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RESEARCH

Combined use of biomarkers to assess the impact of untreated wastewater from the Danube River, Serbia

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Abstract

In this study a battery of bioassays, both in vivo (metals and metalloids concentrations, erythrocyte morphometry, comet assay, micronucleus assay, and histopathological analyses) on vimba bream *Vimba vimba* (L., 1758) and white bream *Blicca bjoerkna* (L., 1758), and in vitro (treatment of HepG2 cells with native water samples) was applied to assess the harmful potential of untreated wastewater. Faecal indicator bacteria were quantified to assess the microbiological water quality. Vimba bream had significantly higher Fe concentrations in both liver and muscle, while white bream had higher Ca and Cu concentrations in liver. Vimba bream had a significantly higher level of DNA damage in both liver and blood cells, in comparison to white bream. Low levels of micronucleus and nuclear abnormalities were observed in both species. Erythrocytes morphometry did not show significant interspecific differences. Histopathological analyses revealed a similar response of the studied species, with a significantly higher presence of ceroid pigments in the liver of vimba bream. Treatment of HepG2 cells revealed the high genotoxic potential of water downstream of the discharge point. The results of this study clearly demonstrate the importance of effect-based monitoring, in order to enforce more efficient management of natural resources and implementation of wastewater treatment systems.

Keywords Metals accumulation · Erythrocyte morphometry · Comet assay · Micronucleus · Histopathology · HepG2

Introduction

Untreated household and industrial wastewater is a major pollutant of surface water worldwide, introducing both pathogenic microorganisms and chemical pollution into the environment (Koopaei and Abdollahi 2017). The Danube River is the most international river in the world and the second-largest river in Europe, which is, unfortunately, along its entire course through Serbia exposed to numerous types of diffuse and point pollution sources (Lenhardt et al. 2016; Kirschner et al. 2017). In Serbia, only about 16% of wastewater discharged into the wastewater collecting system was treated to some extent, while only 2% of industrial wastewater was treated (The Statistical Office of the Republic of Serbia 2017). Many ex situ and in situ studies conducted in this part of the Danube revealed the harmful potential of such exposure, pointing to the importance of monitoring biota in pressured ecosystems (Subotić et al. 2013a, b; Vuković-Gačić et al. 2014; Aborgiba et al. 2016; Marić et al. 2020).

In such ecosystems, fish are one of the most affected organisms, due to the fact that they are at the top of the food

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chain. Besides, they are an interesting choice for indicator organisms due to their role in human nutrition. Vimba bream *Vimba vimba* (L., 1758) and white bream *Blicca bjoerkna* (L., 1758) both belong to the family Cyprinidae, subfamily Leuciscinae, and are widely spread in Europe and Asia (Kottelat and Freyhof 2007). Both species are represented in commercial fisheries yields in the Belgrade sector of the Danube River Basin (Jovičić et al. 2014) and have been used before for assessing the state of fish populations in polluted environments (Miege et al. 2012; Aborgiba et al. 2016; Subotić et al. 2019, 2021).

When creating a battery of bioassays, it is essential to choose a set that on the one hand provides reliable information about the changes at multiple levels of biological organisation, and on the other hand, is relatively simple and economical. Faecal indicator bacteria may quickly identify untreated municipal wastewater, which is the source of both the chemical and microbiological pollution. (Kirschner et al. 2009; König et al. 2017). Assessment of metals and metalloid concentrations in tissues of bioindicator organisms may be considered as a bioaccumulation marker (Van der Oost et al. 2003). In accordance with Houston (1997), for assessment of fish health, classical hematological variables (red blood cells count, hematocrit, and hemoglobin) are not as useful as in mammalian species since the teleost erythrocytes mature within circulation changing physiologically and morphologically. So, the use of erythron profile which represents the relative abundance of immature, intermediary developed and mature erythrocytes was proposed (Houston 1997). The proportion of immature erythrocytes in circulation could be used as an indicator of pollution (Witeska 2013). One of the most sensitive methods for determining the genotoxic potential of an aquatic environment is the comet assay (Frenzilli et al. 2009), which provides information about the state of the examined organisms at a specific moment. If the detected lesions are not repaired or are being repaired in an incorrect manner, this may eventually lead to changes at a higher level. A test that is widely used in ecogenotoxicological research is the micronucleus test, in which clastogenic and aneugenic effects of pollutants in the environment are detected (Bolognesi and Hayashi 2011). As a rule, chromosomal aberrations can further lead to cell death and a number of pathophysiological changes (Guo et al. 2019). Histopathological (HP) alterations in fish are valuable markers for the assessment of fish condition at the organ/tissue level of organisation. Most of these changes may be considered adaptive in order to achieve homeostasis in an environment that is constantly changing due to endogenous and exogenous factors (Nikolić et al. 2021; Santos et al. 2021; da Silva Montes et al. 2022). In order to quickly estimate the harmful potential of untreated wastewater for human health, metabolically competent HepG2 cells are frequently utilized as an in vitro eukaryotic model system due to their endogenous expression of a number of xenobiotic metabolising enzymes (Mersch-Sundermann et al. 2004).

This study examines the use of a battery of bioassays, both in vivo (metals and metalloids concentrations, erythrocyte morphometry, comet assay, micronucleus assay, and HP analyses) on vimba bream and white bream, and in vitro (treatment of HepG2 cells with native water samples). Faecal indicator bacteria were quantified to assess the microbiological water quality. The aim was to get a wider picture of different fish species' vulnerability to the same pressures on site and to eventually single out a more suitable bioindicator. These data are necessary to establish better wastewater management and protection of aquatic ecosystems, especially the ones as large and internationally important as the Danube River.

Materials and methods

The sampling site

The sampling location Novi Banovci is situated on the Danube River (44°57'13.4"N 20°17'16.7"E) (Fig. 1).

This site is affected by the untreated wastewater from the municipalities Indjija and Stara Pazova that share a regional collector. This collector receives wastewater from connected households and industries, discharging untreated wastewater directly into the Danube River. The number of inhabitants that are connected to the public sewer from Indjija municipality is 26,562 and from the municipality Stara Pazova 15,760 (in total 42,322 inhabitants). In the Indjija municipality industrial wastewaters represent 20 %, and the rest is from households. In the Stara Pazova municipality, 33% represent industrial water, and 67% are from households. Nearly 80% of wastewaters from this municipality are discharged in septic tanks that are not watertight. Industries that are present in this region belong to the wood processing industry, metal industry, machine factory, plastic factory, ice cream factory, brickyard, textile, and chemical industries. Besides, this is an area of intensive agricultural activity, with both municipalities having 85% of the total area under agricultural use (JKP "Vodovod i kanalizacija" Indjija i Stara Pazova 2018). A sampling of water and fish tissues was performed on 12/07/2017.

