Bioaccumulation and bioamplification of pharmaceuticals and endocrine disruptors in aquatic insects

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Abstract

Environmental fate of emerging contaminants such as pharmaceuticals and endocrine disrupting compounds at the aquatic terrestrial boundary are largely unexplored. Aquatic insects connect aquatic and terrestrial food webs as their life cycle includes aquatic and terrestrial life stages, thus they represent an important inter-habitat linkage not only for energy and nutrient flow, but also for contaminant transfer to terrestrial environments. We measured the concentrations of pharmaceuticals and endocrine disrupting compounds in the larval and adult tissues (last larval stages and teneral adults) of five Odonata species sampled in a wastewater-impacted river, in order to examine their bioaccumulation and bioamplification at different taxonomic levels. Twenty different compounds were bioaccumulated in insect tissues, with majority having higher concentrations (up to 90% higher) in aquatic larvae compared to terrestrial adults (reaching 88 ng/g for 1H-benzotriazole). However, increased concentration in adults was observed in seven compounds in at least one suborder (41 % of the accumulated), confirming contaminants bioamplification across the metamorphosis. Both, bioaccumulation and bioamplification differed at various taxa levels; the order (Odonata), suborder (Anisoptera and Zygoptera) and species level. Highest variability was observed between Anisoptera and Zygoptera, due to the underlying differences in their ecology. Generally, Zygoptera had higher concentrations of contaminants in both larvae and adults. Additionally, we aimed at predicting effects of contaminant properties on bioaccumulation and bioamplification patterns using the commonly used physicochemical and pharmacokinetic descriptors on both order and suborder levels, however, neither of the two processes could be consistently predicted with simple linear models. Our study highlights the importance of taxonomy in studies aiming at advancing the understanding of contaminant exchange between aquatic and terrestrial food webs, as higher taxonomic categories include ecologically diverse groups, whose contribution to “the dark side of subsidies” could substantially differ.

Keywords: emerging contaminants, aquatic-terrestrial habitat linkage, ecological traits, subsidies, Odonata

1. Introduction

Significant amounts of wastewater are daily discharged into freshwater bodies worldwide, which causes presence of various emerging contaminants (ECs) in surface waters. Pharmaceuticals (PhACs) and endocrine disrupting compounds (EDCs) are very diverse groups of substances used for medical or personal care, as well as in food and manufacturing industry, and are often detected in natural freshwaters polluted with wastewater (Tijani et al., 2013). In fact, more than 600 individual PhACs or their metabolites are found worldwide in many natural habitats, including surface waters (aus der Beek et al., 2016). In spite of the growing number of studies investigating ecological effects of PhACs and EDCs on aquatic ecosystems, due to very large number of compounds and complexity of these ecosystems, specific impacts of individual compounds as well as their mixtures are yet to be discovered (Ebele et al., 2017).

Aquatic organisms can absorb and ingest contaminants by aqueous and dietary exposure (Arnot and Gobas, 2006). Contaminants can be retained in organisms, metabolically transformed or excreted by digestion or respiration (Mandaric et al., 2015). When a certain substance, including PhACs and EDCs, is more retained than excreted from the organism, we observe bioaccumulation (Lagesson et al., 2016; Meredith-Williams et al., 2012; Previšić et al., 2019; Ruhí et al., 2015; Wilkinson et al., 2018). When the organism loses body mass, concentration of contaminants can further increase without additional exposure, hence we observe bioamplification (Kraus et al., 2014b). Bioamplification usually occurs during life stages characterized by the significant developmental changes followed by weight loss and/or decrease in the ability to eliminate pollutants from the body in the same proportion (Daley et al., 2009). For instance, aquatic insect metamorphosis alters metal concentrations in aquatic insects mostly by reducing their body burdens, however, some essential (Cu, Zn, Se) and non-essential metals (Cd, Ag) have shown an opposite trend (Cetinić et al., 2021), confirming bioamplification of metals. Moreover, bioamplification of contaminants in aquatic insects was observed in both hemi- and holometabolous insects, e.g. for polychlorinated biphenyl (PCB) in Ephemeroptera (Daley et al., 2011), organochlorine compounds and polybromodiphenyl ethers in

Diptera and Trichoptera (Bartrons et al., 2007) and PhACs and EDCs in Trichoptera (Previšić et al., 2021).

Aquatic insects connect aquatic and terrestrial food webs as their life cycle includes aquatic (larvae and, in some orders, pupae) and terrestrial (adults) life stage. Thus, they represent an important inter-habitat linkage not only for energy and nutrient flow, but also for contaminant transfer to terrestrial environments (Daley et al., 2011; Kraus et al., 2014a). Various taxa show different trends in contaminant bioaccumulation and bioamplification, implying that ecological traits play a major role in contaminant availability and exposure, and consequently contribute to accumulation and transport of compounds through food webs (Bartrons et al., 2007; Previšić et al., 2021). Knowledge on species ecological traits has been successfully integrated into freshwater ecological assessment systems, however, those developed using autecological information on species level and are not applicable on higher taxonomic groupings (Schmidt-Kloiber and Nijboer, 2004). Analogously, higher taxonomic groupings are potentially composed of ecologically diverse groups whose contribution to "the dark side of subsidies" (Walters et al., 2008) could substantially differ. Existing data on PhACs and EDCs flux are either obtained at the coarse taxonomic resolution, e.g. insect orders (Bartrons et al., 2007; Park et al., 2009) or at the genus or species level (de Solla et al., 2016; Ruhí et al., 2015), without establishing potential hierarchical patterns and their causalities. Even though all Odonata species are predators throughout their life cycle, the two suborders differ in their trophic position, habitat preferences, dispersal behaviour and type of respiration, potentially influencing different bioaccumulation and bioamplification patterns (Corbet, 1999).

Furthermore, besides ecology, prediction of environmental fate of contaminants, i.e. bioaccumulation, has been related to physico-chemical properties of contaminants, such as the octanol–water partition coefficient (log K_{ow}), aqueous solubility (log S) etc. (Du et al., 2014; Franke et al., 1994; Huerta et al., 2012). However, first insights suggest that bioaccumulation of PhACs in aquatic insects is not easily predicted by simple physicochemical descriptors (e.g. log $K_{ow,}$ log D, log S) and models mainly established on persistent organic pollutants (Previšić et al., 2021). Bioamplification of medium-chain chlorinated paraffins (SCCPs and MCCPs) (Liu et al., 2020) as well as persistent halogenated organic pollutants (Liu et al., 2018) in insects has also been related to log K_{ow} values, however, data on PhACs are lacking.

This study aims at improving our knowledge on the fate and behaviour of individual PhACs and EDCs at the aquatic-terrestrial ecosystem boundary. More specifically, we focus on establishing bioaccumulation and bioamplification patterns of PhACs and EDCs at different taxonomic levels of an aquatic insect group, i.e. at the order (Odonata), suborder (Anisoptera and Zygoptera) and species level. We hypothesise that fine scale differences in ecological traits (such as habitat preferences, dispersal behaviour and type of respiration) would influence uptake and bioamplification across metamorphosis of these ECs in Odonata, at least between Anisoptera and Zygoptera. Hence, we compare concentrations of PhACs and EDCs measured across all life stages (aquatic larvae and terrestrial adults) from a location impacted by wastewater effluents at the order, suborder and species level. Moreover, having in mind the very limited knowledge of prediction of environmental fate of PhACs and EDCs, we aimed at assessing the influence of physicochemical descriptors and predictors related to pharmacokinetics of PhACs and EDCs on uptake and bioamplification in Odonata. This was also conducted respective with the taxonomic resolution, i.e. at the order and suborder level.

