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ORIGINAL ARTICLE

Monitoring pollution in river Mureş, Romania, Part III: biochemical effect markers in fish and integrative reflection

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Abstract Along a downstream stretch of River Mureş, Romania, adult males of two feral fish species, European chub (*Leuciscus cephalus*) and sneep (*Chondrostoma nasus*) were sampled at four sites with different levels of contamination. Fish were analysed for the biochemical markers hsp70 (in liver and gills) and hepatic EROD activity, as well as several biometrical parameters (age, length, wet weight, condition factor). None of the biochemical markers correlated with any biometrical parameter, thus biomarker reactions were related to site-specific criteria. While the hepatic hsp70 level did not differ among the sites, significant elevation of the hsp70 level in the gills revealed proteotoxic damage in chub at the most upstream site, where we recorded the highest heavy metal contamination of the investigated stretch, and in both chub and sneep at the site right downstream of the city of Arad. In both species, significantly elevated hepatic EROD activity downstream of Arad indicated that fish from these sites are also exposed to organic chemicals. The results were indicative of impaired fish health at least at three of the four investigated sites. The approach to relate biomarker responses to analytical data on pollution was shown to fit well the recent EU demands on further enhanced efforts in the monitoring of Romanian water quality.

Keywords Biomarker · Chub · Cytochrome P450 · Danube tributary · Hsp70 · Monitoring · Sneep

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1 Introduction

In order to set discharge effluent and surface water quality standards, chemical criteria have originally been developed and applied to natural water bodies for centuries. Relying on chemical criteria alone for assessing the status of surface water integrity can, in many instances inaccurately portray the biological and ecological condition of aquatic ecosystems (Adams, 2002). This is shown by a comparison of the indicative potential of biological and chemical criteria in more than 600 river and stream segments (Yoder and Rankin, 1998). Within this context, chemical criteria indicate contamination but cannot show biological or environmental damage. With a great variety of biological assessment tools now available, an improved understanding of contaminant effects on ecosystem structure and function, and an increased ability to interpret biological data is possible. The use of physiological, cellular and biochemical effects, so-called biomarkers, has become attractive and useful for assessing the effects of environmental stressors on the sub-lethal level of biological systems.

Chemical analyses on sediments of the downstream part of River Mureş, Romania, in a stretch from the Carpathian Mountains through the Plain of Arad to the Romanian-Hungarian border showed high concentrations of cadmium (up to 8.7 mg/kg) and copper (up to 49.2 mg/kg) which surpassed the (exposure defined) quality standards from a number of industrialized countries, most likely due to the mining and metallurgical activities at many tributaries to the Mureş (Sandu *et al.*, 2006, this issue). In addition to the highest measured metal levels, pollution by untreated faecal waste was proven in the stretch of the river course between the Carpathian Mountains and the city of Arad. Despite contamination, however, the species number and biomass of invertebrates and planktonic algae in the river were not affected and the structure of the planktonic communities seemed to be slightly affected at a single site only (Sandu *et al.*, 2006, this issue).

In the present study, we applied biomarkers to assess whether chemically detected pollution of the River Mureş has resulted in effects on fish health. Effects were assessed in organs that are sites of primary attack and accumulation, the gills and the liver. Apart from documenting metal accumulation in fish liver and concomitant histopathological changes in both fish gills and liver which were found all along the downstream stretch of the river Mureş (Triebskorn *et al.*, 2006, this issue), we used the following two biochemical markers for health assessment in two feral fish species, the carnivorous European chub (*Leuciscus cephalus*) and the planctivorous sneep (*Chondrostoma nasus*):

(1) Stress proteins. The best investigated stress protein family, hsp70, is commonly used as a marker which effectively integrates overall adverse effects on protein integrity, hence measures proteotoxicity. Its induction by heavy metals in a variety of species has been shown in numerous studies (for reviews see Schramm *et al.*, 1999; Kammenga *et al.*, 2000).
(2) Hepatic cytochrome P450. The induction of CYP1A, a group of isoforms among more than 900 gene products identified throughout phylogeny, is accepted as a measure of bioavailable arylhydrocarbon receptor (AhR) ligands, such as dioxins, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and structurally similar compounds (Schlenk and Di Giulio, 2002). This marker has been correlated with liver lesions and immune suppression in fish (Collier *et al.*, 1998; Reichert *et al.*, 1998).