Microbiological water analyses

Faecal indicator bacteria were assessed in samples from the wastewater outlet, upstream and downstream of the outlet. Water was collected from a depth of 30 cm in a sterile glass bottles transported to laboratory in dark and cool conditions,

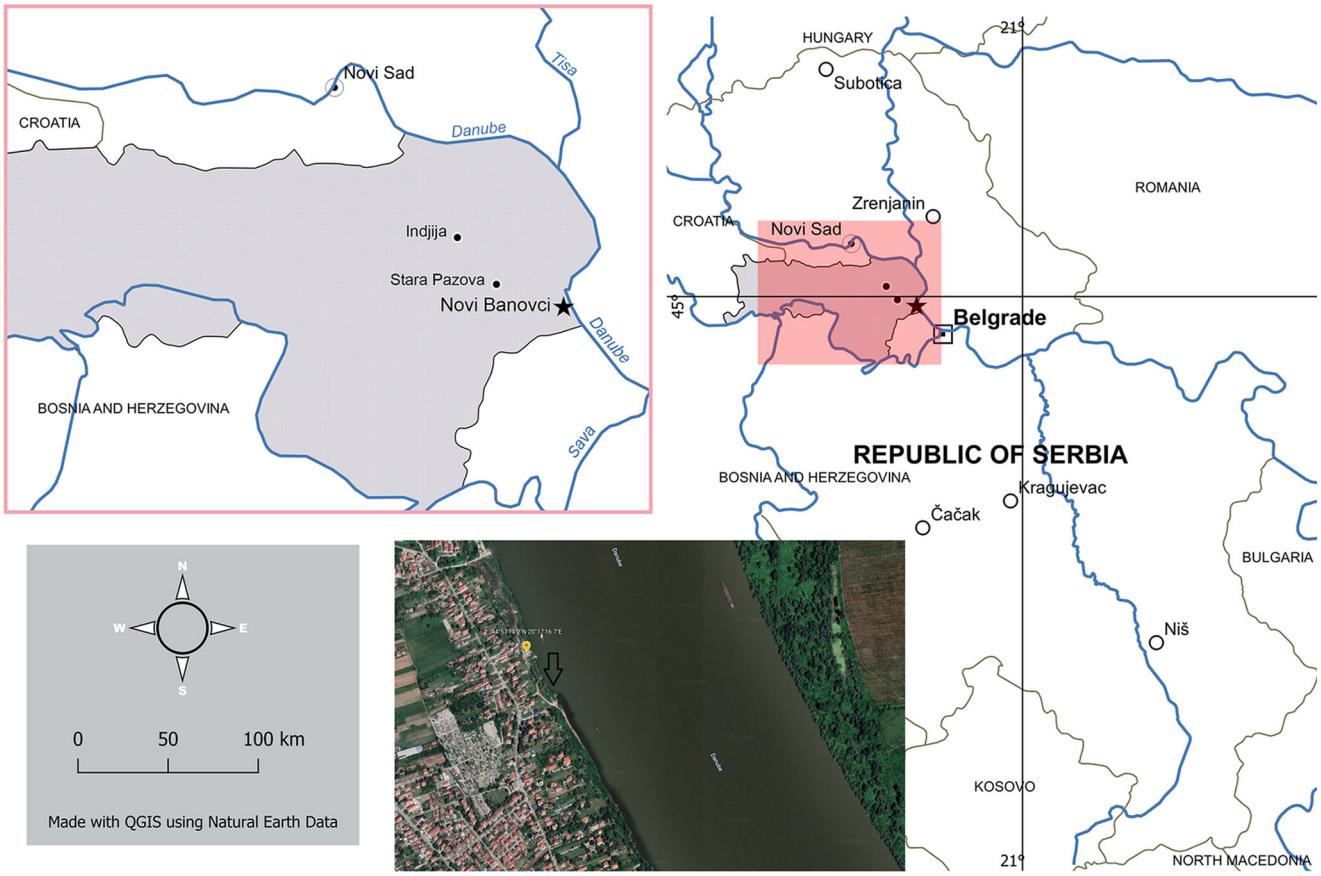

Fig. 1 Sampling site Novi Banovci: Map and satellite image of collector discharge point

and processed within two hours. Enzymatic methods and the MPN approach were applied. For analysis of total coliforms (TC) and *Escherichia coli* (EC) Colilert 18 (IDEXX, US) according to ISO standard 9308-2:2012 was used, and for enterococci (EF) Enterolert (IDEXX, US).

Ecogenotoxicological analysis of water

For the treatment of the HepG2 cells, water was sampled at the wastewater outlet, as well as upstream and downstream. HepG2 (ATCC) cells were kindly provided by Bojana Žegura (National Institute of Biology, Ljubljana, Slovenia). The methodology for HepG2 cell growth and treatment was applied as described in Žegura et al. (2009). The treatment of 10^5/mL HepG cells (from passages 4 and 5) involved exposure to pre-filtered water samples (diameter pores 0.2 µm), for 24 h at 37 °C, where the cell growth medium was replaced with 30% of the corresponding water sample. Positive control was BaP (final concentration of 50 µM), and for the negative control, bidistilled water was used. Thereafter, the treated cells were subjected to a comet assay as described in subsection *Alkaline comet assay*.

Sampling of fish tissue

Fish were caught and provided by local fishermen (fishing nets mesh size 50 mm). Prior to dissection specimens of vimba bream ($n = 5$) and white bream ($n = 5$) were anesthetised with clove oil (final concentration 50 µL/L), and total length (TL in cm) and weight (W in g) were measured. Blood was collected from the heart with heparinised needle and syringe, and used for the erythrocyte morphometry, comet assay, and micronucleus assay. Liver and muscle were dissected for the analysis of metals and metalloids, while the second gill arch from the left side of each fish and another portion of the liver were sampled for the comet assay and histopathological analyses.

