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Bioaccumulation of heavy metals in *Ephemera danica* larvae under influence of a trout farm outlet waters

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Abstract – Trout farms are one of the major sources of pollution of highland streams and rivers. Since river sediment burdened with organic pollution binds greater amounts of heavy metals we investigated the influence of the trout farm on the accumulation of metals in the sediment, water and *Ephemera danica* larvae. Research was conducted seasonally (April, July, and October of 2015 and January of 2016) at one control locality (SK1) and three localities downstream from the farm (SK2–SK4). In agreement with the hypothesis the fish farm discharge induced localized and statistically significant increase of concentrations of Fe, Cu, Cr, Ni, Pb and Cd in sediment and *E. danica* larvae, but not in water at locality SK2 just below the fish farm indicating that sediment is the main source of heavy metals for this organism. However, according to the values of Biota sediment accumulation factor (BSAF) only metals with low sediment concentrations (As, Cd and Cu) accumulated in the larvae. Moreover, BASF values for toxic metals (As, Cd and Cr) showed negative correlation with their concentrations in sediment indicating existence of defense mechanisms in *E. danica* against assimilation of these metals in high concentrations lethal for it. On the other hand, BSAF values for essential microelements (Cu and Fe) were positively correlated with their sediment concentrations. In conclusion the trout farm induced accumulation of heavy metals in river sediment and *E. danica* larvae proved to be a good bioindicators of the pollution of river systems by As, Cd and Cu.

Keywords: Trout farm / sediment / *Ephemera danica* larvae / metals / accumulation

Résumé – Bioaccumulation de métaux lourds dans les larves d'*Ephemera danica* sous l'influence des effluents d'un élevage de truites. L'élevage de truites est l'une des principales sources de pollution des ruisseaux et des rivières des régions montagneuses. Comme les sédiments fluviaux chargés de pollution organique lient de plus grandes quantités de métaux lourds, nous avons étudié l'influence de l'élevage de truites sur l'accumulation des métaux dans les sédiments, l'eau et les larves d'*Ephemera danica*. La recherche a été menée de façon saisonnière (avril, juillet, octobre 2015 et janvier 2016) dans une localité témoin (SK1) et trois localités en aval de la ferme (SK2 – SK4). En accord avec l'hypothèse que le rejet de la pisciculture a induit une augmentation localisée et statistiquement significative des concentrations de Fe, Cu, Cr, Ni, Pb et Cd dans les sédiments et les larves d'*E. danica*, mais pas dans l'eau à la localité SK2 juste sous la pisciculture indiquant que les sédiments sont la principale source des métaux lourds pour cet organisme. Cependant, selon les valeurs du facteur d'accumulation des sédiments du biote (FASB), seuls les métaux à faible concentration sédimentaire (As, Cd et Cu) se sont accumulés dans les larves. De plus, les valeurs de BASF pour les métaux toxiques (As, Cd et Cr) ont montré une corrélation négative avec leurs concentrations dans les sédiments indiquant l'existence de mécanismes de défense chez *E. danica* contre l'assimilation de ces métaux en fortes concentrations mortelles pour lui. D'autre part, les valeurs de BSAF pour les microéléments essentiels (Cu et Fe)

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étaient positivement corrélées avec leurs concentrations dans les sédiments. En conclusion, l'accumulation de métaux lourds dans les sédiments fluviaux et les larves d' *E. danica* s'est avérée être un bon bioindicateur de la pollution des systèmes fluviaux par As, Cd et Cu.

Keywords : Élevage de truites / sédiments / larves d'*Ephemera danicae* / métaux / accumulation

1 Introduction

Strong anthropogenic influence, discharge of industrial and communal wastewaters and runoff of artificial fertilizers from agricultural land are factors that cause accumulation of various substances, including metals, in rivers (Schueler, 2000; Çevik *et al.*, 2009; Triquet-Amiard *et al.*, 2013), which leads to deterioration of the quality of aquatic environments and threatens the survival of aquatic organisms. Together with other animal organisms, insects are constantly exposed to action of the indicated anthropogenic stressors, which cause daily and seasonal fluctuations of water temperature, changes of dissolved oxygen concentrations and food availability and the presence of pollutants (De Almeida *et al.*, 2007; Despotović, 2013). Metals are among the most significant pollutants, in view of their stability in the ecosystem and transport through the food chain (Shahbaz *et al.*, 2013) and considering the fact that they enter aquatic ecosystems via runoff from land subjected to drainage by man or as a consequence of other forms of human activity (Sakan *et al.*, 2011; Waykar and Deshmukh, 2012). Owing to their toxicity and increased concentration in aquatic ecosystems, metals represent a growing problem on the global level, since once they enter an ecosystem they constitute a potential danger to living organisms for a number of years (Geffard *et al.*, 2003; Fernandes *et al.*, 2007; Snodgrass *et al.*, 2008; Besser *et al.*, 2009; Abdel-Baki *et al.*, 2011), in as much as they are deposited both in the sediment (Fernandes *et al.*, 2007; Abdel-Baki *et al.*, 2011) and in tissues of aquatic organisms, where in elevated concentrations they have various toxic effects (Shahbaz *et al.*, 2013: Ezejiofor *et al.*, 2013). It is known that river sediments can receive high concentrations of heavy metals, which remain as difficultly degradable matter in the rivers (Fernandes *et al.*, 2007; Abdel-Baki *et al.*, 2011), and if the sediment contains a large amount of organic material it can bind metals in even greater measure (Zhang *et al.*, 2014; Hsu *et al.*, 2016; Strom *et al.*, 2011). Increase in the amount of organic matter in a rivers sediment can occur under influence of the outlet water from a trout farm, which introduces remains of uneaten food and trout faeces into the recipient stream (Liao, 1970; Weston *et al.*, 1996), with the result that metals accumulate in the sediment directly below the trout farm, enabling aquatic organisms to accumulate them in some measure (Burridge *et al.*, 2010).

The list of significant polluters of aquatic environments includes Zn, Cu, Pb, Cd, Hg, Ni and Cr. Some of them like Cu, Zn and Cr are essential elements because they are co-factors of enzymes or a constituent of the respiratory pigment (haemocyanin). However, certain metals such as As, Hg and Cd in higher concentrations have a toxic effect. Their ions, even in low concentrations, can have serious effects on the health of living beings (Despotović, 2013). The degree of accumulation of metals in the body of organisms is affected both by physiological processes in the organisms themselves and by changes in physical and chemical parameters of the environment (Vicente-Martorell *et al.*, 2009; Shulkin *et al.*, 2003). Feeding also exerts considerable influence on metal accumulation in the body of vertebrates and represents one of the main sources of metals for many aquatic invertebrates (Wang, 2002). Thus, differences in the feeding of organisms of the benthofauna determine differences in the bioaccumulation of metals in the body of aquatic invertebrates (Clements, 1991; Pourang, 1996; Corbi *et al.*, 2010).

