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Chironomus riparius exposure to field-collected contaminated sediments: From subcellular effect to whole-organism response

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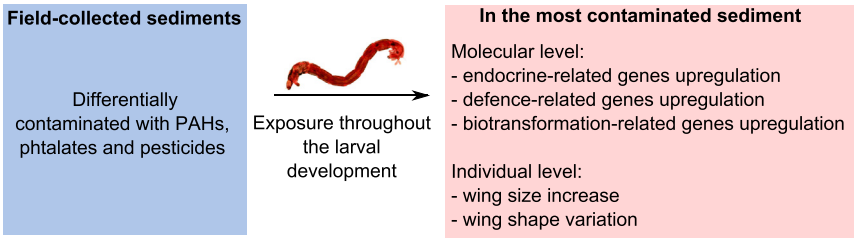
HIGHLIGHTS

- Chironomids larvae were exposed to three field-collected sediments differentially contaminated
- After 15 days of exposure, effects were measured from the molecular to the whole-organism response
- Differential gene expression and wing shape variation were observed in the group exposed to the most contaminated sediment
- This approach contributes to improved assessment of the risk associated with complex mixture exposure

GRAPHICAL ABSTRACT



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ABSTRACT

The toxicity of three field-collected sediments differentially contaminated with pesticides, heavy metals, phtalates and polycyclic aromatic hydrocarbons (PAHs), was assessed in *Chironomus riparius*. For this purpose, *C. riparius* larvae were exposed throughout their entire life cycle to sediments collected in three sites along the Saulx river in France, and the toxic effects were measured at different levels of biological organization: from the molecular (lipidomic analysis and transcriptional variations) to the whole organism response (respiration rate, shape markers and emergence rate). In the sediment characterized by an intermediate level of contamination with PAHs and phtalates, we detected an increase of the cell stress response and delayed emergence of males. In the group exposed to the most contaminated sediment with PAHs, phtalates and pesticides, genes related to endocrine pathways, cell stress response and biotransformation processes were overexpressed, while female wing shape was affected. Field-collected sediment exposure did not induce significant effects on mentum shape markers or on the lipid profile. The present study provides new insights into the multilevel effects of differentially contaminated sediments in insects. This integrative approach will certainly contribute to improved assessment of the risk that complex mixtures of pollutants pose to the aquatic ecosystem.

1. Introduction

Anthropogenic activities release a wide range of chemical compounds into aquatic systems. Exposure to this complex mixture of chemicals can affect the health of aquatic animals from molecules,

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tissues, organs to the whole organism level, and even alter the population dynamics. To better protect aquatic ecosystems, it is now crucial to focus efforts on a better comprehension of the toxic effects induced by complex exposure scenarios. In this context, multi-stressor approach will undoubtedly contribute to bridge the gap between the molecular response and the ecological impacts (Segner et al., 2014). Moreover, the successful integration of genomic data with results from cellular, physiological and organismal studies will contribute to unravel the complexity of the interactions among different levels of biological organization (Kassahn et al., 2007).

Aquatic insects are key elements in the functioning of freshwater ecosystems: they sustain higher trophic levels and contribute to the carbon cycle in streams and rivers. Their health can be strongly affected, from the molecular to the whole organism level, by a toxic exposure. Developmental stages, such as egg, larval and pupal stages, within their life-cycle are particularly vulnerable to environmental stressors, and toxic exposure during this critical period could have irreversible consequences (Weis, 2014). Indeed, early-life exposure can translate into phenotypic variations in postnatal individuals, which can affect the adult fitness. While studies on delayed effects of early-life exposure to toxic compounds are receiving increasing attention in vertebrates, little is known about how development is altered by toxic compounds in insects. Yet, some studies have reported developmental abnormalities in insects, particularly in chironomid larvae (Diptera), exposed to chemical compounds during developmental stages (Martinez et al., 2003; Di Veroli et al., 2014).

Chironomus spp. have an aquatic developmental stage and a terrestrial adult stage, which allow the reproduction and dispersal of the species. *Chironomus* spp. are widely used in ecotoxicology to test water and sediment toxicity. In this way, OECD guidelines (OECD, 2004; OECD, 2010; OECD, 2011) describe three toxicity tests using *Chironomus* sp. based on the measurement of classical endpoints such as survival, growth and reproduction. Complementary to these traditional approaches, in recent years molecular biomarkers including gene transcription and enzyme activity have been demonstrated to be effective for the early detection of exposure to toxic compounds (Planelló et al., 2008; Planelló et al., 2011; Aquilino et al., 2016; Ozáez et al., 2016; Herrero et al., 2018).

Given that molecular defects could have implications at larger scales in terms of morphology and physiology, which may ultimately compromise the survival of the population, we studied the toxic effects not only at the molecular (gene expression and lipidomic) and cellular (enzyme activities and energy reserve contents) levels but also at the individual (respiration rate and morphological abnormalities) level. Through this approach, we aim (i) to determine the effects induced by a multiple toxic exposure at various levels of biological organization, and (ii) to estimate whether probable effect concentrations (MacDonald et al., 2000) are protective enough in view of the effects observed in *Chironomus riparius*.

To meet these objectives, chironomid larvae were exposed throughout their entire larval cycle (from the 1st to the 4th instar) to three sediments collected along the river Saulx (France), contaminated with a mixture of pesticides, phtalates, heavy metals and polycyclic aromatic hydrocarbons (PAHs) and showing a gradient in PAHs and phtalates. In exposed organisms we measured: i) the biochemical response (lipidomic analysis, gene expression related to endocrine pathways, cell stress response and biotransformation processes, enzyme activities and energy reserve contents), ii) the physiological response (respiration rate), iii) the teratogenic effects (shape markers) and vi) life history traits (emergence rate and male/female ratio). Given that lipids can be mobilized for detoxification processes and that toxic exposure can induce lipid peroxidation, we hypothesize that lipid profile will be disturbed by the toxic exposure. In that way, lipid profile was shown affected by copper exposure in oysters (Chan and Wang, 2018) and by tributyltin in *Daphnia magna* (Jordão et al., 2015). Defence mechanisms allowing to cope with a toxic stress, such as heat shock proteins induction (Lee et al., 2006; Planelló et al., 2008), biotransformation processes (Fisher et al., 2003) and anti-oxidant response (Choi et al., 2000), were also studied. Moreover, given that phtalates can affect the endocrine system (Planelló et al., 2011; Herrero et al., 2017), gene expression related to endocrine pathways were measured. Finally, PAHs and heavy metals were shown responsible for teratogenic effects in chironomids (Dickman et al., 1992; Di Veroli et al., 2014). Therefore, morphological abnormalities were investigated.

2. Material and methods

2.1. Chironomid culture

The strain of *C. riparius* was maintained in the laboratory under a 16:8 h light:dark photoperiod at 20 ± 1 °C. Aquaria were composed of 2 cm of Fontainebleau sand layer (Fontainebleau sand Technical, VWR), surrounded by drill water (electrical conductivity 450 μS cm^{-1}, pH 4.47 and alkalinity 219 mg HCO_3^- L^{-1}). Constant oxygenation was provided.

