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RESEARCH ARTICLE

Frequencies of erythrocyte nuclear abnormalities and of leucocytes in the fish *Barbus peloponnesius* correlate with a pollution gradient in the River Bregalnica (Macedonia)

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Abstract Integrated chemical and biomarker approaches were performed to estimate if there is ongoing toxicity in the River Bregalnica, namely connected with the presence of metals. The study was performed in water, sediment, and barbel (*Barbus peloponnesius*), collected in two seasons, from two suspected polluted and one reference zones. The water analyses revealed higher mean values in polluted sites for most of the examined physicochemical parameters. Metal concentrations (Zn, Cu, Cd, Mn, Pb, and Fe) in water were more or less constant, whereas in sediment, they were higher at the two polluted locations. Condition factor (CF), as a general health indicator, revealed better overall condition in barbel from the reference site. In general, blood parameters revealed higher values in the polluted localities. Irrespective of sex and/or season, the frequency of micronuclei (MN) and vacuolated nuclei (VN) were with higher rates in polluted sites. Similarly, the frequencies of the leucocytes (Le), binuclei (BN), and irregularly shaped nuclei (ISN) were also significantly increased in the polluted localities, but they seemed prone to be influenced by sex and/or season. However, strong positive correlations between blood biomarkers and most water physicochemical parameters and metal in sediment were estimated. Our data support that the River Bregalnica's lower course receives significant genotoxic pollution, likely via metal industry effluents, agricultural runoff, and domestic sewage, and reinforced the utility of MN and other nuclear abnormalities as sensitive and suitable biomarkers for genotoxicity when used in monitoring studies.

Keywords Blood · Fish · Genotoxicity · Metals · Micronuclei · Sediment contamination · Water quality

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Introduction

The main water resource in Eastern Macedonia that sustains life in that region is the River Bregalnica and its watershed. The Bregalnica is the second largest river (225 km) in R. Macedonia. The river flows through densely populated areas and receives untreated wastewaters from the industry, mining, and households, which are pumped directly into the river, threatening the drinking water supply, and disturbing the fragile ecological balance. Moreover, the Valey of Kocani, situated in the middle course of the river, is the principal rice production region (Andreevska et al. 2013), which results in additional negative pressure in the middle and lower parts of the river course, namely having in mind the past and current liberal and uncontrolled uses of agrochemicals with unknown effects on local species. Therefore, recent efforts have been focused on studying the pollution of the river, which generally refers to physicochemical and metal contamination (Stafilov et al. 2009; Serafimovska et al. 2011; Balabanova et al. 2011, 2013; Dimitrovska et al. 2012; Ilić Popov et al. 2014; Ramani et al. 2014). However, the investigations concerning the effect of this contamination on the aquatic organisms that inhabit the river ecosystem are scarce (Barišić et al. 2015; Jordanova et al. 2016), although this kind of investigations is essential for the preservation of biodiversity and further deterioration of the ecosystem.

In aquatic eco-toxicological studies, fish are mostly used as a bioindicator organism, since they are at the higher levels of food chain and therefore may bioaccumulate toxicants from the food, water, and sediment (Koca et al. 2005). Barbel (*Barbus peloponnesius*, Valenciennes, 1844) is the dominant species along the river axis (Kostov et al. 2010) and is a typical benthophagous fish (Georgiev 1998), inhabiting all over the water flow of the River Bregalnica. Contrary to other local fish, the barbel is less mobile and colonizes special regions of the river. Consequently, the health status of this fish can better reflect the environmental status of particular extensions of the river. Moreover, as it was outlined by Minissi et al. (1996), the genus *Barbus* have wild geographic distribution all around Europe, which enables comparisons of the in situ monitoring results between different rivers and different countries.

Many from the wide range of hazardous substances or their mixtures that end up into the aquatic ecosystems have cytotoxic potential and could provoke genotoxic damage in biota (Costa et al. 2008; Muranli and Güner 2011), which could be reflected on next generations, generating adverse reproductive effects or even extinction risk (Boettcher et al. 2010). Therefore, the need for the assessment of genotoxic potential in the surface waters becomes one of the necessary tasks in field studies. In this vein, the presence of micronuclei (MN) in erythrocytes from the peripheral blood of fish, as an indicator of chromosomal damage, has been increasingly used (Ali et al. 2008; Strunjak-Perovic et al. 2008; Osman et al. 2010; Fuzinatto et al. 2013). Studies have been showing that exposure of fish to different types of pollutants, under field or laboratory conditions, causes significant increases in the baseline natural incidence of MN in peripheral blood erythrocytes (Al-Sabti and Metcalfe 1995; Ayllon and Garcia-Vezquez 2000; Çavaş and Ergene-Gözükara 2003, 2005; Ergene et al. 2007). Beside MN, nuclear abnormalities could be considered as valuable indicators of genotoxic damage and could be used in routine genotoxicity surveys as complements of the MN frequency (Ergene et al. 2007; Carrola et al. 2014).

On the other hand, hematological assessment of peripheral blood is important in evaluating the health of many organisms, including fish. In fact, knowledge of the baseline blood characteristics and the subsequent hematological investigations offer sensitive indexes that can effectively reflect physiological and pathological states of fish (Kori-Siakpere et al. 2005). For example, altered leukocytic counts were evaluated in fish exposed to various types of pesticides (Das and Mukherjee 2003; Adhikari et al. 2004; Mgbenka et al. 2005), or metals (Nussey et al. 1995; Mazon et al. 2002), and are recognized as a non-specific secondary stress indicator, suitable for assessing fish health and responses to contaminants (Pierson et al. 2004). In this vein, the condition factor is a biometric index that is almost routinely applied in monitoring studies, as a first non-specific indicator of the fish health status, being indicative of cumulative organism-level changes (Facey et al. 2005).

Although studies that include standard hematology and MN scrutiny are widely applied, there are not such investigations for fish in water bodies in the R. Macedonia. Considering the increased pollution investigations for the River Bregalnica, we assume that fish inhabiting the river are facing different levels of stress, as a result of the presence of different types of contaminants, including genotoxic substances. So, this research was designed to study the genotoxicity of the River Bregalnica by evaluating the nuclear damage of the barbel erythrocytes from the peripheral blood, in two seasons and both sexes. Concretely, our aims were to (1) assess the frequency of MN and nuclear abnormalities in barbel erythrocytes; (2) estimate the frequency of blood leucocytes (Le) and the Fulton's condition factor (CF) as complementary proxies of the individual fish's health; (3) examine water and sediment for selected heavy metals, as well as general physicochemical parameters; and (4) perform the correlation analyses for biomonitoring and chemical data sets.

Material and methods

Sampling localities

To collect representative material in accordance with the aims of this study, the sampling of water, sediment, and barbels were carried out on three sampling localities along the River Bregalnica (Fig. 1), chosen in the upper, middle, and lower parts of the river (Milevski et al. 2008), according to the location of potential and known sources of pollutants. First sampling station used as a reference/control area is located before the city of Berovo, in the upper part of the river (22° 51′ 27.9″ E, 41° 41′ 59.8″ N; altitude 856 m), which does not receive effluents of urban, industrial, or agriculture origins. The second sampling zone (site A) is located in the middle part of the River Bregalnica (22° 20′ 58.5″ E, 41° 51′ 45.4″ N; altitude 317 m), which has been subjected to strong pollution from (1) agriculture activities with dominating rice fields in which pesticide use is substantial; (2) mining activities from the active zinc-lead mine which waste material exposed to the rain through the river Zletovica comes into the River Bregalnica; and (3) household water from the town of Kocani and

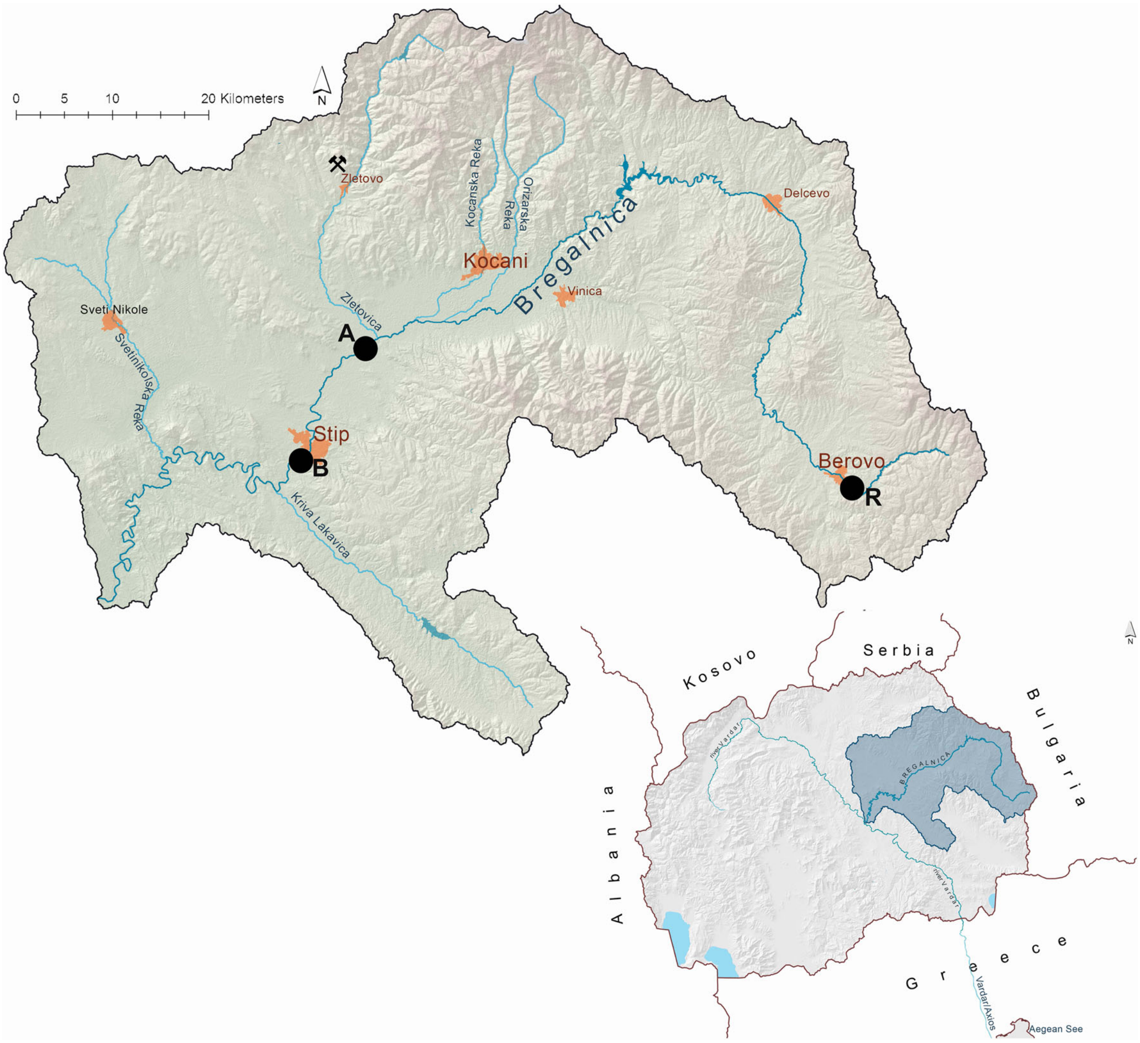


Fig. 1 Location of the sampling sites along the River Bregalnica: reference site (*R*), site A (*A*), and site B (*B*)

surrounding villages directly discharges into the river. The third sampling zone (site B) is located in the lower part of the river, downstream to the city of Stip (22° 10′ 27.0″ E, 41° 43′ 55.0″ N; altitude 250 m)—the largest town in the eastern part of R. Macedonia—being subjected not only to treated sewage, but also to direct untreated household water discharges and waste from textile factories as a potential risk.

