

# HISTOLOGICAL ALTERATIONS UNDER METAL EXPOSURE IN GILLS OF EUROPEAN PERCH (*PERCA FLUVIATILIS* L.) FROM TOPOLNITSA RESERVOIR (BULGARIA)

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**Abstract:** Topolnitsa Reservoir is located in a region of Bulgaria rich in copper mines where intensive mining has been ongoing for several decades. General data on the ecological status of the reservoir and the effects of metal on fish is relatively scarce. The first aim of the study was to measure the concentrations of six metals (As, Cd, Cu, Ni, Pb and Zn) in water samples and in the gills of European perch (*Perca fluviatilis* Linnaeus, 1785.). The second objective was to examine gill structure and determine the severity of histological alteration as a result of metal exposure. Surface water and fish gill samples were collected in spring, summer and autumn in 2012 and metal and histological analyses were performed. Metal concentrations in the water samples varied, but only Cu concentrations were determined in all three seasons and they were higher than the maximum permissible levels. The concentrations of metals in the gills were significantly higher ( $P < 0.05$ ) than in the water. Examination of gill structure revealed the presence of proliferative and degenerative changes, as well as changes in the blood vessels. Histological lesions were similar in their severity in all three seasons. This study provides the first information about metal effects on the morphology of European perch gills from Topolnitsa Reservoir. It can be concluded that the metal contamination of the Topolnitsa Reservoir and fish is chronic and that it can negatively affect the structure and function of fish gills. As metals display a tendency to accumulate in fish gills, their effects are expected to become more severe with time, as they affect gill functions.

**Key words:** Cu; bioaccumulation; fish; gills; perch

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## INTRODUCTION

Pollutants affecting the natural environment include certain chemical elements released into ecosystems as a result of multifaceted activity of humans (Brucka-Jastrzbska and Protasowicki, 2004). The mining and smelting of metals create a potential source of metal pollution in aquatic environments (Moiseenko and Kudryavtseva, 2002; Carrasco et al., 2011). Metals pose a serious threat to aquatic life because of their toxicity, long persistence, bioaccumulation and biomagnification in the food chain (Shah et al., 2009).

The use of living organisms in the study of environmental quality is widely accepted (Stamenković et al., 2013). Fish are sentinel organisms in aquatic ecosystems and despite their high mobility, they are considered as reliable organisms for environmental health assessment (Arnaudov et al., 2008; Antal et al., 2013). Fish can be exposed to heavy metals via two exposure routes, waterborne and dietary (Sloman, 2007). Thus, in teleost fish, the gills are very frequently utilized in bioaccumulation studies (Mansour and Sidky, 2002).

Perch is a species typically found in both pristine and metal-contaminated lakes and it reflects

