ETC-08-413(82)

SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 9999, No. 12, pp. 1–7, 2009

Printed in the USA
DOI: 10.1002/etc.82

Special Section

LIVER, GILLS, AND SKIN HISTOPATHOLOGY AND HEAVY METAL CONTENT OF THE DANUBE STERLET (*ACIPENSER RUTHENUS* LINNAEUS, 1758)

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(*Submitted* 3 *September* 2008; *Returned for Revision* 9 *January* 2009; *Accepted* 20 *March* 2009)

Abstract—The sterlet (*Acipenser ruthenus* L.) is a bottom-feeding fish species with a direct exposure to contaminants from water and sediments. Although heavy metal pollution is believed to be one of the main threats to the sterlet population in the Danube River basin, there is a lack of knowledge of the exact impact of heavy metals on their survival. In the present study, effects of heavy metal pollution on sterlet in the Danube basin were assessed as well as the utility of different sterlet organs and tissues as indicators of heavy metal contamination. The sterlet were sampled at three different sites in the Danube basin, in Hungary and Serbia, isolated from each other by dams. Heavy metal analysis included measurement of Cd, As, Pb, Cr, Hg, Cu, Ni, Fe, Mn, and Zn concentrations in sterlet gills, muscle, liver, and intestine, and histopathological analyses comprised assessment and scoring of the extent and intensity of alterations in skin, gills, and liver tissue. Analysis revealed a significant presence of sublethal histopathological changes that were most pronounced in the liver and skin and increased accumulation of heavy metals, with the highest concentrations in the liver. Canonical discriminant analysis showed significant differentiation among the three studied localities, suggesting that the heavy metal concentrations in sterlet populations were site specific. The present study concludes that the accumulation of heavy metals is a response to the presence of these pollutants in the environment, and, together with other pollutants, it affects the vital organs of natural sterlet populations. Environ. Toxicol. Chem. 2009;9999:1–7. © 2009 SETAC

Keywords—Sterlet Histopathology Heavy metal pollution

INTRODUCTION

Fish are often exposed to highly contaminated water, which leads to different changes, ranging from biochemical alterations in single cells up to changes in the whole population [1]. Measurements of bioaccumulation and biomarker responses in fish from contaminated sites provide information that can contribute to environmental monitoring programs designed for various aspects of environmental risk assessment [2].

The sterlet (*Acipenser ruthenus* L.) is a bottom-feeding sturgeon species. In addition to direct exposure to contaminants from water, it is possible that sterlet are also exposed to contaminants from sediments, because the fish are dependent on invertebrate species for food throughout their life cycle. Therefore, the sterlet could be a good indicator of the quality state of water ecosystems [3].

Pollution is considered to be the main threat to survival of sturgeon species [4–8]. Although water pollution by heavy metals in the Danube is considered to be very high, there are no specific data available on the impact on sturgeons [9]. Compared with reproductive and developmental changes, histological alterations are more sensitive and occur earlier. They provide better evaluation of both the health of the fish and the effects of pollution than any single biochemical parameter [10]. Histopathological changes integrate the impact of a variety of stressors (pathogens, toxic compounds, and unfavorable nutritional and environmental conditions). Moreover, histopathological biomarkers incorporate biotic factors and water quality in a holistic view of fish status, so they are reliable markers of environmental stress [10–13].

The present study was performed to examine the use of bioaccumulation and histopathology as indicators of heavy metal exposure and its effects on the fragile sterlet population. The study was conducted in the Serbian and Hungarian parts of the Danube and Tisza Rivers.

MATERIALS AND METHODS

Sample origin

In total, 39 sterlet individuals were caught by professional fishermen during September and October, 2007, at several different locations (Fig. 1). The sterlet is a migratory species, and maximum migration distance in the Danube River can be more than 300 km [14]. Therefore, sampling sites were defined in such way that they were separated by dams. The first sampling site was located on the Serbian Danube River section upstream from the Djerdap I and II dams (1,319–1,173 km of the river flow). The second sampling site was

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Presented at the 1st International Workshop on Aquatic Toxicology and Biomonitoring, Vodnany, Czech Republic, August 27–29, 2008.

Published online xx Month 2009 in Wiley InterScience (www.interscience.wiley.com).