2 Material and methods

2.1 Sites and sampling

Four sampling sites along a downstream stretch of the River Mureş were investigated (in upstream to downstream order): site 1 (Zam), 107 km upstream of the city of Arad; site 2 (Mândruloc), 15 km upstream of Arad; site 3 (Bodrogu Vechi), right downstream the influx of the municipal wastewater treatment plant of Arad; and site 4 (Pecica), 21 km downstream of Arad and right downstream of an industrialized area (for a map see Sandu *et al.*, 2006, this issue). At each sampling site, 8–10 individuals of *C. nasus* and 6–10 individuals of *L. cephalus* were caught in the open water body by means of electro-fishing from boats during three subsequent days (May 25–27, 2004), in the order site 4 to site 1 opposite to the direction of the water flow. Due to different habitat preferences of male and female fish during spawning, all captured fish

were adult males. Fish were anaesthetized with 0.05% ethyl-4-aminobenzoate (benzocaine) for one minute, killed, measured for length (L [in cm]) and wet weight (wt [in g]), and dissected in the field. The liver was excised immediately and cut into pieces of which two portions were frozen in liquid nitrogen each for stress protein and EROD analysis. The other pieces were used for histopathology and metal analysis (see Triebskorn *et al*., 2006, this issue). Subsequently, the gills were removed and the right branches frozen in liquid nitrogen for stress protein analyses. From each fish, a couple of scales were removed for age analysis. The individual condition factor (cf) was calculated according to the equation

$$cf = wt \cdot L^{-3} \cdot 10^2 \text{ (Fulton, 1902)}$$

2.2 Stress protein analysis

Gill and liver samples were homogenized on ice in a buffer (80 mM potassium acetate, 5 mM magnesium acetate, 20 mM Hepes, pH 7.5) and analyzed by a highly reproducible Western blotting technique and subsequent image analysis. Total protein concentration in the supernatant was determined according to the method of Bradford (1976). Constant protein weights (10 mg of total protein/lane) were analyzed by minigel SDS-PAGE (12% acrylamide, 0.12% bisacrylamide (w/v), 15 min at 80 V, 90 min at 120 V). Every gel contained a standard extract from zebrafish (*Danio rerio*) in order to ensure methodological reproducibility. Protein was transferred to nitrocellulose by semidry blotting and the filter blocked for 2 h in 50% horse serum in TBS (50 mM Tris pH 5.7, 150 mM NaCl). After washing in TBS, monoclonal antibody (mouse anti-human hsp70; Dianova, FRG, dilution 1:5,000 in 10% horse serum/TBS) was added and incubated at room temperature (22°C) overnight. After repeated washing in TBS for 2 min, the nitrocellulose filter was incubated in secondary antibody goat anti-mouse IgG (H + L) coupled to peroxidase (Dianova, FRG, dilution 1:1,000 in 10% horse serum/TBS) at room temperature (22°C) for 2 h. After subsequent TBS washing, the antibody complex was detected by 1 mM 4-chloro (1) naphthol and 0.015% H_2O_2 in 30 mM Tris pH 8.5 containing 6% methanol. The optical volumes (average grey scale value x area) of the Western blot protein bands were measured after background subtraction with a densitometric image analysis system (Herolab E.A.S.Y.). Optical volumes were normalized using the respective *D. rerio* standard on the respective blots as a reference. The methodological variability of this protocol has been shown to be ±2.7% from the mean (Köhler *et al*., 2005).

2.3 Cytochrome p450 analysis

Liver samples were weighed and homogenized in 2 ml ice-cold homogenization buffer (2 M sucrose, 20 mM Mops, 1% EDTA/ethanol, 0.2 mM phenylmethylsulfonylfluorid, 1 mM ε-amino capronic acid, 0.3 M mercaptoethanol, 0.02 mM dithiotreitol) with three strokes of a Potter-Elvehjem homogenizer at 300 rpm. The homogenate was centrifuged for 20 min at 10,000 × g and 4°C and the supernatants were again centrifuged in an ultracentrifuge (Beckman Optima) for 60 min at 100,000 g. After centrifugation, the supernatant was removed and the microsomal pellet was solubilized in 100 µL of homogenization buffer. The microsomal fraction was directly assayed for CYP1A by measuring the catalytic 7-ethoxyresorufin (EROD) activity. Subsequently, the microsomes were frozen at –80°C until protein determination.

