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One health approach through freshwater ecosystem contamination assessment of Sarajevo Region, Bosnia and Herzegovina

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Abstract

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Regarding contamination-burdened aquatic ecosystems, we sought to assess how faecal effluents in rivers affect water quality and how sewage hazards affect *Barbus meridionalis* petenyi Heck.- fish populations. IDEXX defined-substrate assay was applied to enumerate total coliforms, *Escherichia coli*, faecal coliform, and *Enterococci* in water. Collected fish individuals from Miljacka River (Bosnia and Herzegovina) at two sampling sites were analysed for *E. coli*, *Salmonella* spp., *Pseudomonas* spp., *Listeria monocytogenes,* sulfite-reducing clostridia, and *S. aureus*. Analysis of histopathological granuloma formation was also implemented as an immunity response of fish to pathogens. Results revealed that microbial counts for the different microbiological parameters varied between sampling sites, respectively. The results of the histological analysis indicated that the intensity of granulomas in fish liver, muscle tissues, and gills increases with coliform contamination. Hence, histological indicators in the form of granuloma have been established in all analysed organs with a high correlation to the degree of water pollution. Overall the granulomatous lesions in these animals may indicate infectious diseases that can be associated with environmental contamination.

Keywords: contamination; fish; granulomatous inflammation; microbiology; water

Introduction

Freshwater ecosystems play a vital role in preserving life on Earth therefore their worldwide protection and their sustainable use as natural resources are essential. More generally, anthropogenic pressure is becoming a major force in reshaping planet Earth's water quality (Nasir et al., 2021) thus monitoring is extremely significant for the preservation and protection of water resources (Liu et al., 2023). Analysis of individual factors of water quality indicates the current state of the water environment, but more important is the ability of bioindicators to identify indirect biotic effects of pollutants, which is something that many physical or chemical tests are unable to do (Chandel et al., 2024). Moreover, their physiological and pathophysiological states reflect both the current status of the environment and long-term activities (Jafarabadi et al., 2019). In this regard, fish provide an evident biological endpoint of historical exposure to environmental change and fish diseases, and histopathology, with a broad range of causes, is increasingly being used as an indicator of environmental stress (Stentiford et al., 2003). Since many pollutants involve metabolic activation before they induce cellular change in the affected organism, histopathological biomarkers strongly correlate to other stress biomarkers. For example, fish can absorb xenobiotics via their gills, stomach juices, skin, and other organs. Xenobiotics are substances that are not naturally occurring inside the organism but possess the potential for interaction and can cause cancerous consequences, leading to cellular intoxication and death on a cellular level. Hence, tissue necrosis as a histopathological

biomarker can be manifested as a consequence of intoxication (Lal et al., 2023). Apart from xenobiotics, infectious diseases, and parasites may cause histopathological lesions in tissue, which can lead to necrotic and degenerative changes that trigger an inflammatory and defensive response in the host. Furthermore, an increased number of macrophagic aggregates can be found in the liver, kidney, and spleen in fish exposed to bacteria, fungi, or parasites.

The granulomatous fish disease is commonly associated with bacterial, mycotic, or parasitic infections (Karniely et al., 2023). Granulomatous inflammation is a distinctive pattern of chronic inflammatory reaction observed in various bacterial diseases. This is an immune response involving the activation of humoral and cellular elements (Rajme-Manzur et al., 2021). Nevertheless, the host-pathogen interaction depends on the environmental conditions. Microorganisms play a vital role in water and impact the decomposition of nutrients, parameters in water, and water quality (Mishra et al., 2021).

On the other hand, microorganisms can cause fish diseases and the most important fish-associated pathogens are bacteria, parasites, and viruses. Nevertheless, some of the microorganisms are obligatory pathogens, always causing disease while some are potentially (conditionally) pathogens and will cause infection after stress (Yu et al., 2021). Furthermore, the risk of environmental contamination and the development of aquatic-derived zoonoses is increasing (Ziarati et al., 2022). This study explored the reflection of the wastewater-destroyed environment on fish health status through microbiological fish analysis and bacteriological water analysis as well as liver, muscle, and gills granuloma examination in the wild *Barbus meridionalis* Heck. population. This species was selected due to widely distribution in the study area and well-known biology hence it can be considered a key role in understanding the consequences of exposure to xenobiotics (Arias, 2020).

