, and slightly decreased in summer. Since gills respond quickly to environmental changes it is possible that higher response during spring is related to the flooding event and introduction of xenobiotics in the water.

During the study period, the most prevalent alterations in gills were regressive changes, followed by circulatory and progressive disturbances. The most pronounced regressive alterations were epithelial lifting, architectural and structural alterations, and presence of goblet cells in secondary lamellae, particularly during spring and summer. Epithelial lifting represents one of the most common alterations in studies of gill histopathology, and is frequently found in metal exposure studies (Pandey et al., 2008). This rapid and reversible change occurs as a response to stress conditions/presence of contamination, resulting in an increased diffusion distance between water and blood. In newly formed conditions of reduced oxygen concentration, blood flow increases which leads to an increase in the level of circulatory alterations (Schlenk et al., 2008; Rašković et al., 2013). Our study confirmed accompaniment of regressive and circulatory alterations in gills, since we observed significant positive correlation between these two groups of alterations. The most pronounced circulatory alteration was hyperemia, representing an increased blood supply due to disturbed gas exchange (Rašković et al., 2010). Nonetheless, Cr had the highest concentration in gills during spring, while Mn and Ba had significantly higher concentrations in gills during both spring and summer. In the study of Alazemi et al. (1996) exposure of fish to Cr induced extensive alteration of the normal architecture of gill lamellae. Cr and Ba caused gill alterations such as aneurism, dilated and clubbed tips, hyperplasia, oedema, curvature, fusion of lamellae, increase of mucous secretion in zebrafish (Rahmani et al., 2016). The most pronounced progressive alteration was hyperplasia of goblet cells which was the highest during winter. According to the Haaparanta et al. (1997) low temperature has been linked to the proliferation and distribution of fish gill mucous cells. The increase in goblet cells production is also considered as a defense mechanism since mucus contains glycoproteins which may arrest toxicants and prevent their entry in the gill epithelium (Peatman et al., 2015). This may be the reason for the reduced response of gills during winter.

Fish liver is responsible for processing and storage of nutrients, synthesis of enzymes, and metabolism of xenobiotics, due to which it usually represents one of the most frequently damaged organs (Wolf and Wolfe, 2005). According to Marchand et al. (2009) in livers of field specimens histopathological alterations could be induced by a large number of pollutants. Also, gill degeneration caused by oxygen deficiency could make an additional pressure on the induction of changes in the fish liver (Younis et al., 2013). In the present study, liver histopathological index (I_L) showed the highest values in summer, although not significant in comparison to winter and spring. As in the case of DNA damage (comet assay) this finding could be prescribed to a higher metabolic rate of fish liver during warm seasons and flooding. The study of Einsporn et al. (2005) reported about the presence of histopathological alterations in liver of flounder (*Platichthys flesus*) even 5 months after a flooding event.

Circulatory and inflammatory alterations in liver were significantly higher during summer, in comparison to winter and spring. The most prevalent circulatory alterations were sinusoidal congestion and stasis, with significantly higher values during summer in comparison to winter, and in the case of stasis in comparison to spring as well. Circulatory disturbances are frequently reported in fish exposed to organic contaminants (Rašković et al., 2013). The study of Kaoud and El-Dahshan (2010) attributed histopathological alterations such as circulatory impairment to the prolonged exposure to heavy metals, such are Pb, Cu, Cd and Hg. In our study liver is marked as a major organ for Pb, Cu and As accumulation. Atamanalp et al. (2008) reported that treatment of rainbow trout (*Oncorhynchus mykiss*) with copper sulfate induced, among other alterations, congestion of blood vessels in the liver. According to Mohamed (2009) stasis of blood could be responsible for cellular degeneration and necrosis in liver. Sinusoidal congestion is blocking blood from the hepatic artery and the interbiliary portal vein, which has to pass through the sinusoids, on its way to the central vein. This incapacity of blood to reach the central vein forces liver to pump blood harder which may cause the stress in liver (Olojo et al., 2005). This could be further corroborated by the presence of fibrosis in periportal and portal areas, frequently found in similar field studies (Poleksić et al., 2010; Rašković et al., 2015). Within inflammatory alterations the most prevalent was leukocyte infiltration with significantly higher values during summer in comparison to both winter and spring. The study of Paolini et al. (2004) reported leukocyte infiltration in chub (*Leuciscus cephalus*) liver from two Italian rivers exposed to sewage wastewater discharge. According to Secombes and Chappell (1996) fish leukocytes are part of non-specific cellular defense, such as phagocytosis and phagocyte killing. The presence of leukocytes clearly indicates an inflammatory reaction and reflects an immunological response to environmental contaminants (Reite, 1998). Beside the affinity of certain elements to accumulate in fish liver significant seasonal difference in their concentrations in this tissue was not observed. Thus, significant increase of liver histopathological index during summer could be attributed to some other chemical and infectious agents, but also to increased metabolism of fish.

