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Antioxidant enzyme responses of *Ephemera danica* (Insecta: Ephemeroptera) to heavy metal stress

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Abstract: The aim of this study was to evaluate whether *Ephemera danica* Müller, 1764 larvae can serve as bioindicators of heavy metal pollution, using antioxidant enzymes as biomarkers. Larvae, water, and sediment from four localities were analysed for the presence of arsenic, chromium, cadmium, copper, iron, manganese, nickel, and lead. The dependence of SOD, CAT, GPx, and GSH activity in larvae on the concentration of metals in water, sediment, and larval tissue was examined. Heavy metal exposure was closely associated with changes in antioxidant mechanisms, indicating metal-induced oxidative stress. The different responses of SOD, CAT, GPx, and GSH suggest varying sensitivities among components of the antioxidant system. In particular, GPx activity proved to be a reliable indicator of oxidative stress. These results confirm that *E. danica* larvae are a sensitive indicator of heavy metal pollution in aquatic ecosystems.

Keywords: oxidative stress; aquatic insects; environmental contamination; metal toxicity.

INTRODUCTION

Water pollution is a major global and local issue, causing approximately 1.4 million deaths annually.[1] Among various pollutants, heavy metals are particularly significant. They enter aquatic ecosystems naturally through erosion, volcanic activity, and leaching, but their current presence is mainly associated with urbanisation, industrialisation, municipal waste, and intensive agriculture. Interest in their effects arises from their accumulation in the environment and high toxicity to organisms. Although some metals are essential, elevated concentrations present a global problem because they cannot be degraded, accumulate through food

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chains, and disrupt ecosystem balance. They persist in sediments and accumulate in aquatic organisms, causing various toxic effects.[2] Heavy metals also induce oxidative stress in aquatic insects, especially when other prooxidants are present, emphasising the importance of biomonitoring in polluted waters.

Aquatic insects are reliable bioindicators of metal contamination, reflecting metal levels in their habitats. Among them, *E. danica* is notable as a model species due to its morphology, burrowing lifestyle, detritivorous feeding, and relatively large body size. It can also be collected in sufficient numbers for analysis. Its larval stage lasts 1–3 years enabling long-term monitoring of pollution effects.[3]

Heavy metals affect biochemical and physiological processes in organisms. Biomarkers are widely used to assess pollution and its biological effects in both field and laboratory studies.[1] They indicate sublethal molecular, cellular, or physiological changes caused by pollutant exposure or by contaminants present in the environment. In aquatic organisms, oxidative stress indicators – lipid peroxidation and enzymatic or non-enzymatic antioxidants – are useful for evaluating environmental stress. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), along with glutathione (GSH), are established pollution biomarkers in various organisms.[4]

The aim of this study is to evaluate whether *E. danica* larvae can serve as sensitive bioindicators of heavy metal pollution using antioxidant biochemical markers.

EXPERIMENTAL

Study site

The Skrapež River, a left tributary of the Đetinja River in western Serbia, is 48 km long and has a catchment area of 743 km². It originates on the southeastern slopes of the Povlen Mountains at about 1100 m a.s.l., below Mali and Veliki Povlen, and is formed by several springs, the largest of which is Taorsko vrelo. Its upper course is characterised by cold, oxygen-rich water, typical of mountainous streams in Serbia.[5] The trout pond "Kraj vodenice" (production area 588 m²) is located near the village of Radanovci on the left bank of the Skrapež River, approximately 2 km downstream from the Taorsko vrelo springs near Kosjerić. Macrozoobenthos was sampled at four sites: one upstream (S1) and three downstream of the trout pond (S2, S3 and S4) with site characteristics presented in Table I.

Collection of samples

Hydrobiological surveys of the Skrapež River were carried out in April, July, and October 2015, and in January 2016. Larvae of *Ephemera danica* were collected using a benthic sieve and tweezers. Only fourth-instar larvae were used for biochemical analyses in order to minimize variability associated with ontogenetic differences. At each site, 20–25 individuals were cryopreserved in liquid nitrogen for antioxidant enzyme analyses and transported to the Department of Biochemistry and Physiology of Insects, Institute for Biological Research "Siniša Stanković". An additional 20–25 individuals were fixed in 96% ethanol for heavy metal analysis and delivered to the Environmental Protection Agency laboratory. Water and sediment samples were collected simultaneously for heavy metal determination. Several larvae from each

site were preserved in 96% ethanol for identification, which was performed using a Zeiss Discovery V8 stereomicroscope and standard taxonomic keys.

