



Exposure to sediments from polluted rivers has limited phenotypic effects on larvae and adults of *Chironomus riparius*



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ABSTRACT

Laboratory studies have sometimes failed to detect a relationship between toxic stress and morphological defects in invertebrates. Several hypotheses have been proposed to account for this lack of effect. (1) It was suggested that only a combination of stressful conditions – rather than a single one – would affect the phenotype. (2) Phenotypic defects should be detected on adult individuals, rather than on juveniles. (3) Phenotypic abnormalities might mostly affect the progeny of the exposed individuals, some contaminants exhibiting trans-generational effects. In the present study, we test those three hypotheses. We first examined the effects of a multiple exposure by using laboratory *Chironomus riparius* larvae cultured on two sediments sampled in contaminated rivers and those containing a mixture of mineral and organic compounds. On the larvae, we investigated mentum phenotypes: the frequency of phenodeviants, the shape fluctuating asymmetry and the mean shape. To test whether adult's morphology was more sensitive than the larval's, we also measured asymmetry and mean shape of the adult wings. Finally, to test for a trans-generational phenotypic effect, we measured mentum shape variations in the offspring derived from the measured adults. Overall, our results point out a very limited phenotypic response to contaminated sediments, suggesting that a multiple exposure is not necessarily sufficient to generate phenotypic defects. Adult traits were no more affected than larval traits, discarding the hypothesis that adult phenotypes would be more sensitive biomarkers. Finally, no effect was detected on the offspring generation, suggesting that no trans-generational effect occurs. This general lack of effect suggests that the use of phenotypic defects in *C. riparius* as an indicator of sediment contamination should be considered cautiously.

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1. Introduction

Rivers contaminated by metals and organic substances have often been reported to be associated with an increased incidence of phenotypic defects, such as phenodeviation and fluctuating asymmetry (FA), particularly in invertebrates (Al-Shami et al., 2011; Bonada and Williams, 2002; Groenendijk et al., 1998). A phenodeviation is an abnormal and rare expression of a trait (Lerner, 1954; Rasmuson, 1960) while fluctuating asymmetry refers to random and subtle departure from a perfect bilateral symmetry (van Valen, 1962). These morphological defects occur when developmental homeostasis is insufficient to compensate environmental stress (Graham et al., 1993; Palmer and Strobeck, 1992) and have thus been proposed as relevant indicators of such environmental stress (Leary and Allendorf, 1989; Vermeulen, 1995). Particularly, phenodeviations have been widely reported in chironomid

larvae (Diptera) collected in rivers contaminated by metals (Janssens de Bisthoven et al., 1998a; Martinez et al., 2002) or organic compounds (Janssens de Bisthoven et al., 1996; Servia et al., 1998). Among the chironomid larvae, the genus *Chironomus* (Al-Shami et al., 2011; Bird, 1994; Di Veroli et al., 2012; Janssens de Bisthoven et al., 1998a; Lenat, 1993), particularly the species *Chironomus riparius* (Servia et al., 1998), has been shown to be prone to such pollution-induced phenotypes.

To identify the substances involved in phenotypic defects and the concentrations necessary to induce them, numerous bioassays have been performed in *C. riparius* larvae under controlled conditions in the laboratory (Bleeker et al., 1999; Di Veroli et al., 2012; Merregalli et al., 2001; Park et al., 2009). This species has been widely used for sediment bioassay for several reasons. First, the larvae spend the majority of their life in sediment making them particularly relevant for sediment bioassay. Second, the abundance of this species in both preserved and disturbed rivers facilitates the sampling of numerous individuals. Third, *C. riparius* can be easily cultured in the laboratory (short life cycle, resistance to manipulation), allowing to perform bioassay in controlled conditions. Fourth, as *C. riparius* is one of the most commonly used species

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(Groenendijk et al., 1998; Watts et al., 2003; Park et al., 2009; Servia et al., 2004; Vogt et al., 2013), a good comparative framework is available. Despite this common use in ecotoxicological studies, the phenotypic responses of *Chironomus* larvae to pollution are heterogeneous across studies: while some studies have reported increased frequency of deformities and FA following an exposure to mineral (Martinez et al., 2003) or organic compounds (Meregalli et al., 2001; Park et al., 2009), others failed to detect such effects (Arambourou et al., 2012; Bird et al., 1995).

Several hypotheses have been proposed to explain the weak link observed between stress and phenotypic defects in laboratory studies, as discussed in Arambourou et al. (2012). First, developmental stability in aquatic invertebrates could be mainly affected by a combination – rather than a single – of stressful environmental conditions (Campero et al., 2008; Langer-Jaesrich et al., 2010). Indeed, the use of a single pollutant – as it is often the case in the laboratory – could be inefficient in inducing strong phenotypic effects. Second, to explain the weak responses measured in the larval life stage of a damselfly, Campero et al. (2008) proposed that, as metamorphosis is energetically costly and thus comparable to a stressful event, FA could be higher in adults than in larval life stages, suggesting that adults should be preferentially used to detect developmental instability. Third, morphological abnormalities could appear after several generations of exposure. Indeed, it is well known now that some toxics, such as endocrine disruptors, can contribute to trans-generational developmental effects in aquatic organisms, such as in the fish *Oryzias latipes* (Gray et al., 1999; Zhang et al., 2008), leading to an increase of morphological abnormalities in the offspring derived from the exposed parents. In the present study, we test these three hypotheses in *C. riparius* exposed to sediments collected in multi-contaminated rivers.

2. Material and methods

2.1. Experimental design

The experiment consisted of exposing laboratory *C. riparius* larvae during their entire larval life cycle to two sediments sampled in disturbed rivers (Fig. 1). First, to test whether a multiple toxic exposure would have severe phenotypic effects, we exposed larvae to sediments collected in disturbed rivers and those containing a mixture of mineral and organic compounds. In the larvae, after 20 days of exposure, we measured three types of mentum phenotypic variations: the frequency

of phenodeviants, the shape FA and the mean shape changes. Second, to determine whether the metamorphosis could contribute to the increase of phenotypic defects, we measured phenotypic variations in adults derived from the exposed larvae. On the wings, we measured three phenotypic traits: shape FA, centroid size FA and the mean shape changes. Finally, to assess if the chemical exposure was involved in a trans-generational phenotypic effect, we also measured mentum shape variations in the offspring larvae derived from the measured adults.

