Transcriptional Responses, Metabolic Activity and Mouthpart Deformities in Natural Populations of *Chironomus riparius* Larvae Exposed to Environmental Pollutants

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ABSTRACT: Biomarkers are an important tool in laboratory assays that link exposure or effect of specific toxicants to key molecular and cellular events, but they have not been widely used in invertebrate populations exposed to complex mixtures of environmental contaminants in their natural habitats. The present study focused on a battery of biomarkers and their comparative analysis in natural populations of the benthic larvae of *Chironomus riparius (*Diptera*)*, sampled in three differentially polluted rivers (the Con, Sar, and Louro in Galicia, Spain). In our study, some parameters were identified, such as *hsp70* gene activity, GST enzymatic activity, total glycogen content and mouthpart deformities, which showed significant differences among populations from the three rivers that differed in the levels and types of sedimentary contaminants analyzed (metals, organic-chlorine pesticides, alkylphenols, pharmaceutical, and personal care products). In contrast to these sensitive biomarkers, other parameters showed no significant differences (*hsc70* gene, *EcR* gene, *P450* gene, RNA:DNA ratio, total protein content), and were stable even when comparing field and nonexposed laboratory populations. The *hsp70* gene seems to be particularly sensitive to conditions of pollutant exposure, while its constitutive counterpart *hsc70* showed invariable expression, suggesting that the *hsc70*/*hsp70* ratio may be a potential indicator of polluted environments. Although further studies are required to understand the correlation between molecular responses and the ecological effects of pollutants on natural populations, the results provide new data

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about the biological responses to multiple-stressor environments. This field study adds new molecular endpoints, including gene expression, as suitable tools that, complementing other ecotoxicological parameters, may help to improve the methodologies of freshwater monitoring under the increasing burden of xenobiotics. Environ Toxicol 30: 383–395, 2015.
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INTRODUCTION

The potentially adverse effects on wildlife of the increasing number of anthropogenic chemicals released into the environment have become a serious issue. In addition to concerns about exposure to individual substances, there is growing awareness about the importance of mixtures of several chemicals, as actually found in polluted freshwaters. One of the challenges facing environmental toxicologists is to understand the biological responses of organisms living in natural aquatic environments exposed to a large variety of chemical contaminants and to improve early warning biological water quality monitoring tools, capable of detecting sublethal effects on biological communities exposed to toxicants. Traditional approaches have mainly focused on the analysis of survival, growth, development and reproductive success in natural populations from polluted habitats (Faria et al., 2008). Such life-cycle endpoints are also widely used in laboratory investigations with model organisms, for examining the effects of particular toxicants (León et al., 2008). Interesting data are obtained from these studies, but they fail to provide information on the more subtle effects of chemicals on organisms. Hazardous substances can have detrimental effects on aquatic biota at the molecular and cellular levels, later shown at both organismal and population levels. Novel approaches based on suborganismal parameters are providing valuable information about the molecular underpinnings of certain phenomena, such as acute response to toxicants and adaptation to chronic exposure to polluted environments. Moreover, molecular endpoints reveal specific cellular targets of toxicants, highlighting the underlying mechanisms of their mode of action. Biochemical biomarkers, such as enzyme activities (e.g., cholinesterase, glutathione *S*-transferase, among others), have been shown to be useful toxicity tools for the assessment of environmental toxicants (Jemec et al., 2010; Damasio et al., 2010). Recently, the identification and validation of marker genes is gaining acceptance in aquatic toxicology, because these genes can provide a rapid, sensitive, and high throughput assay for testing compounds (Steinberg et al., 2008). Advances in ecotoxicogenomics are being made especially in fish species (Costa et al., 2011), but due to the lack of genomic information there are still few studies in aquatic invertebrates, even though they are predominant in aquatic communities. Moreover, there have been even fewer studies in natural populations collected in the field. This adds to the interest of the present work, as its main focus was to test ecotoxicity biomarkers in natural environments, so as to evaluate if such biomarkers could be used as monitoring tools for assessing the health status of organisms exposed to complex mixtures of pollutants. The biomarkers tested included different gene markers previously used in controlled scenarios, such as laboratory assays.

