Science of the Total Environment 704 (2020) 135333

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Aquatic macroinvertebrates under stress: Bioaccumulation of emerging contaminants and metabolomics implications

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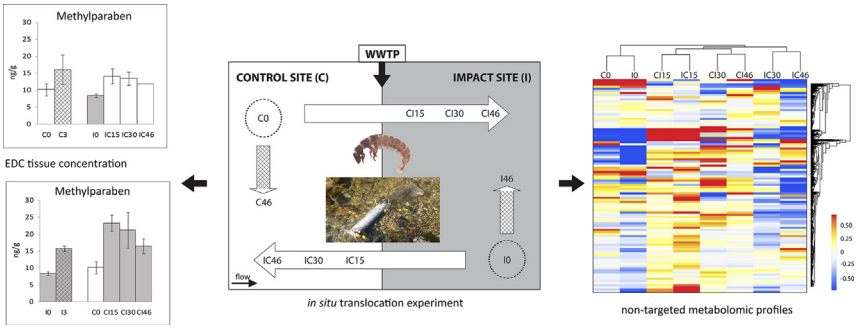
HIGHLIGHTS

- PhACs and EDCs were detected in water but only EDCs were measured in insects.
- Bioaccumulation was temporary and strongly related to varying water concentrations.
- Metabolite profile of insects was altered during the translocation experiment.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 27 September 2019
Received in revised form 24 October 2019
Accepted 31 October 2019
Available online 25 November 2019

Editor: Daqiang Yin

Keywords:
Endocrine-disrupting compounds (EDCs)
Aquatic insects
In situ experiment
Non-targeted metabolomics
Hydropsyche
Urban wastewater

ABSTRACT

The current knowledge on bioaccumulation of emerging contaminants (ECs) in aquatic invertebrates exposed to the realistic environmental concentrations is limited. Even less is known about the effects of chemical pollution exposure on the metabolome of aquatic invertebrates. We conducted an *in situ* translocation experiment with passive filter-feeding caddisfly larvae (*Hydropsyche* sp.) in an effluent-influenced river in order to i) unravel the bioaccumulation (and recovery) dynamics of ECs in aquatic invertebrates, and ii) test whether exposure to environmentally realistic concentrations of ECs will translate into metabolic profile changes in the insects. The experiment was carried out at two sites, upstream and downstream of the discharge of an urban wastewater treatment plant effluent. The translocated animals were collected at 2-week intervals for 46 days. Both pharmaceuticals and endocrine disrupting compounds (EDCs) were detected in water (62 and 7 compounds, respectively), whereas in *Hydropsyche* tissues 5 EDCs accumulated. Overall, specimens from the upstream site translocated to the impacted site reached higher ECs concentrations in their tissues, as a reflection of the contaminants' water concentrations. However, bioaccumulation was a temporary process susceptible to change under lower contaminant concentrations. Non-targeted metabolite profiling detected fine metabolic changes in translocated *Hydropsyche* larvae. Both translocations equally induced stress, but it was higher in animals translocated to the impacted site.

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https://doi.org/10.1016/j.scitotenv.2019.135333
0048-9697/

1. Introduction

Occurrence, fate and impact on the aquatic ecosystems of emerging contaminants (ECs) such as pharmaceutical (PhACs) and endocrine disrupting compounds (EDCs) are increasingly studied (Chang et al., 2019; Comber et al., 2018; Ebele et al., 2017; Pico et al., 2019) in the last years. Aquatic organisms may bioaccumulate these compounds via bioconcentration (uptake as a function of water concentration) and via trophic transfer (uptake via dietary sources) (Ding et al., 2016; Ruhí et al., 2016). However, the current knowledge on bioaccumulation and metabolic effects on aquatic invertebrates is sparse (reviewed by Rodríguez-Mozaz et al., 2016; but also reported by Burket et al., 2019; de Solla et al., 2016; Ruhí et al., 2016; Zhao et al., 2018) in spite of their vital role in freshwater food webs. In particular, passive filter feeders such as the caddisfly *Hydropsyche* larvae are omnivorous, their diet consisting of coarse particulate organic matter (CPOM), algal fragments, or smaller animals (Graf et al., 2008), and therefore are highly susceptible to incorporate contaminants from the other food web components. Some *Hydropsyche* species tolerate high levels of water pollution (Vuori, 1995, 1996), and may bioaccumulate PhACs and EDCs from the contaminated aquatic environment (Ruhí et al., 2016).

The current research on PhACs and EDCs bioaccumulation in aquatic invertebrates suggests their main entrance pathway being through uptake from ambient water (Ding et al., 2016; Huerta et al., 2015). Kinetics of bioaccumulation and depuration of PhACs have been studied in laboratory experiments using high concentrations and short-time exposure to water contaminants (Meredith-Williams et al., 2012) or mixtures of selected compounds in semi-natural systems (Lagesson et al., 2016). Temporal framework required by invertebrate tissues to show appreciative bioaccumulation of ECs *in situ* was mainly acquired from studies in which caged molluscs were exposed to wastewater effluents, e.g. for 6 weeks in the mudsnail *Potamopyrgus antipodarum* (Gray) (Gust et al. 2014) or 4 weeks in the mussel *Lassmigona costata* (Rafinesque) (de Solla et al., 2016). Moreover, data on bioaccumulation and depuration dynamics of accumulated ECs on finer temporal scale show taxa- and compound-specific patterns (Burket et al., 2019; Lagesson et al., 2016). However, data on the dynamics of bioaccumulation and depuration of ECs in aquatic invertebrates *in situ* and under realistic environmental concentrations at the individual and population levels are scarce (Burket et al., 2019).

Here we aim to unravel the bioaccumulation (and recovery) of ECs in aquatic invertebrates submitted to *in situ* exposure, and whether these were reflected on the metabolomic patterns of the individuals. We used a translocation exposure experiment to detect effects on *Hydropsyche* spp. larvae receiving (or having received) urban wastewater pollution. We hypothesised that i) the translocation of *Hydropsyche* spp. individuals from the upstream reach (devoid of direct WWTP effluent) to the impacted reach (downstream the WWTP effluent) would cause accumulation of ECs in their tissues; and ii) the individuals translocated from the impacted to the control site would fully recover from their bioaccumulation, being that effects on the exposed individuals act temporary. We further aimed to test whether exposure to environmentally realistic concentrations of ECs would translate into the metabolic profile of the organisms. We hypothesised that translocation to the impacted reach and the subsequent stress to the individuals would alter the metabolomic profiles of *Hydropsyche* specimens. It has been observed that altered metabolic pathways and metabolite levels occur as a response to chemical pollution in various freshwater organisms and communities (river biofilms; Serra-Compte et al., 2018a, cladoceran *Daphnia magna* Straus; Kovacevic et al., 2016, amphipod *Gammarus pulex* (L.); Gómez-Canela et al., 2016, and fathead minnows *Pimephales promelas* (Rafinesque); Skelton et al., 2014). Thus, it is presumed that chronic chemical pollution would produce changes on the metabolome of aquatic insects. In order to test this, we analysed the physiological changes in *Hydropsyche* specimens during the experiment via non-targeted metabolomics, i.e. by evaluating alterations in the metabolite profiles of translocated animals.

