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Original Article

Gonadal sensitivity of *Barbus balcanicus* Kotlik, Tsigenopoulos, Rab & Berberi, 2002 (Actinopterygii: Cypriniformes) to aquatic pollution in Bosnia and Herzegovina

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Abstract: Growing pressures from aquatic pollution and the increasing vulnerability of aquatic organisms lead to frequent disturbances in the health of aquatic ecosystems. This study aimed to investigate the gonadal tissue of the fish *Barbus balcanicus* as a tool to identify the effects of water contamination on freshwater organisms. Within this context, quantitative analyses of the elements Cd, Pb, Ni, Fe, Mn, Zn, and Cu in water, sediment, and gonads, microbiological analyses of water, and histopathological evaluations of gonad tissue were performed. Two different areas (S1 and S2) of the Miljacka River in Bosnia and Herzegovina were investigated. The results from Colilert-18 and Enterolert revealed that the water quality at S2 poses extremely high risks of total and fecal coliform contamination. The results also showed significant differences in heavy metal levels in the water, sediment, and inside fish gonads. Importantly, average concentrations of toxic heavy metals Cd and Pb in water at S2 were higher than the permissible values, respectively. As a result, higher values of metals in gonadal tissues and the metal pollution index were determined at site S2 in addition to severe histopathological alterations. Higher values of Cu, Fe, Zn, Cd, Ni, and Pb were recorded in both locations in testicle tissue compared to the ovaries. Nearly all bioaccumulation values in the testes are higher than in the ovary. Lastly, histopathological alterations were slightly higher in individuals from S2 than in those from S1. From the perspective of these findings, fish gonad sensitivity to pollutants and environmental disturbances could be useful indicators of environmental health.

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Introduction

Current evidence supports the strong anthropogenic pressure on many natural ecosystems. Observing biological diversity, the freshwater fish fauna often stands out as the most affected by human activities (Zeng et al., 2022). Anthropogenic factors affecting the ichthyofauna refer to agricultural, industrial, and municipal wastewater pollution. Fish, as bioindicator species, play a significant role in assessing and monitoring water quality because they are highly sensitive to changes in aquatic environments. However, bioindicator organisms may indicate long-term changes in water ecosystems (Tokatli et al., 2020). Long-term accumulation of heavy metals from contaminated environments in fish bodies compromises the development and function of numerous tissues and organs, including reproductive organs. For instance, the most severe negative impact on the fish population comes from industrial and communal wastewater sources, which cause significant mortality among sexually mature individuals and juveniles (Lemmers et al., 2023). All these factors should be viewed as integral because they are interconnected, and their negative consequences are unpredictable in the overall evaluation of the fish stock.

Heavy metal pollution has increased considerably in recent years due to global industrialization and urbanization. Water courses are polluted by heavy metals such as copper, mercury, cadmium, zinc, and lead. These metals can cause different physiological and morphological changes. Due to heavy metals and metalloids' tendency to accumulate in water and sediment and to dissolve readily in aquatic

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environments, they pose a significant risk to aquatic organisms and human health. The influence of environmental pollution on fish gonad health has been overlooked. The survival and reproductive capacity of organisms are significantly affected by heavy metal toxicity (Aziz et al., 2023). Similarly, reproductive toxicity due to exposure to heavy metals is also observed, comprising damage to the biological system, of which infertility is one of them (Anyanwu and Orisakwe, 2020). Heavy metals may cause anomalies in reproductive cell/organ development and, consequently, pathological changes of varying severity in the gonads (Taslima et al., 2022). These external stressors considerably impair the immune systems of aquatic animals, making them more vulnerable to invasive infections. Hence, polluted environments always contain disease-spreading pathogens in addition to facultative microbes (Dar et al., 2020).

The ecosystems of the Miljacka River are severely impacted by human activities, including untreated home waste and municipal drainage. Sarajevo (Bosnia and Herzegovina) has experienced rapid urbanization in recent years, making wastewater discharge a major problem. We assume it is still clean in the upper parts, but in the lower parts, it is much more polluted and hazardous to people and all living organisms.

Considering the potential negative impact of water pollution in the Miljacka River (Bosnia and Herzegovina), this research aimed to investigate the effects of a contaminated environment on gonadal alternation and divergence in gonad structure in *Barbus balcanicus* Kotlik, Tsigenopoulos, Rab & Berberi, 2002, as well as the incidence of gonad pathology. This freshwater fish was captured from two locations, Dariva (S1) and Otoka (S2). It was considered because of its wide distribution in the study area, well-known biology, abundance, and benthic behavior. Therefore, this species could play a significant role in understanding the effects of xenobiotics on reproductive organs. Furthermore, this species, which might be regarded as the wild type, has low fishery value and is not targeted for commercial fishing, artificial propagation, or fry release. According to Xue et al. (2021), it is an ideal candidate for investigating the effects of environmental pollution on fish.

Therefore, the aims of this study are (1) to assess the concentrations of copper (Cu), iron (Fe), manganese (Mn), zinc (Zn), cadmium (Cd), chromium (Cr), nickel (Ni), and lead (Pb) in water and sediments and fish gonadal tissue, (2) to determine the water quality status according to microbiological analyses involving Total Coliform (TC), Fecal Coliform (FC), *E. coli,* and intestinal *Enterococci*, (3) to determine the bioaccumulation of heavy metals in the gonads of the studied fish species and, (4) to examine histopathological alterations and variations in the gonad tissues of *B. balcanicus* from two sites.

