Holo- and hemimetabolism of aquatic insects: Implications for a differential cross-ecosystem flux of metals

Katarina A. Cetinić[1], Ana Previšić[2], Marko Rožman[1*]

[1] Ruđer Bošković Institute, Zagreb, Croatia

[2] Department of Biology, Zoology, Faculty of Science, University of Zagreb, Zagreb, Croatia

*Corresponding author:

Marko Rožman

Ruđer Bošković Institute

Bijenička cesta 54, 10000 Zagreb, Croatia

marko.rozman@irb.hr

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Abstract:

Increased metal concentrations in aquatic habitats come as a result of both anthropogenic and natural sources. Emerging aquatic insects that play an indispensable role in these environments, transferring resources and energy to higher trophic levels in both aquatic and terrestrial habitats, may inadvertently also act as biovectors for metals and other contaminants. This study measured levels of 22 different metals detected in biofilm, aquatic and terrestrial life stages of Trichoptera and Odonata, as well as riparian spiders, to examine the uptake and transfer from freshwater to terrestrial ecosystems. We show that emerging insects transfer metals from aquatic to terrestrial ecosystems, however with large losses observed on the boundary of these two environments. Significantly lower concentrations of most metals in adult insects were observed in both hemimetabolous (Odonata) and holometabolous insect orders (Trichoptera). In holometabolous Trichoptera, however, this difference was greater between aquatic life stages (larvae to pupae) compared to that between pupae and adults. Trophic transfer may have also played a role in decreasing metal concentrations, as metal concentrations generally adhered to the following pattern: biofilm > aquatic insects > terrestrial invertebrates. Exceptions to this observation were detected with a handful of essential (Cu, Zn, Se) and non-essential metals (Cd, Ag), which measured higher concentrations in adult aquatic insects compared to their larval counterparts, as well as in aquatic and terrestrial predators compared to their prey. Overall, all metals were found to be bioavailable and biotransferred from contaminated waters to terrestrial invertebrates to some degree, suggesting that risks associated with metal-contaminated freshwaters could extend to terrestrial systems through the emergence of these potential invertebrate biovectors.

Capsule: Emerging insects transfer metals from aquatic to terrestrial ecosystems and metamorphosis plays a significant role in altering metal concentrations in emergent aquatic insects. The ramifications of such findings are that risks associated with metal-contaminated freshwaters are not limited to aquatic ecosystems but can rather extend to terrestrial systems.

1. Introduction

Metal contamination of natural environments as a result of increased anthropogenic activity has become an area of significant concern. Due to the high bioavailability and persistence of metals, combined with their reactivity and toxicity at even very low concentrations, the increased loading of metals into freshwater systems via wastewater effluents and agricultural runoff could have severe ecological implications (Wuana and Okieimen, 2011; Masindi and Muedi, 2018). Once present within the aquatic environment, metals tend to accumulate in high concentrations in biofilm (Farag et al., 1998; Behra et al., 2002; Serra et al., 2009), which can in turn serve as an important exposure pathway for the dietary accumulation of metals and other contaminants in aquatic herbivores, such as Trichoptera larvae, that inhabit and feed on freshwater biofilm and sediments (Munger and Hare, 1997; Cain et al., 2004; Croteau and Luoma, 2008).

As all aquatic insects undergo metamorphosis and many a subsequent change of habitat, this could potentially lead to the movement of bioaccumulated metals and other contaminants across ecosystem boundaries and, in turn, impact riparian predators (Walters et al., 2008). A handful of studies have examined the impact of metamorphosis on metal levels in aquatic insects, with varying results regarding degree of accumulation. While some authors have observed decreases in concentrations of metals following emergence (Harvey, 1971; Timmermans and Walker, 1989; Kraus et al., 2014; Wesner et al., 2017), others have reported similar or elevated concentrations in adults (Kraus et al., 2014; Naslund et al., 2020). To our knowledge, however, none have investigated differences in bioaccumulation and bioamplification of metals between hemi- and holometabolous aquatic insects (i.e. those that undergo incomplete [e.g. Odonata] and complete metamorphosis [e.g. Trichoptera], respectively) in a natural setting. These two insect orders differ not only in type of metamorphosis, but also in their feeding habits. Predatory Odonata feed throughout their lifetimes, both as larvae and adults, while the majority of Trichoptera species only feed during their larval stage, depleting accumulated lipids during their non-feeding pupal and adult stages (Huryn and Wallace, 2000). If accumulated metals are not efficiently excreted, and are instead retained or bioamplified (i.e. exhibiting an increase in concentration with each subsequent life stage as a result of a greater loss in body mass compared to the rate of elimination of contaminants [Daley et al., 2011]), large amounts of metals could be transferred to insectivorous terrestrial predators, especially during periods of mass emergence. As these ubiquitous aquatic insects play a vital role in the transfer of resources and energy to higher order consumers (Vanni, 2002; Thorp and Covich, 2015), exporting millions of tonnes of carbon annually (Bartrons et al., 2013), this could result in significant amounts of metals being transferred between systems, depending on the level of insect production and the metal body burden (Timmermans and Walker, 1989). Furthermore, exposure to contaminants during the larval stages may affect the metamorphosis and adult stages of insects through alterations in behaviour, decreases in immune responses, feeding inhibition, and reductions in emergence rate (Schulz and Liess, 1995; Tomé et al., 2014; Jinguji et al., 2018; Mangahas et al., 2019; Lidman et al., 2020).

A useful concept that aids in the understanding of metal bioavailability, mobility and toxicity in environmental media and biota incorporates a biochemical approach, in which metal ions are Lewis acids and the ligands they complex with are Lewis bases (Pearson, 1963). This concept differentiates two main classes of metals: class A (hard) metals, class B (soft) metals, as well as borderline (intermediate) metals, depending on their affinity for different ligands

(Pearson, 1963; Duffus, 2002; Kinraide, 2008). Hard metals are less toxic and preferentially bind with ligands that contain oxygen, creating relatively weak ionic bonds that are easily broken (Duffus, 2002). Due to this, class A (hard) metals are considered to be more mobile and more easily displaced in environmental media. Class A metals include Li, Be, Cs, Ba, Ti, Sc, Al, Mg, Na, Rb, and K. Soft metals, on the other hand, are more toxic, and tend to form complexes with sulfur-containing ligands, forming strong covalent bonds. When these class B (soft) metals enter biota, they are not easily excreted and tend to bioaccumulate in tissues (Rensing et al., 1999; Duffus, 2002; Sharma and Agrawal, 2005; Kraus et al., 2014). Examples of class B metals include Cu, Pd, Ag, Cd, Pt, Tl, Zn and Se. Borderline metals can exhibit characteristics of either class A or class B metals, depending on the circumstances, and include metals such as Sb, As, V, Cr, Mn, Fe, Co, Ni, and Mo (Duffus, 2002).