2. Materials and methods

2.1. Study area and study organism

The experiment was conducted in the Segre River (Ebro River basin, NE Iberian Peninsula) near the city of Puigcerdà. The city has 8900 inhabitants but the wastewater treatment plant (WWTP) processes effluents of ca 30,000 population equivalents. The river is relatively unimpacted (Ruhí et al., 2016), running through a valley covered with pastures, native forests and small agricultural fields. The river section including the control and impact sites is relatively homogeneous with regards to the main environmental factors such as geomorphology, hydrology, water chemistry, vegetation cover and shading, and streambed substrata (Acuña et al., 2015). The hydrological regime is driven by snowpack production during winter and snowmelt in late spring, with lower water flow during summer. We designed our manipulative experiment during October and November 2015, that is after summer lower flow and before higher flows could re-start. We selected two sites, one located ca 500 m upstream of the WWTP effluent (“control site”) and the other one ca 500 downstream from the WWTP discharge (“impact site”), respectively.

Two *Hydropsyche* species co-occurred during the experiment: *Hydropsyche siltalai* Döhler, 1963 and *Hydropsyche dinarica* Marinković-Gospodnetić, 1979, both accounting for high abundances. *H. siltalai* was dominant in the impact site, while in the control site only *H. dinarica* was present. Most *Hydropsyche* species are relatively tolerant of organic pollution, however, *H. dinarica* is listed as a bioindicator for oligosaprobic to beta-mesosaprobic water classes, and in the Iberic-Macaronesian ecoregion it is listed as a sensitive species (Graf et al., 2008; Graf et al., 2019). These species have a relatively intense growth period in autumn, so the timing was the most adequate for an *in situ* experiment.

2.2. Translocation exposure experiment: experimental design and sample collections

Design of enclosures was based on Vuori (1995). We used PVC tubes, 60 cm long and 12 cm in diameter, with 0.5 mm mesh size nylon nets at both upstream and downstream ends (Fig. 1A & B). In order to provide a net-spinning surface for *Hydropsyche* larvae across the whole diameter of the tube, a structure made of firm, 2 cm mesh size plastic, was inserted in each tube. The tubes were completely submerged and horizontally fixed to the riverbed. In order to prevent debris clogging within the tube, a coarser net was fixed in form of a triangular prism with a tip facing the current (Fig. 1A & B). This net and tube covers were occasionally cleaned or replaced.

Eight enclosures were placed at each site; 6 enclosures with translocated larvae (experimental enclosures) and two control enclosures containing the larvae from that particular site (Fig. 1C). At the control site, 5th instar larvae of *H. dinarica* were collected, and transported to the impact site, to complete up to 70 specimens in each of the tubes. At the same time, a mixture of 5th instar larvae of *H. dinarica* and 3rd instar larvae of *H. siltalai* were collected at the impact site, and transported to the control site, where a total of 120 specimens were placed in each tube. At

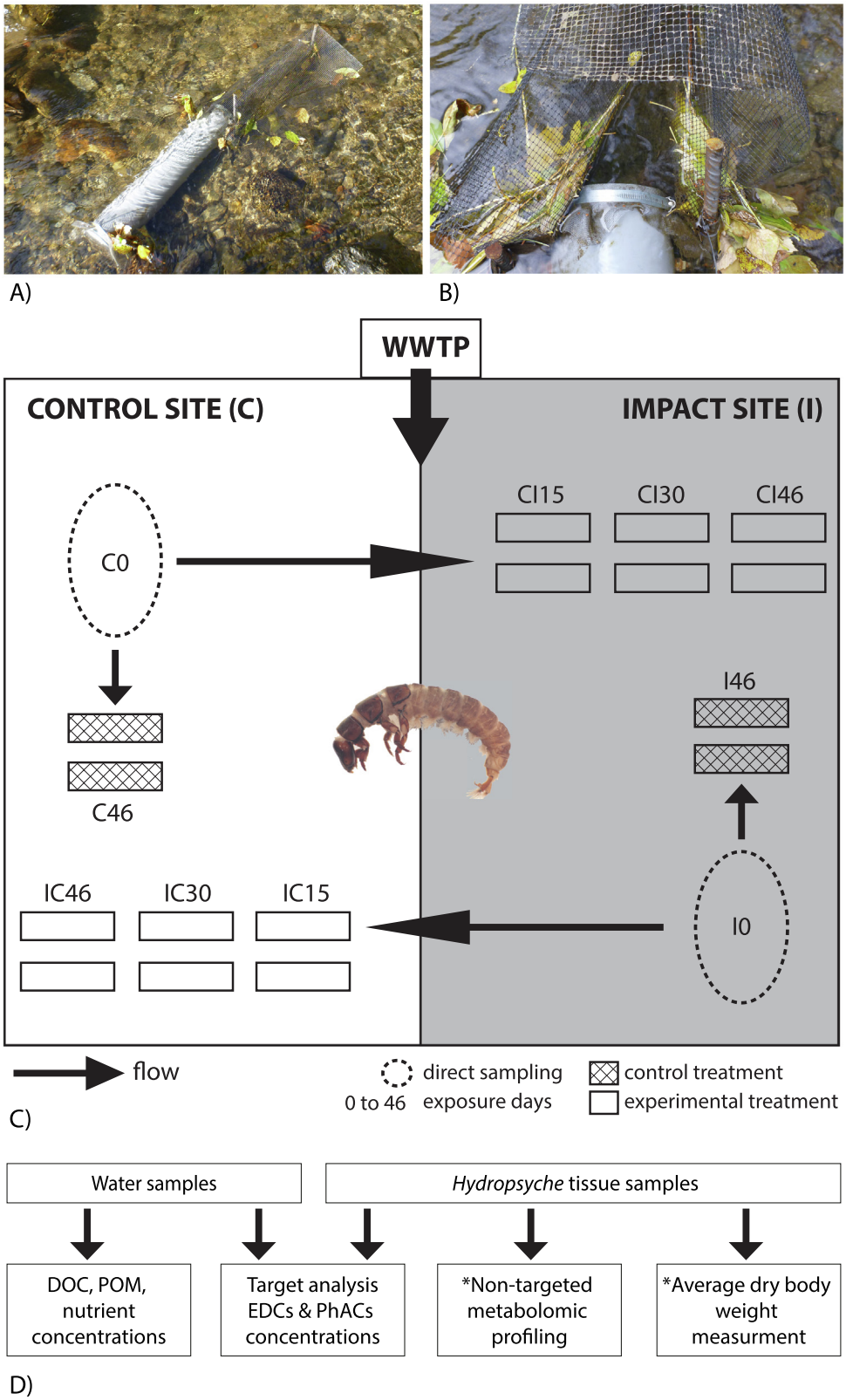

Fig. 1. Translocation experiment with *Hydropsyche* larvae; A) picture of the enclosure at the control site, B) detail of the enclosure; front with leaves and C) experiment design scheme (CI and IC are enclosures with translocated animals, from Control to Impact site and vice versa, respectively; numbers refer to days of exposure; C46 and I46 are control enclosures), and D) flowchart of the methodology applied for the analyses of water and *Hydropsyche* tissue samples (*conducted with *H. dinarica* samples only, for details see Materials and methods).

both sites, one additional empty enclosure was fixed for the collection of water samples used for nutrient and dissolved organic carbon (DOC) concentration analyses (see section 2.3).

The experiment was initiated on the 7–9 Oct 2015 with the collection of larvae and the translocations. At the same time, initial samples of both species were collected directly from the riverbed (C0 and I0, Table 1, Fig. 1C). Three subsequent collections were executed in approximately two-week intervals, so the maximum exposure length was 46 days in total (collection periods at 15, 30 and 46 exposure days; Table 1, Fig. 1C). Upon collection, specimens 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. Larvae from each enclosure were separated based on their respective species, freeze-dried, weighed and kept at −20 °C until analysis. Individuals from the control enclosures were only sampled at the last collection (Fig. 1C).