Aquatic larvae of the midge *Chironomus riparius* (Meigen) have been selected as test organisms to investigate the effects of aquatic sediment-associated contaminants (EPA, 1996; OECD, 2001). Sediments are the sink for contaminants and can serve as a reservoir of toxic compounds that continually threaten the health and viability of aquatic biota. Communities of benthic organisms play a key role in energy, nutrient and contaminant fluxes to higher trophic levels. Therefore, the consequences of exposure to toxic substances on benthic organisms should be studied more closely under realistic scenarios. Bioassays based on *Chironomus* life-cycle parameters, using survival, growth, and development as endpoints, have been employed to assess water quality and contamination in rivers (Faria et al., 2006). Certain cytogenetic effects induced by environmental factors have also been analyzed (Michailova et al., 2006; Planelló et al., 2007). However, to date, analysis of molecular biomarkers in field populations has been limited to a few enzymatic activities (Olsen et al., 2001) and to semiquantitative analysis of membrane multixenobiotic transporters (Moreau et al., 2008; Saez et al., 2008). Recent research in gene expression profiles in this species is adding new endpoints that are now being used in laboratory studies to evaluate the short time effects of specific toxicants (Lee et al., 2006; Park and Kwak, 2008, 2012; Planelló et al., 2008; Morales et al., 2011; Nair et al., 2011; Martínez-Guitarte et al., 2012; Martínez-Paz et al., 2012; Park et al., 2012). Nevertheless, none of the marker genes identified have yet been tested in wildlife populations of *Chironomus*. Standard ecotoxicological tests often cannot handle the complexity of life in nature, and a mismatch between laboratory and field conditions can lead to errors in ecological risk assessment (Laskowsky et al., 2010).

In the present work, a comparative analysis a battery of biomarkers was carried out in natural populations sampled in three different polluted rivers in Galicia (Spain). Physical and chemical characteristics of the sediments were measured at each sampling site. Different biomarkers were selected for this study: the *hsp70* and *hsc70* genes related to the stress response in cells; the ecdysone receptor gene (*EcR*) related to hormonal signaling; the cytochrome *P450* gene (*CYP4G*)

and the glutathione *S*-transferase enzymatic activity related to detoxification pathways; the RNA:DNA ratio, total protein and glycogen content related to growth and metabolic rates; and, finally, the mouthpart deformities related to sublethal effects during larval development. The data obtained in field populations from polluted environments may allow the characterization of sensitive and stable biomarkers, which will assist in improving the definition of the complex scenario that involves adaptation to chronic exposure to xenobiotics of native benthic invertebrate communities.

MATERIALS AND METHODS

Test Animals and Culture Conditions

The experimental animals were the aquatic larvae from the midge *Chironomus riparius*. Field populations were collected in the rivers Con, Sar, and Louro, located in Galicia (Spain) as described below. Fourth instar larvae were collected in October 2010 from one selected area around 50–100 m^2 in each river, with at least four sampling sites in the prospective area. Sampling was performed choosing the most suitable microhabitats for these organisms, that is, places with a very low current velocity, fine sediment and accumulated organic matter. Harvesting was carried out with a small hand net of 15 cm diameter, 20 cm deep, and a mesh size of 250 μm. Larvae were weighed and stored frozen at −80°C until used. Because of the difficulty in finding *C. riparius* in non contaminated areas in the upper course of the rivers, laboratory cultures were used as reference for populations never exposed to toxicants. Larvae used as unexposed controls were obtained from a permanent laboratory culture reared under standard laboratory conditions for several years according to toxicity testing guidelines (EPA, 1996; OECD, 2001). Laboratory larvae were grown in culture medium (0.5 mM $CaCl_2$, 1 mM NaCl, 1 mM $MgSO_4$, 0.1 mM $NaHCO_3$, 0.025 mM KH_2PO_4, 0.01 mM $FeCl_3$) supplemented with nettle leaves, commercial fish food, and cellulose tissue, maintained at 20°C under constant aeration and standard light-dark periods 16:8.