Studies have been conducted in recent years examining the flux of metals both within aquatic and terrestrial systems individually (Peterson et al., 2003; Croteau et al., 2005; Islam et al., 2016), as well as between these two systems (Schmidt et al., 2013; Otter et al., 2013; Kraus et al., 2014; Wesner et al., 2014; Naslund et al., 2020). There are, however, still some gaps in our understanding surrounding the movement of metals between systems following aquatic insect emergence. The objective of the current study was to evaluate class A and B metal flux to better understand to what extent emerging aquatic insects act as biovectors of metal transfer from contaminated freshwaters to terrestrial habitats, and the potential impact this movement of metals could have on terrestrial predators. We set the following hypotheses: (i) Different types of metamorphosis will result in different rates of metal bioaccumulation and transfer between holometabolous Trichoptera (complete metamorphosis) and hemimetabolous Odonata (incomplete metamorphosis), due to differences in life history traits; (ii) Concentrations of class

A metals will decline with each subsequent life stage, due to their higher mobility compared to class B metals. Taking into consideration that insect metamorphosis has been shown to alter the levels of contaminants in organisms (Kraus et al., 2014; Wanty et al., 2017; Wesner et al., 2017), and that Trichoptera only feed during their larval stage (Huryn and Wallace, 2000), we expected any bioaccumulated class A metals to be more efficiently excreted within holometabolous Trichoptera compared to Odonata (as well as class B metals to exhibit greater bioamplification), due to the former consisting of an addition stage in their life cycle, i.e. the pupal stage, during which they do not ingest any additional food. To test our hypotheses, samples of biofilm, as well as aquatic and terrestrial life stages of Trichoptera and Odonata, and riparian spiders were collected across locations impacted by wastewater effluents and agricultural runoff. To investigate the impact aquatic insect emergence has on the transfer of bioavailable metals from aquatic to terrestrial environments, we compared concentrations of metals detected across different life stages of Trichoptera (larvae, pupae, adults) and Odonata (larvae, adults). Furthermore, we compared metal concentrations across different trophic levels, from biofilm to riparian predators, to assess the potential flux of metals via trophic transfer.

2. Materials and methods

2.1. Study sites and sample collections

Sampling was conducted at three sites in NW Croatia, at different running waters impacted by pollution: the mid-sized lowland river Krapina, the lowland stream Bistrec and the hydropower plant drainage ditch Dubrava in Prelog (an artificial habitat very similar to large lowland streams/small-sized lowland rivers). Site selection was based on previous data on the abundance of targeted aquatic insects, pollution and/or increased metal concentrations in aquatic biota (in Bistrec [Kiš-Novak, 2012]). The Bistrec stream and Dubrava drainage ditch are recipients of untreated communal wastewaters (approximate population: 4500 and 7700, respectively). The Krapina river is the recipient of both treated and untreated communal wastewaters, however, the sampling site was positioned downstream of untreated effluent from the town Zabok (approximate population: 8900). Additionally, all sampling sites are influenced by agriculture. Further information on the selected sampling sites and identified taxa, as well as annual range and averages of the main physico-chemical water parameters measured at the sites are listed in Supporting Information (Table S1).

At each site, two collections within maximally 30 days were conducted in April and May 2018, in order to I) collect aquatic (larval and pupal) and terrestrial (adult) stages of insects inhabiting the targeted sites, and II) reduce variability in temporal dynamics of aquatic insect flux (Kato et al., 2003). On each sampling occasion, adult Trichoptera and Odonata were collected with an entomological net by sweeping riparian vegetation along the watercourse (up to 3 m laterally). In order to remove any doubt in the larvae-adults comparisons, we collected exclusively teneral immature adult Anisoptera, as they are known to disperse over relatively long distances (e.g. Corbet et al., 1999). Caddisflies collected as adults in the current study (*Silo* sp., Goeridae and Sericostomatidae) belong to taxa that are known as low dispersers, rarely dispersing more than few meters from the sites of their emergence (Sode & Wiberg-Larsen 1993). Riparian spiders were collected by hand from riparian vegetation directly overhanging the watercourse. Aquatic insect larvae and pupae were collected with a D-net, and biofilm was scraped from stones. Upon collection, aquatic insect larvae were kept in 10 L containers with river water and transported to the lab, where they were placed in filtered river water for 24 h to allow gut clearance. Taxa were separated on their respective groups, freeze-dried and stored at -80°C until further processing.

2.2. Sample laboratory processing and analysis

Biofilm samples, as well as macroinvertebrate samples (all stages of aquatic insects and riparian spiders) were processed following an in-laboratory approved protocol. Briefly, triplicate samples (each weighing approximately 40 mg) were digested with 1.5 ml concentrated nitric acid (HNO_3; *suprapur*) and 0.5 ml hydrogen peroxide (H_2O_2), vortexed, then placed in a drying oven at 85 ºC for 3.5 h. A set of 22 metals grouped within three metal classes (class A, class B, and borderline metals) was preselected for analysis within the biota based on their differences in environmental mobility and toxicity (Pearson, 1963; Duffus, 2002). To analyse for these metals, 1 ml of each sample was diluted with 3.75 ml of Milli-Q water, then acidified with 100 µl of high purity concentrated HNO_3 (Fluka TraceSELECT). Samples were then analyzed for metal content using a high resolution inductively coupled plasma-mass spectrometer (HR ICP-MS, Element 2; Thermo Finnigan, Germany), equipped with an SC-2 DX FAST autosampler (ElementalScientific, USA). All the samples were analysed in triplicate and metal content was presented as an average.

2.3. Quality assurance and quality control (QA/QC)

All standard environmental field and laboratory procedures for QA/QC were followed for this study. Multi-element standard solution for trace elements (100 ± 0.2 mg L^{-1}, Analytika, Czechia), supplemented with Cs solution (Sigma-Aldrich, Germany), was used for the external calibration for the trace element analysis. Platinum and silver standard solutions (1000 mg L^{-1}, Atomic Spectroscopy Standard Solution, Fluka, Germany) were also used for the external calibration. Calibration solutions were prepared at 1, 10 and 100 μg L^{-1} for trace metals, and 1 and 10 μg L^{-1} for Ag and Pt. Indium (Indium Atomic Spectroscopy Standard Solution, Fluka, Germany) was added as an internal standard to a final concentration of 1 μg L^{-1} to monitor and correct for instrument drift. The accuracy and precision of the HR ICP-MS measurements were tested using quality control samples for trace elements (QC Trace Metals, Catalogue Number 8072, UNEP GEMS, Burlington, Canada).

The QA of all metal analyses relied on analytical blanks and the accuracy and reproducibility of data relative to the certified reference materials (CRMs). On the basis of the procedural blank values, the values of detection limits (LOD) and quantification limits (LOQ) were calculated. Detection limits were calculated as three times the standard deviation of ten consecutive measurements of the analyte mass fraction in the procedural blank and multiplied by the dilution factor (Table S1). Blank values were systematically below detectable levels, while CRM values were in accordance with certified values. Mean recovery rates indicated a satisfactory performance of trace metal determination, ranging from 95 – 106% for the majority of metals analysed, with the exception of Al (116%) and Ni (75%).

At all points in time, precautionary measures were taken to prevent possible contamination of the samples. All laboratory glassware and plasticware was thoroughly cleaned by soaking in 1-3M hydrochloric acid (HCL) overnight and rinsing with distilled water prior to use. Reagents were prepared and standardized with care against reliable primary standards.

