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Original article

Metal bioaccumulation in common carp and rudd from the Topolnitsa reservoir, Bulgaria

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Concentrations of arsenic (As), cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb), and zinc (Zn) were determined in water samples and five fish organs (gills, liver, kidney, spleen, and muscle) of common carp (*Cyprinus carpio* L.) and common rudd (*Scardinius erythrophthalmus* L.) from the Topolnitsa reservoir (Bulgaria) in three seasons (spring, summer, and autumn). This water ecosystem is located in a copper mining and metallurgical region. Water metal concentrations were significantly higher in the summer than in the spring ($p<0.05$). Moreover, As, Cd, Cu, and Zn concentrations were higher than the national limits. Qualitative factors "element" and "fish organ" had a stronger influence on metal bioaccumulation than the factors "season" and "fish species". In fish, the highest metal levels were detected in the liver, spleen, kidney and gills, and the lowest in the dorsal muscle. Tissue levels were higher in the summer, but in general they were similar between the two Cyprinid fish. Fish muscles had the lowest metal levels at all times, but As and Pb exceeded the national and international standards. Therefore, we would not recommend fish consumption from Topolnitsa, as continuous metal contamination of the reservoir may seem to present human health risk.

KEY WORDS: *bioaccumulation; freshwater fish; ICP-MS; organs*

Contamination of aquatic ecosystems with metals has received increasing attention worldwide (1, 2). Metal contaminants are of particular concern due to high toxicity, persistence, and ability to accumulate in the food chain and aquatic ecosystems (3, 4). Metals enter the aquatic environment through atmospheric deposition and erosion of the geological matrix. Anthropogenic sources include fuel combustion, industrial effluents, wastewater from smelting, metallurgical or mining enterprises, and leaching from waste rocks and mine tailings (5, 6). After entering the water, metals may precipitate or adsorb on the surface of solids, remain soluble or suspended in it, or may be taken up by flora and fauna (7).

Fish are a major protein source for humans, fish muscle in particular, and health risks of food poisoning with metals have become a major concern in recent decades (8-10). Fish may also serve as reliable indicators of metal contamination because they are more sensitive to changes in the aquatic environment than invertebrates and tend to accumulate metals in concentrations several times higher than that of the ambient medium (11, 12). Fish are exposed to metals through water and food (13). Metal bioaccumulation depends on many factors such as fish species, age, tissue, season, pH, water temperature, and hardness (14). In teleosts, the most common tissues used in toxicology studies are the gills, liver, kidney, and muscles (15, 16). Metal levels in gills reflect metal concentrations in the surrounding water; liver is an organ for storage and detoxification of metals, whereas kidney is involved in the process of excretion (17).

Spleen in fish is a haematopoietic organ with important functions. However, it has not been investigated in bioaccumulation studies as thoroughly as the liver and kidney (18, 19).

Topolnitsa (Bulgaria, 42° 25' 90" N 23° 59' 38" E) is a reservoir built on the Topolnitsa River which runs through a region rich with copper mines, mine tailings, and metallurgy plants. In addition, the reservoir serves as a final sink for all types of contaminants which are carried with the river and its tributaries. Even though these circumstances call for a full investigation and monitoring, no data have been published over the last few decades on metal levels and their effects on fish from this artificial lake. Gecheva et al. (20) recently provided information on metal levels in water and sediment samples along the Topolnista River basin. However, their results cannot be used as reference, as we investigate still water and their study a flowing water.

The aim of our study was to address all of the above issues: 1) to establish current water pollution data on As, Cd, Cu, Ni, Pb, and Zn concentrations of the Topolnitsa reservoir and see if there are seasonal differences; 2) to compare metal bioaccumulation in common carp and common rudd gills, liver, kidney, spleen, and muscle and to see how metal pollution reflects on organs with different structure and functions; and 3) to compare metal levels in the fish muscle with national and international standards for safe consumption.

MATERIALS AND METHODS

Sampling area

The Topolnitsa reservoir (Figure 1) is located near the village of Muhovo, south-west Bulgaria (63 km from the capital Sofia, 444 m above the sea level). The reservoir was built in 1961 and is one of the largest artificial lakes in the country with a total volume of 140 million m^3 of water. All samples were collected near the dam wall (86 m high, 338 m long) in the spring, summer, and autumn of 2012.