5. Conclusions

This study has highlighted the impact of the sampling season and possible significant impact of floods on the variation of the biomarkers response and concentrations of metals and metalloids in the fish tissues. This is reflected in significantly increased DNA damage, a slight increase of histopathological alterations and increased concentrations of metals and metalloids in gills during spring. On the other hand, the response of liver, both in the case of DNA damage and histopathological disturbances was postponed and it was the highest during summer. As expected, gills and liver showed different responses to environmental stress. Gills as the first organ in direct contact with water showed a higher response in terms of DNA damage (molecular level), while the liver as the major organ for processing of xenobiotics both from water and food showed a higher degree of histopathological alterations (tissue/organ level). Without complex assessment of biota response to all field variables, any monitoring program could not be considered appropriate. The use of set (battery) of indicators, as well as examination of different tissues was approved as an effective approach, once again.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.05.273.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

Aborgiba, M., Kostić, J., Kolarević, S., Kračun-Kolarević, M., Elbahi, S., Knežević-Vukčević, J., Lenhardt, M., Paunović, M., Gačić, Z., Vuković-Gačić, B., 2016. Flooding modifies the genotoxic effects of pollution on a worm, a mussel and two fish species from the Sava River. Sci. Total Environ. 540, 358–367.

Alazemi, B.M., Lewis, J.W., Andrews, E.B., 1996. Gill damage in the freshwater fish *Gnathonemus petersii* (family: Mormyridae) exposed to selected pollutants: an ultrastructural study. Environ. Technol. 17 (3), 225–238.

Atamanalp, M., Sisman, T., Geyikoglu, F., Topal, A., 2008. The histopathological effects of copper sulphate on rainbow trout liver (*Oncorhynchus mykiss*). J. Fish. Aquat. Sci. 3, 291–297.

Au, D.W.T., 2004. The application of histo-cytopathological biomarkers in marine pollution monitoring: a review. Mar. Pollut. Bull. 48 (9), 817–834.

Bagenal, T.B., Tesch, F.W., 1978. Age and growth. In: Bagenal, T.B. (Ed.), Methods for Assessment of Fish Production in Fresh Waters. Blackwell Scientific Publications, Oxford, pp. 101–136.

Bartel, R., Wiśniewolski, W., Prus, P., 2007. Impact of the Włocławek dam on migratory fish in the Vistula River. Arch. Pol. Fish. 15 (2), 141–156.

Bernet, D., Schmidt, H., Meier, W., Burkhardt-Holm, P., Wahli, T., 1999. Histopathology in fish: proposal for a protocol to assess aquatic pollution. J. Fish Dis. 22 (1), 25–34.

Bhattacharya, A., Bhattacharya, S., 2007. Induction of oxidative stress by arsenic in *Clarias batrachus*: involvement of peroxisomes. Ecotoxicol. Environ. Saf. 66 (2), 178–187.