Because of characteristics of their life cycle [confinement to the bottom, long duration of the life cycle, limited mobility (Hellawell, 1986; Bonada *et al.*, 2006; De Pauw *et al.*, 2006)], aquatic invertebrates are suitable bioindicators for monitoring the degree of accumulation of heavy metals both from the water and from the sediment (Fialkowski *et al.*, 2003). Moreover, certain responses of aquatic organisms can indicate a danger of contamination by heavy metals in the earliest phases of pollution (Gremyatchikh *et al.*, 2009), and monitoring changes at the individual level is more informative than monitoring changes at the community level for purposes of biomonitoring of aquatic ecosystems (Al-Shami *et al.*, 2010). In view of what has been said and considering the fact that larvae of Ephemeroptera accumulate certain heavy metals in higher concentrations than some other taxa (Burrows and Whitton, 1983; Fialkowski *et al.*, 2003) thanks to their specific morphological and functional characteristics (Cain *et al.*, 1992), we chose the species *Ephemera danica* as a test organism. It is a good candidate for a model organism owing to its burrowing way of life, mode of feeding (an active gatherer of detritus filterers) and relatively large body dimensions (Clements and Kiffney, 1994), as well as because it can be collected in numbers sufficient for analysis (Nummelin *et al.*, 2007). Also, its larval phase lasts one or two years, which makes it possible to monitor accumulation of heavy metals over a certain period of time (Svensson, 1977; Winkelmann and Koop, 2007; Bennett, 2007).

The present work is one piece of a comprehensive investigation of the trout farm effects on the various aspects of *E. danica* larvae biology. The first part of this investigation, dealing with the effects on the larvae antioxidative defense, has already being published (Božanić *et al.*, 2018). In as much as river sediment burdened with organic pollution binds greater amounts of heavy metals than an unburdened recipient stream (Hsu *et al.*, 2016; Burridge *et al.*, 2010), the purpose of the present work was to establish whether an increase in the concentration of certain heavy metals occurs in water and sediment of the Skrapež River, a stream under influence of the outlet water from a trout farm, and ascertain the extent of metal bioaccumulation in the body of *Ephemera danica* larvae so as to determine their potential usefulness as a bioindicator of the pollution of river systems by heavy metals.

2 Material and methods

2.1 Description of investigated localities

Detailed description of the study area (Fig. 1 in Božanić *et al.*, 2018), the trout farm and investigated localities is given in our previous publication (Božanić *et al.*, 2018) therefore, only the basic data are given here. Located in western Serbia, the Skrapež River is 47.7 km long and has a drainage area of 647.65 km^2 (Gavrilović and Dukić, 2002). The Kraj Vodenice (=Millside) Trout Farm is located along its course. Trout are reared in four independent basins on this fish farm, where they are given extruded feed (Skreting Optiline he 3p gal) with 0.9% phosphate content, 40% protein content and 28% fat content. Sampling was conducted at a control locality about 180 m upstream from the fish farm (locality SK1) and at three downstream localities (SK2, 30 m downstream from the fish farm; SK3, 300 m beyond locality SK2; and SK4, 300 m away from locality SK3).

2.2 Sampling of larvae and determination of heavy metal content

As already described in our previous publication (Božanić *et al.*, 2018) specimens of *E. danica* larvae were collected seasonally (in April, July and October of 2015 and in January of 2016) at four localities on the Skrapež River by using a benthos sieve and tweezers. At each locality, 20 specimens of larvae were collected and fixed in the field with 96% alcohol in glass bottles. The samples were transported to a laboratory of the Environmental Protection Agency, where they were analyzed for the presence of heavy metals according to U.S. EPA Method 200.3. This method is applicable for analysis of the following elements: arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), lead (Pb), nickel (Ni) and manganese (Mn).

Samples of *E. danica* larvae were first homogenized and a weighed amount (5 g) of a frozen tissue sample was then transferred to a 125-ml flask. A measured volume (10 ml) of concentrated nitric acid was added and the mixture warmed with gentle swirling of the samples on a hot plate until the tissue was solubilized (U.S. EPA Method 200.3). The temperature was increased to near boiling until the solution began to turn brown. The mixture was cooled, another batch (5 ml) of concentrated nitric acid was added to it and the sample was returned to the hot plate until the solution once again began to turn brown. It was then cooled and yet another batch (2 ml) of concentrated nitric acid was added, after which it was returned to the hot plate until its volume was reduced to 5–10 ml. After this, the mixture was again cooled, a measured volume (2 ml) of 30% hydrogen peroxide was added to it and the sample was returned to the hot plate until its volume was reduced to 5–10 ml. It was then cooled once more, a measured volume (2 ml) of concentrated hydrochloric acid was added to it and the sample was returned to the hot plate until its volume was reduced to 5 ml. The sample was then allowed to cool, after which it was quantitatively transferred to a 100-ml volumetric flask. Finally, the sample was diluted to volume with ASTM type I water and mixed so as to allow any insoluble material to separate (U.S. EPA Method 200.3). The concentration of metals in samples prepared in this way was determined by atomic absorption spectrophotometry (AAS) on a Perkin Elmer Analyst 600 instrument.

2.3 Analysis of heavy metals in the water and sediment

Water samples for analysis of heavy metal content were taken 30 cm below the water surface using plastic bottles with a volume of 500 ml. Detailed procedure for sediment collection is described in Vranković *et al.* (2018). Analysis of heavy metals content in water and sediment was conducted at the Institute of Chemistry, Technology and Metallurgy of Belgrade University's Chemistry Centre. Samples of water and sediment were analysed for the presence of the following elements: As, Cd, Cr, Cu, Fe, Pb, Ni and Mn.

