Nikolić D, Poleksić V, Skorić S, Tasić A, Stanojević S, Rašković B (2022) The European Chub (*Squalius cephalus*) as an indicator of reservoirs pollution and human health risk assessment associated with its consumption. Environmental Pollution, 310: 119871.

https://doi.org/10.1016/j.envpol.2022.119871

URL: https://doi.org/10.1016/j.envpol.2022.119871

THE EUROPEAN CHUB (*Squalius cephalus*) AS AN INDICATOR OF RESERVOIRS POLLUTION AND HUMAN HEALTH RISK ASSESSMENT

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ACKNOWLEDGEMENTS

This study was supported by the Ministry of Education, Science, and Technological Development of the Republic of Serbia [Grant numbers: 451-03-68/2022-14/200053, 451-03-68/2022-14/200116, and 451-03-68/2022-14/200030].

The authors wish to express their gratitude to Dr. Gorčin Cvijanović, Dr. Katarina Jovičić and Stojan Janjić, for their contribution in the fish sampling, Dr. Mirjana Mihailović and Zorica Radović for samples preparation, and Dr. Ljiljana Kostić Kravljanac for her contribution in the ICP-OES analysis. Also, we are grateful for the help and hospitality of our hosts from NP Tara, PIO Vlasina and PIO Medjuvršje.

ABSTRACT

Five reservoirs (Vlasina, Medjuvršje, Zaovine, Perućac, and Garaši) in Serbia were chosen as study sites, which differ by their position, purpose, stages of eutrophication, management policies, and levels of anthropogenic pressure. The objectives of this research were to: determine the concentrations of 26 elements in muscle, gills, and liver of the European chub by inductively-coupled plasma optical emission spectrometry (ICP-OES); determine the concentrations of 17 organochlorine pesticides in fish muscle by gas chromatography with mass spectrometric detection (GC-MS); compare these findings with condition factor (CF) and histopathological (HP) biomarkers; and assess the potential human health risks due to consumption of chub muscle tissue. The highest elemental accumulation was found in the gills. The European chub was not a good indicator of Pb pollution between reservoirs. Concentrations of Hg, As, and Cu were low and did not exceed the proscribed maximum allowed concentrations (MACs). 4,4′-DDE was detected only in individuals from Vlasina, 4,4′-DDD from Perućac and Zaovine, and heptachlor from Zaovine. Low to moderate levels of HP were observed for both gills and liver in all studied reservoirs. HP index for gills was significantly higher for Zaovine compared to Vlasina. Significantly lower HP index for liver and the total HP index value were observed for fish from Vlasina compared to Perućac. No significant human health risks due to the intake of examined pollutants in each reservoir were recorded; women were at higher risk compared to men. A reason for concern is a few muscle samples from Garaši, Vlasina, Perućac, and Medjuvršje in which Cd exceeded the MAC. A reservoir for water supply (Garaši) is generally considered the safest for fish consumption.

Keywords: fish, artificial lakes, toxic elements, organochlorine pesticides, histopathology, health risk

1. INTRODUCTION

Certain chemical pollutants are characterized by: long persistence and long-range transport; ability to resist chemical, photolytic, and biological degradation; capacity to bioaccumulate and biomagnify in the food webs (especially in aquatic ecosystems); as well as their toxic properties, the toxicity of their by-products, and adverse effects on wildlife and human (El-Shahawi et al. 2010; Buah-Kwofie et al. 2018; Rajeshkumar et al. 2018; Sun et al. 2020; Karaouzas et al. 2021). These pollutants are inorganic (i.e. heavy metals) and organic (i.e. persistent organic pollutants – POPs) compounds and are of both natural and anthropogenic origin. The type of the geological substrate, volcanic emissions, as well as atmospheric precipitation are among the natural sources (Mdegela et al. 2009), while processes such as urbanization, industrialization, and agriculture (use of pesticides and fertilizer) are one of the main anthropogenic causes of chemical pollution (El-Shahawi et al. 2010; Nyeste et al. 2019).

As placed in the middle or top of the aquatic food chains, fish are significant indicators of environmental pollution (Di Giulio and Hinton 2008; Nyeste et al. 2019; Nikolić et al. 2021a). As key organs in fish metabolism and target organs in toxicopathology, fish gills and liver are traditionally analyzed in monitoring environmental contamination and fish health (Macêdo et al. 2020; Nikolić et al. 2020a, Nikolić et al. 2021b; Santos et al. 2021). Moreover, muscle tissue is used for evaluation of the safety aspects of fish for human consumption (Djedjibegović et al. 2012; Nikolić et al. 2020a; Subotić et al. 2021). The European chub, *Squalius cephalus* (Linnaeus, 1758), is widely distributed in European fresh and brackish waters (Kottelat and Freyhof 2007). It prefers rivers and streams, but also can be found along the banks of slow-flowing lowland rivers, as well as in reservoirs in which it has acclimatized well, regardless of the altitude (Kottelat and Freyhof 2007). Because of its wide range of distribution and habitat preferences, abundance, trophic position (opportunistic predator) as well as its importance as game fish and human food (Kottelat and Freyhof 2007), European chub was often used as a bioindicator in environmental studies (Winter et al. 2005; Sunjog et al. 2016; Sunjog et al. 2019; Rašković et al. 2018; Nyeste et al. 2019).

As a biomarker of fish general health, we opted for assessing changes in histological structure of gills and liver in sampled fish. This approach is frequently employed in environmental studies as it is known as a good biomarker of effects, especially for chronic pollution (Santos et al. 2019; Teh et al. 2020, Schweizer et al. 2022).

Five reservoirs (Vlasina, Medjuvršje, Zaovine, Perućac, and Garaši) in Serbia were chosen as study sites. They differ by their position (altitudes ranging from 273 to 1213 m a.s.l.), purpose (electricity generation and drinking water supply), stages of eutrophication, management policies and levels of anthropogenic pressure. Garaši serves as a drinking water source and suffers a low anthropogenic impact, through runoff from agricultural land. Other reservoirs were formed for electricity generation. Anthropogenic impact in these reservoirs is reflected as: large variations in water levels (Zaovine), the occasional occurrence of floating debris (Perućac), industrial and urban runoff (Medjuvršje), and wastewater collection from a few nearby camps and settlements (Vlasina), The objectives of this research study were to assess possible environmental risks to the population of European chub using it as a biomarker of effect, as well as to conduct human health risk of consumption of the mentioned species. This was carried out by employing several methodologies, in order to: 1) determine the concentrations of 26 metals and trace elements in muscle, gills, and liver of the European chub; 2) determine the concentrations of 17 organochlorine pesticides – OCPs in fish muscle; 3) determine general fish health status using histopathological (HP) biomarkers in gills and liver; and 4) assess the potential human health risks using target hazard quotient (THQ) and target carcinogenic risk factor (TR) due to consumption of chub muscle tissue, regarding concentrations of metals, trace elements and OCPs. Therefore, we assume that the highest loadings of potentially toxic elements and pesticides (indicating also the highest human health risk from fish consumption and followed by more severe HP alterations in analyzed fish tissues) would be recorded for Medjuvršje, opposite to Garaši where the lowest values are expected.

2. MATERIAL AND METHODS

2.1. Sampling location

The field study was conducted during the summer of 2017 at Garaši (44.287054 N, 20.473708 E), Vlasina (42.727170 N, 22.363471 E), Perućac (43.968131 N, 19.364310 E), Zaovine (43.866337 N, 19.406074 E), and Medjuvršje (43.915620 N, 20.232869 E) (Fig. 1). The properties of these reservoirs, the intensity of anthropogenic impact, as well as sources and forms of their pollution vary considerably (Nikolić et al. 2020a; Nikolić et al. 2020b).

2.2. Fish sampling and sample preparation

Fifty individuals (ten per sampling site) were sampled using standing gillnets (30 m × 2 m, 30–50 mm mesh size). The authentication of fish (*Squalius cephalus*) was performed using Kottelat and Freyhof (2007). Before the dissection, each fish was sacrificed with a quick blow to the head and measured. The total body length (TL, cm) and weight (W, g) were used for the calculation of the condition factor (CF), $CF = W\ TL^{-3} \times 100$ (Ricker 1975).

The samples of muscle, gills, and liver intended for elemental analysis were washed with distilled water and stored at −20 °C. In the laboratory, samples were measured two times – before and after they were dried in the Freeze Dryers Rotational Vacuum Concentrator, GAMMA 1–16 LSC, (Germany). Dried samples portions between 0.2 and 0.4 g were digested at the Dried fish program (200 °C) in a microwave digester (ETHOS EASY, Milestone, Italy), by adding 4 mL of hydrogen peroxide (30%) (Merck, Germany) and 6 mL of nitric acid (65%) (Merck, Germany). Each sample was diluted with distilled water to a total volume of 25 mL before analysis.

The modified QuEChERS technique with acetonitrile was used for the extraction of organochlorine pesticide residues, which were purified using dispersive solid-phase extraction (d-SPE) cleanup. The liquid-liquid partition was conducted using 10 g of fish muscle, 5 mL of water, 10 mL of acetonitrile, 1.6 g of $MgSO_4$, and 0.4 g of NaCl, and the dispersive solid-phase extraction using 80 mg of C18, 80 mg of primary secondary amine and 150 mg of $MgSO_4$. Final extracts of acetonitrile were concentrated using a gentle stream of nitrogen and reconstituted into hexane. To get the best possible extraction results, extracts were re-purified using the C18 column before the analysis.

For histopathological (HP) analysis, samples of gills and liver were quickly removed and fixed in 4% formaldehyde (Lach-Ner, Neratovice, Czech Republic). After 48h, the formaldehyde solution was replaced with 70% ethanol. An automatic tissue processor Leica TP 1020 (Leica, Nussloch, Germany) was used for sample dehydration and clearing using graded ethanol series and xylene, respectively. Later, samples were embedded in paraffin, and paraffin blocks were sectioned using SM 2000R (Leica, Nussloch, Germany) to make 5 μm sections. These sections were transferred to glass slides, deparaffinized, and stained in an automatic slide stainer ST 4040 (Leica, Nussloch, Germany) using haematoxylin and eosin (HE). A Leica DM LB (Leica, Mannheim, Germany) microscope equipped with a DFC 295 camera (Leica, Mannheim, Germany) was used to take micrographs.

2.3. Element analysis

An inductively-coupled plasma optical emission spectrometry (ICP-OES, Spectro Genesis EOP II, Spectro Analytical Instruments DmbH, Germany) was used for measuring concentrations of 26 elements with wavelength lines (λ, nm) given in brackets: Ag (328.068), Al (396.152), As (189.042), B (249.773), Ba (230.424), Ca (317.933), Cd (214.438), Co (228.616), Cr (267.716), Cu (324.754), Fe (238.204), Hg (184.950), K (766.491), Li (460.289), Mg (285.213), Mn (294.921), Mo (202.095), Na (589.592), Ni (231.604), P (214.914), Pb (220.353), S (182.034), Se (196.090), Si (251.612), Sr (460.733), and Zn (213.856). The reference materials BCR-185R Bovine liver (European Commission Joint Research Center) and IAEA-336 Lichen (AQCS, International Atomic Energy Agency) were used to assess the quality control of the analytical process and indicated that the concentrations were within 90–115% of the certified values for all elements included in the study. When the concentration of a certain element was below the detection limits (ND), the value equal to half of the spectrometer sensitivity (ICP-OES) for this element was used.

All element concentrations are expressed as μg g^{-1} dry weight (dw). Wet weight (μg g^{-1} ww) metal concentrations were also used to calculate metal pollution index (MPI), assess human health risk, and compare concentrations of Cd, Hg, Pb, As, Cu, and Zn in fish muscle with the maximum allowed concentrations (MAC) in fish meat set by the national legislation of Serbia (Official Gazette of RS 2018) and the European Union (EU) (EC 2006). According to both legislations, the MACs for Cd, Hg, and Pb are 0.05, 0.50, and 0.30 μg g^{-1} ww, respectively. The MACs for As, Cu, and Zn are 2.0, 30.0, and 100.0 μg g^{-1} ww, respectively (Official Gazette of RS 2018).