Fig. 1. Map of the study area, with three sampling locations. UD, upper Danube section; LD, lower Danube section; TR, Tisza River section.

located downstream from Djerdap I and II dams (861 km of the river flow), also in Serbia, whereas the third sampling site was located on the Tisza River in Hungary, between the two dams (402 km of the river flow). In this way, it was certain that there was no mixing among sterlet specimens from different parts of the Danube River Basin and that investigated sterlet populations were completely isolated. Heavy metal concentrations in the Danube River water and sediment at the three sampling sites are presented in Table 1 [15].

Nineteen individuals were caught in the upper Danube section, 10 individuals in the lower Danube section, and 10 individuals in the Hungarian section of the Tisza River. Total mass (g) and total body length (cm) of each individual were measured, and the age was determined from pectoral fin spine sections using the method of Stevenson and Secor [16].

Histopathological analysis

Specimens were sacrificed with a quick blow to the head, and gill, skin, and liver tissue samples were quickly removed, fixed in 4% formaldehyde, and processed using a standard histological technique: dehydration in an ethanol series, embedding in paraffin, and serially sectioning at 5 μm. Sections were stained with hematoxylin and eosin (H/E) [17]. Microphotographs were taken with a Leica microscope with the Leica DC 300 camera.

Frequency of lesions was expressed by dividing the number of organ samples on which a lesion was found by the total number of organ samples analyzed, i.e., total number of fish, because all organs (gills, skin, liver) were sampled and analyzed from each fish caught. For description of histological changes and assessment of the degree of pollution, a method proposed by

Table 1. Heavy metal concentrations in water and sediment in the three studied localities in Hungary and Serbia during August and September 2007 [15][a]

	Upper Danube		Lower Danube		Tisza River	
	W	S	W	S	W	S
Cr	0.001	42	0.002	49	0.003	42
Hg	0.0001	0.62	0.0001	0.33	ND	0.26
Cd	0.0002	0.6	ND	0.6	0.0008	1
As	0.001	6	0.002	7	0.002	8
Pb	0.005	33	0.009	42	0.023	32
Cu	0.017	21	0.105	26	0.035	32
Ni	0.001	40	0.002	46	0.003	18
Fe	1.410	32,720	0.330	33,480	0.380	39,560
Mn	0.060	1,147	0.010	1,359	0.120	2,103
Zn	0.094	62	0.146	65	0.090	87

[a] W = water (μg/ml), S = sediment (μg/g), ND = value below detection.

Bernet et al. [1] was used. According to this method, pathological changes are classified into five reaction patterns, namely, circulatory, regressive, progressive, inflammatory, and neoplastic. An importance factor ranging from 1 (minimal alteration) to 3 (marked importance) is assigned to each alteration, pointing out the relevance of a lesion and its pathological importance. Depending on the degree and extent of lesions, a score value ranging from 0 (unchanged) to 6 (severe occurrence) is determined. By using the importance factor and score value, an organ index of each organ is determined. The sum of the indices for every organ examined results in a total index for the individual fish.

Heavy metal analysis

Sterlet specimens were dissected, and samples of liver, gills, intestine, and muscle were quickly removed, washed with distilled water, and stored at −20°C prior to analysis. Heavy metal analysis included assessment of Cd, Pb, Hg, Fe, Zn, Mn, Ni, Cu, Cr, and As concentrations. Tissues were measured for their weight and then digested with HNO_3, HF, and H_2O_2 (Merck; high purity), according to U.S. Environmental Protection Agency Method 3052 [18], with the microwave digestion system model MDS2100 (CEM Corporation). Deionized water with a resistance of 18 Ω was used throughout the process. Calibration standards were prepared from multielement standard stock solutions (Atomic Absorption Standard Solutions Plus, Tested vs. National Institute for Standards and Technology Standard Reference Materials). The concentrations of Fe, Mn, Cu, and Zn in fish tissues were determined by atomic absorption spectroscopy—flame (PerkinElmer model 3100); the concentrations of Cd, Pb, Ni, and Cr were determined by atomic absorption spectroscopy—graphite furnace (PerkinElmer model 4100zl); and As and Hg were determined by atomic absorption spectroscopy—hydride system (PerkinElmer model 3100/MHS-10). Heavy metal concentrations were expressed as μg^{-1} frozen weight.

