GC-MS profiling of organic pollutants and physicochemical, histological, microbiological analyses of water and fish samples from Canton Sarajevo (Bosnia and Herzegovina) rivers

Saida Ibragic[1, *], Samir Djug[2], Muamer Dizdar[1], Anela Topcagic[1], Selma Pilic[2], Renata Besta-Gajevic[2], Arijana Nuic[1], Mahir Gajevic[2], Adi Vesnic[2], Senad Sljuka[2], Aldijana Musovic[2], Dusan Culum[1]

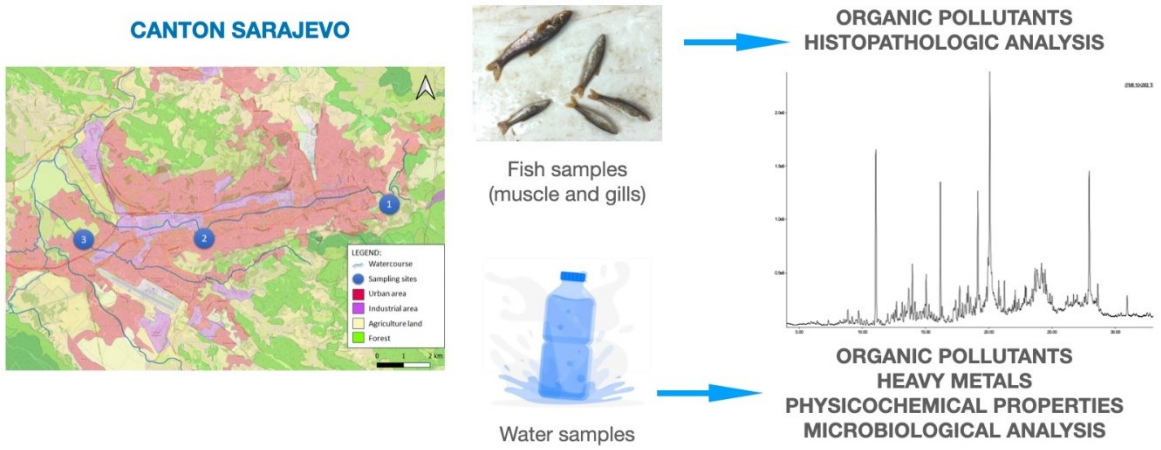
[1]University of Sarajevo – Faculty of Science, Department of Chemistry, Zmaja od Bosne 33-35, 71000 Sarajevo, Bosnia and Herzegovina

[2]University of Sarajevo – Faculty of Science, Department of Biology, Zmaja od Bosne 33-35, 71000 Sarajevo, Bosnia and Herzegovina

[*]Corresponding author:

E-mail: *saidetun19@gmail.com*, tel: +387 33 279 914

GRAPHICAL ABSTRACT



ABSTRACT

Continuous monitoring of the water quality in Canton Sarajevo is one of the top priorities necessary for water management, pollution control and conservation efforts in the region. This study presents a comprehensive assessment of water quality in the interconnected river systems of Miljacka and Zeljeznica, employing a multianalytical approach. Water and fish samples were collected at three sites and analysed for the presence of microorganisms, heavy metals and organic pollutants. Physicochemical parameters were examined to understand the rivers' overall health and ecologic condition. Histopathological analysis of fish species native to these two rivers served as a bioindicator of environmental stressors. In general, there were less anthropogenic pressures found at Site 1 – Kozija ćuprija (river Miljacka – upstream from the city of Sarajevo) and 3 (river Zeljeznica – downstream from the city of Sarajevo). Site 2 – Otoka (river Miljacka, city center) had the highest concentrations of lead, ammonia, orthophosphates and BOD5, as well as total coliform bacteria. This may be linked to the runoff from sewage. The detected histopathological changes are probably associated with the combined microorganisms and organic pollutants, among which plasticizers, emulsifiers, solvents, surfactants and additives were detected by GC-MS.

Keywords: water quality, organic pollutants, microorganisms, histopathology, Canton Sarajevo.

1. Introduction

River ecosystems are fundamentally different from most other ecosystems in that they are (i) open systems with very close functional connections to neighboring ecosystems, (ii) systems with physical and ecological structures and functions that change on a spatiotemporal scale. Their hierarchical organization and close linkages with neighboring terrestrial and subsurface ecosystems have led to the development of the concept of the river continuum. Recent concepts on river functioning consider the importance of physical discontinuity and local processes in rivers as an important contribution to understanding habitat structure and carbon cycling in lotic ecosystems. In this context, it should be emphasized that the loading effects of small rivers on large rivers have been studied only sporadically, although such information is essential to understanding the functioning of a large river (Padmalal et al., 2014).

Pollutants from a variety of sources, including agriculture, industry, or wastewater, enter the aquatic environment and can pose a hazard or risk to the aquatic ecosystem, aquatic organism health, and human health. Systematic studies of water pollution in small river of Bosnia and Herzegovina with pesticides and heavy metals are scarce and provide limited information on water quality and its impact on aquatic life. Riverine communities are sensitive to changes in physical and chemical parameters and to the effects of pollution. Therefore, species composition and abundance in rivers form the basis for characterizing and assessing the ecological status of the river (McCluney et al., 2014).

Sustainable management of water resources requires regular monitoring of water quality and its trends, which allows the identification of hazards and/or risks posed by various anthropogenic stressors. Physical, chemical, biological and bacteriological elements of water quality form the basis of this system, as they provide a wide range of information necessary for sustainable water resources management. Sustainable management of water resources relies on regular monitoring of status and trends of qualities of surface waters, which allows

identification of hazards and or risks posed by multiple anthropogenic stressors (Geissen et al., 2015). Chemical pollution of water resources is considered one of the main causes of degradation of aquatic ecosystems and increasing the pressure on biodiversity (BeyerManica, 2020; Ginebreda et al., 2014; Vörösmarty et al., 2010). The ever-increasing multitude of chemicals entering aquatic environments constitutes a challenge for monitoring schemes, because most of these compounds typically occur at rather small concentrations. Moreover, some chemicals might undergo biotic or abiotic transformation forming very complex environmental mixtures where most individual components can only hardly be identified (Ginebreda et al., 2014). Metals are among the major chemical toxicants polluting the environment due to their prolonged persistence and complex interactions with organisms in aquatic ecosystems (Authman et al., 2015; Boyd, 2010; de Paiva Magalhães et al., 2015). Consequently, changes in metal levels can be reflected in aquatic organisms, which serve as biological indicators of metal exposure. The advantage of applying indicator organisms and organs is that metals are retained in tissues for longer periods than in water, so biological responses indicate long-term metal variability (Samir et al., 2020). This is especially valid for hard tissues, such as fish scales and otoliths, which offer a permanent record of metal exposure over the life span of a fish. Fish hard tissues were mostly applied for stock discrimination, movement studies, and only to a lesser extent as environmental indicators of pollution which were mostly represented by fish soft tissues, such as liver, gills and muscle (Adami et al., 2001; Milton et al., 2008; SawhneyJohal, 1999). Aquatic organisms assimilate pollutants in different ways like intake of particulate matter suspended in water, exchange of dissolved pollutants via lipophilic membranes, and adsorption on the surface of tissues and membranes (Alesci et al., 2022). Consequently, pollutants cause oxidative stress, genotoxicity, cytotoxicity, histopathological alterations, and inflammatory responses in the tissues of fish and aquatic organisms (Bibi et al., 2021; El-Garawani et al., 2022). Therefore, histopathological research