2.3.1 Metal pollution index (MPI)

According to Usero et al. (1997), MPI was used to compare the total metal content in muscle, gills, and liver of European chub from different reservoirs:

$$\mathrm{MPI} = (\mathrm{C}_1 \times \mathrm{C}_2 \times \ldots \times \mathrm{C}_\mathrm{n})^{1/\mathrm{n}} \qquad (1)$$

where C_n refers to the concentration of metal n (μg g^{-1} ww).

2.4. Analysis of organochlorine pesticides residues (OCPs)

OCPs (aldrin, α-HCH, β-HCH, γ-HCH, δ-HCH, 4,4'-DDD, 4,4'-DDE, 4,4'-DDT, dieldrin, endosulfan I, endosulfan II, endosulfan sulfate, endrin, endrin aldehyde, heptachlor, heptachlor epoxide, metoxychlor) in fish muscle (fillet) were analyzed by gas chromatography with mass spectrometric detection (GC-MS). A GC Clarus 680 PerkinElmer system comprising an autosampler and a gas chromatograph interfaced with an MS Clarus SQ8T instrument were used under the following conditions: capillary column Elite-5MS (30 x 0.25 mm ID x 0.25 µm df, composed of 95% dimethyl polysiloxane and 5% Phenyl), operating in the electron impact mode at 70 eV. The temperature of the ion source was 280 ºC. The carrier gas was helium (99.999%) at the constant pressure of 22.5 psi and an injection volume of 2 µL (a split ratio of 50:1) was employed at the injector temperature of 250 ºC. The oven temperature was set at 70 °C for 3 minutes, and increased to 150 °C at a rate of 25 °C min^{-1}, then to 200 °C at 3 °C min^{-1}, and finally to 280 °C at 8 °C min^{-1}, and held for 10 min. Mass spectra were taken at 70 eV, a scan-interval of 0.2 seconds and fragments from 50 to 400 Da were applied. The software Turbo Mass Ver 6.1.0 was used.

Stock standard solutions (10 µg mL^{-1}) were obtained from AccuStandard Inc. (New Haven, USA), and working solutions by appropriate dilutions with hexane. A blank sample of European chub was used for preparation matrix calibration curves by addition calibration standard (0.01, 0.02, 0.03, 0.05, and 0.10 mg kg^{-1}) in European chub before extraction.. We used TPP (triphenyl phosphate) as an internal standard. The method was validated in terms of linearity, limits of detection (LOD), limits of quantification (LOQ), specificity, accuracy and precision based on the SANTE 11312/2021 validation guidelines. Mean recovery values obtained after a spike of 10 µg kg^{-1} and 20 µg kg^{-1} were done in 6 replicates. Spike was initially added to the blank fish sample and a preparation procedure was performed. Also, the preparation method was confirmed by satisfactory results for Endosulfan II (beta) using a certified reference material - oily fish (Fapas, UK), the obtained value was 18.1 µg kg^{-1}, and the assigned value was 17.8 µg kg^{-1} with a permissible range 10.0-25.6 µg kg^{-1}. The linearity of the analytical response across the studied range of concentrations, LOD and LOQ are given in Table 1. The average recoveries of the pesticides from the European chub ranged from 80.6% to 107.5%, for fortification level of 10 µg kg^{-1} and 20 µg kg^{-1}.

All OCPs concentrations are expressed as µg kg^{-1}. Concentrations of 4,4'-DDD, 4,4'-DDE, 4,4'-DDT, heptachlor and heptachlor epoxide in fish muscle were compared with the maximum allowed concentrations (MAC) in fish meat set by the national legislation of Serbia (Official

Gazette of RS 2011). The MAC for DDT and derivatives is 1.0 mg kg^{-1}, and heptachlor and heptachlor epoxide 0.1 mg kg^{-1}.

2.5. Health risk assessment

The health risk was assessed by USEPA (1989) methodology. We assumed that inorganic As was 3% of the total As (Varol and Sünbül 2019), cooking fish meat has no effect on pollution, ingested and absorbed pollutant dose are the same, the average European chub ingestion rate (FIR) is 20 g d^{-1}, the average body weight (BWa) for women is 63 kg and 86 kg for men, the average lifetime or exposure duration (ED) for women is 78.1 years and 73.2 years for men. The data were obtained from the Republic Statistical Office of Serbia. Mean values for each element were used in the human health risk assessment analysis.

2.5.1. Target hazard quotient (THQ)

$$\mathrm{THQ} = ((\mathrm{EFr} \times \mathrm{ED} \times \mathrm{FIR} \times \mathrm{C})/(\mathrm{BWa} \times \mathrm{AT} \times \mathrm{RfD})) \times 10^{-3} \qquad (2)$$

$$\text{Total THQ (TTHQ)} = \sum \mathrm{THQ} \qquad (3)$$

where EFr represents the exposure frequency (365 d $year^{-1}$), C is the concentration of investigated pollutant in fish (μg g^{-1}), AT refers to the averaging exposure time for non-carcinogens (365 d $year^{-1}$ × number of exposure years), and RfD represents the oral reference dose (mg $kg^{-1}d^{-1}$): 0.0003, 0.001, 1.5, 0.00016, 0.004, and 0.3 for As, Cd, Cr, Hg, Pb, and Zn, respectively, as well as 0.0005 for heptachlor (USEPA, 2009). The TTHQ or hazard index lower than 1 indicates the absence of significant non-carcinogenic risk (Wang et al. 2005; Zheng et al. 2007).

2.5.2. Target carcinogenic risk factor (TR)

$$\mathrm{TR} = ((\mathrm{EFr} \times \mathrm{ED} \times \mathrm{FIR} \times \mathrm{C} \times \mathrm{CSF_O})/(\mathrm{BWa} \times \mathrm{AT})) \times 10^{-3} \quad (4)$$

where AT represents the averaging time for carcinogens (365 d year^{-1} × ED), and $\mathrm{CSF_o}$ refers to the oral carcinogenic slope factor (mg kg^{-1} d^{-1}): 1.5 for As, 0.0085 for Pb, 0.24 for 4,4′-DDD, 0.34 for 4,4′-DDE, and 4.5 for heptachlor (USEPA, 2009). In cases where $\mathrm{TR} < 10^{-6}$, cancer risk is considered negligible, while $10^{-6} < \mathrm{TR} < 10^{-4}$ is tolerable (Islam et al. 2014).

2.6. Histopathological (HP) analysis of gills and liver

For the quantification of HP alterations in gill and liver tissue, we used the methodology developed by Bernet et al. (1999). Generally, this semiquantitative scoring system recognizes four reaction patterns (*rp*): inflammatory, circulatory, progressive, and regressive. The importance factor (IF or *w*) was allocated to each HP alteration (Table 3). It ranges from 1 (minimal importance) to 3 (marked importance), pointing out the significance and relevance of a specific alternation(s). A score value (*a*), ranging from 0 (none) to 6 (severe alteration), is determined based on the extent of certain alteration. The following formulas were used:

(a) Reaction index of the organ:

$$\mathrm{I}_{org\ rp} = \sum_{alt}\left(a_{org\ rp\ alt} \times w_{org\ pr\ alt}\right) \quad (5)$$

(b) HP index of the organ:

$$\mathrm{I}_{org} = \sum_{rp}\sum_{alt}\left(a_{org\ rp\ alt} \times w_{org\ rp\ alt}\right) \quad (6)$$

(c) Total index for each individual:

$$\mathrm{I}_{T} = \sum_{org}\sum_{rp}\sum_{alt}\left(a_{org\ rp\ alt} \times w_{org\ rp\ alt}\right) \quad (7)$$

where *org* refers to the organ (I_{G} – gills; I_{L} – liver), and *alt* is to the alteration.

2.7. Statistical analysis

Detected concentrations for all elements are expressed as mean (s) ± standard deviation (SD). First, we tested the normality (Shapiro-Wilk test) and homoscedasticity (Levene's test) of the data. If data followed a normal distribution and passed homoscedasticity assumptions, significant differences among groups (reservoirs) were tested using the one-way ANOVA, followed by Tukey’s HSD *post-hoc* test. On contrary, when data lacked normal distribution we used the non-parametric Kruskal-Wallis *H* test in order to assess differences among groups (reservoirs), followed by the Mann–Whitney *U* test. The Spearman’s rank correlation test was used to investigate the correlation between CF and element concentration as well as the relationship between HP scores for gills and liver. We set the significance level (α) at 5%. For principal components analysis (PCA), the untreated concentrations of elements for gills and liver tissue were used as input variables, as well as each of reaction patterns in both tissues. The analysis was applied to assess the differentiation among the concentrations of elements and histopathological scores and first two principal components were used for creating plots. The Statistica 7.0 Software (StatSoft, Inc., Tulsa, OK, USA) was used for statistical analyses.

3. RESULTS

3.1. Fish condition (CF)

Statistically significant differences in CF, fish length, and weight between the reservoirs are presented in Table 2. Fish from the Garaši reservoir had the smallest body mass, length and CF ($p \leq 0.05$), while fish from Medjuvršje had significantly higher values of CF compared to Garaši, Zaovine, and Vlasina.

3.2. Element analysis

The highest concentrations of S were detected in muscle, Ca, Cd, Mg, Mn, P, Se, Sr, and Zn in gills, and Cr, Cu, Fe, and Mo in the liver at all study sites (Table 2). On the other hand, the lowest concentrations of Cu, Fe, and Na were observed in muscle, as well as Ca, Cd, and Mg in the liver. Concentrations of Hg, Pb, As, and Cu did not exceed the proscribed MACs. However, concentrations of Cd in Garaši in one sample, Vlasina in two samples, Perućac in two samples, and Medjuvršje in two samples exceeded the MACs.

Statistical tests revealed significant differences between reservoirs regarding the concentrations of all elements, except for Ba and Pb (Table 2). In muscle tissue, the highest concentrations were recorded for Ag and B in fish from Zaovine and Perućac, respectively. The highest concentrations of Al and B in gills were recorded in fish from Medjuvršje and Perućac, respectively. In the liver, the highest concentrations were recorded for Ag, B and K in fish from Zaovine, Perućac and Garaši, respectively. On the other hand, individuals from Perućac had significantly lower concentrations of Mg in muscle and Sr in gills compared to fish from other reservoirs. Significant negative correlations were found between CF and Mn (r = -0.576917), Na (r = - 0.730900), P (r = - 0.505498), and Sr (r = - 0.589442) in muscle, Cr (r = - 0.516765) in gills, and K (r = - 0.600576) in liver.

According to MPI, the gills were exposed to the highest pressure of metal pollution at all studied localities (Table 2). The MPI value for muscle was significantly lower for Zaovine compared to other reservoirs. A significantly lower MPI value for the liver was recorded for Međuvršje compared to the Garaši reservoir.

3.3. OCPs analysis

The linearity of the analytical response across the studied range of concentrations was excellent, with very high correlation coefficients (Table 1). The concentrations of aldrin, α-HCH, β-HCH, γ-HCH, δ-HCH, 4,4'-DDT, dieldrin, endosulfan I, endosulfan II, endosulfan sulfate, endrin, endrin aldehyde, heptachlor epoxide, and metoxychlor were not detected at any studied reservoir. On the other hand, we detected concentrations of 4,4′-DDE in four individuals (10 μg kg^{-1}, 15 μg kg^{-1}, 14 μg kg^{-1}, 20 μg kg^{-1}) from Vlasina, 4,4′-DDD in four individuals (10 μg kg^{-1}, 12 μg kg^{-1}, 13 μg kg^{-1}, 15 μg kg^{-1}) from Perućac and four individuals (10 μg kg^{-1}, 12 μg kg^{-1}, 14 μg kg^{-1}, 18 μg kg^{-1}) from Zaovine, as well as heptachlor in four individuals (12 μg kg^{-1}, 13 μg kg^{-1}, 10 μg kg^{-1}, 17 μg kg^{-1}) from Zaovine. Interestingly to mention, individuals from Zaovine in which 4,4′-DDD was detected also had heptachlor in their muscle tissues. Concentrations of analyzed pesticides did not exceed the proscribed MACs.