Analysis of metals and metalloids

The analysis of the concentrations of metals and metalloids in fish tissues was performed according to our previous study (Kostić et al. 2017). After dissection, all samples were immediately washed with distilled water and stored at −18 °C until analysis. Samples were freeze-dried by Freeze Dryers Rotational-Vacuum-Concentrator (GAMMA 1–16

LSC, Germany) and then digested in a microwave (ETHOS EASY Advanced Microwave Digestion System 230 V/50 Hz, Milestone, Italy) using 6 mL of 65% HNO_3 and 4 mL of 30% H_2O_2 (Merck Suprapur, USA). The analysis of 22 elements (Al, Ag, As, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Li, Mg, Mn, Mo, Ni, Pb, Se, Sr, and Zn) was carried out by optical-spectrometric method (ICP-OES, Spectro Genesis EOP II, Spectro Analytical Instruments DmbH, Germany). The following wavelength lines of ICP-OES were used: Al 396.152 nm, Ag 328.068 nm, As 189.042 nm, B 249.773 nm, Ba 230.424 nm, Ca 317.933 nm, Cd 214.438 nm, Co 228.616 nm, Cr 267.716 nm, Cu 324.754 nm, Fe 238.204 nm, Hg 184.950 nm, K 766.491 nm, Li 460.289 nm, Mg 285.213 nm, Mn 294.921 nm, Mo 202.095 nm, Ni 231.604 nm, Pb 220.353 nm, Se 196.090 nm, Sr 460.733 nm, and Zn 213.856 nm. The detection limits for analysed elements were Al 0.00158 µg/L, Ag 0.0032 µg/L, As 0.00282 µg/L, B 0.000931 µg/L, Ba 0.000531 µg/L, Ca 0.00562 µg/L, Cd 0.000132 µg/L, Co 0.00024 µg/L, Cr 0.000366 µg/L, Cu 0.000588 µg/L, Fe 0.000562 µg/L, Hg 0.00553 µg/L, K 0.00179 µg/L, Li 0.042 µg/L, Mg 0.000604 µg/L, Mn 0.000403 µg/L, Mo 0.000784 µg/L, Ni 0.00114 µg/L, Pb 0.00343 µg/L, Se 0.00197 µg/L, Sr 0.00138 µg/L, and Zn 0.000391 µg/L. A blank sample was used to exclude the possible presence of analysed elements in chemicals used for digestion. The analytical process quality was controlled through reference materials of bovine liver (BCR-185R) and lichen reference material (IAEA-336). The found concentrations were within 90–115% of the certified values for all measured elements. All values are expressed in µg/g dry weight (dw). For muscle, the concentrations of elements per tissue wet weight (ww) were calculated for comparisons with the prescribed maximum acceptable concentrations (MACs) in fish meat set both by the European Union (EU) and the national legislation. EU legislation (European Commission Regulation 2006) prescribed the MAC for Cd (0.05 µg/g ww), Hg (0.5 µg/g ww), and Pb (0.3 µg/g ww), while the National legislation prescribed MAC for As (2.0 µg/g ww), Cd (0.1 µg/g ww), Hg (0.5 µg/g ww), Pb (1.0 µg/g ww), Cu (30.0 µg/g ww), Fe (30.0 µg/g ww), and Zn (100 µg/g ww) (Official Gazzette of RS 2011).

Erythrocyte morphometry

Two blood smears on microscopic slides were prepared per specimen. Slides were stained with a Bio-Diff kit (Bio Optica, Italy). Under DM RB photomicroscope (Leica, Germany), random fields of the microscopic slides were observed and photographed. Pictures were analysed using the image analysis software (ImageJ) which automatically calculated cellular parameters (area, perimeter, and length) of erythrocytes. Shape factor was calculated with the following equation:

$$\text{Shape factor} = (4 \times \pi \times \text{CellArea})/\text{CellLength}$$

Development stages of erythrocytes (immature, intermediary, and mature) were categorised based on shape factor (Houston 1997).

Comet assay

Preparation of cell suspension

Liver and gills cell suspensions were prepared as described in our previous study (Kostić et al. 2016). Tissue samples were disintegrated using two scalpels in 200 µL Hank's Balanced Saline Solution (HBSS) and treated with trypsin (final concentration 0.05%, 10 min, room temperature). Blood, liver and gill cell suspensions were centrifuged, and the pellet was resuspended and diluted with HBSS to obtain about 50,000 cells/mL.

Alkaline comet assay

Prior to the comet assay, cell viability was determined by differential staining acridine orange/ethidium bromide (AO /EB) (Squier and Cohen 2001). Samples that showed more than 70% cell viability were tested further. The alkaline version of the comet assay was conducted as described in our previous study (Kostić et al. 2016). In brief, after coating slides with two layers of 1% NMP agarose (normal melting point), cell suspensions were mixed with 1% LMP agarose (low melting point) and applied on slides. For each cell type, one slide with two gel replicas was prepared per specimen. Slides were exposed to ice-cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO- for blood cells, pH 10) at 4 °C for 16–18 h. After lysis, slides were placed in an electrophoresis chamber and covered with cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for performing denaturation (20 min., 4 °C) and electrophoresis (0.75 V/cm, 300 mA, 20 min., 4 °C). This was followed by neutralisation (0.4 M Tris, pH 7.5, 15 min., 4 °C) and fixation in cold methanol (15 min., 4 °C). To visualise comets, slides were stained with acridine orange (final concentration 2 µg/mL) and observed under 400x magnification on a fluorescent microscope (Leica, DMLS, Germany). A total of 250 comets per fish species, for each cell type, were randomly scored and analysed by the Comet IV computer software (Perceptive Instruments, UK). DNA damage level was expressed by the percentage of DNA in the comet tail Tail intensity (TI %) parameter.

Micronucleus MN and nuclear abnormalities NA

For analysis of micronucleus (MN) and nuclear abnormalities (NA), about 50 µL of blood was smeared on a glass slide directly on site. After drying (1 h), microscopic slides were submerged in cold methanol for 30 min. to enable fixation. Smears were stained with 20 µL of acridine orange (final concentration 2 µg/mL) and observed under a fluorescence microscope (Leica, DMLS, Germany). Micronucleus was determined as a structure that is 1/16 to 1/3 of the main nucleus size (Fenech et al. 2003). Nuclear abnormalities were classified according to Carrasco et al. (1990) and included observation for binucleated cells, blebbed nuclei, lobed nuclei, and notched nuclei. The analysis included 5000 randomly selected cells/specimen, at 1000x magnification.

Histopathological analysis

Histopathological analyses were performed based on our previous study (Kostić-Vuković et al. 2021). After 24 h fixation in 4% formaldehyde, samples of liver and gills were handled by the automatic tissue processor Leica TP 1020 (Leica, Germany), dehydrated with graded ethanol series, cleared with xylene, and embedded in paraffin. Paraffin blocks were sectioned at nominal 5 µm thickness using Leica SM 2000R microtome (Leica, Germany). They were dewaxed and stained with haematoxylin and eosin (HE) combination. All slides were blinded and analysed by two experienced histopathologists (B.R. and V.P.) using a semi-quantitative scoring system and any disagreement of histopathological scores is resolved on a basis of a consensual approach. The extent of histopathological scores was given using the following scale: 0 – no alteration present; 2 – mild alteration (< 33 % of the tissue was affected); 4 – moderate alteration (33–66% of the tissue affected); 6 – severe alteration (> 66 % of tissue affected). Histopathological indices for gills (IG) and liver (IL) were calculated according to the work of Bernet et al. (1999), and are described in details in Nikolić et al. (2021). Micrographs were made by using a Leica DFC 310 FX camera on a Leica DM LS microscope (Leica, Germany).