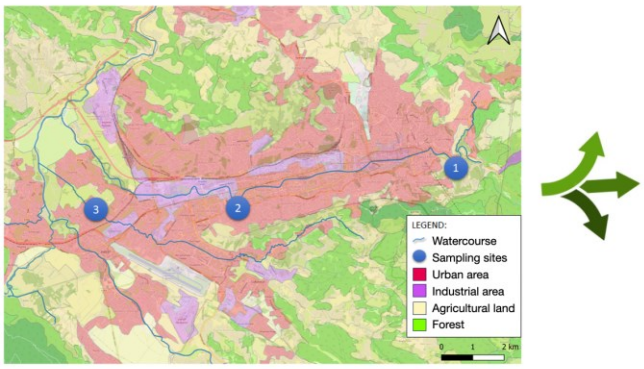
in fish tissues is an important biomarker for assessing the effects of exposure to a variety of anthropogenic pollutants and is a valuable indicator of the general health of fish (Peebua et al., 2008). A number of studies have reported toxicity effects of pesticides and metals, as well as significant histopathological alterations were documented in the gill (hyperplasia, hypertrophy, shortening and edema of secondary lamellae, and necrosis), liver (congestion of blood sinusoids, cytoplasmic vacuolation and necrosis) and muscle tissues as muscle fibers oedema of secondary lamellae, and necrosis), liver (congestion of blood sinusoids, cytoplasmic vacuolation and necrosis) and muscle tissues as muscle fibers splitting and necrosis (Mishra et al., 2023; Nayak et al., 2023; OBAYEMIKOMOLAFE, 2022; Shahid et al., 2022). The action mechanism of pollutants arises from oxidative stress that determines enzymatic and metabolic changes in the affected organism (Anetor et al., 2022). The rates of bioaccumulation in various tissues depend on the type of pollutants and the animal species, and a result of the pollutants' absorption and the mechanisms of regulation, storage, and excretion (Manimekalai et al., 2022; Sayed et al., 2020). Moreover, fish gills are sensitive to water pollution because of the direct contact of toxicants present in water. The gills are the major organs for oxygen uptake and a transport system site engaged in osmoregulation. It has been established that the accumulation of pollutants inside the gills may affect their functioning and transmit pollutants into interior compartments via blood transport (ShahParveen, 2022). Apart from chemical pollutants, biological pollutants primarily microorganisms can cause histopathological lesions in the tissue as a consequence of the inflammatory and defensive response of the organism. Furthermore, faecal water pollution due to numerous pathogens represents a significant risk to human and animal health. The host’s reaction may be in the form of tissue proliferation, cell degeneration, necrosis, and inflammatory cell infiltrating (Chen et al., 2019). Additionally, assessment of the environmental conditions and understanding their potentially deleterious effects on fish are of

the utmost importance because fish populations inhabiting highly polluted water provide precious information on the aetiology of pollutant-mediated diseases (AnvariFar et al., 2018). The absence of continuous monitoring in terms of qualitative and qualitative analyses of both surface and underground waters is listed as one of the top issues concerning water resources in the Sarajevo area in the Cantonal Environmental Protection Plan of Sarajevo Canton. Monitoring activities and intensive research on the effects of specific risk factors on human health are necessary in order to fully comprehend the environmental implications and develop and implement water protection measures. The main watercourse in the Sarajevo city is the river Miljacka which is formed by the confluence of the rivers Paljanska Miljacka and Mokranjska Miljacka. It is 35.9 km long, and runs through the city of Sarajevo, which has 275 500 inhabitants. All municipal waste waters from the city are discharged directly into the river Miljacka without any treatment, and as such pose a very serious threat to the environment and human health (Samir et al., 2020). This study represents the first comprehensive scientific approach to water quality of river Miljacka and Zeljeznica, simultaneously integrating multiple analytical techniques to evaluate microbiological parameters, pollutants and heavy metals offering a holistic under-standing of the ecological health of aquatic ecosystems. For that purpose, water and fish samples were taken from three sites on the river Miljacka and Zeljeznica. The water samples, collected in October 2023, were analysed for their physicochemical characteristics, presence of microorganisms and the content of heavy metals and pollutants. The organic pollutants from the group of pesticides or phthalates were also analysed in fish samples (muscles and gills) and all the results obtained were evaluated as cumulative environmental stressors and additionally sup-ported by histopathological analyses of fish tissues, which serve as bioindicators of water quality.

2. Materials and methods

2.1 Sampling

Water sampling was carried out in accordance with ISO 5667-6:2014, IDT Water quality Sampling Part 6: Guidance on sampling of rivers and streams (Beutler et al., 2014). The three samples of water were collected in sterile 1L polypropylene bottles. After collection, samples were transported and stored on ice before further analysis. Out of a pool of 23 fishes were collected from different sites along the Miljacka river and their muscles and gills were used for analyses. Figure 1 represents the three sites: Kozija cuprija (Site 1, upstream from the city of Sarajevo), Otoka (Site 2, new part of the city) and one site which is on the river Zeljeznica, which is tributary of the river Miljacka (Site 3) (Figure 1). Fish was captured using pulsed direct current backpack electrofishing equipment with a DC500 V generator. Identification of captured fish was done in the field and in the laboratory according to the reference keys for this species (FreyhofKottelat, 2007).



Sample	ID	*Latin name*	Common name	Site
Gills	FG1	*Phoxinus phoxinus*	Common minnow	2
	FG2	*Alburnoides bipunctatus*	Schneider	2
	FG3	*Barbus meridionalis*	Mediterranean barbel	2
	FG4	*Squalius cephalus*	Common chub	2
	FG5	*Gobio gobio*	Gudgeon	1
	FG6	*Barbus meridionalis*	Mediterranean barbel	1
	FG7	*Squalius cephalus*	Common chub	3
	FG8	*Barbus meridionalis*	Mediterranean barbel	3
Muscle	FM1	*Phoxinus phoxinus*	Common minnow	2
	FM2	*Alburnoides bipunctatus*	Schneider	2
	FM3	*Barbus meridionalis*	Mediterranean barbel	2
	FM5		Gudgeon	1
	FM6	*Barbus meridionalis*	Mediterranean barbel	1
	FM7	*Squalius cephalus*	Common chub	3
	FM8	*Barbus meridionalis*	Mediterranean barbel	3
	FM9	*Gobio gobio*	Gudgeon	3
Water	W1	-	-	2
	W2	-	-	1
	W3	-	-	3

Figure 1. Sampling sites on the river Miljacka: Kozija cuprija (Site 1), Otoka (Site 2), and its tributary river Zeljeznica (Site 3).