Miljacka River flows through the capital city of Sarajevo, Bosnia and Herzegovina, and has been subjected to an increasing pollution load principally originating from urban wastewater and sewage. Few studies have dealt with the responses and sensitivity of this species to diverse types of pollutants (Velkova-Jordanoska, 2006; Maceda-Veiga et al., 2010; Blanco et al., 2019; Atli, 2020; Khalid et al., 2023) but this is the first study to focus on *Barbus meridionalis* glomerulation response to polluted environment. Therefore, because of the implication that fish is a practicable tool for health risk analysis and that the water-related exposome is a significant determinant of human health these research results could have broad public health promotion since public health concerns human health, animal health, and the environment.

Material and Methods

Study Sites

Sarajevo is the capital of Bosnia and Herzegovina with a population of 400 000. In terms of hydrography, Bosnia and Herzegovina is a part of the Adriatic Sea basin (30%) and the Black Sea basin (70%). Its southern water bodies flow into the Adriatic Sea, while its northern water bodies flow into the Black Sea basin. The Miljacka River flows through the Sarajevo basins between Bjelasnica and Igman mountain depressions. The Miljacka River has a very steep and wavelike longitudinal profile, while downstream flows are exposed to pollution from urban wastewater and sewage which was discharged without treatment directly to the Miljacka. Our research was undertaken in Sarajevo, Bosnia, and Herzegovina in the summer of 2023, with two stream sampling sites in Miljacka River noted as SS1: Dariva – upstream, and SS2: Hrasno – in the city centre. Sample site SS1 is situated upstream of Sarajevo (location: 43°51′27.40″N, 18°26′56.26″E) and SS2 (location: 43°51′02.79″N, 18°22′43.43″E) is located in a stretch that is highly influenced by untreated wastewater pollution from Sarajevo as discharged into the river Miljacka without any treatment.

Sampling

Water samples for microbiological analysis were done according to standard methods for examining water and wastewater (APHA, 2005). Samples of water were collected in sterile 1l polypropylene bottles. After collection, samples were transported and stored on ice before further analysis. Fish specimens have been collected from two sample sites of the Miljacka River. Fish were captured using pulsed direct current backpack electrofishing equipment with a DC 500 V generator by electrofishing. The captured fish was identified in the field and the laboratory according to the reference keys for this species (Kottelat & Freyhof, 2007). The capture of fish and experimental procedures followed the ethical guidelines of the European Union Council (Guide for Care and Use of Laboratory Animals, Directive 2010/63/EU).

Fish were placed and left in a > 250 mg/L buffered Benzocaine solution for a minimum of 10 min and after cessation of opercular movement, they were decapitated with adequate equipment.

Furthermore, to prevent contamination sampled fish were placed in waterproof plastic bags and sealed.

Microbiological analyses of water

Microbiological analyses of Miljacka River were performed for total and faecal coliforms, *Escherichia coli*, and *Enterococci.* Defined substrate technology tests were used

for bacteriology water quality research, respectively. Two IDEXX kits Colilert-18® which detects total coliforms and *Escherichia coli* in water or faecal coliforms in wastewater, and Enterolert®, which targets *Enterococci,* were used.

Dilution 1:10 and 1: 10 000 freshwater samples made with sterile deionized water. Each sample was mixed with 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). Afterward, 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 41 ± 0.5°C for *Enterococci*. After incubation, the yellow wells were counted as positive for coliforms, and any wells that fluoresced 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 manufacturer-provided MPN table to enumerate coliforms, *E. coli*, and *Enterococci* in terms of MPN/100 mL.