Study Sites

The rivers Con, Sar, and Louro are located in Galicia (northwest Spain). The river Con is a small coastal river (about 10 km in length) that receives the domestic and agricultural effluents of a small rural area, notably pesticides used in potato and corn crops and in vineyards. The sampling site (UTM: 29T 520043E 475571N) was about 1 km from the river mouth. The river Sar receives the effluents of the wastewater treatment plant of Santiago de Compostela (a city with a population of around 100,000 inhabitants), and is considered to be one of the most polluted rivers in Galicia (Prego et al., 2008). The sampling site (UTM: 29T 528728E 4744922N) is located about 4 km downstream of the plant. The river Louro is the most polluted tributary of the river Miño, as it receives untreated or insufficiently treated industrial and municipal wastewaters from the industrial area of Porriño and the city of Tui in Spain. The sampling area (UTM: 29T 530009E 4657720N) presents high levels of organic matter and pesticides (UN Economic and Social Council, 2007).

For each river and condition at least four sampling sites were established within the prospective area and many larvae were collected. Because HSP70 is known to respond to all sorts of stressors, to avoid the stress of larvae until being processed in the laboratory (at the *Encoro do Con Hydrobiological Field Station*, Pontevedra) they were stored into ten-liter containers with water and sediment of the sampling point and transported into polystyrene cubes at a constant low temperature (15°C). Routes did not last more than 30 min in any case. After confirmation of the developmental stage, larvae were frozen at −80°C in dry ice. Sediment samples were taken just after larvae sampling from each of the selected sites within the prospected area from the different rivers. Sediment samples from each river were transported as mentioned above and mixed in the laboratory for further chemical analysis.

Sediment analyses were performed by the Analytical Department of the University of Coruña. The analysis of pesticides was carried out by liquid–liquid extraction, using liquid–solid chromatography for purification of extracts and GC-MS (Thermo Finnigan Polaris Q) for detection. The analytical procedure was validated using the certified reference material NIST 1944 ($n = 6$) and sediments spiked with known concentration of target analytes (10 and 50 μg kg^{-1}, $n = 6$). The quantitation limit was fixed at 0.10 μg kg^{-1} for each analyte. Recoveries were within 88–103% with relative standard deviations (RSDs) of 12–16%. In each run a blank and a quality control of spiked sediment at 50 μg kg^{-1} for each analyte were determined. Blank samples were below the method quantitation limit for all compounds, and recoveries were within the quality control acceptance criteria (80–120%). Environmental concentrations of Pharmaceuticals and Personal Care Products (PPCPs) in the sediments were analyzed by HPLC/MS (Applied Biosystem API 3200 and Thermo LTQ Orbitrap) after ultrasonic solvent extraction. The method was validated by fortifying sediments at 10, 20, and 100 μg kg^{-1} for each analyte ($n = 6$). The quantitation limit was fixed at 0.10 μg kg^{-1} for each analyte. Recoveries were within 82–110% with RSDs of 18–21%. In each run a blank was measured, as well as spiked sediment at 50 μg kg^{-1} for each analyte. Blank samples were below the method quantitation limit for all compounds, and recoveries were within the quality control acceptance criteria (80–120%). Temperature, pH, conductivity and TDS were measured in the field with a multisensor (Crison MM-40) directly at the sampling sites, and the data are shown in Table I. Dissolved oxygen (DO) was measured with an oximeter (YSI 550A). The content of organic matter in sediment was

TABLE I. Physicochemical parameters measured at the sampling sites in the Con, Sar, and Louro rivers and in laboratory cultures