2. Materials and Methods

2.1 Study site and sample collections

Sampling was conducted on Krapina River (Krapina; N45.934457 E15.818039), located in the NW Croatia (Table S1). Krapina River is a medium lowland river with multiple (treated and untreated) wastewater effluents upstream from our sampling location. More than 1100000 m^3 of wastewater was discharged into the Krapina River and its tributaries in 2018, according to Report on data from the Register of Environmental Pollution for 2018. Further information on the selected sampling site and annual range and averages of the main physico-chemical water parameters measured at the sites are listed in Supporting information (Table S1). Two collections within maximally 30 days were conducted in spring 2018 (May & June), in order to collect aquatic (larval [lv]) and terrestrial (adult = imago [im]) stages of Odonata inhabiting the targeted site, and to reduce variability in temporal dynamics of aquatic insect flux (Kato et al., 2003). Water samples were collected in replicates in 1L bottles. Aquatic insects sampling included aquatic and terrestrial stages of the two Odonata suborders, Anisoptera (dragonflies) and Zygoptera (damselflies) (Table 1, Table S1). Adult insects were collected in riparian zones using entomological net, i.e. sweeping riparian vegetation along the watercourse (up to 3 m laterally). In order to remove any doubt in the larvae-adults comparisons, i.e. to avoid specimens that have potentially dispersed from a different locations and/or have fed as adults, we collected exclusively teneral adults, as particularly Anisoptera are known to disperse over relatively long distances (Corbet, 1999). Aquatic stages, i.e. Odonata larvae were sampled with a D-net screening all present freshwater microhabitats. We sampled final instars larvae (i.e. within size ranges listed for last instars for particular taxa (Brochard et al., 2012; Table S1) in order to enable reliable species identification and to reflect effects of bioamplification accurately. Aquatic samples were then transported to the lab in 10 L containers filled with water from respective sites. Before further processing, larval samples were left for 24 hours in river water to encourage gut clearance. All taxa were separated according to species or genera, freeze-dried and stored at -80°C.

2.2 Sample laboratory processing and analysis: PhACs and EDCs analyses

All specimens identified to species level were pooled per species (from both collection dates) to create a composite sample (for Anisoptera 4-6 larvae and 1-3 adults, and for Zygoptera 6 – 15 larvae and 15 - 20 adults/per species) that was shortly grinded by bead beating in a home build bead beater with 2.3-mm-diameter chrome-steel beads at frequency of 20 Hz at 4°C. Three analytical replicates of 50 mg freeze-dried insect tissue were created for each species. Aquatic insect samples were further processed following the methods of (Previšić et al., 2021). Firstly, 1.5 mL ice cold acetonitrile was added to 50 mg freeze-dried insect tissue. Secondly, standard mixture containing all isotopically labelled standards was added as internal standard. Then the tissue was lysed by bead beating at frequency of 20 Hz for 5 min at 4°C. Afterwards, samples were centrifuged at 20 000 x g for 10 min and supernatant 1 was collected. Remaining pellet was re-suspended in 1.5 mL of ice cold acetonitrile and additional lysis was done via ultrasonic probe (Sonoplus HD4050, Bandelin electronic GmbH, Germany) for 1 min at 50% of intensity. Samples were vortexed for 5 min, centrifuged at 20 000 x g for 10 min and supernatant 2 was collected. Supernatants 1 and 2 were evaporated to dryness and dissolved in 1 mL of water.

Both water and biota samples (i.e. supernatants) were additionally cleaned with solid phase extraction using Waters Oasis HLB cartridges (60 mg, 3 mL). Cartridges were conditioned with 3 mL of acetonitrile followed by 3 mL of HPLC-grade water at a flow rate of 1 mL min^{-1}. 100 mL of water sample or 2 mL of biota sample extracts were loaded at 1 mL min^{-1}. Sample were washed with 1mL of water and consequently extracted with 1.5 mL of pure acetonitrile at a flow rate of 1 mL min^{-1}. Final extracts were evaporated to dryness under a gentle nitrogen stream and reconstituted in 0.3 mL methanol/water (50:50, v/v) and used for targeted analysis.

Target analysis was performed using an ultra-performance liquid chromatography (UPLC) system (Waters Milford, USA) coupled to a hybrid quadrupole linear ion trap mass spectrometer Qtrap 5500 (Applied Biosystems, USA) following methods used in our previous publications (Previšić et al., 2021, 2019). Details regarding UPLC separation, and instrument parameters can be found in Supplementary Methods S1. Altogether, aquatic insects and water samples were screened for total of 119 PhACs and 24 EDCs. List of all compounds is provided in Supplementary Methods S1. Instrument control, data acquisition and data analysis were carried out using Analyst 1.5.1 software (Applied Biosystem). Target compounds were quantified using an internal standard method by the Bquant script for batch quantification of liquid chromatography mass spectrometry data using the procedure described (Rožman and Petrović, 2016). Concentrations are presented in ngg^{-1} of dry weight (dw) for insect samples and ngL^{-1} for water samples.

2.3 Data Analysis

In all statistical tests, i.e. comparisons between taxonomic groupings and life stages, all analytical replicates at the species level were included as input (all analytical samples were given the same statistical weight). Differences in total concentrations of ECs, PhACs, EDCs and individual compounds concentrations quantified in Odonata samples between different life stages (larval [lv] and adult stage = imago [im]) were tested within the species/suborder/order level using Mann-Whitney U test (Table S5 and Table S6). The same test was also used to infer differences in ECs concentrations between Zygoptera and Anisoptera larvae and adults. Using Kruskal-Wallis ANOVA test, we also tested differences in totals and individual ECs concentrations within particular life stage/habitat (lv/aquatic and im/terrestrial) for the species and suborder level (Table S7). All tests were conducted in SPSS ver. 27 (IBM).

With the aim of comparing bioaccumulation of PhACs and EDCs between different taxa, bioaccumulation factors (BAFs) were calculated. BAFs were calculated by dividing concentrations of individual compounds in larvae (at both suborder and order levels) with concentrations quantified in water samples from Krapina (Arnot and Gobas, 2006; Ruhí et al., 2015; Sims et al., 2020). Given that certain compounds' concentrations were below the detection limit in water samples, BAF values were calculated for 15 compounds, as shown in Figure 3.

Bioamplification factors (BAMFs) were calculated to evaluate differential cross-ecosystem flux of PhACs and EDCs via aquatic insect emergence. BAMFs were calculated as the ratio of concentrations of PhACs and EDCs between two consecutive life stages (Daley et al., 2011), i.e. between adults and larvae at the suborder and order level. According to Daley, 2013, bioamplification occurs when BAMF exceeds value of 1. As for statistical tests, for calculation of both factors, BAF and BAMF, all analytical replicates at the species level were included as input (all data for Odonata, and separately for Anisoptera and Zygoptera, respectively).