Microbiology fish analysis

In the laboratory, fish were weighed and dissected, and the muscle tissues (skinned) were separated under aseptic conditions. Samples were homogenized with the addition of 180 ml of physiological solution, and basic dilution was made. Each 0.1 ml of a specific dilution was smeared on selective substrates and placed for incubation. For isolation and identification of bacteria from fish samples classic microbiological methods were used according to ISO procedures. The following parameters were analysed: *E. coli*, *Salmonella* spp., *Pseudomonas* spp. *Listeria monocytogenes*, sulfite-reducing clostridia, and *S. aureus*. *Escherichia coli* was determined according to the ISO 16649-2:2001 method. The sample was inoculated to a selective Tryptone Bile X-glucuronide Chromogenic agar – TBX (Conda Pronadisa, Spain) and incubated at 44°C ± 1 at 18 to 24 h. Additionally, for suspected colonies, Coliform ChromoSelect Agar (Merck, Sigma-Aldrich) was used and identification of *E. coli* was conducted based on indole production capacity with Kovac's reagent. The presence of *Salmonella* spp. was determined according to the ISO 6579:2008 method. *Salmonella* spp. was determined by pre-enrichment of the samples in Buffered Peptone Water (Conda Pronadisa, Spain) incubating at 37°C for 24 h and Rappaport Vassiliadis broth (Conda Pronadisa, Spain) at 41.5°C ± 1°C during 24 h ± 3 h. Afterward, streaking was done on Brilliant Green agar and Salmonella-Shigella agar (Conda Pronadisa, Spain). Presumptive *Salmonella* spp. colonies were confirmed through biochemical tests using Triple Sugar Iron (Conda Pronadisa, Spain) and Simmon›s Citrate agar (Conda Pronadisa, Spain) and incubating at 37°C for 24 h. Pseudomonas agar and Cetrimide isolation agar (Conda Pronadisa, Spain) were incubated at 37°C for 24 h for *Pseudomonas* isolation according to ISO 16266:2006. After growing the identification of the isolates followed by ultraviolet (UV) illumination. The presumptive *Pseudomonas* colonies were tested with the Gram staining, oxidase (Merck, Sigma Aldrich), and catalase test. The method according to ISO 11290-1:2017 was used to isolate *L. monocytogenes* in the samples. Broth with a reduced concentration of inhibitors, Fraser's broth ½ (Conda Pronadisa, Spain) was used for revitalization and pre-enrichment of listeria. The next stage involved the use of Fraser broth 1/1 (Conda Pronadisa, Spain) with a full concentration of selective inhibitors. The surface of ALOA agar (Listeria Selective Agar Base according to Ottaviani and Agosti, Conda Pronadisa, Spain) was inoculated from the incubated broth cultures. Sulfite-reducing clostridia *was proven according to the ISO 15213:2004 method in* tryptose sulfite cycloserine agar (Conda Pronadisa, Spain). Coagulase-positive staphylococci were determined according to the ISO 6888-1:2009 method. Using mannitol-salt agar plates (Merck, Sigma Aldrich) were incubated for 24 h ± 2 h, at 37°C the positive samples were then isolated with a culture test on the Baird Parker agar containing tellurite egg yolk supplement (Conda Pronadisa, Spain). To distinguish the *S. aureus* from other *Staphylococcus* species, the sample was then tested on blood agar media, to observe the presence of hemolysis and identified with a catalase, Gram staining, and a coagulase test (Staphylase test, Oxoid).

Histopathological analysis

For histopathological analysis, *Barbus meridionalis* liver, gill (the second right gill arch), and muscles samples were taken and fixed in 10% buffered neutral formalin. Tissues were then routinely dehydrated in an ascending series of alcohol, cleared in xylene, and embedded in paraffin wax. Sections 5–6 μm thick were cut, processed, and stained with hematoxylin and eosin for general structure, and then, Canada balsam was poured and covered with a cover glass (Presnell & Schreibman, 1997). Thereafter, analyses of histological structures were performed using a light microscope BestScope BS-2035DA1 at a magnification of 400× and 1000×, by the program Scopelmage 9.0.

Results and Discussion

Microbiologal analysis of water

The Colilert and Enterolert represent a reliable and effective testing method for water quality in freshwater environments. The data for the analysed parameters is summarized

in Table 1. It should be noted that the impact of sewage on the water quality at SS2 is evident through increases in the number of all analysed faecal indicator bacteria, respectively. In its downstream Miljacka River passes through residential areas and consequently bacterial coliform counts were higher at the SS2 sample site than at the SS1 sample site.

Table 1. Bacteriological analysis of water at SS1 and SS2 sampling sites

Parameter	SS1	SS2
Total coliform MPN/100ml	12 330	488 000
Faecal coliform MPN/100ml	4034	36 200
E. coli MPN/100ml	3314	27 500
Enterococcus MPN/100ml	2755	195 000

When using the obtained data for an indication of the status in the light of faecal contamination (Kavka & Poetsch, 2002; EU directive 2006/7 EEC and 76/160EEC), the SS2 site would be classified as class V (the poorest water quality) according to enterococci and class IV according to other parameters and SS1 would be classified as Class III according to faecal indicator bacteria. As recovery of enterococci gave more reliable results than faecal coliform (Ramoutar, 2020) it can be suggested that SS2 was the poorest water quality. Fish are at constant risk of exposure to pathogens that can quickly disperse in water (Rajme-Manzur et al., 2021). However, isolation of such high concentrations of sewage pollution-indicative bacteria is important for one health approach.