Statistical analysis

Statistical analysis included comparisons of heavy metal concentrations and histopathological changes among different localities within the same tissue as well as among different tissues within the same locality. All variable distributions were evaluated by using the Kolmogorov–Smirnov test for normality. Because the variables lacked normality of distribution, nonparametric tests were applied. Initial assessment of the differences among groups, performed with the Kruskal–Wallis H test, was followed by comparison of particular pairs of samples by Mann–Whitney *U* test.

To assess the differentiation of the three localities based on the heavy metal accumulation and the intensity of histopathological changes, localities were also compared by means of the canonical discriminant analysis. Untreated data for heavy metal concentrations and intensity of histopathological changes in each tissue were used as the input variables.

RESULTS

According to age analysis, specimens belonged to 0, 1, and 2+ year classes. Average body length and mass of individuals and average values of heavy metal concentrations are presented in Table 2. The extent of histopathological changes in different tissues in each of the three localities is presented in Figure 2.

Histological analysis

Gills. Most of the examined gills had normal histological structure, without major pathological alterations. Dominant change in the gills (92.5% of gill samples analyzed) was distended tips of the secondary lamellae, a focal hyperplasia of the respiratory epithelium. Respiratory epithelium hypertrophy, found in 47.5% of the samples, was more pronounced on the distal parts of the primary lamellae and decreased toward proximal parts of the primary lamella. Circulatory changes were found as well: a hyperemia of the secondary lamellae (27.5% of samples) and stases (15% of samples examined). Other changes recorded (frequency of appearance up to 2.5%) were lifting of

Table 2. Total body length (L_t) and weight (M) and heavy metal concentrations in different tissues of sterlet in the three studied localities in Hungary and Serbia (values ± SD)[a]

		Upper Danube section	Lower Danube section	Tisza River
	L_t (cm)	32.19 ± 2.10	44.53 ± 2.62	45.70 ± 2.65
	M (g)	122.32 ± 29.66	273.80 ± 47.18	372.90 ± 55.39
G	Cr	ND	ND	ND
	Hg	0.006[b]	ND	ND
	Cd	0.007 ± 0.012	0.004 ± 0.002	0.012 ± 0.018
	As	0.174 ± 0.208	0.144 ± 0.117	0.177 ± 0.163
	Pb	0.014 ± 0.018	0.030 ± 0.062	0.016 ± 0.012
	Cu	ND	ND	ND
	Ni	0.051 ± 0.092	0.121 ± 0.124	0.079 ± 0.084
	Fe	61.006 ± 18.094	52.590 ± 20.545	62.014 ± 21.955
	Mn	4.047 ± 4.033	3.093 ± 1.395	4.970* ± 1.767
	Zn	15.520 ± 7.301	11.261* ± 2.239	13.473 ± 2.568
M	Cr	ND	ND	ND
	Hg	ND	ND	ND
	Cd	0.003 ± 0.002	0.004 ± 0.005	0.002 ± 0.002
	As	0.136 ± 0.112	0.133 ± 0.131	0.297 ± 0.609
	Pb	0.013 ± 0.022	0.008 ± 0.004	0.008 ± 0.007
	Cu	ND	ND	ND
	Ni	0.125 ± 0.136	0.074 ± 0.087	0.061 ± 0.071
	Fe	13.101 ± 8.641	11.390 ± 8.554	11.837 ± 6.824
	Mn	3.128 ± 2.717	3.277 ± 3.655	3.635 ± 1.246
	Zn	6.924 ± 2.397	8.353 ± 7.531	6.798 ± 0.677
L	Cr	ND	ND	ND
	Hg	0.0002, 0.002[c]	0.0002	ND
	Cd	0.021 ± 0.039	0.233 ± 0.422	0.126 ± 0.345
	As	0.198* ± 0.120	0.086* ± 0.097	0.152 ± 0.086
	Pb	0.060* ± 0.207	0.045 ± 0.049	0.017 ± 0.011
	Cu	23.492 ± 12.460	41.708* ± 24.720	23.047 ± 8.300
	Ni	0.266 ± 0.381	0.277 ± 0.224	0.230 ± 0.202
	Fe	65.043* ± 24.777	353.464* ± 183.825	141.548* ± 75.887
	Mn	2.980 ± 1.596	2.948 ± 0.739	4.324* ± 1.178
	Zn	24.365* ± 3.809	35.586* ± 7.552	28.707* ± 3.900
I	Cr	ND	ND	ND
	Hg	ND	ND	ND
	Cd	0.012 ± 0.029	0.004* ± 0.007	0.006 ± 0.005
	As	0.207* ± 0.123	0.115 ± 0.084	0.093 ± 0.097
	Pb	0.012 ± 0.015	0.008 ± 0.005	0.007 ± 0.005
	Cu	1.575 ± 2.244	0.811 ± 0.986	1.141 ± 1.497
	Ni	0.155 ± 0.180	0.260 ± 0.322	0.133 ± 0.176
	Fe	35.282 ± 59.542	37.817 ± 20.512	16.109* ± 7.570
	Mn	3.785 ± 3.288	2.916 ± 1.811	2.350 ± 1.098
	Zn	17.390 ± 3.876	19.965 ± 5.119	30.145* ± 7.403