Moreover, we aimed at assessing the influence of physicochemical descriptors of PhACs and EDCs on bioaccumulation and bioamplification across metamorphosis in Odonata. For this purpose we used the physicochemical properties of individual ECs compiled using National Institutes of Health (Maryland, USA) PubChem open chemistry database and DrugBank Online (University of Alberta, CA). The most widely used descriptors: the octanol−water partition coefficient (log K_{OW}), polar surface area (PSA), and relative molecular mass (Mr), aqueous solubility (log S), number of rotatable bonds and number of hydrogen bond donors and acceptors were used (Table S3). Octanol−water distribution coefficient (log D_{OW}) and membrane-water distribution coefficient (log D_{MW}) were also considered (Table S3) and methods for estimating the distribution coefficients of studied PhACs and EDCs are summarized in Supplementary Methods Sl. The relationships between physicochemical descriptors and log BAFs as well as log BAMFs (e.g. Liu et al., 2018, 2021)at the order and suborder level in Odonata were analysed by nonparametric correlations and linear regressions in SPSS 27 (IBM).

Additionally, orthogonal partial least squares discriminant analysis (OPLS-DA) was employed to find descriptors responsible for bioaccumulation in aquatic insect tissues. For this purpose, OPLS-DA analysis was performed on two-group data set; ECs quantified in both, larval tissues and water (assumed to be bioaccumulative) versus ECs only detected in water (assumed to be non bioaccumulative). For the OPLS-DA analysis data matrix with already mentioned descriptors was extended by absorption, distribution, metabolism, and excretion (ADME) properties of each compound. ADME descriptors (total of 28 properties) were calculated using pkCSM platform available web interface (Pires et al., 2015). Final data matrix (provided in Supporting Material) contained following descriptors: log K_{OW}, log K_{MW}, log D_{OW}, log D_{MW}, Water solubility, Caco-2 cell permeability, Intestinal absorption (human), Skin Permeability, P-glycoprotein substrate, P-glycoprotein I inhibitor, P-glycoprotein II inhibitor, CYP2D6 substrate, CYP3A4 substrate, CYP1A2 inhibitor, CYP2C19 inhibitor, CYP2C9 inhibitor, CYP2D6 inhibitor, CYP3A4 inhibitor, VDss (human), Fraction unbound (human), BBB permeability, CNS permeability, Total Clearance, Renal OCT2 substrate, Molecular Weight, Rotatable Bonds, Number of Acceptors, Number of Donors, Molecular Surface Area. OPLS-DA analysis was done in R using *ropls* package (Thévenot et al., 2015).

3. Results

Out of 119 different PhACs and 24 EDCs that water and aquatic insect samples were screened for, a total of 37 compounds were quantified in water samples from Krapina (25 PhACs and 12 EDCs; Table 1). In aquatic and terrestrial stages of Odonata, a total of 20 compounds were quantified (8 PhACs and 12 EDCs; Table 1).

[Table 1 – listed after references]

3.1 Bioaccumulation and transport of PhACs and EDCs through life stages in Odonata: differences in taxonomic level

Bioaccumulation of PhACs and EDCs generally varied between taxa and life stages of aquatic insects collected in the current study. Total concentrations of ECs (sum of PhACs & EDCs) and of EDCs are significantly higher in larval stages in Odonata (213 % and 388 % for ECs and EDCs, respectively), as well as in Zygoptera (242 % and 552 % for ECs and EDCs, respectively; Fig. 1 A & E; Mann-Whitney U test Table S5). In Anisoptera, only total EDCs concentration was significantly higher in larval stages compared to adults (233 % higher; Fig. 1 C; Mann-Whitney U test Table S5). Differences between concentrations of individual ECs between nymphs and adults show different trends across all three taxonomic levels of Odonata (Table S7). However, highest variability was observed at the suborder level, i.e. between Anisoptera and Zygoptera (Figs. 1, Table S5 & S7). More precisely, in 11 compounds concentrations significantly differed between life stages in Zygoptera (differences ranging from 41-100 %), and only six in Anisoptera (differences ranging from 37-100 %) (Fig. 1 D&F, Mann-Whitney U test; Table S5). Significantly higher concentrations of eight individual compounds were observed in Zygoptera larvae compared to adults, including antibiotics (e.g. tilmicosin; 363 % higher concentrations in larvae), the nonsteriodal anti-inflammatory drugs (NSAIDs; e.g. salicylic acid; 206 % higher concentration in larvae) and organophosphorus flame retardants (OPFRs; e.g. TCPP 1374 % higher concentration in larvae; Fig. 1F). Similarly, in Anisoptera larvae, concentrations of five individual compounds significantly differ from concentrations in adults, including naproxen and the parabens (the methyl- and propylparabens, 270 % and 319 % higher concentrations in larvae, respectively; Fig. 1D). In contrast, concentration of only one PhAC (the antibiotic azithromycin) was significantly higher in adult terrestrial stages of Anisoptera compared to aquatic larvae (160 % higher), and three individual compounds were significantly higher in adult Zygoptera compared to aquatic larvae (e.g. triclosan, 272% higher; Fig. 1 D&F; Table S5). At the order level, none of the compounds had significantly higher concentration in terrestrial stages compared to larvae (Fig. 1B). Certain variability in bioaccumulation without any clear patterns was recorded among species of both suborders, regarding both, total concentrations and individual compounds (Fig. S1, B, D, F, H, J; Mann-Whitney U test, Table S6).