2.2. Sediments collection and analyses

Sediments were collected in autumn in east of France from three stations located on the Saulx River (Fig. 1), named from upstream to downstream: Echenay (ECH: 5°17′46.7″E 48°27′57.2”N), Paroy-sur-Saulx (PAR: 5°15′20.4″E 48°30′52.6”N) and Montiers-sur-Saulx (MON: 5°15′20.4″E 48°34′3.8”N). Sediments were collected from the upper layer horizon (0–5 cm), sieved at 2 mm and brought back to the laboratory where they were kept at −20 °C until use.

Analyses of 231 chemicals, discharged into the environment through urban, industrial and agricultural activities, were performed by Eurofins (France) in the three study sediments following standard procedures (Appendix, Table A.1). The results are summarized in Table 1.

2.3. Experimental design

The experimental design included three conditions corresponding to the three field-collected sediments: MON, PAR and ECH. Glass jars were maintained at 16 ± 1 °C, in a 16:8 h light:dark photoperiod under constant oxygen supply. As recommended by OECD guideline (OECD, 2010), a 2-cm layer of field-collected sediments was provided as a substrate and a volume of drill water four times greater was added. Water

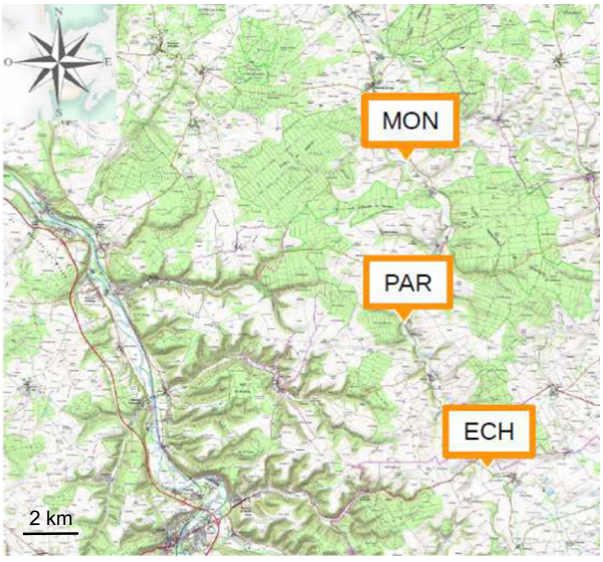

Fig. 1. Location of the three sampling stations (from upstream to downstream: ECH, PAR, MON) on the Saulx River (France).

Table 1
Main chemical compounds measured in the three study sediments and pooled by family.

Compound	MON	PAR	ECH
Σ 8 Metals (mg kg^{-1} dw)	1096.4	1064.5	1058.6
Σ 16 PAHs (mg kg^{-1} dw)	0.4	4.0	11.8
Toluene (mg kg^{-1} dw)	1.4	0.038	<0.001
Σ 7 PCBs (μg kg^{-1} dw)	<QL	<QL	<QL
Σ 6 Phthalates (μg kg^{-1} dw)	34	190	410
Σ 6 Pesticides (μg kg^{-1} dw)	40.1	1.0	140.5

8 metals: Arsenic, cadmium, chromium, copper, nickel, lead, mercury and zinc.
16 PAHs included in the US EPA priority pollutants list: acenaphtene, acenaphtylene, anthracene, benzo(a)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(ghi) perylene, benzo(a)pyrene, chrysene, dibenzo(ah)anthracene, fluoranthene, fluorine, indeno(1,2,3-cd)pyrene, naphthalene, phenanthrene, pyrene.
7 PCBs: PCB 28, PCB 52, PCB 101, PCB 118, PCB 138, PCB 153 and PCB 180.
6 pesticides: Isoproturon, metazachlor, propyzamide, napropamide, prochloraz and anthraquinone.
<QL: Lower than the quantification limit (Appendix, Table A.1).

and sediment were left for 1 day to allow the suspended solids to settle. One hour before adding the larvae, the overlying water was aerated. Measurements of the overlying water were taken for pH, temperature, conductivity, dissolved oxygen and nitrite at zero time and at subsequent 3-day intervals until the end of the test. Twenty newly hatched larvae (first stage) were placed in each glass jar. Per condition, the study design included 20 glass jars ($n_{total} = 400$ larvae). Larvae were fed daily ad libitum with 1 mg Tetramin® per larva.

For each condition, after 15 days of exposure, larvae were removed from 10 glass jars. Larvae mortality was estimated by counting the number of larvae alive. Six replicates consisting of a pool of five larvae were then weighed using an electronic balance (Sartorius CPA 225D).

Three replicates of five pooled larvae were frozen at −80 °C for gene expression analyses. Similarly, three replicates of five pooled larvae were frozen at −80 °C for lipidomic analyses. In addition, head capsules of 60 larvae were removed for mentum shape analyses while bodies were frozen at −80 °C (12 replicates of 5 pooled bodies) for GST activity, phenoloxidase activity and energy reserve content analyses. Moreover, 40 larvae were placed in clean water for respiration rate measurement and 12 larvae were placed in a 96 well-plate for ECOD analysis.

Finally, the other 10 jars were fitted with a cap to retain emerging chironomids.

2.4. Gene expression analysis

Total RNA of three replicates of five pooled larvae per condition was extracted using TRIzol Reagent (Invitrogen, Germany), following the manufacturer's protocol. After that, RNA was treated with RNase-free DNase (Roche, Germany) and purified with phenol:chloroform:isoamyl alcohol (Fluka, Germany) using 5PRIME Phase Lock Gel Light tubes (Quantabio, USA). Purified RNA was resuspended in diethylpyrocarbonate (DEPC) water, quantified by spectrophotometry at 260 nm using a BioPhotometer (Eppendorf, Germany), and stored at −80 °C.

For each condition and sample, 7 μg of isolated RNA were reverse-transcribed using Script Reverse Transcription Supermix for RT-qPCR (Bio-Rad, USA), according to the manufacturer's instructions. The obtained cDNA was conserved at −80 °C and used as the template for subsequent qPCR analyses.

The transcriptional activity of target genes related to endocrine pathways (ecdysone receptor: *EcR*, insuline receptor: *InR* and vitellogenin: *vtg*), cellular stress response (heat shock proteins 70, 40, 10: *hsp70, hsp40, hsp10* and glycoprotein 93: *gp93*) and biotransformation reactions (cytochrome P450: *Cyp4g*, glutathione peroxidase: *GPx*, glutathione-S-transferase: *GST*, catalase: *CAT* and superoxide dismutase: *SOD*) were analyzed. Quantitative real-time RT-PCR (qRT-PCR) was carried out in a CFX96 Real-Time Detection System (Bio-Rad) using the Quantimix Easy Kit (Biotools, Spain). Genes encoding β actin and the 26S ribosomal subunit were used as endogenous references.

Primer sequences are shown in Appendix (Table A.2). The qRT-PCR was run as described in Herrero et al.[13]. A melting curve analysis was performed after amplification to verify the specificity of each fragment. CFX Maestro software (Bio-Rad) was used to determine total mRNA levels by normalizing the expression ($2^{-\Delta Cq}$) of the target genes against the two reference genes. Each sample was run in triplicate.