Water sampling and analysis

Surface water samples for metal analysis were collected in triplicates in prewashed, double-caped polyethylene bottles following the procedures described by the ISO standard 5667-4 (21). Samples

Figure 1 *The location of the Topolnitsa reservoir in Bulgaria*

were acidified with 1 % HNO_3 and stored on ice for as short time as possible to minimise changes in metal physicochemical properties before analysis. We also recorded water pH, temperature (°C), dissolved oxygen, and conductivity ($\mu S\ cm^{-1}$).

For reference we used water from the ponds in which the Institute of Fisheries and Aquaculture in Plovdiv rears fish under strict toxicant-free conditions.

Water was analysed for metals according to the ISO standard 17294-2 (22) with an Agilent 7500ce (Agilent Technologies, Tokyo, Japan) inductively coupled plasma mass spectrometer (ICP-MS), and the findings are reported as $\mu g\ L^{-1}$. The detection limit of the instrument was 0.5 $\mu g\ L^{-1}$ for As, 0.05 $\mu g\ L^{-1}$ for Cd, 0.5 $\mu g\ L^{-1}$ for Cu, 0.5 $\mu g\ L^{-1}$ for Ni, 10 $\mu g\ L^{-1}$ for Pb, and 10 $\mu g\ L^{-1}$ for Zn.

Fish sampling and analysis

Common carp (*Cyprinus carpio*, L.) is spread and farmed all over Bulgaria and Europe and has been used as a test organism in many toxicological assays (23-25) because of its relative resilience even to heavy pollution (26, 27). Common rudd (*Scardinius erythrophtalamus*, L.) is a Cyprinid species of many European rivers and lakes, fished recreationally. Even though metal levels have been reported in common rudd organs, mainly in the gills, liver, and muscle (28, 29), toxicological data on common rudd are quite scarce compared to other Cyprinids.

In parallel with water sampling, ten fish of each species were caught with fishing nets in each season, which totalled 60 fish. All samples were collected following the EMERGE Fish Sampling Manual for Live Fish (30). Prior to dissection, fish length and weight were measured to the nearest millimetre and gram (mean common carp and rudd weight was

101.1±4 g and 55.2±13.2 g and length 15.5±0.7 cm and 15.4±1.2 cm, respectively). Dissection was performed in a provisional field laboratory with clean stainless steel scalpel blades, scissors, and tweezers. We first dissected the second gill arch on the right side and then carefully removed the liver, kidney, and spleen. Finally, we took a small piece of the dorsal muscle. All samples were placed in pre-marked clean polyethylene zip-lock bags and kept on ice until we froze them in our laboratory at -25 °C for analysis.

For reference we obtained from the national Institute of Fisheries and Aquaculture in Plovdiv five healthy fish of each species for each of the three seasons, totalling 15 carps (mean weight 120±1.2 g; mean length 14.9±1.9 cm) and 15 rudds (mean weight 51±13.2 g; mean length 13±1.5 cm).

All experiments were conducted in accordance with the Directive 2010/63/EU on the protection of animals used for scientific purposes (31).

Metal bioaccumulation was analysed in the gills, liver, kidney, spleen, and dorsal muscle of the two fish species. Approximately 1 g of each tissue sample was wet mineralised using a microwave digestion system (Milestone Ethos Plus, Italy) at 200 °C. Digestion solution was prepared with 6 mL of 65 % HNO_3 and 2 mL of 30 % H_2O_2. After mineralisation, samples were diluted to 25 mL by adding ultra-pure water and analysed for metal content using an Agilent 7500ce ICP-MS. Data are reported as μg kg^{-1} wet weight. The detection limit of the instrument was: 10 μg kg^{-1} for As, 1 μg kg^{-1} for Cd, 10 μg kg^{-1} for Cu, 10 μg kg^{-1} for Ni, 30 μg kg^{-1} for Pb, and 30 μg kg^{-1} for Zn.

All analyses were carried out at the regional laboratory of the Executive Environment Agency in Plovdiv, Bulgaria. Reagents were purchased from the Merck Group (Darmstadt, Germany) and were of analytical and Suprapur® quality. Glassware was washed with non-ionic detergent, treated with a solution of 10 % HNO_3 for 48 h, and rinsed thoroughly with deionised water before use to minimise contamination.