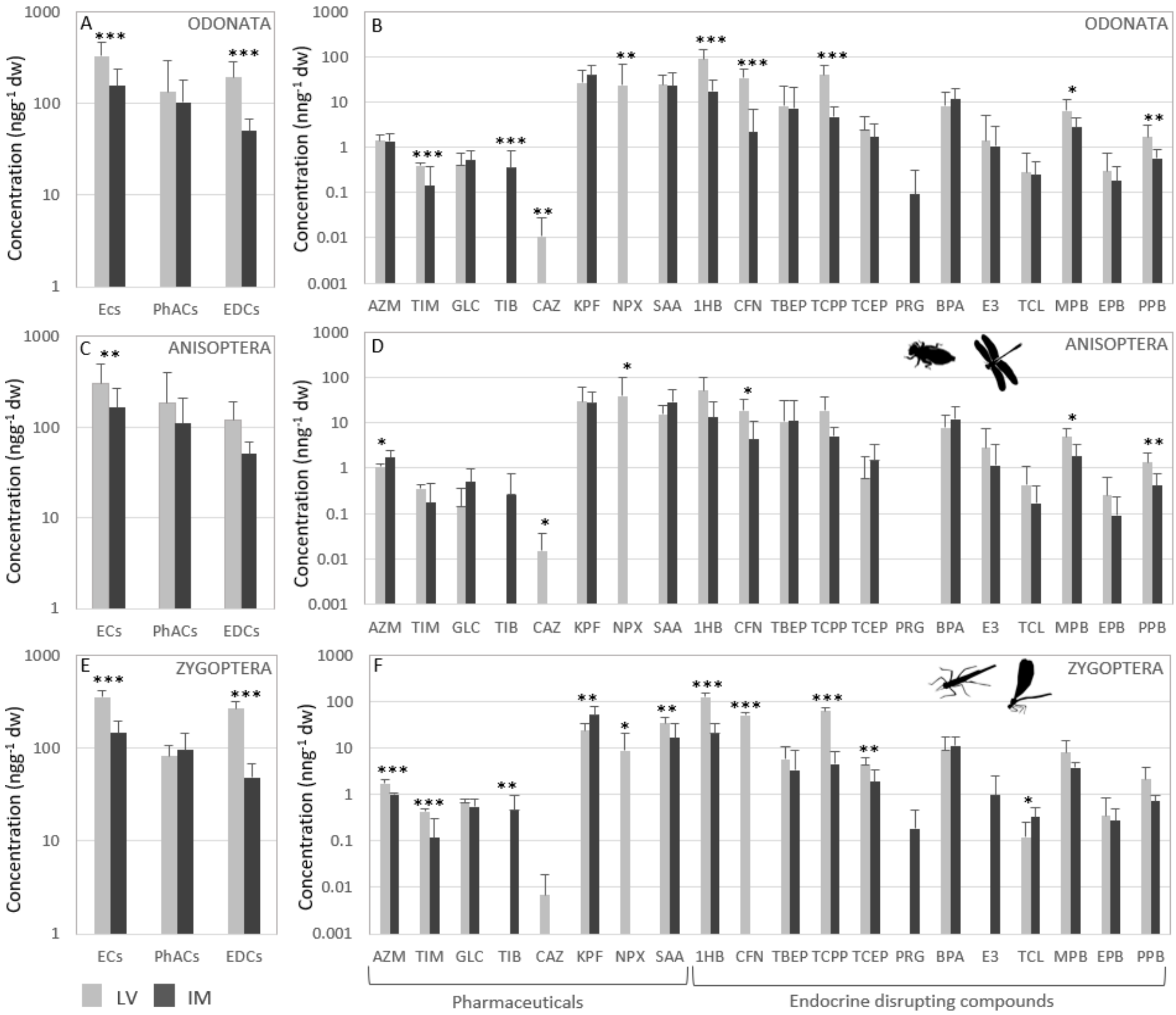

Figure 1. Total concentrations (**A, C & E**) of emerging contaminants (ECs: sum of PhACs & EDCs), pharmaceuticals (PhACs) and endocrine disrupting compounds (EDCs) and individual compounds concentrations (**B, D & F)** in aquatic (LV) and terrestrial (IM) stages of Odonata and separately on suborder taxonomic levels, in aquatic and terrestrial stages Anisoptera and Zygopera from Krapina River – Kupljenovo, Croatia. Concentrations are shown in logarithmic scale and significance is tested with the Mann-Whitney U test, significance is listed in Table S5. Full names of ECs are listed in Table 1 caption.

Comparison of total EC concentrations (sum of PhACs & EDCs) in larvae of the two suborders (Anisoptera and Zygoptera) shows significantly higher values of total ECs and EDCs in zygopteran larvae (115 % and 225 %, respectively; Fig. 2A). Similarly, significant differences were observed for seven individual compounds, all having higher values in zygopteran larvae (from 158 % to 693 %; Fig. 2B). In adults, total EC concentrations (sum of PhACs & EDCs) showed no significant difference between the two suborders (Fig. 2C), whereas in four individual compounds (the NSAID ketoprofen and the three parabens) significantly higher values were measured in zygopteran adults (from171 % to 305 %; Fig. 2D).

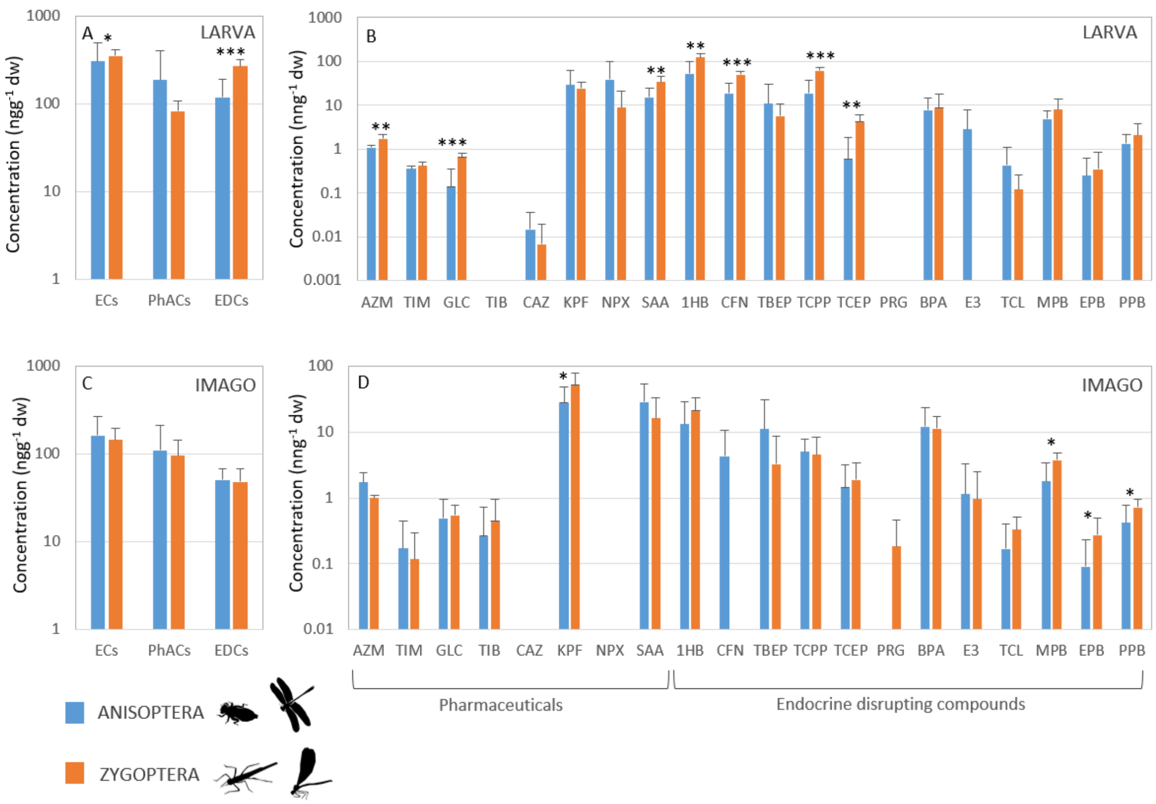

Figure 2. Total concentrations (**A, C**) of emerging contaminants (ECs: sum of PhACs & EDCs), pharmaceuticals (PhACs) and endocrine disrupting compounds (EDCs) and individual compounds concentrations (**B, D**) in aquatic and terrestrial stages of Anisoptera and Zygoptera. Concentrations are shown in logarithmic scale in B) and D), significance is tested with the Mann-Whitney U test and listed in Table S9. Full names of ECs are listed in Table 1 caption.