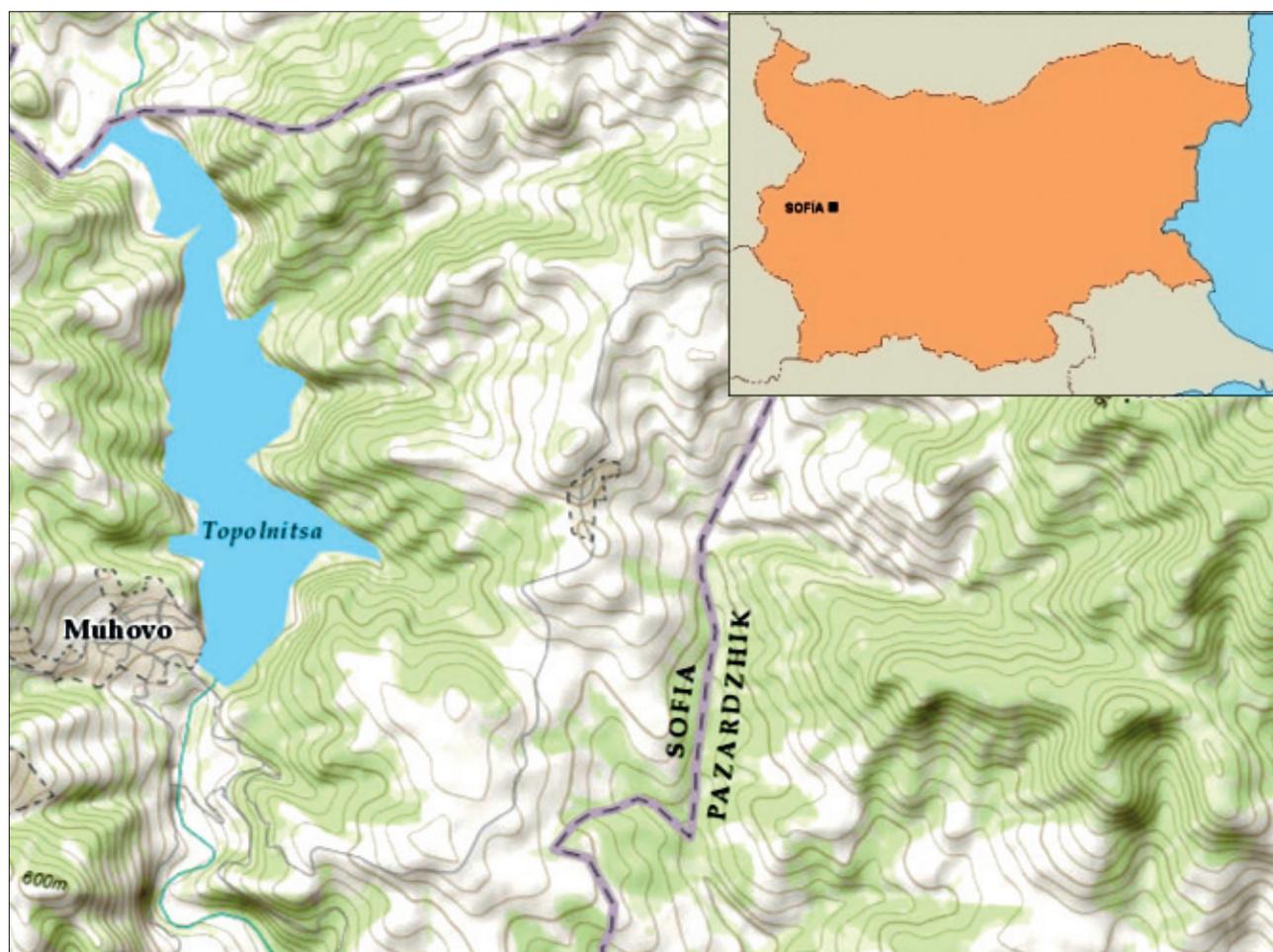


Fig. 1. Location of the Topolnitsa Reservoir in Bulgaria.

the contamination of the sites where it is sampled (Giguère et al., 2004). In Sweden, for example, biomarkers in perch have been used for more than 25 years to evaluate the environmental impact of pulp mills, metal industry, landfills and large population centers (Hansson et al., 2006).

Histological alterations in selected target organs can be used as reliable markers for the effects of sublethal stressors, especially chronic ones, on aquatic biota, and are a reflection of the overall health of the ecosystem (McHugh et al., 2011). Histological alterations are also sensitive, occur earlier, and provide better evaluation of the effects of pollution than any single biochemical parameter (Poleksić et al., 2010).

Topolnitsa Reservoir (42° 25' 90" N 23° 59' 38" E) stands across the Topolnitsa River in western Bulgaria (Fig. 1). It is one of the big artificial reservoirs in the country built in the early 1960s. The Topolnitsa River runs through a region rich in copper ores. In the last few decades, the main sources of pollution have been the copper mines, metallurgy plants and mine tailings. Hence, Topolnitsa Reservoir serves as a final settling tank for many different toxicants, including metals that are carried by the river and its tributaries. Recently Georgieva et al. (2014); Georgieva et al. (2014); Yancheva et al. (2014) and Yancheva et al. (2014) provided some recent data on the effects of metal contamination on different organs of a few fish species from Topolnitsa Reservoir. However, general data on the ecological status of the reservoir and metal effects on fish is relatively scarce or old, and it deals mostly with Topolnitsa River and other species (see Gecheva et al., 2013; Zhelev et al., 2013 and Zhelev et al., 2013).

In the present study our first objective was to measure the metal concentrations of arsenic (As), cadmium (Cd), copper (Cu), nickel (Ni),

lead (Pb) and zinc (Zn) in water samples and in the gills of European perch. We also examined the structure of fish gills and determined the extent and severity of the histological alterations.