The catalytic activity of CYP1A was detected fluorometrically by measuring the conversion of 7-ethoxyresorufin into the fluorescent product, resorufin (Burke and Mayer, 1974). EROD activity was determined in a kinetic microplate assay using 96 well plates and a fluorescent plate reader (Victor2, Wallac, Perkin-Elmer, Freiburg, FRG). The reaction mixture contained ethoxyresorufin dissolved in methanol at a final concentration of 0.5 µM in phosphate buffered saline (PBS – 0.08 M Na_2HPO_4, 0.02 M KH_2PO_4, 0.15 M KCl, pH 7.8) and the reaction was started by the addition of NADPH (47 µM final concentration). Resorufin fluorescence was detected at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The amount of resorufin produced was calculated using a resorufin standard curve. Enzyme activity was expressed in pmol resorufin/mg protein/min.

Protein content of the microsomal cell fraction was measured spectrophotometrically using the Bio-Rad DC protein assay kit which is based on the method of Lowry, Rosebrough, Farr, and Randall, (1951). Bovine serum albumin was used as a standard.

2.4 Statistical analysis

Data were tested for normal distribution using the Shapiro-Wilk W test. Since data were not normally distributed, significance of differences between two respective test groups was tested by the non-parametric Mann Whitney Wilcoxon test. Levels of significance were set to $p \leq 0.01$ (**), and $0.01 < p \leq 0.05$ (*). For correlation analysis, the recorded data for hsp70 in gills, hsp70 in liver, EROD in liver, histopathology of the liver and the gills (both taken from Triebskorn *et al*., 2006, this issue), individual age, wet weight, length, and condition factor were subjected to linear and polynomial (2nd and 3rd degree) regression analysis. 95% confidence intervals for each regression curve and significance at the $p = 0.05$ level (ANOVA) were calculated. All statistical analysis was conducted with SAS JMP 4.0.0.

3 Results

All morphometrical parameters (length, weight, condition factor) and age were found to correlate significantly with one another in both fish species (*L. cephalus* weight vs. condition factor with $p = 0.002$, all other combinations with $p < 0.0001$). In contrast, none of the investigated biochemical markers correlated with age, length, weight, or condition factor of fish (data not shown) and it was therefore concluded that biochemical markers reflected the environmental conditions at the respective sites. Consequently, all biochemical data were analysed in respect to the variable 'site'.

Stress protein (hsp70) induction indicated proteotoxic action of environmental threats in the gills of both fish species. In gills of *L. cephalus*, significantly elevated hsp70 levels were found in individuals from sites 1 and 3. Also *C. nasus* gills showed hsp70 to be significantly induced at site 3 (Fig. 1). In contrast, the stress protein levels in the liver did not reveal any differences between the four sites, neither in *L. cephalus* nor in *C. nasus* (Fig. 2).

Hepatic cytochrome P450 activity, measured as EROD activity and indicative of water pollution with organic compounds like PAHs or coplanar PCBs, was slightly higher in *C. nasus* than in *L. cephalus* at all sites. A significant elevation in EROD activity could be found in fish downstream of the city of Arad, precisely in *L. cephalus* at site 3 and in *C. nasus* at site 4 (Fig. 3).

4 Discussion

Biochemical biomarkers such as stress proteins and cytochrome P450-associated enzyme activity are commonly accepted as sensitive indicators of toxic impact since these molecular responses are typically the first line of defense following exposure to xenobiotics. However, they have been shown to be also extremely variable and plastic among individuals in a given population (Schlenk and DiGiulio, 2002). Nevertheless, both defense systems, the hsp70 stress response and the cytochrome P450-dependent biotransformation system, have been shown to comprise suitable