2.2. Test organisms and culture conditions

We used the same stock culture as in the previous study (Arambourou et al., 2012). The strain of *C. riparius* used was provided by the IRSTEA laboratory (Lyon, France) and was maintained in the CETE IDF laboratory (Trappes, France) since 2004. The stock culture was reared under 16:8 h light:dark photoperiod at 15 ± 1 °C. Aquaria were composed of 2 cm of Fontainebleau sand layer (Fontainebleau sand Technical, VWR) with a median diameter of solid particles ranging from 150 to 300 μm , surrounded by a mixture of deionized water and source water (electrical conductivity = 346 ± 4 $\mu\text{S/cm}$, pH = 7.7 ± 0.1 and hardness = 146 ± 3 mg CaCO_3/l).

2.3. Test sediments

Following OECD recommendations for sediment bioassay (OECD, 2004), the sediment provided as control sediment is an artificial mixture of Fontainebleau sand (75.3%), kaolin (19%), dried crushed peat (5.0%), calcium carbonate (0.7%) and deionized water (33%). The artificial sediment used in the present study was the same as in our previous study focusing on a mono-exposure (Arambourou et al., 2012). As numerous studies reported an increase of morphological abnormalities in *C. riparius* collected in polluted areas (Janssens de Bisthoven et al., 1996; Martinez et al., 2002; Servia et al., 1998), we chose to study two sediments sampled in polluted rivers. The first one was sampled in the small river La Drouette located in the Paris region. The river flow was lower than 10 l/s. This river drains mixed urban, agricultural and forest land. The sediment was collected downstream from a sewage treatment plant designed for treating a load of waste of 400 population equivalent. As the drained watershed was only slightly disturbed by human activities, this station will be referred to as LOW in the text. The second sediment was sampled during a dredging in the Seine river, upstream the Andrézy lock located in the Paris region. The mean

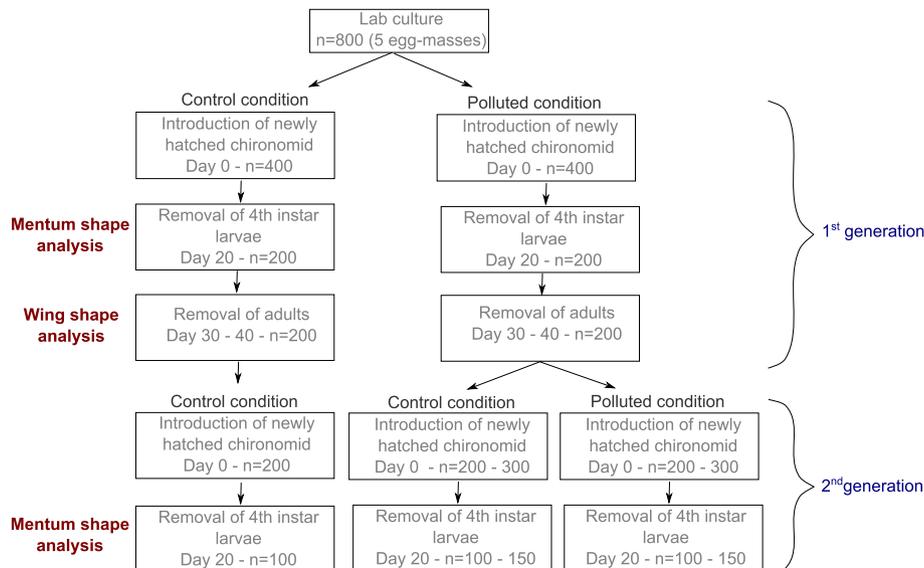


Fig. 1. Experimental design for each bioassay performed on field-collected sediment. n: sample size.

river Seine flow is about 100,000 l/s. This station is located in a densely mixed urban and industrial area. As the drained watershed was very disturbed by industrial and urban activities, this station will be referred to as HIGH in the text. Upon collection, both sediments were passed through a 3 mm sieve and lyophilized until their use for the bioassay.

2.4. Experimental aquaria

Tanks were maintained at 15 ± 1 °C, on a 16:8 h light: dark photoperiod. A 1.5 centimeter layer of either control sediment or field-collected sediment was provided as a substrate and a volume of rearing water four times greater was added. As recommended by OECD guideline for sediment bioassay (OECD, 2004), water and sediment were left for two days to allow toxic equilibration and settlement of suspended solids. One hour before the introduction of the larvae, 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. For the parental generation, the study design included 4 aquaria for each condition tested. After 20 days of larval exposure, aquaria of each condition were fitted with a cap to retain emerging chironomid. After the mating and the oviposition of emerged chironomids, in each replicate egg-masses were collected and grouped together according to the condition (control or exposed). Then, chironomid larvae that hatched at the same date were randomly injected either in control sediment or in field-collected sediment (Fig. 1). For the second generation, depending on the number of larvae that hatched at the same date, each condition tested included 2 or 3 aquaria. Offspring larvae were collected after 20 days of exposure.

2.5. Chironomid sample preparation

Five egg masses (each produced by a different single female and each containing about 300 eggs) were taken from the laboratory culture and placed in Petri dishes filled with rearing water four days before injection. To maximize the number of newly hatched larvae in the study (i.e. 400 larvae by tested condition), the two sediments were studied sequentially. This resulted in two bioassay sessions separated by a period of three months. To account for a possible effect of the experimental session, we included a control group in each experiment. After hatching, 800 larvae were randomly selected from the five egg masses and assigned to one of the 8 test aquaria (4 control and 4 treated). Each larva had a mean of 2 cm² of sediment available. The larvae were fed daily with 0.40 mg of fish food (Tetramin®) per individual. At 15 °C, larvae reach the 4th instar after 15 days. To make sure that individuals were at the 4th instar, we removed half of the larvae (50 individuals) from the four replicate test aquaria after 20 days of exposure. Upon collection, chironomid larvae were rinsed in deionized water bath to remove particles bound to the external surface. Chironomid head capsules were then cut and treated to remove internal tissues as recommended by Epler (2001). Finally, they were mounted to present a ventral view on microscope slide using Eukitt® medium. Head capsules were examined under microscope at 100× and 400× magnification (Microscope: Nikon Eclipse E600) and photographed with a Sony SSC-DC14 camera. We collected adults of the parental generation when all organisms were dead. Upon collection, we separated females from males. Wings were cut off, placed on microscope slides in Eukitt® medium and then scanned using a Plustek OpticFilm 7400 scanner.