Statistical analyses

Statistical analyses were performed in the IBM SPSS Statistics for Windows, version 25 (IBM Corp.). The normality of data distribution was tested using the Kolmogorov-Smirnov test. Differences between the two species (element concentrations, comet assay in vivo, MN and NA, histopathology) were tested using the Mann-Whitney U test. For the analysis of DNA damage of HepG2 cells and difference in erythrocyte parameters between development stages (for each fish species individually), one-way ANOVA was conducted, with subsequent Games-Howell post-hoc test. For all tests, the significance level was set to $p < 0.05$.

Principal Component Analysis (PCA) was used as an unsupervised method for data analysis to see if the grouping of two fish species based on analysed parameters exists. PCA was performed by Eigenvector Solo 7.0 software (Eigenvector Inc., Chelan, WA, USA). The concentration of elements in muscle and liver, an abundance of immature, intermediary, and mature erythrocytes, TI % in blood, liver, and gills, as well as histopathological index of gills and liver were used as input variables.

Results

The mean length and weight of vimba bream was 28.9 ± 1.3 cm and 225.8 ± 27.6 g, and of white bream 24.2 ± 2.9 cm and 177.4 ± 66.2 g respectively (mean ± SD).

Microbiological analyses of water

Results for microbiological indicators of faecal pollution TC, EC, and EF, at wastewater outlet (W), upstream (U), and downstream (D) of the outlet are shown in Table 1. In comparison to the upstream site, all indicators were higher in wastewater outlet and downstream site, thus showing the strong impact of this source of pollution. Water quality classification was performed according to the Directive 2006/7/EC of the European Parliament and of the Council concerning the management of bathing water quality and repealing Directive 76/160/EEC (The European Parliament and the Council of the EU 2006), based on the work of Kavka et al. (2006). According to all indicators of fecal pollution, the quality of water at the outlet and downstream was characterised as strongly and excessively polluted (IV and V class). The quality of water upstream of the outlet was classified as critical based on the number of TC and EC (III class), and as little polluted according to EF (I class).

Table 1 Concentrations of the faecal indicator bacteria (FIB) in the river water samples

FIB (MPN/100 mL)	U	W	D
TC	54,750	325,700	>241,960
EC	3,450	>2,419,600	>241,960
EF	20	141,360	12,997

TC Total coliforms, *EC E. coli*, *EF* Feacal enterococci, *U* Upstream, *W* Wastewater outlet, *D* Downstream

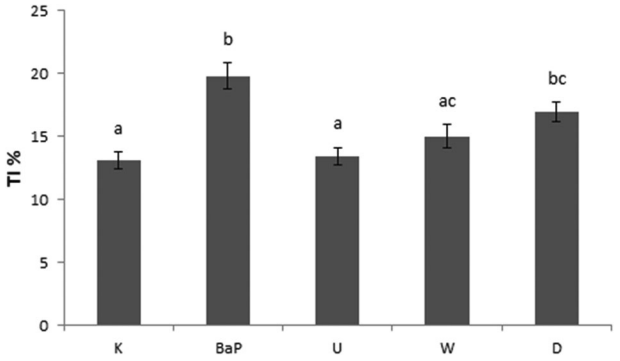

Fig. 2 DNA damage of HepG2 cells treated with river water samples. U Upstream outlet, W Wastewater outlet, D Downstream outlet, K Negative control, BaP Positive control-. a, b, c – different letters denote statistically significant differences between the groups. One-way ANOVA with Games-Howell post hoc ($p < 0.05$); values are presented as mean ± SE

Ecogenotoxicological analysis of water- Comet assay on HepG2 cells

The viability of HepG2 cells after treatment was above 95% in all sample groups. Graphical presentation of data obtained in comet assay on HepG2 cells is shown in Fig. 2. Selected parameter TI%, had a statistically significant higher value at the site (D) downstream in comparison to the site (U) upstream of the wastewater outlet. Site D also showed significant differences in DNA damage level in comparison with the negative control.

Analysis of metals and metalloids in fish tissues

From 22 analysed elements 12 were above the detection limit in the liver (B, Ca, Cr, Cu, Fe, K, Mg, Mn, Mo, Se, Sr, Zn), and 8 (B, Ca, Cr, Fe, K, Mg, Sr, Zn) in muscle, in all specimens for both species. Values are presented in μg/g dry weight (dw) in Table 2, with significant differences observed between the species within a tissue. Significantly higher concentrations of Fe in liver and muscle of vimba bream, and of Cu and Ca in the liver of white bream have been observed.

Out of the elements for which MAC in fish meat are prescribed, As, Cd, Cu, Hg, and Pb were not detected, while concentrations of Fe (white bream 1.44 ± 0.00 μg/g ww; vimba bream 3.18 ± 0.01 μg/g ww) and Zn (white bream 5.69 ± 0.00 μg/g ww; vimba bream 3.90 ± 0.01 μg/g ww) were below MAC, pointing to the relatively safe consumption of these species' meat.

Erythrocyte morphometry

Development stages of erythrocytes (immature, intermediary, and mature) were in similar proportion in both species. Most frequent were immature (48.24% in vimba bream, 49.80% in white bream), followed by an intermediary (31.34% in vimba bream, 26.85% in white bream), and mature (20.42% in vimba bream, 23.35% in white bream) erythrocytes (Fig. 3).

When comparing erythrocyte parameters of different development stages, in vimba bream, one-way ANOVA showed a statistical difference only in the length ($F = 29.154$; $p = 0.000$) of erythrocytes. Post hoc test showed that all three stages are significantly different.

In white bream, there is a statistical difference in perimeter ($F = 16.421$; $p = 0.000$) and length ($F = 105.351$; $p = 0.000$) of erythrocyte development stages. Subsequent post hoc test showed that all three stages differ from each other.

Alkaline comet assay on fish cells

The viability of blood, liver and gill cells was above 80% in all samples. The level of DNA damage in the liver, blood, and gill cells of white bream and vimba bream was expressed with parameter TI% as mean ± SE and shown in Fig. 4. Statistical analyses showed significantly higher DNA damage levels in the vimba bream liver and blood cells, in comparison to the white bream.

Micronucleus and nuclear abnormalities

The overall frequency of MN and NA in both species was low. No significant variation in MN or NA frequency between investigated fish species was observed (Table 3).

Histopathological analysis of fish tissues

Histopathological scores of the gills were generally low (Table 4), meaning that 10 out of 12 alterations found in fish had average scores below 2 (out of 6 which is a maximum possible score). Only hyperaemia and oedema of the secondary epithelium of gill tissue were more frequent and intensive and thus had moderate histopathological scores. No significant differences were observed between the two species for any histopathological alteration in gills.