Chemical and physicochemical analysis of water and sediment

During the 2007–2008, water samples were taken monthly (12 sampling campaigns) from the selected sites. At each occasion, water samples were collected in 2-L polyethylene bottles, kept in portable coolers, and transported to the laboratory for further analysis. The following parameters: pH, water temperature, dissolved oxygen (DO) concentration, and oxygen saturation were measured directly on the field, using a portable digital multiparameter pH meter (WTW pH 197i—3A30-110) and an oxygen meter (YSI Model 51 DO meter). Concerning the sediment samples, they were collected twice during the investigated period, in autumn-winter (AW) and spring-summer (SS). In the laboratory, a standard analytical method recommended by APHA (1992) for the metal analysis, as well as for the investigation of alkalinity, hardness, carbonates (CO_3), hydrogen carbonates (HCO_3), sulfates, nitrates, nitrites, ammonia, total phosphorus, and COD_{Mn}, was used. Concentrations of the metals (Zn, Cu, Pb, Mn, Fe, Cd) in all the samples were measured by a flame atomic absorption spectrometer (Model Varian SpectraAA 55) and electrothermal atomic absorption spectrometry, using Zeeman background correction (Varian SpectraAA 640). Results were expressed in micrograms per liter (μg L^{-1}) and milligrams per kilogram (mg kg^{-1}) for concentration of metals in the water and their content in the sediment, respectively.

The monitored parameters in the water were interpreted according to Macedonian water quality standards (Regulation for Water Classification and Regulation for categorization of Watercourses, Lakes, Accumulations and Underground waters, Official Gazette of the Republic of Macedonia 1999). According to this classification, there are five classes of waters, where the quality of the first class is the best and the water quality of the fifth class is the worst.

Fish collection and sex determination

Fish capture and handling complied with the all the current laws for the animal welfare of the Republic of Macedonia. A total of 107 specimens of the barbel were collected from the selected localities in two seasons: AW and SS. Standardized electric fishing procedures described in the CEN directive, "Water Analysis—Fishing with Electricity" for wadeable and non-wadeable rivers, were used (CEN EN 14011: 2003). The stunned fish were maintained alive by transporting them in water to a local field laboratory. Fish were humanely euthanized by one experienced researcher, via instantaneous cervical transection followed by pithing (Leary et al. 2013). Then, the total length (TL) and fork length (FL) were measured to the nearest millimeter (mm), while body weight (BW) to the nearest gram (g). CF was calculated using the following formula: CF = body weight (g) × 100 / fork length (cm^3). For sex determination, gonads were removed, fixed in Bouin's fixative, embedded in paraffin, cut into 5-μm-thick sections, stained with hematoxylin and eosin, and examined by light microscopy.

Sample preparation and analysis

Peripheral blood was collected from the caudal vein, with a heparinized 2.5-ml disposable sterile plastic syringe and Microlance needles. Immediately after collection, blood smears (five per fish) were manually made, air-dried, fixed 10 min in methanol, and stained with May-Grünwald/Giemsa (MGG) method. From each slide, 20 fields (100 fields per fish) were sampled systematically and analyzed, with an oil immersion lens, at a magnification of ×1000. From each selected field of view, the total numbers of erythrocytes and of Le were counted, and the number of white cells per/1000 red blood cells was estimated. All erythrocytes (8.000 to 16.000 per fish) were inspected for the presence of nuclear abnormalities and scored according to the classification of Carrasco et al. (1990) and Strunjak-Perovic et al. (2008). MN, binuclei (BN), vacuolated nuclei (VN), and irregularly shaped nuclei (ISN) were classified separately. Only round- or ovoid-shaped non-refractory particles, with structure similar to that of chromatin and clearly detached from the main nucleus, with diameters up to one third of the nuclei size, were interpreted as MN (Fig. 2). BN were those that contained two round or ellipsoidal nuclei with approximately the same size (Fig. 3). Blebbed, lobed, and notched nuclei were interpreted together and denoted as ISN (Fig. 4). VN were those that

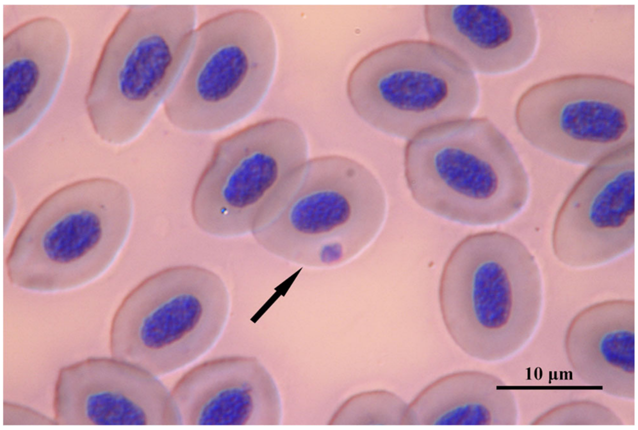


Fig. 2 Light micrograph showing MN (*arrow*) in periphery blood from barbel captured at site A (MGG). *Bar* = 0.01 mm

despite being regularly shaped, nuclei contained one, or rarely two, well-demarcated round vacuoles (Fig. 5). All investigated parameters were calculated as mean frequencies, determined as number of Le, and nuclear abnormalities/1000 erythrocytes (‰).

Statistical analysis

The data for all investigated parameters (except for metal content in the sediment) were presented as mean values, followed by the coefficient of variation (CV = SD / Mean). The data concerning fish parameters additionally were grouped according to sex, locality, and season. The statistics was conducted with the software STATISTICA 7.0 (StatSoft) for Windows (Microsoft). For every parameter, raw data were first log transformed to promote the premises of normality and homoscedasticity and then analyzed by a factorial (three-way) ANOVA, to test the effects of sex, season, sampling site, and their interactions. Post hoc analysis recurred to the Tukey method. Because the transformation could not always statistically grant homoscedasticity and in view of the absence of a non-parametric equivalent to a three-way ANOVA, in what

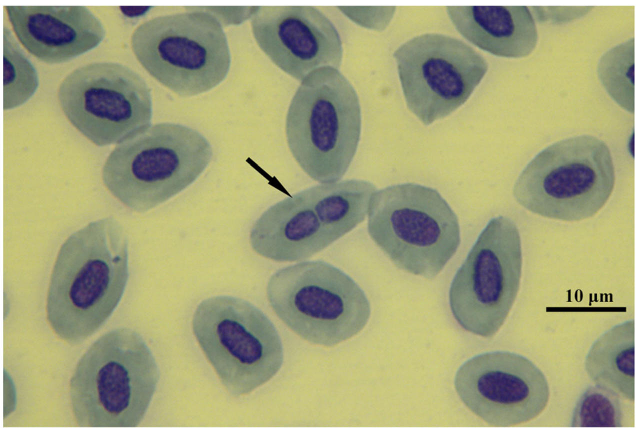


Fig. 3 Light micrograph showing BN (*arrow*) in periphery blood from barbel captured at site A (MGG). *Bar* = 0.01 mm

Fig. 4 Light micrograph showing ISN (*arrow*) in periphery blood from barbel captured at site A (MGG). *Bar* = 0.01 mm

regard selected comparisons between two groups, an additional non-parametric approach was conducted using the Mann-Whitney's *U* test. Results from the post hoc parametric and non-parametric approaches were highly similar. Correlation analyses for selected variables were used to find specific linear associations between average metal concentrations in water and metal content in sediment versus average fish blood parameters (biomarkers), at a particular location and season, using the Spearman rank-order correlation test (as homoscedasticity could not be granted always). The statistical significance level, alpha, was set at 5%, and so, null hypothesis was rejected whenever $p < 0.05$.

Results

Physicochemical parameters of the water

The results of physicochemical analyses of the water at the sampling sites divided by seasons are given in Table 1. In general, multivariate analysis of variance (MANOVA) revealed that locality alone had the strongest influence on almost all water parameters, except for ammonia, total phosphorus, and COD_{Mn}, while temperature, DO, O_2 saturation, sulfates, and total phosphorus were also affected by season alone. Significant temperature fluctuations were recorded in all localities between the seasons. However, in both seasons, the highest mean water temperatures were recorded in lower altitudes of the river (sites A and B), compared with the reference site. Also, average alkalinity, hardness, CO_3, HCO_3, nitrates, and nitrites were significantly greater in middle and lower river flows (sites A and B) compared with the reference one, while sulfates differed between all sites and between seasons in site A.

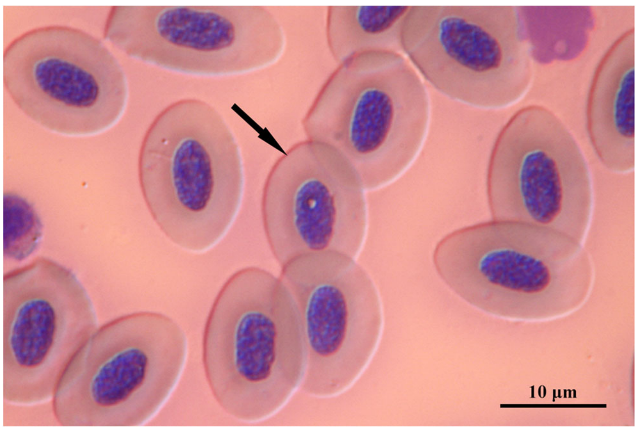


Fig. 5 Light micrograph showing VN (*arrow*) in periphery blood from barbel captured at site A (MGG). *Bar* = 0.01 mm

Metal analyses in the water and the sediment

The metal concentrations in the water were more or less constant, with a trend for higher values of Mn and Fe downstream (Table 2). Statistical analyses showed that season alone had influence on concentration of Cd, with significantly higher values in SS compared with AW (Fig. 6), while Mn concentrations were affected by both factors individually (season and locality) and in interaction (Fig. 7). As for the concentrations of Fe, significantly higher values were observed in AW season for site A (Fig. 8), compared with the reference site, and site B. As for the concentrations of Cu (Fig. 9) and Pb (Fig. 10) in the water, no statistical differences were observed. Furthermore, metal content in the sediment of the three sampling sites varied considerably (Table 2). For such data, no definitive statistical analyses were conducted, because we have only two samples, which are viewed as preliminary and exploratory. Anyway, the fact is that the content of all the metals in the sediment (Zn, Cu, Cd, Mn, Pb and Fe) was higher at site A and site B compared to those in the reference site.