Method validation

Analysis included samples in triplicates and batches with blanks. Accuracy was validated using standard reference materials for trace elements in water SRM 1643e (National Institute of Standards and Technology, Gaithersburg, MD, USA) and fish protein DORM-3 (National Research Council Canada, Ottawa, ON, Canada). All results showed a good agreement with the standards, and recovery ranged between 96 % and 105 % for fish and 92 % and 101 % for water.

Statistical analysis

Raw data on metal concentrations in water and fish samples were distributed normally and analysed using STATISTICA version 7.0 for Windows (StatSoft Inc., New York, NY, USA). Differences between the variables were tested for significance using Student's *t*-test ($p<0.05$). Relationships between metal concentrations in water and in fish tissues were tested using Pearson's product-moment correlation, principal components analysis (PCA) ($p<0.05$). Data are reported as mean±SD.

RESULTS AND DISCUSSION

Metal concentrations in water

Table 1 shows general Topolnitsa water quality parameters, which meet the Basin Directorate for Water Management – East Aegean Region (Bulgaria) requirements for healthy aquatic environment for fish (unpublished data).

Table 2 shows metal concentrations in water samples taken from Topolnitsa. In reference water all

Table 1 *Topolnitsa reservoir water quality parameters*

Season	pH	T (°C)	Conductivity (μS cm^{-1})	Dissolved oxygen (mg L^{-1})
Spring	8.3	5.1	330	8.3
Summer	8	19.4	620	7
Autumn	6.1	4.2	410	9
Mean±SD	8.1±0.2	9.6±8.5	453.3±150	8.1±1

Table 2 *Metal concentrations (mean±SD) in the Topolnitsa reservoir water (n=3 in each season)*

Element	Metal concentrations (µg L^{-1})			
	Spring	Summer	Autumn	Bulgarian limits
As	**200±10***	4±0.1*	10±1*	10
Cd	**0.6±0.1**	0.4±0.1	-*	0.5
Cu	**20±1**	**20±1**	**10±5**	1
Ni	3±1	-	-	20
Pb	-	-	-	7
Zn	8±0.001**	**200±0.01****	*	8

below the detection limit: 0.5 µg L^{-1} for As, 0.05 µg L^{-1} for Cd, 0.5 µg L^{-1} for Cu, 0.5 µg L^{-1} for Ni, 10 µg L^{-1} for Pb, and 10 µg L^{-1} for Zn
**significantly different between the seasons (p<0.05)*
Bold – above the Bulgarian limit

metal concentrations were below the detection limit of the instrument. Lead concentrations in the Topolnitsa water also kept below the detection limit in all three seasons, whereas other metal concentrations varied significantly between the spring and summer (p<0.05). Cadmium concentrations dropped below the detection limit in the autumn, Ni concentrations in the summer and autumn, and Zn concentrations in the autumn. In contrast, As and Cu were measurable in all three seasons, which may indicate chronic exposure of the biota to these toxicants. Spring As and Cd, and summer Zn concentrations exceeded the maximum permissible values set by the Bulgarian regulations (32, 33) based on the Directive 2000/60/EC (34). Autumn As and spring Zn concentrations were borderline. Furthermore, Cu concentrations were above the permissible levels in all three seasons, probably due to intensive copper mining in the region. However, concentrations of the other metals are more likely related to background levels and complex interactions between water, sediment, and biota, or may be caused by anthropogenic factors other than mining (domestic sewage, industrial waste waters and agricultural runoff).

Metal bioaccumulation in fish

We wanted to see which of the selected qualitative factors, namely "element", "fish organ", and "season" had stronger influence on metal bioaccumulation in either of the Cyprinid fish. Our PCA analysis (Figures 2 and 3) determined that "element" had more influence (common carp − 71.73 %; common rudd − 64.05 %) than "season" (common carp − 20.23 %; common

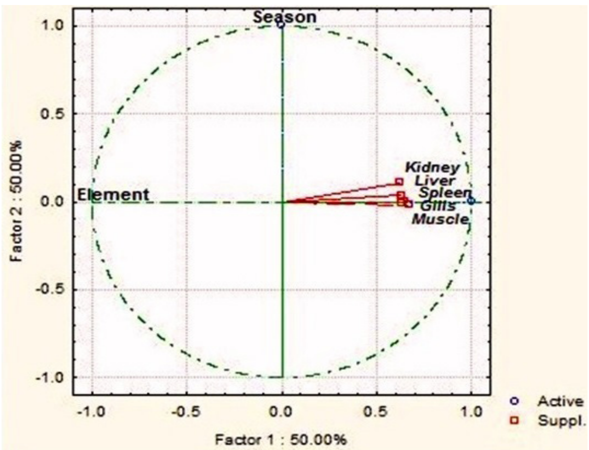

Figure 2 *Relationship between qualitative factors "element" and "season" for common carp: axis x represents the significance of the factor "element" (71.73 %); axis y represents the significance of the factor "season" (20.23 %)*