Materials and Methods

Site description: The Miljacka River runs through the capital, Sarajevo, Bosnia and Herzegovina. This river is a symbol of Sarajevo, and its source is 20 km from the city. The Miljacka River was created by the joining of two rivers - Paljanska Miljacka (locality of Begovina at an altitude of 1,010 meters) and Mokranjska Miljacka (locality of Kadino Selo at an altitude of 1,135 meters). A few kilometers east of Sarajevo in the village of Dovlići, these two rivers merge into one - the Miljacka. Further on, the Miljacka River flows (21.2 km) to the west, to Sarajevo, from where it continues its journey towards the river Bosna, into which it flows. The Mijacka River belongs to the Sava River basin. The central part of Sarajevo is drained mostly towards the Miljacka River, and along its banks, there is also the Smiljevići waste disposal site. In addition to the negative effects of waste as a pollutant on the environment and human health, it is also drained into waterways. The mouth of the Miljacka River carries sediment deposits from the northern part of Ravna Planina and Trebević, the southwestern slopes of Romanija, and from the southern slopes of Bukovik and Crepoljsko. It is a typical mountain watercourse with torrential character and has two minimum (summer and winter) and two maximum (spring and late autumn) flows. The

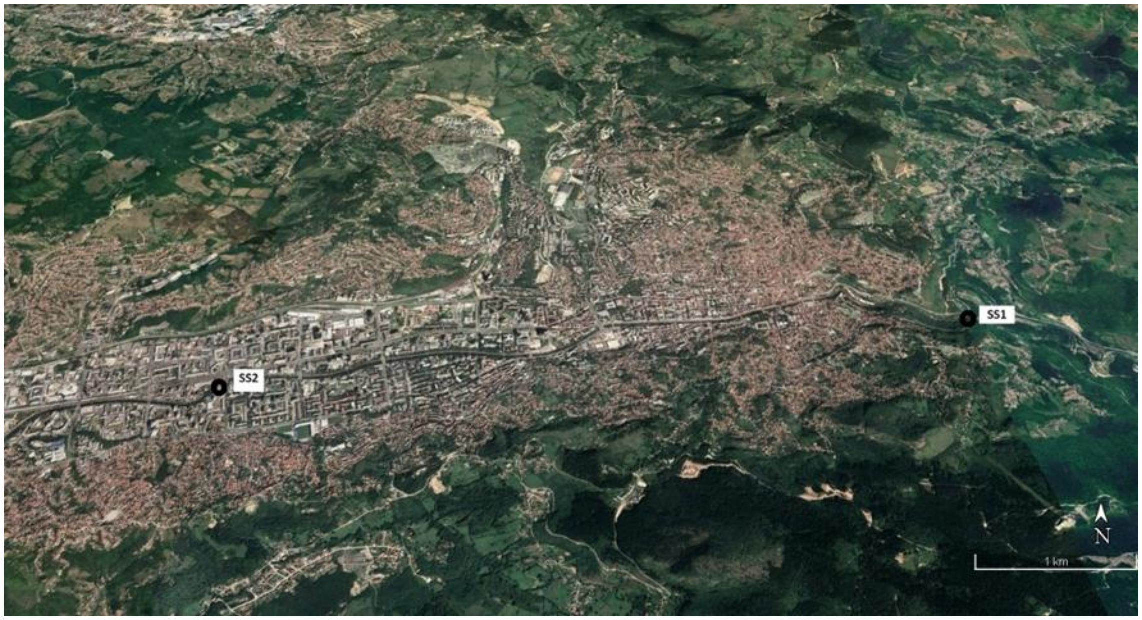

Figure 1. Map of sampling locations in the Miljacka River, Sarajevo.

average annual flow in the city of Sarajevo is 5.7 m^3/s. The largest tributaries of the river Miljacka are: on the left, the river Repašnica; on the right, the rivers Lapišnica, Mošćanica, and Koševski potok. The river Miljacka shows several specific characteristics in each of its three parts of the course, which are reflected in the living world that inhabits it.

Field survey: Sampling was conducted in the summer of 2023. *In situ* measurements of color, odor, temperature, dissolved oxygen, saturation, pH, and electrical conductivity were taken using the Multi 3630 IDS F set multimeter (WTW, Germany). The probe was submerged at a depth of about 15-30 cm below the surface. Water samples for evaluating fecal coliform, *E. coli*, and *Enterococci* were collected according to standard methods for the examination of water and wastewater (APHA, 2005). Samples were aseptically collected in sterile polypropylene bottles from two sampling points on the Miljacka River, at depths of approximately 15-30 cm below the surface at each station. Water samples were transported at 4ºC to the laboratory for further analysis.

Water samples for heavy metal analysis were collected in sterile polypropylene containers (500 mL capacity), and preservation was achieved by adding 0.5 mL of concentrated nitric acid (min. 65,0%, HNO_3). Sediment samples were collected in sterile flasks. Fish (n = 45) were captured using pulsed direct-current backpack electrofishing equipment with a 500 V DC generator at S1 and S2 (Fig. 1). Individuals were identified in the field and in the laboratory using the reference keys for this species (Kottelat and Freyhof, 2007). All methods were carried out in accordance with the relevant guidelines and regulations. This species is widely distributed in the waters of B&H and is not subject to any protection.

Heavy metal analysis: For the experimental investigation, Certipur-grade single-stock heavy-metal solutions (1000 mg/L) from Merck (Darmstadt, Germany) and analytical-grade 65% HNO_3, 37% HCl, and 30% H_2O_2 were used. Quantification of heavy metals (Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn) was conducted using a Varian AA240FS flame atomic absorption spectrometer under operating parameters specified by the instrument manual. Fish tissue samples were placed directly within the disposable polypropylene bags and frozen until analysis. Before metal determination, fish tissue samples were thawed, homogenized, weighted by an analytical balance (±0.01 mg, Mettler Toledo), and wet-digested in a mixture of nitric acid and hydrogen peroxide (v/v 5:1). Sediment samples were air-dried, grounded in a mortar, homogenized, sieved through a standard steel sieve (ϴ < 1 mm), and used for analysis. 1 g (±0.01 mg) of each sediment sample was treated with 10 mL of a 3:1 mixture of hydrochloric (HCl) and nitric

(HNO_3) acids (aqua regia). After digestion, 2 mL of distilled water and 3 mL of 30% H_2O_2 were added to support digestion. Finally, 10 mL of 37% HCl was added, evaporated to approximately 5 mL, cooled, and quantitatively filtered (Whatman 40) into a volumetric flask with distilled water to a final volume of 50 mL.