Histopathological alterations in the liver maintained the same trends as in the gills (Table 4). Average values of histopathological alterations were low and in some cases moderate. The only histopathological alteration in the whole study that showed the significant statistical difference was the presence of ceroid pigments, with higher scores in the liver of vimba bream ($p = 0.037$). Concerning the extent of necrosis of tissue (irreversible histopathological alteration), the average scores amounted to at least 2 in both species and in both sampled tissues, with the exception of the white bream liver, where necrosis was 3.6. Some of the most

Table 2 Concentrations of elements detected in liver and muscle of white bream and vimba bream

	Liver		Muscle	
	White bream	Vimba bream	White bream	Vimba bream
B	3.08 ± 3.11	4.47 ± 4.10	10.14 ± 5.73	14.07 ± 7.17
Ca	172.37 ± 123.66*	42.56 ± 23.04*	1602.49 ± 514.90	958.29 ± 736.23
Cr	0.11 ± 0.03	0.09 ± 0.08	0.10 ± 0.03	0.11 ± 0.11
Cu	48.24 ± 22.02*	9.47 ± 1.20*	nd	nd
Fe	132.27 ± 47.34*	284.40 ± 71.73*	5.02 ± 2.15*	11.57 ± 2.58*
K	2429.63 ± 516.11	2752.95 ± 557.74	6336.24 ± 878.69	5971.43 ± 902.45
Mg	298.12 ± 46.70	352.63 ± 51.93	901.80 ± 158.56	783.76 ± 150.53
Mn	1.65 ± 0.74	1.28 ± 0.73	nd	nd
Mo	0.95 ± 0.16	0.86 ± 0.14	nd	nd
Se	0.98 ± 0.28	0.92 ± 0.16	nd	nd
Sr	0.49 ± 0.29	0.37 ± 0.07	2.64 ± 0.92	1.40 ± 1.01
Zn	59.33 ± 15.14	37.44 ± 5.66	19.49 ± 6.13	14.27 ± 2.50

Values are presented in μg/g dry weight as mean ± SD. *Significant differences between species within a tissue, Mann-Whitney U test ($p < 0.05$). nd nondetected

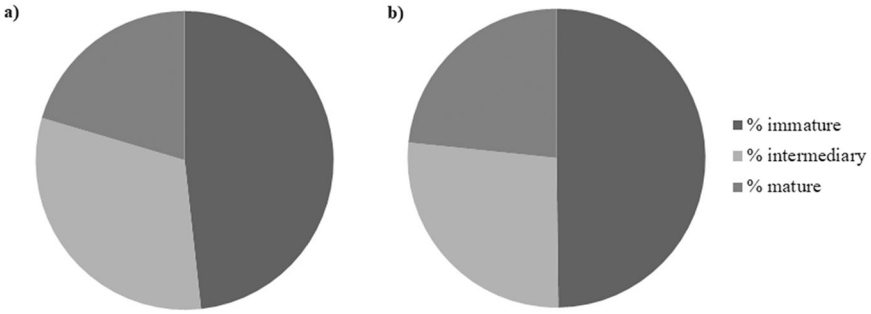

Fig. 3 Percentage of immature, intermediary, and mature erythrocytes in vimba bream (**a**) and white bream (**b**)

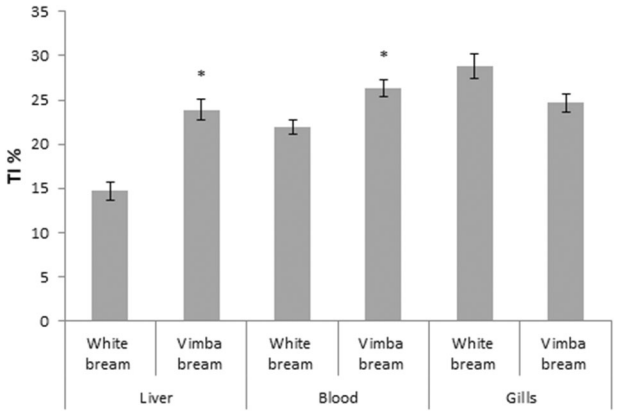

Fig. 4 DNA damage level in vimba bream and white bream liver, blood, and gill cells. *Statistically significant differences between species within a tissue, Mann-Whitney U test ($p < 0.05$)

common pathologies found in both species are shown in Fig. 5.

PCA analysis of combined results

According to the PCA analysis which included: genotoxicity parameter TI% in blood, liver, and gills, toxicity parameters (metals and metalloids in liver and muscle), erythrocyte morphometry, and histopathology score, 3 groups stand out: vimba bream group, white bream group, and a separate group with 1 vimba bream and 1 white bream. The latter group has increased concentrations of boron in the liver and muscle, chromium in muscle, a higher score for histopathology and TI% in blood, as well as a higher number of immature erythrocytes, which all indicate contamination. Vimba bream group is also clustered by higher concentrations of Fe in the liver and muscle, and higher scores for TI% in the liver, which may be related to this species' physiology and behavior (Fig. 6).

Table 3 Micronucleus and nuclear abnormalities frequency in blood of white bream and vimba bream

	MN ‰	NA ‰
White bream	0.32 ± 0.30	0.28 ± 0.44
Vimba bream	0.24 ± 0.22	0.24 ± 0.26

Groups were compared using Mann-Whitney U test ($p < 0.05$); values are presented as mean ± SD

Table 4 The average scores of histopathological alterations in vimba bream and white bream sampled at the locality Novi Banovci using a semi-quantitative scoring method