Breukelaar, A.W., Lammens, E.H., Breteler, J.G.K., Tatrai, I., 1994. Effects of benthivorous bream (*Abramis brama*) and carp (*Cyprinus carpio*) on sediment resuspension and concentrations of nutrients and chlorophyll a. Freshw. Biol. 32 (1), 113–121.

Brinkmann, M., Hudjetz, S., Cofalla, C., Roger, S., Kammann, U., Giesy, J.P., Hecker, M., Wiseman, S., Zhang, X., Wölz, J., Schüttrumpf, H., Hollert, H., 2010. A combined hydraulic and toxicological approach to assess re-suspended sediments during simulated flood events. Part I–multiple biomarkers in rainbow trout. J. Soils Sediments 10 (7), 1347–1361.

Camargo, M.M., Martinez, C.B., 2007. Histopathology of gills, kidney and liver of a Neotropical fish caged in an urban stream. Neotrop. Ichthyol. 5 (3), 327–336.

Clarke, A., Johnston, N.M., 1999. Scaling of metabolic rate with body mass and temperature in teleost fish. J. Anim. Ecol. 68 (5), 893–905.

De Andrade, V.M., Da Silva, J., Da Silva, F.R., Heuser, V.D., Dias, J.F., Yoneama, M.L., De Freitas, T.R., 2004. Fish as bioindicators to assess the effects of pollution in two southern Brazilian rivers using the comet assay and micronucleus test. Environ. Mol. Mutagen. 44 (5), 459–468.

Deutschmann, B., Kolarević, S., Brack, W., Kaisarević, S., Kostić, J., Kračun-Kolarević, M., Liska, I., Paunović, M., Seiler, T.B., Shao, Y., Sipos, S., Slobodnik, J., Teodorović, I., Vuković-Gačić, B., Hollert, H., 2016. Longitudinal profile of the genotoxic potential of the River Danube on erythrocytes of wild common bleak (*Alburnus alburnus*) assessed using the comet and micronucleus assay. Sci. Total Environ. 573, 1441–1449.

Dizer, H., Wittekindt, E., Fischer, B., Hansen, P.D., 2002. The cytotoxic and genotoxic potential of surface water and wastewater effluents as determined by bioluminescence, umu-assays and selected biomarkers. Chemosphere 46 (2), 225–233.

Dural, M., Lugal Göksu, M.Z., Özak, A.A., Derici, B., 2006. Bioaccumulation of some heavy metals in different tissues of *Dicentrarchus labrax* L, 1758, *Sparus aurata* L, 1758 and *Mugil cephalus* L, 1758 from the Camlik lagoon of the eastern cost of mediterranean (Turkey). Environ. Monit. Assess. 118 (1), 65–74.

Einsporn, S., Broeg, K., Köhler, A., 2005. The Elbe flood 2002—toxic effects of transported contaminants in flatfish and mussels of the Wadden Sea. Mar. Pollut. Bull. 50 (4), 423–429.