The method of sampling, identification and quantification of ichthyofauna was based on the European standard EN 14011:2003 (Sykes et al., 2012).

2.2. Physicochemical characterization and determination of heavy metals content

Physicochemical characteristics of water at the investigated sites were analysed in the field using the Multi 3630 IDS F set. Colour, odour, temperature, dissolved oxygen, saturation, pH,

and electrical conductivity have been analysed. In the Laboratory for freshwater ecology the following analyses have been performed: quantification of BOD5, ammonia, orthophosphates and heavy metals (Zn, Pb and Cd). The content of heavy metals in water samples has been deter-mined using Nanocolor UV/VIS II photometric tube tests.

2.3. Microbiological analysis of water

Microbiological analysis of Miljacka and Zeljeznica river samples was performed for total and fecal coliforms, *Escherichia coli*, and *Enterococci*. Defined substrate technology tests were used for bacteriology water quality research. Two IDEXX kits Colilert-18® which detect total coliforms and *E. coli* in water or fecal coliforms in wastewater, and Enterolert®, which targets *Enterococci*, were used. Freshwater samples were diluted (1:10 and 1:10 000) with sterile deionized water. Each sample was mixed with the reagent and placed in Quanti-Tray/2000 then sealed using Quanti-Tray Sealer according to the manufacturer's instructions (IDEXX Laboratories, Inc., Westbrook, ME, USA). Afterwards, trays were incubated for at least 18 h at 35±0.5°C for coliforms and *E. coli*, and 24 h at 44.5±0.5 °C for thermotolerant coliforms as well as at 41±0.5°C for *Enterococci*. After incubation, the yellow wells were counted as positive for coliforms, and any wells that showed fluorescence under ultraviolet (UV) light at 366 nm were quantified as positive *E. coli* (IDEXX Colilert) and *Enterococci* (IDEXX Enterolert). The number of positive wells was compared to the MPN table provided by the manufacturer to enumerate coliforms, *E. coli*, and *Enterococci* in terms of MPN/100 mL.

2.4. Extraction for GC-MS analysis

Water samples were prepared by adding 15 mL dichloromethane and 15 mL hexane to 50 mL sample. Upon extraction, anhydrous magnesium sulphate was added to the organic layer, which was then filtered and the solvent was evaporated. The remaining dry matter was weighed and redissolved in 500 µL hexane. Tissue samples were prepared as homogenates by adding 30 mL mixture of dichloromethane and hexane (1:1, v/v) and 2 g anhydrous magnesium sulphate to 1

g tissue using Sonifier Cell Disruptor B15 (Branson). The tissue was homogenized on ice for 10 minutes with stirring. After the ultrasonic-assisted extraction for 30 min, the samples were filtered and water residues were removed with anhydrous magnesium sulphate. The solvent was evaporated and the remaining dry matter was weighed and redissolved in 500 µL hexane.

2.5. GC-MS analysis

GC-MS analysis was carried out on a Shimadzu QP2010 and the determination method was developed and optimized by our laboratory. The GC conditions were: fused silica HP-5 column (30 m × 0.25 mm i.d., 0.25 µm df), carrier gas He (1.0 mL/min), temperature was programmed from 100°C to 240°C with increments of 3°C/min. The hold time for 100°C was 2 min, the it was increased to 160°C and held for 5 min and finally the temperature of 240°C was held for 7 min. The injection port temperature was 225°C. The detector temperature was 240°C, ionization of the sample components was performed in the EI mode (70 eV), scan range was from 50 to 550 m/z. The identification of volatile constituents was performed using NIST and Wiley databases available as part of the instrument software. All isolated extracts were dissolved in dichloro-methane prior to GC-MS analysis.

2.6. Histopathological analysis

Samples for histological analysis of the gills and muscle tissues of fish were obtained by dissection and then fixed in 10% buffered neutral formalin. After dehydration in graded concentrations of ethanol, samples were cleared in xylene and embedded in paraffin wax. Histological sections of 5-6 µm thickness were stained with hematoxylin and eosin method (Humason, 1962). Sections were analyzed using a light microscope BestScope BS-2035DA1 at different magnifications, then photographed using the program ScopeImage 9.0 and described. Fifteen randomly selected sections of five fish from three sampling sites were selected for semiquantitative scoring of each histopathological parameter. Levels of the histopathological alterations were marked as - for unchanged tissue (0–2%); + for mild alternations (2–10% area

section); ++ for moderate changes (10–40% area section); and +++ for severe tissue alternations (> 40% partition area).

3. Results and Discussion

3.1. Physicochemical characterization and determination of heavy metals content

The physicochemical properties of the collected water samples (Table 1) show similar values for Site 1 and Site 3. The temperature of all samples was around 15°C. The increase of water temperature results with the increased biological activity and reduced dissolved oxygen concentration (Sánchez et al., 2007). The water pH at all sites ranged from 8.02–8.42. The electrical conductivity ranged from 324–380 μS/cm and while this parameter itself does not provide information about specific contaminants - it serves as an indicator of the overall ion concentration in water. Water samples from Site 2 had the highest concentration of ammonia (0.78 mg/L) and orthophosphates (0.11 mg/L) and the highest BOD_5 value (7.98 mgO_2/L). These results indicate a higher concentration of nitrogen-containing compounds, organic pollutants, and phosphorous in the water, which all pose various environmental concerns.

Table 1. Physical and chemical characteristics of water at the investigated sites.

Parameter	Site 1	Site 2	Site 3
Water temperature (°C)	15.6	15.4	15.2
pH	8.03	8.02	8.42
Dissolved oxygen (mg/L)	8.86	8.85	9.45
Electrical conductivity (μS/cm)	324	380	352
NH_3-N (ammonia) (mg/L)	0.07	0.78	0.12
Orthophosphates (mg/L)	0.03	0.11	0.06
BOD_5 (mgO_2/L)	2.02	7.98	2.24

Heavy metals are destabilizing ecosystems due to their negative impacts on biota and their toxic effects on living beings (Zuluaga Rodríguez et al., 2015). They have the ability to bio-accumulate and can slowly release to water bodies causing serious threats to the living organism (Bilal et al., 2021). The collected water samples were analysed for the content of three heavy metals: Zn, Cd and Pb. Among the investigated sites, the highest concentration of all three elements were found at Site 2 (Table 2).