[a] Concentrations are expressed as μg/g frozen weight; G = gills; M = muscle; L = liver; I = intestine; ND = value below detection threshold.
[b] Mercury concentrations above detection threshold only in one sample.
[c] Hg concentrations above detection threshold only in two samples.
* Significant differences between the sites (Mann–Whitney *U* test, $p < 0.05$).

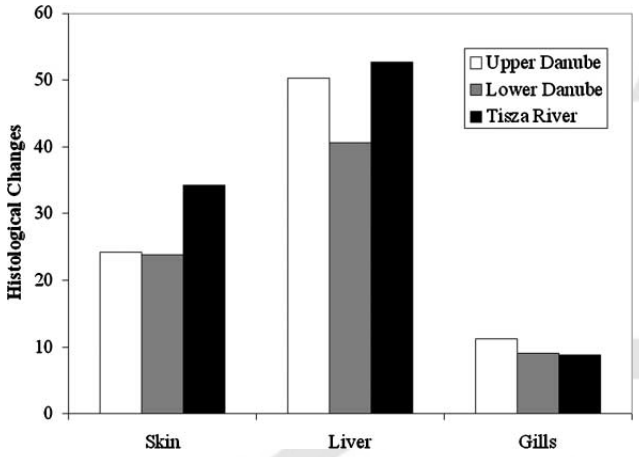

Fig. 2. Average extent of histopathological changes in different tissues of sterlet in the three studied localities. The *y* axis represents score values according to the scoring protocol of Bernet et al. [1].

the respiratory epithelium, fusion of several secondary lamellae, and fusion of the tips of primary lamellae. Parasites found were *Trichodina* (two samples) and Trematodes (one sample).

Skin. Samples of the examined sterlet skin have shown changes only at the level of the epidermis, without major changes in the dermis and hypodermis. Picnotic nuclei in the matrix layer of the epidermis, found in 25% of skin samples, were the most severe lesion recorded. A common feature was erosion with desquamation of epithelium, and rupture (excoriation) of parts of epidermis (frequency of 65% of the skin samples analyzed). The major skin change revealed was a hyperplasia of the epidermal cells (52.5%), including hyperplasia of mucous cells (47.5%). Some mucous cells were emptied, whereas other samples revealed a lack of this cell type, indicating an overproduction of mucous that has exhausted the capacity of the epidermis to divide and differentiate into mucous cells. In addition, a leucocytes infiltration in the epidermis was found (35% of skin samples), indicating a possible inflammatory reaction, especially in parts with excoriated epidermis where lack and/or erosion of the epidermis induced a defense mechanism.

Liver. The change most frequently found was fibrosis (77.5% of examined fish), focal leukocyte infiltration (52.5%), congestion of sinusoids (40%), and vasodilatation (22.5%). Hepatocyte cloudy swelling (47.5 frequency) and melanomacrophage centers (30%) were recorded as well. Other, more severe changes were signs of cirrhosis (20% of samples) and necroses (focal necroses recorded in 50% of samples analyzed). Finally, some signs of regeneration were noted only for samples from Serbia (7.5% of the total number analyzed). Examples of typical lesions found in the present study are shown in Figure 3.

Both histological analysis with frequencies of lesion appearance and scoring of changes according to the protocol proposed by Bernet et al. [1] have revealed that the histopathological changes differed significantly among tissues ($p < 0.05$) and were the most pronounced in liver from all three localities, followed by the changes in skin and gills (Fig. 2). Histopathological changes in the liver and skin differed significantly among localities ($p < 0.05$), with the highest intensity in the Tisza River and the lowest in the downstream Danube section. The changes in gills did not differ significantly among localities (Fig. 2).