2.4. Statistical analysis

To correct for the non-normal distribution of the data, variables were $\log_{10}$ (x+1) transformed. Data were then visually inspected for outliers. Possible outliers were confirmed by using Tukey's fences criterion and removed from the dataset. Principal components analysis (PCA) was used to examine patterns of metal concentrations in Trichoptera and Odonata (aquatic and terrestrial stages) and riparian spiders across sites. To investigate the transfer of metals between various life stages of holometabolous Trichoptera (i.e. complete metamorphosis – larvae, pupae and adults) and hemimetabolous Odonata (i.e. incomplete metamorphosis – larvae and adults), we compared metal concentrations measured in each life stage using a one-way analysis of variance (ANOVA) and a Student's t-test, respectively. In addition, a Student's t-test was used to determine if there were significant differences in metal levels between aquatic insects with incomplete and complete metamorphosis (i.e. between Trichoptera and Odonata). As Bistrec was the only sampling location at which both Trichoptera and Odonata were found, the comparison was conducted using data from that site (i.e. Trichoptera and Odonata larvae and adults). To examine the movement of metals via trophic interactions, we also conducted ANOVAs between available aquatic and terrestrial trophic compartments within their respective sites, i.e. biofilm – Trichoptera larvae (primary consumers) – Trichoptera adults (terrestrial prey) – riparian spiders (terrestrial predators) at Prelog, and biofilm – Trichoptera larvae (primary consumers) – Odonata larvae (aquatic predators) – Trichoptera adults (terrestrial prey) – Odonata adults (terrestrial predators) at Bistrec. Concentrations of metals in biofilm between sites were also compared using a one-way ANOVA. All ANOVAs were followed by Tukey's post-hoc test of multiple comparisons (Zar, 1984) wherever results were found to be significant ($p < 0.05$). *P*-values were adjusted to control for experiment-wise error rate using the Bonferroni method.

Metal-specific bioamplification factors (BAmF) were calculated as the ratio of mean metal concentration (in μg g^{-1} dry weight) between two consecutive life stages in hemimetabolous Odonata (adults/larvae) in Krapina and Bistrec, and holometabolous Trichoptera (adults/pupae and pupae/larvae) in Prelog. Calculated values for BAmF were visually represented only in instances where differences in metal concentrations between life stages were statistically significant; otherwise values were assumed to be 1.0. Metals that exhibited opposing results across sites (i.e. a significant increase at one site, and a significant decline or no change at the other; determined for Odonata adults/larvae at Bistrec and Krapina) were considered to be inconsistent and were omitted from further analyses. Univariate analyses were performed with the software package R (R Development Core Team, 2019) using version 3.6.1., while the PCA was conducted using Primer 7 (Version 7.0.13, PRIMER-e, NZ). The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

3. Results

3.1. Collected aquatic and terrestrial invertebrates

In total, 3 taxa of Trichoptera, 6 taxa of Odonata and 1 taxon of riparian spiders were identified across the three sampled sites. Detailed information regarding the identified taxa and their feeding behaviour and general ecology are presented in Table S2.

3.2. Metal concentrations in biofilm

All 22 tested metals were detected across all sampled biota. For the majority of the metals, the highest concentrations were measured in biofilm, regardless of sampling sites (Table 1). The most abundant metals detected within the biofilm were Fe, Mn and Al, with highest average concentrations measured in Bistrec (Table 1). Overall, biofilm metal burden differed among sites, with concentrations of all metals except Ag, Sr, Tl, Se and Pb highest in Bistrec ($P < 0.05$; ANOVA). Levels of Sr and Pb measured highest in Krapina [all data presented as μg g^{-1} DW mean ± SE] (340.65 ± 7.93 and 27.42 ± 1.37, respectively; Table S3), while Ag measured highest in Prelog (12.11 ± 1.06; Table S3).

3.3. Metal concentrations in aquatic insects (Trichoptera, Odonata) and riparian spiders (Araneae)

In general, both aquatic insects and riparian spiders presented a similar pattern of metal concentrations to those measured in the biofilm. Metals that were present in high concentrations in the biofilm (i.e. Fe, Mn, Al, Ba, Sr and Ti) also accumulated in high levels within these taxa. Although concentrations of Mn, Sr and Fe were much lower compared to those detected in the biofilm (Table 1), they measured similar or elevated concentrations compared to other invertebrates collected from contaminated sites (Table S4). A PCA of all aquatic and terrestrial invertebrate biota sampled within the study revealed four distinct clusters: Trichoptera larvae, Trichoptera pupae – Trichoptera adults, Odonata larvae, and Odonata adults – riparian spiders (Fig. 1). Separation along the first principle component (PC) (accounting for 71.6% of the dataset variance) showed that concentrations of metals distinctly differed between aquatic and terrestrial life stages of invertebrates, indicating a pattern consistent with metamorphosis (i.e. emergence) of these aquatic insects. The second principal axis (explaining 16.6% of the variability) showed effective separation consistent with trophic position, separating Odonata and Araneae as predators and Trichoptera as prey [Fig. 1]. The inclusion of biofilm further emphasized these differences and highlighted the importance of habitat as a determinant of metal concentrations, with PC1 and PC2 explaining 80.1 and 9.0% of total variance (Fig. S1). The most important metals associated with the separation along the first PC were Al, Mn, Ti, Fe, Ba, Pb, and along the second PC were Mo, Mn, Sr, Al, Ba, Cu, Fe. A list of the contributions of all variables accounting for the variability in a given PC is provided in Table S5.

3.3.1. Movement of metals through various life stages of hemi- and holometabolous aquatic insects

For the majority of metals, holometabolous Trichoptera exhibited higher concentrations in their tissues than hemimetabolous Odonata (i.e. Ba, Sr, Fe, Mn, Cr, V, Ti, Al, Pb, Cs, Mo), with only Cu and Ag recorded in higher levels in Odonata (Table S6). In Odonata, patterns were generally consistent across both sites, with the majority of metals measuring significantly higher concentrations in aquatic larvae than terrestrial adults (Table S7). In contrast, only class B metals Ag, Cd and Cu (Krapina) and Ag, Tl and Se (Bistrec) were present in significantly higher concentrations in adults than larvae (Table S7). A similar pattern was observed within holometabolous Trichoptera, where concentrations of most metals (Co, Ni, Ba, Mn, Ti, Pb, Tl, V, Fe, Ag, Cs, Cr, Cd, Al) were highest in the aquatic larval stage, while Mo, Pt, Se and Sr showed no difference between different life stages of Trichoptera (Table S8). Only Cu, Zn and As measured significantly higher concentrations in Trichoptera adults than larval and pupal stages (Table S8; Fig. S2). Mean concentrations of selected metals measured across life stages of Odonata and Trichoptera collected at all three sampling locations are presented in Fig. 2.

Metal-specific BAmFs reflected these results, with the majority of metals detected in hemimetabolous Odonata decreasing in concentration (BAmF < 1.0) between the larval and adult stage (Fig. 3). In holometabolous Trichoptera, a total of 14 metals declined in concentration between larval and pupal stages (BAmF < 1.0), while only 7 declined between pupal and adult stages, with the majority remaining at similar concentrations (BAmF ~ 1.0) to the preceding life stage. Furthermore, three metals exhibited bioamplification (BAmF > 1.0) between the pupal and adult life stages (Cu, Zn, As) compared to only one between the larval and pupal stages (Zn), indicating the tendency of some metals to concentrate in the tissues of Trichoptera during metamorphosis due to the loss of body mass following the cessation of feeding in pupal and adult stages (Huryn and Wallace, 2000). In hemimetabolous Odonata, BAmF measured > 1.0 for adult/larval life stages for Ag across both sites, while BAmFs for Cd, Pb, Cr, Fe, Co, Ni, Cu, Sr, Sb and Se were inconsistent between sites (Fig. 3). The reason for such inconsistency between the two locations was likely a result of different sample sizes in Krapina (n=11) and Bistrec (n=2), with Krapina offering a much more representative result due to its greater number of observations and lower variability. The mean BAmF for Trichoptera adult/pupa in Prelog was 0.85 and 0.64 for pupa/larva. For Odonata, the mean BAmF for adult/larva in Krapina was 0.57 and 0.86 in Bistrec. As none of these mean values surpassed the threshold of BAmF > 1.0, this suggests the overall lack of bioamplification of metals in both hemimetabolous Odonata and holometabolous Trichoptera.