Assessment of metal pollution index and bioaccumulation factors: The metal pollution index (MPI) and bioaccumulation factors (BAFs) were determined to assess the metal pollution in fish gonads (Abdel-Khalek, 2016). The metal pollution index was calculated from the equation of MPI = (CM1 x CM2 x CM3 x . . . x CMn) x 1/n, where CM1 represents the concentration value of the first respective metal, CM2 is the concentration value of the second concerned metal, CM3 is the concentration of the third concerned metal, CMn is the concentration of the nth metal (mg/kg dry wt) in the tissue sample of a certain species. The Metal Pollution Index (MPI) compares the total metal content at different sampling locations.

Bioaccumulation factors (BAFs) were calculated as a ratio between the concentration level of biota (those in water) and the living environment of the specimens, and were expressed as follows by using the equation of BAF = CnBiota/CnWater, where CnBiota represents the concentration of metal in the tissues (mg/kg), and CnWater is the metal concentration in the aquatic environment (mg/L). BAF is categorized as follows: no probability of accumulation (BAF < 1000); bioaccumulative (1000 < BAF < 5000); and extremely bioaccumulative (BAF > 5000) (Arnot and Gobas, 2006).

Defined substrate technology (Quanti-Tray system): Fecal coliforms, *E. coli*, and *Enterococci* were enumerated using IDEXX, following the manufacturer's instructions. Samples were diluted to either 1:10 (S1 water) or 1:1000 (S2 water) in sterile water. Substrate technology using Colilert 18 (IDEXX, USA) and Enterolert-E (IDEXX, USA) was used to identify selected bacterial indicators. For each test, the reagent was dissolved in 100 mL of the water sample. The solution was added to a Quanti-Tray, sealed, and incubated at 35±0.5℃ for 18±1 h for coliforms and *E. coli*, 44.5±0.2℃ for 18±1 h for fecal coliforms, and 41±0.5℃ for 24±1 h for *Enterococci*. After incubation, any wells that turn yellow are considered positive. The number of wells in the Quanti-Tray®/2000 that fluoresced after incubation was counted and used to calculate the most probable number (MPN) of *Enterococcus* and *E. coli*. All parameter values were presented as the most probable number (MPN) per milliliter (mL) of river water.

Histopathological analysis: Following a 72-hour fixation period in 10% neutrally buffered formalin, gonad samples were cleaned for 12 hours under running tap water and prepared for histological examination using conventional methods. Sections 5-6 μm thick were cut, processed, and stained with hematoxylin and eosin for general structure, and then Canada balsam was applied and covered with a coverslip. Samples were examined using a light microscope (BestScope BS-2035DA1) at magnifications of 400× and 1000×, with the ScopeImage 9.0 program.

Analytical quality control: Laboratory quality control for heavy metal determination was performed using analytical-grade reagents (Merck, Germany), triplicate analysis for each sample, calculation of the standard deviation of repeatability (Eurachem Guide, 2014), preparation of blanks for each measurement, and calibration curves. Standard metal solutions traceable to the National Institute of Standards and Technology were used for quality assurance and control (Yang et al., 2016).

Microbiological accuracy was determined by repeated analyses and by analyses that included standard reference materials such as *Enterococcus faecalis* ATCC®TM 19433 and *E. coli* ATCC® 8739TM, and, as a negative control, sterile water was processed. Samples for histopathological analyses underwent comprehensive analytical quality control procedures.

Statistical data analysis: The statistical program Statistics 12 (Copyright © StatSoft, Inc., 1984-2014) was used to analyze the collected data. The mean, standard deviation (SD), minimum (MIN), and maximum (MAX) values were among the descriptive statistics used to characterize the levels of harmful and

Table 1. Results of defined substrate technology for the two water sampling sites from Miljacka River.

Parameter	SS1	SS2
Total coliform MPN/100ml	12033	770 100
Fecal coliform MPN/100ml	4721	48 200
***E. coli* MPN/100ml**	3873	38 300
Enterococcus MPN/100ml	2733	19

essential metals. One-way ANOVA was used for comparative analysis by samples, followed by the post hoc Newman-Keuls test. A statistical significance level of $P<0.05$ was applied to all analyses.

Results and Discussions

Analysis of the physicochemical parameters: The water temperature ranged from 13°C at S1 to 16.2°C at S2, and the pH values ranged from 8.4 at S1 to 8.6 at S2. Dissolved oxygen, as a key factor for healthy fish growth, tissue repair, and reproduction (Wang et al., 2023), was in the range of 6.83 mg/L (S2) to 7.69 mg/L (S1), while saturation was 95 at S1, and 88 at S2, respectively. Additionally, the water's electrical conductivity ranged from 328 to 586 µS/cm, which is within the WHO-recommended range. According to the WHO, the normal range of water pH is 6.5 to 8.5; therefore, the water sample from the S2 location indicates the complete absence of free CO_2, which is directly related to organic or bacteriological pollution. Most organisms are adapted to a pH range of 6.8-7.2, and pH values below 5 can be harmful to aquatic organisms (Leo and Dekkar, 2000).

Microbiological water analysis: Table 1 summarizes fecal indicator bacteria results from two sampling sites obtained using the defined substrate technology method. This study detected total coliforms, fecal coliforms, *E. coli*, and intestinal *Enterococci* in water from both sample stations. The values recorded here showed better water quality at the upstream S1 site than at the downstream S2 sample site. Furthermore, the results revealed a risk of bacterial contamination in water samples from S2. Water quality analysis suggested that S2 declined due to waste sewage discharge in the lower part of the Miljacka River.