Fish condition and morphometric parameters

The fish BW and TL are presented in Figs. 11 and 12, respectively. For both cases, season and sex were significant factors for the observed differences, but for BW, the sampling station and the interaction of season × sex were also significant. Females were overall heavier and longer than males in all localities, although significantly only in the reference site (in AW) and in site A (in SS). As for the localities, the only difference in BW was observed in females from AW, with heavier specimens in reference site, compared with samples collected in site B. Besides that, females collected in AW from the reference site were significantly longer than females from SS at the same site. As for the CF (Fig. 13), significant influences existed for each factor (locality: in both sexes and both seasons; sex: in reference site, in both seasons; and season: between males from the site A), as well as by interaction locality × sex. The CVs showed very low

Table 1 The physicochemical data[1] of the sampling sites in the River Bregalnica in AW and SS seasons

Parameters	Season	Reference site	Site A	Site B
Temperature (°C)	AW	2.1 (1.27) Aa	8.5 (0.39) Ab	7.7 (0.40) Ab
	SS	12.6 (0.26) Ba	16.4 (0.20) Bb	18.1 (0.26) Bab
pH	AW	7.7 (0.02) ab	7.6 (0.02) a	8.1 (0.03) b
	SS	7.8 (0.01) a	7.6 (0.01) b	8.1 (0.02) c
DO (mg L^{-1})	AW	13.2 (0.10) A	10.9 (0.16) A	12.3 (0.15)
	SS	9.8 (0.09) B	8.1 (0.10) B	9.2 (0.11)
O_2 saturation (%)	AW	96.8 (0.04) A	92.4 (0.11)	102.7 (0.13)
	SS	92.2 (0.03) Ba	82.1 (0.05) b	95.9 (0.04) a
Alcalinity (meq L^{-1})	AW	0.8 (0.53) a	1.8 (0.52) b	2.4 (0.44) b
	SS	0.6 (0.39) a	1.7 (0.28) b	2.2 (0.37) b
Hardness (°dH)	AW	2.1 (0.53) a	5.1 (0.52) b	6.7 (0.44) b
	SS	1.6 (0.39) a	4.7 (0.28) b	6.3 (0.37) b
CO_3 (mg L^{-1})	AW	33.6 (0.53) a	79.6 (0.52) b	104.5 (0.44) b
	SS	25.5 (0.39) a	74.3 (0.28) b	98.8 (0.37) b
HCO_3 (mg L^{-1})	AW	46.6 (0.53) a	110.3 (0.52) b	144.8 (0.44) b
	SS	35.3 (0.39) a	103.0 (0.28) b	136.9 (0.37) b
Sulfates (mg L^{-1})	AW	19.6 (0.24) a	28.4 (0.19) Ab	52.6 (0.32) c
	SS	19.9 (0.24) a	39.5 (0.25) Bb	62.3 (0.17) c
Nitrates (mg L^{-1})	AW	1.0 (0.37) a	3.8 (0.30) b	4.2 (0.16) b
	SS	1.6 (0.43) a	3.7 (0.27) b	3.2 (0.25) b
Nitrites (mg L^{-1})	AW	0.03 (1.22) a	0.13 (0.45) b	0.16 (0.37) b
	SS	0.02 (0.70) a	0.16 (0.38) b	0.21 (1.29) b
Ammonia (mg L^{-1})	AW	0.4 (1.22)	0.8 (0.76)	0.4 (1.13)
	SS	0.3 (0.44)	0.2 (0.57)	0.5 (0.89)
Total phosphorus (μg L^{-1})	AW	0.015 (0.57)	0.016 (0.97)	0.010 (1.77)
	SS	0.006 (0.80)	0.008 (1.15)	0.004 (1.23)
Chemical oxygen demand	AW	4.2 (0.41)	4.4 (0.30)	5.1 (0.44)
COD_{Mn} (mg L^{-1} O_2)	SS	5.5 (0.40)	4.1 (0.34)	4.8 (0.27)

[1] The results are presented as mean values accompanied by the coefficient of variation. For every parameter, different uppercase letters represent differences between the seasons within sampling site (read vertical) and different lowercase letters represent differences between sampling localities within each season (read horizontal)

interindividual variability for TL and CF and marked biological unevenness for the BW.

Blood analyses

As to blood analyses, the frequencies of Le, BN, ISN, MN, and VN are presented in Figs. 14, 15, 16, 17, and 18, grouped by locality, season, and sex of the fish. All types of examined nuclear deformities were present in all groups, even in the reference one, although with low frequency. MANOVA showed that locality alone was the only factor that has influence on all parameters. As to interactions, locality × season influenced the Le and MN frequencies, while season × sex affected only the frequencies of BN. Generally speaking, for all parameters, the reference site revealed significantly lower values regarding the downstream parts of the river. However, when analyzing each blood cell parameter separately, some differences in the pattern of variations become apparent.

The frequency of the Le (Fig. 14) in AW in the reference site was 16.01‰ for females and 9.96‰ for males, in site A around 15‰, while in site B, the values were around 23‰ for both sexes. In SS, highest values were observed in site A (30.45‰ in females and 27.39‰ in males) and lowest ones in both the reference site (14.34‰ in females and 12.28‰ in males) and site B (12.39‰ in females and 15.55‰ in males). Statistical analysis of the Le frequency revealed significant differences only in males, but in both seasons: (a) in AW males from the reference site, it revealed significantly lower values than males from site B, and (b) in SS males from the reference site, it had lower values too, differing from males from site A.

Table 2 Metal concentration in the water[1] and metal content in the sediments[2] of the sampling sites in the River Bregalnica in AW and SS seasons

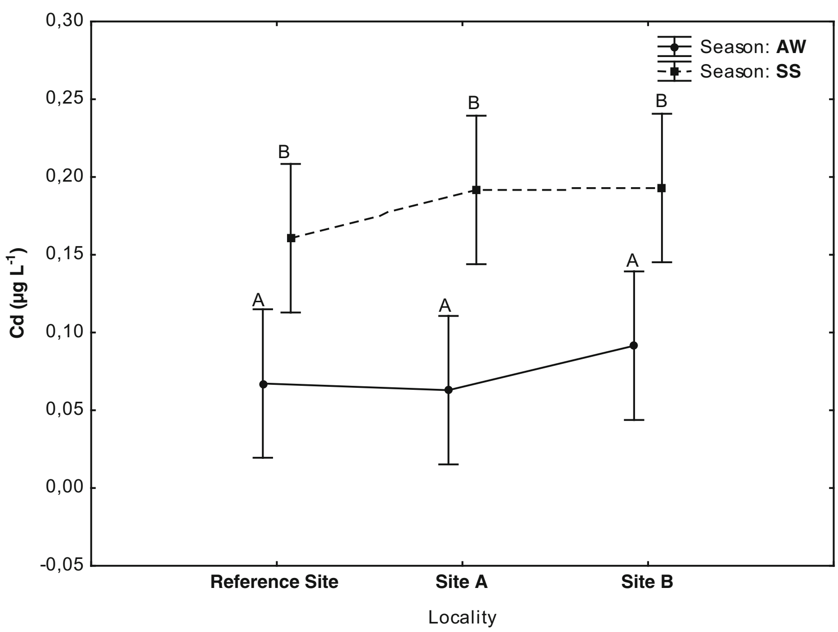
		Water (μg L^{-1})			Sediments (mg kg^{-1})		
		Reference site	Site A	Site B	Reference site	Site A	Site B
Metal	Season						
Zn	AW	<10	<10	<10	65	118	400
	SS	<10	<10	<10	20	272	171
Cu	AW	5.48 (0.39)	4.39 (0.49)	4.34 (0.46)	40.9	77.0	62.7
	SS	7.72 (0.46)	5.53 (0.34)	7.33 (0.68)	7.8	45.6	38.7
Cd	AW	0.07 (0.25) A	0.06 (1.02) A	0.09 (0.69) A	0.16	0.47	0.90
	SS	0.16 (0.39) B	0.19 (0.31) B	0.19 (0.32) B	0.05	1.81	0.45
Mn	AW	20 (1.03)	123 (0.75)	122 (0.72)	404	1011	2687
	SS	16 (0.21)	33 (1.02)	18 (0.50)	84	1866	1311
Pb	AW	6.66 (0.77)	6.49 (0.68)	9.46 (0.30)	6	36	93
	SS	9.05 (0.25)	8.59 (0.49)	7.86 (0.38)	5	124	35
Fe	AW	93 (0.87) a	407 (1.17) b	219 (1.10) ab	33,863	44,422	42,655
	SS	62 (0.64)	145 (0.54)	71 (0.54)	24,756	36,355	30,715

[1] The results are presented as mean values accompanied by the coefficient of variation. [2] The results are presented as single values in appropriated season. Different uppercase letters represent differences between the seasons within sampling site (read vertical), and different lowercase letters represent differences between sampling localities within each season (read horizontal)

As to the MN (Fig. 15), for both sexes and in both seasons, the lowest values were recorded in fish from the reference site (from 0.1 to 0.3‰). The MN frequency was much higher at the site A (from 3.55 to 4.80‰), reaching the highest values at site B (from 5.19 up to 22.17‰). The MN frequency at both polluted sites (A and B) significantly differed from the reference site; such differences were observed in both seasons and were valid for both sexes.