3.4. Distribution of metals across trophic levels

When taking into consideration the trophic level of organisms examined in our study, a similar pattern emerged to that following metamorphosis. In Prelog, riparian spiders measured significantly higher levels of class B and borderline metals (Cu, Zn, Cd and Fe) than Trichoptera adults (Table S9). Only Pt and Se showed no significant differences in concentration between aquatic and terrestrial stages of Trichoptera and riparian spiders, suggesting no relationship with trophic position. A larger portion of the metals (Ti, Pb, Al, V, Cr, Co, Ni) were present in significantly higher concentrations in biofilm than in Trichoptera larvae but exhibited no differences in concentration between the collected terrestrial biota (Table S9), suggesting the possibility of the retainment of these metals in riparian systems, however without biomagnification. All remaining metals declined in concentration from aquatic grazers to riparian predators (Table S9), suggesting that these metals may have the potential of biodilution (i.e. a decrease in concentration of a contaminant with increasing trophic position [Campbell et al., 2005]) through food chains. In Bistrec, highest levels of class B metals (Cu and Se) were measured in terrestrial predators (Odonata adults). Some metals did not differ in concentration, regardless of the trophic position (i.e. Odonata predator or Trichoptera prey) of the organisms collected (Sr, Sb, Pt, Ag Tl, Cd), suggesting that these metals may have biotransferred between trophic levels in respective habitats, but did not biomagnify. Fe, Mo, Cr, Mn, Ti, Ba, and Al measured significantly higher concentrations in each preceding trophic level (Table S10), suggesting possible trophic dilution of these metals in both aquatic and terrestrial environments.

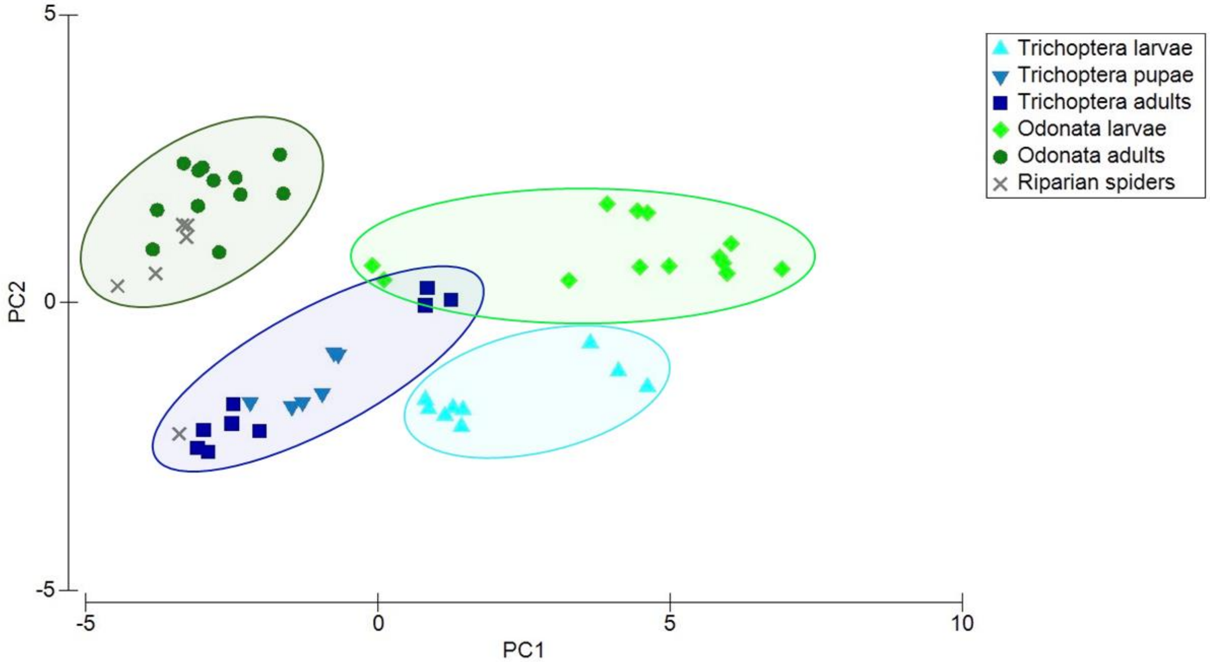

Fig. 1. Principal components analysis (PCA) of metal concentrations measured in tissues of aquatic insects (Trichoptera and Odonata) and riparian spiders (Araneae) collected across three sites in NW Croatia in spring 2018. The ellipses denote the following clusters: Trichoptera larvae (cyan), Trichoptera pupae – Trichoptera adults (indigo), Odonata larvae (light green), and Odonata adults – riparian spiders (olive). The following metals explained over 90% of the variance along PC axis 1: Al, Mn, Ti, Fe, Ba, Pb, Ni, and PC axis 2: Mo, Mn, Sr, Al, Ba, Cu, Fe.