Water samples were stabilized by the addition of 0.5 mL HNO_3 (70 wt.%, ACS reagent, Sigma Aldrich) and stored at 4 °C. Sediments were first homogenized, and the samples were then dried at 105 °C overnight. After this, sediment samples were powdered and passed through a sieve (<500 μm). Batches of samples weighing 0.5 g (accuracy ± 0.1 mg) were measured out into Teflon™ cuvettes and 15 ml of HNO_3 (70 wt.%, ACS reagent, Sigma Aldrich) and HCl (37 wt.%, ACS reagent, Sigma Aldrich) (1:3, v/v) were added. Microwave digestion was done using the ETHOS 1 Advanced Microwave Digestion System (Milestone, Italy) for a period of 20 min at 200 °C. After digestion, the contents of the cuvette were diluted to 25 ml with rinsing of the precipitate (Božanić *et al.*, 2019). Element concentrations were determined by the analytical technique of inductively coupled plasma optical emission spectrometry (ICP-OES) using a Thermo Scientific iCAP 6500 Duo ICP instrument (Thermo Fisher Scientific, Cambridge, UK). For calibration of the instrument, the following plasma standard solutions were used: SS-Low Level Elements ICV Stock (10 mg/L) and ILM 05.2 ICS Stock 1 (500 mg/L) (VHG Labs, Inc., LGC Standards, Manchester, NH 03103 USA); and Multi-Element Plasma Standard Solution 4, Specpure®, 1000 μg/ml (Alfa Aesar GmbH & Co., Germany). Quality control of the analytical process, performed using U.S. EPA Method 200.7 and LPC Solution Certified Reference Material (ULTRA Scientific, USA), indicated that the resulting concentrations were within 98–103%., The measurements were performed in triplicate ($n = 3$) for each digested sample.

2.4 Biota-sediment accumulation factor

Biota-sediment accumulation factor (BSAF) was used to quantify the capacity of *E. danica* larvae to accumulate heavy metals from the sediment (Mackay and Fraser, 2000). BSAF was calculated as the ratio of concentration of a metal in *E. danica* larvae (M_E) and its concentration in the sediment (M_s): BSAF = M_E/M_s.

2.5 Statistical analyses

Data were presented as mean ± standard error. Values of metal concentrations in the sediment, their concentrations in larvae of *E. danica* and BSAF values at the investigated localities were compared statistically using one-way ANOVA followed by the post hoc Fisher least significant difference (LSD) test ($P < 0.05$). The t-test was used to compare metal concentrations in the sediment and *E. danica* at the same locality. The existence of correlation between pairs of variables was tested with Pearson product moment correlation ($P < 0.05$). Statistical tests were performed with the aid of the Sigma Plot 11 software (Systat Software Inc., San Jose, CA, USA).

Co-inertia analysis (CIA) was used to describe the influence of metal concentrations in the sediment on their concentrations in *E. danica* larvae and appropriate BSAF values (Dolédec and Chessel, 1994). CIA was computed using the ADE-4 software (Thioulouse *et al.*, 1997).

3 Results

In the present study, we investigated larvae of *Ephemera danica* for their ability to biologically accumulate in their bodies selected metals from the water and sediment in their characteristic aquatic environment, which is conspicuous for high concentrations of chromium and nickel in the sediment. In addition to this, we examined the influence of wastewater from a trout farm on concentrations of the tested heavy metals, both in water and sediment of the recipient stream and in larvae of *E. danica* dwelling in it.

Co-inertia analysis (CIA) was used to investigate the relationship between concentrations of heavy metals in the sediment on the one hand and their concentrations and capacity for accumulation in larvae of *E. danica* (measured by the biota sediment accumulation factor or BSAF) on the other, as well the influence of wastewater from the trout farm on these processes (Fig. 1). This analysis indicated the existence of a statistically highly significant ($P < 0.001$) co-structure between the PCA correlation matrix of heavy metal concentrations in the sediment and PCA correlation matrix of heavy metal concentrations in *E. danica* on the one hand, and BSAF on the other. In order to graphically represent the observed co-structure (Fig. 1), two factorial axes were retained, with the F1 axis explaining most of the co-structure (85.7%) and the F2 axis only small part of it (7.7%). Great strength of the co-structure was indicated by high values of the coefficients of correlation along the F1 axis (0.83) and F2 axis (0.87), as well as by short length of the arrows representing position of the localities.

3.1 Metal concentrations in the sediment

Analysis of metal concentrations in the sediment indicates that the vectors representing the concentrations of iron (Fe_s), copper (Cu_s), chromium (Cr_s), nickel (Ni_s), lead (Pb_s) and cadmium (Cd_s) are aligned along the F1 axis and are oriented toward its negative end. Situated at the negative end of the F1 axis, the positions of all seasons of locality SK2 (origins of black arrows) which lies directly below the inflow of wastewater from the trout farm stand out clearly from positions of the other localities, which are placed either near the middle of the F1 axis (all seasons of the SK1 control locality and winter and summer of locality SK3) or at its positive end (all seasons of locality SK4 and autumn and

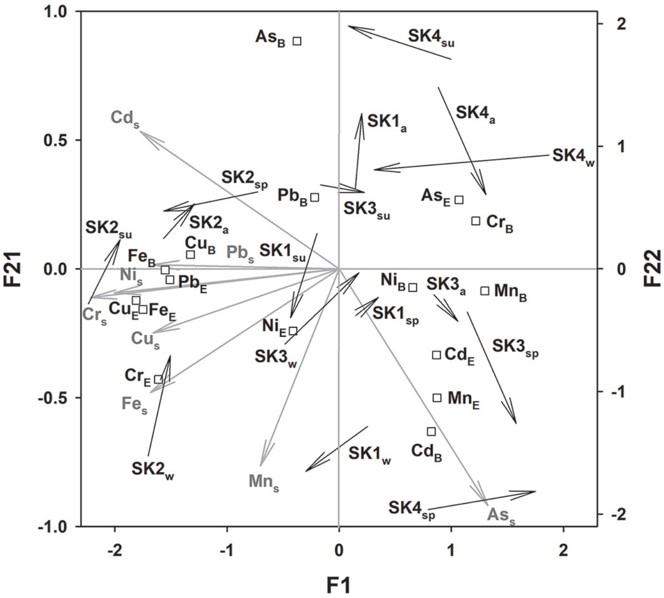

Fig. 1. Triplot presentation of the co-inertia analysis (CIA) of eight metal concentrations in the sediment and *E. danica* and their biota sediment accumulation factor performed on 16 samples [four sites, SK1-SK4, sampled in spring (sp), summer (su), autumn (a), and winter (w)]. The ordination diagram of eight normalized metal concentrations in the sediment (As_s, Cd_s, Cr_s,Cu_s, Fe_s, Mn_s, Ni_s, and Pb_s) in the CIA is represented by grey arrows projected onto the F1 × F21 factorial map. Positions of metal concentrations in *E. danica* (As_E, Cd_E, Cr_s,Cu_E, Fe_E, Mn_E, Ni_E, and Pb_E) and their BSAF (As_B, Cd_B, Cr_B,Cu_B, Fe_B, Mn_B, Ni_B, and Pb_B) are also plotted onto the CIA F1 × F21 plane. Standardized co-inertia scores of sediment and *E. danica* data for each sample (black arrows) are projected onto the F1 × F22 factorial map. Origin of the arrow indicate the position of the sample as ordinated by metal concentrations in the sediment, while the arrowhead indicate its position as ordinated by the values of metal concentrations in *E. danica* and their BSAF.