2.6. Body dry weight, time to emergence and adult emergence rate

Larval dry weight was evaluated after placing the larvae at 30 °C for 24 h. The first day of emergence was recorded for each replicate test aquaria. As endpoints of the toxicity test, the numbers of emerged adults from each replicate test aquaria were counted.

2.7. Determination of trace metals and organic compounds in chironomid larvae bodies and sediments

In order to obtain a sufficient dry chironomid mass for chemical analysis, we pooled one of the replicate with the three other ones. As a consequence, we obtained three values of accumulation for each compound measured. For trace metal analyses, after decapitation chironomid bodies were dried at 30 °C for 24 h. Then, they were digested in a mixture of 6 ml of HNO₃, 2 ml of HCl and 2 ml of H₂O₂ and heated at 125 °C in a fume-hood for 2 h. Therefore, they were diluted to 40 ml with deionized water. Sediment samples were dried at 105 °C for 24 h, digested in the same way as chironomid bodies and diluted to 100 ml with deionized water. Lead, copper, nickel, cadmium and chromium determinations were performed using furnace atomic absorption (Varian, GTA 120). Zinc determination was performed using flame atomic absorption (Varian 280 FS). Single element calibration standards and multi-element quality control standards were used for calibration and quality assurance, respectively.

For organic compound analyses, after decapitation chironomid bodies were lyophilized with a Christ Alpha 1–4 LD freeze dryer. The complete extractions of alkylphenols (AP) and polycyclic aromatic hydrocarbon (PAH) were obtained by mixing chironomid bodies with 5 ml of ultrapure methanol. The mixture was placed two times in sonication bath for 15 min with a 15 minute rest time. Extract was purified by passing the sample through a glass column containing 1 g of silica. Complete extraction for lyophilized sediment was made by Soxhlet extraction with dichloromethane. Extract was purified by passing the sample through a glass column containing 5 g of silica. Analyses were made by HPLC (High Performance Liquid Chromatography) equipped with a fluorescence detector (Waters 2475). The two most commonly detected AP in the environment were analyzed: 4-nonylphenol (4-NP) and 4t-octylphenol (4t-OP). Moreover, we measured 15 PAH: naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(ah)anthracene, benzo(ghi)perylene and indeno(1,2,3-cd)pyrene. To provide quality assurance, a quality control consisting of a solution containing known concentrations of the measured compounds was injected during each analysis session. Aliphatic hydrocarbons between n-C10 and n-C40 in the sediment were analyzed by GC-FID.

2.8. Mentum deformities analysis

We first examined the deformity rate. The mentum was considered as deformed if it had extra tooth, missing tooth, fused teeth and Köhn gap (Fig. 2). If missing teeth had rough surface as opposed to smooth surface, mentum was considered broken and not deformed. A cleft median tooth was difficult to assess in several cases due to the slight size indentation. We thus did not consider this type of phenotypic variation as a deformity.

2.9. Mentum and wing FA analyses

Both mentum and wing shape FA were analyzed by geometric morphometrics. For this, we digitized 29 “type 1” landmarks (Bookstein, 1991) in the mentum and 8 “type 1” landmarks in the wing (Fig. 3) using TPSdig2 software (Rohlf, 2010). Each measure was taken twice in order to assess measurement error. In the present work, for shape FA analysis, two types of object symmetry were considered: object symmetry and matching symmetry. Object symmetry corresponds to the case where the plan of symmetry is contained in the studied objects, which is the case for the mentum in chironomids larvae. Matching symmetry is the symmetry of paired separated structures, as the wings (see Klingenberg et al., 2002 for an introduction and the details of the shape analysis procedures). The size FA can only be determined for objects with matching symmetry (Savriama and Klingenberg,

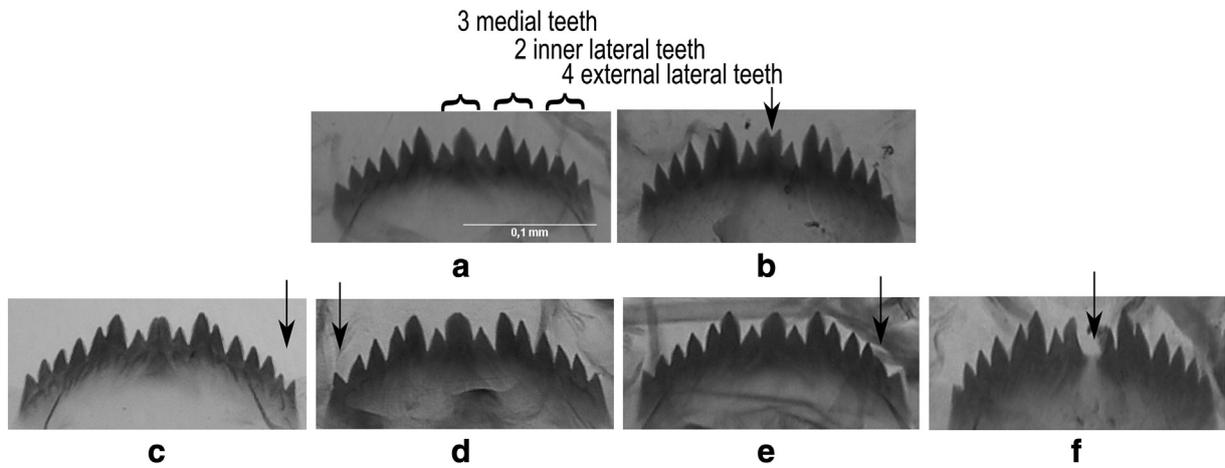


Fig. 2. Normal and deformed mentum observed. Normal mentum (a), cleft medial tooth (b), extra tooth (c), missing tooth (d), fused teeth (e) and Köhn gap (f). Arrows indicate morphological abnormality.