3.4. Human health risk assessment

The TTHQ values indicated that there is no significant non-carcinogenic health risk due to the intake of examined elements and heptachlor (Fig. 2). The highest TTHQ was recorded for Perućac, and the lowest for Zaovine reservoir both for women and men. Higher TTHQ was recorded for women compared to men for all reservoirs. In all studied cases, elements contributed to the TTHQ in the following order: Hg, Cd or Zn, Pb, As, and Cr. There was no THQ for Cu since this element was not detected in the muscle of any fish. On the other hand, USEPA (2009) has not evaluated RfDs for 4,4′-DDD and 4,4′-DDE.

The TR values were lower for Pb than for As (Table 3). Higher TR values for both elements were recorded for Medjuvršje and Vlasina compared to the other reservoirs. For Zaovine, TR values for heptachlor were higher compared to 4,4′-DDD. Additionally, women were at higher cancer risk compared to men, but the risk was considered negligible or tolerable. Garaši reservoir is generally considered as safest for fish consumption.

3.5. Histopathological analysis of gills and liver

Several different histopathological alterations were found in the gill tissue of sampled chub (Table 4; Fig. 3). Some mean histopathological scores in fish had the extent higher than 50% in gill samples (which corresponds with score 3 in the table), such as hyperaemia and the presence of mucous cells in secondary lamellae (Fig. 3a), but the majority of fish had mean histopathological scores less than 3. In addition, those two alterations are reversible if environmental conditions stay the same or do not deteriorate. The alterations with higher importance factor such as infiltration, proliferation of epithelial cells (Fig. 3b) and necrosis (Fig. 3c) had low to moderate mean histopathological scores in fish from all sampled locations. Concerning statistical significance between mean gill scores of fish sampled from different water reservoirs, hypertrophy of respiratory epithelium and proliferation of mucous and epithelial cells were three alterations that showed differences among reservoirs ($p = 0.013$, $p < 0.001$ and $p = 0.009$, respectively), and in both alterations, mean scores were highest in fish from Garaši reservoir, while lowest at Vlasina and Zaovine reservoirs. A various number of parasites from three distinctive groups (oval cysts of organisms from the class of Myxosporea and individuals of *Trichodina* spp. and monogenean flukes) were found in fish gills in the following frequencies: Garaši – 50%, Vlasina – 30%, Perućac – 70%, Zaovine – 20%, Medjuvršje – 80%.

In the liver, there were a higher number of statistically significant differences between mean scores in fish caught at various locations, compared to the gills. Circulatory alterations, such as sinusoidal dilation ($p = 0.002$) and congestion ($p = 0.005$) were both with the lowest scores in fish from the Garaši reservoir, while the highest mean scores were found in fish from Zaovine (dilation; Fig. 3d) and Perućac reservoir (both dilation and congestion). On contrary, fish caught at Zaovine and Vlasina reservoirs had the lowest scores for several histopathological alterations ($p \leq 0.05$): pyknosis of nuclei (Zaovine and Vlasina), focal changes of hepatocytes (Zaovine) and the presence of melanomacrophage centers (MMCs) (Vlasina), while highest mean scores for mentioned alterations were found at Perućac (stasis (Fig. 3e), pyknosis (Fig. 3f), focal changes of hepatocytes, vacuolization of hepatocytes), Garaši (focal changes of hepatocytes, vacuolization of hepatocytes) and Zaovine (MMCs). The general structure of the liver, with branching rows of hepatocytes intermingled with sinusoidal capillaries and with bile ducts and blood vessels as elements of stroma, is not altered.

Reaction patterns of HP alterations were also given in indices, separately for gills (Fig. 4a) and liver (Fig. 4b). It showed that mean index of progressive changes was significantly lower in Vlasina and Perućac reservoirs, comparing to Garaši (I_{LP}; $p \leq 0.05$), but at the same time, index of regressive changes (I_{LP}) was lowest in fish from Garaši reservoir compared to Zaovine ($p \leq 0.05$). In liver, mean values of reaction patterns of circulatory disturbances, regressive changes and inflammation were all highest in fish caught at Perućac reservoir, compared to either Vlasina

(I_{LR} and I_{LI}; $p \leq 0.05$) or to Garaši (I_{LC}; $p \leq 0.05$). HP index for gills (I_G) was significantly higher for Zaovine compared to Vlasina (Fig. 5). Significantly lower HP index for liver (I_L), as well as the total HP index value (I_T) was observed for fish from Vlasina compared to Perućac (Fig. 5).

3.6 Principal components analysis

The results of the PCA are shown at Fig. 6, but in both gills and liver first two principal components are describing relatively low percentage of total variation (32% for gills and 35% for liver). However, elements and histopathological indices in gills revealed association between I_{GC} and B and to less extent with cluster of elements (Hg, Ag, Co, and Si). Since regression changes scores were highest in the majority of samples, they contributed the most to total HP index and that is the reason for close association I_{GR} of and I_{GT}. Both indices were associated with Al, Li, Na, and As. I_{GP} and I_{GI} were associated with Pb, Mn, K, and S. In liver, almost all HP indices (I_{LC}, I_{LP}, I_{LR}, I_{LT}) were associated with Pb and B. I_{LI} was associated with cluster of elements (Li, Al, Mn, Ag, Cr, Fe).

4. DISCUSSION

Differences in CF between fish from studied reservoirs did not reflect the differences in elemental accumulation in their tissues. However, it was observed that the increase of certain elements (i.e. Na, K, Sr, Mn, P, and Cr) in fish tissues was coincident with lower CF values. Kerambrun et al. (2012a, 2012b) noted that CF values decreased after exposure of fish to xenobiotics. On the contrary, there are also cases when fish fitness increased with the increase of some element’s concentration (Fonseca et al. 2015). As the CF index has given variable results in many studies regarding fish responses to pollution, Kroon et al. (2017) suggested that the specificity and applicability of this biomarker should be examined in more detail.

Analyzing the concentrations of Cd, Cu, Hg, and Pb in six fish species from the Neretva River, Djedjibegović et al. (2012) found the highest total heavy metal load in European chub and emphasized its importance as a bioindicator in environmental studies. European chub also accumulated more metals compared to pikeperch (*Sander lucioperca*) and Cactus roach (*Rutilus virgo*) from the Zlatar reservoir (Nikolić et al. 2022). Concentrations of elements in chub tissues varied significantly both among tissues and reservoirs. According to the results, the highest elemental accumulation was found in the gills, followed by the liver. This was the case with similar studies conducted on European chub (Rašković et al. 2018; Nyeste et al. 2019). The gills participate in ion exchange with the environment through transport proteins or ion channels (Wood 2001, 2011; Erickson et al. 2008), while the liver which serves as a detoxification center has the ability to bioaccumulate due to the presence of proteins (e.g. metallothioneins) that can bind elements (Weber et al. 2020). The muscle tissue had the lowest potential for bioaccumulation, and this is common for both chub (Yilmaz et al. 2007; Sunjog et al. 2016; Sunjog et al. 2019; Nyeste et al. 2019) and other fish species (Nikolić et al. 2020a; Nikolić et al. 2021a). However, analysis of elemental concentrations in fish muscle is extremely important since it is found to be a target tissue for the accumulation of Hg (Dušek et al. 2005; Rašković et al. 2018; Łuczyńska et al. 2018; Nikolić et al. 2020a, Nikolić et al. 2021a, Nikolić et al. 2021b).

Compared to other heavy metals investigated in this study, the European chub was not good indicator of Pb pollution between investigated reservoirs. This was not the case with the study on the European perch (*Perca fluviatilis*), where fish from Medjuvršje and Vlasina had lower concentrations of Pb in their tissues compared with the individuals from Garaši, Perućac, and Zaovine (Nikolić et al. 2020a). In all analyzed muscle samples, concentrations of Pb as well as As and Hg were below the prescribed MACs, which was also noted in other studies on the European chub (Yilmaz et al. 2007; Sunjog et al. 2016; Sunjog et al. 2019; Rašković et al. 2018). A reason for concern is a few muscle samples in which Cd exceeded the MAC value. Chronic intoxication of humans with Cd is responsible for kidney, lung, and prostate cancers (Waalkes, 2003), while acute intoxication is associated with testes, lungs, and liver injuries (Bertin and Averbeck 2006). An increased concentration of Cd could be due to a severe sediment enrichment of all studied reservoirs with this element (Nikolić et al. 2020c). Higher concentrations of some elements (i.e. Mn, Mo, Ni) in fish tissues from sites with low or without known anthropogenic pollution were also found in similar studies on the European chub (Sunjog et al. 2016; Rašković et al. 2018). These differences could be a consequence of both biological (physiological acclimation and/or fish diet) and geochemical (composition of the sediments) factors.

The differences in MPI values for muscle and liver between Medjuvršje and Garaši were *inter alia* a consequence of significantly different concentrations of Na and Ni. Ni could be of both natural (rocks and soils, dust, forest fires, volcanic emissions, and vegetation) and anthropogenic origin (sewage and waste, product of fossil fuel and coal combustion), and deposited in the sediments (Cempel and Nikel 2006). As part of the Tara national park, located at almost 900 m a.s.l., the Zaovine reservoir is exposed to a low anthropogenic impact. This could be a reason for significantly lower MPI value for muscle compared to other reservoirs. On the other hand, Ag which has bioaccumulate in higher concentrations in muscle and liver tissues of fish from this reservoir compared to other study sites is of natural origin. It is expected that sediments are sinks for a majority of the silver, where it appears in complexes and compounds (Purcell and Peters

1998). The concentrations of B in all tissues were higher at Perućac compared to other reservoirs, which was also confirmed in the study conducted on the European perch (Nikolić et al. 2020a). This element is used in the production of detergents, bleaches and fertilizers, glass and fiberglass industries, metallurgy, and nuclear shielding, but it is also present in many silicate minerals (Parks and Edwards 2005). Al is the most abundant metallic element of Earth′s crust (Ciacci et al. 2013), and its solubility increases with decreasing water pH (Gensemer and Playle 1999). Although Al causes both negative respiratory and ion regulatory effects, fish can acclimate to this element, but at a metabolic cost (Gensemer and Playle 1999). The increased levels of K in the liver of fish from Garaši are probably due to the fertilization of agricultural fields located on the banks of this reservoir.

Comparing the concentrations of OCPs in muscle tissue in caged and feral chub from rivers in the West Midlands (UK), Winter et al. (2005) emphasized the use of feral fish in biological monitoring programs as they accumulated contaminants at higher levels. Analyzing concentrations of DDTs (pp′-DDT and pp′-DDE) in chub muscle from the Po River in Italy, Viganò et al. (2000) found higher concentrations of these organochlorines compared to our study. Numerous and diffuse sources of these pollutants (i.e. agricultural and urban runoffs, urban wastewater discharges, atmospheric deposition) in the Po River are probably a reason for this. On the other hand, no OCP residues were found in any of the six fish species (including European chub) from the Karakaya Dam Reservoir, Turkey (Varol and Sünbül 2019). Residues of OCPs (isomers of DDT, DDD, DDE, and HCHs) were detected in all samples of chub muscle from the Elbe River in the Czech Republic (Randak et al. 2009). Low levels of pp′-DDT compared to pp′-DDE indicated that DDT-based insecticides were not in use for several decades. However, pp′-DDT and op′-DDT were major contributors to pollution downstream from

Pardubice indicating the possibility of recent release or use of DDT in this area (Randak et al. 2009).