spring of locality SK3). Along the F1 axis, which explains by far the greatest part of variation of the data, seasonal samples for each of the localities (with the exception of SK3) are very close together, which indicates that seasonal variations in concentrations of the aforementioned metals in the sediment are much less pronounced than longitudinal variations. This means that a clear increase in concentrations of the given metals occurs at the locality directly below the trout farm (SK2), the indicated increase being more significant than eventual natural longitudinal changes, but it is strictly localized and complete recovery occurs already at the next locality (SK3). For this reason, the F1 axis can be considered the axis of the trout farm's effect. One-way ANOVA indicated that Fe_s, Cu_s, Cr_s, Ni_s, Pb_s and Cd_s increase statistically significantly at SK2 in relation to SK1 and then decline statistically significantly at SK3 and SK4, except in the case of the Pb_s concentration, where a statistically significant decrease occurs just at S4 (Fig. 2B–E, G, H). Also, the concentrations of these metals in the sediment of SK4 are without exception lower than at S1, although that decrease is statistically significant only in the cases of Cu_s and Cr_s (Fig. 2B, D). There are no statistically significant differences between localities in the cases of the concentrations of manganese and arsenic in the

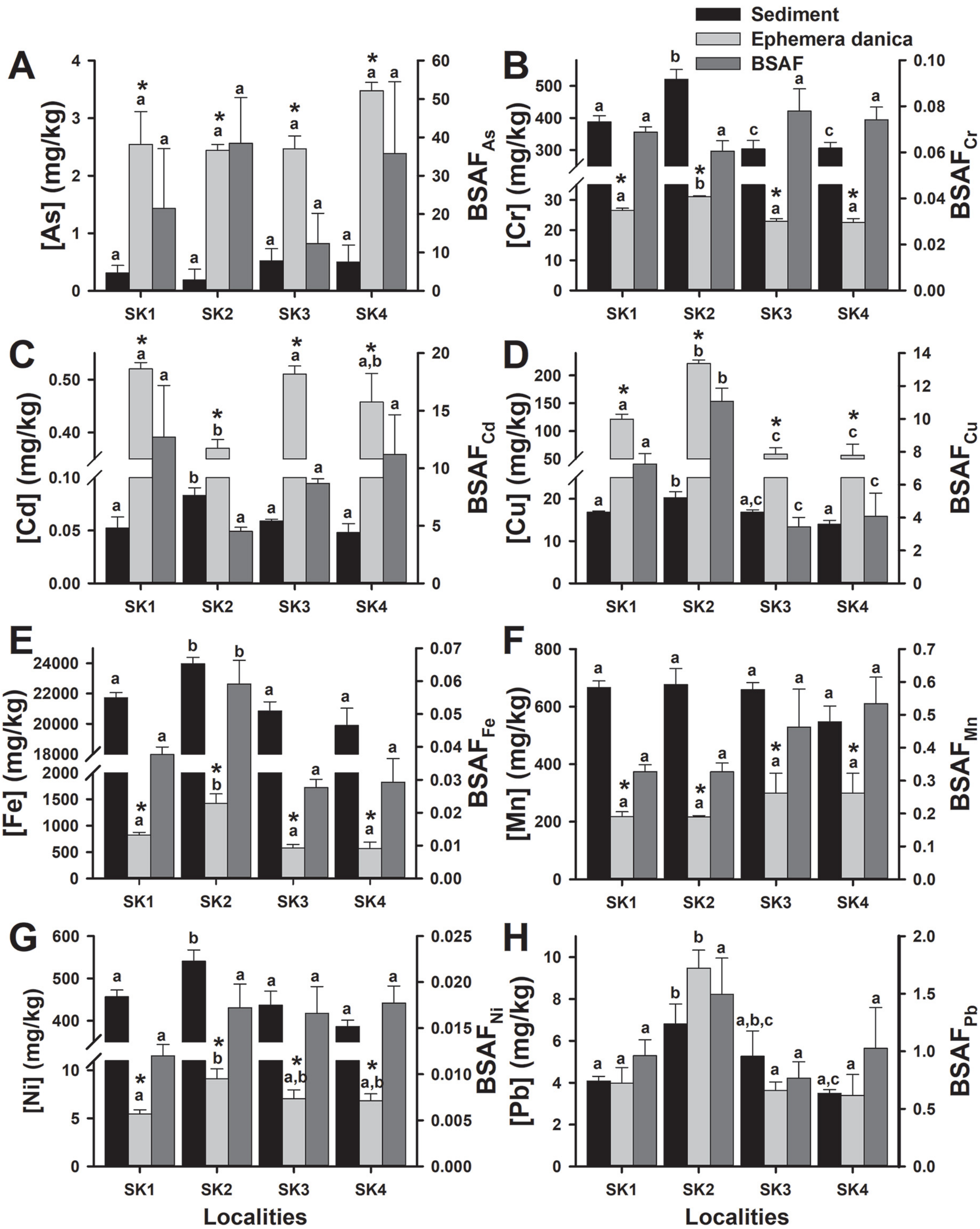

Fig. 2. Changes in average metal concentrations in the sediment (black bars) and *E. danica* (grey bars) and their BSAF (dark-grey bars) for arsenic (A), chromium (B), cadmium (C), copper (D), iron (E), manganese (F), nickel (G) and lead (H) at four investigated localities (SK1, SK2, SK3, and SK4) along the Skrapež River. Samples at different localities for each parameter that are significantly different are marked with different letters (a, b, c). A statistically significant difference between metal concentrations in the sediment and *E. danica* at each locality is marked with an asterisk (*).

sediment (Mn_s and As_s, respectively), and the value of As_s is even somewhat lower at SK2 than at the other localities. This is a consequence of seasonal variations greater than longitudinal ones, as is also indicated by CIA, where the vectors of these two metals are linked with the F2 axis, which represents the axis of seasonal variations.