Histopathological alteration	White bream	Vimba bream	*p*-value
Gills			
Telangiectasia	1.2 ± 1.1	2.0 ± 1.6	0.494
Hyperaemia	4.4 ± 1.7	4.0 ± 0.0	0.661
Hyperthrophy of secondary epithelium	0.4 ± 0.9	1.0 ± 1.2	0.456
Necrosis	2.0 ± 1.4	2.0 ± 1.6	0.892
Oedema of secondary epithelium	4.0 ± 1.4	2.5 ± 1.0	0.140
Aneurism	0.8 ± 1.1	1.5 ± 1.0	0.396
Hyperplasia of epithelial cells	1.6 ± 0.9	1.0 ± 1.2	0.456
Oedema of primary epithelium	0.8 ± 1.1	1.5 ± 1.0	0.396
Proliferation of mucous cells	0.0 ± 0.0	0.5 ± 1.0	0.371
Presence of mucous cells in secondary lamellae	1.2 ± 1.1	0.5 ± 1.0	0.396
Infiltration	1.2 ± 1.1	0.0 ± 0.0	0.101
Atrophy	0.8 ± 1.8	0.5 ± 1.0	0.867
Histopatological index of gills (IG)	*26.0 ± 8.7*	*22.5 ± 5.3*	*0.556*
Liver			
Leukocyte infiltration	1.6 ± 1.7	2.5 ± 1.0	0.416
Necrosis	3.6 ± 1.7	2.0 ± 1.6	0.241
Stasis	2.0 ± 1.4	3.5 ± 1.9	0.281
Presence of MMC	0.0 ± 0.0	1.0 ± 1.2	0.128
Fibrosis of periportal and portal areas	1.6 ± 1.7	1.0 ± 1.2	0.687
Sinusoidal congestion	1.2 ± 1.1	2.0 ± 0.0	0.237
Sinusoidal dilation	2.4 ± 2.2	2.5 ± 1.0	0.771
Presence of ceroid pigments	0.0 ± 0.0*	1.5 ± 1.0*	0.037
Pyknosis of hepatocytes‘ nuclei	2.4 ± 1.7	1.0 ± 1.2	0.239
Increased number of eosinophilic granulocytes	0.8 ± 1.1	2.0 ± 1.6	0.283
Pleomorphic nuclei	1.2 ± 1.1	3.0 ± 1.2	0.078
Hyperplasia of bile ducts	1.2 ± 1.8	0.0 ± 0.0	0.240
Fatty degeneration	0.0 ± 0.0	0.5 ± 1.0	0.371
Presence of granuloma	0.0 ± 0.0	0.5 ± 1.0	0.371
Other nuclear alterations	0.8 ± 1.8	0.0 ± 0.0	0.502
Histopatological index of liver (IL)	*34.0 ± 12.3*	*34.0 ± 9.8*	*0.905*

The following semi-quantitative scores were given to each fish: 2 = low extent of histopathological alteration, 4 = moderate extent of histopathological alteration; 6 = severe extent of histopathological alteration; the groups were compared using Mann-Whitney U test, an asterisk (*) denotes statistically significant difference ($p < 0.05$) in average values of the same alteration among two species; values are presented as mean ± SD

Discussion

This study compared the response of several biomarkers in two autochthonous fish species at the Novi Banovci site on the Danube River, which is under the impact of untreated municipal and industrial wastewater. Also, in vitro treatment with native water samples on HepG2 cells was performed, as well as analyses of faecal indicator bacteria on site. Such an approach provides a complex set of results based on which it is possible to make a clearer assessment of how the same stress conditions affect different fish species, but also a possible health risk for humans. One of the limiting aspects of this study is the lack of a control site, which is a recognised issue in in situ studies, especially those carried out in urban areas (Sandhu and Lower 1989; Liber et al. 2007). On the other hand, it is possible to focus the research on spatial, temporal variations, or different species' responses at the same locality (Qadir and Malik 2011; Jovanović et al. 2018; Kostić-Vuković et al. 2021).

Analysis of microbiological indicators of faecal pollution is a reliable tool for the assessment of untreated municipal wastewater presence at the examined locality. Besides of the health risk that untreated sewage presents, it can also cause eutrophication by introducing a high content of nutrients into natural waters (Rechenburg et al. 2006). High numbers of TC downstream could be related to the increased nutrient concentration under the impact of wastewater discharge (Owili 2003). The group of TC comprises bacteria that are naturally present in the soil, vegetation and water (Edberg et al. 2000), so they are not considered reliable indicators of faecal pollution. On the other hand, the numbers of precise indicators of faecal pollution EC and EF, were the highest at the outlet spot. Since the aquatic environment is not their natural habitat, their number is expected to decrease downstream.

HepG2 cells represent an in vitro eukaryotic model system, considered appropriate for human risk assessment (Baderna et al. 2013). In this study, only the sample downstream of the wastewater outlet distinguished itself statistically from the negative control. Several studies showed genotoxic properties of untreated wastewater using hepatoma cell lines (Žegura et al. 2009; Manzano et al. 2015; Marić et al. 2020). Since these are metabolically active cells, they can inactivate direct acting mutagens. This could be a possible reason why no effect was shown during the treatment with water from the outlet itself. Downstream of the discharge, certain conditions can be established in which the genotoxicity of water increases. The study of Žegura et al. (2009) proposed that during the summer, high UV radiation, as well as eutrophication, may lead to the occurrence of novel genotoxic compounds. That the conditions of eutrophication prevail downstream from the discharge is also confirmed by the results of microbiological analyses with increased number of TC downstream.

Metals and metalloids enter the fish body mainly from water, sediment, and food. After they reach the intestine and liver they are transported to the fish muscle (Çoğun et al. 2006). Due to the use of muscle in human nutrition, analysis of metals in this tissue is necessary to assess the risk of consumption, while other fish tissues are examined to assess how these elements burden the body of fish in a polluted environment (Jovičić et al. 2015). The two studied species are both predominantly benthophagous and reflect the