Elwej, A., Ben Salah, G., Kallel, C., Fakhfakh, F., Zeghal, N., Ben Amara, I., 2016. Protective effects of pomegranate peel against hematotoxicity, chromosomal aberrations, and genotoxicity induced by barium chloride in adult rats. Pharm. Biol. 54 (6), 964–974.
EU, 2013. Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 Amending Directives 2000/60/EC and 2008/105/EC as Regards Priority Substances in the Field of Water Policy (The European Parliament and the Counil of the European Union).
Everaarts, J.M., Shugart, L.R., Gustin, M.K., Hawkins, W.E., Walker, W.W., 1993. Biological markers in fish: DNA integrity, hematological parameters and liver somatic index. Mar. Environ. Res. 35 (1), 101–107.
Farkas, A., Salanki, J., Varanka, I., 2000. Heavy metal concentrations in fish of Lake Balaton. Lakes Reserv. Res. Manag. 5 (4), 271–279.
Farombi, E.O., Adelowo, O.A., Ajimoko, Y.R., 2007. Biomarkers of oxidative stress and heavy metal levels as indicators of environmental pollution in African cat fish (*Clarias gariepinus*) from Nigeria Ogun River. Int. J. Environ. Res. Public Health 4 (2), 158–165.
Fasulo, S., Marino, S., Mauceri, A., Maisano, M., Giannetto, A., D'Agata, A., Parrino, V., Minutoli, R., De Domenico, E., 2010. A multibiomarker approach in *Coris julis* living in a natural environment. Ecotoxicol. Environ. Saf. 73 (7), 1565–1573.
Fatima, M., Usmani, N., Hossain, M.M., 2014. Heavy metal in aquatic ecosystem emphasizing its effect on tissue bioaccumulation and histopathology: a review. J. Environ. Sci. Technol. 7 (1), 1–15.
Fatima, M., Usmani, N., Firdaus, F., Zafeer, M.F., Ahmad, S., Akhtar, K., Husain, D.S.M., Ahmad, M.H., Anis, E., Hossain, M.M., 2015. *In vivo* induction of antioxidant response and oxidative stress associated with genotoxicity and histopathological alteration in two commercial fish species due to heavy metals exposure in northern India (Kali) river. Comp. Biochem. Physiol. C 176, 17–30.
Frenzilli, G., Nigro, M., Lyons, B.P., 2009. The comet assay for the evaluation of genotoxic impact in aquatic environments. Mutat. Res. Rev. Mutat. Res. 681 (1), 80–92.
Haaparanta, A., Valtonen, E.T., Hoffmann, R.W., 1997. Gill anomalies of perch and roach from four lakes differing in water quality. J. Fish Biol. 50 (3), 575–591.
Hollert, H., Duerr, M., Erdinger, L., Braunbeck, T., 2000. Cytotoxicity of settling particulate matter and sediments of the Neckar River (Germany) during a winter flood. Environ. Toxicol. Chem. 19 (3), 528–534.
Jarić, I., Višnjić-Jeftić, Ž., Cvijanović, G., Gačić, Z., Jovanović, L., Skorić, S., Lenhardt, M., 2011. Determination of differential heavy metal and trace element accumulation in liver, gills, intestine and muscle of sterlet (*Acipenser ruthenus*) from the Danube River in Serbia by ICP-OES. Microchem. J. 98 (1), 77–81.
Jezierska, B., Witeska, M., 2006. The metal uptake and accumulation in fish living in polluted waters. In: Twardowska, I., Allen, H., Häggblom, M., Stefaniak, S. (Eds.), Soil and Water Pollution Monitoring Protection and Remediation. Springer, Netherlands, pp. 107–114.
Jha, A.N., 2008. Ecotoxicological applications and significance of the comet assay. Mutagenesis 23 (3), 207–221.