Heavy metal analysis

Heavy metal analysis showed that Cr was below detection thresholds in all analyzed samples, and Hg was above detection thresholds only in four samples: in three livers (two from the lower Danube section and one from the upper Danube section), with concentrations ranging from 0.0002 to 0.002 μg/g frozen weight, and in one sample of gills (upper Danube section), with 0.006 μg/g frozen weight.

Comparison of extent of heavy metal accumulation in different tissues showed that Cu, Fe, Zn, Ni, and Pb had the highest concentrations in liver (with only Zn in the Tisza River having higher concentrations in intestine than in liver). This was statistically significant in most of the localities ($p < 0.05$). Intestine was the tissue with the second highest intensity of Cu, Zn, and Ni accumulation, followed by the gills and muscle, with the lowest concentrations. Iron and Pb accumulation was, after the liver, most pronounced in gills and least pronounced in intestine and muscle. In comparison with the case in other tissues, Cd reached high concentrations in liver, but the differences were not statistically significant because of a relatively high variability of individual observations. However, Cd revealed the lowest concentrations in the muscle ($p < 0.05$). In the samples from the Tisza River and the upper Danube sections, Mn showed the highest concentrations in gills, whereas in the lower Danube section it was in the muscle, but differences were not significant. Differences in the accumulation of As among different tissues also were not significant.

Comparison of the three localities suggested that they differed mostly in concentrations of heavy metals in liver; Fe, Zn, and Cu had significantly highest concentrations ($p < 0.05$) in liver in the lower Danube section and the lowest in the upper Danube section. Cadmium also revealed higher concentrations in liver in the lower Danube section, but differences were not significant. On the other hand, Pb and As in liver showed significantly highest concentrations in the upper Danube section.

Manganese in both the liver and gills had significantly highest concentrations in the Tisza River, whereas Zn in gills had significantly lowest concentrations in the lower Danube section. With regard to the heavy metal accumulation in intestine, As had significantly highest concentrations in the upper Danube section and Zn in the Tisza River, whereas Fe had the lowest concentrations in the Tisza River. Differences in accumulation of Ni among different localities were not significant.

Canonical discriminant analysis showed a high degree of differentiation among the three studied localities (Fig. 4). Two canonical functions (CV) together accounted for 100% of the total heterogeneity (CV1 74.9% and CV2 25.1%). The upper and lower Danube sections were each separated from the other two localities along the first canonical function, and the Tisza River section was separated from the two Danube River sections along the second canonical function. The upper Danube section was differentiated mostly by high concentrations of As in liver and intestine, whereas the lower Danube section was differentiated by high concentrations of Fe, Zn, Cu, and Cd in liver as well as by the low intensity of histopathological changes in the