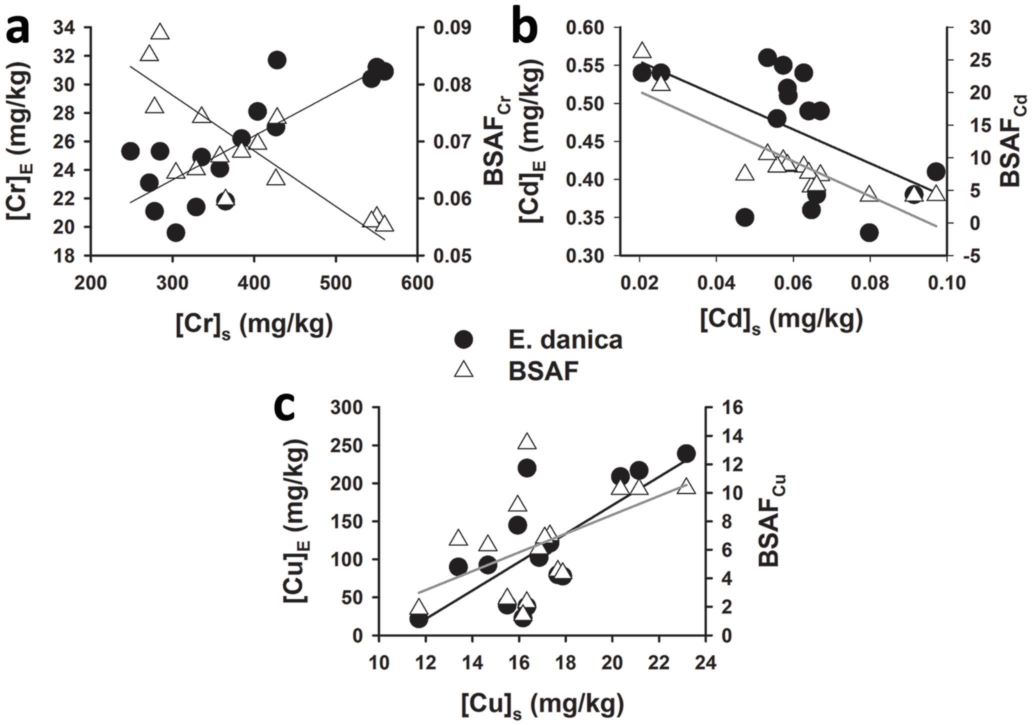

Fig. 3. Effects of metal concentration in the sediment on metal concentration in *E. danica* and its BSAF. (A) Effect of changes in Cr concentration in the sediment (Cr_s) during the investigation period on its concentration in *E. danica* (Cr_E, black circles) and BSAF (Cr_B, open triangles) at the investigated localities on the Skrapež River. The black solid line represents the linear fit of the data in the case of Cr_E ($R = 0.80$, $P < 0.001$) and the grey one in the case of Cr_B ($R = –0.77$, $P < 0.001$). (B) Effect of changes in Cd concentration in the sediment (Cd_s) during the investigation period on its concentration in *E. danica* (Cd_E, black circles) and BSAF (Cd_B, open triangles) at the investigated localities on the Skrapež River. The black solid line represents the linear fit of the data in the case of Cd_E ($R = –0.54$, $P = 0.030$) and the grey one in the case of Cd_B ($R = –0.88$, $P < 0.001$). (C) Effect of changes in Cu concentration in sediment (Cu_s) during the investigation period on its concentration in *E. danica* (Cu_E, black circles) and BSAF (Cu_B, open triangles) at the investigated localities on the Skrapež River. The black solid line represents the linear fit of the data in the case of Cu_E ($R = 0.72$, $P = 0.002$) and the grey one in the case of Cd_B ($R = 0.53$, $P = 0.036$).

3.2 Metal concentrations in larvae of *E. danica*

Analysis of the co-inertia of metal concentrations in larvae of *E.* danica indicates that the concentrations of chromium (Cr_E), copper (Cu_E), iron (Fe_E) and lead (Pb_E) just like the concentrations of these metals in the sediment are linked with the negative end of the F1 axis and are positively correlated with concentrations in the sediment (Fig. 1). As indicated by ANOVA, these metals are characterized by a statistically greater concentration at SK2 in relation to the other localities. Also, the concentrations of all four metals in *E. danica* show a statistically significant positive correlation with the concentration of the corresponding metal in the sediment: for Cr_E, $R = 0.80$ and $P > 0.001$ (Fig. 3A); for Cu_E, $R = 0.72$ and $P = 0.002$, (Fig. 3C); for Fe_E, $R = 0.634$ and $P = 0.008$; and for Pb_E, $R = 0.57$ and $P = 0.021$.

In co-inertia analysis, the concentration of cadmium in *E. danica* (Cd_E) is aligned counter to the vector of Cd_s, which indicates that their values change in opposite directions. Thus, ANOVA showed that Cd_E at SK2 is statistically greater than at SK1 and SK3 (Fig. 2C), while Pearson's test indicated that Cd_s and Cd_E are negatively correlated ($R = –0.54$, $P = 0.030$). On the CIA graph, the positions of concentrations of arsenic, nickel and manganese in *E. danica* (As_E, Ni_E and Mn_E, respectively) are situated virtually at a right angle to the vectors of concentrations of the given metals in the sediment (Fig. 1), and there is no statistically significant correlation between them. Only in the case of Ni_E does a statistically significant increase occur at SK2 in relation to SK1, while there are no significant differences between localities in the cases of As_E and Mn_E. If we compare the concentrations of a given metal in *E. danica* and in the sediment, the *t*-test indicates that a statistically significant difference exists in the cases of all metals except Pb and at each of the investigated localities, whereas in the case of Pb none exists at any of the localities (Fig. 2).

3.3 Biota sediment accumulation factor – BSAF

The values of BSAF indicate the direction of these differences. They are positive in the cases of arsenic (As_B), cadmium (Cd_B) and copper (Cu_B), which indicates that *E. danica* accumulates these metals in relation to the sediment (Fig. 2A, C, D). Accumulation is most pronounced in the case of arsenic, where $As_B = 27 \pm 7$, whereas somewhat lower (but still significant) values were obtained for cadmium ($Cd_B = 9.3 \pm 1.5$) and copper ($Cu_B = 6.5 \pm 0.9$). In the case of lead, Pb_B is close to one (Fig. 2H), which means that its concentrations are the same in the sediment and in *E. danica*. As for the other metals, the value of this index is less than one (Fig. 2B, E, F, G),

Table 1. Average water concentrations of heavy metals (in mg/kg) at the localities of the Skrapež river.