Table 2. Concentrations of heavy metals in water at the investigated sites.

Parameter (µg/L)	Site 1	Site 2	Site 3
Lead (Pb)	0.51	1.52	0.47
Cadmium (Cd)	2.88	4.48	3.20
Zinc (Zn)	0.25	0.54	0.22

Because metals are naturally occurring and their ecotoxicology is influenced by the physicochemical conditions of the water body in which they are present, they provide a variety of special issues for environmental regulators (Comber et al., 2008). According to the EU Water Framework Directive (2000/60/EC), the Environmental Quality Standard (EQS) values for cadmium are between 0.08–0.25 µg/L depending on water hardness and for lead the EQS is 1.2 µg/L. The obtained results show that the detected Cd concentrations at all three sites exceeded the EQS values. The detected Pb concentrations are above the threshold in the water sample from Site 2. Such findings indicate a worrisome state of the water quality considering the toxic effects of heavy metals. Zinc concentrations in water samples varied between 0.22 – 0.54 µg/L and as such they are within the national EQS values for zinc in surface waters, which range from 3.1 -1300 µg/L in different EU member states (VorkampSanderson, 2016).

3.2. Microbiological analyses of water

The measurements of enteric bacteria at each of the three sampling sites are shown in Table 3. *E. coli*, intestinal *Enterococci*, total coliforms, and faecal coliforms were found in all of the water samples. Overall, these data demonstrated that Site 1 had the best water quality. Additionally, water samples from Sites 2 and 3 had greater levels of faecal contamination, according to microbiological water analysis. The sewage discharge at Site 2 is the most probable reason for the lowest water quality.

Table 3. Results of faecal indicator bacteria in water at watercourse sampling sites.

Parameter	Site 1	Site 2	Site 3
Total coliform MPN/100mL	12997	116 900	93000
Faecal coliform MPN/100mL	9804	31 400	23 800
E. coli MPN/100mL	8664	24600	18900
Intestinal *Enterococci* MPN/100mL	41	8600	2909

A maximum of 10,000 cfu/100 mL for faecal coliform bacteria (FC) and a maximum of 4,000 cfu/100 mL for faecal enterococci were set by Kavka and Poetsch (2002) and the EU directive (2006/7 EEC and 76/160EEC) for class III water. Our study's findings indicate that the water at Site 1 is classified as Class II due to intestinal *Enterococci* and Class III due to faecal coliforms. Water samples from Site 2 and Site 3 belong to Class IV. Intestinal *Enterococci* are far less numerous in both mammalian faeces and sewage and usually detected in small numbers compared to *E. coli.* Nevertheless, in recent years intestinal *Enterococci* have been considered a primary indicator for bacterial contamination as it is slightly more resistant to environmental stresses and disinfection than *E. coli* (Fitzmorris-Brisolara et al., 2022). Thus, it was evident from the data that Site 2 water had the highest concentration of Enterococci. Results in our study suggest that lower water quality in Site 2 is most likely linked to intensive runoff from sewage into Miljacka River. Furthermore, since they may point to the presence of pathogens in natural waters, faecal markers also require attention (Cho et al., 2022).

3.3. GC-MS analysis

The GC-MS analysis aimed to establish the presence of organochlorine pesticides and other organic pollutants in the water and fish samples. Table 4 summarises the identified pollutants in all fish and water samples, specifies their use and describes the reported toxicity and hazard statements according to the globally harmonised system of classification and labelling of chemicals. All hazard statements and toxicity information available in Table 4 have been collected from Pubchem – National Institute of Health (Kim et al., 2016).

Based on the obtained results, organochlorine pesticides and their metabolites were not detected in any of the samples. More generally observed organic compounds like long chain alkanes, fatty acids and sterols were present in the majority of samples. Various environmental pollutants ranging from plasticisers, emulsifiers, solvents, surfactants and additives used in pharmaceutical and cosmetic industries have been encountered in the collected water and fish

samples. The mentioned types of compounds were more frequent in gill and water sample than in the muscle tissue.

Water pollution has often been linked to phthalates, also known as phthalic acid esters (PAEs), which are widely distributed. They are frequently employed as additives and plasticizers to increase the mechanical extensibility and flexibility of a variety of products. Synthetic PAEs are currently easily detectable in the atmosphere, water, soil, and sediments. They are thought to pose a risk to public health and ecological functioning (Huang et al., 2021). They can enter water sources through industrial discharges, runoff, or leaching from plastic materials. Like with many other organic chemicals, the aquatic toxicity of phthalate esters is strongly influenced by water solubility. According to Bradlee and Thomas (2003) higher phthalate esters with alkyl chain lengths ≥C6 do not pose intrinsic toxicity to aquatic organisms. The fact that PAEs are regularly found in sources of plants and microorganisms is noteworthy since it raises the prospect that they may be biosynthesized in the natural world (Huang et al., 2021). Diisobutyl phthalate, dinonyl phthalate, diisodecylphthalate, and phthalic acid, belong to the identified phtalate compounds.

Diisobuthyl phthalate was identified in water samples in the range of 1.68-6.4%. Dinonyl phthalate was detected in FG3 and FG7 samples in reaching amounts of 6.83 and 22.86%, respectively. A high percentage of phthalate, diisodecyl phthalate (43%) was detected in FG5 sample.

Some phthalates with no reported toxicity were also detected in significant amounts (over 10%), such as butyl isodecyl phthalate.

Benzophenone was found in a rather high percentage (17.47%) in a water sample (W1) and while it is considered a plant metabolite it is also used in many different industries (ManderLiu, 2010). The same water sample contained tributyl phosphate which is mainly used as a flame retardant. Aircraft hydraulic fluids can contain 25 to 75% TBP. It is also used as a solvent,

plasticizer, carrier for fluorescent dyes and its toxicity has been studied and tested on rainbow trout.

Table 4. Main compounds identified as potential organic pollutants in water and fish samples collected from Miljacka and Zeljeznica river.