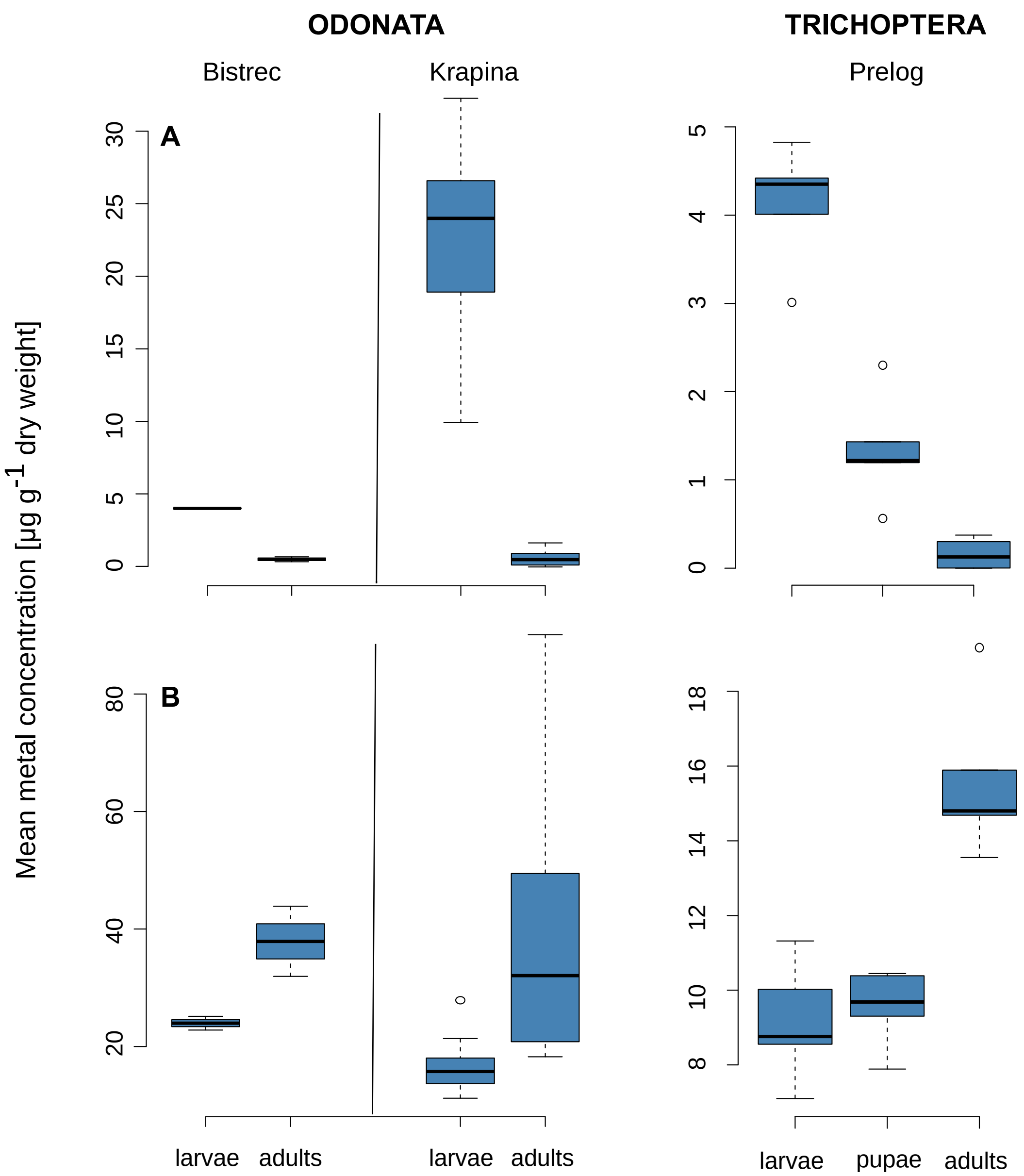

Fig. 2. Mean metal concentrations (µg g^{-1} dry weight ± SD) of (A) titanium (Ti) and (B) copper (Cu) measured across different life stages of Odonata (larvae and adults) and Trichoptera (larvae, pupae and adults) from three sites in NW Croatia in spring 2018. The two selected metals are representative of the following groupings: (A) biodilution, i.e. the reduction in metal concentration through metamorphosis (e.g. Ti, V, Al, Pb, Ba, Fe) and (B) bioamplification, i.e. the increase in metal concentration through metamorphosis (e.g. Ag, Cu). Note: y-axes scales differ between panels.

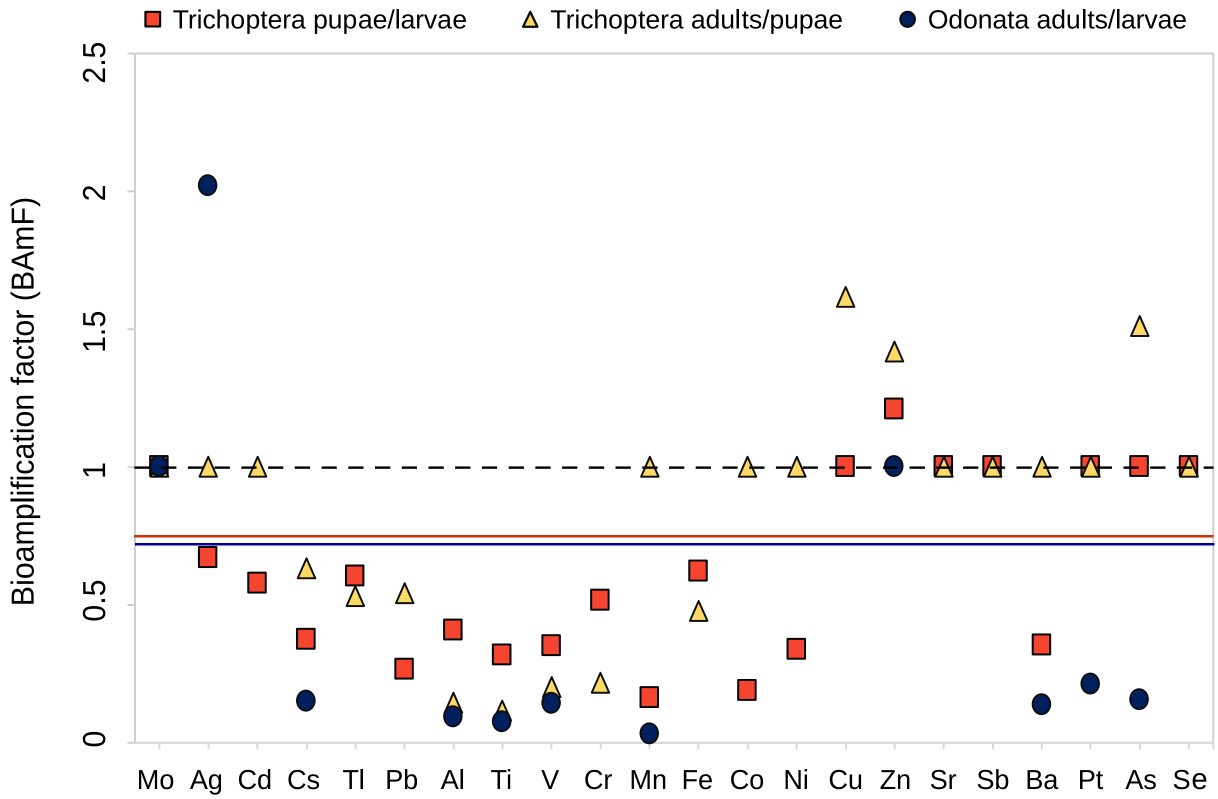

Fig. 3. Metal-specific bioamplification factors (BAmFs) for Trichoptera pupae/larvae and Trichoptera adults/pupae in Prelog, and Odonata adults/larvae in Krapina and Bistrec. The dashed line represents a bioamplification equilibrium of 1.0, with BAmF values > 1.0 indicating bioamplification, and BAmF values < 1.0 indicating reduction of a specific metal between two life stages. BAmFs that were inconsistent between sites for Odonata adults/larvae were omitted from the plot (Cd, Pb, Cr, Fe, Co, Ni, Cu, Sr, Sb, Se). The solid red and blue lines represent the mean bioamplification factors across life stages of Trichoptera (larvae, pupae and adults [0.736]) and sites for Odonata (Krapina and Bistrec [0.714]), respectively.

Table 1. Mean concentrations (μg g^{-1} dry weight) and associated standard errors (in parentheses) of trace metals in tissues of aquatic and terrestrial stages of aquatic insects (Trichoptera and Odonata) and riparian spiders (Araneae) collected across three sites in NW Croatia in spring 2018.