2.3. Environmental characterisation of sampling sites

Water temperature (°C), oxygen content (mg L^{-1}) and oxygen saturation (%), pH and electrical conductivity (μS cm^{-1}) (using WTW probes, Weilheim, Germany) were measured in the two sites. The river width, mean depth, mean current velocity and discharge were measured using Flowtracker (SonTek, USA) at the collection periods 0, 30 and 46. Water samples were collected on all occasions for nutrient (total nitrogen, nitrite, nitrate, ammonium, total phosphorus, and phosphate), and DOC, POM, PhACs and EDCs analyses (Fig. 1D). Water samples collected for nutrient analysis and DOC concentration measurement (3 × 60 mL per site per collection) were filtered in the field using 0.7 μm GF/F filters (Whatman Int. Ltd., Maidstone, UK) and stored at −20 °C until analysis. POM concentration was obtained after filtering water through pre-weighed filters, through drying at 30 °C for 72 h, weighing, and then ashing at 450 °C for 4 h to obtain ash dry mass. For of PhACs and EDCs analysis, 3 replicates of 1L of water were collected per site per collection and stored at −20 °C until analysis.

2.4. Analysis of PhACs and EDCs in water and biota samples

Water samples (Fig. 1D) were processed using methods described in detail in Gros et al., 2012. Insect tissues were processed using modified methods of Huerta et al. (2015). Briefly, 0.1 g of freeze-dried *Hydropsyche* samples was extracted using an ultrasonic probe (Branson Digital Sonifier, model 102C; 3 cycles of 120 s at 30% of intensity) with methanol as extracting solvent. Extract (1.5 mL) was evaporated to dryness and dissolved in 2 mL of water with EDTA at 1%. *Hydropsyche* samples were extracted using Oasis HLB cartridges (60 mg, 3 mL). 60 mg SPE cartridges were conditioned with 5 mL of methanol followed by 5 mL of HPLC- grade water at a flow rate of 2 mL min^{-1}. 250 mL water sample and 2 mL insect sample extracts were loaded at 2 mL min^{-1}. Analytes were eluted with 2 mL of pure methanol at a flow rate of 1 mL min^{-1}. Extracts were evaporated to dryness under a gentle nitrogen stream and reconstituted with 1 mL of methanol/water (50:50, v/v). Finally, standard mixture containing all isotopically labelled standards was added in the extract as internal standard. Obtained extracts were used for both target and non-target analysis.

Target analysis was performed using an ultra-performance liquid chromatography (UPLC) system (Waters Milford, MA, USA) coupled to a hybrid quadrupole linear ion trap mass spectrometer Qtrap 5500 (Applied Biosystems, Foster City, CA, USA). Details regarding UPLC separation can be found in Supporting information file while instrument depended and scheduled MRM parameters are provided in references (Gros et al., 2012; Huerta et al., 2015). The sample volume injected was 5 μL for all analyses. Samples were screened for 21 EDCs and 41 PhACs. 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 by Rožman and Petrović (2016).

2.5. Non-target analysis for metabolome profiling of H. dinarica

Non-target high resolution metabolomic profiling was performed on an LTQ-Orbitrap Velos™ coupled with the Aria TLX-1 HPLC system (Thermo Fisher Scientific, USA). The system was controlled via Xcalibur 2.2 software (Thermo Fisher Scientific, USA). The chromatographic separation was achieved using KINETEX EVO C18 (50 mm × 2.1 mm i.d., 1.7 μmm particle size, Phenomenex Inc., USA) chromatographic column in both the positive

Table 1
Physico-chemical parameters of water at control and impact site of the Segre River where translocation experiment was conducted in October and November 2015. C - control site, I - impact site, 0 to 46 refer to days of exposure.

Environmental variable	Control site				Impact site			
	C0	C15	C30	C46	I0	I15	I30	I46
Discharge (m^3 s^{-1})	1.010		0.544	0.736	1.309		0.892	0.672
Temperature (°C) (time of measurement)	8.8 (10.00)	8 (15.15)	9.3 (14.30)	2.5 (15.00)	10 (12.45)	6.6 (12.15)	2.5 (15.00)	1 (10.50)
Conductivity (μS cm^{-1})	145	143.7	124.8	133	160	171.9	125.7	156.3
Oxygen content (mg L^{-1})	9.3	9.9	10.27	12.34	8.81	9.62	10.57	12.4
Oxygen saturation (%)	91.4	95.1	101.1	102.7	89.7	89.2	100.3	98.7
pH	7.46	7.622	7.474	7.513	7.403	7.554	7.142	7.261
POM: mean AFDW (mg L^{-1})	16.388	2.300	2.434	1.035	53.277	21.600	5.299	1.714
DOC (mg L^{-1}) (s.d.)	3.207 (0.065)	3.199 (0.069)	2.848 (0.040)	1.965 (0.342)	3.121 (0.051)	3.513 (0.048)	3.348 (0.329)	2.636 (0.076)
TN (μg L^{-1}) (s.d.)	57.9 (0.267)	44.397 (2.117)	42.460 (14.934)	50.133 (0.264)	157.433 (0.907)	230.767 (4.594)	160.5 (0.781)	91.04 (15.873)
N-NH_4^+ (μg L^{-1}) (s.d.)	0.367 (0.014)	<LOQ	0.558 (0.180)	0.711 (0.050)	56.714 (0.444)	120.962 (1.197)	77.573 (0.161)	33.656 (3.900)
N-NO_2^- (μg L^{-1}) (s.d.)	0.423 (0.160)	<LOQ	<LOQ	<LOQ	2.008 (0.020)	0.951 (0.014)	<LOQ	<LOQ
N-NO_3^- (μg L^{-1}) (s.d.)	47.390 (0.797)	33.023 (0.197)	46.224 (0.365)	39.493 (0.252)	62.390 (1.261)	47.098 (0.536)	47.018 (0.473)	40.228 (8.269)
TP (μg L^{-1}) (s.d.)	4.052 (0.047)	3.285 (0.068)	2.442 (0.441)	2.703 (0.041)	10.241 (1.630)	16.316 (0.228)	8.065 (0.139)	3.105 (0.372)
P-PO_4^{3-} (μg L^{-1}) (s.d.)	3.263 (0.166)	2.513 (0.166)	2.012 (0.321)	2.214 (0.025)	8.201 (0.352)	13.841 (0.492)	7.163 (0.086)	2.562 (0.311)

ionization (PI) and the negative ionization (NI) modes. Solvents used for PI mode separation were methanol (solvent A) and formic acid 0.1% (solvent B) while for NI mode acetonitrile (solvent A) and 10 mM ammonium acetate at pH 9 (solvent B) were used. In both modes, metabolites were separated using the following 13.5 min gradient: 0.0 – 0.5 min, 2% A; 0.5 – 8.5 min, 2–98% A; 8.5 – 11.5 min, 98% A; 11.5 – 13.5 min, 2% A. The sample injection (20 μL), separation and spectra acquisition were carried out automatically. The electrospray capillary voltage was set as (±)4 kV, capillary temperature was at 300 °C, *m*/*z* range from 100 to 1000, the instrument resolution was 100,000 at 400 *m*/*z*, and mass accuracy within error of ±5 ppm. Data extraction and analysis was done using Thermo Xcalibur 2.2 SP1.48 (Thermo Fisher Scientific Inc., USA), MZmine2.17 (Katajamaa et al., 2006) and parts of Bqunat script (Rožman et al., 2018; Rožman and Petrović, 2016).