Differences in the BN frequency (Fig. 16) were observed in males from both seasons, with significant differences between males from the reference site (in AW—0.09, SS—0.14‰) and site B (in AW—0.34, SS—0.60‰). As for the ISN, although the locality was the only influencing factor, different modes of variation were observed in females and males (Fig. 17). Namely, in males from both seasons, a significantly lower frequency was observed in the reference site (in AW—0.44, SS—0.69‰) compared with both polluted sites (site A in AW—1.30, SS—1.90‰, site B in AW—1.50, SS—3.16‰). In females, only SS individuals differed significantly. The highest values were noted in the site A (1.83‰), somewhat

Fig. 6 The concentration of Cd in the water of the sampling sites in the River Bregalnica in AW and SS seasons. The data are expressed as mean, and *vertical bars* denote a 0.95 confidence interval



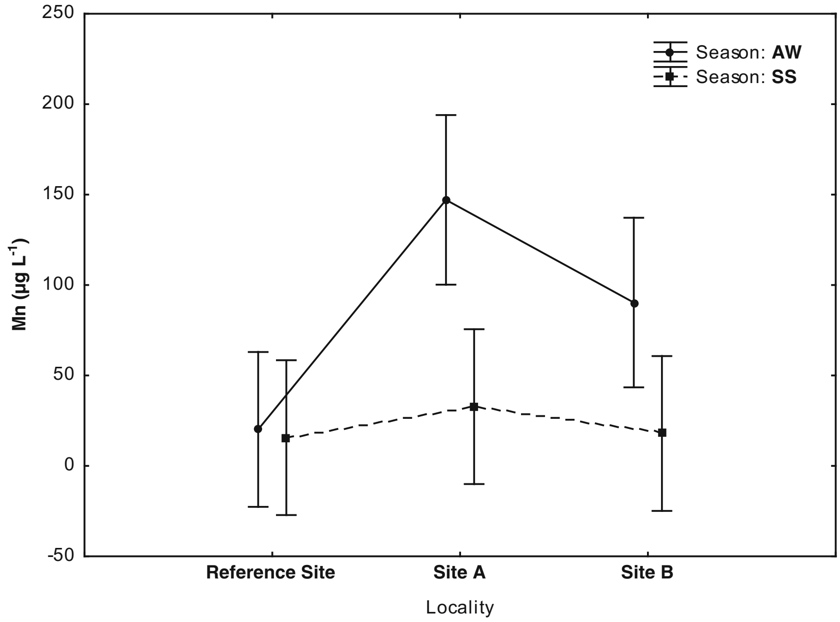


Fig. 7 The concentration of Mn in the water of the sampling sites in the River Bregalnica in AW and SS seasons. The data are expressed as mean, and *vertical bars* denote a 0.95 confidence interval

lower in site B (1.19‰) and lowest in the reference site (0.39‰). However, statistical calculations revealed significant differences only between females from the reference site and those from site B. Concerning VN (Fig. 18), the lowest values for that type of nuclear abnormality were recorded in the reference site, for both sexes in both seasons (female from AW—0.12, SS—0.03; male from AW—0.04, SS—0.06‰), while the highest values were noted in AW female (19.57‰) and SS male (20.13‰) from the site A and AW male (23.71‰) and SS female (25.03‰) from the site B. Regardless these facts, the observed values in the reference site were always significantly lower compared with both other sites (site A and site B), for both sexes and seasons.

In general, the CVs showed high interindividual variability for all examined parameters. The CVs for Le ranged from 40 to 133%, values that were also observed for ISN (from 43 to 133%). Variation of VN was even somewhat higher than that of the two previous parameters (from 53 to 162%). The MN and BN revealed the highest variance; CVs for MN ranged from 95 up to 400%, while for BN from 75 to 331%.

Correlation analyses

Linear correlation analyses were investigated in females and males separately. This type of analyses was conducted to check associations between average values of blood

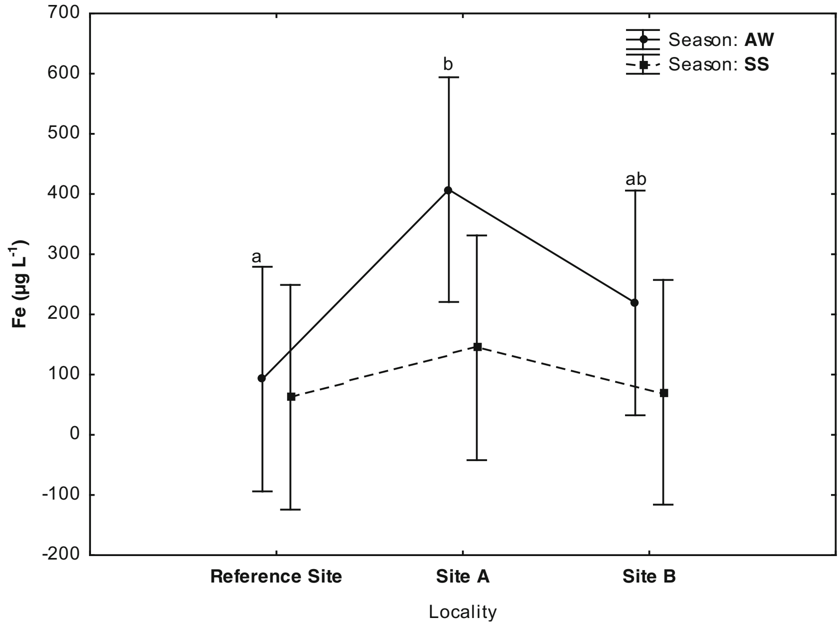


Fig. 8 The concentration of Fe in the water of the sampling sites in the River Bregalnica in AW and SS seasons. The data are expressed as mean, and *vertical bars* denote a 0.95 confidence interval

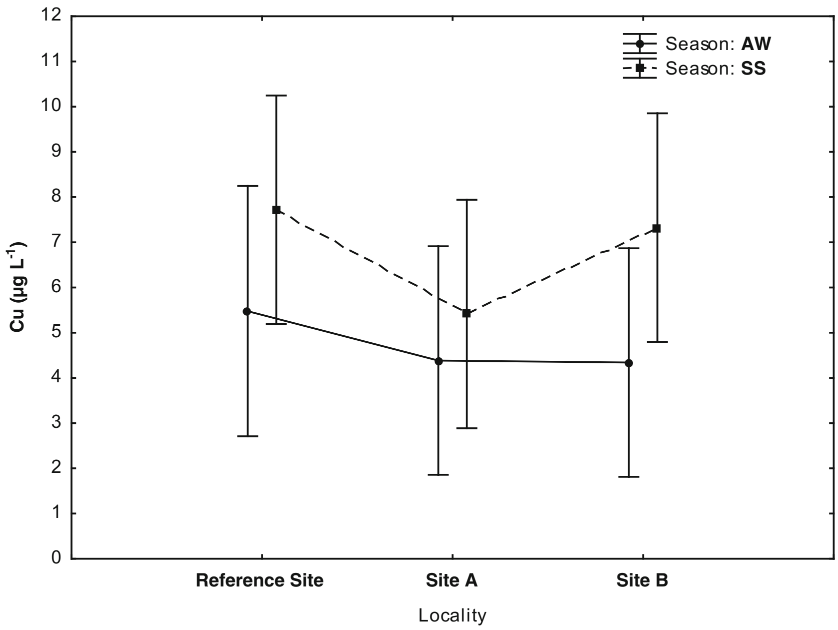


Fig. 9 The concentration of Cu in the water of the sampling sites in the River Bregalnica in AW and SS seasons. The data are expressed as mean, and *vertical bars* denote a 0.95 confidence interval

parameters vs. average values of physicochemical parameters of the water. We also investigated correlation between average values of blood parameters vs. average values of concentration of metals in the water and metals in sediments. All linear correlation analyses were made regarding the localities and seasons. The correlation coefficients and respective statistical significance are displayed in Table 3. In summary, in females, data show that (1) MN was strongly and positively correlated with sulfates, nitrates, nitrites, alkalinity, CO_3, HCO_3, and hardness; (2) VN were positively correlated only with nitrites; and (3) ISN was strongly correlated with the content of Cd, Mn, Pb, and Zn in the sediment. In males, (1) MN was strongly positively correlated with sulfates, nitrites, alkalinity, CO_3, HCO_3, hardness, and contents of Mn and Zn in the sediment; (2) ISN also strongly positively correlated with nitrites and sulfates; (3) Le revealed strong positive correlations with the content of Cd, Mn, Pb, and Zn in the sediment; and (4) VN was strongly (but now negatively) correlated with Cu in the water.

Discussion

The River Bregalnica is considered one of the most polluted rivers in the Republic of Macedonia, due to collection of the wastewater from households and industry from the bigger

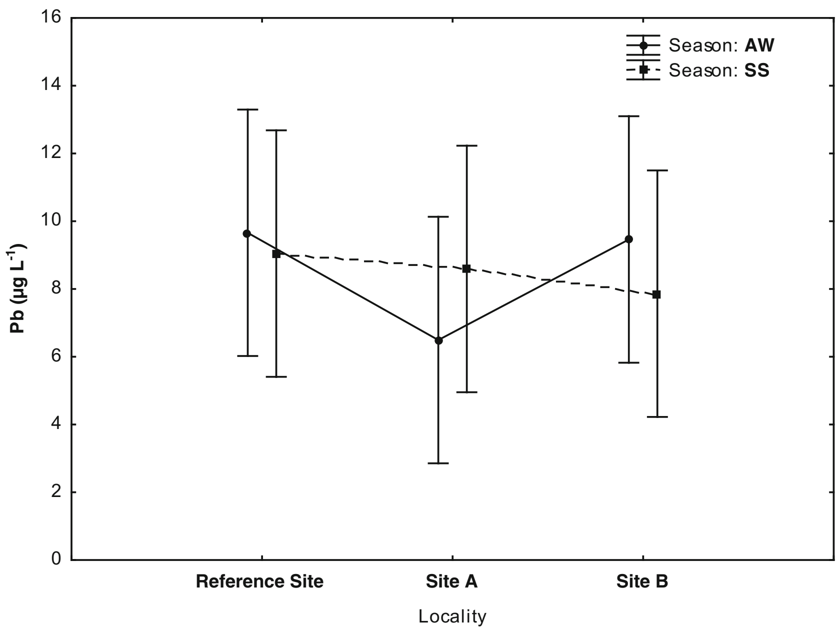


Fig. 10 The concentration of Pb in the water of the sampling sites in the River Bregalnica in AW and SS seasons. The data are expressed as mean, and *vertical bars* denote a 0.95 confidence interval

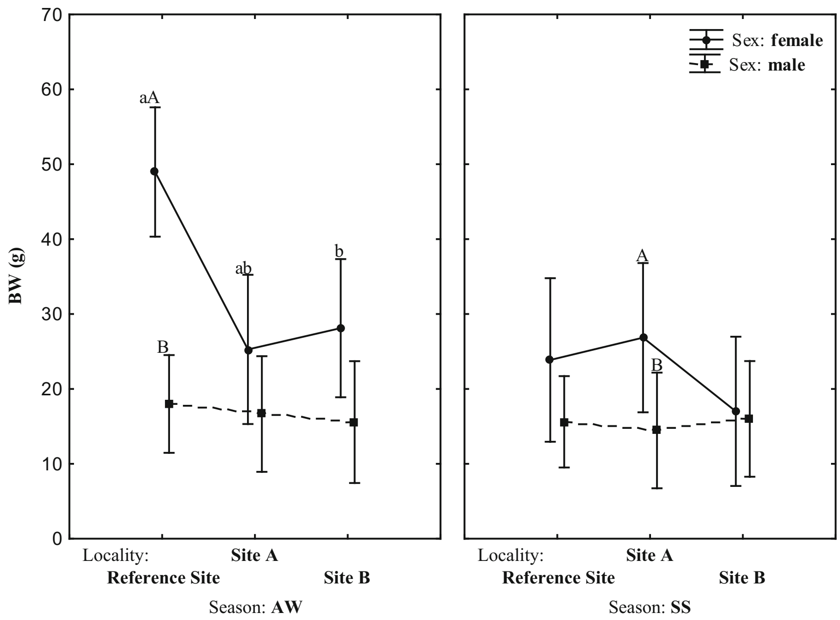


Fig. 11 Changes in the BW, of the female and male barbels sampled from the selected sites and in different seasons. The data are expressed as mean, and *vertical bars* denote a 0.95 confidence interval

cities, such as Berovo, Delcevo, Kocani, and Stip, and according to Dimitrovska et al. (2012), the water quality was below the stipulated national regulation. In line with the latter, the use of Macedonian water quality standards (Regulation for Water Classification and Regulation for Categorization of Watercourses, Lakes, Accumulations and Underground Waters, Official Gazette of the Republic of Macedonia, 1999) allowed a rating of second category for the upper part of the River Bregalnica (reference site) and of the third category for the middle and lower parts of the river (sites A and B).