Localities	As	Cr	Cd	Cu	Fe	Mn	Ni	Pb
SK1	1.48 ± 0.73^a	4.05 ± 0.21^a	0.012 ± 0.012^a	1.83 ± 0.88^a	23.91 ± 13.75^a	1.77 ± 0.80^a	2.26 ± 0.31^a	1.13 ± 0.28^a
SK2	0.87 ± 0.50^a	4.24 ± 0.88^a	0.023 ± 0.023^a	2.79 ± 1.75^a	83.57 ± 60.85^a	5.76 ± 3.27^a	3.84 ± 1.63^a	1.53 ± 0.13^a
SK3	1.79 ± 0.67^a	4.21 ± 0.34^a	0.029 ± 0.029^a	3.65 ± 1.71^a	59.30 ± 26.37^a	3.77 ± 1.36^a	3.22 ± 0.59^a	1.41 ± 0.17^a
SK4	1.19 ± 0.44^a	4.15 ± 0.23^a	0.056 ± 0.056^a	1.98 ± 0.85^a	28.61 ± 13.74^a	2.20 ± 0.85^a	2.61 ± 0.30^a	1.32 ± 0.20^a

[a] Differences between values within the same column marked with a different superscript letter are statistically significant ($P < 0.05$).

which means that they do not accumulate in larvae of *E. danica*. This is expressed especially strongly in the case of Ni, where the concentrations in *E. danica* are 60 times lower than in the sediment ($Ni_B = 0.016 \pm 0.001$).

In analysis of the relationship between the concentrations of metals in the sediment and their BSAF, CIA indicates that Cu_B and Fe_B are linked with the negative end of the F1 axis (Fig. 1) and that they are characterized by a positive correlation with concentrations in the sediment ($R = 0.53$ and $P = 0.036$ for Cu_B, Fig. 3C; $R = 0.51$ and $P = 0.044$ for Fe_B). Moreover, ANOVA showed that the values of Cu_B and Fe_B attain a statistically significant maximum at SK2 (Fig. 2D, E). As for As_B, Cr_B and Cd_B, they are aligned counter to the vectors of their concentrations in the sediment and are characterized by a negative correlation ($R = -0.80$ and $P < 0.001$ for As_B; $R = -0.77$ and $P < 0.001$ for Cr_B, Fig. 3A; and $R = -0.88$ and $P < 0.001$ for Cd_b, Fig. 3B).

3.4 Metal concentrations in the water

Co-inertia analysis (CIA) was used to analyze concentrations of the tested metals in the water and estimate their influence on the concentration and capacity for accumulation in *E. danica,* although this time it was shown that no significant ($P = 0.7$) co-structure exists between the PCA correlation matrix of heavy metal concentrations in the water and the PCA correlation matrix of heavy metal concentrations in *E. danica* and BSAF, so the analysis could not be completed (Tab. 1). This indicates that there is no significant influence of the concentration of heavy metals in the water and *E. danica,* a conclusion additionally confirmed by the absence of any statistically significant correlation between the concentration of metals in the water and *E. danica* [except in the case of iron ($R = 0.52$ and $P = 0.037$)], as well as by the absence of statistically significant variation of any of the metals at the investigated localities (Tab. 1).

4 Discussion

Determination of the total concentration of metals in the sediment and water does not give a precise estimate of their influence on the environment, and it is therefore necessary to monitor how much a certain element accumulates in organisms, *i.e.*, how great is its bioavailabilty. Absorption of metals by aquatic invertebrates leads to accumulation of the metals in their bodies, which through the food chain further affects all consumers (including man) with potential long-term implications for human health (Notten *et al.*, 2005; Fernandes *et al.*, 2007; Corbi *et al.*, 2008; Agah *et al.*, 2009) and causes pollution of aquatic ecosystems (Malik *et al.*, 2010). In order to understand the influence of a trout farm on accumulation of heavy metals in aquatic invertebrates, we investigated larvae of *E. danica* as a model organism.

4.1 Analysis of heavy metal concentrations in the sediment and in the body of *E. danica* larvae

Very high concentrations of Cr and Ni are a basic characteristic of the sediment at the investigated localities on the Skrapež River. According to published reference values for quality of the sediment in freshwater ecosystems (MacDonald *et al.*, 2000), the lowest concentration of Ni is 7.4 times greater and that of Cr 2.45 times greater than PEC (the probable effect concentration, 48.6 and 111 mg/kg, respectively). A similar conclusion can be drawn if we compare the concentrations of these two metals with the maximum permissible concentrations (MPC) (Official Gazette No. 50/2012), which are significantly lower than the concentrations of these elements in the Skrapež sediment (the MPC for nickel is 44 mg/kg, while that for chromium is 240 mg/kg). Chromium is rarely found in natural waters (Mertz, 1986), but it can enter them and be deposited as a pollutant in the sediment as a result of various human activities. It is sometimes included as a micronutrient in fertilizers used in growing different cereals. According to the results obtained by Santos *et al.* (2009), soils are polluted with different concentrations of chromium depending on agricultural activity. Because the valley of the Skrapež is under agricultural crops, that is probably one of the reasons for the higher concentration of chromium in the rivers sediment. Chromium concentrations are higher at locality SK2 than at the other localities. There is more organic matter at that locality than at the other localities due to effluents from the trout farm, and the content of organic matter can have decisive influence on binding of chromium in the substrate, as was demonstrated by Santos *et al.* (2009) in their research. With respect to the concentration of nickel in the sediment, it is most likely of geochemical origin: the elevated concentration of this element in the sediment can be attributed to the fact that geological composition of the Skrapež basin includes magmatic rocks, metamorphic rocks and schists in which nickel is concentrated (Cannon *et al.*, 1978; Kovačević-Majkić, 2009). Moreover, there are veins of nickel on Mt. Povlen, on which the Skrapež River arises (Kovačević-Majkić, 2009), and this is an additional reason for its high concentration. As in the case of Cr, an increase in the concentration of Ni occurs at the locality directly below the trout farm. This is probably a consequence of its elevated concentration in the outlet water due to the use of chemical substances in agricultural processes, which causes residues of the metal to be found in the raw materials used in production of trout feed.