2011). Consequently, shape FA was measured for both the mentum and the wings while size FA was only measured for the wings. Moreover, for wing FA analysis, we distinguished the males from the females. Therefore, to obtain a number of individuals per sex sufficient for FA analysis, replicates were pooled. In order to get rid of the differences among configurations other than shape differences – position, orientation and size – all configurations were superimposed using generalized Procrustes superimposition method (Rohlf and Slice, 1990). This method combines three transformations: (1) scaling: all configurations are scaled to a unit centroid size; (2) translation: all configurations centroids are superimposed; and (3) rotation: the configurations are rotated around their centroid to achieve an optimal fit of corresponding landmarks. Analysis of shape FA was performed as recommended by Palmer and Strobeck (2003) and by Klingenberg and McIntyre (1998) using a Procrustes ANOVA (also see Klingenberg et al., 2002 and Savriama and Klingenberg, 2011 for a general discussion). Because measurement error can contribute in a large proportion to FA variation, we tested for the significance of FA relative to measurement error (interaction term in the ANOVA). Departure from ideal FA (the occurrence of either Directional Asymmetry (DA) or Antisymmetry (AS), two other kinds of asymmetry) was also tested. The occurrence of AS was examined by scatter plots of vectors of shape asymmetry: a clustering would indicate AS phenomenon. To avoid making the assumption of isotropic variation at each landmark, the occurrence of shape DA was tested using MANOVA statistics (Klingenberg and McIntyre, 1998). To test for size effects on shape asymmetry, we performed a regression between mean centroid size and vectors of shape asymmetry. To test the null hypothesis of the independence of these two variables, we performed a permutation test (10,000 permutations). As wings have matching symmetry, we also measured wing size FA using centroid size. The occurrence of AS was examined by testing the occurrence of bimodality in the distribution of $(R - L)$ values. DA was tested by comparing the side effect with the interaction effect in the ANOVA. To test

for allometric effect, we assessed the relationship between absolute asymmetry $(|R - L|)$ and trait size $((R + L) / 2)$ by non-parametric Spearman correlation test. A non-parametric test was preferred because it does not assume homogeneity of variance and it is less sensitive to outliers (Palmer and Strobeck, 2003). For shape and size FA calculation, we used FA10 index (Palmer and Strobeck, 2003). This index describes the average difference between sides after measurement error has been partitioned out. Because FA10 is a variance estimate, we used F-test to compare differences in FA between treatments.

2.10. Mean mentum and wing shape variation

Both mean mentum and wing shape variation among treatment groups were analyzed by Canonical Variate Analysis (CVA) on Procrustes coordinates. To obtain a number of individuals much larger than the number of landmarks, replicate test aquaria were pooled. The Procrustes distance between each group was calculated and the significance was assessed by a permutation test (10,000 permutations). Morphometric and statistical analyses were performed with R software (Development Core Team, 2012) and Morpho J (Klingenberg et al., 2002) software.

3. Results

3.1. Characterization of the three sediments studied

By comparison with both the control and the LOW sediment (Supplementary material 1, Table A), the HIGH sediment showed high levels of organic matter, organic carbon, organic nitrogen and total phosphorus. The ratio between organic carbon and organic nitrogen is between 8 and 14 in LOW and HIGH sediments. This result suggested that unlike the control sediment, a part of the organic matter is available for the feeding of the chironomid larvae (Péry et al., 2003) in the field-

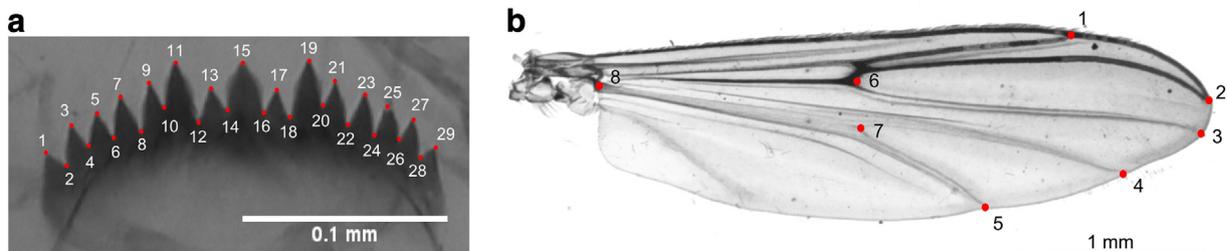


Fig. 3. The 29 landmarks digitized on the mentum (a) and the 8 landmarks digitized on the wing (b).

Table 1
Concentrations in mineral and organic compounds measured at the start of the bioassays in the four sediments studied (mg/kg dw). Mean and standard deviation between the four replicate test aquaria.

	Control 1	LOW	Control 2	HIGH	Seine river background (Meybeck et al., 2004)
Pb	1.6 ± 0.1	19.4 ± 1.0	2.4 ± 0.2	195.5 ± 4.9	20
Cu	3.1 ± 0.4	18.0 ± 2.0	2.5 ± 0.2	210.5 ± 11.0	15
Zn	<10	75.8 ± 9.3	<10	809.5 ± 16.4	60
Cr	0.7 ± 0.2	6.2 ± 2.0	0.2 ± 0.0	97.1 ± 4.0	40
Ni	0.4 ± 0.3	14.0 ± 3.0	0.2 ± 0.0	63.4 ± 7.8	16
Cd	<0.04	0.2 ± 0.0	<0.04	14.0 ± 0.6	0.22
Mn	2.7 ± 0.1	107.5 ± 20.2	3.0 ± 0.2	391.6 ± 18.3	
Ba	13.2 ± 1.2	35.7 ± 5.9	21.0 ± 1.0	1257.6 ± 21.2	
As	0.9 ± 0.1	6.0 ± 2.0	1.0 ± 0.0	7.6 ± 0.3	
Se	<0.4	<0.4	<0.2	<0.4	
Hg	<0.2	1.0 ± 0.8	<0.2	1.0 ± 0.1	
4 t-OP	<0.02	0.10 ± 0.13	<0.02	0.62 ± 0.12	
4-NP	<0.02	0.05 ± 0.01	<0.02	0.32 ± 0.26	
Aliphatic hydrocarbon	<100	<100	<100	2528.0 ± 120.7	
Σ15 PAH	0.01 ± 0.01	1.24 ± 0.46	0.01 ± 0.01	17.19 ± 3.41	

collected sediments. By comparison with the control sediment, coarse sand fraction (from 0.5 to 3.0 mm) was higher in LOW sediment while fine fraction (<0.06 mm) was higher in HIGH sediment (Supplementary material 1, Table A). By comparison with control sediment, the concentrations of organic and mineral compounds measured in this study were higher in field-collected sediments. Nevertheless, for Pb, Cu, Cd, Hg, and Zn, concentrations in the LOW sediment remained in the same order of magnitude than the Seine river background levels (Meybeck et al., 2004) (Table 1). On the contrary, they were several times higher than the Seine river background levels for the HIGH sediment. Furthermore, in the latter, the concentrations of Cu, Ni, Cd and fluoranthene were 1.02, 1.33, 1.20 and 1.05, respectively, times higher than the effect range median concentrations in *C. riparius* larvae established by Ingersoll et al. (1996).