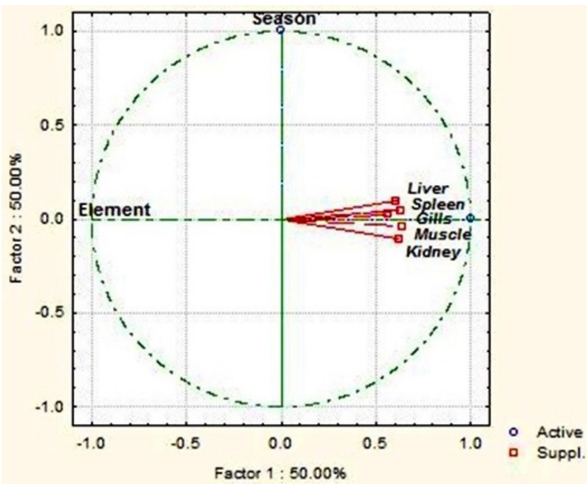

Figure 3 *Relationship between qualitative factors "element" and "season" for common rudd: axis x represents the significance of the factor "element" (64.05 %); axis y represents the significance of the factor "season" (18.09 %)*

rudd – 18.09 %), which suggests that metal properties and bioavailability in the aquatic environment are important for metal bioaccumulation.

Qualitative factor "fish organ" also proved important for metal bioaccumulation (Figure 4), as the studied organs differed in their affinity to metals. Internal organs (liver, kidney, and spleen) showed the highest affinity, followed by the gills and muscles. Qualitative factor "season" had less influence on metal bioaccumulation.

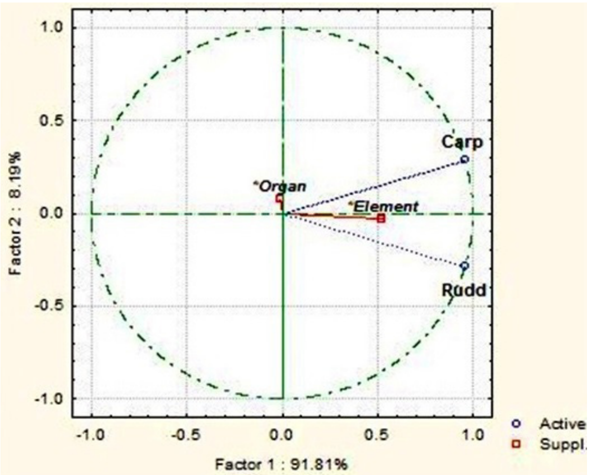

Figure 4 *Relationship between qualitative factors "element" and "organ" for common carp and common rudd: axis x represents the significance of the factor "element" (64.05 %); axis y represents the significance of the factor "organ" (8.09 %)*

Metal mass fractions in the organs of common carp and common rudd from Topolnitsa were higher than the reservoir water concentrations (Table 3), whereas all reference fish metal levels were below the detection limit of the instrument. The highest metal mass fractions in both fish species were measured in the summer, even though not all seasonal differences were significant.

Gills are considered an important point of entry into the organism for essential (Cu, Zn, Se, Mn, Fe) and non-essential elements (Al, As, Cd, Cr, Pb) (35) and are a useful tool for assessing metal bioavailability and accumulation in water (17). We found a positive correlation between water and spring gill As concentrations irrespective of the fish species (r=0.84 for carp and r=0.63 for rudd), as both had similar metal concentrations. The same is true for the summer gill Zn concentrations (r=0.72 for carp and r=0.64 for rudd). Even though statistics did not establish significant correlations between other gill metal levels and water metal levels, we believe that the correlations established for As and Zn sufficiently support that the Cyprinid fish gills can accurately reflect water metal contamination. We share the opinion of Teien et al. (36), Hansen et al. (37), and Terra et al. (38), who think that the reason for this is that the gills, being negatively charged, bind positively charged metal species in the water. Our results confirm other reports that fish gills reflect lower water quality (39-41).