A maximum of 10,000 cfu/100 mL for fecal coliform bacteria (FC) and a maximum of 4,000 cfu/100 mL for fecal *Enterococci* were set by Kavka and Poetsch (2002) and the EU directive (2006/7 EEC and 76/160 EEC) for class III water. Overall, our findings suggest that the S1 water microbial quality is classified as Class II for intestinal *Enterococci* and Class III for fecal coliforms, while water samples from S2 are classified as Class IV. Water contaminated with fecal coliform indicates the presence of pathogenic microbes. Furthermore, *Enterococci* have recently become increasingly popular as a primary indicator since they are considerably more resistant to chlorination and other environmental conditions than *E. coli*. Our results are consistent with Shankar and Ponnusamy's (2023) findings, which revealed a relationship between fecal coliform concentrations in the river and runoff. Such unfavorable water quality increases stress in aquatic organisms and suppresses their immune function, thereby increasing their susceptibility to opportunistic bacterial pathogens.

Heavy metal analysis in water, sediment, and gonads: The total length of sampled fish individuals ranged from 13.60 to 23.60 cm. The studied water, sediment, and fish specimens' testicles and ovaries exhibited a wide range of heavy metal concentrations (Table 2). The distribution of eight selected heavy metals at the S1 sample points followed the decreasing order: Fe > Mn > Zn > Pb > Cr > Cd > Cu > Ni. In contrast, in S2 water, the metal content was ordered as Pb > Fe > Zn > Mn > Cr > Cd > Ni > Cu, based on their averages. In decreasing order, the contamination levels in S1 sediment were Fe > Mn > Cd > Cr > Pb > Zn > Ni > Cu, while in the sediment from the S2 pattern, they were Fe > Mn > Pb > Cr > Zn > Cd > Cu > Ni. The contamination levels in fish testicle specimens from S1 and S2 organs had the same order: Zn > Fe > Cu > Cr > Mn > Ni > Pb > Cd. In ovary samples from individuals in S1, the average metal concentrations, in descending order, were Zn > Fe > Cu > Cr > Mn > Ni > Pb > Cd, whereas in S2 they

Table 2. Heavy metals concentrations in water, sediment, and reproductive organs of *Barbus balcanicus* collected from Miljacka River.

Studied metals	Sites/ Samples	Water (mg/L)		Sediment (mg/kg)		Testicle (mg/kg)		Ovary (mg/kg)	
		Mean±SD	(Min, Max)	Mean±SD	(Min, Max)	Mean±SD	(Min, Max)	Mean±SD	(Min, Max)
Cu	S1	0±0.00^{f}	0.00, 0.00	0.95±0.05^{c}	0.90, 1.00	0.50±0.04^{d}	0.47, 0.54	0.43±0.04^{e}	0.39, 0.47
	S2	0±0.00^{f}	0.00, 0.00	3.15±0.02^{a}	3.14, 3.17	1.41±0.04^{b}	1.37, 1.44	0.51±0.03^{d}	0.49, 0.54
Fe	S1	0.18±0.01^{f}	0.17, 0.19	22213.97±3.00^{a}	22211.00, 22217.00	5.93±0.25^{e}	5.67, 6.16	5.04±0.19^{e}	4.86, 5.23
	S2	0.15±0.01^{f}	0.15, 0.16	12020.33±0.12^{b}	12020.20, 12020.40	14.51±0.56^{c}	13.90, 15.01	7.89±0.35^{d}	7.67, 8.30
Mn	S1	0.05±0.00^{c}	0.04, 0.05	1571.16±2.02^{a}	1569.00, 1573.00	0.12±0.01^{c}	0.11, 0.13	0.13±0.01^{c}	0.12, 0.13
	S2	0.07±0.01^{c}	0.06, 0.08	655.71±1.49^{b}	654.00, 656.57	0.16±0.03^{c}	0.13, 0.18	0.09±0.02^{c}	0.07, 0.11
Zn	S1	0.04±0.00^{e}	0.03, 0.04	6.21±0.08^{d}	6.17, 6.30	19.04±2.94^{cb}	16.09, 21.96	17.35±3.15^{c}	13.93, 20.14
	S2	0.08±0.01^{e}	0.08, 0.10	8.62±0.10^{d}	8.50, 8.68	30.39±1.58^{a}	28.68, 31.78	21.06±1.85^{b}	19.21, 22.90
Cd	S1	0.01±0.00^{c}	0.01, 0.01	39.62±0.65^{a}	39.00, 40.30	0.01±0.01^{c}	0.01, 0.02	0.01±0.01^{c}	0.01, 0.02
	S2	0.02±0.00^{c}	0.02, 0.02	4.63±0.31^{b}	4.30, 4.92	0.02±0.01^{c}	0.01, 0.02	0.02±0.01^{c}	0.01, 0.02
Cr	S1	0.03±0.01^{c}	0.03, 0.04	25.11±0.10^{a}	25.00, 25.17	0.19±0.03^{c}	0.16, 0.21	0.17±0.02^{c}	0.15, 0.19
	S2	0.06±0.01^{c}	0.05, 0.07	18.79±0.96^{b}	18.02, 19.87	0.23±0.01^{c}	0.22, 0.24	0.25±0.02^{c}	0.23, 0.27
Ni	S1	0±0.00^{c}	0.00, 0.00	1.79±0.05^{a}	1.74, 1.83	0.10±0.02^{b}	0.09, 0.12	0.10±0.01^{b}	0.09, 0.11
	S2	0.01±0.00^{c}	0.01, 0.01	1.32±0.09^{b}	1.27, 1.43	0.16±0.02^{b}	0.14, 0.17	0.12±0.03^{b}	0.10, 0.15
Pb	S1	0.04±0.01^{c}	0.03, 0.05	20.10±0.09^{b}	20.00, 20.15	0.04±0.03^{c}	0.02, 0.07	0.02±0.02^{c}	0.00, 0.04
	S2	0.16±0.01^{c}	0.15, 0.16	28.36±1.13^{a}	27.27, 29.53	0.12±0.01^{c}	0.11, 0.13	0.09±0.03^{c}	0.06, 0.11

All values are presented as mean ± standard deviation. Values that share the same letter do not differ statistically significantly within one parameter at a significance level of $p < 0.05$ after post hoc analysis of variance by the Newman-Keuls test.

were Zn > Fe > Cu > Cr > Ni > Pb > Mn > Cd.