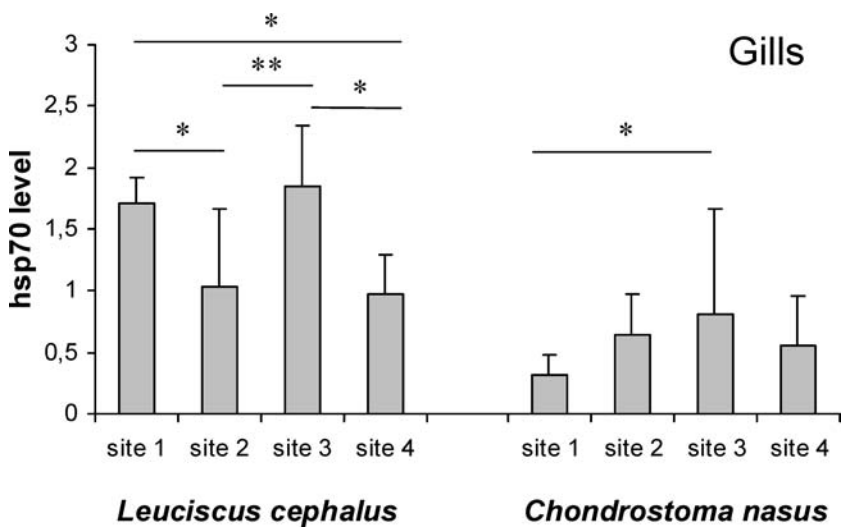

Fig. 1 Hsp70 levels (optical volume relative to a standard) in the gills of *L. cephalus* and *C. nasus* sampled at the four sites at River Mureş. Means and SD. Significance at $p \leq 0.01$ (**) and $0.01 < p \leq 0.05$ (*)

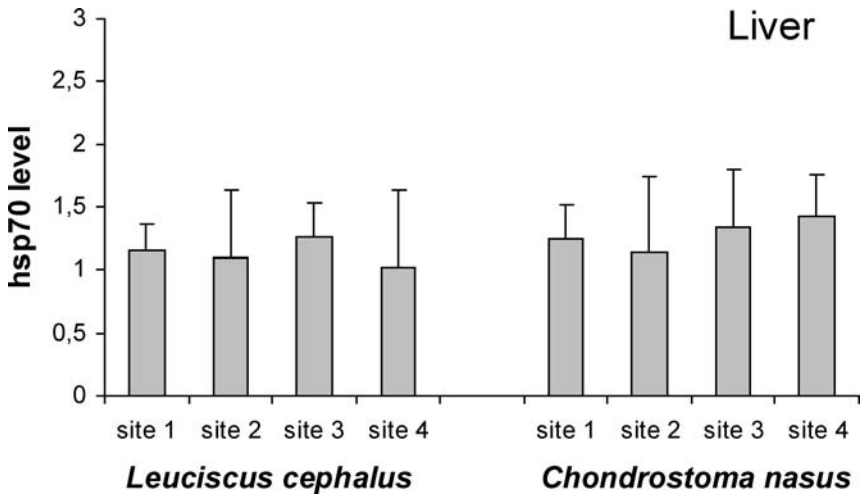

Fig. 2 Hsp70 levels (optical volume relative to a standard) in the liver of *L. cephalus* and *C. nasus* sampled at the four sites at River Mureş. Means and SD. No correlation was found

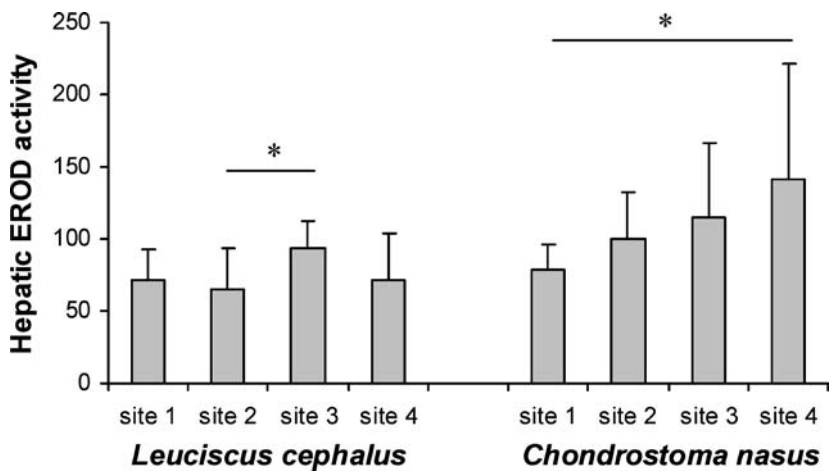

Fig. 3 EROD activity (pmol · min^{-1} per mg microsomal protein) in the liver of *L. cephalus* and *C. nasus* sampled at the four sites at River Mureş. Means and SD. Significance at $0.01 < p \leq 0.05$ (*)