2.6. Data analysis and statistics

2.6.1. Variability of environmental conditions between sites and collections

The variability of environmental and chemical characteristics (nutrients, DOC and concentration of EDCs in water) during the experiment was examined at spatial (across-site) and temporal (across-collection) levels using repeated measures ANOVA. The analysis was conducted on log-transformed data of nutrients, DOC and concentration of EDCs in water using the IBM SPSS Statistics 22.0 (IBM Corporation). Pairwise comparisons were conducted with Bonferroni adjustment for multiple comparisons.

2.6.2. Bioaccumulation of EDCs in Hydropsyche tissues

Differences between the two sites of accumulated compounds in *Hydropsche* tissues were tested for *H. dinarica* vs *H. siltalai*, when these species were co-existing within the same experimental tubes. T-tests did not show significant differences between the species or between the two tubes used in each site at each collection. We used the genus level and sampling site/collection as data units for the subsequent statistical analyses (Fig. 1D). The bioaccumulation and depuration evolution of EDCs in translocated *Hydropsyche* was tested using repeated measures ANOVA. The analysis was conducted using the IBM SPSS Statistics 22.0 (IBM Corporation). Analyses were executed with log-transformed data of total concentration of EDCs, and separately for the concentration of each compound accumulated in *Hydropsyche* tissues. Pairwise comparisons were conducted with Bonferroni adjustment for multiple comparisons. Bioaccumulation of EDCs in the control enclosures was assessed with the paired T- test using the same program.

2.6.3. Average dry weight

The potential changes in body mass (growth or loss of body weight) during the experiment were evaluated individually in *H. dinarica* specimens (Fig. 1D). Average individual body mass (dry weight) was calculated for each experimental and control enclosure at each collection (from weighted freeze-dried sample per enclosure; N = 6 – 108). Further, we correlated the changes in body mass to the observed bioaccumulation and depuration of total EDC concentrations using Pearson correlation analysis IBM SPSS Statistics 22.0 (IBM Corporation).

2.6.4. Data extraction and analysis of non-target metabolomic profiles of H. dinarica

Metabolomic profiles were restricted to *H. dinarica* (Fig. 1D). There were at least 2 replicate samples for the mass spectrometry analysis in all the cases, except in the control samples, which were thus excluded from further analyses of the metabolome.

Obtained raw mass spectra were recalibrated by least square fitting using a set of internal standards and common contaminants (e.g. surfactant 4-decylbenzenesulfonic acid) according to (Zubarev and Mann, 2007). Obtained standard deviations were 0.00082amu for the positive ion mode and 0.00063amu for the negative ion mode. Accordingly, maximum mass deviation was set at 2σ to minimize false positive identifications and balance between sensitivity and specificity. Data extraction and formula assignments were done using MZmine program (Katajamaa et al., 2006). Details about the procedure and parameters are provided in Supporting information. Peak areas of the obtained matrix were normalised and corrected for features detected in blank samples by removing features with an intensity ratio sample:blank < 20. Subsequently, data were power transformed (cube root) and centred. Obtained data matrix was analysed using principal component analysis (PCA) with covariance method. Hierarchical clustering was attempted based on Euclidean distance and complete linkage.

Identification of possible molecules of biological reference was performed by searching acquired masses against the Human Metabolome Database (HMDB), Lipid Maps (LM) Database and KEGG database. Obtained results were filtered according to following criteria: accurate mass, acquisition ion mode, log$P_{octanol/water}$ - retention time relationship allowing only tentative identification. It is important to stress that mentioned databases only cover metabolites of a key species and while some metabolites are "shared" one should bear in mind that each organism has its own unique set of metabolites. Identification and functional characterisation of unidentified metabolite species were out of the scope of this study.

Molecular categories were created to obtain an approximate overview of the likely structures behind the identified molecular formulae. Molecular categories were assigned on a basis of the elemental composition and the corresponding modified aromaticity index (AI_{mod}) (Koch and Dittmar, 2006) details can be found in Supporting information (Methods details, A1).

3. Results

3.1. Environmental conditions and contaminants in the Segre river

Concentrations of ECs, nutrients and organic matter content in river water differed significantly between sites as well as between collection dates (Tables 1, A1). A total of 69 ECs (62 PhACs and 7 EDCs), were detected in water samples, 66 (59 PhACs and 7 EDCs) at the impact site, and 41 PhACs and 7 EDCs at the control site (Table A2). Caffeine, salycilic acid and iopromide had the highest concentrations across sites and collecting dates (Table A2).

Water concentrations of EDCs were significantly different between sites and collection dates, with the exception of methylparaben (Table A1). While most EDCs were higher in the impact site, the highest concentrations of methylparaben, propylparaben and TCEP were recorded at the control site (Table A2). The concentration of nutrients and DOC were as well higher at the impact site (Table A1). A decreasing trend of EDCs water concentrations, DOC and nutrient concentrations occurred as the experiment progressed (Tables 1, A1 & A2). Accordingly, the interaction of site*collections was significant for DOC, the concentrations of nutrients and water EDCs (except methylparaben; Table A1). The *Hydropsyche* larvae in the experiment were therefore exposed to different concentrations and combinations of pollutants and environmental variables between sites and collection periods.

3.2. Body weight changes

The average dry weight (ADW) of individual *H. dinarica* specimens increased for both the control (C) and impact sites (I) between the initial and final experiment stages. Specimens

translocated from control to impact site (C-to-I) increased only slightly at the end, but those translocated from impact to control (I-to-C) experienced an immediate increase (Fig. A1).

3.3. Bioaccumulation of PhACs and EDCs in Hydropsyche spp. tissues

3.3.1. In situ bioaccumulation

PhACs did not accumulate in *Hydropsyche* spp. individuals, but EDCs accumulated in animals from the two sites. *Hydropsyche* tissues accumulated two parabens (methylparaben and ethylparaben), caffeine and two organophosphate flame retardants (OPFRs: TCEP (Tris(2-chloroethyl) phosphate) and TCPP (tris(chloroisopropyl)phosphate) (Table 2). The highest individual compound concentrations were recorded for TCPP, ranging from 21.8 ng g^{-1} in C0 to 47.3 ng g^{-1} dw in I46. The highest mean concentrations of parabens were 1.7 ngg^{-1} of ethylparaben at I46 and 23.8 ng g^{-1} dw of methylparaben at CI15 (Table 2). Values of caffeine were overall similar across sites and treatments and ranged from 9.2 ng g^{-1} dw at C0 and 16.3 ng g^{-1} dw at I46 (Table 2). The lowest total EDC concentration was in specimens from the control site, while the highest mean concentrations occurred in the *Hydropsyche* from the impact site (Table 2). Initial and final total concentrations of EDCs were positively correlated to average dry weight (ADW) of *H. dinarica* ($r = 0.823$, $N = 8$, $p = 0.05$). In control enclosures, the total concentration of EDCs and all compounds except TCEP had higher concentrations at the end of the experiment, regardless of the site (Table 2). However, according to the paired T-test, this bioaccumulation was significant only in the case of parabens at the impact site ($t(2) = -15.96$, $p < 0.005$ for ethylparaben, and $t(2) = -13.64$, $p = 0.005$ for methylparaben).

3.3.2. Bioaccumulation dynamics after translocation

Total EDCs concentration in *Hydropsyche* larvae showed a similar pattern in the two translocations; the initial bioaccumulation was fast between the first two collections (C0 to CI15 and I0 to IC15), followed by loss of bioaccumulated EDCs from 15 days of exposure onwards (Fig. 2 A). There was a significant main effect of days of exposure on total concentration of EDCs in *Hydropsyche* tissues (repeated measures ANOVA, $F_{1,2} = 18.29$, $p < 0.0001$). Changes in total concentration of EDCs were significant between the first two collections (C0 to CI15 and I0 to IC15; i.e. the described initial bioaccumulation) as well as between 30 and 46 days of exposure (CI15 to CI46 and IC15 to IC46; i.e. the described loss of bioaccumulated EDCs, Table A3).