Microbiologal analysis of fish

After the incubation period, the bacterial isolation on selective media revealed many bacterial isolates: *Salmonella* spp., *Pseudomonas* spp., *E. coli*, *Staphylococcus aureus*, *Listeria monocytogenes,* and sulfite-reducing clostridia. Colony characters and biochemical studies confirm the results. The microbiological analysis of individual samples varied between sampling sites, respectively. It is interesting to find that fish collected from SS2 have been contaminated with all investigating microorganisms, while fish from SS1 have been contaminated with *Salmonella*, *E. coli*, and *S. aureus* but significantly lower. The results of the bacteriological examination are summarized in Table 2.

The data in Table 2 showed that samples collected from the SS1 site were found to be contaminated with *Salmonella* spp. *Pseudomonas* spp*., E. coli,* and *S. aureus,* and none of the examined fish samples from this sample site contained *Listeria monocytogenes* and sulfite-reducing clostridia. Additionally, the illustrated results in Table 2 revealed that samples from SS2 were contaminated with all investigated bacteria and our findings regarding *L. monocytogenes*, *S. aureus*, *Pseudomonas* spp., *Salmonella* spp., and *E. coli* are notably higher than those found in fish samples from SS1, respectively. However, all the samples from SS1 were negative for sulfite-reducing clostridia and four fish samples from S2 were positive. Also, the isolation of pathogenic microorganisms from fish such as *Salmonella* spp*., E. coli, Listeria monocytogenes, Clostridium* spp., and potentially pathogenic microorganisms such as *S. aureus* and *Pseudomonas* spp. could be attributed to the continued environmental faecal pollution. Serious diseases can be caused by water contaminated with feces and in this concern, some of these bacterial isolates have a significant risk to human health. Furthermore, the 95th percentile gastrointestinal illness risk resulted mostly from exposure to pathogenic *E. coli* and *Salmonella.* When discussing sewage discharge as a vector we have to consider *E. coli* O157 which can cause life-threatening hemolytic uremic syndrome (HUS) with hemolytic anemia, acute kidney failure, and thrombocytopenia in humans (Kolodziejek et al., 2022; Megantara et al., 2023).

Histological analysis of tissues

Histological examination revealed a significant presence of many types of pathological lesions in fish gills, liver, and muscles. Figure 1 shows microscopic preparations of the liver with pronounced granulomatous inflammation at the level of hepatocellular parenchyma. Furthermore, a serious presence of granulomatous formations of varied sizes and compositions was observed in fish samples collected at SS2. The occurrence of more serious changes in fish liver from SS2 can be correlated with worse water quality at this sample site. A similar assessment was obtained by Velkova-Jordanoska et al. (2012) and Flores-Lopes et al. (2019) who demonstrated that hepatic changes can be a response to environmental quality. The formation of granulomas is a process induced

Table 2. Occurrence of bacterial species in *Barbus meridionalis* from SS1 and SS2 of Miljacka River

Sampling sites	Number of samples	Number of positive samples, %					
		Salmonella spp.	*Pseudomonas* spp.	*E. coli*	*S. aureus*	*Listeria monocytogenes*	*Sulphite-reducing clostridia*
SS1	20	10	30	20	40	0	0
SS-2	20	60	90	60	100	100	10

by the persistence of pathogens in the host tissues. These changes develop as proliferative and multifocal nodular lesions, whose composition and subsistence depend on several factors, including the interaction between macrophages and T lymphocytes, the recruitment of inflammatory effector cells, the cell proliferation at the formation site, and the survival of accumulated cells into the granuloma (Novotny et al., 2010). Correspondingly, Mahmoud et al. (2016) and Rajme-Manzur et al. (2021) reported that bacteria belonging to genera *Streptococcus*, *Staphylococcus, Mycobacterium, Nocardia, Francisella*, *Salmonella*, *Pseudomonas* and *Vibrio* have been implicated in the development of granulomatous processes. In the present study, fish samples were found to be contaminated with *Salmonella* spp., *Pseudomonas* spp., *E coli*, *Listeria monocytogenes*, *Clostridium* spp., and *S. aureus.* Our findings regarding *L. monocytogenes*, *S. aureus*, *Pseudomonas*, *Salmonella*, and *E. coli* are notably higher in fish samples from SS2 than those found in fish samples from SS1. However, bacteria causing granulomatous diseases can be persistent microorganisms, difficult to eliminate that can cause chronic diseases, even using some immune system components to survive (Zhu et al., 2015).