Jovičić, K., Lenhardt, M., Višnjić-Jeftić, Ž., Đikanović, V., Skorić, S., Smederevac-Lalić, M., Jaćimović, M., Gačić, Z., Jarić, I., Hegediš, A., 2014. Assessment of fish stocks and elemental pollution in the Danube, Sava and Kolubara rivers on the territory of the city of Belgrade, Serbia. Acta. Zool. Bulg. 2014 (Suppl. 7), 179–184.
Kaoud, H.A., El-Dahshan, A.R., 2010. Bioaccumulation and histopathological alterations of the heavy metals in *Oreochromis niloticus* fish. Nat. Sci. 8 (4), 147–156.
Kirschner, A.K., Kavka, G.G., Velimirov, B., Mach, R.L., Sommer, R., Farnleitner, A.H., 2009. Microbiological water quality along the Danube River: integrating data from two whole-river surveys and a transnational monitoring network. Water Res. 43 (15), 3673–3684.
Kolarević, S., Aborgiba, M., Kračun-Kolarević, M., Kostić, J., Simonović, P., Simić, V., Milošković, A., Reischer, G., Farnleitner, A., Gačić, Z., Milačič, R., Zuliani, T., Vidmar, J., Pergal, M., Piria, M., Paunović, M., Vuković-Gačić, B., 2016. Evaluation of genotoxic pressure along the Sava River. PLoS One 11 (9), e0162450.
Kostić, J., Kolarević, S., Kračun-Kolarević, M., Aborgiba, M., Gačić, Z., Lenhardt, M., Vuković-Gačić, B., 2016. Genotoxicity assessment of the Danube River using tissues of freshwater bream (*Abramis brama*). Environ. Sci. Pollut. Res. 23 (20), 20783–20795.
Kraal, M.H., Kraak, M.H., DeGroot, C.J., Davids, C., 1995. Uptake and tissue distribution of dietary and aqueous cadmium by carp (*Cyprinus carpio*). Ecotoxicol. Environ. Saf. 31 (2), 179–183.
Kumar, P., Kumar, R., Nagpure, N.S., Nautiyal, P., Kushwaha, B., Dabas, A., 2013. Genotoxicity and antioxidant enzyme activity induced by hexavalent chromium in *Cyprinus carpio* after *in vivo* exposure. Drug Chem. Toxicol. 36 (4), 451–460.
Lenhardt, M., Jarić, I., Višnjić-Jeftić, Ž., Skorić, S., Gačić, Z., Pucar, M., Hegediš, A., 2012. Concentrations of 17 elements in muscle, gills, liver and gonads of five economically important fish species from the Danube River. Knowl. Manag. Aquat. Ecosyst. 407, 02.
Loos, R., Gawlik, B.M., Locoro, G., Rimaviciute, E., Contini, S., Bidoglio, G., 2009. EU-wide survey of polar organic persistent pollutants in European river waters. Environ. Pollut. 157 (2), 561–568.
Marchand, M.J., Van Dyk, J.C., Pieterse, G.M., Barnhoorn, I.E.J., Bornman, M.S., 2009. Histopathological alterations in the liver of the sharptooth catfish *Clarias gariepinus* from polluted aquatic systems in South Africa. Environ. Toxicol. 24 (2), 133–147.
Mohamed, F.A., 2009. Histopathological studies on *Tilapia zillii* and *Solea vulgaris* from Lake Qarun, Egypt. World J. Fish Mar. Sci. 1 (1), 29–39.
Morillo, J., Usero, J., 2008. Trace metal bioavailability in the waters of two different habitats in Spain: Huelva estuary and Algeciras Bay. Ecotoxicol. Environ. Saf. 71 (3), 851–859.
Navarro-Ortega, A., Acuña, V., Bellin, A., Burek, P., Cassiani, G., Choukr-Allah, R., Dolédec, S., Elosegi, A., Ferrari, F., Ginebreda, A., Grathwohl, P., Jones, C., Rault, P.K., Kok, K., Koundouri, P., Ludwig, R.P., Merz, R., Milacic, R., Munoz, I., Nikulin, G., Paniconi, C., Paunović, M., Petrović, M., Sabater, L., Sabaterb, S., Skoulikidis, N.T., Slob, A., Teutsch, G., Voulvoulis, N., Barcelo, D., 2015. Managing the effects of multiple stressors on aquatic ecosystems under water scarcity. The GLOBAQUA project. Sci. Total Environ. 503, 3–9.