Once metals cross the biological barrier and enter the bloodstream, they will reach and accumulate in the internal organs of fish, which explains the significantly higher internal organ metal levels in our study. Another reason for higher metal levels in the internal organs may be gastrointestinal route of exposure (13), rendering the liver and the kidney additionally vulnerable to chronic metal exposure (42). Similar to Falfushynska and Stoliar (43) and Siscar et al. (44), we think that metal accumulation in internal organs is associated not only with organ function such as haematopoiesis, antioxidant defence, detoxification, and excretion, but also with metallothionein synthesis. Our results are also in agreement with those of Shinn et al. (45) and Poleksić et al. (46), who measured the highest metal levels in the internal organs. All metal variations in our study may be related to changes in the abiotic factors and fish metabolic activity or to individual fish susceptibility and features in the biology of common carp and common rudd. We also agree with Sokolova and Lannig (47) that toxicant bioaccumulation increases with water temperature, as it accelerates the crossing of metals over biological barriers, which may account for seasonal variations. The positive correlation we found between water and carp liver Cu (r=0.80) and water and rudd liver Cu (r=0.72) in all three seasons as well as between water and carp kidney Cd (r=0.65) support the leading role these organs have for metal storage.

The spleen also showed high metal levels in both fish species. Cd and Cu in common rudd spleen significantly varied between some of the seasons and were significantly higher than carp findings (Table 3). Summer Pb and Zn in common carp spleen were significantly higher than in the spring and autumn. In addition, our results suggest that Zn has strong affinity for this organ (r=0.72 for carp; r=0.63 for rudd) and that the spleen is as important depot for metals as the liver and kidney. It carries important functions such as new lymphocyte formation, breakdown of old red blood cells, and overall immune response. We therefore think that toxicology studies should stop underestimating this organ and use it more often to assess transfer and distribution of metals in fish, as

Table 3 *Metal mass fractions (mean±SD) in common carp and common rudd organs from the Topolnitsa reservoir (ten samples per fish species per season)*

Season	Fish	Organ	Element (µg kg^{-1})					
			As	Cd	Cu	Ni	Pb	Zn
Spring	Carp	gills	60±10*	700±30	900±50	300±10	400±10§	126000±1000
		liver	900±10	900*±50	16000*±300	1700±50*	2400±300	109000±100
		kidney	1200±300	200±10*,§	1600±300§	1100±50*	1000±500*	134000±500
		spleen	1100±300	100±10§	2000±500	1500±50*	1700±500*	397000±300
		muscles	70±10	1±0.5	500±30↑	400±10	300±10↑	8000±500
	Rudd	gills	100±10	100±10	1300±200	100±10	11000±300§	40000±500
		liver	1300±500	1300±10§	8000±500§	1400±10	1300±300§	170000±500
		kidney	1140±500	2100±300*,§	4000±300	1100±10	2300±500	290000±3500
		spleen	1200±30	500±10	1500*±10	1500±10	2000±20	198000±500§
		muscles	100±10↑	10±1	300±10	40±10	60±10	7000±500
Summer	Carp	gills	90±20	700±30	2800±300	300±1	300±10§	152000±730
		liver	1300±10	8800±500*,§	53000±200*,§	5500±300*,§	5500±500§	174000±500
		kidney	1600±400	2000±50*,§	3000±500	3300±500*,§	3300±100*	313000±1500
		spleen	1900±500	200±10	2000±500	3900±500*,§	3900±300*	654000±550*,§
		muscles	60±1	30±10	300±10	200±10	200±10↑	8200±300
	Rudd	gills	100±10	300±0.01	1800±200	200±10	1200±50	50000±200
		liver	2000±700	1600±0.05§	17000±1000§	1500±10	1300±10§	56000±500
		kidney	2000±500	5500±0.2*	6000±500§	1400±500§	2100±500	283500±1000
		spleen	1400±300	800±0.01§	5000±500*,§	1500±300	2100±100	46000±1500§
		muscles	100±1↑	20±0.001	300±10	100±3	30±1	8000±300
Autumn	Carp	gills	700±30*	200±10	1900±400	400±10	500±1	124000±250
		liver	900±50	1300±50*,§	19000±2000*,§	2500±300	2500±100	134000±2500
		kidney	1000±200	800±50*,§	1300±50	1900±500	2000±100	287000±2500
		spleen	1300±500	150±30	1500±300§	2200±200	1900±500*	385000±5*
		muscles	70±2	40±1	260±40	150±10	150±30	7800±300
	Rudd	gills	100±10	100±1	2000±500	200±10	800±10	50000±500
		liver	1300±500	1100±1§	16000±1500§	2000±10	1600±300	263000±500
		kidney	1140±500	3000±500§	3000±500	1200±10§	2400±400	243000±300
		spleen	1200±300	100±1§	2000±500	1400±10	2700±500	272000±300§
		muscles	100±10↑	10±1	200±50	15±1	20±1	5600±100