Sites	*T* (°C)	pH	Dissolved Oxygen (mg L^{-1})	Oxygen Saturation (%)	Conductivity[a] (μS cm^{-1})	TDS[b] (mg L^{-1})	Organic Matter (%)
Lab	20	7.7	7.75	84.7	530	339	–
Con	15.8	6.5	9.33	94.3	136	87.3	1.43
Sar	16.4	6.8	6.16	84.1	228[b]	145.8	2.3
Louro	16.2	6.3	7.42	75.4	121.5	77.8	1.85

[a]Conductivity in noncalcareous unpolluted Galician rivers ranges from about 40–100 μS cm^{-1} (Membiela et al., 1991).
[b]TDS: Total dissolved solids.

calculated by the loss of weight of samples after calcination for 4 h at 450°C.

RNA Extraction

RNA was extracted from a total of 20 frozen larvae for each population, divided into groups of five, using a guanidine isothiocyanate based method, performed with a commercial kit (TRIzol, Invitrogen). Briefly, frozen material was homogenated in one volume of TRIzol and left for 5 min at room temperature. Then, 0.2 volumes of chloroform were added to each sample, mixed and left for 5 min at room temperature. Subsequently, the samples were centrifuged for 15 min at 4°C and 15000*g*. Following transfer of the aqueous phase, the RNA was finally recovered by isopropyl alcohol precipitation (0.5v/v), washed with 70% ethanol, and resuspended in DEPC water. The RNA was then treated with RNase-free DNase (Roche). The quality and quantity of total RNA were determined by agarose electrophoresis and absorbance spectrophotometry (Nanodrop1000, Thermo), and the purified RNA was finally stored at −80°C.

DNA Extraction

After complete removal of the aqueous phase, as described in the RNA isolation protocol, the DNA in the interphase and phenol phase from the initial homogenate was isolated according to the manufacturer's protocol. Following precipitation in 100% ethanol, the DNA pellet was washed twice in a solution containing 0.1 M sodium citrate in 10% ethanol, once with 70% ethanol and, finally, resuspended in water. The DNA was then treated with DNase-free RNase (Roche). The use of TRIZOL® Reagent (Invitrogen) allowed the extraction of RNA and DNA content from the same samples. Simultaneous extraction of genomic DNA and RNA was used for calculating the RNA:DNA ratio.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR was used to evaluate the mRNA expression profiles of the different selected genes in larvae sampled in laboratory and wildlife populations from the three selected rivers. Reverse transcription was performed with 1 μg of the isolated RNA. An oligo dT primer (Invitrogen) was used with the M-MLV enzyme (Invitrogen) following the manufacturer's instructions. The cDNAs obtained were stored at −20°C until being used as templates for PCR reactions. Amplification reactions were carried out using gene-specific primers, with *GAPDH* and *26s* as reference genes (Martínez-Paz et al., 2012). The *EcR*, *hsp70*, *hsc70*, and *CYP4G* gene primers are described in Planelló et al. (2008); Morales et al. (2011) and Martínez-Paz et al. (2012). Sequences and fragment size of each gene-specific pair of primers are shown in Table II. *Chironomus riparius* gene

TABLE II. Primers used for semiquantitative RT-PCR of genes studied from *C. riparius*

Primer Name	Primer Sequence	Fragment Size
EcR forward	5' - AGACGGTTATGAACAGCC - 3'	240 bp
EcR reverse	5' - CGAGCCATGCGCAACATC - 3'	
CYP4G forward	5' - GACATTGATGAGAATGATGTTGGTG- 3'	340 bp
CYP4G reverse	5' -TAAGTGGAACTGGTGGGTACAT - 3'	
Hsp70 forward	5' - CATGTGAACGAGCCAAGAGA - 3'	274 bp
Hsp70 reverse	5' - TTGCCACAGAAGAAATCTTG - 3'	
Hsc70 forward	5' - CGTGCTATGACTAAGGACAA - 3'	239 bp
Hsc70 reverse	5'- GCTTCATTGACCATACGTTC - 3'	
GAPDH forward	5' - TCATCAAAGCCGTTGTCT - 3'	243 bp
GAPDH reverse	5' - AATCGAATTGCAAACACC - 3'	
26S forward	5' - TTCGCGACCTCAACTCATGT - 3'	220 bp
26S reverse	5' - CCGCATTCAAGCTGGACTTA - 3'	