In this study, we reported granulomas that are histologically characterized by a necrotic "core" where extracellular bacteria persist surrounded by a cellular homogeneous necrotic debris (Figure 1g). While the disease is active, the lesion enlarges with progressive necrosis, resulting in tissue erosion and dispersal of infectious material (Martínez-Lara et al., 2021). However, granuloma can become a restricted area reinforced with organized fibrotic tissue (Figure 1e), which favors the containment of necrosis and, consequently, the pathology (Martinot, 2018). In addition, many studies also revealed granulomatous formations that were associated with diverse types of parasitic infections (Mahmoud et al., 2016; Mandrioli et al., 2022). In this context, we have noted parasite-related granulomatous inflammation changes (Figure 1c). Similar changes were reported by Roganovic-Zafirova et al. (2003) and Velkova-Jordanoska (2006) who revealed that these changes were associated with *Capillaria* sp. eggs.

Histopathological analysis of the gills showed changes such as leukocyte infiltration, shortening, degeneration, and fusion of lamellae. The presence of granulomas was observed in the lamellar epithelium, or embedded deep in the branchial tissue (Figure 2). The granulomas were spherical to ovoid in shape and completely enclosed within the epithelial wall. Hence, our findings are in correspondence with Dar et al. (2020) who reported a correlation between microbiological water quality and tissue lesions in gills from *Oreochromis niloticus*. In many studies, these formations are attributed to various parasitic infections (Azevedo et al., 2009; Banerjee et al., 2015; Molnár et al., 2018). A parasite, probably Nematoda, was also observed (Figure 2h). During investigation samples collected from the SS2 site showed more serious changes. In addition, our results especially from SS2 correspond with Mandrioli et al. (2022) who state a significant relationship between bacteriological parameters in water and inflammatory processes in the analysed fish species. In our study, SS2 contamination is evident through increases in the number of all analysed faecal indicator bacteria.

Histopathological examination of the muscle tissue showed the presence of cracks within myofibers, fragmentation, and degeneration of myofibers, and granulomas in the muscle fibres or embedded in the surrounding connective tissue (Figure 3). This is consistent with Zhuang et al. (2023) who revealed the mechanisms of bacteria-induced grass carp protein degradation. Results of this study showed that *Pseudomonas putida*, *Shewanella putrefaciens,* and *Aeromonas rivipollensis* caused disarrangement of myofilaments and fragmented myofibers. Results of microbiological examination in our study revealed the presence of *Pseudomonas* spp. in fish samples. Dar et al. (2020) found that *Pseudomonas aeruginosa* infected fish developed clinical abnormalities like skin darkness, scale detachment, blindness large irregular hemorrhages on the body surface, fin necrosis, etc. Ulcerous disease is a common fish disease caused by several members of the family *Pseudomonadaceae* but *P. fluorescens,* and *P. aeruginosa* are the most common opportunistic pathogens affecting fish (Ndi & Barton, 2012). In addition, histological analysis of samples collected from SS2 showed more severe histopathological changes as well as congestion and dilation of blood vessels in the connective tissue. There was also infiltration of blood cells into the connective tissue. In the histological sections of skeletal muscles, the presence of many granulomas in the muscle fibers was observed (Figure 3ef). The granulomas consist of foci containing a granular crystallogenous matrix located in an irregular muscle fiber with pyknotic nuclei or complete absence of nuclei and surrounded by fibroblastic tissue. Related results are given by a study conducted by Sindeaux-Neto et al. (2017) describing aspects of parasitic infections caused by myxosporidia *Kudoa orbicularis* in the muscle tissue of *Chaetobranchopsis orbicularis*. The infected specimens had grossly abnormal muscle texture with fragile tissue and inconsistent features.