Site	Sample	Mo	Ag	Cd	Cs	Tl	Pb	Al	Ti	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	Sr	Sb	Ba	Pt	As	Se
PRELOG	**BIO**	0.71 (0.11)	12.11 (1.06)	0.43 (0.07)	0.51 (0.20)	0.1 (0.01)	15.03 (2.04)	1759.04 (139.59)	37.15 (2.44)	4.69 (0.30)	5.76 (0.68)	670.30 (90.69)	4114.78 (454.89)	1.72 (0.17)	4.29 (0.43)	10.89 (1.33)	86.29 (12.65)	127.66 (29.27)	0.12 (0.01)	51.98 (7.69)	0.00	5.86 (0.70)	1.51 (0.25)
	TR – Larva	6.03 (0.49)	0.01	0.10 (0.01)	0.02	0.02	1.81 (0.06)	57.86 (4.67)	4.16 (0.25)	0.44 (0.03)	0.54 (0.03)	442.75 (52.28)	242.18 (10.49)	0.32 (0.04)	0.42 (0.04)	9.09 (0.59)	39.70 (2.39)	14.65 (1.15)	0.06	16.79 (1.13)	0.01	1.29 (0.10)	3.22 (0.20)
	TR – Pupa	6.19 (0.37)	0.01	0.06	0.01 (0.01)	0.01	0.48 (0.07)	23.66 (5.35)	1.32 (0.23)	0.15 (0.04)	0.28 (0.05)	72.58 (4.67)	150.75 (34.27)	0.06 (0.01)	0.10 (0.01)	9.57 (0.38)	48.09 (1.45)	15.22 (2.79)	0.05 (0.01)	5.96 (0.09)	0.00	1.21 (0.05)	4.48 (0.34)
	TR – Imago	6.26 (0.48)	0.01	0.06 (0.01)	0.01	0.01	0.26 (0.01)	3.42 (0.94)	0.15 (0.06)	0.03	0.06 (0.01)	74.90 (5.35)	71.93 (6.99)	0.03	0.09 (0.01)	15.48 (0.80)	68.27 (3.68)	16.99 (0.85)	0.05	6.15 (0.49)	0.01	1.82 (0.19)	4.25 (0.53)
	ARAN	1.17 (0.73)	0.07 (0.01)	1.07 (0.24)	0.01	0.01	0.15 (0.02)	3.40 (1.24)	0.09 (0.03)	0.04 (0.01)	0.02 (0.01)	25.04 (7.08)	172.98 (27.77)	0.03 (0.01)	0.09 (0.01)	47.53 (9.95)	134.36 (24.83)	4.34 (1.30)	0.02	1.73 (0.82)	0.00	0.46 (0.20)	3.51 (0.48)
BISTREC	**BIO**	2.24 (0.08)	6.32 (0.75)	0.92 (0.04)	0.30 (0.01)	0.06	14.21 (0.45)	3222.54 (116.17)	190.27 (5.96)	25.74 (1.49)	53.86 (1.56)	25444.62 (1815.59)	28806.56 (1321.19)	21.30 (1.32)	38.04 (2.14)	12.04 (1.29)	115.59 (4.98)	173.38 (31.30)	0.26 (0.03)	782.33 (44.01)	0.01	44.80 (3.01)	1.01 (0.03)
	TR – Larva	2.54 (0.08)	0.02	0.42 (0.10)	0.06	0.01	0.70 (0.02)	210.99 (13.76)	13.60 (1.09)	1.65 (0.31)	1.30 (0.06)	1736.14 (478.06)	789.21 (88.15)	3.33 (0.74)	10.25 (2.70)	16.17 (2.30)	122.12 (9.84)	5.40 (1.44)	0.06 (0.01)	61.35 (12.80)	0.01	4.29 (1.13)	3.18 (0.39)
	TR – Imago	0.50 (0.01)	0.02 (0.01)	0.09 (0.04)	0.03	0.01 (0.01)	0.36 (0.10)	97.03 (10.48)	5.82 (1.07)	0.52 (0.13)	0.57 (0.11)	139.39 (17.80)	269.63 (12.47)	0.16	0.34 (0.02)	18.16 (0.66)	76.00 (6.36)	4.81 (0.20)	0.01	35.86 (2.66)	0.01	0.15 (0.01)	0.53 (0.02)
	OD – Larva	0.23 (0.01)	0.03	0.23 (0.01)	0.02	0.01	0.24 (0.02)	57.96 (0.93)	4.00 (0.03)	0.35 (0.01)	0.27 (0.05)	192.38 (11.26)	221.47 (3.74)	0.30 (0.01)	0.39 (0.01)	23.97 (0.68)	35.31 (1.28)	3.23 (0.5)	0.01	3.45 (0.06)	0.01	0.53 (0.01)	1.78 (0.08)
	OD – Imago	0.15 (0.01)	0.07	0.01	0.01	0.02	0.15	8.82 (1.78)	0.49 (0.10)	0.08 (0.01)	0.14 (0.02)	8.97 (0.49)	128.64 (11.33)	0.31 (0.06)	0.12 (0.04)	37.90 (3.45)	103.96 (14.12)	1.44 (0.01)	0.01	0.82 (0.12)	0.00	0.15 (0.01)	3.32 (0.02)
KRAPINA	**BIO**	0.36 (0.03)	0.79 (0.05)	0.12	0.48 (0.04)	0.04	27.42 (1.37)	2315.18 (192.79)	38.54 (1.28)	5.64 (0.44)	6.66 (0.60)	1645.43 (13.25)	5135.85 (409.56)	3.01 (0.15)	7.01 (0.45)	5.62 (0.28)	30.00 (1.87)	340.65 (7.93)	0.07	149.66 (3.17)	0.00	6.13 (0.18)	0.43 (0.02)
	OD – Larva	0.27 (0.04)	0.03	0.09 (0.02)	0.13 (0.02)	0.014 (0.002)	12.78 (2.03)	861.07 (88.96)	22.69 (2.00)	2.65 (0.30)	2.90 (0.37)	788.04 (147.45)	2823.34 (518.68)	2.28 (0.25)	3.22 (0.33)	16.71 (1.43)	65.69 (4.66)	7.76 (0.95)	0.07 (0.01)	24.02 (4.37)	0.00	4.24 (1.21)	1.71 (0.19)
	OD - Imago	0.33 (0.05)	0.06 (0.01)	0.39 (0.10)	0.01	0.005 (0.001)	0.28 (0.04)	30.78 (6.12)	0.59 (0.19)	0.13 (0.02)	0.21 (0.03)	12.78 (2.18)	153.92 (20.14)	0.08 (0.01)	0.19 (0.02)	38.13 (7.13)	71.82 (7.77)	1.44 (0.40)	0.01	0.89 (0.19)	0.00	0.14 (0.02)	1.88 (0.41)

4. Discussion

In this study, we examined the bioaccumulation and biotransfer of 22 different metals within aquatic and associated terrestrial systems. We found that biofilm did indeed act as a natural sink for metals present within the sampled freshwater streams (Croteau and Luoma, 2008; Xie et al., 2009; Cain et al., 2011), at times measuring up to 2837-fold higher concentrations than all other collected aquatic and terrestrial life stages of Trichoptera and Odonata, as well as riparian spiders. This is to be expected, as the freshwaters in our study were impacted by a gradient of communal and industrial wastewater effluents and agricultural runoff, with measured biofilm levels of Cr, Mn, Fe, Ni, As, Sr and Pb of the same order of magnitude as those recorded in the highly polluted Tisza River in Hungary (Mages et al., 2004).

Trichoptera larvae measured highest concentrations of most metals among invertebrates (albeit at much lower concentrations than those measured in biofilm), suggesting that diet may have served as an important exposure pathway for the bioaccumulation of metals in these aquatic grazers. This is in accordance with findings from Cain et al. (2011) who found that, under natural conditions, feeding on Cu- and Cd-contaminated biofilm resulted in increased metal body burdens of aquatic mayflies. Similarly, Xie et al. (2009) showed that diet was a significant Cd exposure route for the grazing mayfly *Centroptilum triangulifer*, while Timmermans and Walker (1989) noted that midge larvae accumulated considerable amounts of Zn and Cd from metal-contaminated substrate. Although aquatic organisms may also uptake metals through aqueous exposure (Wilding and Maltby, 2006; Sevilla et al, 2014), due to the tendency of sediments and biofilm to act as a sink for metals, holding much higher concentrations of metals compared to the surrounding water, the benthic (i.e. dietary) route is likely to present the dominant route of uptake (Barranguet et al., 2000; Morrison et al., 2000; Farag et al., 2006; Mebane et al., 2020).