TABLE I. Basic characteristics of the investigated localities of the river Skrapež

Locality	S1	S2	S3	S4
Location of the site	180m upstream of the pond	30m downstream of the pond	300 m from the previous location	300 m from the previous location
Geographic coordinates	44°04'00.8" 19°50'13.3"	44°03'55.9" 19°50'21.2"	44°03'47.8" 19°50'28.6"	44°03'40.9" 19°50'28.1"
Elevation, m	573	566	552	550
Average flow depth, cm	23.08	17.25	16.5	21.24
Stream width, m	4	1.39	6.7	8.12
Average water temperature, °C	12.1	11.9	11.8	11.4
Average flow rate, m s^{-1}	0.27	0.55	0.35	0.34
Riparian vegetation	Field and forest	Meadow and field	Field and forest	Field and forest
Flow regulation	Land flow regulation	Land flow regulation	Land flow regulation	Land flow regulation

Analysis of heavy metals in the water, sediment and in the body of Ephemera danica

A Van Veen dredge with a catchment area of 260 cm^2 was used to remove 1000 g of sediment from the soft riverbed, while a rake and shovel were used to collect sediment from the hard riverbed. For the analysis of heavy metal content, water samples were taken at all four sites 30 cm below the water surface in plastic bottles with a volume of 500 ml. To preserve these samples (pH < 2), 0.5 ml of concentrated HNO_3 was added to each water bottle. The labelled bottles containing the sediment and water samples were taken to the laboratory of the Institute of Chemistry, Technology and Metallurgy in Belgrade, where the analysis for heavy metals was carried out. The concentrations of the following elements were measured: arsenic (As), chromium (Cr), cadmium (Cd), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni) and lead (Pb), using the technique of inductively coupled plasma optical emission spectrometry (ICP-OES). More detailed descriptions of the methods can be found in Vranković *et al*.[6] The collected larvae of *E. danica* were taken to the chemical laboratory of the Environmental Protection Agency to determine the concentration of heavy metals in their bodies. The larvae were analysed as individual samples, enabling assessment of variability within the population and increasing the statistical reliability of the results. The individuals were analysed for the presence of the heavy metals mentioned above according to the standard method.[7]

Biochemical analysis (SOD, CAT, GPx activity and GSH content)

The larvae of *E. danica*, transported from the field to the laboratory in a cryocontainer, were weighed on a balance with an accuracy of 0.0001 g (AE 163; Mettler-Toledo International) and stored at -80 °C until the homogenate was prepared. Homogenates of whole larvae were used to determine and analyse the components of the antioxidant protection system. The larvae

were homogenised on ice in sucrose buffer, pH 7.4 (0.25 M sucrose, 0.05 M TRIS, 0.10 M EDTA; Serva, Heidelberg, Germany), so that the tissue concentration was 100 mg mL^{-1}. Homogenisation was performed in three cycles of 10 seconds, with 15-second pauses at 2000 rpm. The resulting homogenates were sonicated (3 × 10 seconds with 10-second pauses at a frequency of 20 kHz; Sonicator model HD2070, Bandelin, Berlin, Germany) and then centrifuged for 100 minutes at 37,000 g and 4 °C (Beckman L7-55 ultracentrifuge). For the determination of total glutathione (GSH), a portion of the sonicated homogenates was centrifuged for 10 minutes at 5000 rpm (centrifuge model 5417R, Eppendorf, Hamburg, Germany) after the addition of 5% sulfosalicylic acid (Sigma-Aldrich Chemie, Steinheim, Germany), which was used to precipitate proteins. After centrifugation, the supernatants were isolated and frozen at -80 °C. The total protein concentration in the samples was determined according to the Bradford method using bovine serum albumin (Sigma-Aldrich Chemie, Steinheim, Germany) as the standard.[8] Superoxide dismutase (SOD) activity was determined according to the standard method.[9] The coupled enzyme method was used to determine glutathione peroxidase activity.[10] In homogenates in which proteins were precipitated with sulfosalicylic acid, the amount of total glutathione was determined.[11] Catalase activity was determined by measuring the decomposition of the substrate H_2O_2.[12]