Sample	Compound name	Content (%)	Use, toxicity and hazard (PubChem)
W1, W2, W3	Diisobutyl phthalate	1.68-6.40	USE: plasticizer; insect repellant and solvent; solvent in cellulose acetate, fragrances, and cosmetics TOXICITY: Phtalate esters are endocrine disruptors. Animal studies have shown that these effects disrupt reproducitve development and can cause a number of malformations in affected young.
FG3, FG7	Diisononyl ester = dinonyl phthalate	6.83-22.86	USE: low volatility plasticizer for vinyl resins; stationary liquid phase in chromatography TOXICITY: Occupational hepatotoxin - Secondary hepatotoxins: the potential for toxic effect in the occupational setting is based on cases of poisoning by human ingestion or animal experimentation.
FG5	Diisodecyl phthalate	43.00	USE: plasticizer for polyvinyl chloride in calendered film, coated fabrics, building wire jackets, wire and cable extrusion, and other applications;
FG4	Phthalic acid, didecyl ester	20.33	USE: plasticizer
W1	Benzophenone	17.47	ROLE: photosensitizing agent and a plant metabolite. USE: in perfumes, flavoring agents, and organic synthesis; inhibitor of styrene polymerization; a photoinitiator; an ultraviolet curing agent; and an additive (plastics, cosmetics, coatings, and adhesives). TOXICITY: The lethal and sublethal effects of six monosubstituted derivatives of benzene were measured by using the 7 day test with fathead minnow larvae. The LC50s for larvae were compared to those derived from the acute test. The larvae were more sensitive than juvenile fish, yet the toxicity order of the six monosubstituted derivatives of benzene was the same for both life stages, that is, butylphenylether > benzophenone > toluene = benzene > nitrobenzene > aniline.
W1	Phosphoric acid, tributyl ester (TBP)	1.39	USE: in many different industries as extracting solvent, defoaming agent, flame retardant and plasticizer. TOXICITY STUDIES: Rainbow trout treated with tributyl phosphate had severe balance disturbances, which included highly atypical movements like darting, coiling swimming, and backward somersaults. At higher concentrations the fish were immobilized, lying on their sides at the bottom of the water, and some of them died.
FM5, FM6, FM7, FM8	Oleamide	1.13-8.39	USE: a variety of industrial uses including as a slip agent a lubricant, a corrosion inhibitor. Construction and building materials, opacifying, slip additives in carpet backing, viscosity controlling. HAZARD: H413 (70.27%)
FG4	Behenic alcohol	2.29	USE: emollient; emulsifier; thickener in cosmetics; nutritional supplement HAZARD: H400 (42.31%), H410 (28.21%), H411 (42.31%), H412 (28.21%)

FG1	2-Tetradecyloxyethanol	1.50	USE: surfactant; emulsifyer HAAZARD: H400 (99.29%), H410 (62.86%), H412 (62.86%)
FG3	Decane, 1-iodo-	2.64	HAZARD: H413 (84.44%)
FG3	Decanedioic acid, didecyl ester	1.62	USE: synthetic intermediates; low temperature plasticizers; emollients; plastic additives and coating additives
FG4	2,6,10,15-Tetramethylheptadecane	0.46	Metabolite observed in cancer metabolism. It has a role as a human metabolite
FG2, FG4, FM9	2,6-Diisopropylnaphthalene	0.16-1.34	USE: Plant growth retardant and an agrochemical.

H400: Very toxic to aquatic life [Warning Hazardous to the aquatic environment, acute hazard]
H410: Very toxic to aquatic life with long lasting effects [Warning Hazardous to the aquatic environment, long-term hazard]
H411: Toxic to aquatic life with long lasting effects [Hazardous to the aquatic environment, long-term hazard]
H412: Harmful to aquatic life with long lasting effects [Hazardous to the aquatic environment, long-term hazard]
H413: May cause long lasting harmful effects to aquatic life [Hazardous to the aquatic environment, long-term hazard]

Oleamide is naturally found in the human body and certain plants but it was also found to be leaching out of polypropylene plastics (Naumoska et al., 2020). Fish muscle tisssue samples FM5-FM7 were found to contain oleamide. In this case, the detection of oleamide does not necessarily imply aquatic pollution. The presence of oleamide in fish may be the result of dietary sources, as fish can accumulate variouss lipids and fatty acids from their food (Kim et al., 2012).

A few more compounds were detected in gill samples (FG1, FG3 and FG4) for which harmful effects were found. Their representation ranged from to 0.46 zo 2.29%.

As previously mentioned, organochlorine pesticides were not detected, but a naphthalene derivative, 2,6-diisopropylnaphthalene was found in several fish samples (0.16-1.34 %). 2,6-Diisopropylnaphthalene is a plant growth regulator and has been classified as a biochemical pesticide and a non-toxic mode of action (Lee et al., 2021).

In light of the findings, it is recommended that phthalates and other contaminants be continuously monitored. Some phthalates such as, bis(2-ethylhexyl) phthalate are already regarded as priority pollutants by the EU Directive 2008/105/EC (European Commission 2008) due to their harmful health effects (Peñalver et al., 2021).

3.4. Histopathological analysis

The important histopathological alterations that were observed in the gills samples include lifting of the respiratory epithelium, lamella shortening, leukocyte infiltration into the secondary lamella, complete fusion of several lamellae, degeneration and necrosis in the gill tissues (Figure 2). In the muscles, histopathological lesions can be described as splitting of muscle fibers, broken myofibrils, inflammatory cell infiltration, degeneration, and necrosis (Figure 3). The histologic changes recorded in the present study suggest variable severity in sample tissues from different sample sites. Increased levels of tissue damage in the gills and muscles were recorded in fish from Site 2. Samples from Site 1 showed less prominent alternations as well as the absence of some changes (Table 5).

Table 5. Semiquantitative scoring of the histopathology in the fish gills and muscles at the investigated watercourse sites.

	Histopathological lesion	Sites		
		Site 1	**Site 2**	**Site 3**
Gill	Lifting of the respiratory epithelium	+	++	++
	Lamella shortening	+	+++	++
	Leukocyte infiltration into the secondary lamella	++	++	++
	Complete fusion of several lamellae	-	++	+
	Degeneration and necrosis	-	++	++
Muscle	Splitting of muscle fibers	++	+++	++
	Broken myofibrils	-	++	++
	Degeneration (D) and vacuolar degeneration	+	++	++
	Inflammatory cell infiltration	++	++	++
	Necrosis	-	++	+

Score: (-) No alteration, (+) Mild alteration, (++) moderate alteration, (+++) severe alteration

As compared to the reports of studies conducted by Rajme-Manzur et al. (2021) and Rediet et al. (2022) lesions with infiltration of inflammatory cells were indications of bacterial diseases. In our results, inflammatory responses such as infiltration of inflammatory cells could have been influenced by the high microbial load in water, particularly in Site 2 and Site 3 water sources. Many histopathological alterations in our study are probably associated with the combined microorganisms and toxicity of phthalates and other organic pollutants.