According to the physicochemical parameters in our study, the values obtained for some of the measured parameters are in accordance with the Macedonian standards. For example, pH, DO, and O_2 saturation were within the allowed values for this watercourse category. However, this investigation revealed that several measured parameters, such as the concentration of the nitrites, and Cd, Mn, and Fe in the water exceeded the legal requirements. Namely, there was an increased concentration of nitrogen compounds (mainly nitrites) in sites A and B that indicated a presence of organic compounds and/or unsatisfactory microbiological quality in the water. The intensive agricultural activities along the middle and lower courses of the water could be contributing to the higher concentration of nitrogen compounds. Moreover, the middle and lower parts of the River Bregalnica were characterized with increased hardness and sulfates, which could be

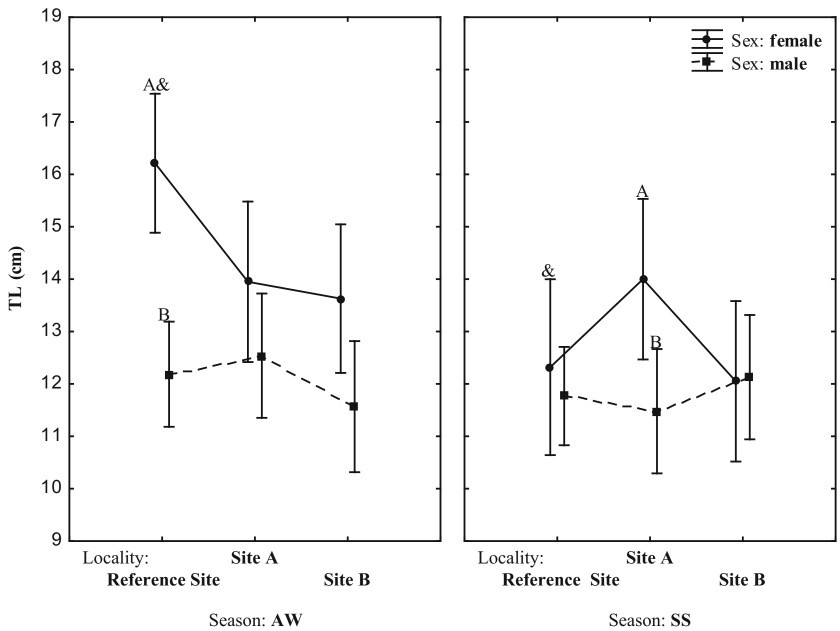


Fig. 12 Changes in the TL, of the female and male barbels sampled from the selected sites and in different seasons. The data are expressed as mean, and *vertical bars* denote a 0.95 confidence interval

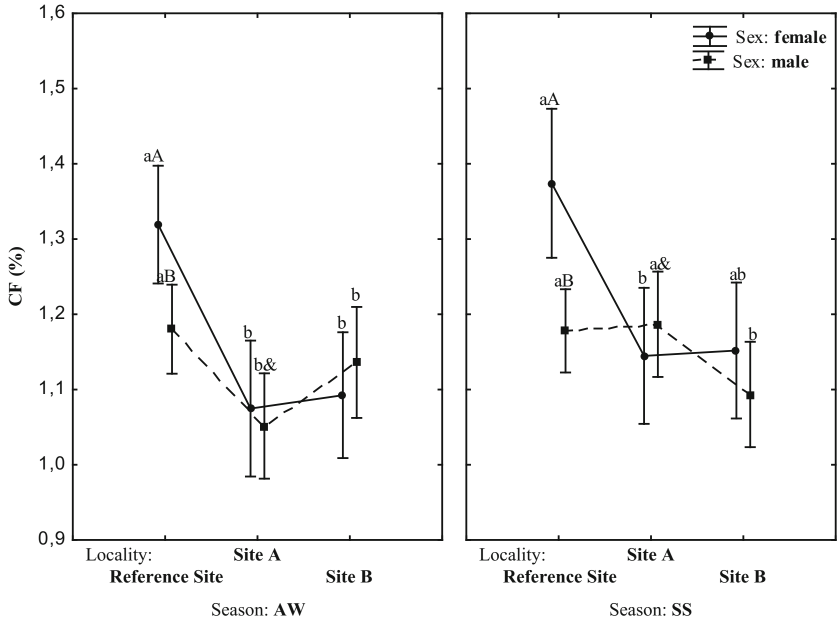


Fig. 13 Changes in the CF, of the female and male barbels sampled from the selected sites and in different seasons. The data are expressed as mean, and *vertical bars* denote a 0.95 confidence interval

interpreted as a mineral component of the river water, because these variables had a common origin in rock dissolution and soils, but higher concentrations for the sulfates also indicated anthropogenic inputs to total solutes (Li et al. 2011).

There are investigations in which differences in morphometric parameters were observed between fish from polluted and unpolluted sites. For example, in the study of George-Nascimento et al. (2000), *Paralichthys* spp. collected from the control site were significantly greater in length and BW than those collected from polluted sites. In our study, lower water quality recorded in lower parts of the river was associated with the reduced BW, noted in females from site B, compared with females from the reference site in AW season. In the investigated barbel, beside locality, seasonal and sex influence was noticed for the morphometric parameters (BW and TL). As it was expected, females were heavier and longer than males in all localities, although significantly only in the reference site in AW and in site A in SS. The same differences in the length and weight of the females and males barbel were already noticed (Šorić and Janković 1989; Šorić 1992; Vasiliou and Economidis 2005). In barbel, sexual maturation in males takes place earlier than in females, which may be the reason for the smaller body size of the males (Vasiliou and Economidis 2005). In fact, earlier maturation and smaller body size in males than in females are common in many fish species (Vasiliou and Economidis 2005). Unlike the

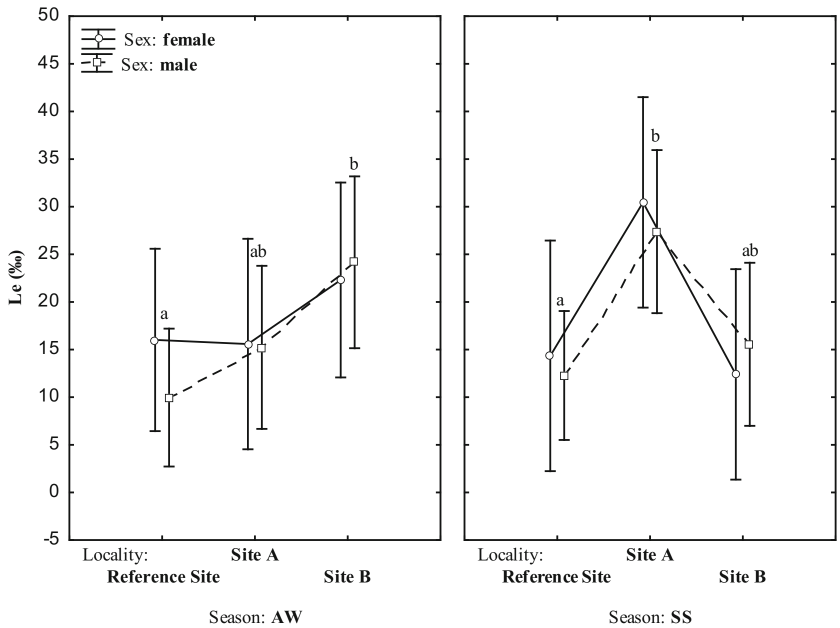


Fig. 14 Changes in the Le frequency of the female and male barbels sampled from the selected sites and in different seasons. The data are expressed as mean, and *vertical bars* denote a 0.95 confidence interval

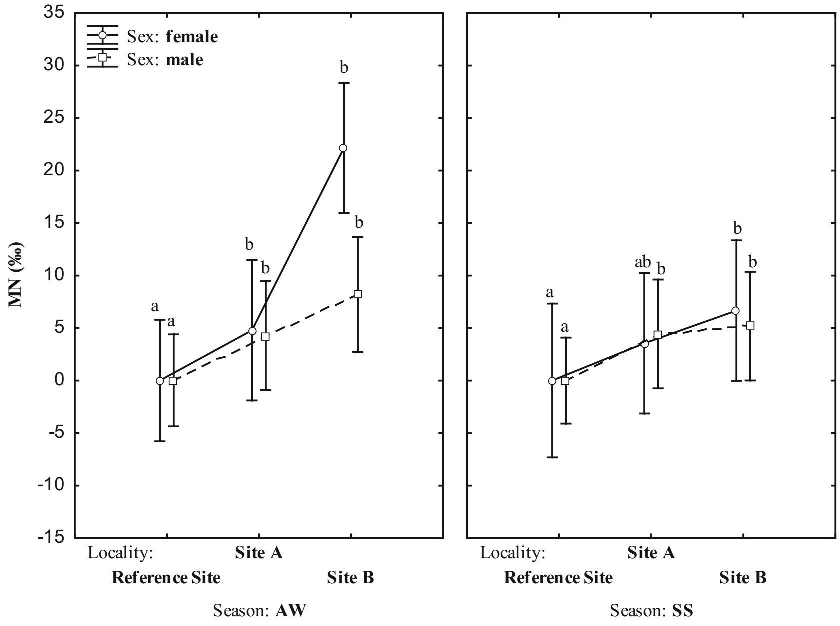


Fig. 15 Changes in the MN frequency of the female and male barbels sampled from the selected sites and in different seasons. The data are expressed as mean, and *vertical bars* denote a 0.95 confidence interval

morphometric parameters, fish from polluted sites (sites A and B) revealed significantly lower condition regardless the season or sex. This is not an isolated case since decreased CF was also reported for other barbel species collected from the polluted areas. For example, significantly lower CF was detected for *Barbus bocagei* collected from river Tajo, which has been under influence of industrial, urban, and agricultural activities (Carballo et al. 2004), and for *Barbus meridionalis* collected from the river Ripoll, at sites located after point sources of urban and metallurgical industry effluents (Maceda-Veiga et al. 2010). As it can be noticed, the sources of pollution in the aforementioned investigations have similarities with the kind of pollution in our polluted localities, so we can assume that lower CF in our barbel is, at least partly, provoked by direct pollution input in the river.