In accordance with patterns observed for concentrations of individual compounds, both BAF and BAMF values also show variability between insect taxa, more precisely between two suborders (Fig. 3). Comparing BAFs of ECs for aquatic larval stages of Zygoptera and Anisoptera, the following highest values stand out: propylparaben and salicylic acid for Zygoptera and propyparaben and triclosan for Anisoptera (Fig. 3). Generally, Zygoptera BAF values are higher for 10 out of 15 compounds, with five compounds having at least double the BAF values of Anisoptera. Moreover, organophosphorus flame retardants TCPP and TCEP, have three times and almost seven times higher BAFs in Zygoptera compared to Anisoptera, respectively.

BAMF values patterns for Zygoptera and Anisoptera are not consistent (Fig. 3B). Overall, seven compounds (41 %) have BAMF values ≥1 for at least one suborder, whereas two compounds, TBEP and bisphenol-A show bioamplification in both, Anisoptera and Zygoptera (Fig. 3B). Azitromycin, glibenclamide and salicylic acid BAMFs indicate bioamplification in Anisoptera solely. On the other hand, ketoprofen and triclosan have BAMF values ≥1 only in Zygoptera (Fig. 3B).

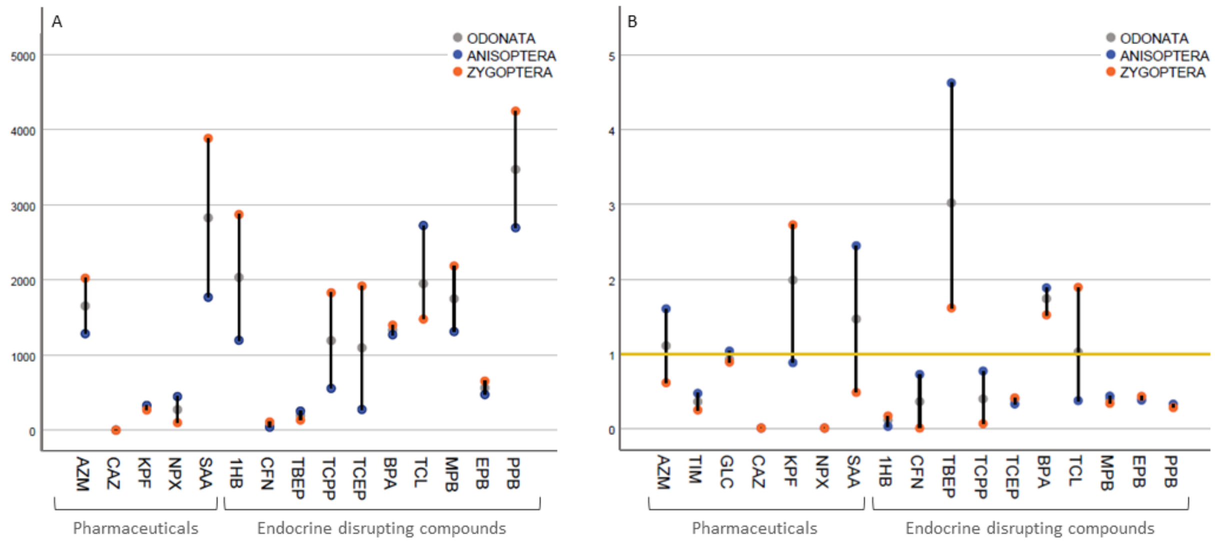

Figure 3. Bioaccumulation factors (A; BAFs; L/kg dw) and bioamplification factors (B; BAMF) of emerging contaminants for aquatic stages of Odonata and each suborder separately (Anisoptera and Zygopera). Full names of ECs are listed in Table 1 caption.

3.2 Influence of physicochemical and pharmacokinetic descriptors of PhACs and EDCs on bioaccumulation and bioamplification in Odonata

OPSL-DA classification model was computed to pinpoint specific descriptors of PhACs and EDCs influencing differential bioaccumulation behaviour in aquatic insects. However, OPLS-DA failed to expose group separation suggesting that no variation in the descriptor data matrix correlates with group membership, i.e. the ECs bioaccumulated in insect tissues could not be distinguished from those present only in the water based on the employed predictors (Fig. S2).

Similarly, Spearman's rank correlations and linear regressions conducted with each of the descriptors and BAFs and BAMFs for both levels, Odonata and suborders (Anisoptera and Zygoptera) did not enable predictions of bioaccumulative behaviour of ECs. More specifically, no statistically significant correlations between physicochemical (Table S8) and pharmacokinetic descriptors and BAF and BAMF values were inferred (data not shown). The only exception was positive relationship inferred between BAMF values in Odonata and log K_{ow} using linear regression (Figure S3).

4. Discussion

The present study confirms the presence of PhACs and EDCs of aquatic origin in all stages of aquatic insects inhabiting both aquatic and terrestrial habitats. More importantly, the current study implies differences in fate and behaviour of bioaccumulated PhACs and EDCs at the aquatic-terrestrial ecosystem boundary depending on insect taxa and/or life history traits. In accordance with the previous findings (Previšić et al., 2021), both Odonata suborders had generally higher concentrations of ECs in aquatic larval stages, however, considerable differences in both bioaccumulation and bioamplification patterns were observed between the two suborders.

All BAFs of PhACs and EDCs detected in this study are likely to be lower than limit value of 5000 L/kg wet weight (ww), suggesting that none of the measured compounds is very bioaccumulative in observed aquatic insect larvae (Arnot and Gobas, 2006; Borgå, 2013). Despite the fact that BAF values were expressed on dry weight (dw) basis, these are still comparable, as dw based values are 3-10 times higher than those based on ww (Karlsson et al., 2002). Nevertheless, some studies have shown that certain compounds can be bioaccumulative in aquatic insects, e.g. hydroxyzine in Zygoptera (Lagesson et al., 2016) and azithromycin in Trichoptera (Grabicova et al., 2015), having BAF values over 5000 L/kg ww. Comparing bioaccumulation for detected PhACs and EDCs at both order and suborder level observed in this study, Zygoptera stand out having overall highest concentrations of ECs and consequently highest BAF values. These differences between the two suborders could be related to their larval ecological traits and preferences like dietary preferences, feeding behaviour, and habitat distribution, which directly determine available routes of contaminant exposure in macroinvertebrates (Ducrot et al., 2005; Sidney et al., 2016). Both Zygoptera and Anisoptera larvae are predators, thus all taxa observed in this study (Anisoptera - *Gomphus vulgatissimus*, *Onychogomphus forcipatus*, *Orthetrum albistylum*; Zygoptera - *Calopteryx splendens*, *Platycnemis pennipes*; as listed in Table 1) are also predators (Dijkstra et al., 2022). Even so, larvae of the two suborders occupy different trophic positions, i.e. Anisoptera larvae can prey on Zygoptera and/or smaller Anisoptera (Johansson, 1991), implying that the trophic position could also cause differences in bioaccumulation of ECs between them. However, establishing potential direct trophic linkages was beyond the scope of the current study. Furthermore, habitat preferences differ between taxa, which can also affect availability to contaminants (Mayer-Pinto et al., 2016). Anisopteran larvae of the genera *Gomphus* and *Orthretum* like to burrow themselves in substrates, which could potentially increase their exposure to contaminants adsorbed on sediment particles (Simon et al., 2019). On the other hand, zygopteran *Calopteryx* sp. and *Platycnemis pennipes* larvae prefer slow flowing parts of streams and parts with standing water. Furthermore, larvae of the two suborders also have considerable differences in respiration organs, with zygopteran larvae having external sets of gills at the abdomen tip, and anisopteran larvae having internal rectal gills (Corbet and Brooks, 2008). It has been recorded that different types of respiration in aquatic invertebrates affect exposure to dissolved contaminants in water (Baird and Van den Brink, 2007). For example, plastron breathing in Hemiptera (e.g. *Notonecta glauca*) can reduce availability of contaminants, compared to biota with gills and breathing oxygen from water (Meredith-Williams et al., 2012). Furthermore, it has been confirmed that at least in specific conditions, such as hypoxia, Anisoptera larvae can be air-breathing, as in their last stages of nymphal development their imaginal respiratory systems are already developed (de Pennart and Matthews, 2020; Gaino et al., 2007; Kriska, 2013; Ubhi and Matthews, 2018). Moreover, certain aeshnid larvae (family Aeshnidae) can also use their nymphal rectal gills for breathing air outside of water (de Pennart and Matthews, 2020). Hence, larvae with the ability to breathe air instead of using gills and filtrating oxygen dissolved in water, could result in considerably lower exposure and uptake levels of contaminants in polluted aquatic environments.