Total THQ and TR indicated that there is no significant non-carcinogenic nor carcinogenic health risk due to the intake of examined pollutants in each reservoir. A major total THQ contributor for all reservoirs, for both men and women, was Hg. This element contributed the most to total THQ values from similar studies conducted on fish (Storelli 2008; Nikolić et al. 2020b; Nikolić et al. 2021b; Nikolić et al. 2022). The primary way of Hg exposure for humans is nutrition with contaminated fish (Tchounwou et al. 2003; Storelli 2008). Intoxications of human with this element lead to kidney injuries, stomatitis, and permanent damage to the CNS (Gochfeld, 2003; Tchounwou et al., 2003). The probability of a person to develop cancer over a lifetime is indicated by the TR, and based on animal studies, Pb is recognized as a Class-B2 carcinogen, and As as a Class-A human carcinogen (USEPA, 2009). In addition, the tolerance to contaminants depends on human body weight and lifetime.

Semi-quantitative scoring of histopathological changes in gills showed three changes that differed between fish caught at studied reservoirs: hypertrophy and hyperplasia of epithelial cells, as well as hyperplasia of mucous cells. This is in line with other published studies on European chub or closely related species Vardar chub (*Squalius vardarensis*), which reported more pronounced lamellar hypertrophy and epithelial hyperplasia in natural ecosystems of the Balkan peninsula polluted with metals (Barišić et al. 2015; Dane and Sisman 2017; Hermenean et al. 2017; Rašković et al. 2018). Both alterations represent efficient mechanisms for the physiological adaptation of fish to the presence of pollutants in the water, since they result in increased physical barrier between water and blood (Baberschke et al. 2019). There are several possible reasons for the proliferation of mucous cells in fish gills. It is established that salinity and in general alteration in any kind of ionic stress or even temperature of water influence the density of mucous and chloride cells in freshwater fish (Moron et al. 2009; Cabillon and Lazado 2019). On the other hand, parasites and other pathogens can also increase the number of mucous cells in branchial tissue (Dang et al. 2020; Dezfuli et al. 2021). Concerning parasites, half of the gill samples analyzed were infested with parasites, sometimes two to three species of parasites were found, particularly in Medjuvršje where 8 out of 10 samples were infested. This histopathological alteration is also reported earlier in European chub sampled from polluted rivers, and it is usually accompanied by the transfer of mucous cells to secondary lamellae (Triebskorn er al. 2008; Rašković et al. 2018). We can only speculate about the cause of described histopathological alterations in European chub, because there are no exposure laboratory trials involving histopathology in this species, so there is also a lack of baseline studies. Concerning other histopathological alterations that did not show significant difference among sampling sites, the extent of necrotic tissue in the gills of fish from the Zaovine reservoir is of importance, since this alteration is irreversible and part of the branchial tissue that was affected is impossible to be remodeled and provide normal respiration (Huang et al. 2020).

Compared to the gills, the liver was a more responsive organ, in terms of the higher number of histopathological alterations that differed among fish inhabiting studied reservoirs. Changes that differed among reservoirs were mainly from two distinctive groups: circulatory changes and alterations of hepatocytes.

The increased blood flow in the liver is one of the factors for circulatory changes in the tissue. When blood flow increases, stasis and congestion of sinusoids can occur, which can eventually lead to physical damage of blood vessel cells and cause hemorrhage (Costa et al. 2011; Rodrigues et al. 2019)? The presence of hemorrhage was not confirmed in the present study, so circulatory alterations probably had a minor negative influence on the metabolism of the liver, depicted in the increased presence of stasis and congestion of sinusoids. Dilation of sinusoids is an enlargement of capillaries in the hepatic stroma, and in mammals, in the majority of cases, it is a direct consequence of hepatic venous outflow obstruction, although the causes can also be contributed to a vascular cause, the presence of neoplasia or the systemic inflammatory disorders (Kakar et al. 2004). Moreover, when hepatic venous outflow obstruction is present, it also causes vascular stasis and congestion of hepatic parenchyma (Brancatelli et al. 2018). So, this characteristic pattern of histopathological alterations is caused either by increased blood flow in the liver or slower outflow of blood from it.

In general, the morphological aspect of hepatocytes is very plastic and the depends on presence of pollutants in the environment and the nutritional status of fish. When a fish organism is burdened with xenobiotics, hepatocytes are the main cells involved in biotransformation processes. Two alterations that contributed the most to the high histopathological score in fish from Perućac are focal changes of hepatocytes and pyknosis of their nuclei. Pyknosis of nuclei is one of the phases in the cascade of cellular necrosis or apoptosis. This histopathological alteration is irreversible and observed in various fish species exposed to mine tailings, including European chub (Rašković et al. 2018; Weber et al. 2020) and organic contamination (Lukin et al. 2011). Focal changes of hepatocytes included different staining properties of one part of hepatic tissue, with hepatocytes stained either more basophilic or eosinophilic than the rest of the section. Different causes can induce the presence of this alteration and it can be found even in fish with no apparent pathology, although the cause could also be an endocrine disruption or early neoplastic process (Wolf and Wolfe 2005; Wolf and Wheeler 2018). The higher presence of MMCs in fish sampled from Zaovine could be the effect of the presence of anthropogenic pollutants, as their presence is frequently used as a marker in field studies (Basilone et al. 2018; Carreras-Colom et al. 2022). However, these results should be taken with caution, since the presence of MMCs varies in numbers and position within the liver and is dependent also on age, sex, diet, season and temperature, so only stereological studies could be deemed as relevant (Steinel and Bolnick 2018; Matsche et al. 2019).

However, a fish diet can induce some changes on hepatocytes that are similar to hepatotoxicity, in the first place vacuolation. In the nutritional pathology vacuolation of hepatocytes is caused by an unbalanced diet which leads to lipid infiltration (Figueiredo-Silva et al. 2005; Sirri et al. 2018; Roh et al. 2020; Khieokhajonkhet et al. 2022), but that appearance of hepatocytes is similar in fish exposed to pollutants such as pesticide DDT (Badamasi et al. 2019) or metals, such as Cr (Mohamed et al. 2020; Zulkipli et al. 2021). Vacuolation of hepatocytes in common carp can also have a seasonal aspects, with vacuoles within the hepatocytes enlarging from spring to autumn, when they have a characteristic "clear" appearance of cytoplasm, with the lack of lipid droplets inside (Rašković et al. 2016). Therefore, caution has to be taken when vacuolization is reported in environmental studies (Wolf and Wolfe 2005; Imentai et al. 2020) and we decided to exclude it from the semi-quantitative scoring system used in the present study.

Even though the reaction pattern of histopathological alterations varies among organs, it is obvious that fish caught in the Vlasina reservoir have the lowest histopathological scores in both gills and the liver. This was not followed by elemental accumulation, MAC and MPI values for gills and liver. A false-positive result could be caused by the fish diet (Wolf and Wolfe 2005), especially in the summer period (Nikolić et al. 2020a). PCA showed a potential correlation between reaction patterns and specific elements. In liver almost all HP alterations were associated with Pb and B, while in gills there was larger variation among reaction patterns. A multivariate analysis approach is already being used to explore possible correlation between elemental concentrations and histopathological alterations (Riba et al. 2005; Rašković et al. 2015; Gusso-Choueri et al. 2022), but results can hardly be compared among studies, due to the differences in fish species, environment (marine/freshwater) and the level of contamination. In example, in both Riba et al. (2005) and Gusso-Choueri et al. (2022) the loading matrix and factor scores did not show positive correlation between concentrations of Pb in liver and histopathological alterations, which is in contrast with results from the current study, even though Pb is known to induce necrosis in fish liver upon exposure (Jantawongsri et al. 2021). Moreover, histopathological findings did not reflect contamination levels in chub muscle and thus the level of human health risk due to the consumption of fish meat.

5. CONCLUSIONS

Considerable variations in the accumulation of elements were recorded among tissues as well as among reservoirs. Compared to other heavy metals investigated in this study, the European chub was not a good indicator of Pb pollution between investigated reservoirs. Concentrations of Cd in Garaši in one sample, Vlasina in two samples, Perućac in two samples, and Medjuvršje in two samples exceeded the MACs. Due to the absence of recent use, concentrations of analyzed pesticides were low or under the detection limits, and consequently did not exceed the proscribed MACs. The TTHQ values indicated that there is no significant non-carcinogenic health risk due to the intake of examined elements and heptachlor. In most cases, low to moderate levels of pathology were observed for both gills and liver and their appearance is due to the presence of chronic pollution, which significantly vary in studied reservoirs.

In general, chub meat can be safely used in human nutrition, both for women and men. Due to low anthropogenic pressure, reservoir for water supply (Garaši) is generally considered as safest for fish consumption. On contrary, fish from Medjuvršje and Perućac were less safe for human consumption according to the TR and TTHQ values, respectively.

STATEMENTS & DECLARATIONS

Funding

This study was supported by the Ministry of Education, Science, and Technological Development of the Republic of Serbia [Grant numbers: 451-03-68/2022-14/200053, 451-03-68/2022-14/200116, and 451-03-68/2022-14/200030].

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Dušan Nikolić: Conceptualization; Investigation; Formal Analysis; Visualization; Writing – Original Draft;.**Vesna Poleksić**: Formal Analysis; Supervision; Writing – Review & Editing; **Stefan Skorić**: Investigation; Visualization; **Aleksandra Tasić**: Formal Analysis; Writing –

Review & Editing; **Slobodan Stanojević**: Supervision; **Božidar Rašković**: Formal Analysis; Visualization; Supervision; Writing – Review & Editing.

Data Availability

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Ethical approval

There was no need for the approval from an Ethical Committee for this study, because this species is used in commercial fishing. Moreover, authors had approval of the Ministry of Environmental Protection and Environmental Protection Agency for conducting the study.

Consent to Participate

Not applicable.

Consent to Publish

Not applicable.

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Table 1. Linear regression parameters of the calibration curve of OCPs, correlation coefficient (R^2), limits of detection (LOD), and limits of quantification (LOQ).

Compound	Regression equation	R^2	LOD (μg/kg)	LOQ (μg/kg)
Aldrin	y = 1.08677x + (-44.9351)	0.994454	1.8	6.1
α-HCH	y = 1.07604x + (-45.2940)	0.994873	0.8	2.5
β-HCH	y = 0.675262x + (-41.2580)	0.992612	0.7	2.3
γ-HCH	y = 1.10742x + (-60.2206)	0.997591	2.0	6.7
δ-HCH	y = 0.825525x + (-34.7934)	0.994498	0.9	2.9
4,4’-DDD	y = 2.83435x + (-127.385)	0.993733	1.7	5.6
4,4’-DDE	y = 1.90671x + (-82.0592)	0.994457	1.0	3.3
4,4’-DDT	y = 2.16509x + (-105.751)	0.996042	1.0	3.3
Dieldrin	y = 1.72220x + (-69.3284)	0.994395	2.0	6.7
Endosulfan I	y = 0.277833x + (-16.6105)	0.998058	2.4	7.9
Endosulfan II	y = 0.227093x + (-9.26686)	0.995752	2.0	6.7
Endosulfan sulfate	y = 0.396962x + (-14.4923)	0.993422	1.1	3.6
Endrin	y = 0.274841x + (-13.8913)	0.995432	0.8	2.8
Endrin aldehyde	y = 0.800986x + (-30.7287)	0.994615	0.6	2.0
Heptachlor	y = 1.23478x + (-54.3179)	0.994144	1.6	5.4
Heptachlor epoxide	y = 0.427601x + (-18.8694)	0.994398	1.0	3.2
Metoxychlor	y = 4.25909x + (-194.094)	0.993330	0.3	1.0

Table 2. Total length (TL), weight (W) and Fulton's condition factor (CF) of European chub individuals from five studied reservoirs, and MPI and elemental concentrations (μg g^{-1} dw) in muscle, gills, and liver tissue. Values are presented as mean ± SD; ND indicates values below the detection threshold (0.00471, 0.00165, 0.0151, 0.0325, and 0.00312 μg g^{-1} for Ag, Ba, Cr, Cu, and Sr, respectively).