** – significant differences in metal concentrations for the same species between different seasons*
§ – significant differences between metal concentrations in common carp and common rudd in the same/or different seasons
↑ – borderline or above the Bulgarian limit and FAO/WHO guidelines

high metal levels in this organ can point to oxidative stress, morphological alterations, or biochemical disturbances.

Dorsal muscles of both species had the lowest metal concentrations, and our findings are in agreement with earlier reports (48, 49). It is well known that the muscle does not actively accumulate metals and seems to have a very fast decontamination rate. The increasing concern about the health risks of food poisoning (50) due to consumption of fish meat contaminated with metals has prompted Bulgaria to adopt several standards and recommendations (51-53) into national legislation (Table 4). However, there are huge differences between the maximum permissible metal levels in fish meat set by the Bulgarian regulations and the Joint FAO/WHO standards (53). In addition, the European Commission has omitted As, Cu, Ni, and Zn from its regulations, and the FAO/WHO have omitted Cd, Ni, and Zn. This calls for crucial changes in environmental legislation.

Table 4 *National and international standards and guidelines on maximum permissible metal concentrations (mass fractions) in fish meat.*

Regulation/Guideline	Limit concentration (μg kg^{-1})					
	As	**Cd**	**Cu**	**Ni**	**Pb**	**Zn**
Bulgarian regulation (2004)	1000	50	10000	50	200	50000
EC Regulation (2006)	-	50	-	-	300	-
FAO/WHO (2014)	100	-	400	-	300	-

Therefore, even though our measurements show borderline or excessive levels only for Pb in carp and As in rudd dorsal muscle, we would not recommend Topolnitsa fish consumption, having in mind the toxicity and the ability of these metals to bioaccumulate in the food web. Moreover, Topolnitsa has constantly been contaminated with a cocktail of metals since the early 1960s, and the anthropogenic pressure has not faltered. We therefore propose regular monitoring that would involve metal analysis in both the abiotic and biotic compartments (fish muscles).

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Sažetak

Akumulacija metala u šarana i crvenperki iz umjetnog jezera Topolnitsa u Bugarskoj

Izmjerena je koncentracija arsena (As), kadmija (Cd), bakra (Cu), nikla (Ni), olova (Pb) i cinka (Zn) u uzorcima vode te škrgama, jetrima, bubregu, slezeni i leđnom mišiću šarana (*Cyprinus carpio* L.) i crvenperke (*Scardinius erythrophthalmus* L.) iz umjetnog jezera Topolnitsa (Bugarska) tijekom tri godišnja doba (proljeće, ljeto i jesen) 2012. Taj se ekosustav nalazi u regiji poznatoj po rudnicima bakra i metalurgiji. Koncentracija metala u vodi bila je značajno viša u ljeto nego u proljeće (p<0.05), a koncentracija As, Cd, Cu i Zn prelazila je razinu dopuštenu državnim odredbama. Kvalitativni čimbenici "element" i "riblji organ" jače su utjecali na akumulaciju metala od čimbenika "godišnje doba" i "riblja vrsta". Najviša razina metala u riba izmjerena je u jetrima, slezeni, bubregu i škrgama, a najniža u leđnom mišiću. Razina u tkivima bila je malo viša u ljeto, ali se uglavnom nije razlikovala između ribljih vrsta. Mišićna je razina cijelo vrijeme bila niska, ali su zato As i Pb bili iznad domaćih i međunarodnih normi. Stoga ne preporučujemo konzumaciju ribe iz Topolnitse s obzirom na to da stalno zagađenje umjetnog jezera za sobom povlači zdravstvene rizike.

KLJUČNE RIJEČI: *bioakumulacija; ICP-MS; slatkovodna riba; unutrašnji organi*

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