Biota-sediment accumulation factor

The biota–sediment accumulation factor (BSAF) was used to assess the ability of *E. danica* larvae to accumulate heavy metals from sediment. BSAF was calculated as the ratio of metal concentrations in larval tissue to those measured in the sediment.[13]

Statistical analysis

To analyse the data, univariate and multivariate statistical methods were applied. Based on the Shapiro–Wilk test and normal probability plots, either Pearson or Spearman correlation analyses were used to assess relationships between metals and arsenic from different sources (water, tissue, and BSAF) and enzyme activities. Linear regression was used to further illustrate significant correlations. The influence of elements on enzyme activities was also examined using canonical correspondence analysis (CCA) and redundancy analysis (RDA) with elements as independent variables and enzyme activities as dependent variables. All analyses were performed using PAST and STATISTICA 8.[14]

RESULTS AND DISCUSSION

The dependence of SOD, GPx, and GSH activities in the tissue of *Ephemera danica* on the concentrations of heavy metals in water, sediment, ephemeral tissue, and BSAF was investigated (Table II). Catalase activity was below detection limits, so these data were not included in the analysis. Significant correlations ($p < 0.05$) were observed between several metals and enzyme activities (Table II), whereas metals in the sediment showed no significant relationships with the measured biomarkers. The activities of SOD and GSH are dependent on the arsenic concentration in the water (negative correlation), while SOD activity is negatively correlated with the Cd concentration in the water. GPx activity is positively correlated with the concentrations of Ni and Pb in the tissues of *E. danica*, while the correlation with Cd is negative. GPx activity was also positively correlated with Ni and Pb concentrations in BSAF (Table II). To show relationships between

correlated metals and enzyme activities in more details, linear regression plots are provided (Figure 1).

TABLE II. Correlations among activities of SOD, GPX and GSH enzymes in *E. danica* and heavy metals concentrations in *E. danica*'s tissue, water, BSAF and sediment. Values in bold are statistically significant ($p < 0.05$).

E. danica larvae	As	Cr	Cd	Cu	Fe	Mn*	Ni	Pb
SOD	0.14	0.11	0.00	0.05	0.10	-0.04	0.10	0.01
GPx*	0.11	0.29	-0.70	0.29	0.36	0.12	0.75	0.62
GSH	0.09	-0.04	0.16	-0.15	-0.04	0.08	0.01	-0.11
Water	As	Cr*	Cd*	Cu*	Fe*	Mn*	Ni*	Pb*
SOD	-0.64	0.43	-0.62	-0.37	0.47	0.42	0.36	0.42
GPx*	-0.24	-0.20	0.06	-0.11	-0.06	0.03	0.00	0.17
GSH	-0.59	0.36	-0.34	-0.36	0.36	0.32	0.25	0.20
Sediment	As*	Cr	Cd	Cu	Fe	Mn	Ni	Pb*
SOD	-0.13	0.12	0.25	0.12	0.12	0.11	-0.09	0.09
GPx*	0.02	0.38	0.27	-0.05	0.07	-0.38	0.03	0.12
GSH	0.08	0.01	-0.02	-0.11	0.03	0.15	-0.20	-0.03
BSAF	As*	Cr	Cd*	Cu	Fe	Mn*	Ni	Pb
SOD	0.32	-0.12	-0.28	0.04	0.10	-0.01	0.11	-0.11
GPx*	0.05	-0.20	-0.50	0.30	0.39	0.21	0.70	0.76
GSH	-0.18	-0.09	0.00	-0.12	-0.03	0.10	0.10	-0.11

With "*" are marked elements tested with Spearman correlations. The rest are tested with Pearsons parametric correlations.

Correlation matrices of metals showed that there are no highly correlated elements in *E. danica* tissue and BSAF, while highly correlated elements (r greater than 0.9, for p = 0.05) were found in water samples: Fe with Mn and Ni, and Mn with Ni. The presence of highly correlated elements among the hypothesised independent variables indicates collinearity in the data set. Therefore, in the multivariate analysis testing the influence of metals from water on enzymes, RDA was chosen instead of CCA. As shown in Figures 2 and 3, the multivariate analyses support the results of the univariate tests and provide additional information on the relationships between metals and enzymes at the investigated sites. The situation for *E. danica* tissue and BSAF is very similar (Figure 2). Along the first axis (positive gradient of Ni and Pb and negative gradient of Cd), increased activity of GPx can be observed on the left side of the graph (samples from S2 and some samples from S4), and decreased activity of the same enzyme with increasing Cd concentration (samples from S1 and S3 and some samples from S4). The only slight difference is that in *E. danica* tissue, Ni has the strongest influence on the activity of the respective enzyme, while in BSAF it is Pb. In contrast to *E. danica* tissue and BSAF and their influence on GPx activity, metals from the water do not appear to influence the activity of this enzyme (Figure 3). However, the influence of As and Cd from water on the activities of two other enzymes (SOD and GSH) was observed. Along the first canonical axis, determined by the As and Cd