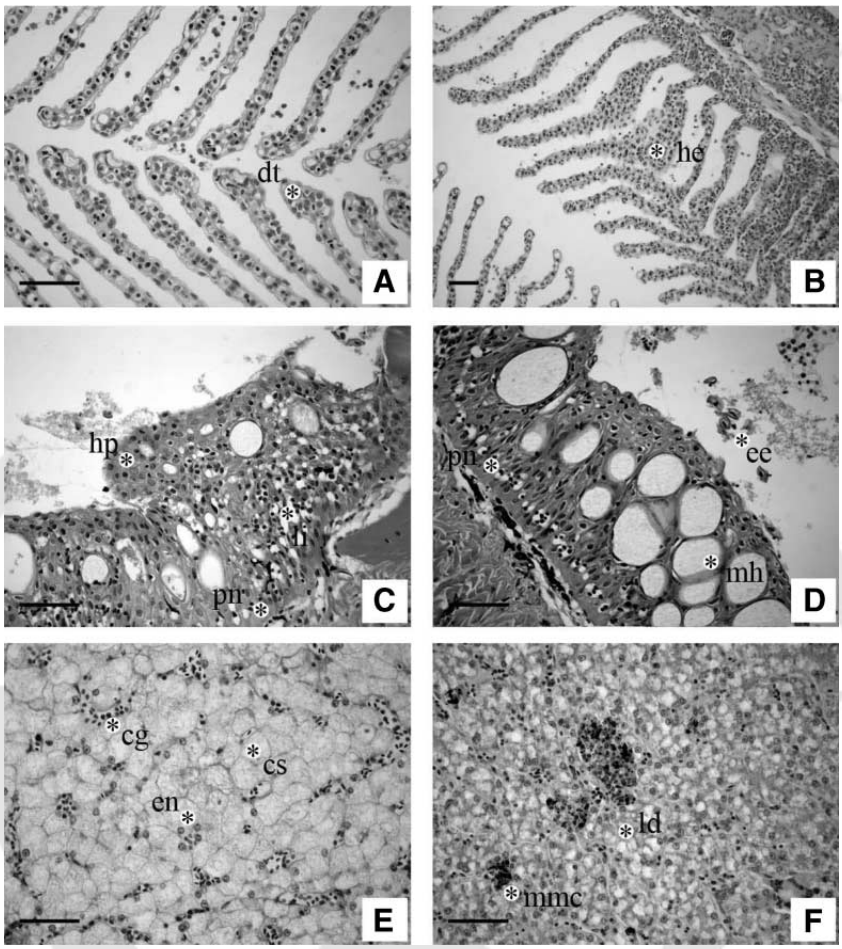

Fig. 3. Examples of histopathological alterations in different sterlet tissues found in the study. (**A**) Gills, Tisza River section (H/E × 40). (**B**) Gills, Tisza River section (H/E × 20). (**C**) Skin, upper Danube section (H/E × 40). (**D**) Skin, upper Danube section (H/E × 40). (**E**) Liver, upper Danube section (H/E × 40). (**F**) Liver, Tisza River section (H/E × 40). dt = Distended tips of secondary lamellae; he = hyperemia; hp = hyperplasia in skin epidermis; li = leukocyte infiltration; pn = picnotic nuclei; ee = excoriated epidermis; mh = mucous cell hyperplasia; en = excentric nuclei; cs = cloudy swelling; cg = congestion of sinusoids; ld = lipid degeneration; mmc = melanomacrophage center. Scale bar = 50 μm.

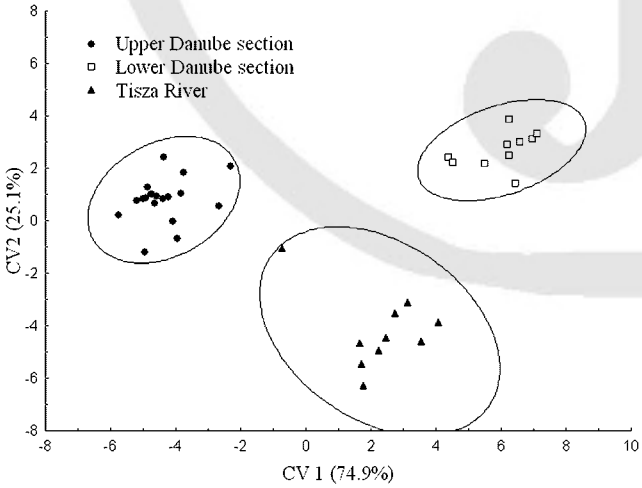

Fig. 4. Results of canonical discriminant analysis applied to the three studied localities (ellipses show 95% prediction intervals). The input variables are the heavy metal concentrations and intensity of histopathological changes in each sterlet tissue.

liver. The Tisza River section was differentiated by a high intensity of histopathological changes in liver and skin as well as by high concentrations of Zn in intestine and Mn in the liver.

DISCUSSION

Fish are exposed to heavy metals both directly from water and sediment and indirectly through the food chain, especially Cu, Cd, and Zn [19]. Heavy metal bioaccumulation affects the structure and function of fish vital organs. In the present study, to compare heavy metal concentrations with the degree of histopathological alterations of Danube sterlet liver, skin, and gills, changes were scored and compared with the level of heavy metals found in caught specimens' vital organs.

Gills of examined sterlet were not heavily changed. Hypertrophy of respiratory epithelial cells, the second most frequently encountered alteration in the present study, is a mild and reparable change, regularly found when heavy metal concentrations in the aquatic environment are increased [20]. Both focal hyperplasia and lamellar hyperemia are reparable alterations. All mentioned gill changes, because they were mostly moderate, could indicate an acceptable water quality. However,

it must be taken into consideration that histopathological changes of gills and other vital organs are not irritant specific, particularly in natural waters, where both mixed pollution and varying environmental conditions could affect fish tissues and organ systems.