Methylparaben showed the highest bioaccumulation pattern in the two sets of translocated insects. Moreover, the bioaccumulation was much higher (average of 2.4×) in C-to-I than in I-to-C animals (average of 1.6×, Fig. 2B). Differences between sites and days of exposure were statistically significant, i.e. bioaccumulation of methylparaben was significant between 0–15, 0–30 and 0–46 days of exposure (Table A3). Patterns of TCEP and TCPP concentrations were not so evident, however, for both compounds differences were statistically significant between sites and days of exposure. TCEP did not bioaccumulate in any of the two sites, and loss of bioaccumulated TCEP was significant between 0 and 15 days of exposure (Fig. 2C, Table A3). In TCPP, bioaccumulation was moderate and occurred between 0 and 15 days of exposure, with different patterns at each site in subsequent collections (Fig. 2D), therefore, there was a significant main effect of site*days of exposure interaction (Table A3). Patterns of caffeine differed between sites, with bioaccumulation only in C-to-I animals (Table 2), and therefore significant differences inferred for site and exposure days (Table A3). For ethylparaben differences in bioaccumulation were not inferred significant for any level and are hence not presented in detail.

3.4. Changes in metabolome profiles of H. dinarica due to pollution and experimental conditions

Principal component (PCA) and hierarchical cluster analyses (Figs. 3 and 4) based on non-target metabolic profiles of *H. dinarica* revealed clear separation of the samples into four major clusters: animals collected at both sites at the start (cluster A), and after 15 days of exposure (cluster C), animals translocated from impact to control site (cluster B), and translocated from control to impact site (cluster D) after 30 and 46 days of exposure, respectively. Metabolome profiles of animals in clusters A and C were clustered together according to days of exposure (0 and 15), whereas animals related to clusters B and D were grouped according to the translocations, i.e. I-to-C and C-to-I, respectively (Figs. 3 and 4). First principal component (PC) axis and initial dendrogram branching is related to separation of metabolic profiles of the animals directly sampled from the riverbed (days of exposure 0, i.e. cluster A, Fig. 3). Internal nodes as well as second and third PC axes are showing distinct partitioning between clusters B, C and D. The PCA results can account for most of the variability between the sample clusters. In particular, the total variance accounted by the three components shown in Fig. 3 was ~78% (variance from [PC1, PC2, PC3] ≈ [36%, 24%, 18%]).

Dendrogram adjacent to the heat map (Fig. 4) represents the abundance of metabolite features (rows, and represented as colours) across a number of comparable samples (as columns). Four major groups with distinct metabolite variation patterns

Table 2
Concentrations of 5 EDCs accumulated in *Hydropschye* spp. tissues (in ng g^{-1} dw) during the translocation experiment at two sites (C - control and I - impact) in the Segre River. Shown are mean values and standard deviation, 0–46 refer to exposure days. Shown are also limits of detection (LOD) and limits of quantification (LOQ) for EDCs in *Hydropsyche* spp. tissues.

	Direct sample		Experimental enclosures – Translocated						Control enclosures		LOD ng g^{-1}	LOQ ng g^{-1}
	C0	I0	IC15	IC30	IC46	CI15	CI30	CI46	C46	I46		
Ethylparaben (s.d.)	1.294	1.162	1.247	1.405	1.158	1.284	1.301	1.320	1.406	1.709	0.18	0.59
	0.309	0.156	0.413	0.675		0.279	0.144	0.283	0.499	0.499		
Methylparaben (s.d.)	10.145	8.387	13.667	12.709	11.888	23.804	21.193	16.541	16.094	15.762	0.44	1.47
	1.846	0.568	3.616	3.231		1.949	5.166	2.589	4.292	0.848		
Caffeine (s.d.)	9.231	12.234	15.175	11.928	13.387	16.066	16.014	14.483	14.656	16.350	2.66	8.87
		2.771	2.660	1.945		3.971	3.021	6.192	5.192	7.933		
TCEP (s.d.)	5.712	6.223	6.111	4.333	4.544	3.436	3.156	4.101	3.559	4.659	0.23	0.77
	0.176	0.718	2.037	2.140		0.456	0.901	0.699	0.864	1.167		
TCPP (s.d.)	21.851	27.972	32.632	29.697	23.898	27.306	21.680	29.336	26.462	47.317	5.57	18.58
	1.756	3.142	3.181	2.895		4.353		9.052	5.045	9.355		
Total concentration (s.d.)	48.233	55.978	69.145	63.041	54.875	71.896	63.798	65.782	62.177	86.022		
	1.277	6.606	9.603	3.844		8.994	8.589	17.271	11.937	19.990		