gradients, the distribution of samples and enzyme activities can be followed. On the right side of the graph, higher concentrations of As and Cd and lower activities of SOD and GSH are found in samples from the autumn and winter seasons, while on the opposite side are samples from spring and summer (lower concentrations of As and Cd and higher activities of SOD and GSH). Unlike *E. danica* tissue and BSAF elements and enzyme activities, where spatial variability could be observed (Figure 2), for elements from water and their relation to enzyme activities, seasonal variability could be observed (Figure 3).

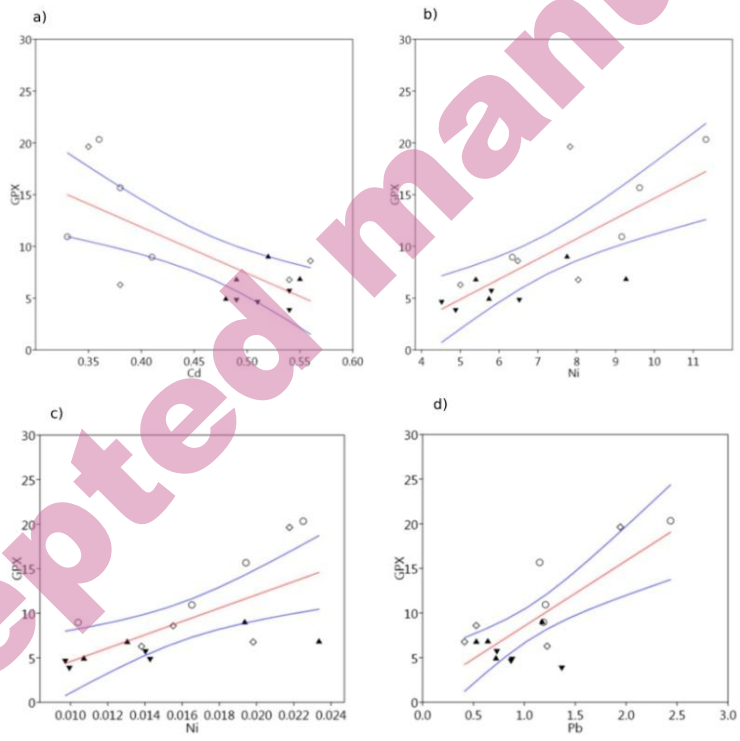

Figure 1. Effects of heavy metals on antioxidant enzyme activities in *Ephemera danica* from the Skrapež River. The straight line represents the best linear fit of the data. The curved lines represent 95% regression fit. a) Effect of Cd from *E.danica* tissue on GPx activity (R=-0.7, R^2=0.49, P=0.002); b) Effect of Ni from *E.danica* tissue on GPx activity.(R=0.75, R^2=0.56, P=0.001); c) Effect of Ni from BSAF on GPx activity.(R=0.7, R^2=0.49, P=0.004); d) Effect of Pb from BSAF on GPx activity.(R=0.76, R^2=0.58, P=0.001); Enzymes activities from *E.danica* and BSAF from four investigated localities are presented with the following simbols: filled inverted triangle (S1), circle (S2), filled triangle (S3) and diamond/square (S4).