Official Gazette of FRY No 28/2011, 2011. Regulation on Quantity of Pesticides, Metals, Metalloids, and Other Toxic Substances, Chemotherapeutics, Anabolics, and Other Substances Which can be Found in Food.
Olojo, E.A.A., Olurin, K.B., Oluwemimo, A.D., 2005. Histopathology of the gill and liver tissues of the African catfish *Clarias gariepinus* exposed to lead. Afr. J. Biotechnol. 4 (1), 117.
Pacheco, M., Santos, M.A., 2002. Biotransformation, genotoxic, and histopathological effects of environmental contaminants in European eel (*Anguilla anguilla* L.). Ecotoxicol. Environ. Saf. 53 (3), 331–347.
Pailler, J.Y., Guignard, C., Meyer, B., Iffly, J.F., Pfister, L., Hoffmann, L., Krein, A., 2009. Behaviour and fluxes of dissolved antibiotics, analgesics and hormones during flood events in a small heterogeneous catchment in the Grand Duchy of Luxembourg. Water Air Soil Pollut. 203 (1–4), 79–98.
Pandey, S., Parvez, S., Ansari, R.A., Ali, M., Kaur, M., Hayat, F., Ahmad, F., Raisuddin, S., 2008. Effects of exposure to multiple trace metals on biochemical, histological and ultrastructural features of gills of a freshwater fish, *Channa punctata* Bloch. Chem. Biol. Interact. 174 (3), 183–192.
Paolini, A., Berti, M., D'Angelo, A., Scortichini, G., Giansante, C., 2004. Use of histopathological indicators on chub (*Leuciscus cephalus*) and brown trout (*Salmo trutta fario*) in evaluating river environments. Vet. Ital. 41 (3), 189–198.
Peatman, E., Lange, M., Zhao, H., Beck, B.H., 2015. Physiology and immunology of mucosal barriers in catfish (*Ictalurus spp.*). Tissue Barriers 3 (4), e1068907.
Pereira, P., De Pablo, H., Vale, C., Pacheco, M., 2010. Combined use of environmental data and biomarkers in fish (*Liza aurata*) inhabiting a eutrophic and metal - contaminated coastal system–gills reflect environmental contamination. Mar. Environ. Res. 69 (2), 53–62.
Polard, T., Jean, S., Gauthier, L., Laplanche, C., Merlina, G., Sanchez-Pérez, J.M., Pinelli, E., 2011. Mutagenic impact on fish of runoff events in agricultural areas in south-west France. Aquat. Toxicol. 101 (1), 126–134.
Poleksić, V., Lenhardt, M., Jarić, I., Djordjević, D., Gačić, Z., Cvijanović, G., Rašković, B., 2010. Liver, gills and skin histopathology and heavy metal content of the Danube sterlet (*Acipenser ruthenus* Linnaeus, 1758). Environ. Toxicol. Chem. 29, 515–521.
Rahmani, R., Mansouri, B., Azadi, N.A., Davari, B., Johari, S.A., Maleki, A., Pordel, M.A., 2016. Histopathological alterations in the gill of zebrafish (*Danio rerio*) exposed to Cr and Ba doped TiO 2 nanoparticles. Int. J. Bioflux Soc. 9 (4).
Rashed, M.N., 2001. Monitoring of environmental heavy metals in fish from Nasser Lake. Environ. Int. 27 (1), 27–33.
Rašković, B., Poleksić, V., Živić, I., Spasić, M., 2010. Histology of carp (*Cyprinus carpio* L.) gills and pond water quality in semiintensive production. Bulg. J. Agric. Sci. 16 (3), 253–262.