Phthalate exposure leads to adverse effects on reproduction, as well as to the liver, kidney, and

other organs (Gao et al., 2018). Our results regarding epithelial lifting, fusion, and curling of lamellae and shortening of primary lamellae in the fish gills can be linked to the toxic effects of detected diisodecyl phthalate (DiDP) in water and fish samples. This finding is in agreement with studies of DiDP-exposed fish (LatifFaheem, 2020; Obiezue et al., 2014; ShincyChitra, 2020; Zhang et al., 2021). Nayak et al. (2023) reported loss of gill structure and secondary lamellar fusion in fishes exposed to various concentrations of naphthalene, as well as Revathy and Chitra (2015) found damage in gill epithelium, degeneration of gill arches and secondary lamellae and erythrocyte infiltration exposed to Di(2-ethylhexyl)phthalate. We found histological alterations in muscles as the splitting of muscle fibers and broken myofibrils which has been confirmed in studies by Latif et al. (2021). Nevertheless, Revathy and Chitra (2016) described disorganized muscle fibers in fish exposed to diisononyl phthalate (DINP). In this context, Sree et al. (2023) summarize the mechanism of phthalates and their biomedical consequences on various tissues or organs. In conclusion, the results of our study indicate that histological parameters could be used as biomarkers for phthalate toxicity monitoring in the aquatic ecosystem.

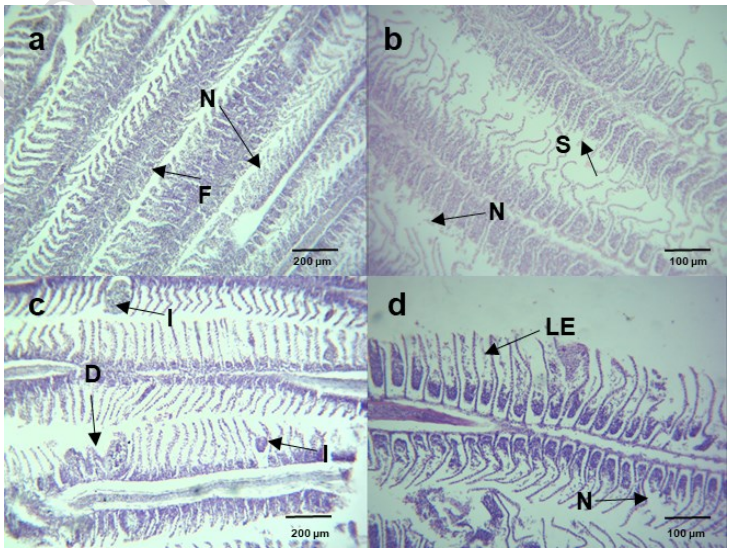


Figure 2. Histopathological changes (a) *Squalius cephalus* gill showing: necrosis (N) and complete fusion of several lamellae (F), (b) *Gobio gobio* gill showing: lamella shortening (S) and necrosis (N), (c) *Barbus meridionalis* gill showing: leukocyte infiltration into the secondary lamella (I) and degeneration (D) (d) *Alburnoides bipunctatus* gill showing: lifting of the respiratory epithelium (LE) and necrosis (N).

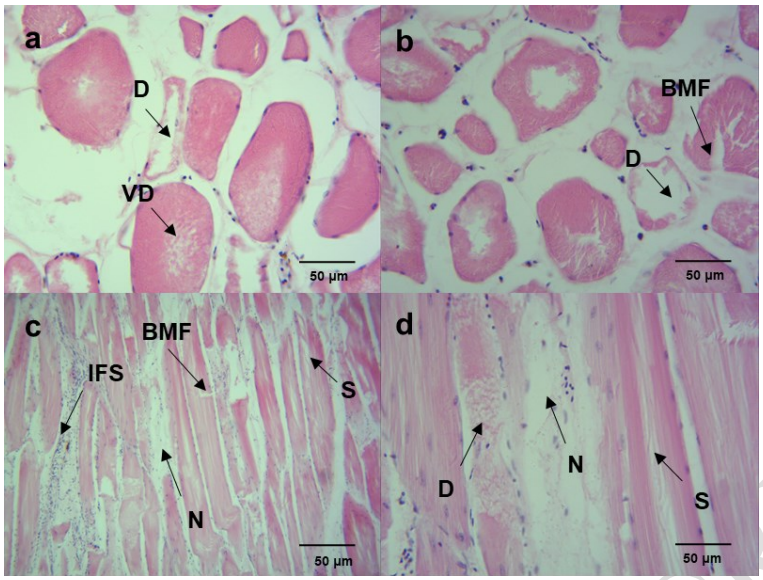


Figure 3. Histopathology lesion of muscle tissues: (a) Transverse sections muscle tissues *Gobio gobio* showing: degeneration (D) and vacuolar degeneration (VD), (b) Transverse sections muscle tissues *Barbus meridionalis* showing: broken myofibrils (BMF) and degeneration (D), (c) Longitudinal sections muscle tissues *Phoxinus phoxinus* showing: splitting of muscle fibers (S), broken myofibrils (BMF), inflammatory cell infiltration (IFC) and necrosis (N), (d) Longitudinal sections muscle tissues *Squalius cephalus* showing: splitting of muscle fibers (S), degeneration (D) and necrosis (N).

4. Conclusions

This study analysed water and fish samples from river Miljacka and Zeljeznica through the evaluation of physicochemical parameters, identification of organic pollutants, microbiological and histopathological findings. The degree of pollution varied among the investigates sites. The results showed that, at Site 2, which is also connected to sewage effluents, anthropogenic influences were particularly apparent. Many of the identified compounds are widely used in diverse industries, which suggest the necessity of a better wastewaters control. Cumulatively, the obtained findings contribute to the ongoing dialogue on sustainable water management practices, emphasizing the need for proactive measures to improve the ecological integrity of the investigated rivers. The present study does not ascertain possible health and environmental hazards posed by the identified contaminants, as such conclusions require more extensive qualitative and quantitative analyses.

Funding: This research was funded by the Ministry of Education, Science and Youth of the

Sarajevo Canton, grant number 27-02-35-37082-31/23.

Conflicts of Interest: The authors declare no conflict of interest.

References

Adami G., Miletić M., Siviero P., Barbieri P. and Reisenhofer E. (2001), Metal contents in tench otoliths: relationships to the aquatic environment, *Annali di chimica*, **91**, 401-408.

Alesci A., Cicero N., Fumia A., Petrarca C., Mangifesta R., Nava V., Lo Cascio P., Gangemi S., Di Gioacchino M. and Lauriano E. R. (2022), Histological and chemical analysis of heavy metals in kidney and gills of boops boops: melanomacrophages centers and rodlet cells as environmental biomarkers, *Toxics*, **10**, 218.

Anetor G. O., Nwobi N. L., Sonuga O. O. and Anetor J. I. (2022), Environmental pollutants and oxidative stress in terrestrial and aquatic organisms: examination of the total picture and implications for human health, *Frontiers in physiology*, **13**, 931386.