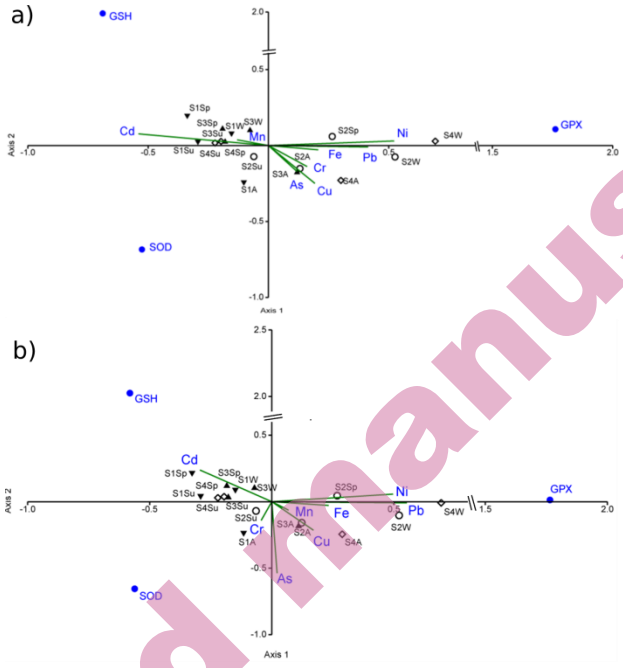

Figure 2. CCA triplot summarising the enzymes activities along heavy metals gradients from various sources: a) Heavy metals in *Ephemera danica* tissue (Axys 1; 95.6 % of variability explained; Ni, Pb and Cd gradient); b) Heavy metals as BSAF (Axys 1; 92.3 % of variability explained; Ni and Pb gradient); Enzymes activities from *E.danica* from four investigated localities are presented with the following simbols: filled inverted triangle (S1), circle (S2), filled triangle (S3) and diamond/square (S4); Measured concentracions of heavy metals at investigated localities are presented as vectors originating from the centre of respective ordination diagrams.

Detailed concentrations of heavy metals in water, sediment, and *Ephemera danica* larvae have been reported in our previous study [15, 16] therefore, only the main findings are presented here. Among the metals analysed, cadmium showed the lowest concentrations in both *E. danica* larvae and water samples, with the minimum value recorded in larvae at site S2 during autumn (0.33 mg/kg), while concentrations in water remained below 0.1 mg/L at all sampling sites throughout the study period. In contrast, iron was the dominant metal in both matrices, reaching its highest concentration in larvae at site S2 during summer (1933.5 mg/kg) and in water at site S2 during autumn (259.3 mg/L). Sediment was characterised by elevated concentrations of iron, chromium, manganese, and nickel, with maximum values of 25,000 µg/g (site S2, winter), 763 µg/g (site S1, winter), 789 µg/g (site S2, winter), and 609 µg/g (site S2, summer), respectively.

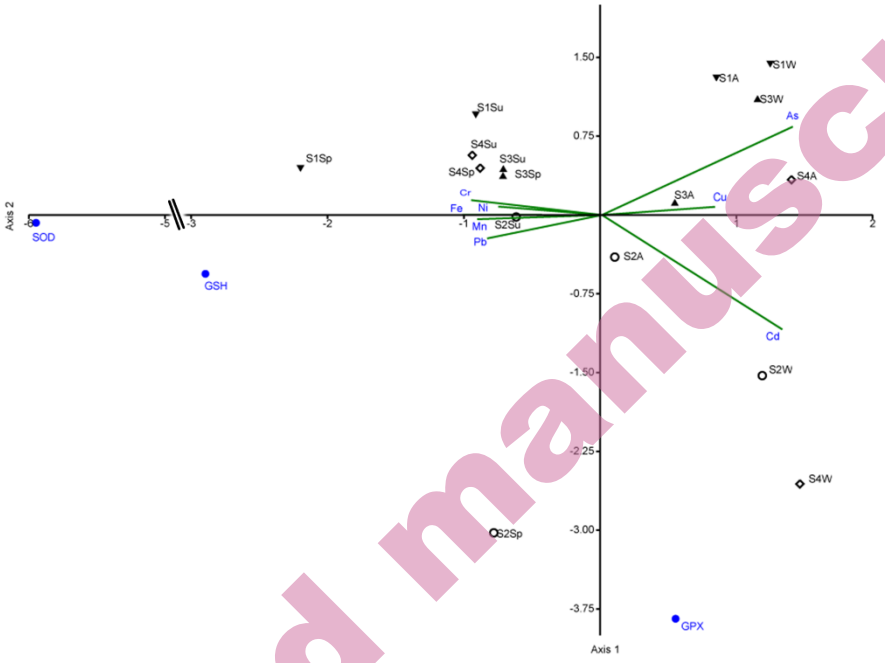

Figure 3. RDA triplot summarising the enzymes activities along heavy metals from water (Axys 1 52.3 % of variability; As and Cd gradient); Enzymes activities from *E. danica* from four investigated localities are presented with the following simbols: filled inverted triangle (S1), circle (S2), filled triangle (S3) and diamond/square (S4); Measured concentracions of heavy metals at investigated localities are presented as vectors originating from the centre of respective ordination diagrams.