Bioaccumulation patterns for certain compounds in aquatic insects could also depend on taxa specific contaminant elimination mechanisms. However, Heynen et al., (2016) showed that prompt response to changes in water concentrations of pharmaceutical oxazepam in dragonfly *Aeshna grandis* (Anisoptera) with fast uptake and elimination rates, could be because of the compounds adsorption processes happening on the surface of the body, rather than true uptake and metabolic eliminations. That increases the importance of the contaminant’s properties in defining bioaccumulation patterns in biota. After entering natural waters, contaminants differ in regard with potential for biodegradation, solubility, adsorption, persistence, mobility etc. (Stasinakis, 2012). Most of the ECs quantified in this study show low or low to moderate potential for bioconcentration (contaminant absorption from water via body surface and respiration, excluding dietary exposure) according to models based on their log K_{ow} values (PubChem Database; Arnot and Gobas, 2006; Borgå, 2013), which is in line with our observations. Consequently, none of the physicochemical and pharmacokinetic descriptors could be used to predict bioaccumulation in Odonata suggesting that used descriptors poorly reflect the underlying biochemistry of bioaccumulation. In line with (Previšić et al., 2021), this study further suggests that previous models mainly established on persistent organic pollutants may not be easily applied to predict bioaccumulation and bioaccumulation potential of ionized compounds such as PhACs in aquatic environments (Ismail et al., 2014; Puckowski et al., 2016).

The current study further confirms the bioamplification across the metamorphosis of some PhACs and EDCs in Odonata, in line with (Previšić et al., 2021). However, here we present considerable differences at different taxonomic levels, i.e. inconsistent patterns between Anisoptera and Zygoptera (i.e. at the suborder level) and among species. The differences between taxa are most likely also attributable to specific life history traits. Odonatan larval development includes individually specific, variable number of molts (*ecdyses*) – u to up to 30 molts over a time period of 3 months to 10 years depending upon species (Corbet and Brooks, 2008). Recent research points out the *ecdyses* as valuable pathway for contaminant elimination in Odonata (Liu et al., 2021), thus the number of molts most likely determines bioaccumulation rates, and subsequently bioamplification. Hence, lower bioaccumulation observed in Anisoptera could influence lower bioamplification and explain observed variations between taxa.

Possible differences in biotransformation and contaminant metabolic degradation processes between these two suborders as well as differences in metamorphosis processes could play an important role in determining bioamplification of PhACs and EDCs. Although little is known about biotransformation of PhACs and EDCs in aquatic organisms, it does seem to affect accumulation of PhACs in freshwater fish (Cerveny et al., 2021) and amphipods (Fu et al., 2020; Miller et al., 2017). The data exist only for a few compounds and show considerable differences between fish and aquatic insects, e.g. biotransformation of temazepam in perch and dragonfly larvae (Cerveny et al., 2021). Furthermore, differences in biotransformation of polybrominated diphenyl ethers (PBDEs) were assumed to affect differences in bioamplification rates between Diptera and Trichoptera (Bartrons et al., 2007). Therefore, different metabolic efficiency to biotransform PhACs and EDCs in Odonata suborders cannot be ruled out as the reason for different PhACs and EDCs bioaccumulation patterns on suborder level.

Potential differences of body mass loss during the metamorphosis process (from larvae to teneral adults) could also drive concentration differences between larvae and adults, as very large differences in the mass loss were reported for various insect taxa (e.g. 90% loss in Lepidoptera, 20% loss in Ephemeroptera, Kraus et al., 2014b). Moreover, in the experiments with the caddisfly *Micropterna nycterobia* in multiple stressor conditions, we have observed considerable variability in mass loss during metamorphosis in respect to environmental conditions (e.g. increased water temperature and pollution with PhACs & EDCs compared to controls) and sex (Previšić et al., unpublished data). To our knowledge, no data on the body mass loss during metamorphosis of Odonata species included in the current study exist. Nevertheless, taking into account the observed variability, literature data could provide only limited information, particularly if obtained from unimpacted sites.

Similar to bioaccumulation, physicochemical descriptors poorly reflected the underlying biochemistry of bioamplification of PhACs and EDCs. However, the linear positive relationship of BAMF and log K_{ow} at the order level (Odonata) found here, is in line with observations for persistent organic pollutants (e.g. PCB), where bioamplification was shown to be log K_{OW} dependent with higher BAMFs for more hydrophobic chemicals (Daley et al., 2012; Kraus, 2019). However, predicting such a process involving complex biochemical changes during insect metamorphosis requires evaluation on a larger dataset and probably more complex models.

Our study shows that taxonomic groupings and underlying biological traits are important when determining the resolution at which we assess, evaluate and predict rates of bioaccumulation and bioamplification of PhACs and EDCs in aquatic insects. Emergence of aquatic insects accumulating PhACs and EDCs during the aquatic phase, represents pathway for these compounds to be further transferred through aquatic food webs (Ruhí et al., 2015), and from aquatic to terrestrial food webs

(Previšić et al., 2021; Richmond et al., 2018). Given that adult aquatic insects are generally small, high prey consumption rates in predators like bats and birds can result in PhAC and EDCs concentrations exposures significantly higher than in their prey (Guigueno and Fernie, 2017; Markman et al., 2011; Secord et al., 2015). Consequently, a comprehensive understanding of these processes linking the two ecosystems merit further attention.