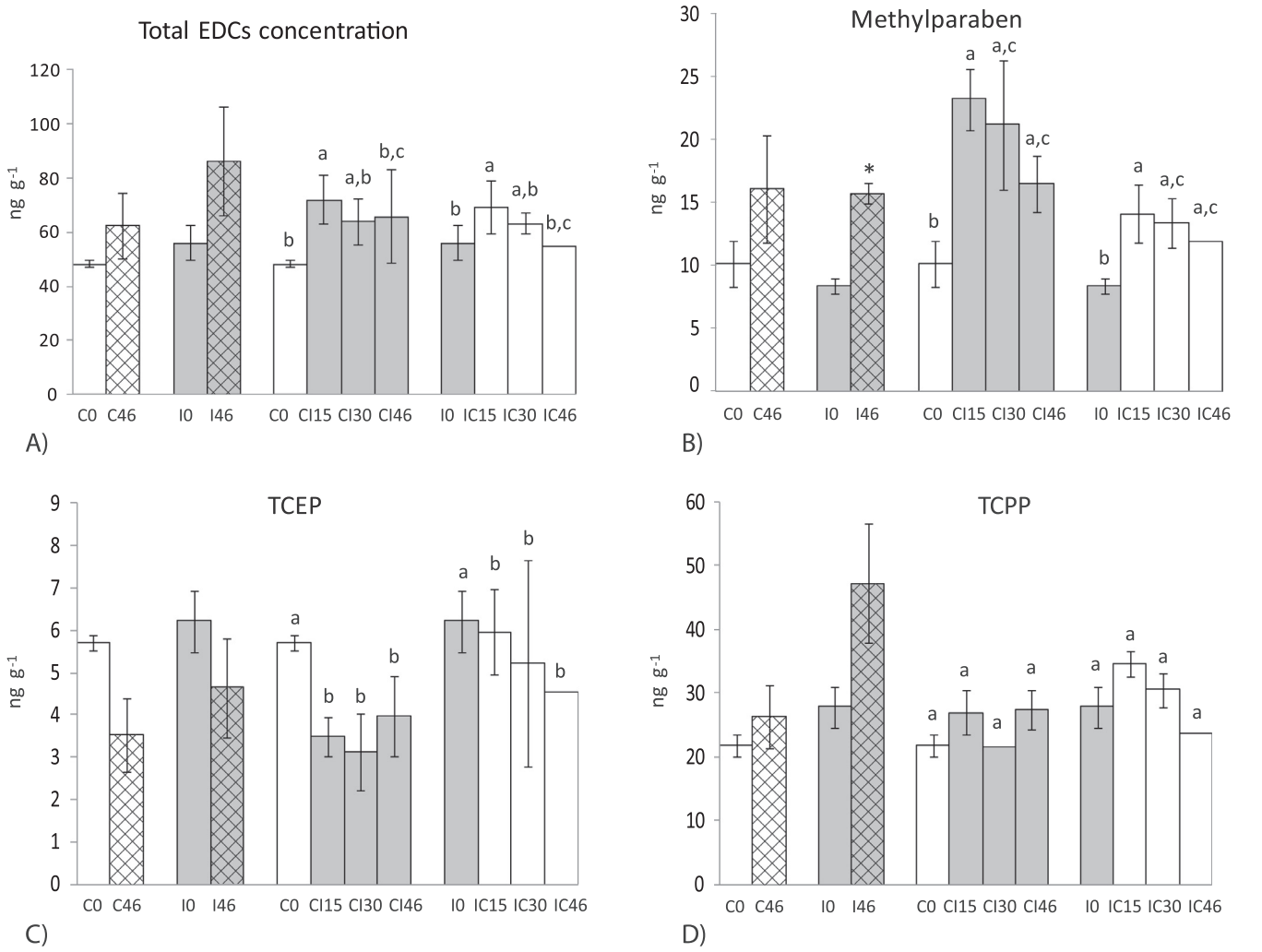

Fig. 2. Bioaccumulation of emerging compounds in *Hydropsyche* tissues (in ng g^{-1} of dry tissue) in control tubes (C0-C46 and I0-I46) and translocated animals (CI - translocated from C to I, IC - translocated from I to C). A) Total concentration of EDCs and B – D) selected compounds. C - control site, I - impact site; 0 to 46 – days of exposure. Different letters show significant differences between collection dates in translocated animals according to repeated measures ANOVA ($p < 0.05$; pairwise comparisons were conducted with Bonferroni adjustment for multiple comparisons), and an asterisk indicates significant difference between start and end point for control enclosures according to the paired T-test ($p < 0.05$).

associated with certain clusters were identified (groups 1–4). Group 1 pattern represents increased metabolite levels of the samples related to cluster A. Group 3 shows decreased metabolite levels of the samples related to cluster A (Fig. 4). Group 2 exhibits decreased metabolite levels in clusters A and B compared to clusters C and D, respectively, while group 4 exhibits lower metabolite levels in samples IC30 and IC46 thus contributing to formation of cluster B (Fig. 4).

The composition of molecular categories (MC) was similar across all samples (Table 3). Altogether, eight MC were identified, with polyphenols, highly unsaturated compounds and peptides amounting for more than 80% in total (mean values 47.3%, 21.8% and 14.7%, respectively, Table 3). List of tentatively identified metabolites associated with groups 1–4 can be found in supporting Table A4.

4. Discussion

4.1. Spatial and temporal variability of environmental conditions

PhACs and EDCs were recorded in water at both the control and impact sites, and complement the results of previous studies which detected very low concentrations of ECs in the control site (Huerta et al., 2015; Ruhí et al., 2016). Noticeable concentrations in the control site observed during our experiment were likely related to the inputs from households upstream from the main city and the WWTP entrance. Even so, total concentrations were considerably higher in the impact site. Most ECs observed are commonly detected in wastewaters and surface waters worldwide, including salicylic acid, caffeine and methylparaben (Ebele et al., 2017; Peng et al., 2017). The variable ECs concentrations within the 6 weeks period of the experiment is common in rivers worldwide (e.g. Acuña et al., 2015; de Solla et al., 2016; Golovko et al., 2014).

4.2. Bioaccumulation dynamics of EDCs in macroinvertebrate tissues

4.2.1. Bioaccumulation of EDCs in Hydropsyche spp. tissues

Five EDCs were recorded in *Hydropsyche* tissues: methylparaben, ethylparaben, caffeine, TCEP and TCPP. These compounds bioaccumulated both in the control and impact sites, and matched the concentrations arriving to each of them. Methylparaben was the compound showing the most noticeable bioaccumulation at the impacted site in both, control enclosures and translocated

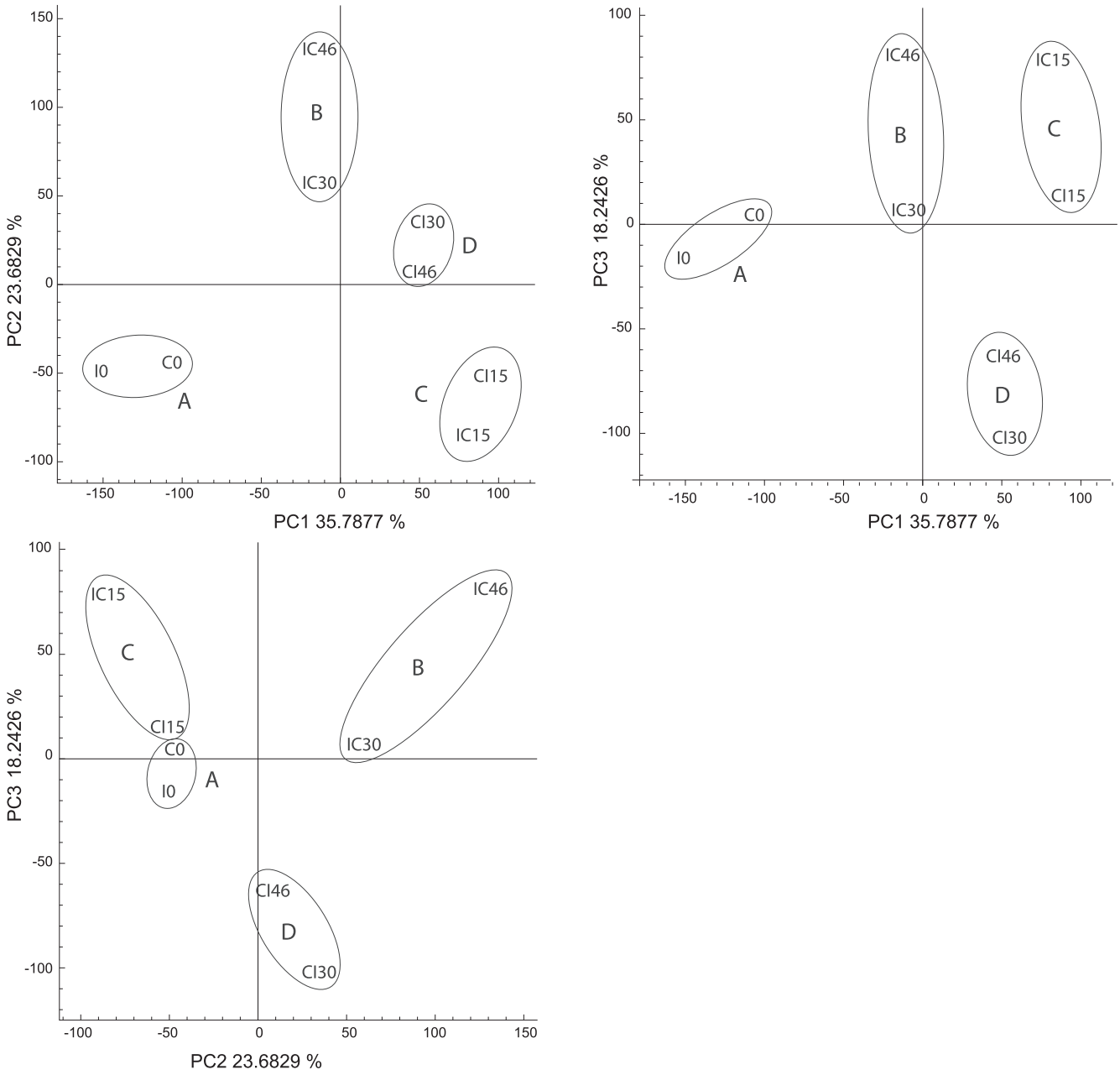

Fig. 3. PCA score plots for samples of *Hydropsyche dinarica* during the translocation experiment. Clusters A-D correspond to clusters in the Fig. 4. For abbreviations of samples see Fig. 1.

animals. Bioaccumulation of parabens has been observed in experimental conditions in Mediterranean marine mussels (*Mytilus galloprovincialis* Lamarck; Serra-Compte et al., 2018b), however, after 40 days the concentrations were lower than in the current study. Mean concentrations of organophosphate flame retardants bioaccumulated in *Hydropsyche* tissues in the current study (up to 47.3 ngg^{-1} dw of TCPP) are in line with previous data from the same river (Huerta et al., 2015; Ruhí et al., 2016), but are higher than those observed in lacustrine benthic invertebrates (freshwater mussels Lamellibranchia; Zhao et al., 2018). Since all these compounds have low octanol-water partition coefficient (log K_{ow} < 3), and therefore possess a low bioaccumulation potential in aquatic organisms (Franke et al., 1994), the occurring bioaccumulation is most likely a direct consequence of the continuous presence of these products in the river water.