2.5. Enzyme activities and energy reserves content

The activity of cytochrome P450, an enzyme involved in biotransformation processes, was indirectly measured in living larva using the ECOD marker. For that purpose, for each condition, twelve 4th instar larvae were individually placed in wells (96-well plate) containing 300 μL of culture water with 7-ethoxycoumarin (400 μM). Blanks were made with the medium containing no organisms. Formation of 7-hydroxycoumarin (excitation: 380 nm, emission: 445 nm) was measured every 5 min for 3 h at 23 °C with a microplate reader (TECAN). ECOD was expressed in pmol 7-hydroxycoumarin formed per min per larval surface (mm^2).

To measure GST activity, phenoloxidase activity and energy reserve content, 12 replicates of five pooled larvae were considered for each condition. To assess the antioxidative response, GST activity was measured according to Habig et al. (1976). GST activity was expressed in nanomoles glutathione-1-chloro-2,4-dinitrobenzene conjugate formed per minute per milligram of protein. Phenoloxidase activity, an enzyme involved in the immune response, was measured as described in Arambourou and Stoks (2015). Phenoloxidase activity was expressed in nanomoles dopachrome formed per minute per milligram of protein. The total protein content was quantified according to Bradford (1976) with bovine serum albumin as the standard. Lipid extraction was performed in a mixture of chloroform and methanol (Bligh and Dyer, 1959) and lipid content was analyzed using the method reported by Marsh and Weinstein (1966) with cholesterol as the standard. Lipid content was expressed as micrograms lipid per milligram wet mass. Glycogen content was measured as described in Masuko et al. (2005) with glucose as the standard. Glycogen content was expressed as μmicrograms glycogen per milligram of wet mass.

The integrated biomarker response index (IBR) (Beliaeff and Burgeot, 2002) was used to describe biomarkers' variation in the three study conditions. Enzyme activation was considered positively (defence mechanism) while energy reserve contents were considered negatively (energy consumption to counteract the toxic stress). The overall IBR for each condition was calculated as the area of the polygon constituted with the scores of each study biomarker.

2.6. Lipidomic analysis

Lipidomic analysis was performed as described by Jordão et al. (2015) with minor modifications. Three replicates, consisting of a pool of five calibrated larvae, per condition were analyzed. Larvae were calibrated by mass to discard differences among conditions due to larvae growth.

Briefly, each replicate was homogenized in 1 mL of chloroform: methanol (2:1) with 2,6-di-*tert*-butyl-4-methylphenol (BHT; 0.01%) as an antioxidant (Jordão et al., 2015; Arambourou et al., 2018). Lipid extraction was performed using a modification of Folch's method (Folch et al., 1957). Briefly, 100 μL of the homogenized sample was mixed with 750 μL of chloroform, 250 μL of methanol and 250 μL of KCl. To semi-quantify the lipids, internal standards (Supplementary material, Table S3) were also added. Samples were then dried under N_2. Lipid extracts were solubilized in 200 μL methanol. The LC-MS/MS consisted of a Waters Aquity UPLC system connected to a LCT premier orthogonal accelerated time-of-flight mass spectrometer (Waters) operated in positive and negative ESI mode. Full-scan spectra from 50 to 1800 Da were

obtained. Mass accuracy and reproducibility were maintained using an independent reference spray (LockSpray; Waters). A 100-mm × 2.1-mm i.d., 1.7-μm C8 Acquity UPLC BEH (Waters) analytical column was used.

A total of 200 lipids, distributed as follows, were identified and semi-quantified: 68 triacylglycerols (TG), 21 diacylglycerols (DG), 4 monoacylglycerols (MG), 44 phosphatidylcholines (PC), 11 lysophosphatidylcholines (LPC), 9 sphingomyelins (SM), 23 phosphatidylethanolamines (PE), 7 lysophosphatidylethanolamines (LPE) and 14 phosphatidylserine (PS).

2.7. Respiration rate

To discard differences among conditions due to bacterial respiration in the sediment, the same protocol using clean sand layer was applied in each condition. Briefly, larvae were left for 24 h in clean water to remove the gut content. Then, 10 larvae were placed in a glass jar containing a 1.5-cm layer of clean sand surrounded by drill water. Jars were then hermetically sealed to allow CO_2 to accumulate in the headspace. After 5 h, the CO_2 content in the headspace was measured by gas chromatography (490 MicroGC, Agilent Technologies). Four replicates per condition were considered. A blank with no larvae was also performed. The respiration rate was expressed as ppm CO_2/min/μg fresh mass.

2.8. Shape markers

In each condition, the head capsules of 60 individuals were mounted to present a ventral view on microscope slide using Eukitt® medium. Head capsules were examined under microscope at ×100 magnification (Microscope:Leica DM 2000 LED) and photographed with a Leica MC 170 HD camera. Mentum deformities and mentum length fluctuating asymmetry were then evaluated as described in Arambourou et al. (2014).

After emergence, adults were collected and sexed. Wings were cut off, placed on microscope slides in Eukitt® medium and then scanned using a Plustek OpticFilm 7400 scanner. Wing shape variations were measured by geometric morphometrics, after digitizing eight type 1 landmarks for describing wing shape, according to Arambourou et al. (2014).

2.9. Statistical analysis

Statistical analyses were carried out using R. 3.4.3 software (R development Core Team, 2017). Transcript gene expression were analyzed using mixed model ANOVA (library lme4) with sediment as the fixed effect and analytical replicate as the random effect. Llipids grouped by family were analyzed with a one-way ANOVA with sediment as the independent variable. Differences in life history traits and biochemical markers (enzymes activity and energy reserves) were evaluated using a nonparametric Kruskal-Wallis test followed by a post hoc pairwise Mann-Whitney-Wilcoxon test with Holm p-value adjustment method. Times of emergence were compared using a Kaplan-Meier model. Mentum deformity rate variations were studied using a χ^2 proportion test. For length FA calculation, we used the FA10 index. This index describes the average difference between sides after measurement error has been partitioned out (Palmer and Strobeck, 2003). Since FA10 is a variance estimate, we used the F-test to compare differences in FA in the different treatments (Palmer and Strobeck, 2003). Wing shape variation among treatment groups were analized by canonical variate analysis on Procrustes coordinates using MorphoJ software (Klingenberg, 2011). The Procrustes distance between each group was calculated and the significance was assessed by a permutation test (10,000 permutations) (Klingenberg, 2011). A p-value <0.05 was used as a cutoff for statistical significance of differences among treatments.

3. Results

3.1. Characterization of sediment contamination

The three study sediments exhibited heavy metal contamination on the same order of magnitude. On the contrary, we observed a gradient of contamination from downstream (MON station) to upstream (ECH station), increasing for both PAHs and phthalates and decreasing for the toluene compound (Table 1). Higher concentrations of pesticides (mainly herbicides) were also detected in the ECH sediment. No PCBs were detected (Table 1). For eight heavy metals and nine PAHs, we compared the measured concentration to the probable effect concentrations reported in MacDonald et al. (2000). The probable effect concentration was only exceeded for chromium in the PAR sediment and for pyrene and fluoranthene in the ECH sediment. In addition, concentrations in phenanthrene and benzo(a)pyrene in the ECH sediment were very close to the probable effect concentrations.

3.2. Gene expression analysis

Genes coding for the hormone receptor *EcR* and for the protein *vtg* were significantly overexpressed in larvae exposed to the ECH sediment relative to the MON condition, with a mean increase of the transcriptional activity of 1.4-fold and 1.5-fold, respectively (mixed-effects model ANOVAs, *EcR*: $t = 2.48$, $p = 0.01$ and *vtg*: $t = 2.35$, $p = 0.02$; Fig. 2A and B). The expression of the *InR* gene did not vary significantly among the three study conditions (mixed-effects model ANOVA, both $p \geq 0.05$; Fig. 2C).