CRediT authorship contribution statement

Marina Veseli: Data curation, Investigation, Methodology, Visualization, Writing – original draft,Writing – review & editing**. Marko Rožman:** Data curation, Funding acquisition, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. **Marina Vilenica:** Investigation, Methodology. **Mira Petrović:** Conceptualization, Funding acquisition. **Ana Previšić:** Data curation, Investigation,Methodology, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition.

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Table 1. Mean concentrations (ng g^{-1} dry weight) and associated standard deviation (in parentheses) of PhACs and EDCs in tissues of aquatic and terrestrial stages of Odonata collected at the Krapina River (NW Croatia) in spring 2018. LV – larval stage, IM – adult stage, A – aquatic habitat, T – terrestrial habitat. AZM – azithromycin, TIM – tilmicosin, GLC – glibenclamide, TIB – thiabendazole, CAZ – carbamazepine, KPF – ketoprofen, NPX – naproxen, SAA – salicylic acid; EDCs: 1HB – 1H-benzotriazole, CFN – caffeine, TBEP-tris(2-butoxyethyl)phosphate, TCPP – tris(1-chloro-2-propyl)phosphate, TCEP – tris(2-carboxyethyl)phosphine, PRG – progesterone, BPA – bisphenol-A, E3 – estriol, TCL – triclosan, MPB – methylparaben, EPB – ethylparaben, PBB – propylparaben.

	Odonata		**Anisoptera**		***Gomphus vulgatissimus***		***Onychogomphus forcipatus***		***Orthetrum albistylum***		**Zygoptera**		***Calopteryx splendens***		***Platycnemis pennipes***	
	LV / A	**IM / T**	**LV / A**	**IM / T**	**LV / A**	**IM / T**	**LV / A**	**IM / T**	**LV / A**	**IM / T**	**LV / A**	**IM / T**	**LV / A**	**IM / T**	**LV / A**	**IM / T**
Ecs	328.290 (139.385)	154.266 (80.835)	305,138 (190,877)	163,400 (105,618)	294.710 (103.458)	261.614 (144.958)	384.631 (341.968)	137.933	236.071 (35.962)	90.652 (15.101)	351,443 (60,372)	145,132 (50,421)	370.243 (80.411)	123.955 (44.363)	369.547 (38.173)	175.152 (23.574)
PhACs	133.429 (161.188)	102.995 (76.634)	184,864 (20,480)	110,412 (100,334)	155.222 (97.136)	198.346 (149.577)	350.678 (338.193)	76.688	48.692 (16.170)	56.203 (13.485)	81,993 (25,435)	95,577 (47,843)	102.243 (38.560)	82.474 (48.546)	69.794 (8.146)	104.533 (27.604)
EDCs	193.070 (95.699)	49.759 (17.548)	118,828 (69,357)	51,079 (17,169)	137.985 (15.149)	60.594 (19.350)	32.522 (5.576)	59.450	185.975 (22.234)	33.193 (9.162)	267,313 (47,426)	48,438 (18,858)	265.610 (49.339)	40.324 (4.266)	298.120 (36.345)	69.640 (4.261)
AZM	1.401 (0.438)	1.369 (0.606)	1,088 (0,137)	1,737 (0,683)	1.117 (0.220)	2.417 (0.568)	1.074 (0.014)	1.795	1.074 (0.155)	1.000 (0.167)	1,714 (0,411)	1,000 (0,088)	1.925 (0.222)	1.055 (0.018)	1.257 (0.105)	0.979 (0.149)
TIM	0.390 (0.075)	0.144 (0.230)	0,357 (0,064)	0,171 (0,280)	0.386 (0.091)	0.257 (0.445)	0.356 (0.045)	n.d.	0.330 (0.061)	0.257 (0.224)	0,422 (0,074)	0,116 (0,180)	0.464 (0.098)	0.102 (0.177)	0.376 (0.047)	n.d.
GLC	0.402 (0.323)	0.517 (0.350)	0,141 (0,211)	0,494 (0,454)	n.d.	n.d.	n.d.	1.044	0.422 (0.021)	0.437 (0.022)	0,664 (0,150)	0,541 (0,230)	0.733 (0.101)	0.452 (0.417)	0.481 (0.039)	0.616 (0.012)
TIB	n.d.	0.358 (0.475)	n.d.	0,263 (0,474)	n.d.	0.790 (0.524)	n.d.	n.d.	n.d.	n.d.	n.d.	0,453 (0,485)	n.d.	0.218 (0.377)	n.d.	0.376 (0.180)
CAZ	0.011 (0.017)	n.d.	0,015 (0,022)	n.d.	0.0129 (0.016)	n.d.	n.d.	n.d.	0.032 (0.029)	n.d.	0,007 (0,012)	n.d.	n.d.	n.d.	0.020 (0.013)	n.d.
KPF	26.755 (23.103)	40.378 (25.737)	29,471 (31,974)	28,010 (20,084)	65.117 (28.045)	40.914 (34.787)	n.d.	19.603	23.296 (6.060)	23.514 (4.148)	24,039 (9,762)	52,746 (25,692)	31.154 (12.733)	43.272 (21.275)	18.822 (4.806)	78.664 (22.623)
NPX	23.866 (45.982)	0.000 0.000	38,958 (61,973)	n.d.	n.d.	n.d.	113.454 (53.218)	n.d.	3.419 (5.922)	n.d.	8,774 (11,826)	n.d.	9.419 (16.315)	n.d.	16.903 (8.835)	n.d.
SAA	24.567 (14.241)	22.705 (22.314)	15,379 (9,582)	29,016 (25,998)	21.287 (11.095)	26.448 (45.809)	17.573 (8.696)	43.034	7.279 (3.252)	17.566 (10.188)	33,754 (12,215)	16,395 (17,106)	39.796 (11.282)	15.451 (9.181)	20.097 (6.827)	8.477 (0.784)
1HB	88.981 (53.238)	17.227 (14.130)	52,389 (46,924)	13,299 (15,743)	65.631 (44.639)	n.d.	n.d.	33.993	91.535 (11.670)	5.905 (1.276)	125,573 (28,429)	21,154 (11,905)	127.324 (28.265)	13.607 (4.119)	131.030 (28.510)	34.894 (4.185)
CFN	34.510 19.798	2.157 (4.963)	18,572 (13,829)	4,314 (6,470)	13.779 (7.927)	n.d.	6.695 (2.695)	12.941	35.243 (5.656)	n.d.	50,448 (8,378)	n.d.	56.293 (6.378)	n.d.	48.992 (11.149)	n.d.
TBEP	8.256 (14.563)	7.236 (14.538)	10,780 (20,236)	11,169 (19,635)	22.911 (35.468)	26.686 (31.585)	2.771 (4.799)	3.266	6.659 (3.815)	3.555 (1.649)	5,732 (5,180)	3,304 (5,368)	6.057 (5.197)	6.120 (9.173)	5.176 (5.391)	2.356 (3.263)
TCPP	40.475 (26.109)	4.761 (3.361)	18,889 (17,424)	5,004 (2,808)	20.339 (18.894)	3.085 (3.113)	1.891 (1.953)	5.583	34.438 (7.371)	6.344 (3.624)	62,060 (9,829)	4,518 (3,999)	57.953 (3.659)	4.177 (3.160)	72.687 (10.521)	7.282 (4.513)
TCEP	2.399 (2.406)	1.676 (1.609)	0,605 (1,222)	1,456 (1,792)	n.d.	n.d.	n.d.	3.667	1.816 (1.635)	0.701 (1.214)	4,193 (1,888)	1,897 (1,478)	2.906 (2.642)	1.884 (1.668)	5.247 (1.188)	2.694 (0.609)
PRG	n.d.	0.092 (0.213)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0,183 (0,279)	n.d.	n.d.	n.d.	0.550 (0.092)
BPA	8.263	11.773	7,866	12,212	12.086	22.605	4.083	n.d.	7.431	14.031	8,659	11,333	4.888	8.905	17.452	15.415