4.2.2. Bioaccumulation dynamics of ECs in translocated Hydropsyche larvae

Bioaccumulation occurred after only 15 days of translocation to new conditions, irrespective on the direction of translocation that was performed. However, bioaccumulation rate was higher when the translocation was in the direction of the impact site receiving the WWTP effluent. The comparison between the initial and final stages (after 46 days) of the translocation only suggests that accumulation of EDCs was growth-dependent, in line with previous observations in fish (in the crucian carp *Carassius auratus* (L.); Choo et al., 2018; various carp species; Peng et al., 2017). However, the comparative sequence of bioaccumulation through time provides a different, more accurate pattern. The initial, fast, EDCs bioaccumulation after 15 days in individuals translocated to the impact site is attributable to their higher aqueous exposure, and

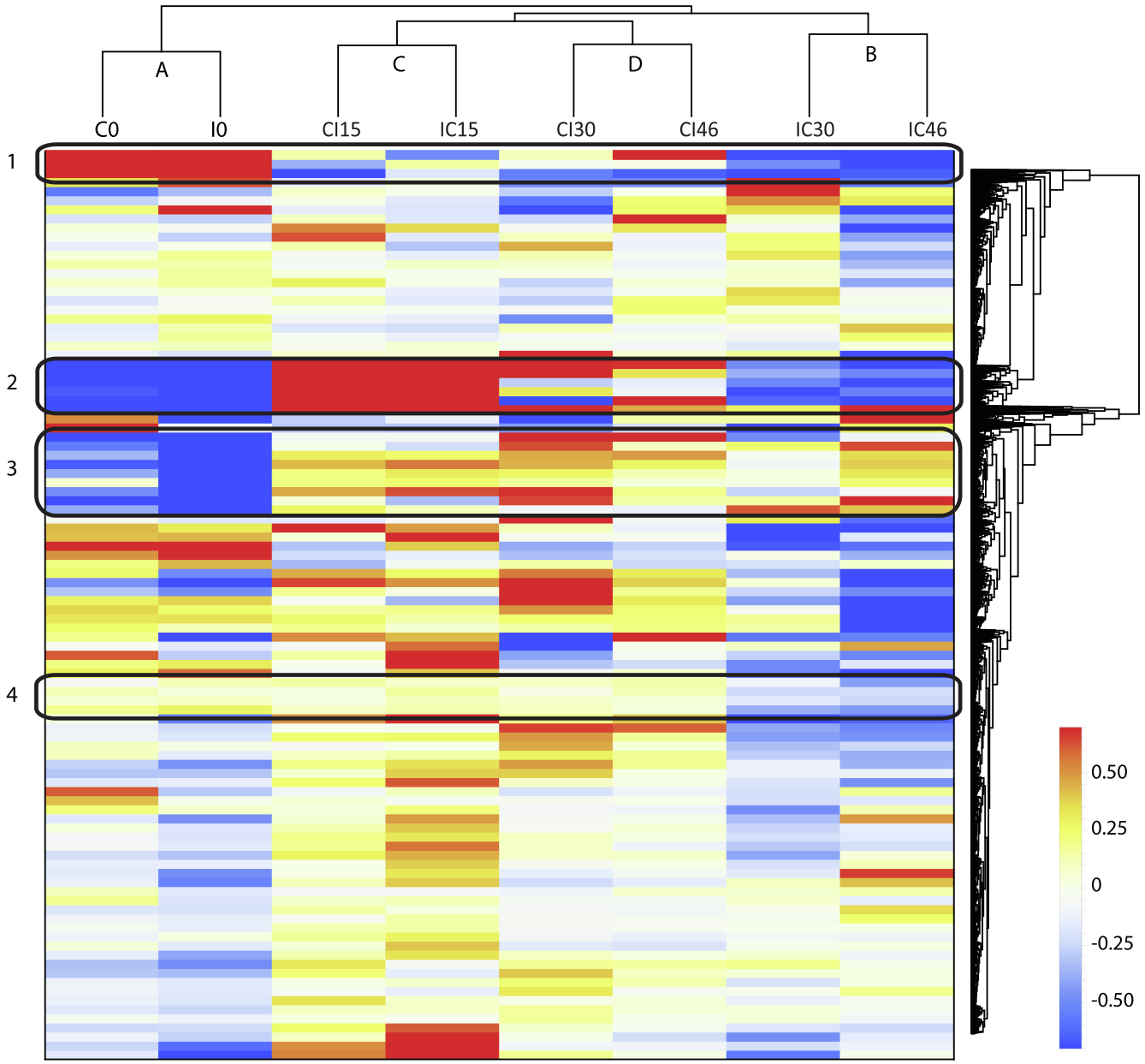

Fig. 4. Hierarchical clustering of samples and heat map of metabolite changes in *Hydropsyche dinarica* during the translocation experiment. Samples are shown in columns, abbreviations are given in Fig. 1, clusters A-D correspond to clusters in the Fig. 3. Major groups (1–4) with increased/decreased metabolite levels cause differentiation of samples at spatial and temporal scales.

Table 3
Percentages of molecular formulae attributed to molecular categories samples of *Hydropsyche dinarica* during translocation experiment. For abbreviations of samples see Fig. 1.

	C0	CI15	CI30	CI46	I0	IC15	IC30	IC46
carbohydrates	1.6	1.6	1.5	1.5	1.5	1.6	1.6	1.5
highly unsaturated compounds	21.8	21.8	21.9	21.8	21.9	21.9	21.7	21.9
peptides	14.7	14.7	14.7	14.7	14.5	14.7	14.8	14.7
phenols	4.1	4.2	4.2	4.2	4.3	4.2	4.2	4.2
polyphenols	47.3	47.3	47.1	47.3	47.4	47.2	47.3	47.3
saturated fatty acids	3.5	3.4	3.4	3.4	3.4	3.4	3.4	3.4
unsaturated aliphatic compounds	2.1	2.1	2.1	2.1	2.0	2.0	2.1	2.0
unknown	4.9	5.0	5.0	5.0	5.0	5.0	5.0	5.0

not so much to growth-dependent accumulation. In this period, the AFDW of *H. dinarica* individuals did not increase, particularly in the case of C0-CI15 larvae (Fig. A2). Additionally, the decreasing ECs water concentrations throughout the experiment match the decreased bioaccumulation rate (de Solla et al., 2016) through the whole period. It is therefore evident that bioaccumulation in *Hydrophsyche* tissues is the result of a transient dynamics, mostly associated to the toxicants occurring in the aqueous media. This

is further stressed by previous observations in the Segre River, on which *Hydropsyche* tissues bioaccumulated PhACs, including diclofenac and ibuprofen (Huerta et al., 2015; Ruhí et al., 2016). The concentrations of ibuprofen in water during the current experiment were almost 3 times lower than those previously reported, and diclofenac was not present (Table A1). This contrasting difference with past observations stresses that specific bioaccumulation patterns occur according to the ECs occurrence in the water medium.