Conclusion

The presented results indicate a serious public health problem of Miljacka River contamination, particularly in

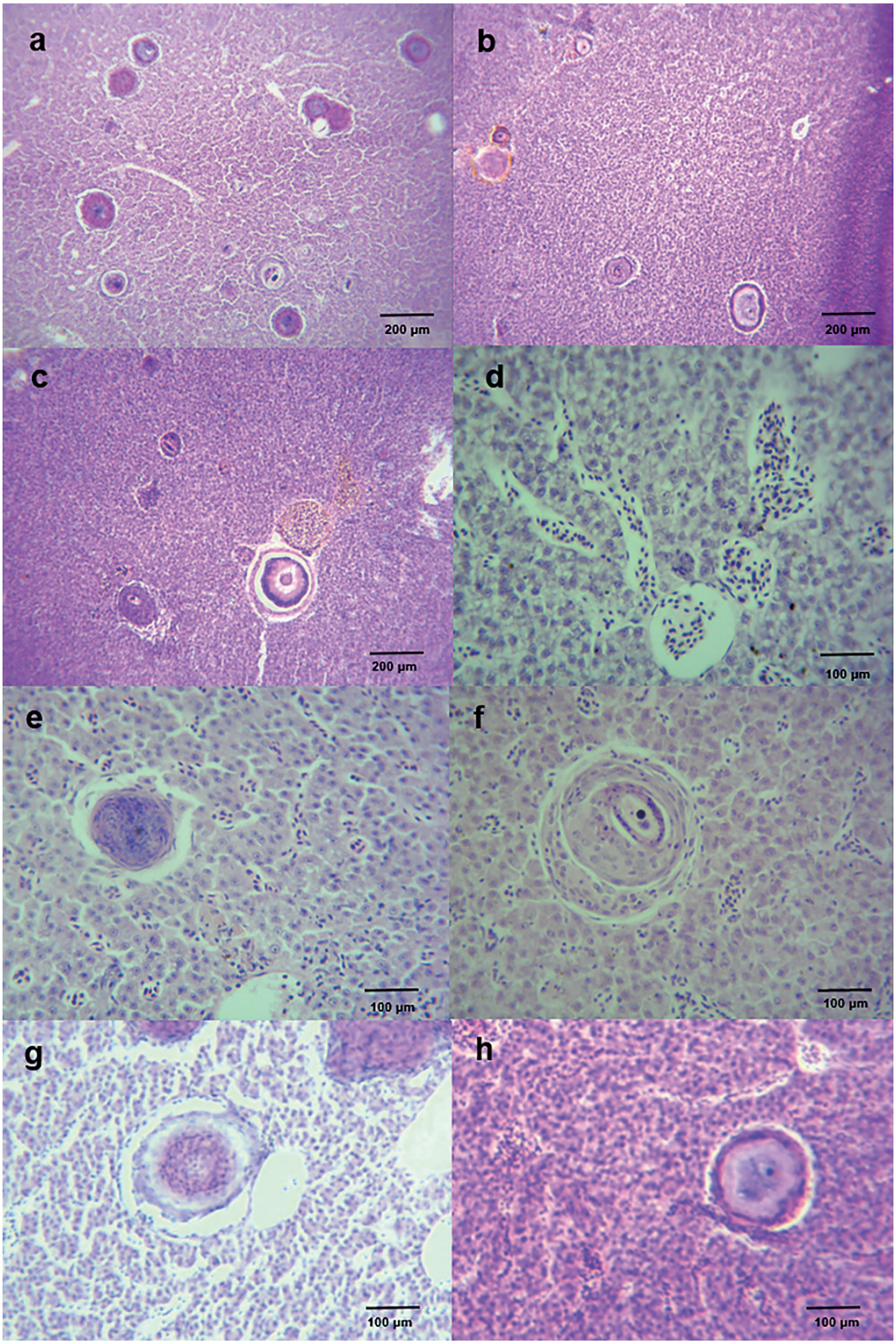

Fig. 1. Histopathological changes in *Barbus meridionalis* liver: (a, b, c) Granulomes of different etiology, (d) Congestion and dilation of the sinusoid, (e, f, h) Granuloma with organized fibrotic tissue, (g) Granulomas characterized by a necrotic "core"