The blood analyses are in line with the CF data and respective conclusions. The frequencies of Le in the blood smears do not seem to result from influences of natural factors as sex or season. Our results have parallels in the literature, as for example, no significant sex differences in blood parameters were observed in *Cichlasoma dimerus* (Vázquez and Guerrero 2007), *Centropomus parallelus* (Santos et al. 2009), and *Rutilus kutum* (Nikoo et al. 2010). However, the results from our study show that regarding the localities, the fish likely experienced stimuli that elevated the Le frequency, despite being statistically significantly only in males. Males from SS

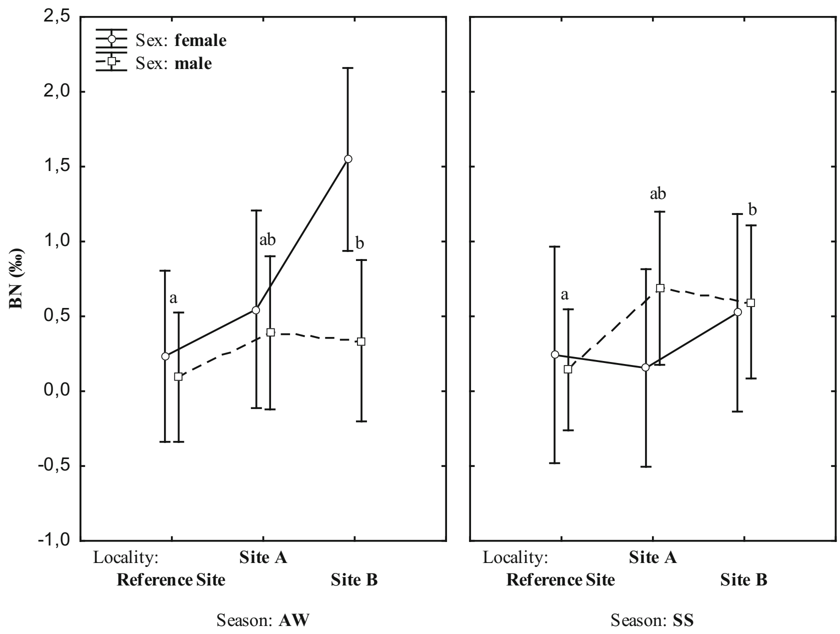


Fig. 16 Changes in the BN frequency of the female and male barbels sampled from the selected sites and in different seasons. The data are expressed as mean, and *vertical bars* denote a 0.95 confidence interval

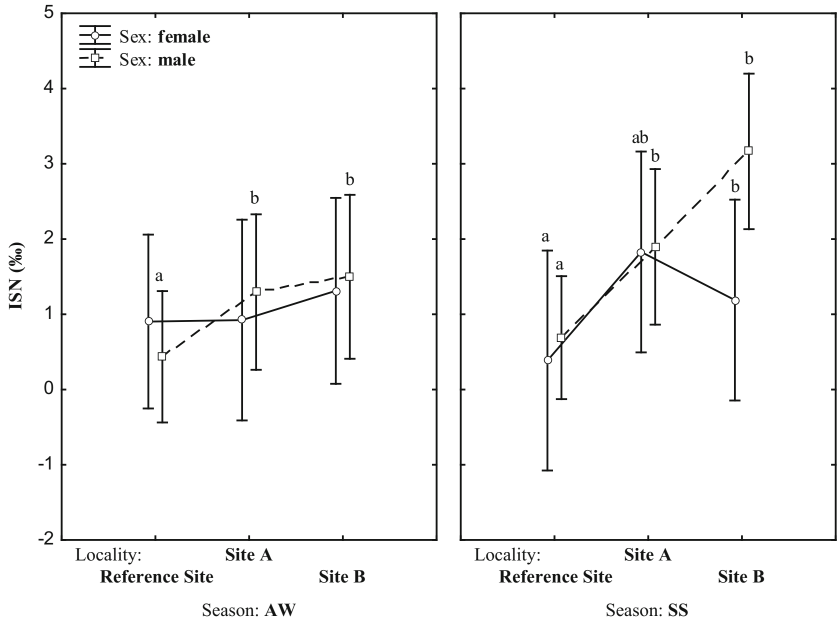


Fig. 17 Changes in the ISN frequency of the female and male barbels sampled from the selected sites and in different seasons. The data are expressed as mean, and *vertical bars* denote a 0.95 confidence interval

at site A and AW at site B revealed significantly higher frequencies of Le than those from the reference site. Those greater values could be at least partly attributed to the increased water temperature, especially at site B, where the geothermal system of Kezhovica-Ldzhi is situated, at the right bank of the River Bregalnica (Ramani et al. 2014). In some other fish species, significant seasonal variations were associated with increased water temperature and reduced photoperiod, both of which inducing leukocytosis (Guijarro et al. 2003; Kavadias et al. 2004).

Although the influence of natural factors cannot be excluded, the anthropogenic pressure and associated pollution impacts present at sites A and B offer a most logical causative explanation for the changes. Indeed, we found a strong positive correlation between male total Le frequency and the content of the heavy metals (Cd, Mn, Pb, and Zn) in the sediment. Significant differences registered in males could be connected with the animal feeding status (Joshi 1980) or species-specific characteristic (Pavlidis et al. 2007). Despite correlation is not necessarily causation, it is apparent that among all other factors, the elevated metal content in the sediment registered in middle and lower parts of the river may be one causative agent for the increased Le. It is well established that white blood cells act as a primary defense line (Tierney et al. 2004), namely for fighting infections (Mekkawey et al. 2011). According to Ates et al. (2007), metal toxicity can stimulate the immune

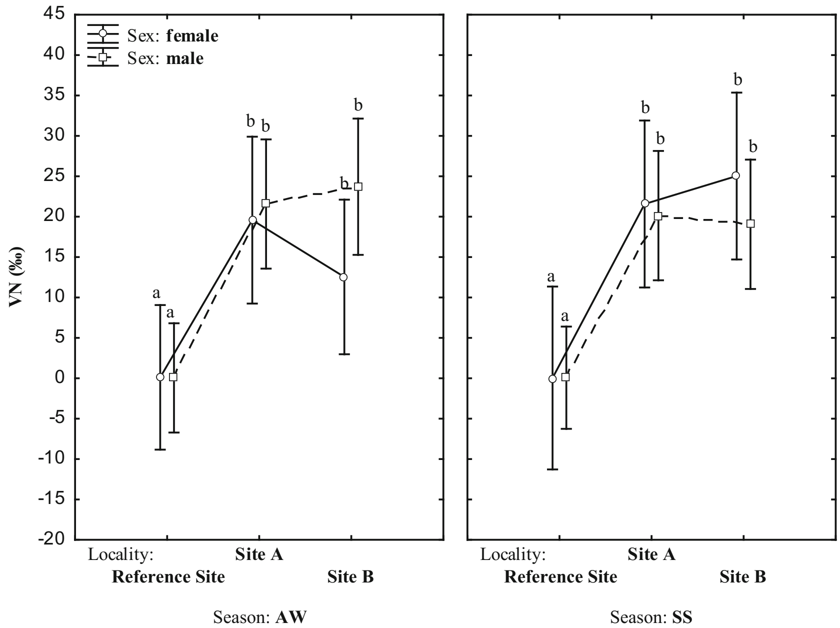


Fig. 18 Changes in the VN frequency of the female and male barbels sampled from the selected sites and in different seasons. The data are expressed as mean, and *vertical bars* denote a 0.95 confidence interval

Table 3 The results from correlation analyses[1] between blood parameters in female and male barbels vs. physicochemical and metal analyses from the water and the sediment

Variables	Spearman's Rho	*p* value
	Female barbel	
MN (‰) vs. Sulfates (mg L^{-1})	+0.89	<0.05
MN (‰) vs. nitrates (mg L^{-1})	+0.83	<0.05
MN (‰) vs. nitrites (mg L^{-1})	+0.83	<0.05
MN (‰) vs. alcalinity (meq L^{-1})	+0.94	<0.01
MN (‰) vs. CO_3 (mg L^{-1})	+0.94	<0.01
MN (‰) vs. HCO_3 (mg L^{-1})	+0.94	<0.01
MN (‰) vs. hardness (°dH)	+0.94	<0.01
VN (‰) vs. nitrites (mg L^{-1})	+0.83	<0.05
ISN (‰) vs. Cd (mg kg^{-1})	+0.94	<0.01
ISN (‰) vs. Mn (mg kg^{-1})	+0.94	<0.01
ISN (‰) vs. Pb (mg kg^{-1})	+0.94	<0.01
ISN (‰) vs. Zn (mg kg^{-1})	+0.94	<0.01
	Male barbel	
MN (‰) vs. sulfates (mg L^{-1})	+0.89	<0.05
MN (‰) vs. nitrites (mg L^{-1})	+0.94	<0.01
MN (‰) vs. alcalinity (meq L^{-1})	+0.94	<0.01
MN (‰) vs. CO_3 (mg L^{-1})	+0.94	<0.01
MN (‰) vs. HCO_3 (mg L^{-1})	+0.94	<0.01
MN (‰) vs. hardness (°dH)	+0.94	<0.01
MN (‰) vs. Mn (mg kg^{-1})	+0.94	<0.01
MN (‰) vs. Zn (mg kg^{-1})	+0.94	<0.01
VN (‰) vs. Cu (μg L^{-1})	-0.83	<0.05
ISN (‰) vs. sulfates (mg L^{-1})	+0.94	<0.01
ISN (‰) vs. nitrites (mg L^{-1})	+0.89	<0.05
Le (‰) vs. Cd (mg kg^{-1})	+0.89	<0.05
Le (‰) vs. Mn (mg kg^{-1})	+0.89	<0.05
Le (‰) vs. Pb (mg kg^{-1})	+0.89	<0.05
Le (‰) vs. Zn (mg kg^{-1})	+0.89	<0.05

[1] Only significant correlations are presented

system and trigger blood leukocytosis. Also, an increase in the number of Le was deemed to be a normal reaction of fish against exposure to foreign substances, such as copper (Nussey et al. 1995; Mazon et al. 2002). However, we should bear in mind that in sites A and B, agriculture additionally burdens the river with pesticides (Stipaničev et al. 2017) and that many investigations revealed altered Le counts derived of pollution from agricultural activities. A rise in the total Le count has been reported for *Heteropneustes fossilis*, exposed to sublethal amounts of fenthion (Srivastava and Mishra 1983) and deltamethrin (Kumar et al. 1999). The same rise was reported for *Labeo rohita* fingerlings, exposed to sublethal concentrations of cypermethrin and carbofuran (Das and Mukherjee 2003; Adhikari et al. 2004), and for *Clarias albopunctatus* fingerlings, exposed to sublethal doses of the insecticide actellic (Mgbenka et al. 2005). However, exposure of teleosts to different classes of pesticides may cause not only increases, but also decreases in the differential count of Le (Singh and Srivastava 2010). Anyway, taking all the facts, the elevated Le frequency herein more likely resulted from cumulative effects of local toxicant stressors.