Regardless of the bioaccumulation dynamics occurring in each individual, biological implications should not be neglected. Cannibalism within enclosures might account for the higher AFDW in I-to-C than in C-to-I (between 0 and 30 days of exposure; Fig. A1). Avoiding these complications is difficult in such experiments, since invertebrates become cannibalistic because of their proximity.

Overall, the current experiment proved that individuals translocated to the impact reach would bioaccumulate higher EDCs concentrations in their tissues, as a reflection of the current contaminant concentrations occurring in its waters. Temporal variability of bioaccumulation patterns within only 46 days indicates that the bioaccumulation was a transient process. In line with some recent findings showing rapid bioaccumulation of PhACs in freshwater filterers (bivalve *C. fluminea;* Burket et al., 2019), the current experiment highlights the importance of investigating *in situ* bioaccumulation of ECs at finer temporal scale.

4.3. Changes in metabolome profiles of H. dinarica

The metabolome profiles of *H. dinarica* larvae were altered as a consequence of the translocation into environment with higher concentrations of ECs, in concord with experiments on other aquatic invertebrates (e.g. caged marine mussels; Cappello et al., 2015). Moreover, the latter could be separated from the effects of the translocation itself (i.e. stress due to experimental conditions). Generally, the composition of MC, i.e. the number of molecular formulae, did not differ across samples. Hence, the origin of sample dissimilarities was in the metabolite regulation (i.e. abundances) rather than in change of metabolite composition.

Metabolome profiles of animals collected directly from the riverbed at both sites (C0&I0, cluster A; Fig. 4) were separated from all other samples, suggesting a different degree of stress between those and the translocated specimens. Overall, the differences in their metabolomic profiles were minor compared to those caused by the experiment. This finding is in contrast with data on indicator values of *H. dinarica*, which is obviously more tolerant to pollution than recognised (Graf et al., 2008, 2019). Cluster A, which was composed of direct samples (C0&I0) was separated through groups 1 and 3 (identified within heat map, Fig. 4), showing upregulation or downregulation of certain metabolites, respectively. Compounds tentatively identified as eicosanoids were found in both groups. Prostaglandins and related eicosanoids are oxygenated metabolites of several polyunsaturated fatty acids (FAs), which play an important role in immune reactions to infection and invasion in insects (Stanley and Kim, 2011). Glycerophospholipids, containing unsaturated FAs, were also found in group 1 (Table A4). Glycerolipid metabolism has been identified as a vital part of the metabolism employed for the generation of lipid energy sources in *Anopheles* mosquitoes and as a signalling pathway modulator (Hoxmeier et al., 2015). The observed decrease of some glycerolipid molecules in translocated animals could indicate stress and reduced immunity. On the other hand, fatty amides (part of the group 3, e.g. palmitoleamide, oleamide and palmitoylethanolamide) were increased in translocated animals. These compounds are important signalling molecules in animals, demonstrating control over sleep and anti-inflammatory activity (Salzet and Stefano, 2002). Increased abundance of some fatty amides may go in line with the *Hydropsyche* mechanism of adaptation to new environmental conditions. Correspondingly, stress related to new habitat could be reflected as a demand for additional energy. Increased levels of glutaric acid, a group 3 identified molecule, contribute to that assumption, since glutaric acid is naturally produced during the degradation of fatty acids and lysine, generating acetyl-CoA, the entry molecule for the citric acid cycle, the main energy supply of animals (Nelson and Cox, 2005).

Immediate effects of translocation (i.e. experimental conditions) are evident as increased metabolite levels in group 2, resulting in the clustering of samples from both sites after 15 days of exposure (CI15 & IC15; cluster C, Fig. 4). Furthermore, levels remained high in C-to-I translocated animals (cluster D), whereas their relative abundance returned to initial levels in I-to-C translocated animals (cluster B, Fig. 4). Translocation itself caused stress to *Hydropsyche* larvae, but conditions were more stressful for animals translocated to impact site, causing elevated metabolite levels to persist. Examples of particular metabolites identified in the group 2 are amino acid metabolites, monosaccharides, succinate and hydroxypyruvate/malonic acid (Table A4). Succinate and hydroxypyruvate/malonic acid are metabolites involved in various essential metabolic pathways such as TCA cycle and oxidative phosphorilation (Nelson and Cox, 2005). Similarly, impairments in energy metabolism reflected in increased levels of TCA cycle intermediates (e.g. succinate, malonate) were observed in caged marine mussels (*M. galloprovincialis*) exposed to pollution (Cappello et al., 2013; Nelson and Cox, 2005).

Group 4 shows lower levels of metabolites in cluster B (I-to-C animals, exposure days 30 and 46). Low level metabolites can be mainly associated with acetylated amino acids and proteolytic breakdown products. Degradation of proteins can be interpreted as a means to increase the availability of amino acids required for the synthesis of new proteins (Willetts, 1967). This observation can be linked with reduced growth of animals related to cluster B and goes in line with observed AFDW loss in I-to-C animals in the second part of the experiment.

By using non-targeted metabolite profiling, we were able to detect small metabolic changes of *H. dinarica* during the course of the experiment. These changes in metabolite profiles could indicate disturbance in signalling pathways, anti-inflammatory activity and energy metabolism. Our initial hypothesis that translocation to the impacted reach caused stressful conditions altering metabolomic profiles of *Hydropsyche* was partly true. Both translocations (I-to-C and C-to-I) equally induced stress, however conditions were more stressful for the animals translocated to the impact site.

5. Conclusion

In the present study, we used a translocation experiment to provide novel insights into ECs bioaccumulation dynamics and stress response of non-model aquatic insects. Bioaccumulation of 5 EDCs were recorded in *Hydropsyche* tissues after only 15 days of translocation to new conditions. We demonstrated that bioaccumulation of ECs in aquatic insect tissues is a temporary process reflecting the current concentrations of contaminants in the water at a relatively fine temporal scale. Furthermore, stress induced by translocations and particularly by pollution, was detected using the non-targeted metabolite profiling. Thus, the current study gives some of the first data on the metabolomic profiles of aquatic insects under stress due to environmentally realistic chemical pollution. Overall, this pilot study highlights the potential of the non-targeted profiling for the measurement of the dynamic multiparametric metabolic response of aquatic invertebrates to environmental stressors.

CRediT authorship contribution statement

Ana Previšić: 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. **Jordi-René Mor:** Investigation, Methodology. **Vicenç Acuña:** Conceptualization, Investigation, Methodology. **Albert Serra-Compte:** Investigation, Methodology. **Mira Petrović:** Conceptualization, Funding acquisition. **Sergi Sabater:** Conceptualization, Investigation, Funding acquisition, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This manuscript is part of the outcomes of the GLOBAQUA project (FP7, No 603629) and TRANSFORMER (H2020 MSCA IF to M.R.), and was completed through the funding of projects CLIMALERT (PCIN-2017- 068) and SPACESTREAM (CGL2017-88640-C2-1-R). Maria Casellas and Ladislav Mandarić (ICRA) are gratefully acknowledged for their assistance in the field collections and lab processing of the samples, and Sara Rodríguez-Mozaz (ICRA) is thanked for her comments and suggestions on the earlier version of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.135333.

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