	(7.887)	(8.661)	(7,007)	(11,066)	(11.667)	(9.138)	(0.726)		(3.356)	(3.953)	(9,096)	(6,041)	(4.255)	(4.123)	(9.902)	(3.834)
E3	1.427 (3.627)	1.059 (1.841)	2,854 (4,835)	1,148 (2,213)	n.d.	3.443 (2.781)	8.561 (4.495)	n.d.	n.d.	n.d.	n.d.	0,971 (1,513)	n.d.	0.962 (1.665)	n.d.	n.d.
TCL	0.277 (0.478)	0.249 (0.221)	0,433 (0,642)	0,170 (0,235)	0.765 (1.174)	0.463 (0.159)	0.297 (0.137)	n.d.	0.237 (0.059)	0.046 (0.042)	0,121 (0,133)	0,328 (0,184)	0.037 (0.032)	0.190 (0.173)	0.233 (0.119)	0.364 (0.034)
MPB	6.473 (4.900)	2.783 (1.657)	4,859 (2,606)	1,801 (1,633)	1.932 (1.677)	3.649 (0.446)	7.036 (0.968)	n.d.	5.608 (1.616)	1.754 (0.691)	8,086 (6,195)	3,764 (0,998)	8.259 (7.315)	3.611 (0.121)	12.652 (1.171)	4.696 (0.198)
EPB	0.298 (0.446)	0.182 (0.201)	0,251 (0,385)	0,090 (0,139)	n.d.	n.d.	n.d.	n.d.	0.752 (0.162)	0.269 (0.068)	0,345 (0,519)	0,274 (0,217)	n.d.	0.247 (0.219)	1.035 (0.068)	0.453 (0.109)
PPB	1.712 (1.324)	0.565 (0.337)	1,330 (0,842)	0,417 (0,355)	0.543 (0.477)	0.663 (0.075)	1.189 (0.132)	n.d.	2.257 (0.588)	0.587 (0.320)	2,095 (1,639)	0,713 (0,257)	1.893 (1.650)	0.622 (0.264)	3.616 (0.269)	0.936 (0.050)

Supplementary Information files

Supplementary Methods S1 – UPCL method details and list of compounds screened for in water and insect samples from the Krapina River, NW Croatia.

Table S1 Information on sampling site in NW Croatia were *in situ* sampling was conducted in 2018, and on taxa collected.

Table S2 Full names of compounds (PhACs and EDCs) and abbreviations used in the study.

Table S3 Absorption, distribution, metabolism, and excretion (ADME) properties and physicochemical descriptors (the octanol−water partition coefficient (log K_{OW}), octanol−water distribution coefficient (log D_{OW}), membrane-water distribution coefficient (log D_{MW}), aqueous solubility (log S), relative molecular mass (Mr), number of rotatable bonds, number of hydrogen bond donors and acceptors and polar surface area (PSA) of ECs used for orthogonal partial least squares discriminant analysis.

Table S4 Total concentrations (ngL^{-1}) of emerging contaminants (ECs), pharmaceuticals (PhACs) and endocrine disrupting and individual compounds concentrations (ngL^{-1}) measured in water samples from Krapina river shown as mean value ± standard deviation; n.d. - compounds not detected (below detection limit).

Table S5 Significance of differences of total concentrations and concentrations of individual compounds (pharmaceuticals [PhACs] and endocrine disrupting compounds [EDCs]) between life stages on order and suborder level (Odonata, Anisoptera, Zygoptera); according to the Mann-Whitney U tests. Significant p values are shown in red. Full names of compounds and abbreviations are listed in Table S2.

Table S6 Significance of differences of total concentrations and concentrations of individual compounds (pharmaceuticals [PhACs] and endocrine disrupting compounds [EDCs]) between life stages (larvae and adults) on species level (*Gomphus vulgatissimus*, *Orthetrum albistylum*, *Onychogomphus forcipatus*, *Calopteryx splendens*, *Platycnemis pennipes*); according to the Mann-Whitney U tests.

Table S7 Significance of differences of total concentrations and concentrations of individual compounds (pharmaceuticals [PhACs] and endocrine disrupting compounds [EDCs]) within same life stage/habitat (A9) lv/aquatic and B) im/terrestrial) among different taxa levels (order Odonata, suborders Anisoptera and Zygoptera, and species *Gomphus vulgatissimus*, *Orthetrum albistylum*, *Onychogomphus forcipatus*, *Calopteryx splendens*, *Platycnemis pennipes*); according to the Kruskal Wallis ANOVA test. Significant *p* values are shown in red. Full names of compounds and abbreviations are listed in Table S2.

Table S8 Spearman's rank correlation between bioaccumulation factors (log BAF) and bioamplification factors (log BAMF) and physicochemical descriptors of ECs used: the octanol−water partition coefficient (log K_{OW}), octanol−water distribution coefficient (log D_{OW}), membrane-water distribution coefficient (log D_{MW}), aqueous solubility (log S), relative molecular mass (Mr), number of rotatable bonds, number of hydrogen bond donors and acceptors and polar surface area (PSA). Significant values in bold.

Table S9 Significance of differences of total concentrations and concentrations of individual compounds (pharmaceuticals [PhACs] and endocrine disrupting compounds [EDCs]) of larvae and adult stages between two suborders - Anisoptera and Zygoptera; according to the Mann-Whitney U tests. Significant p values are shown in red. Full names of compounds and abbreviations are listed in Table S2.

Figure S1 Total concentrations (A, C, E, G & I) of emerging contaminants (ECs: sum of PhACs & EDCs), pharmaceuticals (PhACs) and endocrine disrupting compounds (EDCs) and individual compounds concentrations (B, D, F, H & J) in aquatic (LV) and terrestrial (IM) stages of five Odonata species from Krapina River – Kupljenovo, Croatia. Concentrations (ngg-1 dw) are shown in logarithmic scale and significance is tested with the Mann-Whitney U test, significance is listed in Table S6. Full names of ECs are listed in Table S2.

Figure S2 OPLS-DA analysis of bioaccumulative vs. non-bioaccumulative ECs. OPLS-DA fails to expose group separation (top left plot) as suggested by low variation of ECs explained by the model ($R2Y = 0.37$), poor prediction performance ($Q2Y = -0.072$) and p-values > 0.05.

Figure S3 Relationship (linear regressions) between bioamplification factor (BAMF) and octanol-water partition coefficient (log Kow) for Odonata (a), Anisoptera (b) and Zygoptera (c).