Skin changes recorded in the present study were restricted to the epidermis, and there were no deeper changes in the dermis and hypodermis. Picnosis in the epidermal matrix indicates toxic conditions with no possibility of repair [20]. An increased mucous production is a first defense mechanism. It is often followed by lack of production manifested by emptied, exhausted mucous cells, and finally complete lack of this cell type, showing continual degradation of the environmental conditions that lead to chronic changes. Excoriation of large parts of or the whole epidermis could depend on the sampling technique.

The majority of liver changes that were associated with fibrosis, such as inflammation, vascular alteration, and cloudy swelling/vacuolization, indicated a chronic degradation of environmental conditions and the resulting typical defense mechanisms. Fibrosis is a metaplastic change. It is a repair response that represents a substitution of a highly differentiated tissue, such as hepatocytes, by connective tissue, which is much less vulnerable [21]. Melanomacrophage centers appear when fish store lipids in the liver but can also occur in toxic conditions or with vitamin deficiency [20]. For example, Kruse and Scarnecchia [22] have found sturgeon liver samples with increased levels of melanin, which may be associated with increasing melanomacrophage activity induced by toxic agents. Moreover, both drastic portobiliary cirrhosis, i.e., a hepatorenal syndrome as described by Roberts [20], and melanomacrophage centers could point to increased heavy metal concentrations that, together with chronic levels of other pollutants, could lead to severe liver alterations. In the present study, heavy metal concentrations in different tissues revealed that Cu, Fe, Zn, Ni, and Pb had the highest, statistically significant ($p < 0.05$), concentrations in the liver. The liver tissue is highly active in the uptake and storage of heavy metals. In the present study, the heavy metal concentrations were lower in muscle compared with other tissues, as reported by Bevoets and Blust [23] for gudgeon in metal-polluted lowland rivers in Flanders. Concentrations of Zn, Fe, and Cu were highest in livers of sterlet caught in the Danube River downstream from the Djerdap II dam. This could be explained as a result of pollution in the lower Danube River section, coming from copper and gold mining (city of Bor in Serbia). Wastewater from this mine, which contains increased concentrations of Cu, Fe, Pb, Zn, and Cd, affects the Kriveljska River, Timok River, and the Danube River as the final recipient [24]. The Tisza River was contaminated with numerous industrial accidents from the Carpathian mountain region in Romania, which has a long tradition of mining, especially of Au, Ag, Pb, Zn, Cu, and Mn [25].

Concentrations of some heavy metals, such as Mn and Zn, were in relative accordance regarding their levels in sterlet tissues and in the water and sediment. However, the sterlet is a highly migrating species, which resides for a longer time only on certain sites where it can find enough food, so a better connection between the metal uptake and the site-specific concentrations could thus be obtained only through the use of telemetry monitoring.

In an attempt to connect the intensity/extent of alterations to toxicants concentration in fish tissues, different scoring methods have recently been used [13,26,27]. In the present study, histopathological changes were the most pronounced in liver, followed by the changes in skin and finally in the gills, in all three localities. The changes in liver and skin were the most evident in the Tisza, and the least evident in the lower Danube section. Changes in gills did not differ significantly among localities (Fig. 2).

The present study confirms the value of histopathology in assessing effects of heavy metals in the aquatic environment. As suggested by Van der Oost et al. [2], in addition to simple chemical data, the use of different biomarkers, histology being one of them, could contribute to environmental risk assessment.

Results of canonical discriminant analysis showed that concentrations of As, Fe Zn, Cu, and Mn and the extent of histopathological changes differed significantly among localities and that heavy metal concentrations in sterlet populations were site specific. Accumulation of heavy metals in each of the three assessed populations can be probably attributed to a response to presence of these pollutants in the environment. The increased level of heavy metals recorded in the present study, together with other pollutants probably present in sublethal concentrations, affected vital organs of natural sterlet populations.

Acknowledgement—The present study was supported by project 05SER03/03/007 financed by European Agency for Reconstruction, project 143045 funded by Ministry of Science of Republic Serbia, and project 205135 EU FP7 REGPOT-3. The authors thank Karoly Gyore for the material collected from the Tisza River in Hungary, as well as the editor and two anonymous referees for providing helpful comments and suggestions.

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