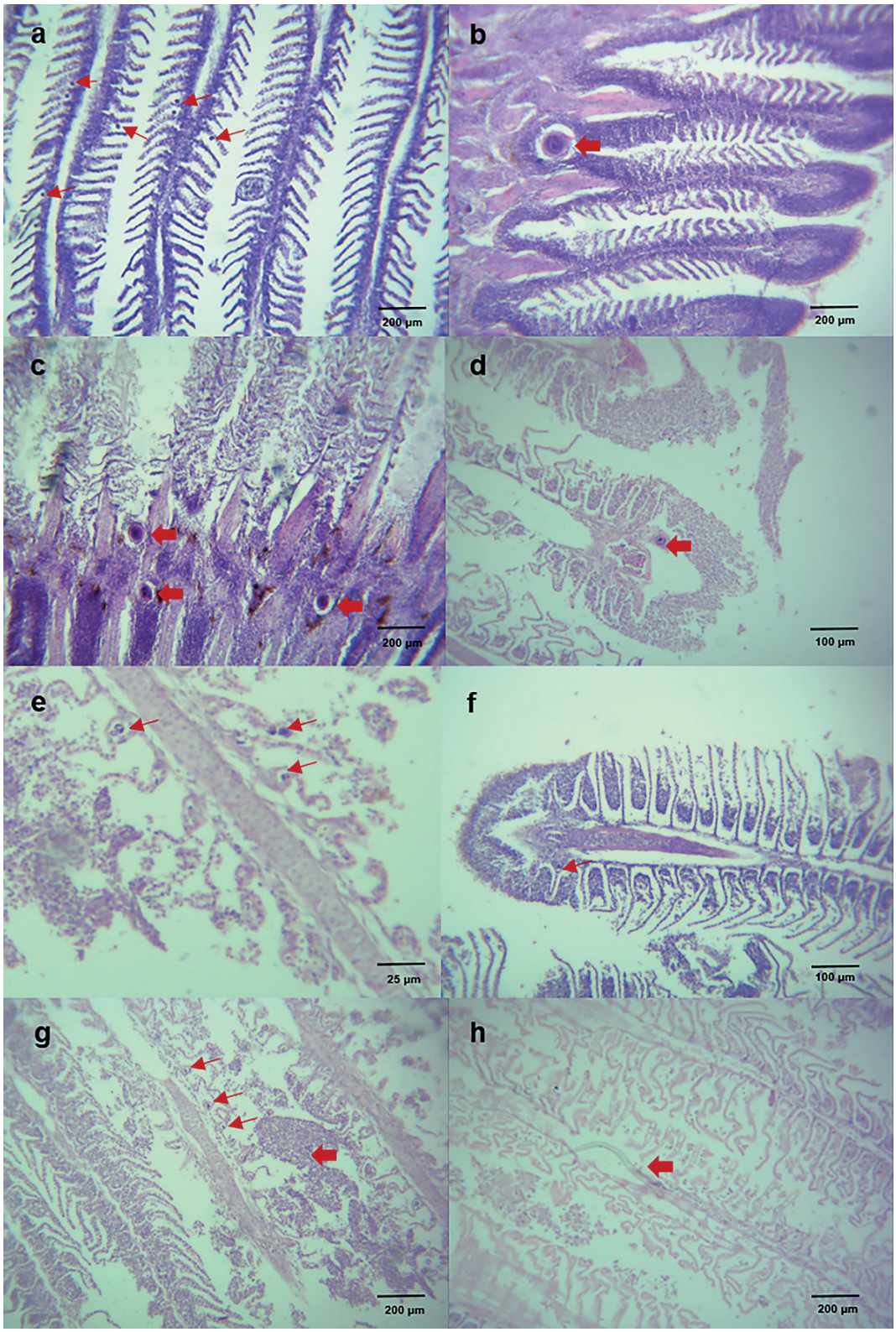

Fig. 2. Histopathological changes in *Barbus meridionalis* gill: (a) Parasitic pathogen along the lamellar epithelium and infiltration of leukocytes into the secondary lamella, (b, c, d, e, f, g) Granuloma of different etiology, (c) Fusion of lamellae, (d) Shortening and degeneration of lamellae, (g) Leukocyte infiltration, (h) Presence of parasite in gill (probably Nematoda)