The results indicated significant differences in most heavy metal concentrations in the analyzed samples. The metal loads in water were much lower than in sediments and showed no significant variability among sites. The results revealed that the average concentrations of the toxic heavy metals Cd and Pb in water exceeded the WHO (2004) permissible values. Considering these findings, Cd could exert toxic effects on both target and nontarget aquatic organisms, as it is among the most harmful heavy metal pollutants in ecosystems. A freshwater fish population affected by Cd, an endocrine-disrupting metal, could experience reproductive impairment and altered natural population dynamics (Vinanthi Rajalakshmi et al., 2023). Higher concentrations of these metals can be due to atmospheric deposition from traffic and industrial sources and atmospheric deposition. The results in Table 2 revealed significant variation ($P<0.05$) in all heavy metals except Zn in the sediment among the sites. According to the EPA (1996) sediment quality guidelines, S1 samples were moderately polluted by Cr and heavily polluted by Cd and Mn (Table 3). On the other hand, S2 sediment was heavily polluted by Mn, and Pb values were near-moderately polluted and above the average shale value (20 mg/kg) according to Turekian and Wedepohl (1961).

The results detected all eight metal elements analyzed in the male and female gonads of fish. However, a slight difference between testicles and ovaries in the concentration of metals was noticed. Except for Zn, the determined concentrations of heavy metals in tissues are lower than in sediment and higher than in water. Except for Pb, all the metals were found to accumulate in the gonads at two locations at higher concentrations than in the water samples. These findings were also previously identified by El-Shenawy et al. (2021). Higher values of all metals in the tissue of the testicle and ovaries (except Mn) were

Table 3. EPA guidelines for sediment quality, compared with the present study, in mg/kg (EPA, 1996).

Heavy metals	Not polluted	Moderately polluted	Heavily polluted	Present study	
				S1	S2
Cu	<25	25-50	>50	0.95	3.15
Fe	ND[a]	ND	ND	22213.97	12020.33
Mn	<300	300-500	>500	1571.16	655.71
Zn	<90	90-200	>200	6.21	8.62
Cd	-	<6	>6	39.62	4.63
Cr	<25	25-75	>75	25.11	18.79
Ni	<20	20-50	>50	1.79	1.32
Pb	<40	40-60	>60	20.10	28.36

[a]ND-not detected

determined at site S2. Higher values of Cu, Fe, Zn, Cd, Ni, and Pb were recorded in both locations in testicle tissue compared to the ovaries. Statistically significant values of metals in testicular and ovarian tissues between localities were determined for Cu, Fe, and Zn.

The Cu concentration in the testicles recorded in the present study ranged from 0.50±0.04 mg/kg to 1.41±0.04 mg/kg, and in the ovaries, it ranged from 0.43±0.04 mg/kg to 0.51±0.03 mg/kg, with the highest concentration observed in S2. Even though the average Cu content in tissues is similar to that reported in earlier research, it did not exceed the FAO/World Health Organization (1989) permissible limit (30 µg/g). Copper is an essential element for various physiological processes; it is a crucial cofactor for enzymes and structural proteins. However, high concentrations of Cu may become toxic in the environment or organisms (Xiao et al., 2023).

In the present study, the maximum concentration of Fe was recorded in the testicles of individuals from S2 (14.51±0.56 mg/kg), and the lowest values were recorded in the ovaries (5.04±0.19 mg/kg) in specimens from S1. Astani et al. (2018) reported an Fe concentration of 35.99±8.59 mg/kg in the gonad tissues of *Alosa braschnikowi*. Similarly, our findings showed the maximum concentration of Mn in fish testicles (0.16±0.03 mg/kg) from S2 and the minimum concentration in ovaries (0.09±0.02 mg/kg) from S2 individuals. These results are consistent with studies of *Colomesus asellus* collected from the Amazon River, where Viana et al. (2023) also found an Mn concentration of 0.50 µg/g in the ovaries. As a result of our study, Zn concentration in gonads ranged from 17.35±3.15 to 30.39±1.58 mg/kg. Regarding the results of Zn concentration reported in the organs, it was below the FAO and WHO (1989) maximum permissible limit (150 µg/g).

The mean concentration of Cr in the testicles of *B. balcanicus* was 0.19±0.03 mg/kg in individuals from S1 and 0.23±0.01 mg/kg for S2, while for the ovaries, it was 0.17±0.02 for S1 and 0.25±0.02 for S2. The findings showed that Cr concentration exceeded the FAO and WHO (1989) allowable limit (0.05 µg/g). Variations in Cr concentration in testicles and ovaries among sampling stations may reflect fish body condition and immunity, given that Cr concentrations in water and sediment were higher in the S1 study area. Water from S2 was microbiologically polluted, which could affect the body status of fish. While Cr is a necessary metal for the metabolism of proteins, fats, and carbohydrates, elevated Cr concentrations harm the kidneys, liver, and endocrine system (Akila et al., 2022).

The mean concentration of Cd in the fish testicles and ovaries was 0.01±0.01 mg/kg in fish from S1 and 0.02±0.01 mg/kg in samples from S2. Sattari et al. (2020) detected the same concentrations of Cd in the gonads of *Rutilus kutum* from the South Caspian Sea. Furthermore, Franco-Fuentes (2021) reported a Cd content range of 0.1-0.6 mg/kg in fish gonads. However, the detected concentration was below the 0.2 mg/kg limit set by the WHO international standard. Cadmium may accumulate in aquatic organisms and, after absorption, is transported to the liver and bones, where it is stored, or to other organs such as the gonads.