ecotoxicological markers whenever a sufficient number of individuals is analyzed. The CYP1A catalytic activity, measured by means of the EROD assay is accepted to indicate exposure to important organic environmental contaminants such as PAHs, coplanar polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins and – furanes (PCDDs, PCDFs), and a number of pesticides (Stegeman and Hahn, 1994; Van Veld *et al*., 1997; Whyte *et al*., 2000; Navas *et al*., 2003). On the other hand, the level of the stress protein hsp70 is a typical effect marker, integrating overall proteotoxic impact of stressors regardless of their nature (Schramm *et al.*, 1999; Kammenga *et al.*, 2000). Despite the particular advantages of these markers, it has become common sense that modern ecotoxicological monitoring programmes must combine a selection of markers at different levels of biological organization, chemical analytics, conventional limnochemistry, and community-level indicators (Triebskorn *et al.*, 2001), together with an array of physiological covariates (Hodson, 2002). This approach has been realized in the present Mureş River study.

Even though our results represent a temporal snapshot of summer 2004 only, we have also included measures which integrate over a longer period of time. Thus, on the basis of our data, we are able to draw up a series of conclusions on the quality of the downstream part of the River Mureş.

(1) Fish health. Significantly elevated stress protein levels in the gills of *L. cephalus* indicated acute

effects at sites 1 and 3, the most upstream site and the site right downstream the city of Arad. Also the sneep, *C. nasus*, showed its highest hsp70 levels in its gills at site 3 and a remarkably low hsp70 level at site 1. The latter likely has to be attributed to an inhibition of the stress response due to pathologic damage of the hsp system as reported e.g. by Köhler *et al*. (2001) after exposure to high concentrations of pollutants. On the basis of stress protein analysis, proteotoxic action of environmental compounds must be considered at least for these two sites. As well, histopathology indicated significantly impaired integrity of the gills for the sites 1 and 2, and also considerable, though not significant impairment at site 3. It is known that gills are particularly sensitive to metals (Mallat, 1985) since they are the target organ for their uptake. Chemical analytics have shown extreme contamination of these sites with cadmium and copper (up to 8.7 and 49.2 mg per kg sediment, respectively; Sandu *et al.*, 2006, this issue) and, thus, it is likely that proteotoxicity and subsequent disintegration of cellular structures in the gills of fish were exerted by these metals. Nevertheless, fish seemed to be able to cope with this burden to some extent and to accumulate these two metals in their livers as symbolized by the exceptionally high concentrations measured in this organ (Triebskorn *et al.*, 2006, this issue). This sequestration over time seems to level out the differences in concentrations in the outer environment at the different sites for some part, since all fish showed equally high stress protein levels in their livers. Therefore, it has to be assumed that hepatic metal accumulation took place in a way which largely removes the stored metals from physiological acute impact, e.g. by protein-mediated sequestration or precipitation as insoluble salts. Even though background data for hepatic hsp70 are lacking for European chub and sneep, absolute values corresponded to the highest gill hsp70 levels measured in this study. Liver histopathology also revealed strong impairment of health, predominantly at site 1 but also at sites 2 and 3 (Triebskorn *et al.*, 2006, this issue). As well as biochemical markers, also the histopathology data taken from Triebskorn *et al.* (2006, this issue) did not correlate with morphometrical parameters with the exception of gill pathology in *L. cephalus*, which slightly correlated in a negative way with the individual age ($p = 0.034$).

(2) Exposure. As mentioned earlier, massive metal contamination presumably deriving from mining activities and metallurgical processing in Western Transsylvania (Sandu *et al.*, 2006, this issue) is ecotoxicologically crucial for the investigated stretch of the River Mureş. The most relevant metals were cadmium and copper the concentration of which in the sediment surpassed the quality criteria of a number of Western states. Both metals were found to accumulate in the liver of abundant fish (Triebskorn *et al.*, 2006, this issue). Moreover, significantly elevated hepatic EROD activities indicated the presence of aromatic organics in fish caught downstream the city of Arad. However, the absolute values measured for EROD activity were not extremely high and, thus, the influence of PAHs, PCBs and other CYP1A-inducing organics seemed to be limited as indicated by their low concentrations in the sediment (Sandu *et al.*, 2006, this issue). The differences in EROD activity between the two fish species may be a consequence of their feeding behaviour: *L. cephalus*, which feeds mainly on plankton in the water column, seems to be less affected by organic pollution than *C. nasus,* which feeds on periphyton and detritus. Due to their high affinity to organic matter, organic pollutants tend to associate with other suspended particles settling to the sediment and, thus, the accumulation of organic pollutants in sediment makes benthic species feeding on contaminated algae or detritus more vulnerable than pelagic ones. At sites 1 and 2, microbiological analyses revealed an additional impact of faecal waste being acutely released into the Mureş (Sandu *et al.*, 2006, this issue) which may have substantially contributed to the fact that the fish caught at site 1 in early summer of 2004 were not suitable for human consumption (Mureş Sampling Consortium, personal experience).