Heavy metal exposure induces oxidative stress, and the resulting biochemical responses in aquatic organisms can serve as early indicators of pollution.[1,17,18,19,20] Comparison of measured metal concentrations with the maximum permissible concentrations[21] indicated that only Ni and Cr exceeded this concentrations (44 mg/kg and 240 mg/kg, respectively), while concentrations of all other analysed metals remained below the permitted values for surface water sediments. Considering the ecology of *Ephemera danica* and the obtained BSAF values, sediment likely represents an important pathway for metal uptake, as larvae spend most of their life cycle burrowing within the substrate and feeding on detritus.

Superoxide dismutase (SOD) is a sensitive biomarker and the first line of defence against reactive oxygen species (ROS), indicating oxidative stress and toxic effects. In our study, SOD activity was negatively correlated with cadmium and arsenic, common water contaminants that induce oxidative stress in aquatic insects. Although initial exposure can increase SOD activity, prolonged high concentrations may inhibit the enzyme due to depletion of antioxidant defences or

direct damage, which is consistent with our results. In the study by Toto *et al.*[1] larvae of *C. pipiens* collected from heavy metal-polluted sites also showed significantly reduced SOD activity. The results of Žikić *et al.*[17] showed that SOD activity in the erythrocytes of *Carassius auratus gibelio* decreased significantly after 24 hours of exposure to cadmium. Azam *et al.*[18] also indicated that long-term exposure to metals decreases the activity of this enzyme along with antioxidant capacity. SOD enzyme activity was significantly lower in mayflies found at polluted sites compared to other sampling sites, suggesting some degree of inhibition. Our results contradict the findings of Islam *et al.*[19] who observed increased SOD activity in *Antheraea assamensis* due to exposure to heavy metals. In addition, Patang and Soegianto[20] detected higher concentrations of SOD in Chironominae and *Gomphus*. In addition to SOD which is crucial for converting superoxide into hydrogen peroxide and oxygen, the enzyme catalase (CAT) also plays an important role in detoxifying hydrogen peroxide in water and within the cellular environment. In our study, CAT activity in *E. danica* was below the detection limit. This lack of CAT activity was unexpected, as significant changes in CAT activity have been documented in aquatic organisms, under the influence of various pollutants.[22] This reduction may result from enzyme inhibition or decreased expression, explaining the lack of correlation between antioxidant activities and chromium, manganese, and copper in sediment and water. Other studies have also shown that exposure to chromium can significantly reduce the activities of important antioxidant enzymes such as CAT in aquatic insects, leading to increased ROS accumulation and oxidative damage.[23]

Copper is an essential trace element that can induce oxidative stress in aquatic insects by disrupting the balance between ROS production and antioxidant defences.[24] Copper and high manganese concentrations, as found in Skrapež River sediment, can inhibit or impair catalase activity.[25] Toto *et al.*[1] observed that reduced SOD activity led to significantly reduced catalase activity in larvae of the mosquito *C. pipiens* exposed to heavy metals. Changes in the activity of antioxidant enzymes can be balanced among different enzymes, as demonstrated by the reduction in catalase (CAT) activity in *E.danica* larvae, which can be compensated by increased glutathione peroxidase (GPx) activity.[26] The absence of detectable CAT activity may be due to low concentrations of hydrogen peroxide, which activates glutathione peroxidase as the primary protection against oxidative stress. The results of our study indicate a positive correlation of GPx activity with lead and nickel in *E. danica* larvae, as well as with BSAF, while a negative correlation was found with cadmium. Lead, a non-essential element primarily originating from human activities, is strongly bound to suspended particles, which reduces its bioavailability.[27] Exposure can induce oxidative stress, increase ROS and biomolecular damage, and have complex effects on antioxidant systems,

growth, development, reproduction, and the overall physiological state of aquatic insects.