3.2. Bioaccumulation

In the larvae from the control groups, we detected weak metal concentrations, except for Zn and Cu, which are essential metals (Fig. 4). In contrast to the control group, Ni was detected in the tissues of larvae from the LOW sediment. Metal concentrations measured in the larvae exposed to the HIGH sediment were high not only for toxic metals but also for essential metals. Indeed, by comparison with control groups, concentrations measured were 27 times higher for lead, 8 times higher

for copper and 5 times higher for zinc. AP and PAH body concentrations did not differ significantly between the three groups studied.

3.3. Body dry weight, time to emergence and adult emergence rate

Emergence rates did not differ significantly between control and exposed groups (Mann–Whitney–Wilcoxon test, LOW bioassay: $W = 6.5$ and HIGH bioassay: $W = 11.5$, $p > 0.05$) (Table 2). By comparison with the respective control group, the HIGH condition however showed a slight emergence decrease (mean emergence rate: HIGH: 75%, $n = 200$ and CONTROL: 87%, $n = 200$). The emergence time was delayed in the HIGH sediment, while it was shortened in the LOW sediment, compared to their respective control group (Mann–Whitney–Wilcoxon test, LOW bioassay: $W = 1$ and HIGH bioassay: $W = 15$, $p \leq 0.05$). The dry weight masses of the fourth instar larvae of the four groups studied differed significantly (pairwise Mann–Whitney–Wilcoxon test, all $p \leq 0.05$): the LOW group had the highest mass while the HIGH group had the lowest.

3.4. Frequency of mentum phenodeviants in parents and offspring

For deformity analysis, no difference was found among the replicates (pairwise proportion tests, all $p > 0.05$), which were thus pooled. The frequency of mentum phenodeviants varied between 3.7 and 9.6% (Fig. 5). In the parental generation, the higher deformity rate was

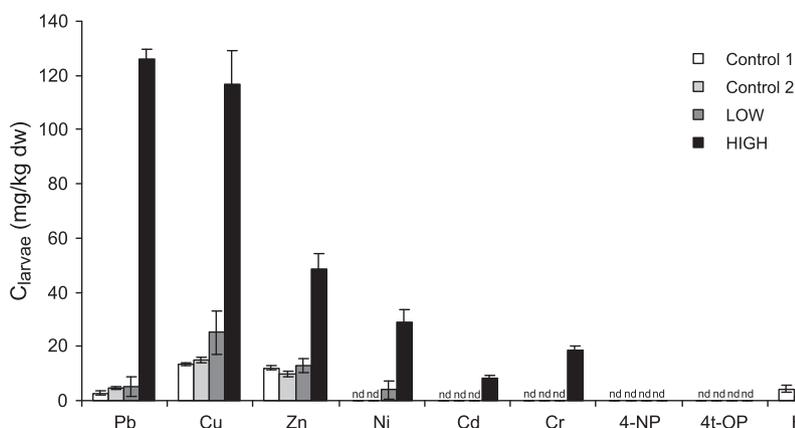


Fig. 4. Bioaccumulation of some mineral and organic compounds in control and exposed larvae. Mean and standard deviation between three samples for analysis. For further clarity, zinc concentrations were divided by 10. nd: not detected.

Table 2

Percentage of emergence, duration before emergence and dry weight of the fourth instar larvae of the four conditions studied. Duration before emergence and emergence rate: mean and standard deviation between the four replicate test aquaria. Dry weight: mean and standard deviation between three samples for analysis.

Condition	LOW bioassay		HIGH bioassay	
	Control 1	LOW	Control 2	HIGH
Emergence (%)	92 ± 4	94 ± 8	87 ± 9	75 ± 16
Duration before emergence (days)	25 ± 1	23 ± 0 *	26 ± 0	28 ± 1 *
Dry weight (mg)	1.0 ± 0.1	1.6 ± 0.2 *	0.8 ± 0.1	0.6 ± 0.1 *

* Significantly different by comparison with the respective control group (Mann-Whitney–Wilcoxon test, $p \leq 0.05$).

detected in the group exposed to the LOW sediment (proportion test, $\chi^2 = 4.0$, $df = 1$, $p \leq 0.05$), while no significant difference was observed in the group exposed to the HIGH sediment (proportion test, $\chi^2 = 0.1$, $df = 1$, $p > 0.05$). We did not observe any significant variation in mentum deformities between parents and offspring (pairwise proportion test, all $p \leq 0.05$). We noticed phenodeviations consisting of missing tooth in all groups studied (Table 3). In contrast, the Köhn gap only appeared in the groups exposed to field-collected sediments.

3.5. Fluctuating asymmetry of mentum shape in parents and offspring

As for deformities, no difference in shape FA was found among replicates (pairwise F tests, all $p > 0.05$), which were thus pooled. Shape DA was detected in all parental groups and most offspring groups (except for control offspring group derived from control 1) (Supplementary material 2, Table B. 1). Scatter plots of vectors of shape asymmetry did not suggest the occurrence of AS for any group. Permutation test performed on the regression between mean centroid size and vectors of shape asymmetry did not indicate any allometric effect on shape FA (permutation test, all $p > 0.05$). In the parental generation, we observed (Fig. 6) a significant decrease of the level of mentum shape FA in exposed groups (F test, LOW bioassay: $F_{3934, 4018} = 1.17$ and HIGH bioassay: $F_{3636, 3244} = 1.10$, $p \leq 0.05$). For the LOW bioassay, we noticed slight variations between shape FA in parental generation and shape FA in offspring. On the contrary, we measured a significant shape FA increase in the offspring derived from HIGH group and reared in control sediment (F test, $F_{4018, 2822} = 1.21$, $p \leq 0.05$).