(3) Community integrity. Community-level indicators measure the state of an ecosystem. These indicators are of highest ecological relevance but rather insensitive compared to subindividual markers, and responses to pollution occur either very slowly or are camouflaged by background noise. Even though community indices have little plausibility for cause-effect mechanisms, they may provide

evidence of cause through association with a sampling location. In this monitoring, the diversity of plankton was affected by a recent flood two weeks before sampling, and the structural parameters did not vary significantly among the investigated sites. Still, a slight decrease of diversity index was recorded at site 1 (Sandu *et al.*, 2006, this issue) which spatially corresponds to the highest concentrations of metals. Since logistic constraints only allowed to sample the sites once, the sampling design was particularly critical for a community survey in a variable environment and, thus, the existing dataset seems not to be robust enough for a final conclusion.

5 Conclusions and outlook

Applying an approach that integrates indicators and markers of different character at different levels of biological organization, we showed that a single cross-survey of even larger stream stretches can provide a refined view of the situation in the aquatic environment. This approach turned out to be especially suitable for regions where pollution can be anticipated but ecotoxicological data are scarce. This is particularly true for the countries of Eastern Europe, as the economy and hence the pollution both quantitatively and qualitatively is very much different from the industrialized countries in Western Europe and North America. It its recent report on the structural development of future member states, the European Commission has included environmental pollution in the list of areas of serious concern for Romania (European Commission, 2005). This report stated that "the capacity to issue integrated permits of a sufficient quality (...) for all industrial installations (...) represents a major challenge and requires serious efforts". Furthermore, "serious concerns exist in relation to industrial pollution. Considerable efforts are required to ensure that relevant permits are issued at local and regional level. (...) The monitoring of water quality requires further enhanced efforts." Not much work is being done to detect pollution effects in the catchment area of the Lower Danube by applying biomarkers and using fish as biomonitors as suggested earlier by Burkhardt-Holm and Bloesch (2000). To the best of our knowledge, a single biomarker study has been conducted in the Danube tributary, River Drava (Croatia) which revealed an inhibition on acetylcholine esterase and an increase in EROD activity in the Prussian and the common carp (*Carassius auratus gibelio* and *Cyprinus carpio*) indicating pollution by organophosphates and polyaromatic and/or polychlorinated hydrocarbons (Jaric and Stepic, 2005). Also in the Mureş case, our results indicated a situation of concern, at least in view to impaired fish health. In this situation, the advantage of biomarkers as sensitive early-warning sentinels becomes clear, particularly since they are able to integrate the effects of the entirety of contaminants, not only of those, respectively, selected for chemical analysis. Conventional limnochemical analyses and macrozoobenthos faunistics, commonly used in water quality assessment, were not relevant in this case, stressing the importance of applying combined approaches like the present one. In combination with the results presented by Sandu *et al.* (2006, this issue) and Triebskorn *et al.* (2006, this issue) this study could reveal both the character of (a number of) discharged substances and resulting effects, exhibiting ways to terminate further pollution in view to a restoration of the system. On the basis of our entire study, not only on the basis of the biomarkers presented in this paper, we propose that the metal pollution of the River Mureş derives from the adjacent mining and metallurgical activity and that its ecological impact probably is much more severe in those upstream tributaries which directly pass the industrial areas. The spatial limitation of sampling and the fact that our initiative was the first of its kind in Romania, however, characterizes this monitoring as a pilot study. Nevertheless, it should be considered as a starting point for larger surveys of the Danube River system including its tributaries in Eastern Europe. Profound ecotoxicological information on the situation in the downstream part and the catchment area of one of Europe's largest streams is urgently needed.

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