		Garaši	Vlasina	Perućac	Zaovine	Medjuvršje
TL (cm)		22.3 ± 4.8^{a}	31.4 ± 2.0^{b}	29.2 ± 3.2^{b}	29.4 ± 3.5^{b}	27.2 ± 4.7^{b}
(min – max)		18.0 – 29.8	27.5 – 34.2	25.6 – 33.6	25.5 – 38.0	22.6 – 37.0
BW (g)		126.0 ± 98.0^{a}	343.7 ± 70.6^{c}	304.8 ± 116.9^{bc}	274.0 ± 122.4^{bc}	268.2 ± 178.6^{b}
(min – max)		53.0 – 303.0	231.0 – 444.0	195.0 – 498.0	178.0 – 612.0	150.0 – 705.0
CF		0.96 ± 0.10^{a}	1.08 ± 0.08^{b}	1.16 ± 0.08^{bc}	1.06 ± 0.06^{ab}	1.24 ± 0.14^{c}
(min – max)		0.86 – 1.18	0.98 – 1.20	1.04 –1.31	0.96 – 1.16	0.95 – 1.40
MPI	Muscle*	1.57 ± 0.35^{c}	2.37 ± 1.70^{bc}	1.34 ± 0.42^{bc}	1.08 ± 0.79^{a}	1.27 ± 0.25^{b}
	Gills	6.71 ± 4.35	5.18 ± 2.62	5.87 ± 3.37	6.30 ± 3.73	6.01 ± 2.65
	Liver*	4.81 ± 6.58^{b}	2.13 ± 1.58^{ab}	2.18 ± 1.18^{ab}	3.15 ± 1.29^{ab}	1.83 ± 1.14^{a}
Ag	Muscle*	0.004 ± 0.007^{a}	0.005 ± 0.010^{ab}	0.005 ± 0.007^{abc}	0.013 ± 0.001^{c}	0.012 ± 0.004^{bc}
	Gills*	ND^{a}	0.058**^{ab}	0.113 ± 0.265^{b}	0.120, 0.121***^{ab}	0.012 ± 0.025^{ab}
	Liver*	0.006**^{a}	0.007, 0.008***^{a}	0.010 ± 0.013^{abc}	0.026 ± 0.014^{c}	0.012 ± 0.004^{b}
Al	Muscle*	17.25 ± 18.73^{b}	26.51 ± 28.25^{b}	9.65 ± 14.80^{a}	10.90 ± 8.87^{ab}	25.69 ± 23.94^{b}
	Gills*	81.85 ± 115.64^{a}	78.18 ± 99.10^{a}	70.27 ± 105.03^{a}	72.26 ± 120.71^{a}	194.09 ± 156.86^{b}
	Liver	138.97 ± 244.90	25.32 ± 30.18	39.02 ± 43.29	73.27 ± 97.53	58.96 ± 75.89
As	Muscle*	0.28 ± 0.22^{a}	0.28 ± 0.20^{ab}	0.20 ± 0.19^{a}	0.15 ± 0.18^{a}	0.52 ± 0.25^{b}
	Gills	0.29 ± 0.26	0.42 ± 0.28	0.23 ± 0.33	0.33 ± 0.47	0.62 ± 0.34
	Liver	0.66 ± 0.60	0.28 ± 0.18	0.36 ± 0.33	0.61 ± 1.16	0.46 ± 0.32
B	Muscle*	0.33 ± 0.85^{ab}	0.77 ± 0.90^{c}	24.36 ± 17.13^{d}	0.22**^{a}	2.57 ± 7.60^{bc}

	Gills*	0.62 ± 0.44^{a}	0.96 ± 0.53^{a}	15.67 ± 16.01^{b}	0.62 ± 0.51^{a}	1.15 ± 0.65^{a}
	Liver*	2.01 ± 2.45^{a}	1.27 ± 0.97^{a}	48.34 ± 71.55^{b}	1.10 ± 0.69^{a}	0.83 ± 0.97^{a}
Ba	Muscle	1.38 ± 0.75	0.94 ± 0.92	1.27 ± 0.80	0.94 ± 0.75	1.08 ± 0.83
	Gills	2.27 ± 3.55	0.91 ± 1.39	ND	ND	ND
	Liver	1.82 ± 2.15	1.39 ± 1.08	3.26 ± 1.76	3.69 ± 3.38	1.68 ± 0.73
Ca	Muscle*	10798.68 ± 6992.55^{c}	11878.75 ± 8749.14^{bc}	2476.42 ± 1755.25^{a}	4504.43 ± 4543.11^{ab}	4526.12 ± 4899.50^{abc}
	Gills	33774.07 ± 15748.91	23903.28 ± 14434.20	27594.05 ± 12567.67	25659.53 ± 5724.88	22518.90 ± 1983.63
	Liver	418.30 ± 333.39	559.46 ± 882.96	548.70 ± 710.81	1490.02 ± 1919.30	702.63 ± 1466.73
Cd	Muscle	0.17 ± 0.03	0.17 ± 0.04	0.15 ± 0.04	0.17 ± 0.01	0.15 ± 0.04
	Gills	0.33 ± 0.17	0.24 ± 0.13	0.22 ±0.09	0.25 ± 0.10	0.25 ± 0.04
	Liver*	0.12 ± 0.08^{b}	0.04 ± 0.05^{a}	0.14 ± 0.12^{b}	0.06 ± 0.08^{ab}	0.06 ± 0.04^{ab}
Co	Muscle*	0.04 ± 0.02^{b}	0.03 ± 0.03^{ab}	0.06 ± 0.04^{b}	0.03 ± 0.03^{a}	0.04 ± 0.03^{ab}
	Gills*	0.03 ± 0.04^{ab}	0.07, 0.08*** ^{ab}	0.10 ± 0.27^{b}	0.03** ^{a}	0.02, 0.02*** ^{ab}
	Liver	0.03 ± 0.02	0.02 ± 0.02	0.04 ± 0.01	0.03 ± 0.04	0.02 ± 0.01
Cr	Muscle*	0.15 ± 0.10^{ab}	0.07 ± 0.11^{a}	0.21 ± 0.11^{b}	0.08 ± 0.08^{a}	0.12 ± 0.08^{ab}
	Gills*	0.18 ± 0.28^{b}	0.02, 0.07*** ^{ab}	ND^{a}	ND^{a}	ND^{a}
	Liver	0.52 ± 0.25	0.26 ± 0.13	0.40 ± 0.30	0.57 ± 0.57	0.19 ± 0.11
Cu	Muscle	ND	ND	ND	ND	ND
	Gills	10.20**	19.18, 66.20***	ND	ND	7.17**
	Liver*	26.76 ± 19.92^{ab}	21.18 ± 20.91^{ab}	15.42 ± 14.85^{a}	35.15 ± 14.80^{b}	35.96 ± 19.47^{b}
Fe	Muscle	28.26 ± 30.39	32.99 ± 28.16	19.05 ± 16.06	24.58 ± 11.43	17.64 ± 6.90
	Gills	310.93 ± 126.74	231.66 ± 122.98	217.85 ± 293.54	258.74 ± 136.18	270.42 ± 158.77
	Liver*	563.42 ± 393.38^{bc}	249.92 ± 146.66^{ab}	309.20 ± 214.59^{ab}	522.05 ± 208.91^{c}	298.57 ± 321.44^{a}
Hg	Muscle*	0.43 ± 0.18^{ab}	0.34 ± 0.48^{a}	1.09 ± 0.84^{d}	0.42 ± 0.10^{bc}	0.64 ± 0.31^{bcd}
	Gills	0.21 ± 0.13	0.17 ± 0.15	21.53 ± 60.46	0.25 ± 0.31	0.38 ± 0.43
	Liver*	0.26 ± 0.23^{ab}	0.12 ± 0.11^{a}	0.59 ± 0.32^{c}	0.27 ± 0.24^{a}	0.44 ± 0.10^{bc}
K	Muscle	8986.70 ± 1932.34	8612.74 ± 1494.90	9094.59 ± 1040.25	10092.53 ± 551.38	9797.07 ± 1135.47
	Gills*	7974.71 ± 862.86^{c}	5835.55 ± 936.22^{a}	6436.54 ± 882.92^{ab}	6750.21 ± 694.49^{ab}	7220.05 ± 766.09^{bc}
	Liver*	9070.43 ± 3274.03^{c}	5672.13 ± 2570.96^{ab}	5815.53 ± 787.66^{a}	6766.78 ± 1002.36^{b}	6479.91 ± 1281.02^{ab}

Li	Muscle	0.22 ± 0.35	0.93 ± 1.53	0.49 ± 0.31	0.38 ± 0.33	0.35 ± 0.29
	Gills*	0.31 ± 0.52^{a}	0.41 ± 0.83^{a}	0.65 ± 1.24^{ab}	1.21 ± 1.04^{bc}	1.11 ± 1.00^{c}
	Liver	0.43 ± 1.06	0.27 ± 0.38	0.27, 0.34***	0.23 ± 0.32	0.44 ± 1.06
Mg	Muscle*	1326.48 ± 158.60^{b}	1494.36 ± 383.23^{bc}	1096.39 ± 176.42^{a}	1450.71 ± 137.96^{c}	1455.13 ± 163.56^{bc}
	Gills*	1912.87 ± 463.98^{ab}	2024.24 ± 815.82^{abc}	1669.90 ± 500.41^{a}	2430.00 ± 357.48^{c}	2252.89 ± 430.19^{bc}
	Liver	1037.34 ± 564.86	660.15 ± 392.35	625.81 ± 87.94	771.41 ± 156.14	757.21 ± 213.41
Mn	Muscle*	7.72 ± 10.80^{b}	15.64 ± 30.52^{b}	0.14** ^{a}	0.07, 0.22*** ^{a}	0.15 ± 0.32^{a}
	Gills*	62.95 ± 45.50^{b}	34.26 ± 20.05^{b}	13.61 ± 7.25^{a}	12.58 ± 3.91^{a}	30.96 ± 15.46^{b}
	Liver*	44.41 ± 114.16^{c}	3.67 ± 5.28^{ab}	0.44 ± 0.59^{a}	1.71 ± 2.51^{ab}	3.42 ± 2.82^{bc}
Mo	Muscle	0.27 ± 0.68	0.10 ± 0.11	0.11 ± 0.09	0.08 ± 0.09	0.13 ± 0.07
	Gills*	0.29 ± 0.17^{b}	0.22 ± 0.36^{ab}	0.13 ± 0.05^{a}	0.13 ± 0.07^{a}	0.10 ± 0.06^{a}
	Liver*	1.18 ± 0.89^{ab}	0.59 ± 0.52^{a}	0.67 ± 0.30^{a}	1.42 ± 0.62^{b}	0.78 ± 0.30^{a}
Na	Muscle*	3066.78 ± 445.05^{d}	2620.43 ± 368.25^{cd}	1978.63 ± 467.13^{ab}	2416.14 ± 359.44^{bc}	1785.80 ± 446.25^{a}
	Gills	5449.98 ± 1876.71	4019.58 ± 1392.69	3499.15 ± 1392.21	3561.15 ± 760.34	3128.69 ± 251.09
	Liver*	4091.42 ± 1184.57^{b}	2648.91 ± 931.90^{a}	3885.23 ± 918.24^{b}	4185.83 ± 1337.89^{b}	2995.82 ± 769.09^{a}
Ni	Muscle*	1.01 ± 0.33^{bc}	0.75 ± 0.32^{b}	1.28 ± 0.22^{c}	0.29 ± 0.27^{a}	0.35 ± 0.31^{a}
	Gills*	2.23 ± 1.18^{c}	1.46 ± 0.60^{bc}	1.13 ± 0.59 ^{ab}	0.98 ± 0.92^{ab}	0.89 ± 1.21^{a}
	Liver*	2.01 ± 0.79^{c}	1.11 ± 0.40^{b}	1.70 ± 0.91^{bc}	1.14 ± 1.05^{abc}	0.69 ± 0.39^{a}
P	Muscle*	10168.73 ± 2521.14^{b}	13944.92 ± 7262.23^{b}	7837.92 ± 1117.45^{a}	9922.99 ± 1727.99^{b}	9619.44 ± 1847.66^{ab}
	Gills*	24628.57 ± 5830.49^{a}	23832.26 ± 9005.16^{ab}	26156.03 ± 4670.34^{ab}	31694.34 ± 5483.75^{c}	30179.07 ± 6066.91^{bc}
	Liver*	12083.53 ± 5213.42^{c}	7067.87 ± 3164.97^{a}	8751.66 ± 1157.81^{bc}	9882.50 ± 1533.12^{abc}	9971.27 ± 2380.87^{b}
Pb	Muscle	0.22 ± 0.18	0.22 ± 0.21	0.25 ± 0.17	0.36 ± 0.18	0.25 ± 0.18
	Gills	0.69 ± 0.53	0.44 ± 0.42	0.29 ± 0.32	0.31 ± 0.28	0.48 ± 0.26
	Liver	0.19 ± 0.26	0.17 ± 0.14	0.42 ± 0.40	0.35 ± 0.38	0.12 ± 0.10
S	Muscle*	7038.48 ± 1797.89^{ab}	7792.44 ± 1110.28^{bc}	5422.95 ± 791.39^{a}	7005.31 ± 1375.38^{ab}	8929.36 ± 1431.55^{c}
	Gills*	6303.57 ± 732.19^{b}	6167.39 ± 698.67^{b}	4992.36 ± 568.03^{a}	5701.64 ± 817.04^{ab}	6393.79 ± 659.16^{b}
	Liver	6505.29 ± 3245.08	4917.71 ± 3018.86	5089.45 ± 991.82	5569.62 ± 1662.41	5666.47 ± 1038.17
Se	Muscle*	4.46 ± 1.14^{a}	6.53 ± 3.14^{ab}	4.94 ± 0.94^{a}	5.91 ± 1.35^{ab}	7.05 ± 1.16^{b}
	Gills	9.91 ± 3.98	10.64 ± 3.19	9.98 ± 2.94	12.24 ± 1.83	12.08 ± 1.32