fragments were cloned and sequenced to ensure that they matched the selected genes. PCR was performed in 20 μL with 2 mM of $MgCl_2$, 0.2 mM dNTPs (Biotools, Spain), 0.4 μM of each primer and 0.2 μL of Taq polymerase (Biotools, Spain) under the following conditions: denaturation for 30 s, annealing for 30 s and elongation for 40 s. A MiniOpticon Thermocycler (Bio-Rad) was used. After several trials to ensure log-phase amplification, 24 and 30 cycles were carried out depending on the gene, with an annealing temperature of 55°C to amplify the fragments corresponding to *EcR*, *hsp70*, *hsc70*, *26s*, *GAPDH*, and *CYP4G*. The amplified PCR products were run through a 9% acrylamide gel at 60 V for 3 h in 1x TGE buffer (40 mM Tris-Cl (pH 8.5), 200 mM glycine, and 2.5 mM EDTA), visualized after ethidium bromide staining and quantified with Chemigenius3 (Syngene), using GeneSnap 6.05 and GeneTools 3.06 software. Values of density across the whole bands were normalized against the median values of *GAPDH* and *26s* standards, and the relative expression levels were then calculated. A total of 20 larvae for each population were used for gene expression analysis, divided into four groups (five larvae each) to minimize intrinsic differences to the larvae being sampled. To avoid variations caused by experimental procedures each group was analyzed four times (four replicates).

Protein Extraction

Frozen larvae were homogenized in 0.5 mL of Tris-EDTA buffer (40 mM Tris, 1 mM EDTA, pH 7.8) with 7x complete EDTA-Free protease inhibitor (Roche), with a pellet mixer (VWR). Crude homogenate was then centrifuged for 15 min at 500 rpm at 4°C. Subsequently, the supernatant was centrifuged for 30 min at 10,000 rpm at 4°C. Total protein was quantified with BCA Protein Assay Reagent (Thermo Scientific) and 25 μg of total protein was used for the enzymatic assay.

Glutathione S-Transferase (GST) Activity

To evaluate GST activity, protein extraction from 20 larvae was performed as described above. Total protein was quantified with BCA Protein Assay Reagent (Thermo Scientific) and 25 μg of total protein were used for the enzymatic assay. The GST activity was assessed spectrophotometrically with the kit GST (Sigma) in a Jasco V-530 spectrophotometer. This kit uses 1-chloro-2,4-dinitrobenzene (CDNB), which is suitable for the broadest range of GST isozymes. Conjugation of the thiol group of the glutathione to the CDNB substrate produces an increase of absorbance at 340 nm. The change in absorbance at 340 nm is used to calculate GST activity.

Estimation of Glycogen

Glycogen was extracted by the boiling alkali method determined by the anthrone method as described in Servia et al. (2006). Glycogen was determined spectrophotometrically at 620 nm (UV–Visible spectrophotometer, UVI-1002E model, Thermo Electron Corporation, England) using type VIII glycogen from *Crepidula fornicata* (Sigma) as the standard.

Evaluation of Mouthpart Deformities

To remove excess soft tissue, the head capsules of each sample (Con: 182; Sar: 174; Louro: 152; Laboratory: 152) was removed from the bodies and cleaned for 2 h in 10% KOH at 50°C. These capsules were subsequently mounted on microscope slides using glycerin gel mounting medium (gelatine powder 10 g, glycerol 54 mL, distilled water 60 mL, crystal phenol 0.5 g). Mentum deformities were identified by the criteria used by Servia et al. (2000). A split or bifid central tooth of the mentum was not recorded as a deformity, because this characteristic has generated considerable controversy among researchers regarding its origin, which may not be related to pollutants (Servia et al., 2002 and references therein).