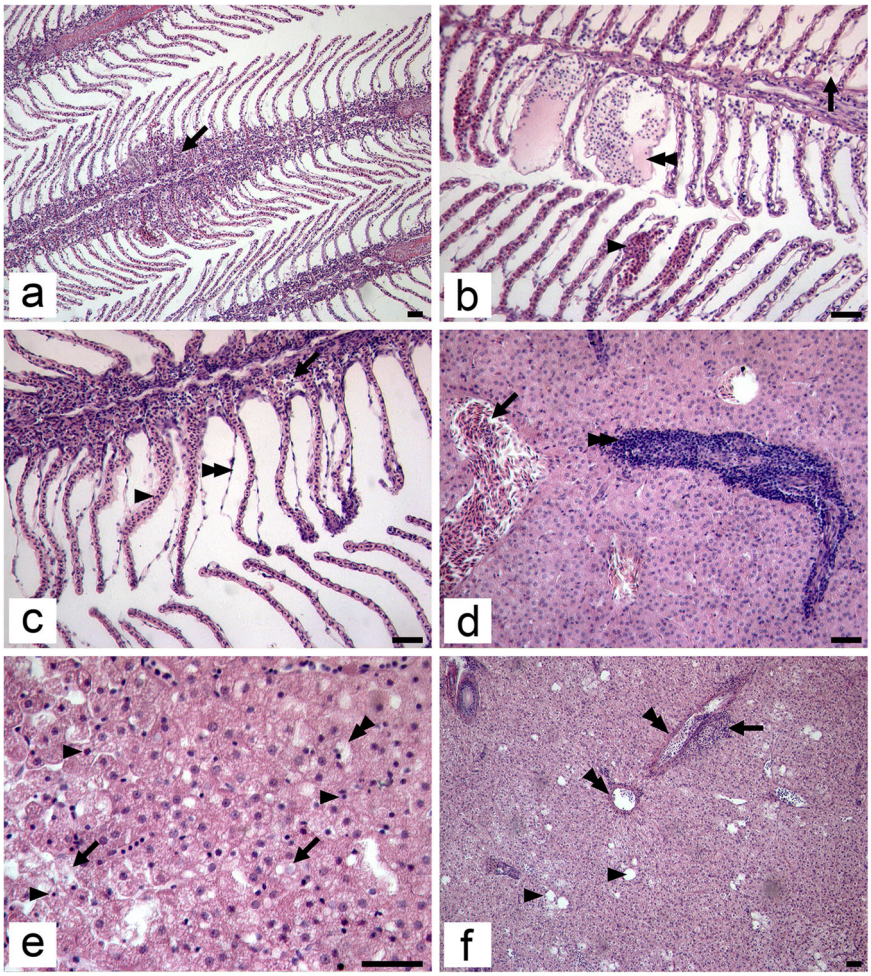

Fig. 5 Histopathological alterations found in gills (**a**–**c**) and liver (**d**–**f**) of the white bream (wb) and the vimba bream (vb) sampled from Novi Banovci site: **a** Proliferation of the interlamellar cell mass (arrow) in one primary lamella of the branchial apparatus (×100 HE) (wb). **b** Intense oedema of primary (arrow) and secondary epithelium, necrosis and presence of exudate in single secondary lamella (double arrowhead) and aneurism (arrowhead) of secondary lamella (×200 HE) (wb). **c** Presence of hyperaemia (arrowhead) and oedema of respiratory epithelium (double arrowhead) in secondary lamellae and infiltration of eosinophilic granulocytes (arrow) in primary lamella (×200 HE) (vb). **d** Stasis (arrow) in the larger blood vessel in the liver and heavy infiltration (double arrowhead) of portal and periportal areas (×200 HE) (vb). **e** Changes in liver parenchyma: condensed and pyknotic nuclei (arrowhead), necrosis of single hepatocytes (arrow) and vacuolation of hepatocytes' cytoplasm (double arrowhead) (×400 HE) (wb). **f** Dilation of sinusoidal capillaries (arrowhead), fibrosis of larger blood vessels (double arrowhead) and infiltration of leukocytes in the hepatic parenchyma (arrow) (×100 HE) (wb); bar = 50 µm

content of metals in the sediment (Dus et al. 2005; Rajkowska and Protasowicki 2013). Concentrations of examined elements did not differ significantly between the species, with exception of higher concentrations of Fe in both liver and muscle of vimba bream, and Cu and Ca in the liver of white bream. The observed differences could be attributed to species specific physiology, feeding habit, habitat preference, specimen size, etc. (Zhao et al. 2012; Yi and Zhang 2012). Many studies demonstrated the affinity of Fe and Cu to accumulate in liver, due to their role in metabolism and intensive blood supply of liver (Subotić et al. 2013a; Vilizzi and Tarkan 2016; Sunjog et al. 2016; Jia et al. 2017; Kostić-Vuković et al. 2021). The study of Das et al. (2021) reported that the stress hormone cortisol stimulates the rise of cytosolic free Ca^{2+} in the hepatocytes of rainbow trout. Nevertheless, many functions of the liver, such as bile secretion, glucose and energy metabolism, cell proliferation, and apoptosis are regulated by the increased Ca^{2+} concentrations (Amaya and Nathanson 2013). Furthermore, significantly higher concentrations of Fe and amounts of ceroid pigments in the liver of vimba bream could be related to their mutual existence in MMC (Viana et al. 2021). It is interesting to note that both species had Cu concentrations below 50 µg/g, which is in agreement with Cu homeostatic control mechanisms (Pyle et al. 2005). Fallah et al. (2011) proposed that difference in Fe muscle content in fish may be related to the different dominance of dark and light muscles. Unfortunately, to our knowledge,

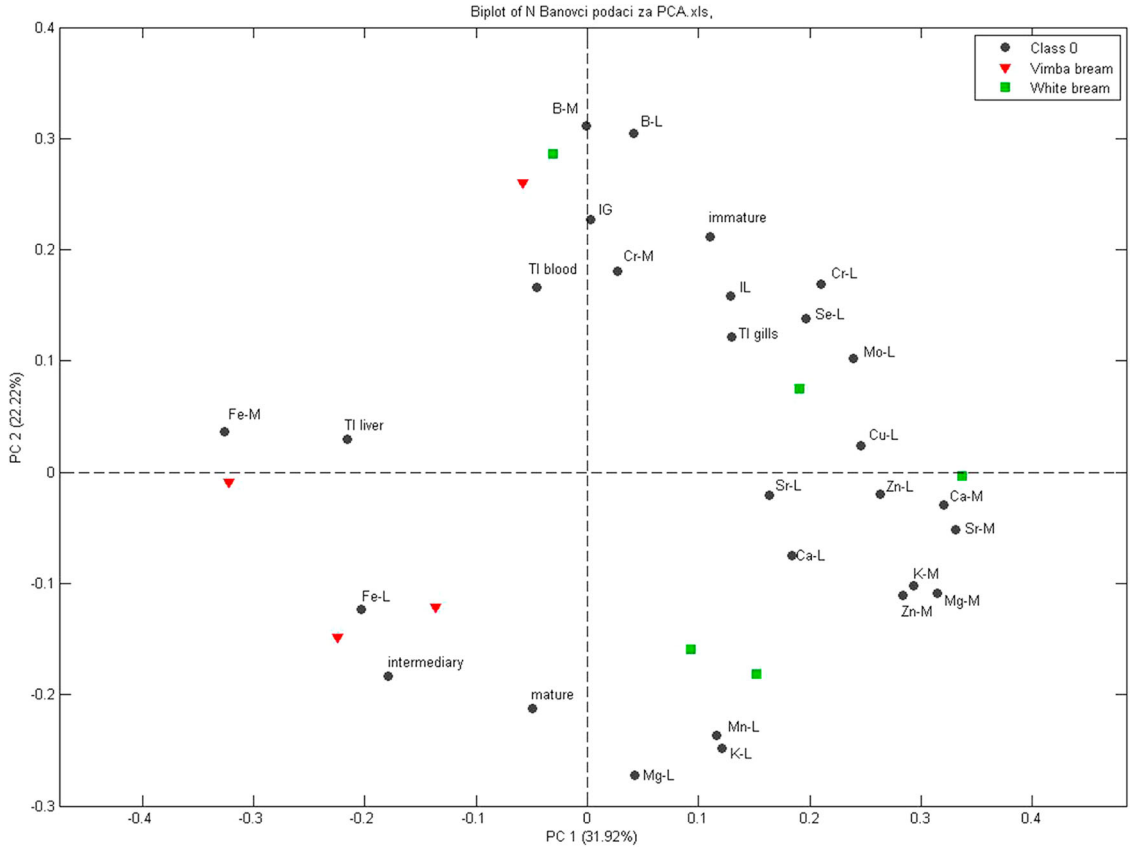

Fig. 6 PCA graph and grouping of analysed fish specimens using as input variables: genotoxicity parameters (TI% in blood, liver and gills), element concentration (B, Ca, Cr, Cu, Fe, K, Mg, Mn, Mo, Se, Sr, Zn) in muscle (M) and liver (L), histopathological index of gills (IG) and liver (IL) and abundance of immature, intermediary and mature erythrocytes

there are no studies related to this issue for the two examined species. Higher concentrations of macroelements Ca, K, and Mg were observed in muscles of both species. The propensity of these elements to accumulate in fish muscle was observed previously (Carvalho et al. 2005; Martins et al. 2017; Varol and Sünbül 2020; Subotić et al. 2021).