Rašković, B., Jarić, I., Koko, V., Spasić, M., Dulić, Z., Marković, Z., Poleksić, V., 2013. Histopathological indicators: a useful fish health monitoring tool in common carp (*Cyprinus carpio* Linnaeus, 1758) culture. Cent. Eur. J. Biol. 8 (10), 975–985.
Rašković, B., Poleksić, V., Višnjić-Jeftić, Ž., Skorić, S., Gačić, Z., Djikanović, V., Jarić, I., Lenhardt, M., 2015. Use of histopathology and elemental accumulation in different organs of two benthophagous fish species as indicators of river pollution. Environ. Toxicol. 30, 1153–1161.
Regoli, F., 1998. Trace metals and antioxidant enzymes in gills and digestive gland of the Mediterranean mussel *Mytilus galloprovincialis*. Arch. Environ. Contam. Toxicol. 34 (1), 48–63.
Reid, S.D., 2002. Physiological impact of acute molybdenum exposure in juvenile kokanee salmon (*Oncorhynchus nerka*). Comp. Biochem. Physiol. C 133 (3), 355–367.
Reite, O.B., 1998. Mast cells/eosinophilic granule cells of teleostean fish: a review focusing on staining properties and functional responses. Fish Shellfish Immunol. 8 (7), 489–513.
Salem, Z.B., Capelli, N., Laffray, X., Elise, G., Ayadi, H., Aleya, L., 2014. Seasonal variation of heavy metals in water, sediment and roach tissues in a landfill draining system pond (Etueffont, France). Ecol. Eng. 69, 25–37.
Schlenk, D., Handy, R., Steinert, S., Depledge, M.H., Benson, W., 2008. Biomarkers. In: Di Giulio, R.T., Hinton, D.E. (Eds.), The Toxicology of Fishes. CRC Press, Taylor & Francis Group, Boca Raton, pp. 683–731.
Secombes, C.J., Chappell, L.H., 1996. Fish immune responses to experimental and natural infection with helminth parasites. Annu. Rev. Fish Dis. 6, 167–177.
Sevcikova, M., Modra, H., Slaninova, A., Svobodova, Z., 2011. Metals as a cause of oxidative stress in fish: a review. Vet. Med. 56 (11), 537–546.
Simonović, P., 2001. Ribe Srbije [Fishes of Serbia]. NNK International, Zavod za zaštitu prirode Srbije, Belgrade (Biološki fakultet (126, 127, 140 pp)).
Skorić, S., Višnjić-Jeftić, Ž., Jarić, I., Djikanović, V., Mićković, B., Nikčević, M., Lenhardt, M., 2012. Accumulation of 20 elements in great cormorant (*Phalacrocorax carbo*) and its main prey, common carp (*Cyprinus carpio*) and Prussian carp (*Carassius gibelio*). Ecotoxicol. Environ. Saf. 80, 244–251.
Sorensen, E.M., Henry, R.E., Ramirez-Mitchell, R., 1979. Arsenic accumulation, tissue distribution and cytotoxicity in teleosts following indirect aqueous exposures. B. Environ. Contam. Toxicol. 21 (1), 162–169.
Sorensen, E.M., Ramirez-Mitchell, R., Harlan, C.W., Bell, J.S., 1980. Cytological changes in the fish liver following chronic, environmental arsenic exposure. B. Environ. Contam. Toxicol. 25 (1), 93–99.
Squier, M.K., Cohen, J.J., 2001. Standard quantitative assays for apoptosis. Mol. Biotechnol. 19 (3), 305–312.
Štrbac, S., Kašanin-Grubin, M., Jovančićević, B., Simonović, P., 2015. Bioaccumulation of heavy metals and microelements in silver bream (*Brama brama* L.), northern pike (*Esox lucius* L.), sterlet (*Acipenser ruthenus* L.), and common carp (*Cyprinus carpio* L.) from Tisza River, Serbia. J. Toxic. Environ. Health A 78 (11), 663–665.