Statistical Analysis

Normality and homoscedasticity of data were tested using the Shapiro-Wilk and Levene tests, respectively. The normalized levels of the specific gene transcripts, GST enzyme activity, the RNA:DNA ratio, and protein and glycogen content were analyzed with ANOVA, followed by Games Howell's or Tukey's post Hoc tests, using SPSS 19 (IBM), when appropriate. Additionally, ANOVA residuals were also tested for normality and homocedasticity. If data were not homogeneous or normally distributed the Kruskal–Wallis test was used, and the differences between pairs were analyzed with Mann–Whitney U tests. Probabilities were corrected using the Bonferroni correction. The frequency of deformed individuals was compared by a Chi-square test. The differences were considered significant at $P < 0.05$.

To study the eventual relationship among contaminants and biomarkers, a multidimensional scaling (MDS) analysis was used. MDS allows displaying the structure of distance-like data as a geometrical picture (Kotz et al., 1985). Each object (variable) is represented by a point in a multidimensional (two-dimensional in our case) space. The points are arranged in this space, so that the distances between pairs of points have the strongest possible relation to the similarities among the pairs of objects. That is, two similar objects are represented by two points that are close together, and two dissimilar objects are represented by two points that are far apart. The Euclidean distance has been used as a measure of dissimilarity between variables. This procedure is free of the distributional problems of the variables. To avoid problems due to the scale of measurement of the different variables, the standardized values were used.

TABLE III. Organochlorine pesticides, alkylphenols, pharmaceutical products, fragrances, and metals measured in sediments collected at the three investigated rivers

Organochlorine Pesticides (ng g^{-1})	Con	Sar	Louro	Pharmaceutical Products (ng g^{-1})	Con	Sar	Louro
a-HCH	<0.10	0.13	1.26	Ibuprofen	3.73	25.62	14.41
b-HCH	<0.10	<0.10	0.32	Diclofenac	1.06	0.85	0.86
g-HCH	<0.10	0.12	<0.10	Carbamazepine	<0.10	<0.10	0.23
d-HCH	0.36	<0.10	1.85	Atenolol	9.84	18.07	8.36
Heptachlor	0.1	0.26	0.11	Caffeine	0.62	0.94	0.65
Aldrin	0.16	0.2	<0.10	Enrofloxacin	0.17	<0.10	<0.10
Heptachlor epoxide	<0.10	<0.10	<0.10	**Fragances (ng g^{-1})**	**Con**	**Sar**	**Louro**
g-Chlordane	0.17	0.1	0.37	Galaxolide	5.53	7.90	3.02
a-Chlordane	0.12	< 0.10	0.37	Tonalide	6.28	7.57	6.33
a-Endosulfan	0.83	0.72	< 0.10	**Metals (μg g^{-1})**	**Con**	**Sar**	**Louro**
p.p'-DDE	0.2	0.3	0.76	Cd	<0.10	<0.10	<0.10
Dieldrin	<0.10	<0.10	<0.10	Pb	8.28	11.52	5.96
Endrin	<0.10	0.64	1.18	Cr	4.15	19.83	5.02
b-Endosulfan	6.27	19.28	18.25	Ni	3.62	10.50	2.99
p.p'-DDD	1.1	0.27	1.7	Cu	5.81	20.37	3.13
Endrin aldehyde	2.28	3.29	<0.10	As	4.24	2.35	3.01
Endosulfan sulfate	4.28	0.62	4.13	Hg	<0.05	0.05	<0.05
p.p'-DDT	4.01	1.93	3.21	Mn	210.00	96.31	128.46
Endrin ketone	4.19	0.92	6.41	Zn	56.14	84.86	31.47
Methoxychlor	2.21	1.99	1.16				
Alkylphenols (ng g^{-1})	**Con**	**Sar**	**Louro**	**Metals (mg g^{-1})**	**Con**	**Sar**	**Louro**
Bisphenol A	0.77	18.84	7.44	Al	9.90	8.34	6.47
Nonilphenol	0.58	0.77	0.31	Fe	17.14	9.19	8.03