## MATERIALS AND METHODS

All experiments were conducted in accordance with national and international guidelines of the European Parliament and the Council on the protection of animals used for scientific purposes (Directive 2010/63/EU).

### Water sampling

Water samples for metal analysis were collected according to ISO 5667-6:2012 from the subsurface layer at a water depth of 1 m in triplicate in every season – spring, summer and autumn – in 2012. Prewashed double-capped polyethylene bottles were used. They were rinsed three times with the water to be sampled prior to sampling. Samples were acidified with 1% HNO<sub>3</sub> and stored on ice for as short time as possible to minimize changes in the metal physicochemical characteristics before analysis. During the field trip, pH, temperature (°C), dissolved oxygen (mg/l) and conductivity (µS/cm) were recorded simultaneously, using a field kit-meter (Multi 340i, WTW).

### Fish sampling

In total, 45 fish (15 in each season) were collected using a boat and fishnets. Fish samples were collected according to the EMERGE protocol (Roseland et al., 2001). Prior to dissection, the fish fork length and weight were measured to the nearest millimeter and gram (mean weight 53 g ± 5.5; mean length 17.5 cm ± 1.5). For metal analysis, the gill filament of the second right

gill arch of each fish was collected and packed in polyethylene bags on ice, transported to the laboratory on the same day and then deep frozen (-25°C). For histological observation, the samples were fixed in 10% neutral formaldehyde (Merck Group, Darmstadt, Germany). Fifteen healthy fish (mean weight 54.5 g ± 3.5; mean length 15.5 cm ± 2.3) and water were obtained from the National Institute of Fisheries and Aquaculture in Plovdiv, Bulgaria, where fish are reared under strict controlled toxicant-free conditions, and used as control (5 in each season).

### Metal analysis

All metal analyses were carried out at the accredited regional laboratory of the Executive Environment Agency in Plovdiv, Bulgaria. Chemicals were purchased from Merck Group (Darmstadt, Germany). They were of analytical grade and Suprapur® quality. Materials were made of Pyrex and high-density polyethylene, washed with 30% HNO<sub>3</sub>, rinsed three times with deionized water and allowed to dry in an oven at 105°C for 2 h (Ross, 1986). Analytical procedures and quality control were checked using replicate blanks and internationally certified reference materials for metals in waters – SRM 1643e (National Institute of Standards and Technology, USA) and fish – DORM-3 (National Research Council Canada, Ottawa, Ontario, Canada) with every ten samples. Replicate analysis of these reference materials showed good accuracy with recovery rates from 95% to 115%.

Water chemistry analysis was carried out according to ISO 17294-2:2005. Metal content in the water was analyzed by using ICP-MS (Agilent 7500ce, Japan) and reported as µg/l. The detection limits of the instrument for different metals were as follows: As – 0.5 µg/l, Cd – 0.05 µg/l, Cu – 0.5 µg/l, Ni – 0.5 µg/l, Pb – 10 µg/l and Zn – 10 µg/l.

Prior to the actual assay, approximately 1 g of each gill sample was mineralized using a microwave digestion system (Milestone Ethos Plus, Italy). The digestion solution was prepared with 6 ml of 65% HNO<sub>3</sub> and 2 ml of 30% H<sub>2</sub>O<sub>2</sub> at 200°C. After mineralization, the samples were brought to 25 ml by the addition of ultra pure water and subjected to metal analysis. Metal content was analyzed using ICP-MS (Agilent 7500ce, Japan) and reported as µg/kg wet weight. The detection limits of the instrument were as follows: As – 10 µg/kg, Cd – 1 µg/kg, Cu – 10 µg/kg, Ni – 10 µg/kg, Pb – 30 µg/kg and Zn – 30 µg/kg.