By comparison to the MON condition, significantly higher levels of *hsp40* gene expression were observed in both PAR and ECH exposed groups (mixed-effects model ANOVAs, both $p < 0.05$; Fig. 2D). A significant upregulation of *hsp70* was also found in ECH exposed group (mixed-effects model ANOVA, $t = 2.68$, $p = 0.04$; Fig. 2E). In contrast, the expression of *hsp10* and *gp93* remained constant in the three study groups (mixed-effects model ANOVA, all $p \geq 0.05$; Fig. 2F and G).

The expression levels of *CAT* and *Cyp4g* genes involved in biotransformation processes were upregulated after exposure to the ECH sediment in comparison to both the MON (mixed-effects model ANOVAs, *Cyp4g*: $t = 4.29$ and *CAT*: $t = 8.19$, both $p < 0.001$; Fig. 2G and H) and the PAR (mixed-effects model ANOVAs, *Cyp4g*: $t = 2.92$ and *CAT*: $t = 6.99$, both $p < 0.05$; Fig. 2G and H) conditions. The expression of genes involved in the antioxidant response (*GPx*, *GST* and *SOD*) was not significantly different in the three study conditions (mixed-effects model ANOVAs, all $p \geq 0.05$; Fig. 2J, K and L).

3.3. Lipidomic analysis

In the three study conditions, PE is the major component of phospholipids (about 58%), followed by PC (about 30%) (Table 2). We did not detect any significant differences in terms of the PC/PE ratio (membrane fluidity index) and the unsaturated/saturated ratios in the three study conditions (ANOVA, all $p \geq 0.05$, Appendix, Table A.4).

No significant differences were observed in terms of TAG, DAG, MAG, PC, LPC, PEA, LPEA, PS and SM contents in the three study conditions (ANOVAs, all $p \geq 0.05$) (Appendix, Table A.5).

3.4. Enzyme activities and energy reserves content

A significant increase of ECOD activity was detected in the PAR exposed group (Pairwise Mann-Whitney-Wilcoxon test, $p = 0.038$). No significant differences were detected in terms of phenoloxidase activity, GST activity, proteins, glycogen and lipid content (Kruskal-Wallis tests, all $p \geq 0.05$; Appendix, Fig. A.1). The IBR increased according to the level of PAHs and phtalates contamination: 6.0 in the MON group, 7.6 in the PAR group and 10.2 in the ECH group (Fig. 3).

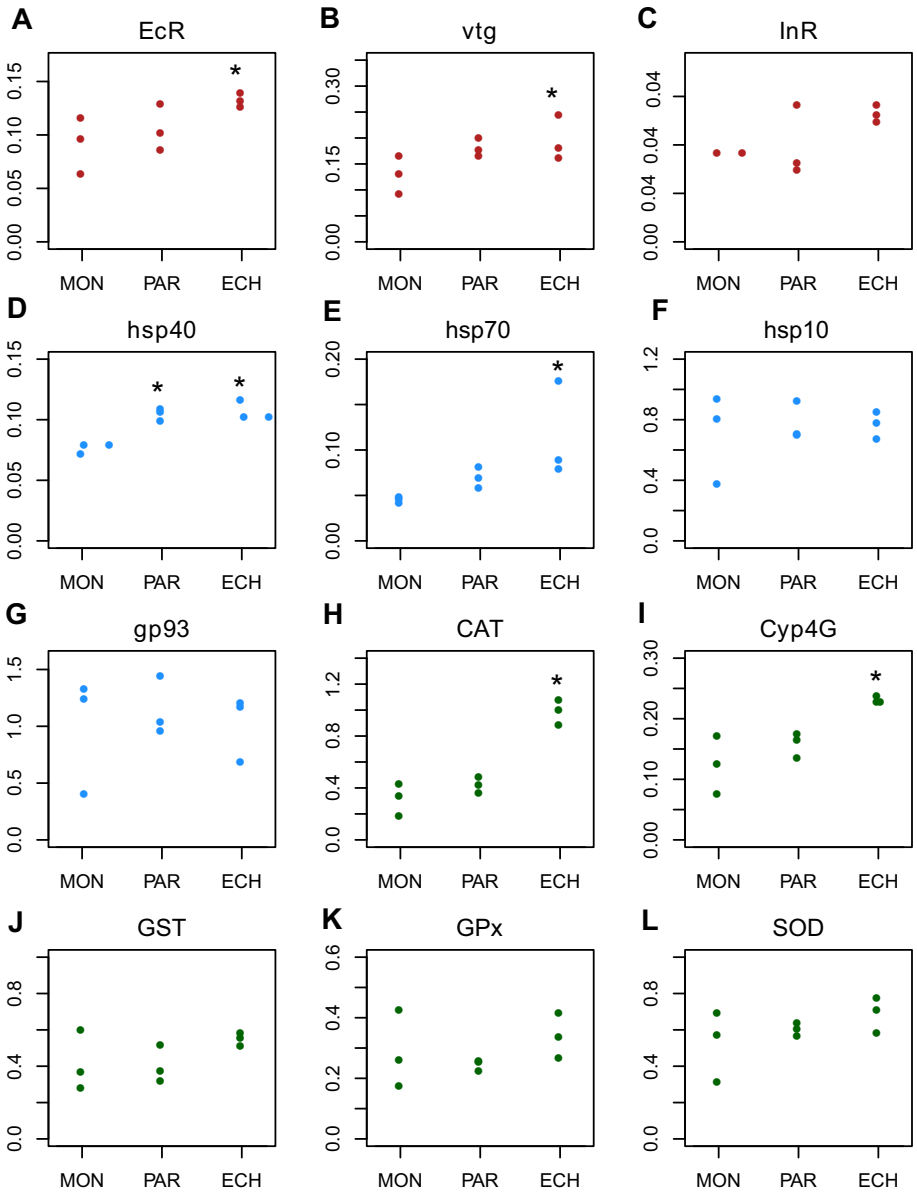

Fig. 2. Boxplot of relative mRNA expression of the 12 study genes classified according to their function (endocrine-related genes A, B and C; defence mechanisms D, E, F and G; biotransformation processes and antioxidant response H, I, J, K and L). *: significantly different from the MON condition (mixed-effects model ANOVA, $p < 0.05$). Dots represent the mean value of three measurements of a pooled sample of five larvae.

3.5. Life-history traits

In the three study conditions, the larvae survival rate varied between 93% and 94% and the success of emergence varied between 78% and 89%. Differences in survival and emergence rates were not statistically significant (Kruskal-Wallis test, both $p \geq 0.05$). No significant differences were detected among groups in terms of larval respiration rate (Kruskal-Wallis test, $W = 3.1$, $p = 0.21$; Fig. 4A). The larvae mass was significantly higher in the group exposed to the ECH sediment by comparison with both the MON and the PAR conditions (Pairwise-Mann-Whitney-

Table 2
Fraction of phospholipids. Mean value ± standard deviation (three replicates of five pooled larvae).