AnvariFar H., Amirkolaie A. K., Jalali A. M., Miandare H., Sayed A. H., Üçüncü S. İ., Ouraji H., Ceci M. and Romano N. (2018), Environmental pollution and toxic substances: Cellular apoptosis as a key parameter in a sensible model like fish, *Aquatic toxicology*, **204**, 144-159.

Authman M. M., Zaki M. S., Khallaf E. A. and Abbas H. H. (2015), Use of fish as bio-indicator of the effects of heavy metals pollution, *Journal of Aquaculture Research & Development*, **6**, 1-13.

Beutler M., Wiltshire K., Meyer B., Moldaenke C., Luring C., Meyerhofer M. and Hansen U. (2014), APHA (2005), Standard Methods for the Examination of Water and Wastewater, Washington DC: American Public Health Association. Ahmad, SR, and DM Reynolds (1999), Monitoring of water quality using fluorescence technique: Prospect of on-line process control, *Dissolved Oxyg. Dyn. Model. Case Study A Subtrop. Shallow Lake*, **217**, 95.

Beyer R. M. and Manica A. (2020), Historical and projected future range sizes of the world's mammals, birds, and amphibians, *Nature Communications*, **11**, 5633.

Bibi S., Naz S., Saeed S. and Chatha A. (2021), A review on histopathological alterations induced by heavy metals (Cd, Ni, Cr, Hg) in different fish species, *Punjab Univ J Zool*, **36**, 81-89.

Bilal M., Ali H., Ahmad A., Khan F. A. and Bashir K. (2021), Effects of heavy metal Cd on essential metal Zn levels in freshwater fish Channa gachua, *Int. J. of Aquatic Science*, **12**, 687-698.

Boyd R. S. (2010), Heavy metal pollutants and chemical ecology: exploring new frontiers, *Journal of chemical ecology*, **36**, 46-58.

Bradlee C. A. and Thomas P. (2003), Aquatic toxicity of phthalate esters, *Series Anthropogenic compounds: phtalate esters,* 263-298.

Chen F., Sun J., Han Z., Yang X., Xian J.-a., Lv A., Hu X. and Shi H. (2019), Isolation, identification and characteristics of Aeromonas veronii from diseased crucian carp (Carassius auratus gibelio), *Frontiers in microbiology*, **10**, 2742.

Cho K. H., Wolny J., Kase J. A., Unno T. and Pachepsky Y. (2022), Interactions of E. coli with algae and aquatic vegetation in natural waters, *Water Research*, **209**, 117952.

Comber S., Merrington G., Sturdy L., Delbeke K. and Van Assche F. (2008), Copper and zinc water quality standards under the EU Water Framework Directive: The use of a tiered approach to estimate the levels of failure, *Science of the total environment*, **403**, 12-22.

de Paiva Magalhães D., da Costa Marques M. R., Baptista D. F. and Buss D. F. (2015), Metal bioavailability and toxicity in freshwaters, *Environmental Chemistry Letters*, **13**, 69-87.

El-Garawani I. M., Khallaf E. A., Alne-Na-Ei A. A., Elgendy R. G., Sobhy H. M., Khairallah A., Hathout H. M., Malhat F. and Nofal A. E. (2022), The effect of neonicotinoids exposure on Oreochromis niloticus histopathological alterations and genotoxicity, *Bulletin of environmental contamination and toxicology*, **109**, 1001-1009.

Fitzmorris-Brisolara K., Maal-Bared R., Worley-Morse T., Danley-Thomson A. and Sobsey M. (2022), Monitoring coliphages to reduce waterborne infectious disease transmission in the One Water framework, *International Journal of Hygiene and Environmental Health*, **240**, 113921.

Freyhof J. and Kottelat M. (2007), Handbook of European freshwater fishes, Maurice Kottelat.

Gao D., Li Z., Wang H. and Liang H. (2018), An overview of phthalate acid ester pollution in China over the last decade: Environmental occurrence and human exposure, *Science of the total environment*, **645**, 1400-1409.

Geissen V., Mol H., Klumpp E., Umlauf G., Nadal M., van der Ploeg M., van de Zee S. E. and Ritsema C. J. (2015), Emerging pollutants in the environment: a challenge for water resource management, *International soil and water conservation research*, **3**, 57-65.

Ginebreda A., Kuzmanovic M., Guasch H., de Alda M. L., López-Doval J. C., Muñoz I., Ricart M., Romaní A. M., Sabater S. and Barceló D. (2014), Assessment of multi-chemical

pollution in aquatic ecosystems using toxic units: compound prioritization, mixture characterization and relationships with biological descriptors, *Science of the total environment*, **468**, 715-723.

Huang L., Zhu X., Zhou S., Cheng Z., Shi K., Zhang C. and Shao H. (2021), Phthalic acid esters: Natural sources and biological activities, *Toxins*, **13**, 495.

Humason G. L. (1962), *Animal tissue techniques*, Freeman and Company.

Kavka G. and Poetsch E. (2002), Microbiology–In: Technical Report of the International Commission for the Protection of the Danube River, Eds, *Peter Literathy, Veronika Koller Kreimal, Igor Liska,* 138-150.

Kim D.-K., Kim K.-D., Seo J.-Y. and Lee S.-M. (2012), Effects of dietary lipid source and level on growth performance, blood parameters and flesh quality of sub-adult olive flounder (Paralichthys olivaceus), *Asian-Australasian journal of animal sciences*, **25**, 869.

Kim S., Thiessen P. A., Bolton E. E., Chen J., Fu G., Gindulyte A., Han L., He J., He S. and Shoemaker B. A. (2016), PubChem substance and compound databases, *Nucleic acids research*, **44**, D1202-D1213.

Latif M. and Faheem M. (2020), Study of Oxidative Stress and Histo-Biochemical Biomarkers of Diethyl Phthalate Induced Toxicity in a Cultureable Fish, Labeo rohita, *Pakistan veterinary journal*, **40**, 1-2.

Latif M., Faheem M., Hoseinifar S. H. and Van Doan H. (2021), Protective efficacy of Nigella sativa seeds against diethyl phthalate induced growth retardation, oxidative stress and histo-biochemical damages in Labeo rohita, *Aquaculture*, **533**, 736065.

Lee H. S., Park J.-S., Lee S. J., Shin H.-S., mi Chung Y., na Choi H., Yun S. S., Jung Y.-h. and Oh J.-H. (2021), Development and validation of an analytical method for the quantification of 2, 6-diisopropylnaphthalene in agricultural products using GC-MS/MS, *분석과학*, **34**, 1-8.

Mander L. and Liu H.-W. (2010), *Comprehensive natural products II: chemistry and biology* (Vol. 1), Elsevier.