### Histological analysis

After fixation was completed (minimum time 12-24 h), the gills were processed by a routine histological method (Humason, 1979). They were then sectioned to a thickness of 5-7 µm at different levels of the tissue using a semi-automated microtome (Leica RM2245, Nussloch, Germany) and mounted on sterilized glass slides. Sections were stained with conventional hematoxylin and eosin (H&E) for histological examinations and prepared for light microscopy analysis. Histological changes in the gills were observed by light microscopy (Nikon, Japan) and photographed with a mounted digital camera (DCE-2, AMCAP software version 1.0.2). The morphology of the gills of all specimens, including control fish gills, was appraised individually and semi-quantitatively using the grading system of Peebua et al. (2006) which we slightly modified. Evaluation of the histological changes was carried out and presented as an average value in percentages. Each grade represents specific histological characteristics and was categorized as follows: no histological alterations – (-); mild histological alterations – 10-20% - (+/-); moderate histological alterations – 30-50% - (+); severe histological alterations – 60-80% - (++); and very severe histological alterations – above 80% - (+++) of gill architecture.

## Statistical analysis

Statistical analysis of raw metal concentration data in the water and gills was performed using the software program STATISTICA (version 7.0 for Windows, StatSoft, 2004). Differences between the individual variables were

tested for significance using the Student's t-test ( $P < 0.05$ ). Relationships between the metal concentrations in the collected water and gill samples were tested using Pearson's Product-Moment correlation ( $P < 0.05$ ). Data were reported as mean  $\pm$  SD.

**Table 1.** Metal concentrations in Topolnitsa Reservoir water, mg/l (n=5 in each season).

Metal	Spring	Summer	Autumn	Bulgarian legislation
As	<b>0.20<sup>§</sup></b> $\pm$ 0.01	0.004 <sup>§</sup> $\pm$ 0.001	<b>0.010</b> $\pm$ 0.001	0.01
Cd	<b>0.0006</b> $\pm$ 0.0001	0.0004 $\pm$ 0.0001	<0.00005*	0.0005
Cu	<b>0.020</b> $\pm$ 0.001	<b>0.02</b> $\pm$ 0.01	<b>0.010</b> $\pm$ 0.005	0.001
Ni	0.003 $\pm$ 0.001	<0.0005*	<0.0005*	0.02
Pb	<0.001*	<0.001*	<0.001*	0.007
Zn	<b>0.008<sup>§</sup></b> $\pm$ 0.001	<b>0.20<sup>§</sup></b> $\pm$ 0.01	<0.001*	0.008

\*less than the detection limit of the instrument; §significant differences between seasons; **bold** – borderline or above the maximum permissible levels set by the Bulgarian legislation

**Table 2.** Metal concentrations in European perch gills from Topolnitsa Reservoir, expressed as mg/kg wet weight (n=15 in each season).

Metal	Spring	Summer	Autumn
As	0.14 $\pm$ 0.30	0.12 $\pm$ 0.05	0.14 $\pm$ 0.05
Cd	0.09 $\pm$ 0.01	0.30 $\pm$ 0.01	0.10 $\pm$ 0.05
Cu	1.66 $\pm$ 0.40	1.45 $\pm$ 0.50	1.89 $\pm$ 0.60
Ni	0.12 $\pm$ 0.05	0.13 $\pm$ 0.01	0.90 $\pm$ 0.05
Pb	2.0 $\pm$ 0.2	<b>0.40</b> $\pm$ 0.03	1.01 $\pm$ 0.30
Zn	213 $\pm$ 16.1	<b>123</b> $\pm$ 13.7	230 $\pm$ 19

**Bold** – significantly lower metal concentration than in other seasons.

**Table 3.** Histological alterations in European perch gills from Topolnitsa Reservoir.

Histological alteration	Spring	Summer	Autumn
Lamellar lifting	+/-	+/-	+/-
Edema	+/-	+/-	+/-
Proliferation of gill epithelium	+/-	+/-	+/-
Fusion	-	+/-	-
Degeneration of gill lamellae	+	-	-
Vasodilatation in the main venous sinus	-	-	-
Vasodilatation in the secondary lamellae	+/-	+/-	+/-
Aneurysms	-	+/-	-

\*no histological alterations, representing normal histological structure – (-); mild histopathological alterations – 10-20% - (+/-); moderate histopathological alterations – 30-50% - (+); severe histopathological alterations – 60-80% - (++) and very severe histopathological alterations – above 80% - (+++).