	PE fraction (%)	PC fraction (%)	PS fraction (%)	LPE fraction (%)	LPC fraction (%)
MON	57.7 ± 0.7	30.5 ± 1.5	9.3 ± 1.1	1.5 ± 0.7	1.1 ± 0.5
PAR	58.0 ± 1.4	29.9 ± 0.9	9.6 ± 0.7	1.5 ± 0.1	1.1 ± 0.1
ECH	58.0 ± 0.4	29.1 ± 0.9	9.6 ± 0.5	2.0 ± 0.9	1.3 ± 0.6

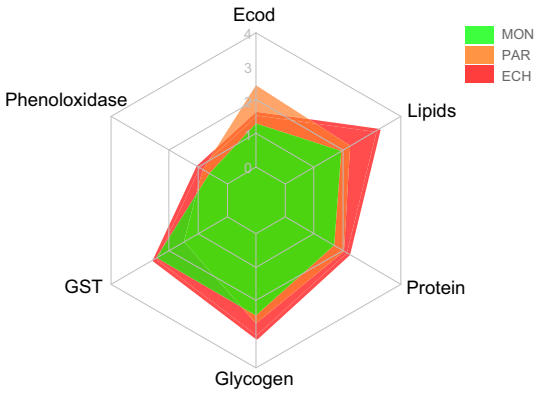

Fig. 3. Radar chart of the IBR in the three study conditions.

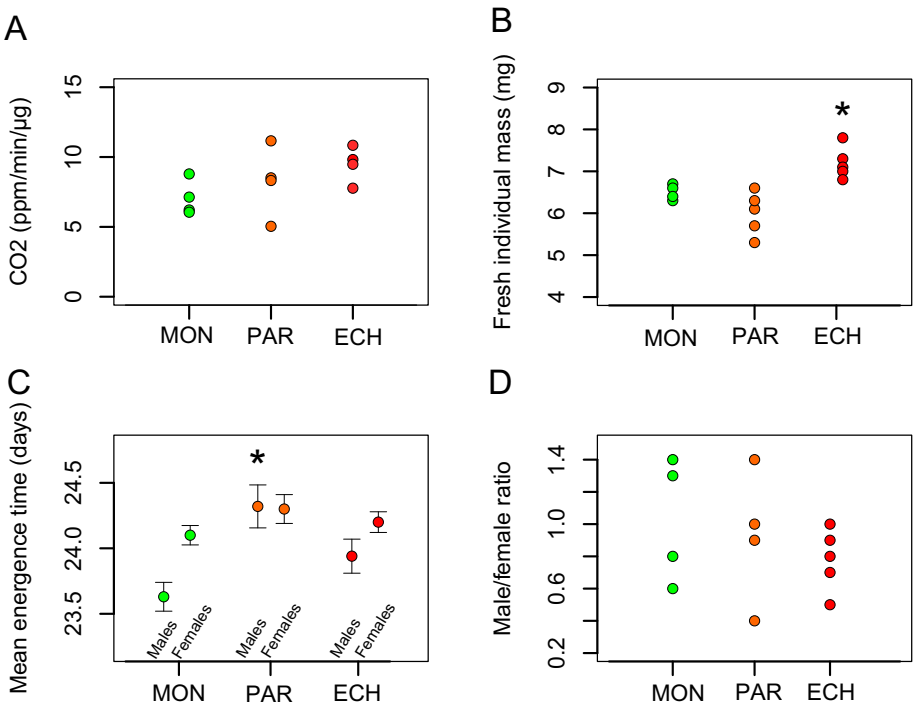

Fig. 4. Life history traits in *C. riparius* exposed to field-collected sediments. Respiration rate (four replicates of 10 pooled larvae) (A), individual larvae mass (six replicates of five pooled larvae per condition) (B), mean emergence time ± standard deviation (10 replicates of 20 larvae per condition) (C) and male/female ratio (five replicates of 20 larvae per condition) (D).*: significantly different from the MON group.

Wilcoxon test, both $p < 0.05$; Fig. 4B). By comparison with the MON condition only, males' emergence was significantly delayed in the PAR group (Kaplan-Meier model, $z = 3.5$, $p < 0.001$; Fig. 4C). No significant difference in the male/female ratio was observed (Kruskal-Wallis test, $W = 2.0$, $p = 0.37$; Fig. 4D).

3.6. Shape markers

We did not detect any significant difference of mentum deformity rates (proportion test, $\chi^2 = 5.5$, $p = 0.06$; Table 3). No significant allometry (Spearman test between length and asymmetry, all $p \geq 0.05$) or side effect (ANOVA, side effect, all $p \geq 0.05$) was observed. Therefore, all the observed asymmetries were considered as genuine mentum fluctuating asymmetry. Measurement error was lower than 20%. We did not detect any significant difference in terms of mentum length (Kruskal Wallis test, $\chi^2 = 0.62$, $p = 0.73$) and mentum fluctuating asymmetry (F tests, both $p \geq 0.05$; Table 3) in the three study conditions.

By comparison with the MON condition, wing centroid size, which is a proxy of wing size, was significantly higher in the PAR and ECH conditions for both males and females (pairwise Mann-Whitney-Wilcoxon test, both $p < 0.05$. Table 3). A significant allometry, i.e., a significant correlation between shape and centroid size, was detected for both males and females (permutation test, $p < 0.001$). Therefore, for shape analysis we only considered the nonallometric component using the residuals of the regression between shape and centroid size.

Table 3
Results of shape analysis of the mentum and the wings. Different letters indicate significant differences (Mann-Whitney-Wilcoxon test, $p < 0.05$).

Condition	Mentum deformity rate (%)	Mentum fluctuating asymmetry index (FA10)	Females' wing centroid size (mm)	Males' wing centroid size (mm)
MON	2^a	2.69^a	3.72 ± 0.09^a	3.41 ± 0.09^a
PAR	14^a	3.24^a	3.77 ± 0.12^b	3.51 ± 0.09^b
ECH	6^a	3.28^a	3.82 ± 0.08^b	3.54 ± 0.07^b

On the non-allometric component, we observed a significant difference of the wing shape between the MON and the ECH conditions in females only (Procrustes distance between Reference and High groups = 0.009, Permutation test, $p = 0.003$), with individuals from the ECH group exhibiting an increased posterior and apex areas (Fig. 5).

4. Discussion

The purpose of this study was to assess the effects of environmental contamination on *C. riparius*, a model aquatic insect species for sediment ecotoxicology, through a multidisciplinary strategy that combines classical ecotoxicological parameters (survival and development) with morphological (shape markers), physiological (respiration rate) and molecular responses (transcriptional, lipidomic, enzymatic). This study points out that an exposure to three field-collected sediments differentially contaminated with pesticides, PAHs and phtalates induced differential responses at various levels of biological organization in

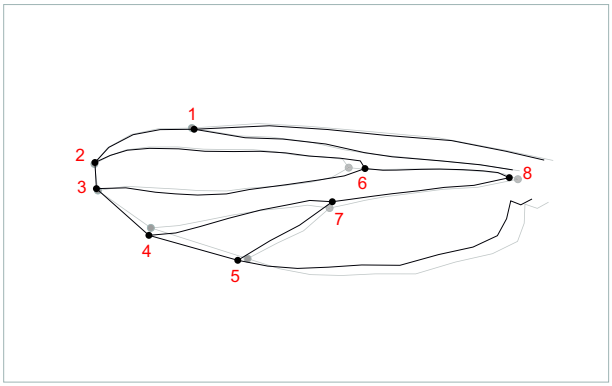

Fig. 5. Nonallometric shape variation in females wings associated with the first component of the canonical variate analysis, separating the ECH group from the MON group. Dots are the eight landmarks used to describe the wing shape. In grey the extreme individual from the MON group and in black the extreme individual from the ECH group.