Manimekalai D., Srinivasan A., Padmavathy P., Jawahar P., George R. and Arisekar U. (2022), Acute and chronic toxicity effects of the heptachlor pesticide on Tilapia (Oreochromis mossambicus): impact to behavioral patterns and histopathological responses, *Journal of Coastal Research*, **38**, 999-1010.

McCluney K. E., Poff N. L., Palmer M. A., Thorp J. H., Poole G. C., Williams B. S., Williams M. R. and Baron J. S. (2014), Riverine macrosystems ecology: sensitivity, resistance, and

resilience of whole river basins with human alterations, *Frontiers in Ecology and the Environment*, **12**, 48-58.

Milton D., Halliday I., Sellin M., Marsh R., Staunton-Smith J. and Woodhead J. (2008), The effect of habitat and environmental history on otolith chemistry of barramundi Lates calcarifer in estuarine populations of a regulated tropical river, *Estuarine, Coastal and Shelf Science*, **78**, 301-315.

Mishra B., Gautam G. J., Chaturvedi R. K., Ansari N. G. and Mishra V. (2023), Ecological and health risk assessment of heavy metals bioaccumulation in Ganges fish near Varanasi, India,

Naumoska K., Jug U., Metličar V. and Vovk I. (2020), Oleamide, a bioactive compound, unwittingly introduced into the human body through some plastic food/beverages and medicine containers, *Foods*, **9**, 549.

Nayak S., Das S., Kumar R., Das I. I., Mohanty A. K., Sahoo L., Gokulakrishnan M. and Sundaray J. K. (2023), Biochemical and histopathological alterations in freshwater fish, Labeo rohita (Hamilton, 1822) upon chronic exposure to a commonly used hopper insecticide, triflumezopyrim, *Chemosphere*, **337**, 139128.

Obayemi O. E. and Komolafe O. O. (2022), Comparative histopathological studies of selected organs of Oreochromis niloticus (Linnaeus, 1758) from Igun and Opa Reservoirs, Southwestern Nigeria, *Notulae Scientia Biologicae*, **14**, 1-14.

Obiezue R. N., Ikele C. B., Mgbenka B. O., Okoye I. C., Attamah G. N., Uchendu C., Ezeamachi E. and Onyia C. Q. (2014), Toxicity study of diethyl phthalate on Clarias gariepinus fingerlings, *African Journal of Biotechnology*, **13**, 884-896.

Padmalal D., Maya K., Padmalal D. and Maya K. (2014), Rivers-structure and functions, *Sand Mining: Environmental Impacts and Selected Case Studies,* 9-22.

Peebua P., Kruatrachue M., Pokethitiyook P. and Singhakaew S. (2008), Histopathological alterations of Nile tilapia, Oreochromis niloticus in acute and subchronic alachlor exposure, *Journal of environmental biology*, **29**, 325.

Peñalver R., Jacobs M. R., Hegarty S. and Regan F. (2021), Assessment of anthropogenic pollution by monitoring occurrence and distribution of chemicals in the river Liffey in Dublin, *Environmental Science and Pollution Research*, **28**, 53754-53766.

Rajme-Manzur D., Gollas-Galván T., Vargas-Albores F., Martínez-Porchas M., Hernández-Oñate M. Á. and Hernández-López J. (2021), Granulomatous bacterial diseases in fish: An overview of the host's immune response, *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, **261**, 111058.

Rediet W., Jirata S., Yacob H. and Abdi F. (2022), Characterization of Lesions in Diseased Fishes, Isolation and Identification of Edwardsiella Tarda from Selected Lakes and Ponds of Ethiopia, *American Journal of Biomedical Science & Research,* 1-17.

Revathy V. and Chitra K. (2015), Di (2-ethylhexyl) phthalate-induced histopathological changes in gill and liver of freshwater fish, Oreochromis mossambicus (Peters, 1852), *International Journal Advanced Research*, **3**, 263-270.

Samir Đ., Nusret D., Sadbera T.-B., Aldijana M., Adi V., Sabina T., Mahir G., Renata B.-G., Senad Š., Ranko M., Enad K. and Rifat Š. (2020), *Biomonitoring akvatičnih ekosistema*, Univerzitet u Sarajevu.

Sánchez E., Colmenarejo M. F., Vicente J., Rubio A., García M. G., Travieso L. and Borja R. (2007), Use of the water quality index and dissolved oxygen deficit as simple indicators of watersheds pollution, *Ecological indicators*, **7**, 315-328.

Sawhney A. and Johal M. (1999), Potential application of elemental analysis of fish otoliths as pollution indicator, *Bulletin of environmental contamination and toxicology*, **63**, 698-702.

Sayed A. E.-D. H., Mekkawy I. A., Mahmoud U. M. and Nagiub M. (2020), Histopathological and histochemical effects of silver nanoparticles on the gills and muscles of African catfish (Clarias garepinus), *Scientific African*, **7**, e00230.

Shah Z. U. and Parveen S. (2022), Oxidative, biochemical and histopathological alterations in fishes from pesticide contaminated river Ganga, India, *Scientific Reports*, **12**, 3628.

Shahid S., Sultana T., Sultana S., Hussain B., Al-Ghanim K. A., Al-Bashir F., Riaz M. N. and Mahboob S. (2022), Detecting aquatic pollution using histological investigations of the gills, liver, kidney, and muscles of Oreochromis niloticus, *Toxics*, **10**, 564.

Shincy F. and Chitra K. (2020), Impacts of Phthalate Plasticisers—An Emerging Issues and Concerns, *Journal of Veterinary Science & Technology*, **9**, 2-3.

Sree C. G., Buddolla V., Lakshmi B. A. and Kim Y.-J. (2023), Phthalate toxicity mechanisms: An update, *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, **263**, 109498.

Sykes A. V., Baptista F. D., Gonçalves R. A. and Andrade J. P. (2012), Directive 2010/63/EU on animal welfare: a review on the existing scientific knowledge and implications in cephalopod aquaculture research, *Reviews in Aquaculture*, **4**, 142-162.

Vorkamp K. and Sanderson H. (2016), European Environmental quality standards (EQS) variability study, *Scientific Report from DCE.*

Vörösmarty C. J., McIntyre P. B., Gessner M. O., Dudgeon D., Prusevich A., Green P., Glidden S., Bunn S. E., Sullivan C. A. and Liermann C. R. (2010), Global threats to human water security and river biodiversity, *nature*, **467**, 555-561.

Zhang Y., Jiao Y., Li Z., Tao Y. and Yang Y. (2021), Hazards of phthalates (PAEs) exposure: A review of aquatic animal toxicology studies, *Science of the total environment*, **771**, 145418.

Zuluaga Rodríguez J., Gallego Ríos S. E. and Ramírez Botero C. M. (2015), Content of Hg, Cd, Pb and As in fish species: a review, *Vitae*, **22**, 148-149.

List of Figures

List of Tables