The mean concentration of Ni in the testicles and

Table 4. Values of bioaccumulation factors in the two studied sites of Miljacka River.

Site	S1		S2	
Metal	**Testicle**	**Ovary**	**Testicle**	**Ovary**
Cu	0.00	0.00	0.00	0.00
Fe	32.90	28.00	96.70	52.60
Mn	2.40	2.60	2.30	1.20
Zn	476.00	433.70	379.80	263.25
Cd	1.00	1.00	0.50	0.50
Cr	6.30	5.60	3.80	4.10
Ni	0.00	0.00	16.00	12.00
Pb	1.00	0.50	0.70	0.50

ovaries from S1 was the same 0.10±0.02 mg/kg, and for individuals from S2, the concentration of Ni in the testicles was 0.16±0.02 mg/kg, while for the ovaries, it was 0.12±0.03 mg/kg. Similar results for Ni contents in female gonads and testicles were reported by Pokorska-Niewiada et al. (2022) for freshwater fish species in Poland, such as common bream (*Abramis brama*), European perch (*Perca fluviatilis*), common roach (*Rutilus rutilus*), and northern pike (*Esox lucius*). Unfortunately, there was a lack of other reports in the literature on nickel contents in female gonads and testicles. Also, no limit has been suggested by FAO for Ni. Nickel is a trace element necessary for both human and animal health, but excessive amounts can be harmful. Endocrine hormones in humans and animals are significantly affected by nickel exposure, which may lead to abnormal secretion and alter the structure and function of endocrine organs.

In the present research, the mean concentrations of Pb in the female gonads and testicles of the fishes examined ranged from 0.02±0.02 mg/kg, with the maximum level recorded in the testicles of S2 at 0.12±0.01 mg/kg. This result agrees with our previous finding (Pokorska-Niewiada et al., 2022), which also reported similar Pb values of 0.028±0.018 mg/kg in female fish gonads and testicles. In the present study, the Pb concentration in testicle S2 of all the organisms was not above the FAO and WHO (1989) maximum permissible limit (0.5 μg/g). As a hazardous nonessential metal, Pb causes behavioral abnormalities, reduced growth and survival rates, decreased metabolism, and nephrotoxicity in organisms due to its significant bioaccumulation (Yap and Al-Mutairi, 2023).

MPI and BAFS: Testes and ovary metal pollution indices (MPI) were 1.63 and 1.41 at sampling site 1 (S1) and 2.85 and 4.62 at sampling site 2 (S2), respectively (Fig. 2). It is suggested that the higher the value of the estimated MPI, the higher the degree of contamination in the tissue is considered. The results obtained at S1 are consistent with Abdel-Kader and Mourad's (2019) findings, but at S2, mean estimations of MPI indicated that ovaries had higher metal contamination. According to the results of Baptista et al. (2021), when comparing those tissues, Mn, Cu, and Zn showed increased ovarian levels.

Table 4 lists the bioaccumulation factor of heavy metal elements in testes and ovaries concerning metal concentrations in water. Fishes' bioaccumulation of heavy metals of gonad tissue from the S1 area was in the following sequence: Zn > Fe > Cr > Mn > Cd > Pb > Ni > Cu, and Zn > Fe > Ni > Cr > Mn > Pb > Cd > Cu at the S2 area. Nearly all BAFs in the testes are higher than in the ovary. It was observed that the highest Zn in the gonads of *B. balcanicus* had the highest BAF values among all investigated metals.

Gonad histopathological analysis: The impact of pollution on the histological structure of *B. balcanicus* testis was assessed using light microscopy. The testicular tissues of both S1 and S2 individuals showed congestion of blood vessels, thick tunica albuginea, reduction of germ cells, vacuolization, and apoptosis (Fig. 3). Also, the structure of the germ cells and spermatids revealed reduced spermatogenesis and spermiogenesis in histology. This could be based on the above results that showed increased concentrations of highly toxic elements cadmium and lead in water and testes, as they result in infertility.

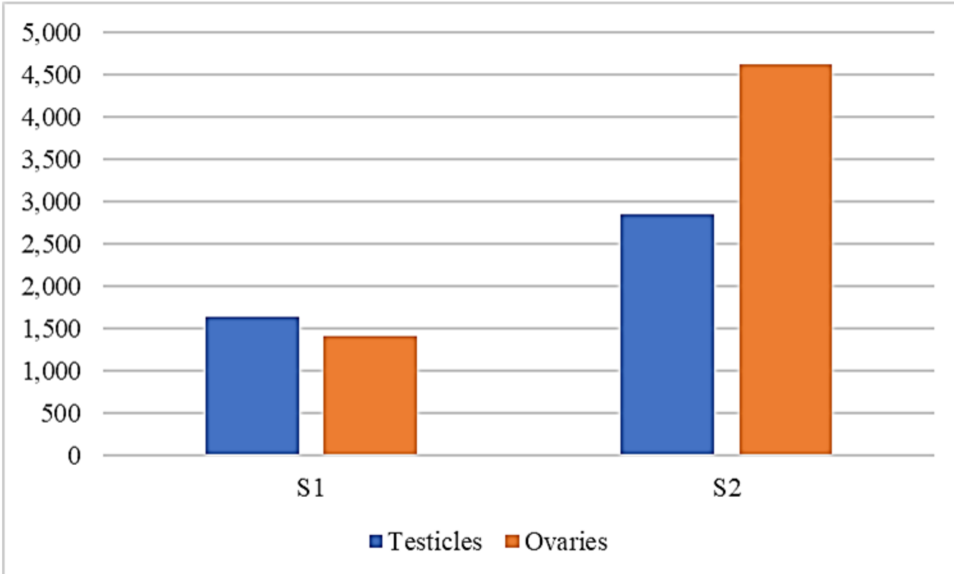

Figure 2. MPI values for testicles and ovaries at sampling sites in the Miljacka River, Sarajevo.