## RESULTS

Topolnitsa water quality data (average for all three seasons) were as follows: mean pH  $8.5 \pm 2.3$ , mean  $t$   $15.5^{\circ}\text{C} \pm 5.5$ , mean conductivity  $420 \mu\text{S}/\text{cm} \pm 15.3$ , mean dissolved oxygen  $11.3 \text{ mg}/\text{l} \pm 2.5$ . In the water from which the control fish were taken, all metal concentrations were below the detection limit of the instrument. Metal concentrations in Topolnitsa Reservoir surface water are presented in Table 1. Generally, they varied in the spring, summer and autumn. In addition, Pb concentrations were below detection limit of the instrument in all three seasons, but Cu concentrations were measured during the whole period of study.

In the gills of control fish, all metal concentrations were below the detection limit of the instrument. Metal concentrations in the European perch gills are presented in Table 2. Similar to the metal concentrations in the water, the metal concentration in fish gills varied between the spring, summer and autumn, but in general, they were similar. Only Pb and Zn concentrations in the summer were significantly lower than the respective concentrations in the spring and autumn ( $P < 0.05$ ).

Control fish gills presented normal distribution of the cellular constituents and organization pattern of the primary and secondary lamellae and blood vessels. Hence, with regard to the semi-quantitative system, control fish gill characteristics were evaluated as normal without alterations (Fig. 2). In contrast, the fish collected from Topolnitsa Reservoir had serious alterations in the gill structure in all three seasons (Fig. 3). We classified the histological lesions in the gills into three main groups: (i) proliferative changes – lamellar lifting, edema, proliferation

of epithelial cells and lamellar fusion; (ii) degenerative changes of gill lamellae, and (iii) changes in the blood vessels – vasodilatation in the main blood sinus and secondary lamellae, and aneurysms. In general, the extent and severity of each particular lesion were similar in the spring, summer and autumn (Table 3). However, degeneration of gill lamellae was observed to a moderate degree of expression only in the spring; fusion and aneurysms were observed to a mild degree in the summer.

## DISCUSSION

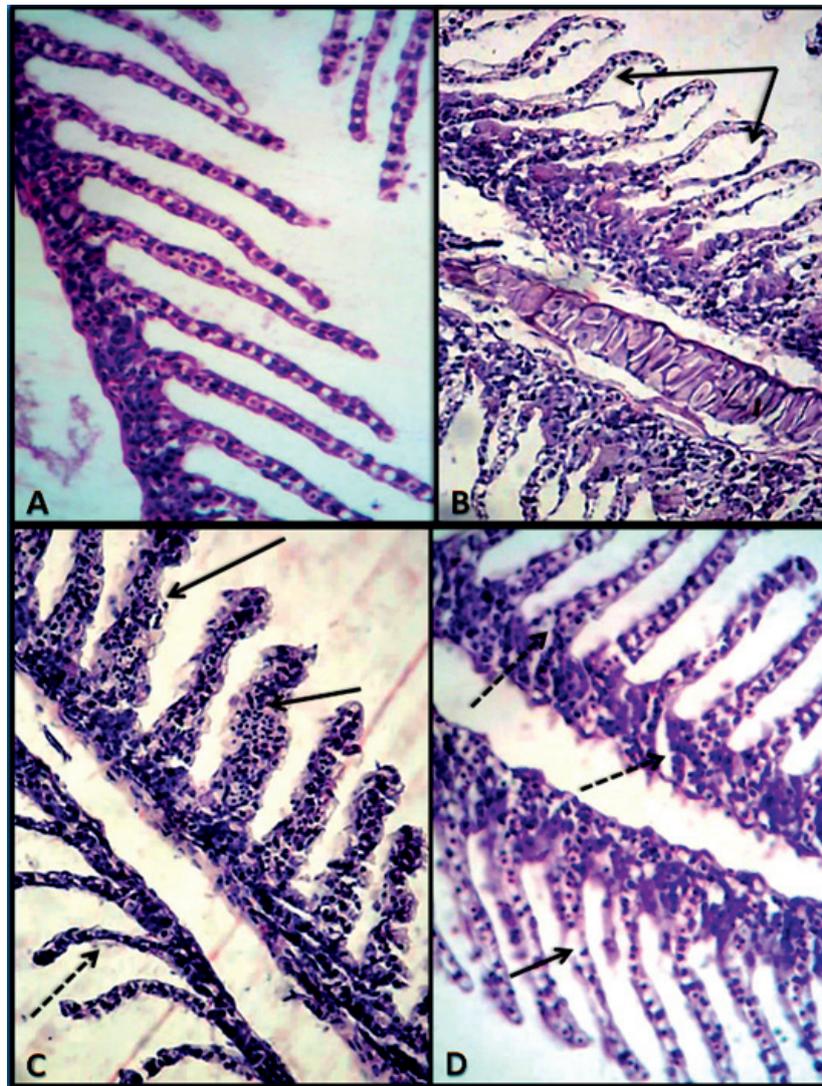
In this work we compared the metal concentrations in Topolnitsa water with the maximum permissible levels set by Bulgarian legislation based on Directive 2013/39/EU. Cu concentrations in all three seasons, As concentrations in the spring, and Zn concentrations in the summer were above the maximum allowable limits set by law. Hence, we think that the contamination by these three metals is chronic and due to the intensive and ongoing copper extraction in the region. On the other hand, concentrations of the other studied metals are more likely related to their background levels and complex interactions between water, sediment and biota, or may be caused by anthropogenic factors such as industrial wastewaters and agricultural runoff.