Exposure to metals and other contaminants during the larval stages of development may negatively impact the metamorphosis and adult stages of insects. This can occur through the triggering of behavioural changes that can in turn compromise their foraging and predation likelihood (Tomé et al., 2014; Jinguji et al., 2018) or by decreasing the immune response of the organisms, therefore increasing their susceptibility to parasites (Mangahas et al., 2019). Furthermore, contaminant exposure has been shown to lead to the reduced emergence of adults (Schulz and Liess, 1995; Lidman et al., 2020), the reduction of survival to the adult stage, as well as the shortening of the life span of adults (Bahadorani and Hilliker, 2009).

Almost all metals detected within our study biotransferred from the aquatic to the terrestrial ecosystem, however, concentrations were lower in the terrestrial ecosystem relative to the aquatic ecosystem. This clear distinction between metal concentrations measured in biota in aquatic and terrestrial habitats was especially highlighted within the multivariate ordination [Fig. 1]). An important underlying factor impeding the transfer of metals between these two systems was the metamorphosis of aquatic insects (both complete and incomplete). Accordingly, BAmFs of total metal concentrations calculated for Odonata and Trichoptera were below the 1.0 threshold (~0.66), indicating that metals were lost during the metamorphosis of both hemimetabolous and holometabolous insect orders. Although the effect of metamorphosis on metal concentrations in aquatic insects is well known (Kraus et al., 2014), an interesting finding of our study is that the pattern of metal loss appeared to differ with type of metamorphosis. In holometabolous Trichoptera, the loss of metals was separated into two steps, with the first step of metamorphosis (larva/pupa) exhibiting a greater decrease in metal concentration, seen as a total BAmF of 0.64. The second step that exhibited a lower rate of decline, with a total BAmF of 0.85, overlapped with the change from pupal to adult stage. This two-step process was reflected in the co-clustering of Trichoptera pupae, an aquatic life stage, together with terrestrial Trichoptera adults (Fig. 1), which can be explained by greater morphological similarities exhibited between these two life stages compared to between the pupal and larval stage, despite differences in habitat (Rolff et al., 2019). A similar finding was reported by Wanty et al. (2017) who examined the movement of Zn through different life stages of the mayfly *Neocloeon triangulifer* and found that overall body concentrations declined significantly between larvae and subimagos but remained similar between subimagos and adults, thereby suggesting that only metabolically useful Zn was retained to adulthood. This detoxification and loss of excess Zn during metamorphosis was also constrained by isotope effects, favouring the elimination of isotopically lighter Zn and the retainment of isotopically heavier Zn, however this process is not yet well understood (Wanty et al., 2017). Furthermore, despite Odonata containing higher overall concentrations of Cu and Ag, we found that more class B and borderline metals exhibited bioamplification between aquatic and terrestrial stages of Trichoptera (Cu, Zn, As) than Odonata (Ag), which is in accordance with our expectations.

The trophic position of organisms collected within our study may have also played a role in shaping the flux of metals, as aquatic and terrestrial prey (i.e. Trichoptera larvae and adults) exhibited a distinct separation from aquatic and terrestrial predators (i.e. Odonata larvae and adults, as well as riparian spiders) on the multivariate ordination. An important finding of our study was that trophic position appeared to be subordinate to metamorphosis in altering metal concentrations, suggesting that metamorphosis could potentially be responsible for greater declines in metal levels than trophic transfer. We must note, however, that although our study assumes a major role of dietary transfer of metals to aquatic larvae, the possibility that aqueous exposure (that we were unable to account for within the scope of this study) played an equal or similar role in the uptake of metals within these aquatic environments must be considered (Sevilla et al, 2014).

Research has shown that some class A metals (i.e. hard metals [Kinraide, 2008]) may have adverse effects on freshwater organisms (Gensemer and Playle, 1999; Golding et al., 2018); however, our findings suggest that these metals, even when accumulated, were relatively quickly lost from within the biota. A possible explanation for the lack of bioamplification of these metals seen within our study may have been low metal assimilation efficiencies and/or high rates of elimination within our collected organisms, most likely due to the softness of individual metals (Williams, 1982; Croteau et al., 2007; Kraus et al., 2014). For example, the reduction in metal concentrations through metamorphosis may have occurred through the shedding of the exoskeleton during molts (Timmermans and Walker, 1989; Bardeggia and Alikhan, 1991; Dallinger and Rainbow, 1993; Kraus et al., 2014; Simon et al., 2019). Simon et al. (2019) found significantly higher levels of the class A and borderline metals Al, Fe and Mn in dragonfly exuviae than in larvae and adults. In addition to these findings, Kraus et al. (2014) noted that deposition into the meconium during metamorphosis into adults could account for over 50% of metal loss in invertebrates, indicating that this process may have in part been responsible for the observed difference in metal concentrations between larval and adult stages of Trichoptera and Odonata within our study. However, it is important to note that studies researching the meconium as a means for elimination of contaminants have focused solely on terrestrial environments (Dallinger and Rainbow, 1993; Kraus et al., 2014), whereas strong evidence supporting this argument for aquatic invertebrates is currently lacking.

Class B metals, on the other hand, were found to bioamplify across Trichoptera and Odonata life stages. As elevated levels of these metals were measured in higher trophic level organisms (i.e. predators), it is possible that class B metals may also exhibit biomagnification within terrestrial food chains. Although the assessment of direct trophic linkages was beyond the scope of this study, Odonata adults and riparian spiders have been shown to feed primarily on aquatic insects – a diet which can account for up to 80 and 90% of their mass, respectively (Paetzold et al., 2005; Jackson et al 2016; Chari et al., 2017). It is therefore possible that the majority of their metal exposure was due to feeding on metal-contaminated prey emerging from the contaminated freshwaters (i.e. Trichoptera adults). As both adult Odonata and riparian spiders are an important transmitting trophic link between small insects and larger terrestrial predators (e.g. birds and bats), they may contribute to the transfer of metals and other contaminants higher up the trophic food web (Popova and Kharitonov, 2012; Richmond, 2018). Furthermore, it is possible that biomagnification of class B metals may also occur within the aquatic environment in higher trophic level predators (e.g. fish). Based on their position in the food web, and that Zygoptera (damselflies) are often prey for Anisoptera (dragonflies), we may also expect differences in metal concentrations between these two suborders in the order Odonata, i.e. it is possible that Anisoptera may exhibit higher levels of metals. However, as the investigation of trophically-linked aquatic predators and prey exceeded the range of this paper, we were unable to assess this. An important implication of these findings is that lower trophic level aquatic organisms (i.e. grazers) may be at greater risk than terrestrial invertebrates regarding the bioaccumulation of class A metals from contaminated aquatic environments, as Trichoptera larvae measured highest levels of these metals at all sampled freshwater sites. However, for class B metals that exhibited increased concentrations following emergence (Cu, Ag, Zn) or trophic transfer (Cd, Cu, Zn, Se), terrestrial invertebrates in adjacent riparian habitats may be most at risk.