Nevertheless, individuals from S1 displayed slightly severe histological alterations. Furthermore, our results are consistent with recent work suggesting increased vacuolation of spermatozoa in response to Cd exposure (Su et al., 2023). Furthermore, Ferreira et al. (2023) showed that Pb in *Astyanax bimaculatus* impairs gametogenesis, disrupts estrogen receptor signaling, and alters the expression of key reproductive biomarkers.

The histological analysis and morphological features of *B. balcanicus* ovaries from two different locations revealed oocytes at various developmental stages. Based on the histological examination, the ovaries from S1 and S2 revealed an increased number of atretic follicles. Furthermore, several types of histological alterations were also observed in the ovaries, such as capsulate atresia (Fig. 4a), alternation of germinal epithelium (Fig. 4b), atretic oocyte (Fig. 4b), alternation in follicular epithelium (Fig. 4c), lymphocytic infiltration (Fig. 4d), stromal hemorrhage (Fig. 4d), cystic atresia with a reduction in size and previtelline space between zona radiata and oocyte content (Fig. 4e), atretic follicles (Fig. 4f), etc. Some extent of damage could be seen in the architecture of the tunica albuginea (Fig. 4f). According to findings, ovaries from S2 showed a noticeably higher degree of malformation than S1 in all oocytes' developmental stages. This alternation might be related to heavy metal pollutants in the river environment (Srivastava et al., 2023) and the microbial load in the water, which can lead to microbial imbalance (dysbiosis) and suppress the fish immune system (Khalefa et al., 2021). On the other hand, there is a correlation between the prevalence of fish dysbiosis and increased heavy metal accumulation in tissues (Dane and Şişman, 2017). The results above do not account for other pollutants, such as pesticides, cosmetics, herbicides, and cleaning materials, which could also contribute to adverse effects on the reproductive system.

Conclusions

In conclusion, the analyses presented in this study revealed that *B. balcanicus* gonads are sensitive to environmental pollution. Therefore, among the measured heavy metals, Pb and Cd in water affected the permission levels and Cr in gonads. Microbiological water analysis results confirmed the high potential risk to the environment and human health in the study downstream area. The results of histopathological analysis showed that *B. balcanicus* gonads can serve as a bioindicator, but further research is required. Our statements regarding the impact of environmental pollution on fish gonads should motivate relevant authorities to address environmental problems effectively. Hence, it is essential to

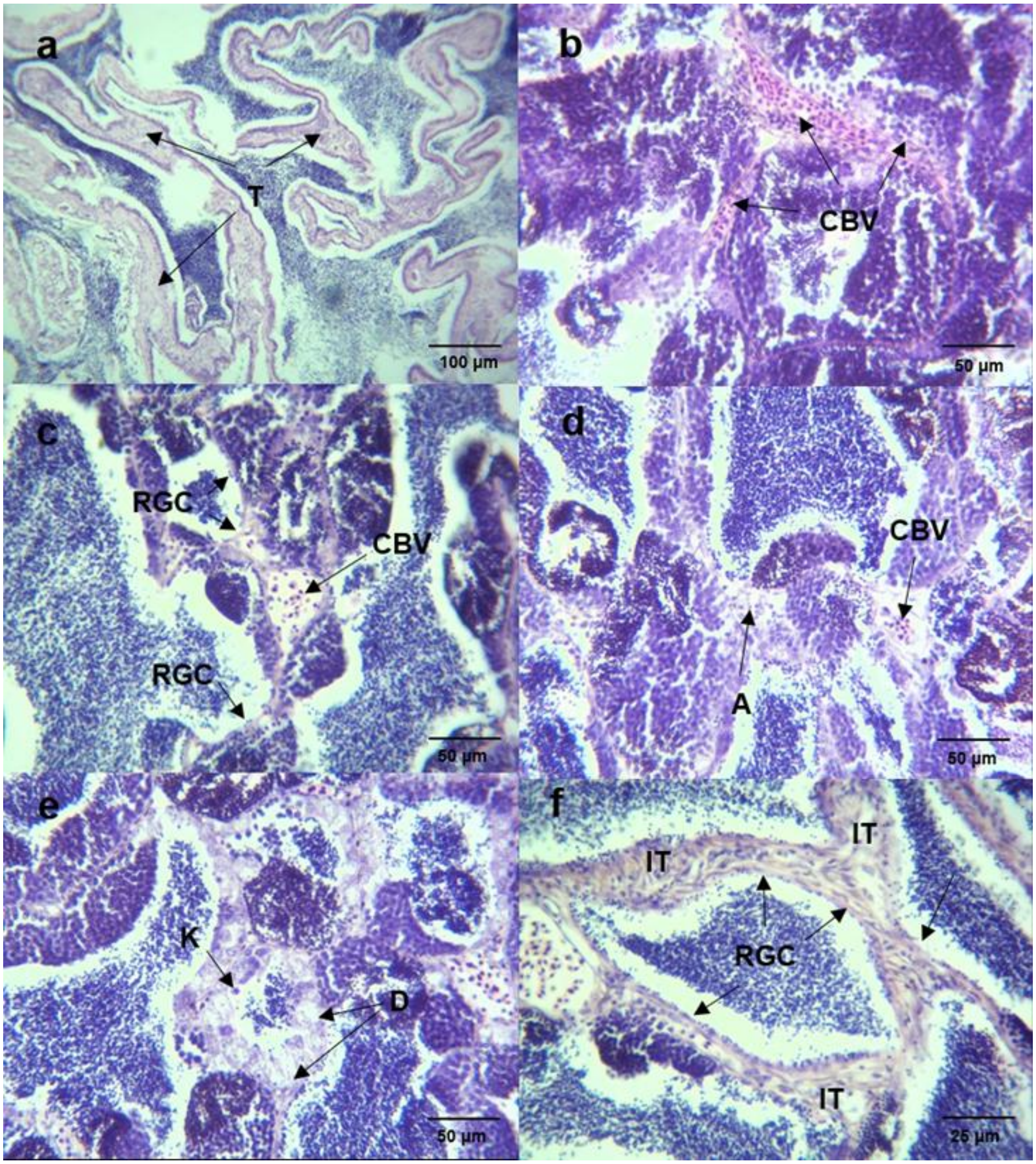

Figure 3. Histological section testicle of *Barbus balcanicus* stained with haematoxylin and eosin: (a) seminiferous tubuli with thick tunica albuginea (T), (b) congestion of blood vessels (CBV), (c) reduction of germ cells (RGC) and congestion of blood vessels (CBV), (d) signs of apoptosis (A) and congestion of blood vessels (CBV), (e) signs of apoptosis, karyopyknotic nuclei (K) and dead cells, vacuolated and pale (D), (f) proliferation of interstitial tissue (IT) and reduction of germ cells (RGC).