The gills are considered an important point of entry into the organism for essential (Cu, Zn, Se, Mn, Fe) and non-essential (Al, As, Cd, Cr, Pb) elements, and they are a useful tool for assessing metal bioavailability and accumulation in water (Ural et al., 2012). Statistical analysis of the data showed that the gill metal concentrations were significantly higher than those in the water were ( $P < 0.05$ ). Even though Pb concentrations

in the water were below the detection limit of the instrument, we found a positive correlation between the concentrations of Pb in the water and gills in the spring and autumn ( $r=0.74$ ;  $r=0.65$ ). A similar correlation was established for As and Cu concentrations in the water and gills in all three seasons ( $r=1$ ), and for Zn in the water and gills in the spring and summer ( $r=0.63$ ;  $r=0.82$ ). Therefore, we consider that perch gills accurately

reflect the degree of metal pollution of Topolnitsa Reservoir. We share the opinion of Terra et al. (2008) who think the reason for this is that the gills, being negatively charged, bind positively charged metal species in the water.

Metal bioaccumulation leads to disturbances in cell metabolism and negative changes in the morphological structure of organs. There



**Fig. 2.** Histological alterations in the gills of European perch from Topolnitsa Reservoir H&E, x 400. A – control gills; B – lamellar lifting (black arrow); C – vasodilatation in secondary lamellae (black arrow); dashed arrow – degeneration of secondary lamellae; D – lamellar lifting (black arrow); edema (dashed arrow).

are reports on the effects of metals on histological changes in gills, both in field and laboratory conditions (Fontainhas-Fernandes et al., 2008; Mohamed, 2009), however, it is often difficult to decide whether morphological alterations are adaptive or destructive (Tkacheva et al., 2004). We presume that alterations in gills, such as lamellar lifting and edema, could serve as a protective mechanism of the fish organism against the metal-contaminated waters of Topolnitsa Reservoir. Velcheva et al. (2010) found similar histological changes in the gills of perch from another reservoir that is subjected to serious heavy metal contamination in Bulgaria, but they were not as severe as in our study. However, more severe structural changes such as fusion and aneurysms can lead to disturbances in fish gill metabolism and hence, to impaired respiratory functions. In our study, we found that hyperplastic alterations in fish gills were predominant. Therefore, we consider that the adaptive and protective mechanisms in the fish have promoted cell division instead of cell death.

## CONCLUSIONS

Cu contamination in Topolnitsa Reservoir is chronic. Gills of European perch accumulated Cu and other investigated metals; their concentrations were similar in all three seasons. Histological alterations in fish gills were more proliferative in nature than degenerative. These alterations could be used as reliable biomarkers for metal pollution. Further investigations in this area should be carried out to improve our understanding of the impact of metal effects on fish gills.

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**Authors' contributions:** I.V. and E.G. designed the research; V.Y. and S.S. performed the research; I.V. and E.G. analyzed the data; V.Y., S.S. and M.B. wrote the paper.

**Conflict of interest disclosure:** The authors state that there is no conflict of interest.

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