	Liver*	5.73 ± 3.13^{ab}	4.71 ± 2.04^{a}	7.18 ± 2.57^{bc}	10.23 ± 5.61^{c}	8.02 ± 2.25^{bc}
Si	Muscle	24.45 ± 12.61	136.68 ± 265.30	591.94 ± 981.98	16.09 ± 13.23	32.76 ± 16.04
	Gills	144.60 ± 174.01	102.49 ± 102.80	105.32 ± 115.48	160.53 ± 206.07	308.24 ± 211.92
	Liver*	173.61 ± 316.53^{b}	13.87 ± 15.91^{a}	75.70 ± 156.56^{ab}	12.44 ± 12.12^{a}	66.72 ± 132.34^{b}
Sr	Muscle	6.09 ± 11.61	22.44 ± 34.68	ND	ND	5.68**
	Gills*	63.16 ± 31.86^{bc}	89.01 ± 55.61^{bc}	36.06 ± 14.52^{a}	55.43 ± 14.01^{b}	77.22 ± 29.07^{c}
	Liver	0.58, 3.09***	ND	ND	ND	ND
Zn	Muscle	66.93 ± 50.58	44.64 ± 17.73	38.70 ± 14.18	31.97 ± 7.83	29.80 ± 6.45
	Gills	360.97 ± 153.73	507.62 ± 297.78	279.70 ±63.69	408.34 ± 110.34	286.99 ± 113.18
	Liver*	125.49 ± 98.13^{bc}	64.82 ± 39.66^{a}	97.11 ± 69.62^{ab}	117.27 ± 36.88^{c}	98.75 ± 32.32^{bc}

^{a,b,c,d} Values with different letters in the same row are different (Mann-Whitney *U* Test, $p \leq 0.05$ or Tukey HSD *post-hoc* test, $p \leq 0.05$).

* Significant differences among reservoirs (Kruskal-Wallis *H* Test, $p \leq 0.05$ or one-way ANOVA $p \leq 0.05$).

** Concentrations above detection threshold only in a single sample.

*** Concentrations above detection threshold only in two samples.

Table 3. Target carcinogenic risk factor (TR) of As, Pb, 4,4’-DDD, 4,4’-DDE, and heptachlor for men and women due to consumption of European chub from five reservoirs.

	As		Pb		4,4′-DDD		4,4′-DDE		Heptachlor	
	Men	Women	Men	Women	Men	Women	Men	Women	Men	Women
Garaši	6.35×10^{-7}	9.47×10^{-7}	1.29×10^{-7}	1.76×10^{-7}	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
Vlasina	9.21×10^{-7}	1.26×10^{-6}	1.73×10^{-7}	2.35×10^{-7}	n.r.	n.r.	4.67×10^{-7}	6.37×10^{-7}	n.r.	n.r.
Perućac	6.70×10^{-7}	9.14×10^{-7}	1.23×10^{-7}	1.68×10^{-7}	2.79×10^{-7}	3.81×10^{-7}	n.r.	n.r.	n.r.	n.r.
Zaovine	6.65×10^{-7}	9.08×10^{-7}	1.50×10^{-7}	2.05×10^{-7}	3.01×10^{-7}	4.11×10^{-7}	n.r.	n.r.	5.44×10^{-6}	7.43×10^{-6}
Medjuvršje	1.45×10^{-6}	1.97×10^{-6}	1.60×10^{-7}	2.18×10^{-7}	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.

n.r. – no risk.

Table 4. Mean HP scores presented ± SD, with importance factor (IF) given per alteration of gills and liver in European chub specimens caught at five reservoirs sites. HP scores ranged from 0 (no alteration) to 6 (severe alteration occurrence). Subscripted letters meaning: G – gills, L – liver, P – progressive, C – circulatory, R – regressive, I – inflammatory.

HP alteration / Sampling site	**IF**	**Garaši**	**Vlasina**	**Perućac**	**Zaovine**	**Medjuvršje**	**p-value**
Gills							
Hyperaemia	1	4.0 ± 1.3	4.2 ± 1.5	5.0 ± 1.9	4.6 ± 1.9	3.2 ± 2.5	0.212
Telangiectasia	1	0.0 ± 0.0	1.4 ± 1.6	1.0 ± 1.1	0.6 ± 1.3	0.8 ± 1.4	0.099
Stasis	1	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 1.3	0.0 ± 0.0	0.6 ± 1.3	0.240
Aneurism	1	0.6 ± 1.3	2.0 ± 1.6	1.2 ± 1.9	1.8 ± 2.0	0.6 ± 1.3	0.142
Oedema of primary epithelium	1	0.2 ± 0.6	0.8 ± 1.7	1.2 ± 1.7	0.4 ± 1.3	0.0 ± 0.0	0.164
Oedema of secondary epithelium	1	2.6 ± 1.6	2.4 ± 1.8	1.4 ± 1.0	2.2 ± 2.2	1.4 ± 1.9	0.390
Structural and architectural alternations	1	0.4 ± 0.8	0.6 ± 1.0	0.6 ± 1.9	1.8 ± 2.4	1.0 ± 1.4	0.318
Presence of mucous cells in secondary lamellae	1	2.2 ± 1.1	3.2 ± 1.4	4.0 ± 2.3	3.2 ± 2.3	3.8 ± 2.0	0.131
Proliferation of mucous cells	1	3.0 ± 1.9^{b}	0.4 ± 0.8^{a}	1.0 ± 1.4^{ab}	0.0 ± 0.0^{a}	1.0 ± 1.7^{ab}	<0.001
Hyperplasia of epithelial cells	2	2.6 ± 1.3^{b}	0.6 ± 1.0^{a}	0.6 ± 1.0^{a}	2.6 ± 1.9^{ab}	2.4 ± 1.3^{ab}	0.009
Hyperplasia of complete primary lamellae	2	0.6 ± 1.3	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 1.3	0.2 ± 0.6	0.444
Hypertrophy of secondary epithelium	1	3.0 ± 1.7^{b}	0.6 ± 1.0^{a}	1.6 ± 1.6^{ab}	1.0 ± 1.1^{ab}	2.0 ± 1.6^{ab}	0.013
Infiltration	2	0.2 ± 0.6	0.4 ± 0.8	0.2 ± 0.6	0.6 ± 1.3	1.4 ± 1.6	0.151
Necrosis	3	0.6 ± 1.0	1.2 ± 1.4	2.2 ± 2.6	3.0 ± 2.2	1.0 ± 1.9	0.043
Liver							
Stasis	1	1.6 ± 2.3^{a}	2.2 ± 1.8^{a}	4.2 ± 1.9^{b}	3.8 ± 2.5^{ab}	1.8 ± 2.0^{a}	0.035
Sinusoidal congestion	1	0.2 ± 0.6^{a}	2.0 ± 2.1^{ab}	2.4 ± 1.7^{b}	0.4 ± 0.9^{ab}	2.6 ± 2.3^{ab}	0.005
Sinusoidal dilation	1	0.2 ± 0.6^{a}	1.6 ± 1.8^{ab}	4.0 ± 2.4^{b}	3.6 ± 2.4^{b}	1.4 ± 1.3^{ab}	0.002
Presence of melanomacrophage centers	1	0.8 ± 1.4^{ab}	0.0 ± 0.0^{a}	2.0 ± 1.7^{ab}	2.2 ± 1.6^{b}	0.6 ± 1.0^{ab}	0.030
Leukocyte infiltration	2	1.4 ± 2.0	0.6 ± 1.0	2.0 ± 1.0	2.0 ± 2.2	2.0 ± 0.9	0.060
Pyknosis of hepatocytes` nuclei	2	1.0 ± 1.4^{ab}	0.4 ± 0.8^{a}	3.1 ± 1.5^{b}	0.2 ± 0.7^{a}	1.2 ± 1.4^{ab}	<0.001
Focal changes of hepatocytes	1	3.8 ± 1.8^{b}	1.8 ± 1.8^{ab}	3.6 ± 1.3^{b}	0.7 ± 1.4^{a}	2.2 ± 1.8^{ab}	0.002
Vacuolization of hepatocytes	-	5.0 ± 1.7^{b}	1.4 ± 2.3^{a}	5.3 ± 1.0^{b}	2.9 ± 1.5^{ab}	3.0 ± 1.7^{ab}	<0.001
Presence of ceroid pigment	1	0.4 ± 1.3	1.2 ± 1.7	0.4 ± 0.9	1.1 ± 1.5	0.4 ± 0.8	0.406
Fibrosis	2	2.0 ± 1.3	2.0 ± 1.9	1.8 ± 1.2	1.6 ± 1.7	2.2 ± 2.0	0.947
Necrosis	3	1.2 ± 1.0	0.6 ± 1.3	1.6 ± 1.9	0.0 ± 0.0	1.6 ± 1.8	0.072

[a, b, c, d] Values with different letters in the same row are different (Mann-Whitney U Test, $p \leq 0.05$), while showed p-value is overall from Kruskal-Wallis H test.