recognize and understand the major problem of pollution in aquatic environments, which has detrimental effects on fish, water quality, and non-target aquatic organisms. Overall, accounting for fish sensitivity in assessments of pollution impacts on other organisms is crucial if we are to better understand and counteract the increasing pressures on the environment.

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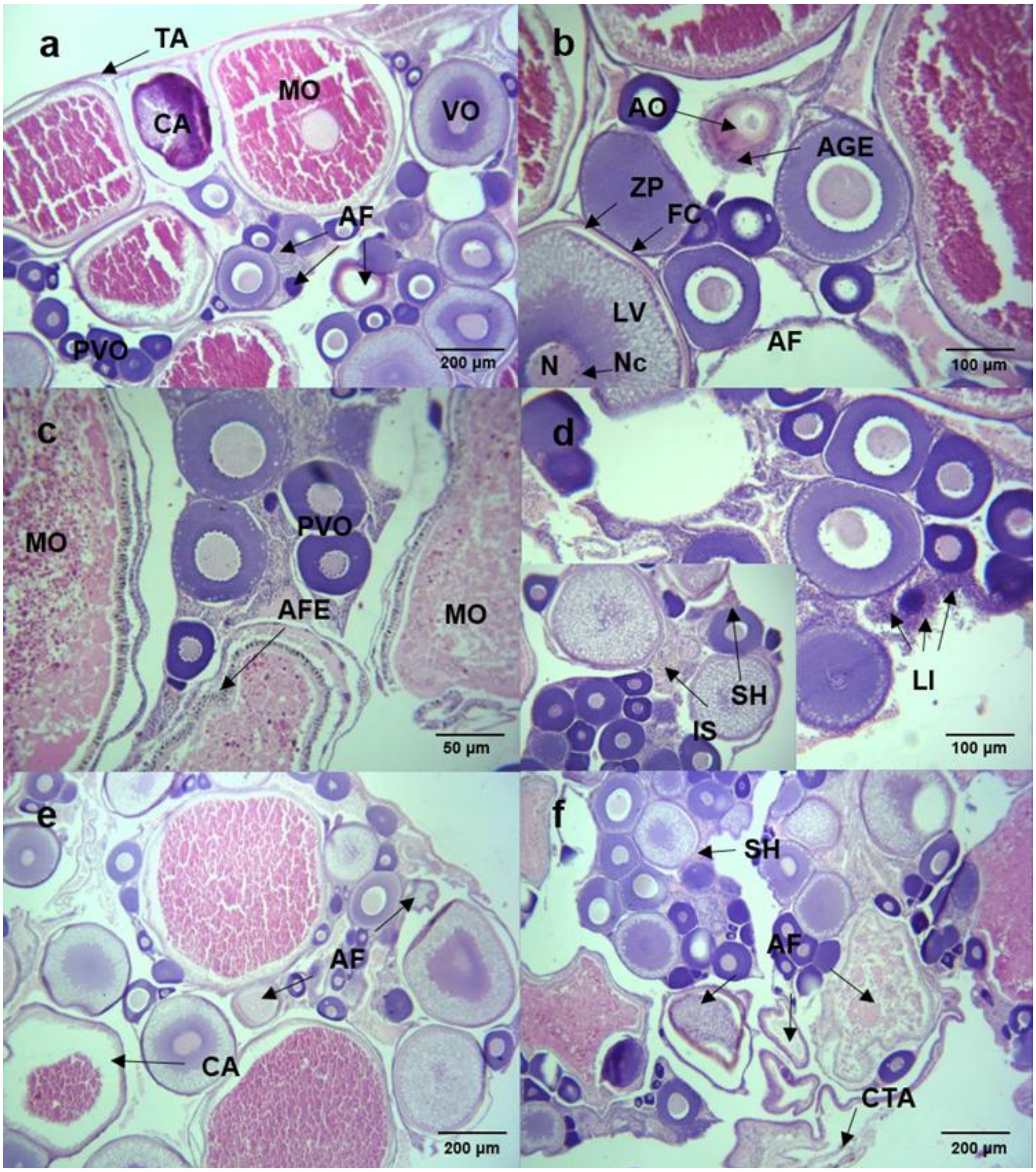

Figure 4. Histological section ovary of *Barbus balcanicus* stained with haematoxylin and eosin: (a) previtellogenic oocytes (PVO), vitellogenic oocytes (VO), mature oocyte (MO), the tunica albuginea (TA), atretic follicles (AF), capsulate atresia (CA), (b) zona pellucida (ZP), follicular cell (FC), nucleus (N), nucleolus (Nc), lipid vacuole (LV), alteration in the germinal epithelium (AGE), atretic oocyte (AO), (c) previtellogenic oocytes (PVO), mature oocyte (MO), alteration in follicular epithelium (AFE) (d) lymphocytic infiltration (LI), increased amount of stroma (IS), stromal hemorrhage (SH), (e) cystic atresia (CA), atretic follicles (AF), (f) atretic follicles (AF), stromal hemorrhage (SH), changes in the architecture of tunica albuginea (CTA).

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