For the other investigated metals in the Skrapež River, even the highest concentrations registered in the sediment are below their TEC (threshold effect concentration, MacDonald *et al.*, 2000) values. That is especially clearly expressed in the cases of As and Cd, whose contamination factors are the lowest and whose highest registered concentrations are nine and 10 times lower, respectively, than the TEC (9.79 and 0.99 mg/kg respectively). Moreover, according to No. 50/2012 of the Official Gazette, the concentrations of all metals with the exceptions of Ni and Cr are far below the maximum permissible concentrations in surface waters.

In regard to the trout farḿs effect on heavy metal concentrations in the sediment, CIA (Fig. 1) and ANOVA (Fig. 2) clearly showed that the concentrations of Fe_s, Cu_s, Cr_s, Ni_s, Pb_s and Cd_s at locality SK2 just below the trout farm attain a distinct and statistically significant maximum with very little seasonal variation, which indicates that the trout farm is the direct cause of this effect. This can be explained by the fact that release of effluents, which contain remains of trout feed and faeces (Kronvang *et al.*, 1993; Iversen, 1995), from the trout farm into the recipient stream causes accumulation of the organic matter in the sediment immediately below the trout farm thus increasing its metal binding capacity (Zhang *et al.*, 2014; Hsu *et al.*, 2016; Burridge *et al.*, 2010). Increase in the concentration of biogenic metals like Cu and Fe below the trout farm can also be attributed to their presence in trout feed (which is rich in trace elements of the iron and copper type), whose uneaten remains enter the recipient stream with the outlet water and accumulate in the sediment directly below the fish farm (Miller *et al.*, 1993; Kamunde *et al.*, 2002). Also, pollution of the environment with copper can arise through the use of copper-based pesticides, and copper salts in this way enter the water and then accumulate in the sediment (deOliviera-Filho et al., 2004; Flemming and Trevors, 1989).

As for concentrations of the tested metals in the body of *E. danica* larvae, co-inertia analysis and ANOVA showed that the concentrations of chromium, copper, iron, nickel and lead in the body of *E. danica* larvae (Cr_E, Cu_E, Fe_E, Ni_E, Pb_E) attain a statistically significant maximum at locality SK2. Except in the case of Ni_E, they exhibit a statistically significant positive correlation with concentrations of the same metals in the sediment (Fig. 3A and 3C). In contrast to them, Cd_E attains a minimum at SK2 and is characterized by a negative correlation with Cd_s (Fig. 3B). Previously published data on the relationship of heavy metal concentrations in the sediment and aquatic invertebrates are fairly contradictory. Thus, Harrahy and Clements (1997) noticed a significant increase of concentrations in the body of *Chironomus tentans* with increase of concentrations in the sediment for all metals (Cd, Zn and Pb) except copper. In keeping with this, in rivers receiving organic pollution it was established that the concentration of copper in *Epobdella octoculata* and *Asellus aquaticus* declines with an increase in its concentration in the sediment, while that of lead increases (Eyres and Pugh-Thomas, 1978). Meanwhile, Goodyear and McNeill (1999) assert that all invertebrates have a body concentration of copper in positive correlation with concentrations in the sediment. Hare *et al.* (1991) found Cu and Pb concentrations in the body of *Hexagenia* to be in proportion with those in the sediment. Corbi *et al.* (2010) state that iron is accumulated in insects in correlation with its concentrations in the sediment. Jop (1991) monitored the concentrations of cadmium in *E. danica*, *E. vulgata*, *Leptophlebia vespertina* and *Baetis vernus* and contrary to our results concluded that the concentration of cadmium in them is proportional to concentrations in the sediment. Contrary to this, Corbi *et al.* (2010) report that Cd was not registered in insect larvae even when present in the sediment.

4.2 Biota sediment accumulation factor – BSAF

Every aquatic invertebrate assimilates metals from its surroundings, such assimilation depending upon the species of invertebrate and the relative biological capabilities of the metal (Rainbow and Wang, 2001). Factors that influence the fate of a metal are physiology of the invertebrate, whether the metal is used for an essential physiological function, whether it is excreted or stored in the body, and whether it gains access to a "wrong" biomolecule and thereby has a toxic effect (Rainbow, 2002). Our research indicates that metals characterized by a high concentration in the sediment (Ni, Cr, Fe and Mn) have a BASF value of less than one, that is their bioaccumulation is absent in *E. danica*, whereas elements characterized by a low concentration in the sediment have a BASF value greater than one, which is to say that their bioaccumulation occurs in *E. danica*. Meanwhile, if the relationship of concentrations of these elements in the sediment and their BSAF values is examined within the framework of these two groups, it is possible to discern further distinctions, namely that Fe and Cu are characterized by a positive correlation of these two values, whereas Cr, As and Cd are characterized by a negative one. Moreover, Cu_B and Fe_B attain a statistically significant maximum at SK2 (Fig. 2D, E). Only Pb, whose concentration in the sediment is also relatively low, is characterized by a BSAF value of about one. In the cases of Ni and Cr, which are toxic for living beings (Poulton *et al.*, 1989; Eisler, 1998; Kotas and Stasicka, 2000) and which are present in the sediment in exceptionally high concentrations, such a result could be expected and probably represents a defense mechanism of *E. danica* against assimilation of these metals in high concentrations lethal for it. This is confirmed by the aforementioned negative correlation between Cr_B and Cr_s (Fig. 3A). Furthermore, in comparison with the other metals, nickel and chromium are less mobile, which means that they are less available to organisms of the bottom fauna and have smaller chances of entering the food chain (Leonard and Wood, 2013). Our results are in agreement with the findings of Shulkin *et al.* (2003), who reported an increase in concentration of all metals except nickel in the body of the shellfish *Crenomitllis grayanus.* Also, De Jonge *et al.* (2014) noted that burdening of the body in *Leuctra* sp., *Rhithrogena* sp., Simuliidae and Perlodidae is not linked with the level of total Ni. Watras *et al.* (1985) measured the concentration of nickel in *Daphnia magna* and noticed that ingested nickel either is not incorporated or else is quickly eliminated. Muyssen *et al.* (2004) concluded that the BSAF value for Ni is almost always less than one, *i.e.*, the concentrations of nickel in benthic organisms are lower than concentrations in the sediment. In the case of chromium, Hamidian *et al.* (2016) noticed that slight bioaccumulation of this metal occurs in chironomids in the Shoor River (Iran), but its concentrations in the sediment were 5–10 times lower than in the Skrapež River, which in view of the negative correlation between Cr_B and Cr_s observed in our study can explain such a result in the Shoor River. All of this supports our conclusion that aquatic invertebrates probably develop defense mechanisms against this toxic metal, and that the greater its content in the sediment, the less it is assimilated by benthic organisms.