Several of the class B metals that were present in higher concentrations in terrestrial organisms (i.e. Ag, Cd) are non-essential and can be highly toxic to organisms (Ali et al., 2019). Genchi et al. (2020) demonstrated the propensity of Cd to easily transfer between biota due to its high solubility and mobility in environmental media. In our study, Cd was present at highest concentrations in riparian spiders, at levels ~20-fold higher than that measured in adult Trichoptera at the same site. Similar results were seen in Croteau et al. (2005), who reported 15-fold higher Cd concentrations between two trophic links in freshwater invertebrate food webs. Although concentrations recorded in our study did not exceed no-observed-effect-concentrations for Cd (Gintenreiter et al., 1993), this non-essential metal has been shown to be highly toxic (Vijver and Peijnenburg, 2011; Genchi et al., 2020) and its potential to biomagnify in the riparian food web prompts further investigation. On the other hand, Ag has not been found to biomagnify across aquatic or terrestrial food webs (Ratte, 1999; Watanabe et al., 2008; Yoo-iam et al., 2014). Although our findings suggest possible bioamplification of Ag in Odonata, as well as the biomagnification in riparian predators, maximum measured concentrations in invertebrate tissues in our study were still relatively low (~0.07 μg Ag g^{-1} DW in Odonata adults) compared to those measured in natural uncontaminated environments (~0.1 μg Ag g^{-1} DW), and 200-fold lower than those found in primary producers within contaminated environments (~14 μg Ag g^{-1} DW [Eisler, 2007]). It is therefore not likely that Ag concentrations within this range would have an adverse impact on higher trophic level consumers (e.g. vertebrates), as they are not considered to be highly sensitive to Ag (Ratte, 1999).

There is still much information lacking regarding how individual metals move within and between ecosystems. Our study provides evidence for the bioamplification and biotransfer of class B (soft) metals (i.e. Ag, Cd, Cu, Zn, Se) from contaminated aquatic systems to terrestrial systems, suggesting that the behaviour of their ions in aqueous solution may play a role in determining the mobility and accumulation of metals in the environment (Pearson, 1963; Duffus, 2002; Kraus et al., 2014). Due to this, we strongly suggest incorporating softness as a quantitative metric when considering the bioaccumulation and/or toxicity of metals in natural environments. An important implication of the findings of our study is that risks associated with metal-contaminated freshwaters are not limited to aquatic systems but can rather extend to terrestrial systems through the uptake and retention of metals in aquatic insects and their distribution via adult emergence.

5. Conclusion

This study provides a comprehensive overview of the impact of complete and incomplete metamorphosis on the cross-ecosystem flux of metals by investigating a suite of metals that have not been strongly represented in literature (i.e. Tl, Al, Mo, Ti, V, Mn, Sr, Sb, Pt, Se), but that have shown to be bioavailable and toxic (Gensemer and Playle, 1999; Golding et al., 2018). We demonstrated that metamorphosis may have played a strong role in altering the concentrations of metals detected in aquatic insects, with levels of most metals declining with each subsequent life stage in both holometabolous Trichoptera and hemimetabolous Odonata. This decline, however, occurred in two steps in the holometabolous order, where a greater decrease was observed between larval and pupal stage compared to that from pupal to adult stage.

Although we did not directly measure trophic linkages within this study, we found that the majority of metals similarly declined in concentration from primary consumers (Trichoptera) to upper trophic level predators (Odonata adults and riparian spiders). Exceptions to this observation were seen with several class B (soft) metals – both essential (Cu, Zn, Se) and non-essential (Cd) – that exhibited bioamplification, and potentially biomagnification. This could in turn have far-reaching impacts on higher level terrestrial predators, particularly during periods of mass emergence of these potential invertebrate biovectors. As emerging aquatic insects can compose up to 100% of the diet of riparian consumers (Likens, 2010), understanding the impact of metamorphosis and trophic transfer on the behaviour of metals is a crucial step in assessing and predicting risks to both freshwater and riparian ecosystems.

Supporting Information

Table S1. Metal concentrations detected in the blanks (μg L^{-1}) and the calculated limits of detection (LOD) and quantification (LOQ) (μg g^{-1}) for the sampled biota.

Table S2. Main characteristics of the three sampled sites in NW Croatia. Data includes annual ranges and averages (mean ± SE) of measured physico-chemical parameters, along with identified taxa and their feeding behaviour. Measurements from Prelog were collected in 2018, while those from Bistrec and Krapina were collected in 2011 and 2012, respectively.

Table S3. Results of the one-way ANOVA comparing detected metal concentrations in biofilm across three sampling locations in NW Croatia (Prelog, Krapina and Bistrec), followed by Tukey's HSD post-hoc analysis of multiple comparisons. *df* indicated degrees of freedom for the sources of variation.

Table S4. A selection of metal concentrations (μg g^{-1} dry weight) detected in macroinvertebrates collected from clean and contaminated* sites.

Table S5. Percentage of variance explained along the first two principal component (PC) axes. The principal component analysis (PCA) was conducted on metal concentrations measured in tissues of aquatic insects (Trichoptera and Odonata) and riparian spiders (Araneae) collected across three sites in NW Croatia in spring 2018.

Table S6. Results of Student's t-test comparing detected metal concentrations in hemimetabolous Odonata and holometabolous Trichoptera in Bistrec, followed by Tukey's HSD post-hoc analysis of multiple comparisons. *df* indicated degrees of freedom for the sources of variation.

Table S7. Results of Student's t-tests comparing detected metal concentrations in various life stages of hemimetabolous Odonata (larvae and adults) in Bistrec and Krapina, followed by Tukey's HSD post-hoc analysis of multiple comparisons. *df* indicated degrees of freedom for the sources of variation.

Table S8. Results of the one-way ANOVA comparing detected metal concentrations in various life stages of holometabolous Trichoptera (larvae, pupae and adults) in Prelog, followed by Tukey's HSD post-hoc analysis of multiple comparisons. *df* indicated degrees of freedom for the sources of variation.

Table S9. Results of the one-way ANOVA comparing detected metal concentrations in aquatic and terrestrial trophic compartments at Prelog (i.e. biofilm – Trichoptera larvae – Trichoptera adults – riparian spiders), followed by Tukey's HSD post-hoc analysis of multiple comparisons. *df* indicated degrees of freedom for the sources of variation.

Table S10. Results of the one-way ANOVA comparing detected metal concentrations in aquatic and terrestrial trophic compartments at Bistrec (i.e. biofilm – Trichoptera larvae – Odonata larvae – Trichoptera adults – Odonata adults), followed by Tukey's HSD post-hoc analysis of multiple comparisons. *df* indicated degrees of freedom for the sources of variation.

Figure S1. PCA of metal concentrations measured in biofilm, along with tissues of aquatic insects (Trichoptera and Odonata) and riparian spiders (Araneae). All samples were collected across three sites in NW Croatia in spring 2018.

Figure S2. Mean concentrations of metals (µg g^{-1} dry weight ± SD) measured across different life stages of Odonata (larvae and adults) and Trichoptera (larvae, pupae and adults) from three sites in NW Croatia in spring 2018. Note: y-axes scales differ between panels.

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Credit Authorship Contribution Statement

Katarina Cetinić: Data curation, Investigation, Visualization, Writing - original draft, Writing - review & editing. Ana Previšić: Data curation, Conceptualization, Funding acquisition, Investigation, Methodology, Writing - review & editing. Marko Rožman: Data curation, Conceptualization, Funding acquisition, Investigation, Methodology, Software, Visualization, Supervision, Writing - review & editing.

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