Unlike Le, the barbel erythrocyte MN frequency had stronger variations along the river course, with higher values seen in both seasons and sexes at sites A and B. Accordingly, barbel collected from the reference site revealed the lowest frequency of the MN in the erythrocytes, which varied within narrow limits (from 0.1 to 0.3‰). Anyway, when interpreting the trend, we must bear in mind that in a field study of wild fish, an inherent weakness is the lack of a true negative control (Çavaş and Ergene-Gözükara 2005). A rate of MN spontaneous induction very close to our findings was registered for *Barbus plebejus* (0.5‰) in a control fish group obtained by artificial reproduction and kept in well water (open system) (Minissi et al. 1996). Also, in *Barbus barbus* from the Danube River, a baseline rate of only 0.6‰ was reported (Boettcher et al. 2010). The low spontaneous MN frequency at our reference site thus supports that the barbels from that location constitute an appropriate control group.

As for other biomarkers of pollution, besides knowing the normal range of spontaneous MN induction, the variations associated with natural factors should also be taken into consideration, when doing applications in situ (Baršienė et al. 2006). Herein, season and fish sex seem not to have major influences on the frequency of erythrocyte MN in any of the investigated localities. The most remarkable result was that the MN frequency appears to be related to the localities examined and consequently suggesting that poorer water and sediment quality may be the basis of the increased values. As we already mentioned, the source of pollution in sites A and B originates from multiple toxic entries in those parts of the river. So, different types of pollution at each site may be the reason for the observed trends in the MN frequencies. Site A is considered the most polluted locality in our study, having the highest heavy metal content in sediments (especially in SS) and being subjected to an additional input of domestic wastewater and agricultural runoff. Yet, the MN‰ at site A (despite seeming lower on a first glance) did not significantly differ from site B. The no difference may be because under field conditions, genotoxicants tend to adsorb onto particulate matter, and so, the sediment compartment is the intermediate or final receptor of insoluble or slightly soluble pollutants (Rocha et al. 2009; Boettcher et al. 2010). Additionally, the impact of mixtures can blur simplistic dose-response patterns. For instance, Costa et al. (2008) noticed that organic contaminants in sediments have stronger genotoxic potency than heavy metals, but mixed influences of these two pollutants may result in lower genotoxic stress. These authors suggested that metals (such as Cu, As, Cd, and Pb) may have an inhibitory effect on genotoxicity when interacting with organic contaminants, at least during early exposure. Also, Ossana and Salibián (2013) stated that incidentally increasing of both organic and inorganic contaminants in addition to metal pollution could contribute to increases in the frequency of MN. In summary, the MN frequency may vary according to the type of pollution (Ali et al. 2008). Here, analyses suggest that increased metal content in sediments was probably not the only reason for more numerous MN. We can suspect about this from the positive correlations between the MN and sulfates, nitrites, alkalinity, CO_3, HCO_3, and hardness, for both sexes, and nitrates for females.

Our results for erythrocyte nuclear abnormalities (BN, ISN, and VN) followed the MN trends, namely because they were influenced by locality only. In general, there were higher frequencies of abnormalities in localities downstream from the reference site. Such pattern attained all the abnormalities, in males, and for both seasons. In females, higher values in middle and lower parts of the river existed for VN (both seasons) and ISN (SS). These somewhat different patterns of males and females were noted also in correlation analyses, where various types of associations exist, especially for ISN (with metals from the sediment in females and with nitrites and sulfates in males). The various patterns of males versus females need future investigations, namely considering the scarcity of data about sex differences in the frequency of nuclear abnormalities other than MN. To our knowledge, variations between sexes were observed only for MN—being attributed to female hormonal status (Al-Sabti and Metcalfe 1995)—as a result of decreasing the body burden of lipophilic mutagens during the spawning period (Huges and Hebert 1991) or as a result of higher susceptibility of males (Liu et al. 2013).

Irrespective of the previously mentioned and considering the use of erythrocyte nuclear abnormalities as proxies of genotoxicity (Carrola et al. 2014), our global data support that harmful substances entering the studied lower part of the river evoke genotoxic stress in barbel (and likely in other fish). Anyway, unlike the MN, the mechanisms underlying the emergence of most nuclear abnormalities in fish erythrocytes have not yet been explained. Likewise, there is still no consensus about the type of morphological nuclear irregularities that may also result from genotoxic stress and that should be considered as MN analogous (Ayllón and Garcia-Vazquez 2000), making the results obtained in different investigations difficult to compare. In spite of this, some authors (Ayllón and Garcia-Vazquez 2000; Çavaş and Ergene-Gözükara 2003, 2005; Baršienė et al. 2006, 2015) defend that morphological nuclear alterations other than MN are offering complementary information for genotoxicity analyses in blood erythrocytes and in other cell types and should be included in routine genotoxicity tests.

Conclusions

In conclusion, increased frequency of MN and of other nuclear abnormalities in blood erythrocytes, together with that of Le at the most polluted sites A and B, as a prime consequence of the influence of the localities, and not of the animals' sex or year season, confirms that lower course of the River Bregalnica receives significant genotoxic pollution. Along the similar line are higher metal concentrations of Mn, Cd, and Fe in the water in sites A and B than allowed third category rating according to the Macedonian Water Quality Standards (Official Gazette, 1999). Supplementary, in these localities, agricultural activities as well as urban and industrial runoff contribute to the overall genotoxic effect. Moreover, the correlation analyses revealed that higher values for blood parameters are, at least in part, linearly associated with metals in the sediment and increased values for most water physicochemical parameters. In this vein, the results of this study further support published works stating that the frequency of MN and nuclear abnormalities are sensitive and suitable biomarkers for genotoxic pollution when used in monitoring studies. Finally, we can state that the benthophagous barbel, abundant in a wide zoogeographic distribution, being in close contact with sediments, and showing some good biomarker correlations with loads of some heavy metals, is here reinforced as a suitable sentinel fish for studying in situ genotoxic pollution in the Balkans.

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References

[APHA] American Public Health Association (1992) Standard methods for the examination of water and wastewater, 18th edn. Washington (DC)

Adhikari S, Sarkar B, Chatterjee A, Mahapatra CT, Ayyappan S (2004) Effects of cypermethrin and carbofuran on certain hematological parameters and prediction of their recovery in a freshwater teleost, *Labeo rohita* (Hamilton). Ecotox Environ Safe 58:220–226

AI-Sabti K, Metcalfe CD (1995) Fish micronuclei for assessing genotoxicity in water. Mutat Res 343:121–135

Ali FK, El-Shehawi AM, Seehy MA (2008) Micronucleus test in fish genome: a sensitive monitor for aquatic pollution. Afr J Biotech 7(5):606–612

Andreevska D, Menkovska M, Andov D (2013) Overview of the current condition, in production, consumption and the research potential of the rice crop in the Republic of Macedonia. Macedonian J Anim Sci 3(2):219–228

Ates B, Orun I, Talas ZS, Durmaz G, Yilmaz I (2007) Effects of sodium selenite on some biochemical and hematological parameters of rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) exposed to Pb^{2+} and Cu^{2+}. Fish Physiol Biochem 34(1):53–59

Ayllón F, Garcia-Vazquez E (2000) Induction of micronuclei and other nuclear abnormalities in European minnow *Phoxinus phoxinus* and mollie *Poecilia latipinna*: an assessment of the fish micronucleus test. Mutat Res 467:177–186

Balabanova B, Stafilov T, Šajn R, Bačeva K (2011) Distribution of chemical elements in attic dust as reflection of lithology and anthropogenic influence in the vicinity of copper mine and flotation. Arch Environ Contam Toxicol 61(2):173–184

Balabanova B, Stafilov T, Šajn R, Bačeva K (2013) Spatial distribution and characterization of some toxic metals and lithogenic elements in topsoil and subsoil from copper mine environs. Int J Environ Prot 3(9):1–9

Barišić J, Dragun Z, Ramani S, Filipović Marijić V, Krasnići N, Čož-Rakovac R, Kostov V, Rebok K, Jordanova M (2015) Evaluation of histopathological alterations in the gills of Vardar chub (*Squalius vardarensis* Karaman) as an indicator of river pollution. Ecotox Environ Safe 118:158–166

Baršiené J, Butrimavičiené L, Michailovas A, Grygiel W (2015) Assessing the environmental genotoxicity risk in the Baltic Sea: frequencies of nuclear buds in blood erythrocytes of three native fish species. Environ Monit Assess 187(1):4078. doi:10.1007/s10661-014-4078-x

Baršiené J, Dedonytė V, Rybakovas A, Andreikėnaitė L, Andersen OK (2006) Investigation of micronuclei and other nuclear abnormalities in peripheral blood and kidney of marine fish treated with crude oil. Aquat Toxicol 785:599–5104

Boettcher M, Grund S, Keiter S, Kosmehl T, Reifferscheid G, Seitz N, Rocha PS, Hollert H, Braunbeck T (2010) Comparison of *in vitro* and *in situ* genotoxicity in the Danube River by means of the comet assay and the micronucleus test. Mutat Res Gen Toxicol Environ Mutagen 700(1–2):11–17

Carballo M, Jiménez JA, de la Torre A, Roset J, Muñoz MJ (2004) A survey of potential stressor-induced physiological changes in carp (*Cyprinus carpio*) and barbel (*Barbus bocagei*) along the Tajo River. Environ Toxicol 20(2):119–125

Carrasco K, Tilbury KL, Myers MS (1990) Assessment of the piscine micronucleus test as an in situ biological indicator of chemical contaminant effects. Can J Fish Aquat Sci 47:2123–2136

Carrola J, Santos N, Rocha MJ, Fontainhas-Fernandes A, Pardal MA, Monteiro RA, Rocha E (2014) Frequency of micronuclei and of other nuclear abnormalities in erythrocytes of the grey mullet from the Mondego, Douro and Ave estuaries—Portugal. Environ Sci Pollut Res Int 21(9):6057–6068

Çavaş T, Ergene-Gözükara S (2003) Evaluation of the genotoxic potential of lambda-cyhalothrin using nuclear and nucleolar biomarkers on fish cells. Mutat Res 534:93–99

Çavaş T, Ergene-Gözükara S (2005) Micronucleus test in fish cells: a bioassay for in situ monitoring of genotoxic pollution in the marine environment. Environ Mol Mutagen 46:64–70

CEN EN 14011:2003 Water analysis—fishing with electricity for wadable and non-wadable rivers. European Committee for Standardization

Costa PM, Lobo J, Caeiro S, Martins M, Ferreira AM, Caetano M, Vale C, DelValls Á, Costa MH (2008) Genotoxic damage in *Solea senegalensis* exposed to sediments from the Sado Estuary (Portugal): effects of metallic and organic contaminants. Mutat Res 654:29–37

Das BK, Mukherjee SC (2003) Toxicity of cypermethrin in *Labeo rohita* fingerlings: biochemical, enzymatic and haematological consequences. Comp Biochem Physiol C 134:109–121

Dimitrovska O, Markoski B, Apostolovska Toshevska B, Milevski I, Gorin S (2012) Surface water pollution of major rivers in the Republic of Macedonia. Procedia Environ Sci 14:32–40

Ergene S, Çavaş T, Çelik A, Köleli N, Kaya F, Karahan A (2007) Monitoring of nuclear abnormalities in peripheral erythrocytes of three fish species from the Goksu Delta (Turkey): genotoxic damage in relation to water pollution. Ecotoxicology 16:385–391