RESULTS

Chemical Analysis of Sediments

To quantify the levels of known toxicants, targeted chemical analysis of the sediments in each sampling site was performed from samples of sediments collected simultaneously with the larvae in the rivers Con, Sar, and Louro. As shown in Table III, all the eleven investigated metals were found in the three rivers sampled and, although the river Sar showed high levels of Cr, Ni, and Cu, the total metal content was higher in the sediments of the river Con, mainly due to the presence of high levels of Mn, As and Fe. All 20 organochlorine pesticides (OCPs) investigated were present in the three rivers, in particular beta-endosulfan, endosulfan sulfate, endrin ketone, 4-4'-DDD, methoxychlor and 4,4'-DDT (Table III). The river Louro presented the highest total content of OCPs. Alkylphenols and pharmaceutical and personal care products (PPCPs) were the other toxic compounds analyzed, and the results are shown in Table III. For both alkylphenols and PPCPs, the highest levels for total content were reached in the river Sar. This may well be due to the presence of the hospital and the wastewater treatment plant of Santiago de Compostela, which are located upstream of our sampling site. Figure 1 summarizes the differences found in the total content of metals and organic compounds in the sediments of the three rivers analyzed.

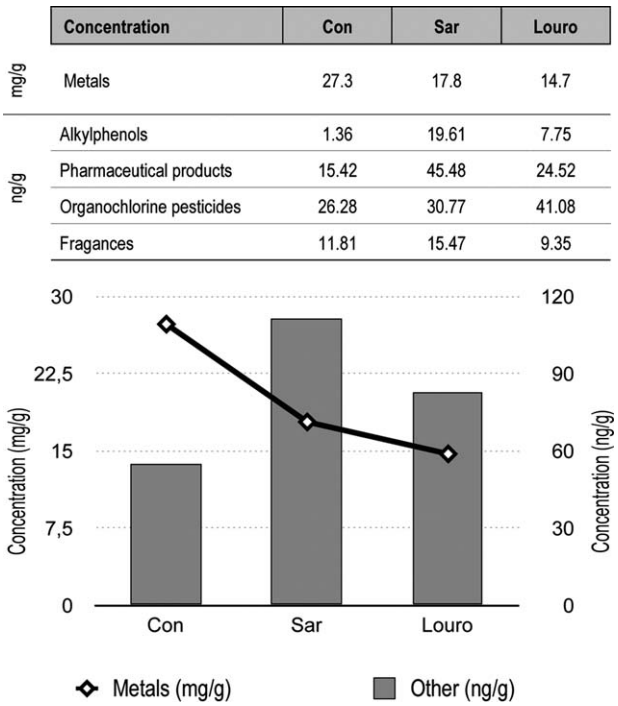

Fig. 1. Total content of metals and organic compounds (organochlorine pesticides, alkylphenols, pharmaceutical and personal care products) in sediment samples from the rivers Con, Sar, and Louro.