Regarding the erythrocytes morphometry, results show significant differences in analysed parameters (area, perimeter, length) of erythrocyte morphometry, which is in accordance with research conducted by Lahnsteiner (2021). A statistically significant increase in cell length in analysed species, between development stages, is a result of cell maturation, which was also reported by Rowan (2007). The proportion of immature erythrocytes, in both species, is in line with the results of Houston and Murad (1991), who reported that between 23% and 56.5% of circulating erythrocytes of goldfish (*Carassius auratus*) were immature. The proportion of immature erythrocytes was higher than those reported for brown bullhead (*Ameiurus nebulosus*) which was in the range of 4–37% (Rowan 2007). The author also observed that the ratio of immature erythrocytes had a positive association with a concentration of sediment contaminants and stated that pollution stress can stimulate erythropoiesis and increase the number of immature erythrocytes in circulation (Rowan 2007). Due to the high pollution output of the sampling site, there is a possibility that the higher proportion of immature erythrocytes is the result of stress induced erythropoiesis, however, further research is needed.

In general, there is a lack of studies in which genotoxic potential was assessed simultaneously in individuals of different species under the same stress conditions in situ. Comet assay is a sensitive tool for genotoxicity detection, revealing differences between seasons, sites, as well as species specific response (De Andrade et al. 2004; Kousar and Javed 2015; Kostić et al. 2016). To our knowledge, this is the first study that applied comet assay on vimba bream. The results revealed significantly higher DNA damage levels in blood and liver cells of vimba bream in comparison to white bream. This implies a higher sensitivity of this species as a bioindicator, indicating either increased exposure to genotoxic compounds through food and habitat selection or weakened mechanisms of DNA repair. Since the increased frequency of MN and NA were not shown in vimba bream, then disturbance of repair mechanisms may be excluded. The study of Grisolia et al. (2009) observed

high frequencies of MN only in top predator fish. Our findings are consistent with previous research that showed higher sensitivity of comet assay in comparison to MN to estimate the genotoxic potential and discriminate fine differences in the response of ecologically similar species (De Andrade et al. 2004; Grisolia et al. 2009; Kolarević et al. 2016).

Histopathological analyses provide fair information about the morphology of investigated organs. The analysis of HP alterations, together with their scoring and statistical analysis, can show a distinctive pattern of response. This pattern in some studies is species (or genus) specific (Nero et al. 2006; Van Dyk et al. 2009; Rašković et al. 2015), but in some studies, it is not (Dang et al. 2017). Yet, no difference in response pattern between different species sampled from the same site was also reported (Fonseca et al. 2016; Nørregaard et al. 2022). This is in line with the present study, as almost no differences in histopathological scores exist between vimba bream and white bream. Only the presence of intracellular pigment (ceroid) in the liver statistically differed between species. Concerning the investigated organs, the two most frequent alterations in the gills with moderate mean scores were oedema of secondary epithelium and hyperemia. Both alterations are among the most frequently encountered HP changes of the gills (Mallatt (1985); Poleksić and Mitrović-Tutundžić 1994; Roberts et al. 2004), that often occur as initial, and considered of"low importance" since they are reversible if water quality improves and do not represent a significant risk for the proper function of the branchial apparatus (Bernet et al. 1999; Rodrigues et al. 2019). Alterations in the liver with moderate mean scores were more numerous compared to the gills and of particular concern are alterations of hepatocytes: pleomorphic and pyknotic nuclei and cellular necrosis. These changes are depicting different aspects of apoptotic/necrotic cascade of hepatocytes deterioration and are irreversible in terms that repair of hepatic tissue in such stage is not possible. They are in line with many studies of either active or passive sampling of fish exposed to urban wastewaters runoff (Bernet et al. 2004; Camargo and Martinez 2007; Dane and Şişman 2015; Vincze et al. 2015; Pérez et al. 2018). The high frequency of necrosis in both investigated species is particularly pointing out the deleterious impact of untreated wastewaters on the biota of the Danube River, even before it enters the city of Belgrade with 1.7 million inhabitants. The city of Belgrade does not have a wastewater treatment plant (WWTP) and fish populations are in great danger from urban wastewater, which is burdened with a mixture of various pollutants. Beneficial effects of WWTP are documented elsewhere, with significant improvement of fish health, demonstrated by histopathology as the main biomarker (Wilhelm et al. 2017).

Conclusion

Fish as mobile bioindicators are able to avoid unfavorable conditions in the environment, still, they are undoubtedly influenced by the constant discharge of untreated wastewater that inhibits the natural ability of rivers to self-clean and biota to recover. Since the studied species are closely related and have a similar ecology, it is not unusual that no drastic differences in their response to the same pressures were observed. It should be emphasized that differences at lower levels of the biological organisation were more apparent, such as DNA damage assessed by comet assay. We could hypothesize that excessive pressure is needed to induce interspecific differences at higher levels of biological organisation, such as histological. However, it is worrying that in both species, an irreversible change of necrosis is already present in both the liver and the gills, pointing to chronic exposure. The results of this study unequivocally show the importance of effect-based monitoring, in order to enforce more effective management of natural resources and implementation of wastewater treatment systems. Regarding the results of this study, vimba bream could be singled out as a more sensitive indicator organism, but more studies are needed.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions ML and BV-G contributed to the study design, conceptualisation, and resources. JK-V and SK contributed to material preparation, data collection, and analysis of the comet assay on HepG2 and fish cells, as well as microbiological analysis. KS performed a micronucleus assay. SS conducted a study of erythrocyte morphometry. ŽV-J performed the analysis of metals and metalloids concentrations in fish tissues. VP and BR were responsible for the histopathological analyses of fish tissues. The first draft of the manuscript was written by JK-V. All authors commented on previous versions. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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