Facey DE, Blazer VS, Gasper MM, Turcotte CL (2005) Using fish biomarkers to monitor improvements in environmental quality. J Aquat Anim Health 17:263–266

Fuzinatto CF, Flohr L, Melegari SP, Matias WG (2013) Induction of micronucleus of *Oreochromis niloticus* exposed to waters from the Cubatão do Sul River, southern Brazil. Ecotox Environ Safe 98: 103–109

George-Nascimento M, Khan RA, Garcias F, Lobes V, Murñoz G, Valdebenito V (2000) Impaired health in flounder, *Paralichthys* spp. inhabiting coastal Chile. Bull Environ Contam Toxicol 64: 184–190

Georgiev S (1998) Key for determination of fish (Osteichthyes) and cephalospidomorphs (Cephalaspidomorpha) from R. Macedonia. Institute of Animal Sciences, Skopje, p 178 (in Macedonian)

Guijarro AI, Lopez-Patinõ MA, Pinillos ML, Isorna E, De Pedro N, Alonso-Gomez A-BM, Delgado MJ (2003) Seasonal changes in haematology and metabolic resources in the tench. J Fish Biol 62: 803–815

Huges JB, Hebert AT (1991) Erythrocyte micronuclei in winter flounder (*Pseudopleuronectes americanus*): results of field surveys during 1980-1988 from Virginia to Nova Scotia and in Long Island Sound. Arch Environ Contam Toxicol 20:474–479

Ilić Popov S, Stafilov T, Šajn R, Tánáselia C, Bačeva K (2014) Applying of factor analyses for determination of trace elements distribution in water from river Vardar and its tributaries, Macedonia/Greece. Sci World J Article ID 809253:1–11. doi:10.1155/2014/809253

Jordanova M, Rebok K, Dragun Z, Ramani S, Ivanova L, Kostov V, Valić D, Krasnići N, Filipović Marijić V, Kapetanović D (2016) Histopathology investigation on the Vardar chub (*Squalius vardarensis*) populations captured from the rivers impacted by mining activities. Ecotox Environ Safe 129:35–42

Joshi BD (1980) Sex-related variations in some haematologic values of certain freshwater teleosts. Japanese J Ichthyol 27(2):139–143

Kavadias S, Castritsi-Catharios J, Dessypris A, Milious H (2004) Seasonal variation in steroid hormones and blood parameters in cage-farmed European sea bass (*Dicentrarchus labrax* L.) J Appl Ichthyol 20:58–63

Koca YB, Koca S, Yildiz Ş, Gürcü B, Osanç E, Tunçbaş O, Aksoy G (2005) Investigation of histopathological and cytogenetic effects on *Lepomis gibbosus* (Pisces: Perciformes) in the Çine stream (Aydın/Turkey) with determination of water pollution. Environ Toxicol 20(6):560–571

Kori-Siakpere O, Ake JEG, Idoge E (2005) Haematological characteristics of the African snakehead, *Parachanna obscura*. Afr J Biotechnol 4(6):527–530

Kostov V, Rebok K, Slavevska-Stamenković V, Ristovska M (2010) Fish fauna of River Bregalnica (R. Macedonia)—composition, abundance and longitudinal distribution. Conference BALWOIS 2010. Proceedings of papers. Topics 6 Lakes and Wetlands, 1–8

Kumar S, Lata S, Gopal K (1999) Deltamethrin induced physiological changes in freshwater cat fish *Heteropneustes fossilis*. Bull Environ Contam Toxicol 62:254–258

Leary S, Underwood W, Anthony R, Cartner S, Corey D, Grandin T, Greenacre C, Gwaltney-Brant S, McCrackin MA, Meyer R, Miller D, Shearer J, Yanong R (2013) AVMA guidelines for the euthanasia of animals: 2013 edition. American Veterinary Medical Association, Schaumburg, IL

Li S, Li J, Zhang Q (2011) Water quality assessment in the rivers along the water conveyance system of the Middle Route of the South to North Water Transfer Project (China) using multivariate statistical techniques and receptor modeling. J Hazard Mat 195:306–317

Liu N, Ma M, Xu Y, Zha J, Rao K, Wang Z (2013) Susceptibility of male and female Japanese medaka (*Oryzias latipes*) to 2,4,6-

trichlorophenol-induced micronuclei in peripheral erythrocytes. Front Environ Sci Eng 7(1):77–84
Maceda-Veiga A, Monroy M, Viscor G, De Sostoa A (2010) Changes in non-specific biomarkers in the Mediterranean barbel (*Barbus meridionalis*) exposed to sewage effluents in a Mediterranean stream (Catalonia, NE Spain). Aquat Toxicol 100:229–237
Mazon AF, Monteiro EAS, Pinheiro GHD, Fernandes MN (2002) Hematological and physiological changes induced by short-term exposure to copper in the freshwater fish, *Prochilodus scrofa*. Braz J Biol 62(4A):621–631
Mekkawey IA, Mahmoud UM, Sayed AEH (2011) Effects of 4-nonylphenol on blood cells of the African catfish *Clarias gariepinus* (Burchell, 1822). Tiss Cell 43:223–229
Mgbenka BO, Oluah NS, Arungwa AA (2005) Erythropoietic response and hematological parameters in the catfish *Clarias albopunctatus* exposed to sublethal concentrations of actellic. Ecotoxicol Environ Safe 62:436–440
Milevski I, Blinkov I, Trendafilov A (2008) Soil erosion processes and modeling in the upper Bregalnica catchment. XXIVth Conference of Danubian Countries on the Hydrological Forecasting and Hydrological Bases of Water Management, Bled, Slovenia, Proceedings 1–10
Minissi S, Ciccotti E, Rizzoni M (1996) Micronucleus test in erythrocytes of *Barbus plebejus* (Teleostei, Pisces) from two natural environments: a bioassay for the in situ detection of mutagens in freshwater. Mutat Res 367:245–251
Muranli FDG, Güner U (2011) Induction of micronuclei and nuclear abnormalities in erythrocytes of mosquito fish (*Gambusia affinis*) following exposure to the pyrethroid insecticide lambda-cyhalothrin. Mutat Res Genet Toxicol Environ Mutagen 726:104–108
Nikoo M, Falahatkar B, Rahmani H (2010) Blood parameters of Southern Caspian kutum *Rutilus kutum*. J Appl Ichthyol:1–3. doi:10.1111/j.1439-0426.2010.01588.x
Nussey G, Van Vuren JHJ, Du Preez HH (1995) Effect of copper on blood coagulation of *Oreochromis mossambicus* (Cichlidae). Comp Biochem Physiol C 111:359–367
Osman A, Ali E, Hashem M, Mostafa M, Mekkawy I (2010) Genotoxicity of two pathogenic strains of zoosporic fungi (*Achlya klebsiana* and *Aphanomyces laevis*) on erythrocytes of Nile tilapia *Oreochromis niloticus niloticus*. Ecotoxicol Environ Safe 73:24–31
Ossana NA, Salibián A (2013) Micronucleus test for monitoring the genotoxic potential of the surface water of Luján River (Argentina) using erythrocytes of *Lithobates catesbeianus* tadpoles. Ecotoxicol Environ Contam 8(1):67–74. doi:10.5132/eec.2013.01.010
Pavlidis M, Futter W, Katharios P, Divanach P (2007) Blood cell profile of six Mediterranean mariculture fish species. J Appl Ichthyol 23:70–73
Pierson PM, Lamers A, Flik G, Mayer-Gostan N (2004) The stress axis, stanniocalcin, and ion balance in rainbow trout. Gen Comp Endocrinol 137:263–271
Ramani S, Dragun Z, Kapetanović D, Kostov V, Jordanova M, Erk M, Hajrulai-Musliu Z (2014) Surface water characterization of three rivers in the lead/zinc mining region of northeastern Macedonia. Arch Environ Contam Toxicol 66:514–528
Regulation for Water Classification and Regulation for categorization of Watercourses, Lakes, Accumulations and Underground waters (1999) Official Gazette of Republic of Macedonia 18/99
Rocha E, Rocha JM, Galante HM, Silva WM, Monteiro AFR (2009) The hepatocytes of the brown trout (*Salmo trutta* F. *fario*): a stereological study of their number and size during the breeding cycle. Ichthyol Res 56:43–54
Santos AA, Egami MI, Ranzani-Paiva MJT, Juliano Y (2009) Hematological parameters and phagocytic activity in fat snook (*Centropomus parallelus*): seasonal variation, sex and gonadal maturation. Aquaculture 296:359–366
Serafimovska JM, Arpadjan S, Stafilov T, Popov IS (2011) Dissolved inorganic antimony, selenium and tin species in water samples from various sampling sites of river Vardar in Macedonia and Greece. Maced J Chem Chem Eng 30(2):181–188
Singh NN, Srivastava AK (2010) Haematological parameters as bioindicators of insecticide exposure in teleosts. Ecotoxicol 19:838–854
Šorić V (1992) The structure of population and reproductive potential of *Barbus peloponnesius* petenyi (Cyprinidae, Pisces) in the Gruza River. Collection of Scientific Papers, Faculty of Science Kragujevac 13:65–75 (In Serbian)
Šorić V, Janković D (1989) Characteristics of growth and sexual maturity of *Barbus meridionalis*. Ichthyologia 21(1):27–37 (In Serbian)
Srivastava AK, Mishra J (1983) Effects of fenthion on the blood and tissue chemistry of a teleost fish (*Heteropneustes fossilis*). J Comp Path 93:27–31
Stafilov T, Peeva L, Nikov B, De Koning A (2009) Industrial hazardous waste in the Republic of Macedonia. Applied environmental geochemistry—anthropogenic impact on human environment in the SE Europe, Ljubljana, Proceedings Book, Šajn R, Žilbert G, Alijagić J (ed), ISBN 978–961-6498-18-0. 108–112
Stipaničev D, Dragun Z, Repec S, Rebok K, Jordanova M (2017) Broad spectrum screening of 463 organic contaminants in rivers in Macedonia. Ecotoxicol Environ Safe 135:48–59
Strunjak-Perovic I, Coz-Rakovac R, Topic Popovic N, Jadan M (2008) Seasonality of nuclear abnormalities in gilthead sea bream *Sparus aurata* (L.) erythrocytes. Fish Physiol Biochem 35(2):287–291
Tierney KB, Farrell AP, Kennedy CJ (2004) The differential leucocyte landscape of four teleosts: juvenile *Oncorhynchus kisutch*, *Clupea pallasi*, *Culaea inconstans* and *Pimephales promelas*. J Fish Biol 65:906–919
Vasiliou A, Economidis PS (2005) On the life-history of *Barbus peloponnesius* and *Barbus cyclolepis* in Macedonia, Greece. Folia Zool 54(3):316–336
Vázquez GR, Guerrero GA (2007) Characterization of blood cells and hematological parameters in *Cichlasoma dimerus* (Teleostei, Perciformes). Tiss Cell 39:151–160