C. riparius. Indeed, individuals exposed to both the ECH and the PAR sediments with the highest levels in PAHs and phtalates had defence mechanisms activated and a larger adult wing size relative to those exposed to the less contaminated MON sediment (Table 4). Nevertheless, no obvious links among molecular, cellular, shape and physiological markers were detected (Table 4).

4.1. Exposure to the most contaminated sediment induced endocrine disruption

The general transcriptional profiles of genes, especially those involved in endocrine pathways, were found to be affected by exposure to the ECH sediment.

Exposure to the ECH sediment led to an increase of the transcriptional activity of *EcR*, suggesting the ability of some contaminants present in the sediment to mimic the action of the hormone ecdysone and possibly disrupting the genetic cascades of this hormonal pathway. Given that ecdysone is implicated in insect growth and development (Nijhout et al., 2014), it is conceivable that the increased mass detected in the ECH group was related to ecdysone signaling pathway disruption. The upregulation observed in the mRNA levels of *vtg* in the ECH condition, suggested that the contaminants might also affect the ecdysone signaling pathway downstream. In the literature, the induction of vitellogenin has been described in *C. riparius* exposed to tributyltin (Morales et al., 2013), nonylphenol (Hahn et al., 2002), bishenol A, phthalates, cadmium, fenbendazole and 2,4-D (Park and Kwak, 2012). In the present study, PAHs, and particularly pyrene, might be responsible for *vtg* induction as was reported in the crustacean *Palaemonetes pugio* exposed to pyrene (Oberdörster et al., 2000). The *vtg* upregulation might be related to the activation of the ecdysone receptor, as reported by Roy et al. (2007) in the *Aedes aegypti* mosquito. The reproductive success of insects relies on vitellogenin biosynthesis and its uptake in the developing oocytes. Given that vitellus is the main energy source that fuels embryo development, vitellogenesis disruption may ultimately affect the embryogenesis of the following generation.

4.2. Contaminated sediments induced cell defence and biotransformation processes

Defence mechanisms and biotransformation processes were activated in larvae exposed to the ECH sediment and to a lesser extend to the PAR sediment.

In that way, *hsp40* transcripts were increased in groups exposed to both PAR and ECH sediments, while *hsp70* transcripts were only upregulated in the ECH condition. Hsp40 and hsp70 proteins belong to the family of heat shock proteins chaperones involved in cell viability. They are important for protein translation, folding, unfolding, translocation, and degradation. In *C. riparius* larvae, hsp40 and hsp70 were shown to respond to toxic compounds exposure such as di(2-ethylhexyl) phthalate and fenbendazole (Choi et al., 2000; Park et al., 2009). Heat shock protein-mediated processes are regarded as fundamental protective mechanisms that decrease cellular sensitivity to damaging events (Padmini, 2010; Planelló et al., 2015). In light of this, heat shock proteins upregulation observed in the present study may protect organisms from the toxic-induced effects and particularly from conformational damages to proteins.

The CAT enzyme is described as the main enzyme implicated in antioxidant protection in insects (Felton and Summers, 1995). In the ECH group, CAT was transcribed 3.1-fold above the MON condition. In the literature, not only upregulation of genes encoding for CAT (Nair et al., 2011) but also an increase of CAT enzyme activity (Lee and Choi, 2006) have been reported in *C. riparius* after toxic exposures. The ECH sediment was also able to alter *Cyp4G* gene expression. In the literature, upregulation of cytochrome *p*450 genes were reported in dipterans after exposure to various hydrophobic compounds (Gopalakrishnan-Nair et al., 2013; Dos Santos Moysés et al., 2017; Zhang et al., 2018). Cytochromes p450 are involved in detoxification mechanisms allowing the transformation of lipophilic xenobiotics, such as PAHs, to more water-soluble substances in order to facilitate their excretion. ECOD activity, which is a proxy of cytochrome *p*450 metabolism, was increased in the PAR sediment while the greater induction observed in *Cyp4G* gene was in the ECH sediment. The discrepancy between *Cyp4G* gene induction in the ECH group and ECOD induction in the PAR group could be explained by the fact that, unlike *Cyp4G* gene expression, ECOD methods allows to detect the activity of various cytochromes p450. In addition, ECOD activity might be reduced in the ECH group due to the high levels of PAHs, phtalates and pesticides contamination. In that way, a decrease of the transcription levels of cytochrome *p*450 was reported after exposure to high concentrations of phenol in *Chironomus kiiensis* (Zhang et al., 2018).

Lipid profiles were not significantly different in the three study groups. Contamination levels might not be sufficient to induce lipids profile disruption and/or to produce lipid peroxidation. In other words, the activation of defence mechanisms we observed through gene expression, might have protected larvae from oxidative damage and lipid membrane impairment.

We observed a delayed emergence time in males from the PAR sediment. This response was widely observed in chironomids exposed to contaminants (Bleeker et al., 1999; Arambourou et al., 2014) and might be due to an increase of energy consumption at the cost of growth in order to counteract the toxic effects. In *Chironomus* sp., as in numerous insects, males emerged before females to increase the success of reproduction. This phenomenon is known as protandry (Bulmer, 1983). In the PAR condition, this protandry disappeared (i.e., males and females emerged at the same time), which could have repercussions on the reproductive success and hence on the population dynamics.

The IBR index, which integrates six biomarkers related to detoxication processes (enzyme activities) and energy reserve content, were higher in both the PAR and ECH conditions, demonstrating metabolism disruption in these conditions.

4.3. Exposure to field-collected sediments affected adult morphogenesis

Wing size significantly increased in adults from larvae exposed to both the PAR and ECH sediments. An increase of appendage size was reported after exposure to the insecticide fenoxycarb in the crustacean *Gammarus fossarum* (Arambourou et al., 2017a). In the ECH sediment, this increase might partly compensate for the increased mass of individuals. This increase might also be related to the disruption of ecdysteroid pathways, which control morphogenesis in arthropods, as suggested by Laufer et al. (2002) in crab.

As observed in our previous study in *C. riparius* exposed to field-collected sediments (Arambourou et al., 2014), female wing shape

Table 4
Effects of an exposure to field-collected sediments on various markers in *C. riparius* by comparison to the MON sediment: =: no significant difference, +: significant difference.

		PAR	ECH
Molecular markers	Endocrine-related genes	=	+
	Defence-related genes	+	+
	Biotransformation-related genes	=	+
	Lipid profile	=	=
Cellular markers	Biotransformation enzyme activity	+	=
	Energy reserve content	=	=
Shape markers	Mentum size	=	=
	Mentum shape variations	=	=
	Wing size	+	+
	Wing shape variations	=	+
Physiology	Respiration rate	=	=
Life history-traits	Mortality	=	=
	Mass	=	+
	Emergence rate	=	=
	Emergence time	+	=
	Males/females ratio	=	=

was altered following larval exposure to the ECH sediment. Contaminants might be responsible for the disruption of morphogenesis and this effect appears to be sex-dependent. This could affect the locomotor capacity, as was observed in damselfly exposed to a heat stress (Arambourou et al., 2017a, 2017b), and hence, the reproduction and species dispersal. Mentum shape was not significantly altered by the exposure to the field-collected sediments. Given that this marker was shown to be poorly sensitive to heavy metals and PAHs exposures (Arambourou et al., 2014; Gagliardi et al., 2016), the level of contamination might not be sufficient to induce such defects.