3.6. Mean mentum shape changes in parents and offspring

The shape changes among control and treated individuals were very weak, although statistically significant. The first axis (50% of the variation) of the CVA contrasted mostly LOW group from the control 1 group (Fig. 7). The pattern of variation consisted of a reduction of teeth size in individuals exposed to the LOW sediment. The second axis (accounting for 38% of the variation) separated the HIGH bioassay from the LOW bioassay. The third axis (not shown) (12% of the

variation) contrasted mostly the HIGH group from the control 2 group. As observed in our previous study (Arambourou et al., 2012), the mean shape of the mentum between the control groups of the two experimental sessions differed significantly (permutation test on the procrustes distance, $p \leq 0.0001$). We also performed a CVA between parents and offspring separately on LOW and HIGH bioassays (Supplementary material 3, Fig. C. 1 A and B). In both analyses, the first axis (accounting for 62% of the variation for the LOW bioassay and for 48% for the HIGH bioassay) allowed to separate individuals of control sediments from exposed individuals. As observed for the parents, the mean mentum shape change consisted of a reduction of teeth size in groups reared in LOW sediment. For the HIGH bioassay, the second axis (39% of the variation) contrasted the parental generation on the positive side from the offspring generation on the negative side. For the LOW bioassay, the parental generation was separated from the offspring generation on the third axis (not shown) accounting for 9% of the total variation observed.

3.7. Fluctuating asymmetry of wing centroid size and wing shape in parents

Wing shape DA was only detected in males from control 2 (Supplementary material 2, Table B. 2). Scatter plots of vectors of shape asymmetry did not reveal AS for any group (not shown). Permutation test performed on the regression between mean centroid size and vectors of shape asymmetry did not indicate any allometric effect on FA (permutation test, all $p > 0.05$). No statistical differences were detected for the wing shape FA between any of the condition tested neither for females nor for males (test F, all $p \leq 0.05$). No departure from normality for any condition tested was detected for the difference between left and right centroid size. Significant side effect in the ANOVA (Supplementary material 2, Table B. 3) was measured only for the female group from HIGH sediment. Spearman test performed between the absolute asymmetry and the mean centroid size did not reveal any allometric effect (Spearman test, all $p > 0.05$). Individual and side \times individual interaction were significant for all tested conditions (Table 6). No statistical differences were detected for the wing centroid size FA between any of the condition tested neither for females nor for males (test F, all $p \leq 0.05$) (Fig. 8).

3.8. Mean wing shape changes in parents and offspring

Only very limited wing shape changes between control and treated groups were detected, mostly in females. Indeed, for the females, the second axis (18% of the variation) tends to separate exposed individuals from control ones. This subtle shape change is nevertheless inconsistent across treatments, as the treated group being on the positive side for the HIGH bioassay and on the negative side for the LOW bioassay. For the males the separation between control and stressed group was only detected for the LOW bioassay. As observed in our previous study (Arambourou et al., 2012), the first axis (accounting for 68% of the variation for the female and for 52% for the male) of the CVA (Supplementary material 3, Fig. C. 2) mostly contrasted the two experimental sessions performed.

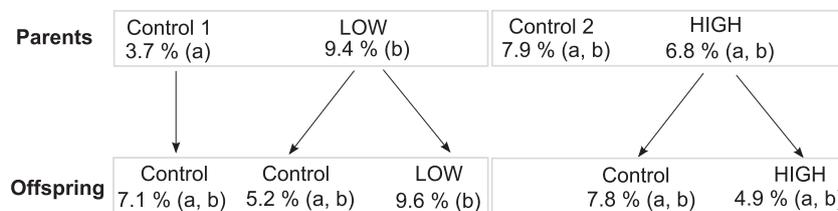


Fig. 5. Total frequency of mentum phenodeviants in parents and offspring. As replicate test aquaria behave similarly, they were pooled. Different letters in brackets indicate significantly different values (pairwise proportion test, $p \leq 0.05$).

Table 3

Proportion of each type of phenodeviation in parents and offspring. For the offspring generation: the first name indicates the parental group while the second name refers to the offspring group. As replicate test aquaria behave similarly, they were pooled.

	Sample size	Extra tooth (%)	Missing tooth (%)	Fused teeth (%)	Köhn gap (%)
<i>Parents</i>					
Control 1	187	1.0	3.6	0.0	0.0
Control 2	191	5.2	1.0	1.0	0.0
LOW	192	4.1	3.6	1.5	0.0
HIGH	205	3.4	1.5	1.0	1.0
<i>Offspring</i>					
Control 1 → control	97	1.0	4.1	2.0	0.0
LOW → control	94	0.0	2.1	3.1	0.0
HIGH → control	141	6.4	1.4	0.0	0.0
LOW → LOW	98	2.1	5.3	1.1	1.1
HIGH → HIGH	143	2.1	2.1	0.7	0.0

4. Discussion

4.1. Polluted sediments can impact body dry weight and induce growth retardation

We detected high heavy metal concentrations in the body of the larvae from the HIGH sediment (similar concentrations were reported in the literature; Di Veroli et al., 2012; Krantzberg and Stockes, 1989; Roulier et al., 2008). These high concentrations were accompanied by a reduced larval dry weight and a delayed development (longer time to emergence). Similar results have been obtained in *Chironomus* larvae exposed to organic (Watts et al., 2003) or mineral (Pascoe et al., 1989; Postma et al., 1995; Servia et al., 2006; Timmermans et al., 1992) compounds. This reduced larval dry weight in the HIGH group might be explained by an increase of the energy used for detoxification processes (Arambourou et al., 2013; Choi et al., 2001). On the contrary, the weak contamination of the LOW sediment is probably not sufficient to induce growth retardation.

4.2. No effect of a multiple-exposure on developmental stability?

A previous study performed on artificial sediment spiked either with heavy metal (Pb) or with organic compound (nonylphenol) failed to detect any strong effect on the phenotype of *C. riparius* larvae (Arambourou et al., 2012). To explain this result, we had hypothesized that a single pollutant could be inefficient in inducing phenotypic effect and only a multiple exposure would be needed to generate a strong response. Nevertheless, in the present study, we obtained FA and deformity values close to those measured in individuals exposed to artificial sediment spiked with a single toxic compound (Arambourou et al., 2012). The very limited response of the frequency of mouthpart deformities was particularly surprising considering the high concentrations of heavy metals detected in the bodies of the larvae from the HIGH sediment. Similar concentrations in heavy metals had indeed been shown to induce strong phenotypic defects in *Chironomus* larvae (Di Veroli et al., 2012;