Subotić, S., Spasić, S., Višnjić-Jeftić, Ž., Hegediš, A., Krpo-Ćetković, J., Mićković, B., Skorić, S., Lenhardt, M., 2013. Heavy metal and trace element bioaccumulation in target tissues of four edible fish species from the Danube River (Serbia). Ecotoxicol. Environ. Saf. 98, 196–202.

Sunjog, K., Gačić, Z., Kolarević, S., Višnjić-Jeftić, Ž., Jarić, I., Knežević-Vukčević, J., Vuković-Gačić, B., Lenhardt, M., 2012. Heavy metal accumulation and the genotoxicity in barbel (*Barbus barbus*) as indicators of the Danube River pollution. Sci. World J. http://dx.doi.org/10.1100/2012/351074.

Sunjog, K., Kolarević, S., Kračun-Kolarević, M., Gačić, Z., Skorić, S., Đikanović, V., Lenhardt, M., Vuković-Gačić, B., 2014. Variability in DNA damage of chub (*Squalius cephalus* L.) blood, gill and liver cells during the annual cycle. Environ. Toxicol. Pharmacol. 37 (3), 967–974.

Sunjog, K., Kolarević, S., Kračun-Kolarević, M., Višnjić-Jeftić, Ž., Skorić, S., Gačić, Z., Lenhardt, M., Vasić, N., Vuković-Gačić, B., 2016. Assessment of status of three water bodies in Serbia based on tissue metal and metalloid concentration (ICP-OES) and genotoxicity (comet assay). Environ. Pollut. 213, 600–607.

Teh, S.J., Adams, S.M., Hinton, D.E., 1997. Histopathologic biomarkers in feral freshwater fish populations exposed to different types of contaminant stress. Aquat. Toxicol. 37 (1), 51–70.

Usero, J., Gonzalez-Regalado, E., Gracia, I., 1997. Trace metals in the bivalve molluscs *Ruditapes decussatus* and *Ruditapes philippinarum* from the Atlantic Coast of Southern Spain. Environ. Int. 23 (3), 291–298.

Van der Oost, R., Beyer, J., Vermeulen, N.P., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13 (2), 57–149.

Van Dyk, J.C., Marchand, M.J., Smit, N.J., Pieterse, G.M., 2009. A histology-based fish health assessment of four commercially and ecologically important species from the Okavango Delta panhandle, Botswana. Afr. J. Aquat. Sci. 34 (3), 273–282.

Velma, V., Tchounwou, P.B., 2010. Chromium-induced biochemical, genotoxic and histopathologic effects in liver and kidney of goldfish, *Carassius auratus*. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 698 (1), 43–51.

Vieira, M.C., Torronteras, R., Córdoba, F., Canalejo, A., 2012. Acute toxicity of manganese in goldfish *Carassius auratus* is associated with oxidative stress and organ specific antioxidant responses. Ecotoxicol. Environ. Saf. 78, 212–217.

Vinodhini, R., Narayanan, M., 2008. Bioaccumulation of heavy metals in organs of fresh water fish *Cyprinus carpio* (Common carp). Int. J. Environ. Sci. Technol. 5 (2), 179–182.

Višnjić-Jeftić, Ž., Jarić, I., Jovanović, L., Skorić, S., Smederevac-Lalić, M., Nikčević, M., Lenhardt, M., 2010. Heavy metal and trace element accumulation in muscle, liver and gills of the Pontic shad (*Alosa immaculata* Bennet 1835) from the Danube River (Serbia). Microchem. J. 95 (2), 341–344.

Vrzel, J., Vuković-Gačić, B., Kolarević, S., Gačić, Z., Kračun-Kolarević, M., Kostić, J., Aborgiba, M., Farnleitner, A., Reischer, G., Linke, R., Paunović, M., Ogrinc, N., 2016. Determination of the sources of nitrate and the microbiological sources of pollution in the Sava River Basin. Sci. Total Environ. 573, 1460–1471.

Wolf, J.C., Wolfe, M.J., 2005. A brief overview of nonneoplastic hepatic toxicity in fish. Toxicol. Pathol. 33 (1), 75–85.

Younis, E.M., Abdel-Warith, A.A., Al-Asgah, N.A., Ebaid, H., Mubarak, M., 2013. Histological changes in the liver and intestine of Nile Tilapia, *Oreochromis niloticus*, exposed to sublethal concentrations of cadmium. Pak. J. Zool. 45 (3), 833–841.