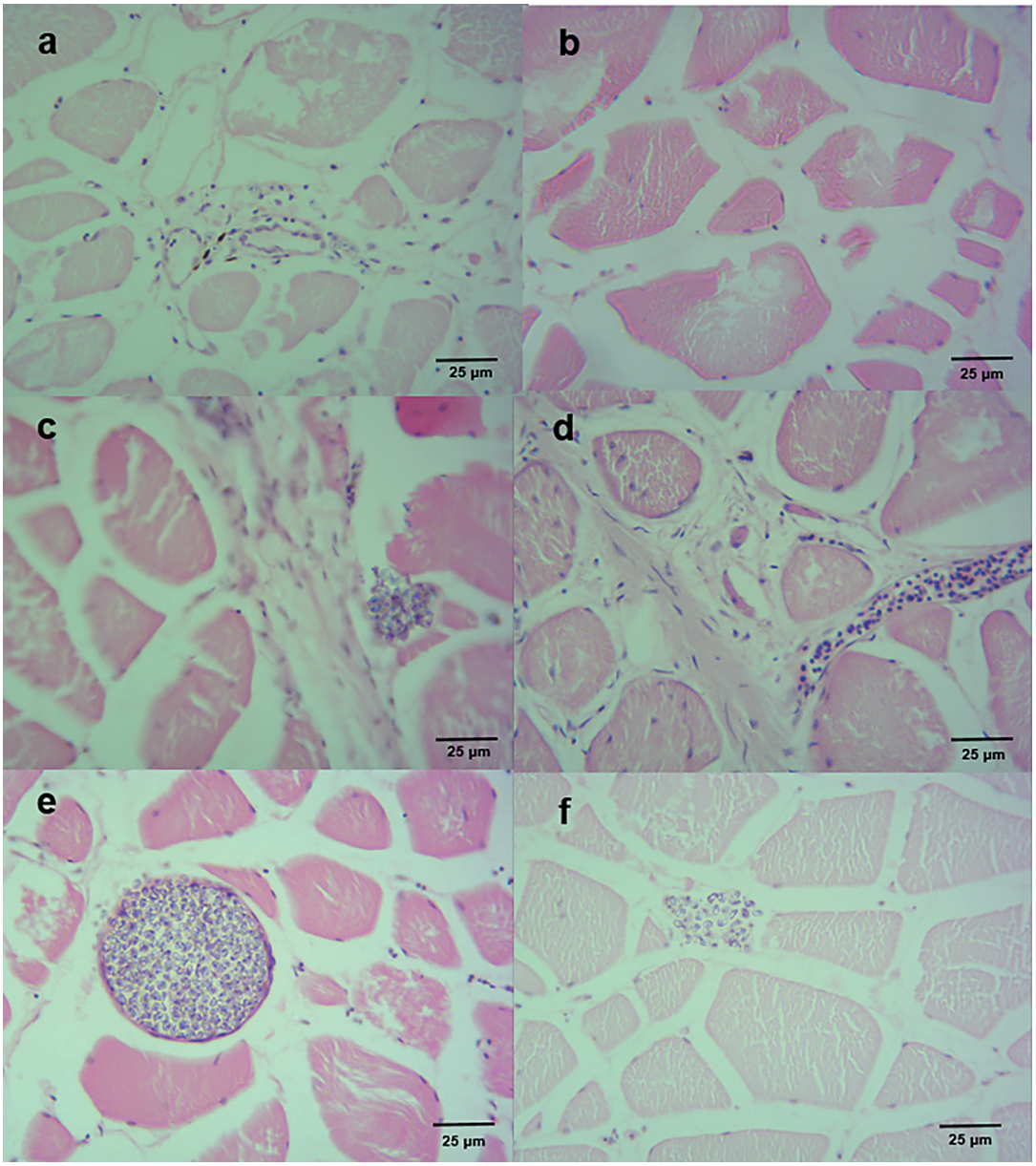

Fig. 3. Histopathological changes in *Barbus meridionalis* muscle tissues: (a, b, c) Cracks within myofibers, fragmentation, and degeneration of myofibers, (d) Congestion and dilation of blood vessels and infiltration of blood cells into the connective tissue (c, e, f) Granulomas located in the cytoplasm of the muscle fibers

downstream reaches. Our data highlight that contamination associated with high concentrations of enteric microorganisms results from runoff from sewage, one of the highest contributors to the overall coliform count. Finding *E. coli*, *Salmonella* spp., *Listeria monocytogenes*, *Clostridium* spp. and *S. aureus* in wild fish is overly concerning. Therefore, an abundance of these pathogens in environmental water should be further explored. Notwithstanding the foregoing, the analysis demonstrated that SS1 water samples showed better microbiological quality than SS2 samples. Importantly *L. monocytogenes* and *Clostridium* spp. were detected only in fish from SS 2. Establishing various pathohistological granulomatous inflammatory responses in the fish investigated in this study is crucial in assessing their health and also provides the opportunity to expand the use of some fish as models for human disease. Furthermore, a serious presence of granulomatous formations of different sizes and compositions was observed in fish samples collected at more polluted sites. Our findings have broad public health implications as fish populations inhabiting highly polluted water provide

precious information on the etiology of pollutant-mediated diseases and seem to be the practicable tool for health risk analysis.

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Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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