Janssens de Bisthoven et al., 1998b; Martinez et al., 2002). Nevertheless, in some studies reporting a strong effect of toxic exposure on *Chironomus* phenotype, deformity frequencies higher than 10% were also detected in the control group (Di Veroli et al., 2012; Meregalli et al., 2001). In the literature, these high deformity rates in control group were associated with inbreeding (Vogt et al., 2013), which could result in an increased sensitivity to toxic exposure (Nowak et al., 2007). It is well known that inbreeding can affect the developmental stability (see Alibert and Auffray, 2003 for a review). In the present study, as the majority of laboratory strains (Nowak et al., 2008), our laboratory culture is probably not free from inbreeding. Nevertheless, phenodeviation rates remained lower than 8% in control groups suggesting a limited inbreeding depression. We measured a slight but significant increase of the frequency of deformities in the group exposed to the LOW sediment that could suggest a toxic effect. As observed by Janssens de Bisthoven et al. (1998b) and Groenendijk et al. (1998), we noted that the phenodeviation called Köhn gap only appeared in exposed groups. So unlike the other type of phenodeviation, this deformity might be preferentially induced by a toxic exposure. We observed a difference in the phenodeviation rates – although not significant – between the two control groups. This difference might be explained by the stochasticity of random genetic drift in inbred laboratory culture of *C. riparius* (Nowak et al., 2007). In our experiment, test organisms could be particularly prone to genetic drift because they derived from only few egg-masses. This result underlined the need, in ecotoxicological studies, for comparing stressed animals with control animals deriving from the same egg-masses.

In addition, we did not observe any significant mentum FA increase in exposed groups. On the contrary, in comparison with their respective control group, we observed a significant decrease of mentum FA in both exposed groups. The level of FA was particularly low in organisms exposed to the HIGH sediment. As the percentage of emergence was slightly – although not significantly – reduced in this group (emergence decrease of 14%), one possible explanation is that selection might have removed the developmentally less stable individuals and thus biasing the estimation of FA. Polak et al. (2002) observed a similar effect in *Drosophila melanogaster* exposed to lethal concentration (inducing an emergence decrease of 38%) of arsenic. They showed that sternopleural bristle asymmetry decreased as mortality increased in relation to arsenic dose. Recently, Polak and Tomkins (2013) provided a direct demonstration of the developmental selection hypothesis by measuring the level of phenodeviations in dead males of *Drosophila bipunctinata* submitted to temperature stress. They indeed showed that dead males were more developmentally unstable than their surviving counterparts. Such analysis is unfortunately impossible in *C. riparius* because larvae died during the most sensitive first instars (see Williams et al., 1986) making the comparison with the last instar surviving fraction impossible.

4.3. The lack of effect is not limited to larval trait

It was envisaged that phenotypic defects should mostly be detected on adult life stage, the metamorphosis adding an energetic cost that would combine to the impact of toxic exposure (Campero et al.,

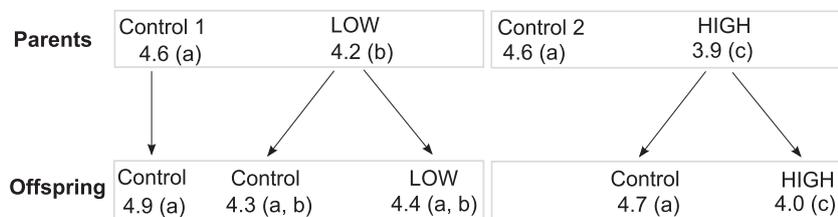


Fig. 6. Mentum shape FA levels in parents and offspring. As replicate test aquaria behave similarly, they were pooled. Different letters in brackets indicate significantly different values (pairwise F test, $p \leq 0.05$).

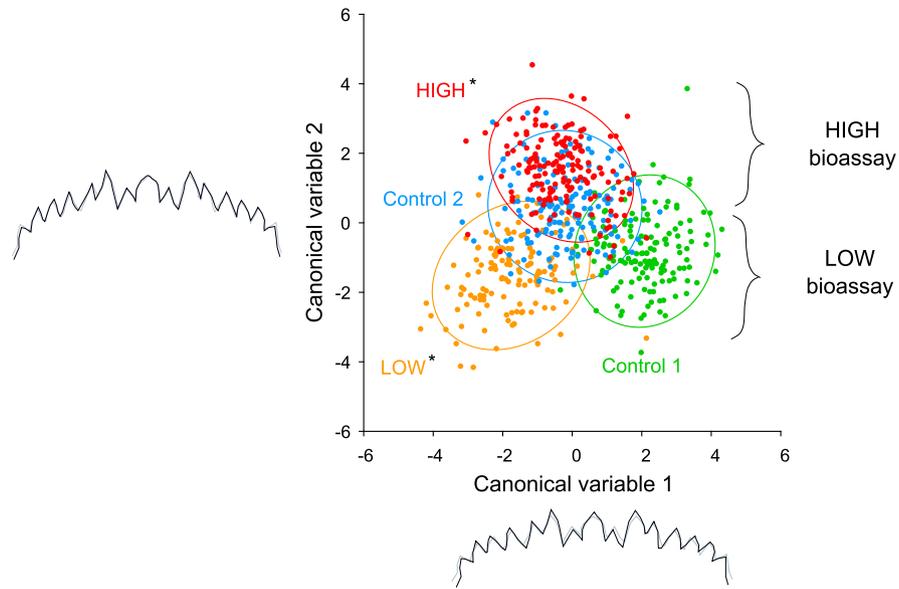


Fig. 7. CVA on parents shape data for the LOW and the HIGH bioassays and associated patterns of mentum shape variation on the axis 1 and the axis 2. Replicate test aquaria were pooled. Mentum shape in gray: extreme individual in negative side, mentum shape in black: extreme individual in positive side. *: Procrustes distance significant between stressed group and the respective control group (permutation test, $p \leq 0.05$).

2008). The alleged additional cost of the metamorphosis does not appear to affect developmental stability. The analysis of adult wing phenotype did not suggest any wing FA differences between control and exposed groups. Note that a similar result was already reported by Hoffmann et al. (2005) in *Chironomus tepperi* exposed to a pesticide. Moreover, the mean shape changes detected between control and treated groups were very slight. Therefore, wings of *C. riparius* do not appear as a particularly sensitive biomarker of a toxic exposure. It is nevertheless conceivable that due to its functional importance for flight, the wing could be more stable than the other traits (Clarke, 1998; Debat et al., 2000).