FIGURE LEGEND

Fig. 1 Map of the sampling sites (Serbia): 1 – Garaši (44.287054 N, 20.473708 E); 2 – Vlasina (42.727170 N, 22.363471 E); 3 – Perućac (43.968131 N, 19.364310 E); 4 – Zaovine (43.866337 N, 19.406074 E); 5 – Medjuvršje (43.915620 N, 20.232869 E).

Fig. 2 Total elemental THQ values due to consumption of fish from five reservoirs for men (A) and women (B). Data for Cu are not shown since this element was not detected in the muscle of any fish.

Fig. 3 Illustration of histopathological alterations found in gills (a-c) and liver (d-f) of fish from the present study: (a) numerous mucous cells present in the secondary lamellae (arrow; fish caught at Perućac reservoir; ×400, HE); (b) infiltration of leukocytes in the primary epithelium (arrow), oedema (arrowhead) and hypertrophy (double arrowhead) of secondary epithelium (fish caught at Garaši reservoir; ×400, HE); (c) complete disruption of branchial tissue: oedema, desquamation and necrosis of primary and secondary epithelium (arrow; fish caught at Zaovine reservoir; ×200, HE); (d) dilation of sinusoid capillaries (double arrowhead; fish caught at Vlasina reservoir; ×400, HE); (e) stasis of blood in larger vessel (arrow) and infiltration of leukocytes (arrowhead; fish caught at Medjuvršje reservoir; ×200, HE); (f) lipid droplets in cytoplasm of hepatocytes, pyknosis of nuclei (arrow) and single cell necrosis of hepatocytes (arrowhead; fish caught at Perućac reservoir; ×400, HE); bar = 50 μm.

Fig. 4 An overview of histopathological scores of reaction patterns in (a) gills and (b) liver. I_G – histopathological index in gills; I_L – histopathological index in liver; reaction patterns: P – progressive changes; C – circulatory disturbances, R – regressive changes, I – inflammation;

sampling locations: M – Medjuvršje, Z – Zaovine, V – Vlasina, P – Perućac, G – Garaši; data are presented as mean values± SD; mean values followed by different superscript letters among reservoirs and within each category were significantly different (Kruskal–Wallis H test, followed by Mann-Whitney U Test, $p \leq 0.05$).

Fig. 5 Mean histopathological indices of gills (I_G), liver (I_L) and total histopathological index value of chub (I_T) caught at five sampling locations; sampling locations: M – Medjuvršje, Z – Zaovine, V – Vlasina, P – Perućac, G – Garaši; data are presented as mean values±SD; mean values followed by different superscript letters among reservoirs and within each category were significantly different (Kruskal–Wallis H test, followed by Mann-Whitney U Test, $p \leq 0.05$).

Fig. 6 Principal component analysis (PCA) of elemental concentrations and histopathological changes in European chub tissues: (a) gills - IG; (b) liver - IL; input variables were concentrations of chemical elements and reaction pattern of histopathological scores: P – progressive changes; C – circulatory disturbances, R – regressive changes, I – inflammation; T – total scores.

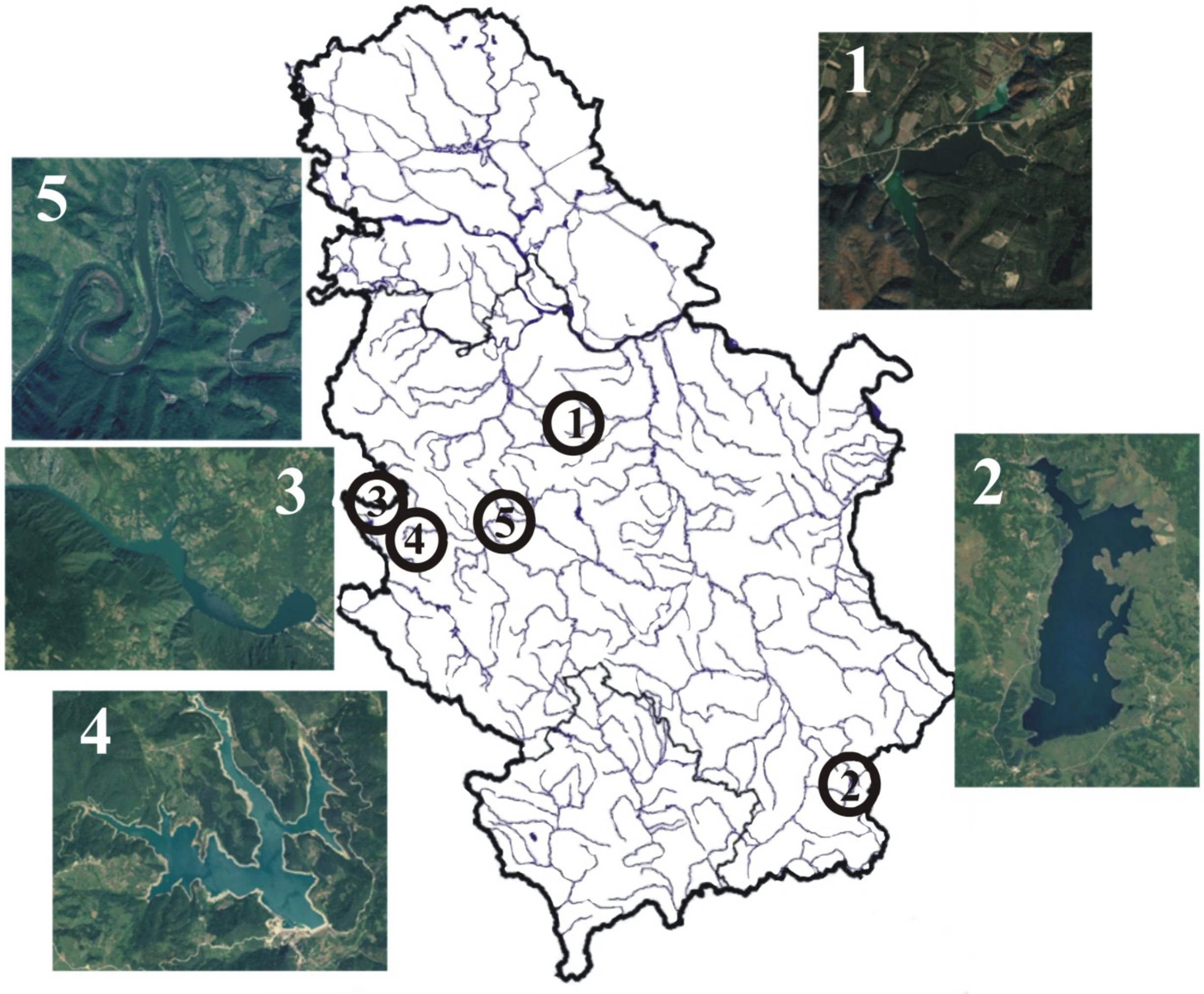
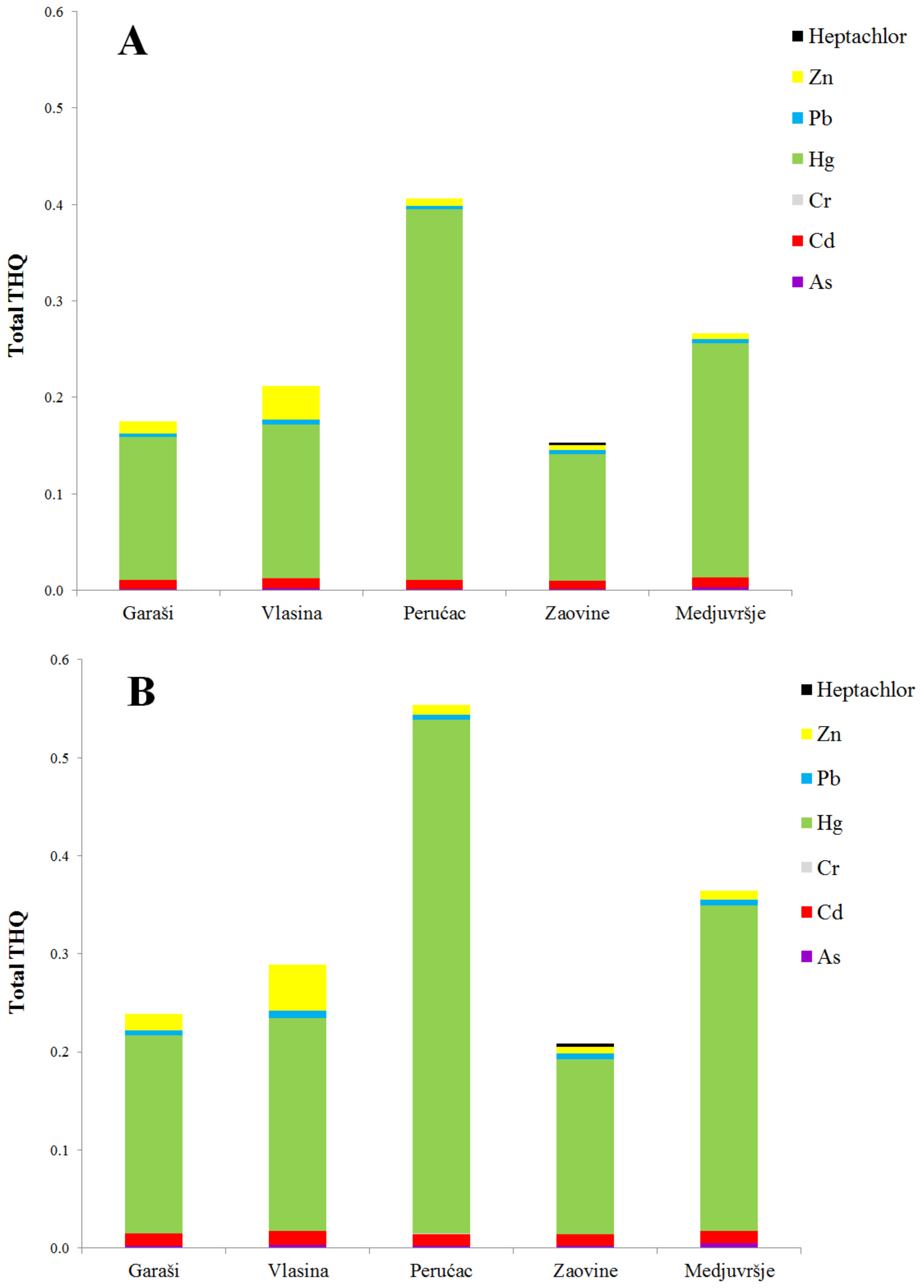


Fig. 1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Medjuvršje
■ Heptachlor
■ Zn
■ Pb
■ Hg
■ Cr
■ Cd
■ As
B
Total THQ
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0.1
0.2
0.3
0.4
0.5
0.6
Garaši
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■ Heptachlor
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■ As