The positive correlation between accumulation of copper and iron in the sediment and in the body of *E. danica* can be attributed to the fact that these metals are essential and necessary for metabolic activity of the organism (Cohen *et al.*, 2001). As has already been said, Goodyear and McNeill (1999) in their study also found a positive correlation between the concentration of copper in the investigated invertebrates and its concentration in the sediment. Moreover, they concluded that copper concentrations in the organisms increase in proportion with increase of copper concentration in the sediment, without signs of biological limitation. These results were corroborated by Gundacker (2000), who compared the concentration of metals in the sediment and in the bodies of shellfish and concluded that bioaccumulation of copper occurred in them, in contrast to lead. It can be asserted that the situation is similar with iron, since Corbi *et al.* (2010) found the concentrations of iron in the sediment to be very high and in positive correlation with its accumulation in insects, especially in species of the genus *Chironomus*.

As for bioaccumulation of Cd and As, a negative correlation is evident between the levels of these elements in the sediment and in the body of *E. danica*. To be specific, although the level of bioaccumulation of Cd and especially As is very high, its value in both cases decreases with increase in concentrations of the elements in the sediment. In view of the fact that we are dealing here with exceptionally toxic substances, this negative correlation probably represents a defense mechanism of *E. danica* against their excessive accumulation. In the case of the level of Cd bioaccumulation, our results corroborate those obtained in the investigations Girgin *et al.* (2010), where it is stated that representatives of the order Ephemeroptera accumulate greater concentrations of cadmium than do members of other orders of aquatic invertebrates. In addition, Gundacker (2000) reported a significant level of Cd bioaccumulation in shellfish. On the other hand, research on bioaccumulation of heavy metals in chironomids in the Shoor (Iran) indicated that it is lacking in the cases of As and Cd (Hamidian *et al.*, 2016). In the case of Cd, that can be explained by the fact that its concentration in the sediment of the Shoor River was below the limit of detection, while the concentration of As in the sediment was more than 10 greater than in our investigation, which in view of the observed negative correlation between As_B and As_s can explain the absence of bioaccumulation in the Shoor River.

4.3 Analysis of concentrations of heavy metals in the water in relation to concentrations in *E. danica*

All of the analyses performed in the present study unambiguously indicate that concentrations of the investigated metals in the water have no significant influence on their concentrations in larvae of *E. danica*. The absence of influence could be a consequence of the way of life of these organisms. As larvae they burrow into the substrate making tunnels in the process, and feed by filtering organic detritus (Clements and Kiffney, 1994). For this reason, there exists a positive correlation between concentrations in the sediment and in the body of the larvae, but not between the latter and metal concentrations in the water. Similarly, Bervoets *et al.* (1997) indicated that the concentrations of lead and cadmium in bodies of the organisms they investigated are in correlation with total lead concentration in the sediment, but not with their concentration in poor water. In contrast to our results, in the study of Brown (1977) who investigated a river polluted by waste materials from an abandoned mine a significant positive correlation was recorded between the concentration of copper in the body of Trichoptera larvae and in the water. Also, a correlation was found to exist between the concentrations of one or more of the studied metals in the bodies of *Ecdyonurus venosus*, *Brachyptera risi*, *Leuctra* spp., *Perla bipunctata*, *Rhyacophila dorsalis* and *Dicranota* sp. and their concentrations in the water (Burrows and Whitton, 1983).

4.4 Effects of metals on the activity of antioxidative enzymes in *E. danica* larvae

In parallel with this investigation we used the same system (Skrapež River and The Kraj Vodenice Trout Farm) to monitor the effects of changes in water chemistry on the activity of enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) and concentration of total glutathione (GSH) in *E. danica* larvae (Božanić *et al.*, 2018). SOD, GPx and GSH are important constitutens of the anioxidant defence system (Valko *et al.*, 2016). It is well documented that one of the mechanisms of metal-induced toxicity is the generation of reactive oxygen species (ROS) (Valko *et al.*, 2016). For example, metals, including Fe, Cu, Cr, and V undergo redox cycling, while As, Cd, Hg, Ni, and Pb, deplete glutathione and protein-bound sulfhydryl groups, resulting in the production of ROS and altering the activity of antioxidative enzymes responsible for ROS degradation (Stohs and Bagchi, 1995; Valko *et al.*, 2005; Gomes *et al.*, 2013).

Comparison of metal concentrations in the sediment with the activities of SOD and GPx and GSH concentration in *E. danica* larvae showed no statisticaly significant correlation (data not shown) which is in agreement with results obtained on *Gammarus dulensis* in the Crnica River (Vranković *et al.*, 2018). However, GPx activity in *E. danica* larvae was stimulated by the increase in Ni_E ($R = 0.746$, $P < 0.001$) and Pb_E ($R = 0.617$, $P = 0.011$) and the decrease in Cd_E ($R = –0.702$, $P = 0.002$), indicating that apart from the concentrations of NO_3^-, NH_4^+ and value of DO% in the water of the Skrapež River, Ni_E Pb_E and Cd_E could olso influence GPx activity. As expected, metals accumulated in the larval body exerted greater influence on the antioxidant defence system compared to those in sediment.

5 Conclusions

This is a rear study investigating the influence of the trout farm effluents on the accumulation of heavy metals in the sediment of the recipient stream, and macrozoobenthos organisms. We clearly showed that trout farm discharge induced localized and statistically significant increase of concentrations of Fe, Cu, Cr, Ni, Pb and Cd in sediment and *Ephemera danica* larvae, but not in water indicating that sediment is the main source of heavy metals for this organism. In addition, evaluation of *E. danica* as a potential bioindicator of heavy metal pollution showed that only metals with low values of contamination factor (As, Cd and Cu) accumulated in the larvae. Moreover, values of Biota sediment accumulation factor for toxic metals (As, Cd and Cr) showed negative correlation with their concentrations in sediment indicating existence of defense mechanisms in *E. danica* against assimilation of these metals in high concentrations. In conclusion *E. danica* larvae proved to be good bioindicators of the pollution of river systems by As, Cd and Cu.

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