4.4. A very limited trans-generational effect

Despite the presence of some endocrine disruptors in the sediment (HAP and AP), especially in the HIGH sediment, we did not observe any trans-generational effect in the offspring. Nevertheless, we observed a slight decrease – although not statistically significant – of deformity rate in the offspring group derived from the LOW parental group and reared in control sediment. This observation was also made by Groenendijk et al. (1998) in the offspring of *C. riparius* reared in clean sediment and coming from heavy metal contaminated station. Similarly, Martinez et al. (2004) observed in the laboratory a decrease

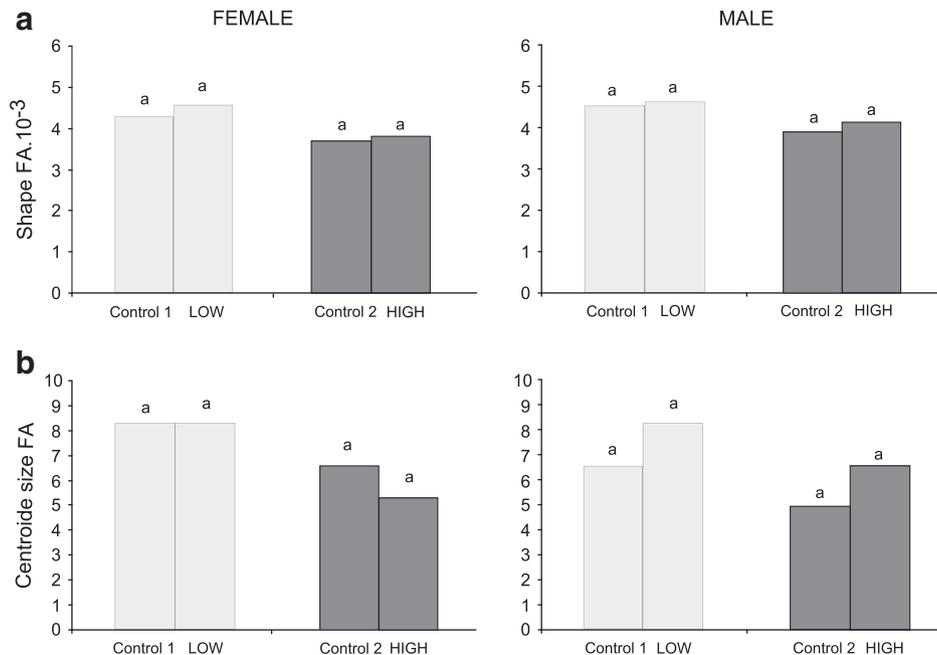


Fig. 8. Wing FA in females and males. Replicate test aquaria were pooled. Shape FA (a) and centroid size FA (b). Different letters above the bar indicate significant different values (F test, $p \leq 0.05$).

of the frequency of mentum abnormalities in the offspring of *Chironomus tentans* placed in control sediment and derived from parents exposed to zinc. As we cannot measure morphological abnormalities with non-destructive method, our experimental design did not allow to assess the inheritance of deformities. Moreover, we only collected few egg-masses from the parental generation, and thus the probability to collect offspring from deformed parents is quite low. Consequently, as outlined by Janssens de Bisthoven et al. (2001), if a genetic component is involved in deformity induction, it is probably masked by experimentally induced genetical drift. Considering these, it seems premature to conclude about the origin of the phenodeviations observed in the offspring derived from the LOW parental group.

We detected weak but significant mean mentum shape changes between parents and offspring and between control and stressed groups. The mean mentum shape observed was mostly explained by the sediment in which larvae grow. Nevertheless, at this stage, we cannot conclude about the main sediment parameters that could influence the shape observed. For the LOW sediment, the change consisted of a decrease of the size of the teeth. Considering the grain size distribution and the sandy nature of this sediment, the mentum shape change was probably mainly due to a physical abrasion. The effect of the substrate on the mentum wear was also reported by Bird (1997). Moreover, we observed a significant increase of FA in the offspring group derived from the HIGH parental group and reared in control sediment. This result could be considered in support of the developmental selection hypothesis: The increased asymmetry detected in the offspring might reflect the level of developmental instability in the absence of selection pressure. This result is of importance because this means that, in case of lethal stress, FA and phenodeviation could be under-estimated.

5. Conclusions

In conclusion, as observed in a previous study (Arambourou et al., 2012), we detected only limited phenotypic effects in *C. riparius* exposed to contaminated sediments. To explain the weak phenotypic response observed in the present study, four hypotheses could be proposed. First, FA and deformities might not be the relevant indicators of toxic exposure that they are often claimed. This idea has been already proposed to explain the contradictory results observed in the literature (e.g. Leung et al., 2003). Second, developmental selection could result in the under-estimation of developmental stability of the stressed individuals. This hypothesis however remained very difficult to test in the *Chironomus* larvae. Third, the studied traits – the mentum in larvae and the wings in adults – could be quite insensitive to toxic exposure, as a result of a putative stabilizing selection due to their functional importance. In particular, it is conceivable that their symmetry itself would be under particular selection, limiting its variability (as suggested by Debat et al., 2000). Nevertheless, for the mentum, this hypothesis contrasts with several studies reporting increasing mentum phenotypic defects in larvae exposed to contaminated sediment (Al-Shami et al., 2011; Di Veroli et al., 2012). Fourth, the species used – *C. riparius* – could be quite insensitive to a toxic exposure. Indeed, *Chironomus* spp. are well-known to be adapted to highly polluted areas (Friberg et al., 2010; Gower and Buckland, 1978; Krantzberg and Stockes, 1989). This however is in contradiction with recently published results which show that field-collected sediments with similar heavy metal concentrations induced a significant increase of mouthpart deformities in *C. riparius* larvae (Di Veroli et al., 2012). As stated above, the use of laboratory strain exhibiting high inbreeding rate – resulting in high rates of mentum deformities in control groups (Vogt et al., 2013) – could partly explain the high sensitivity of phenotypic markers in some laboratory studies. Overall, our results highlight the fact that the studied phenotypic markers are not sensitive enough in *C. riparius* to be used as relevant indicators of toxic exposure. Further investigations on other species – known to be more sensitive to toxic exposure than *C. riparius* – are

now necessary to test if the choice of the model organism is responsible for the limited response that we observed in this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scitotenv.2014.03.010>.

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