4.4. Are probable effect concentration values protective for C. riparius?

Probable effect concentration values were exceeded in the PAR sediment for chromium and in the ECH sediment for pyrene and fluoranthene, suggesting potential adverse effects in these conditions. In accordance with this, upregulation of gene expression, delayed emergence time and increased appendage sizes were observed. Nonetheless, these effects might also be caused by the mixture of contaminants (Segner et al., 2014) and by other contaminants such as pesticides. Studying toxic effects in organisms and at multiple levels of biological organization remain indispensable to have an overview of the global toxicity of complex matrixes such as field-collected sediments.

Even if we were not able to bring out links among the study biomarkers, the effects observed through the different organizational levels were in accordance with the levels of PAHs, phtalates and pesticides observed in the sediments. Indeed, stronger effects were reported on defence- and endocrine-related gene expression and on wing shape variations in organisms from the sediment exhibiting the highest contamination (ECH sediment).

5. Conclusions

This study has pointed out the differential responses at various levels of biological organization in *C. riparius* exposed to three field-collected sediments exhibiting distinct profiles of contamination. These results suggest that exposure to PAHs, phtalates and pesticides, may not only activate stress and defence mechanisms but also cause the disruption of endocrine-related functions and morphogenesis. Further studies, under laboratory controlled conditions, are now needed not only to isolate the chemical compounds responsible for these biological effects but also to assess whether the observed effects have repercussions on the chironomid fitness. Using solely chemical criteria to determine the toxicity of a complex matrix, such as field-collected sediment, is not sufficiently informative about the long-term effects on population dynamics. Combining chemical techniques with biological approaches will be certainly useful to better assess the risk that aquatic sediments pose to aquatic ecosystems.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.03.384.

References

Aquilino, M., Sánchez-Argüello, P., Martínez-Guitarte, J.-L., 2016. Vinclozolin alters the expression of hormonal and stress genes in the midge *Chironomus riparius*. Aquat. Toxicol. 174, 179–187.

Arambourou, H., Stoks, R. 2015. Warmer winters modulate life history and energy storage but do not affect sensitivity to a widespread pesticide in an aquatic insect.

Arambourou, H., Beisel, J.-N., Branchu, P., Debat, V., 2014. Exposure to sediments from polluted rivers has limited phenotypic effects on larvae and adults of *Chironomus riparius*. Sci. Total Environ. 484, 92–101.

Arambourou, H., Chaumot, A., Vulliet, E., Daniele, G., Delorme, N., Abbaci, K., Debat, V., 2017a. Phenotypic defects in newborn *Gammarus fossarum* (Amphipoda) following embryonic exposure to fenoxycarb. Ecotoxicol. Environ. Saf. 144, 193–199.

Arambourou, H., Sanmartín-Villar, I., Stoks, R., 2017b. Wing shape-mediated carry-over effects of a heat wave during the larval stage on post-metamorphic locomotor ability. Oecologia 184, 279–291.

Arambourou, H., Fuertes, I., Vulliet, E., Daniele, G., Noury, P., Delorme, N., Abbaci, K., Barata, C., 2018. Fenoxycarb exposure disrupted the reproductive success of the amphipod *Gammarus fossarum* with limited effects on the lipid profile. PLoS One 13 (4), e0196461.

Beliaeff, B., Burgeot, T., 2002. Integrated biomarker response: a useful tool for ecological risk assessment. Environ. Toxicol. Chem. 21, 1316–1322.

Bleeker, E.A.J., Leslie, H.A., Groenendijk, D., Plans, M., Admiraal, W., 1999. Effects of exposure to azaarenes on emergence and mouthpart development in the midge *Chironomus riparius* (Diptera: Chironomidae). Environ. Toxicol. Chem. 18, 1829–1834.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction andpurification. Can. J. Biochem. Physiol. 37, 911–917.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Bulmer, M.G., 1983. Models for the evolution of protandry in insects. Theor. Popul. Biol. 23, 314–322.

Chan, C. Y.n, Wang, W.X., 2018. A lipidomic approach to understand copper resilience in oyster *Crassostrea hongkongensis*. Aquat. Toxicol. 204, 160–170.

Choi, J., Roche, H., Caquet, T., 2000. Effects of physical (hypoxia, hyperoxia) and chemical (potassium dichromate, fenitrothion) stress on antioxidant enzyme activities in *Chironomus riparius* mg. (diptera, chironomidae) larvae: potential biomarkers. Environ. Toxicol. Chem. 19, 495–500.

Di Veroli, A., Santoro, F., Pallottini, M., Selvaggi, R., Scardazza, F., Cappelletti, D., Goretti, E., 2014. Deformities of chironomid larvae and heavy metal pollution: from laboratory to field studies. Chemosphere 112, 9–17.

Dickman, M., Brindle, I., Benson, M., 1992. Evidence of teratogens in sediments of the Niagara River watershed as reflected by chironomid (Diptera: Chironomidae) deformities. J. Great Lakes Res. 18, 467–480.

Dos Santos Moysés, F., Bertoldi, K., Lovatel, G., Vaz, S., Ferreira, K., Junqueira, J., Bagatini, P.B., Rodrigues, M.A.S., Xavier, L.L., Siqueira, I.R., 2017. Effects of tannery wastewater exposure on adult *Drosophila melanogaster*. Environ. Sci. Pollut. Res. 24, 26387–26395.

Felton, G.W., Summers, C.B., 1995. Antioxidant systems in insects. Arch. Insect Biochem. Physiol. 29, 187–197.

Fisher, T., Crane, M., Callaghan, A., 2003. Induction of cytochrome P-450 activity in individual *Chironomus riparius* Meigen larvae exposed to xenobiotics. Ecotoxicol. Environ. Saf. 54, 1–6.

Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226, 497–509.

Gagliardi, B.S., Pettigrove, V.J., Long, S.M., Hoffmann, A.A., 2016. A meta-analysis evaluating the relationship between aquatic contaminants and Chironomid larval deformities in laboratory studies. Environ. Sci. Technol. 50, 12903–12911.

Gopalakrishnan-Nair, P.M.G., Park, S.Y., Choi, J., 2013. Characterization and expression of cytochrome p450 cDNA (CYP9AT2) in *Chironomus riparius* fourth instar larvae exposed to multiple xenobiotics. Environ. Toxicol. Pharmacol. 36, 1133–1140.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1976. Glutathione S-transferase AA from rat liver. Arch. Biochem. Biophys. 175, 710–716.

Hahn, T., Schenk, K., Schulz, R., 2002. Environmental chemicals with known endocrine potential affect yolk protein content in the aquatic insect *Chironomus riparius*. Environ. Pollut. 120, 525–528.