Nickel (Ni) is toxic and induces oxidative stress in aquatic organisms, affecting antioxidant enzymes such as GPx. In *Carassius auratus*, Ni exposure suppressed GPx activity, leading to cellular damage and apoptosis.[28] Similarly, in *Hydropsyche exocellata* larvae, increased metal contamination, including Ni, altered the activities of SOD, CAT, GPx, and GST, indicating oxidative stress from combined metal exposure.[29]

Cadmium (Cd) is a highly toxic, non-essential element that damages cell membranes and inhibits glutathione-related antioxidant enzymes, depleting cellular glutathione and potentially leading to cell death. [30]Reduced GPx activity may result from the direct effects of pesticides or heavy metals, often serving as an initial response to pollutants. [31,32] GPx plays a key role in the antioxidant network, interacting with other enzymes such as SOD, and serves as a sensitive indicator of oxidative stress, being particularly responsive to various pollutants beyond heavy metals.[33,34] Toto *et al.*[1] also report a decrease in GPx activity in larvae at polluted sites. Barata *et al.*[29] states that no GPx activity was detected in *Hydropsyche* larvae. Borković-Mitić *et al.*[35] found positive correlations between GPx activity and Cd concentration. Glutathione peroxidase (GPx) uses GSH to reduce hydrogen peroxide and organic hydroperoxides, while GSH protects against metal-induced toxicity. Depletion of GSH and accumulation of GSSG indicate oxidative stress.[36, 37] Our research results show a negative correlation between GSH concentration and arsenic in water. Glutathione (GSH) plays a crucial role in maintaining cellular redox status, especially when cells are exposed to arsenic. GSH acts as an electron donor in the reduction of arsenate (As^{5+}) to arsenite (As^{3+}), a key process in arsenic detoxification. During this reduction, GSH is oxidised to glutathione disulphide (GSSG), which is then converted back to GSH by the enzyme glutathione reductase using NADPH as a reducing agent. Several studies have demonstrated the role of GSH in reducing arsenic toxicity. For example, elevated GSH levels have been observed as a protective mechanism against arsenic-induced oxidative stress. However, under severe oxidative stress, GSH reserves may be depleted, resulting in failure of the cellular antioxidant defence system and increased susceptibility to arsenic toxicity.[38] Its fluctuations, in relation to glutathione-dependent enzymes, can serve as an early indicator of physiological changes and provide insight into xenobiotic-induced oxidative stress. Studies have shown that exposure to heavy metals can reduce GSH levels in aquatic insects. Increased GSH levels have been observed in some species as an adaptive response to chronic exposure to low concentrations of pollutants, increasing resistance to oxidative stress.[39] While antioxidant protection persists under mild oxidative stress, GSH reserves are depleted under intense stress.

This study highlights the complex interactions between heavy metals and antioxidant mechanisms in aquatic insects, which are crucial for understanding their responses to pollution. Fluctuations in oxidant and antioxidant biomarkers can indicate metal pollution and other environmental stressors, including oxygen, temperature, salinity, and organic pollutants.[34] Seasonal variations and long-term exposure may affect biomarker interpretation, emphasising the value of controlled laboratory studies to assess pollution effects accurately.

CONCLUSION

Alterations in antioxidant defences of *Ephemera danica* larvae were closely linked to heavy metal exposure, reflecting metal-induced oxidative stress. Variations in SOD, CAT, GPx, and glutathione indicate differing susceptibility of antioxidant components, with GPx showing strong potential as a biomarker. These results confirm that *E. danica* larvae are sensitive bioindicators of freshwater metal pollution, and controlled laboratory studies are recommended to clarify causal relationships.

И З В О Д

ОДГОВОР АНТИОКСИДАТИВНИХ ЕНЗИМА КОД *EPHEMERA DANICA* НА ИЗЛОЖЕНОСТ ТЕШКИМ МЕТАЛИМА

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Циљ овог рада био је да се процени да ли ларве *E. danica* могу послужити као биоиндикатори загађења тешким металима коришћењем антиоксидативних ензима као биомаркера. Анализирани су узорци ларви, воде и седимента са четири локалитета на присуство арсена, хрома, кадмијума, бакра, гвожђа, мангана, никла и олова. Испитана је зависност активности SOD, CAT, GPx и GSH у ларвама од концентрација метала у води, седименту и ткиву ларви. Изложеност тешким металима била је уско повезана са променама у антиоксидативним механизмима, што указује на оксидативни стрес изазван металима. Различити одговори антиоксидативних ензима сугеришу различиту осетљивост компоненти антиоксидативног система. Посебно се глутатион-пероксидаза показала као поуздан индикатор оксидативног стреса. Ови резултати потврђују да су ларве *E. danica* осетљив показатељ загађења тешким металима у воденим екосистемима.

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