Fig. 2

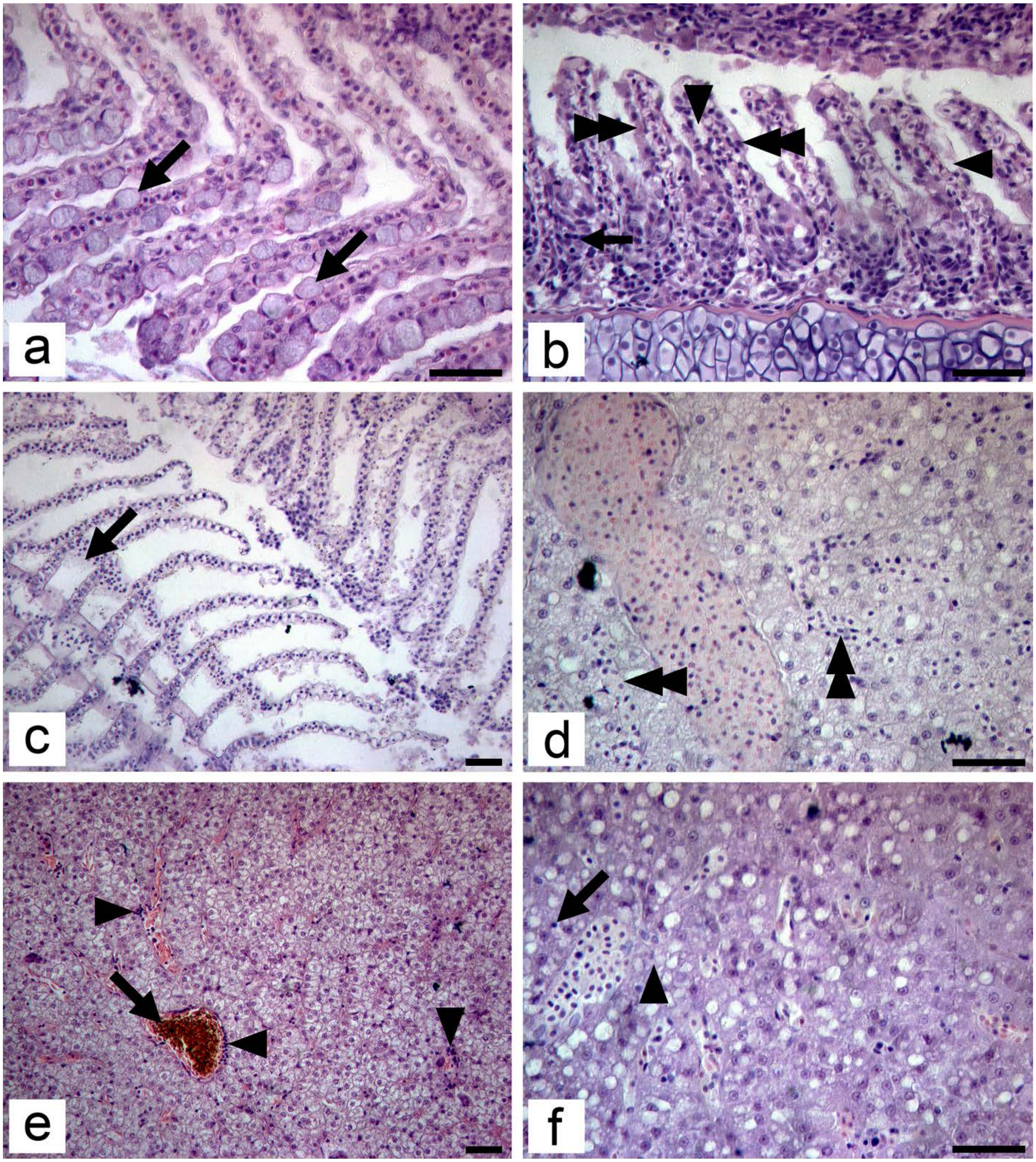
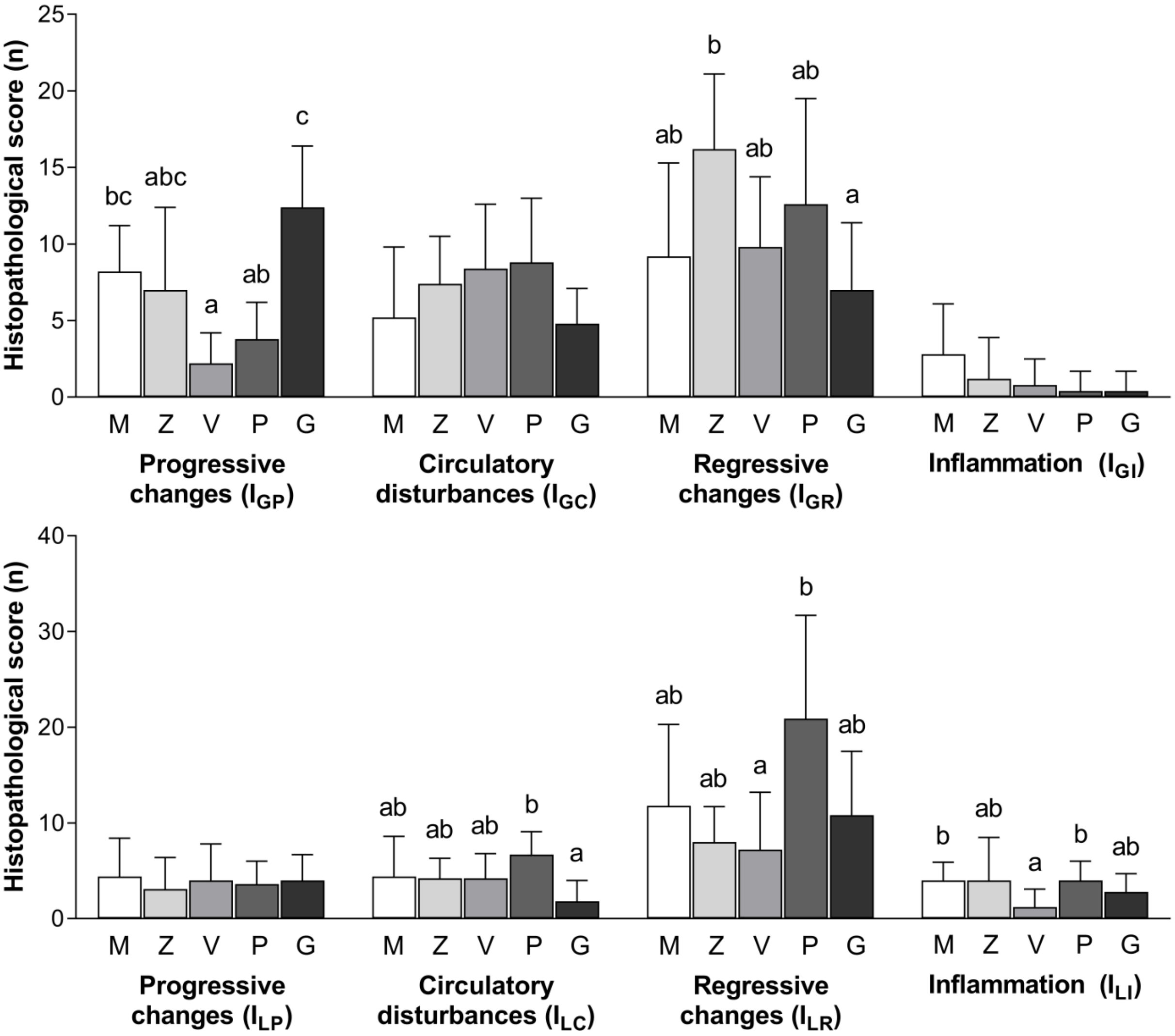


Fig. 3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Circulatory disturbances (I_{GC})
Regressive changes (I_{GR})
Inflammation (I_{GI})
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20
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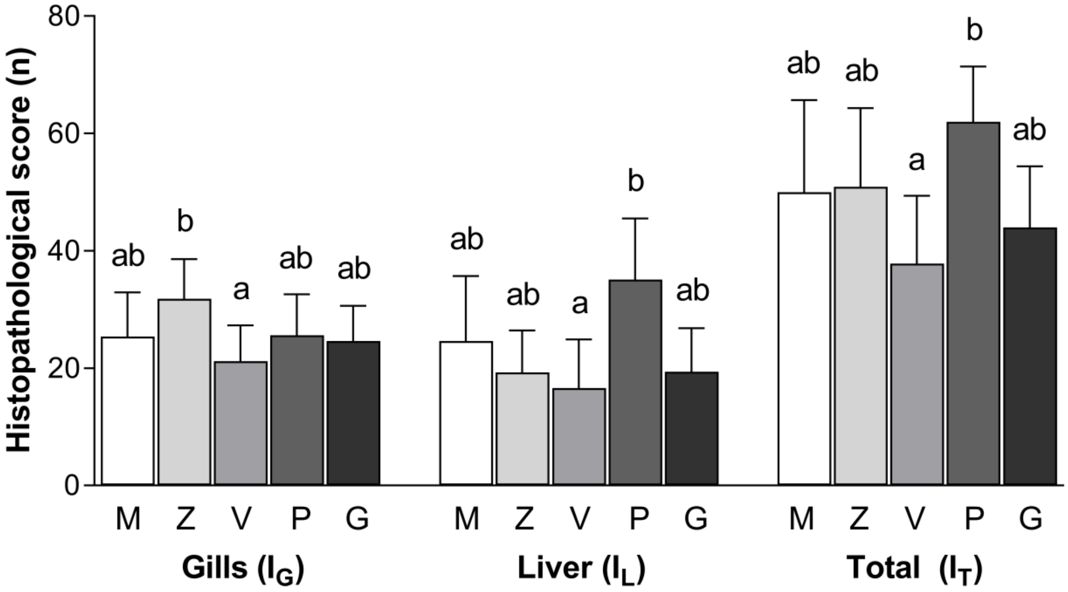
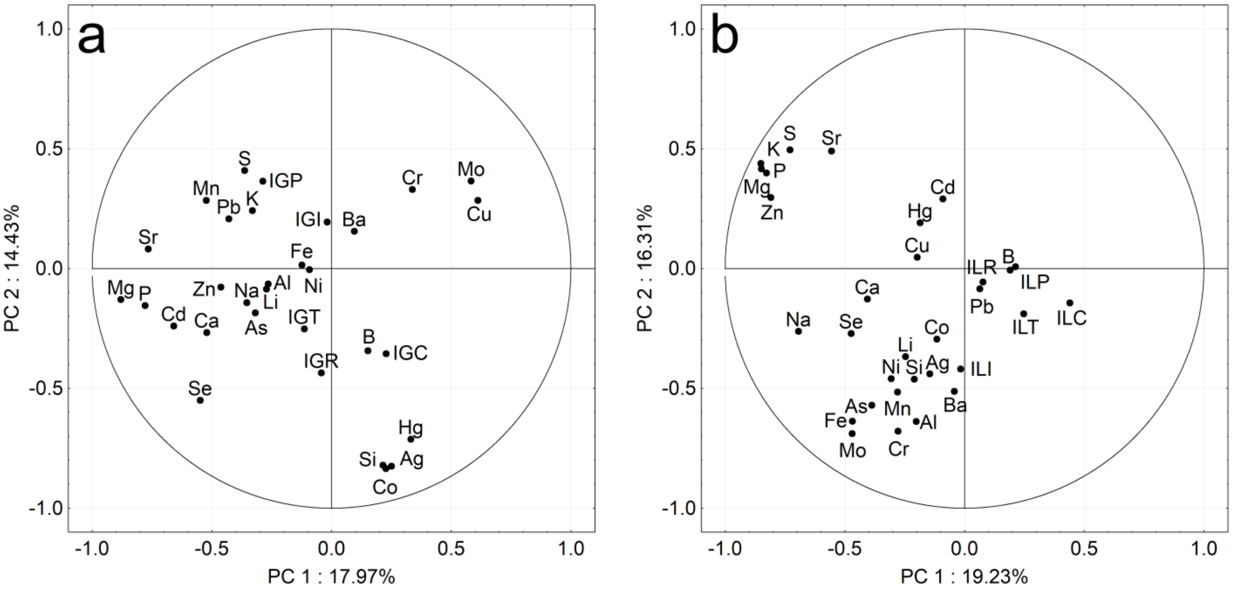
Fig. 4



1041

1042 **Fig. 5**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B
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-0.5
0.0
0.5
1.0
PC 1 : 17.97%
b
1.0
0.5
0.0
-0.5
-1.0
PC 2 : 16.31%
S
Sr
K
P
Mg
Zn
Cd
Hg
Cu
ILR
B
ILP
Pb
ILT
ILC
Ca
Na
Se
Co
Li
Ni
Si
Ag
ILI
As
Mn
Ba
Fe
Al
Mo
Cr
-1.0
-0.5
0.0
0.5
1.0
PC 1 : 19.23%

Fig. 6