Herrero, Ó., Morcillo, G., Planelló, R., 2017. Transcriptional deregulation of genetic biomarkers in *Chironomus riparius* larvae exposed to ecologically relevant concentrations of di(2-ethylhexyl) phthalate (DEHP). PLoS One 12, e0171719.

Herrero, Ó., Aquilino, M., Sánchez-Argüello, P., Planelló, R., 2018. The BPA-substitute bisphenol S alters the transcription of genes related to endocrine, stress response and biotransformation pathways in the aquatic midge *Chironomus riparius* (Diptera, Chironomidae). PLoS One 13 (2), e0193387.

Jordão, R., Casa, J., Fabrias, G., Campos, B., Piña, B., Lemos, M.F.L., Soares, A.M.V.M., Tauler, M., Barata, C., 2015. Obesogens beyond vertebrates: lipid perturbation by tributyltin in the crustacean *Daphnia magna*. Environ. Health Perspect. 123, 813–819.

Kassahn, K.S., Crozier, R.H., Ward, A.C., Stone, G., Caley, M.J., 2007. From transcriptome to biological function: environmental stress in an ectothermic vertebrate, the coral reef fish *Pomacentrus moluccensis*. BMC Genomics 8, 358.

Klingenberg, C.P., 2011. MorphoJ: an integrated software package for geometric morphometrics. Mol. Ecol. Resour. 11, 353–357.

Laufer, H., Ahl, J., Rotllant, G., Baclaski, B., 2002. Evidence that ecdysteroids and methyl farnesoate control allometric growth and differentiation in a crustacean. Insect Biochem. Mol. Biol. 32, 205–210.

Lee, S.-B., Choi, J., 2006. Multilevel evaluation of nonylphenol toxicity in fourth-instar larvae of *Chironomus riparius* (Diptera, Chironomidae). Environ. Toxicol. Chem. 25, 3006–3014.

Lee, S.-M., Lee, S.-B., Park, C.H., 2006. Expression of heat shock protein and hemoglobin genes in *Chironomus tentans* (Diptera, chironomidae) larvae exposed to various environmental pollutants: a potential biomarker of freshwater monitoring. Chemosphere 65, 1074–1081.

MacDonald, D.D., Ingersoll, C.G., Berger, T.A., 2000. Development and evaluation of consensus-based sediment quality guidelines for freshwater ecosystems. Arch. Environ. Contam. Toxicol. 39, 20–31.
Marsh, J.B., Weinstein, D.B., 1966. Simple charring method for determination of lipids. J. Lipid Res. 7, 574–576.
Martinez, E.A., Moore, B.C., Schaumloffel, J., Dasgupta, N., 2003. Morphological abnormalities in *Chironomus tentans* exposed to cadmium—and copper-spiked sediments. Ecotoxicol. Environ. Saf. 55, 204–212.
Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S.-I., Lee, Y.C., 2005. Carbohydrate analysis by a phenol–sulfuric acid method in microplate format. Anal. Biochem. 339, 69–72.
Morales, M., Martínez-Paz, P., Ozáez, I., Martínez-Guitarte, J.L., Morcillo, G., 2013. DNA damage and transcriptional changes induced by tributyltin (TBT) after short in vivo exposures of *Chironomus riparius* (Diptera) larvae. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 158, 57–63.
Nair, P.M.G., Park, S.Y., Choi, J., 2011. Expression of catalase and glutathione S-transferase genes in *Chironomus riparius* on exposure to cadmium and nonylphenol. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 154, 399–408.
Nijhout, H.F., Riddiford, L.M., Mirth, C., Shingleton, A.W., Suzuki, Y., Callier, V., 2014. The developmental control of size in insects. Wiley Interdiscip. Rev. Dev. Biol. 3, 113–134.
Oberdörster, E., Brouwer, M., Hoexum-Brouwer, T., Manning, S., McLachlan, J.A., 2000. Long-term pyrene exposure of grass shrimp, *Palaemonetes pugio*, affects molting and reproduction of exposed males and offspring of exposed females. Environ. Health Perspect. 108, 641–646.
OECD, 2004. Test No. 218: Sediment-Water Chironomid Toxicity Using Spiked Sediment.
OECD, 2010. Test No. 233: Sediment-water Chironomid Life-cycle Toxicity Test Using Spiked Water or Spiked Sediment.
OECD, 2011. Test No. 235: *Chironomus* sp., Acute Immobilisation Test.
Ozáez, I., Morcillo, G., Martínez-Guitarte, J.-L., 2016. Ultraviolet filters differentially impact the expression of key endocrine and stress genes in embryos and larvae of *Chironomus riparius*. Sci. Total Environ. 557–558, 240–247.
Padmini, E. 2010. Physiological adaptations of stressed fish to polluted environments: role of heat shock proteins Rev. Environ. Contam. Toxicol. 206, 1–27, Whitacre, D. M., editor, Springer New York.
Palmer, A., Strobeck, C., 2003. Fluctuating Asymmetry Analyses Revisited in Developmental Instability: Causes and Consequences. Oxford University Press, pp. 279–319 Michal Polak editor.
Park, K., Kwak, I.-S., 2012. Assessment of potential biomarkers, metallothionein and vitellogenin mRNA expressions in various chemically exposed benthic *Chironomus riparius* larvae. Ocean Sci. J. 47, 435–444.
Park, K., Bang, H.W., Park, J., Kwak, I.-S., 2009. Ecotoxicological multilevel-evaluation of the effects of fenbendazole exposure to *Chironomus riparius* larvae. Chemosphere 77, 359–367.
Planelló, R., Martínez-Guitarte, J.L., Morcillo, G., 2008. The endocrine disruptor bisphenol a increases the expression of HSP70 and ecdysone receptor genes in the aquatic larvae of *Chironomus riparius*. Chemosphere 71, 1870–1876.
Planelló, R., Herrero, O., Martínez-Guitarte, J.L., Morcillo, G., 2011. Comparative effects of butyl benzyl phthalate (BBP) and di(2-ethylhexyl) phthalate (DEHP) on the aquatic larvae of *Chironomus riparius* based on gene expression assays related to the endocrine system, the stress response and ribosomes. Aquat. Toxicol. 105, 62–70.
Planelló, R., Servia, M.J., Gómez-Sande, P., Herrero, O., Cobo, F., Morcillo, G., 2015. Transcriptional responses, metabolic activity and mouthpart deformities in natural populations of *Chironomus riparius* larvae exposed to environmental pollutants. Environ. Toxicol. 30, 383–395.
R development Core Team, 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria Available online at. https://www.R-project.org/.
Roy, S.G., Hansen, I.A., Raikhel, A.S., 2007. Effect of insulin and 20-hydroxyecdysone in the fat body of the yellow fever mosquito, *Aedes aegypti*. Insect Biochem. Mol. Biol. 37, 1317–1326.
Segner, H., Shmitt-Jansen, M., Sabater, S., 2014. Assessing the impact of multiple stressors on aquatic biota: the receptor's side matter. Environ. Sci. Technol. 48, 7690–7696.
Weis, J.S., 2014. Larval Development in Physiological, Developmental and Behavioral Effects of Marine Pollution. Springer, Dordrecht, pp. 215–251.
Zhang, Q., Chu, D., Sun, L., Cao, C., 2018. Cytochrome P450 CYP6EV11 in *Chironomus kiiensis* larvae involved in phenol stress. Int. J. Mol. Sci. 19, 1119.