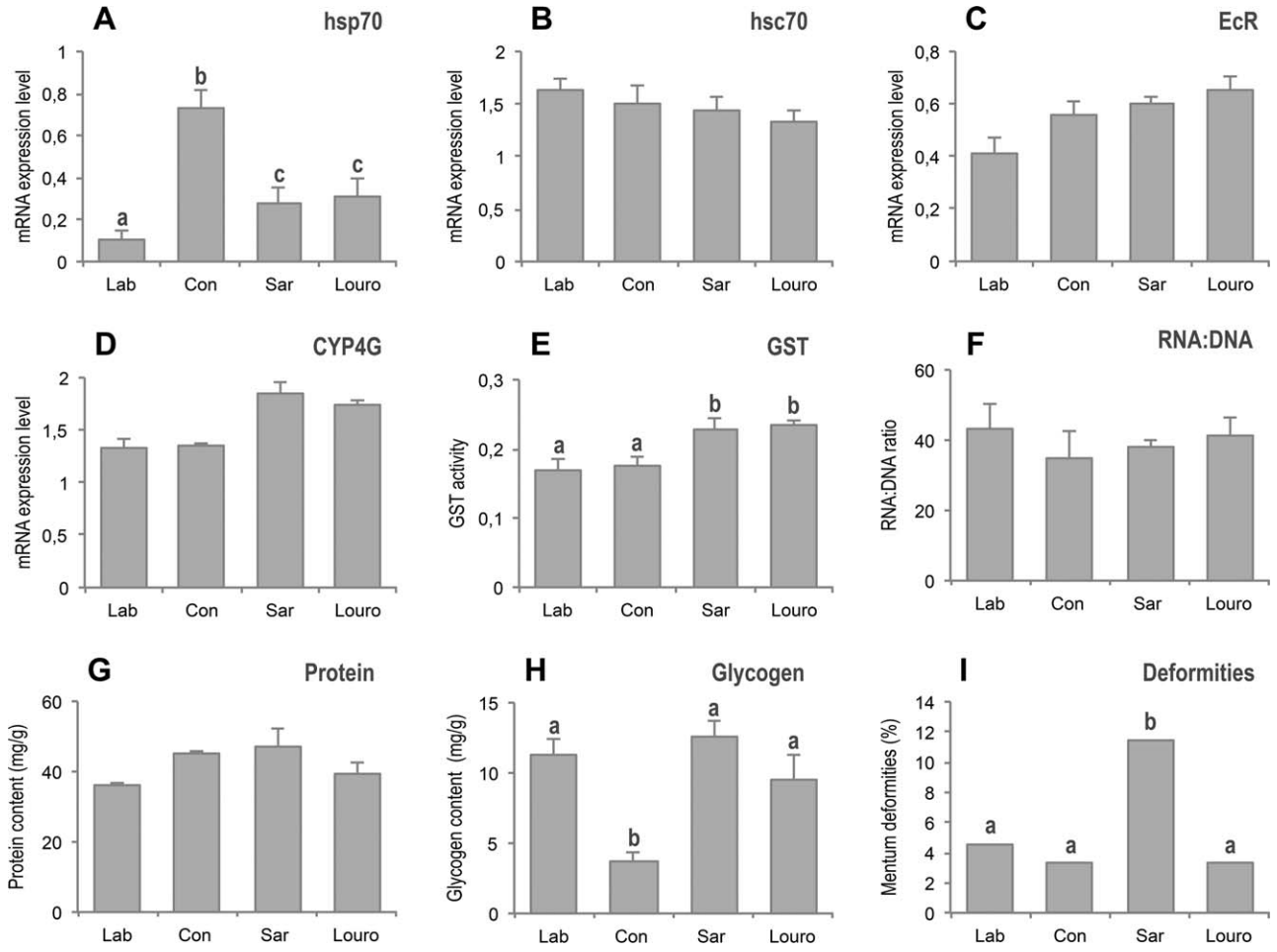

Fig. 2. Expression levels of the *hsp70* gene (A), *hsc70* gene (B), *EcR* gene (C), and *CYP4G* gene (D) in *C. riparius* larvae from laboratory cultures (Lab) and wildlife populations sampled in the rivers Con, Sar, and Louro. The mRNA densitrometic values were calculated relative to *GAPDH* and *26s* as reference genes. Analysis of glutathione *S*-transferase activity (E), RNA: DNA ratio total (F), protein content (G) and total glycogen content (H), measured in laboratory populations and *C. riparius* larvae from laboratory cultures and field populations sampled in the rivers Con, Sar, and Louro. Each bar is the mean ± SE. For each location and method 20 larvae were used, separated into groups of five, and four experimental replicates were made for each group. (I) Percentage of mouthpart deformities in *C. riparius* fourth instar larvae from laboratory cultures ($n = 152$) and wildlife populations sampled in the rivers Con ($n = 182$), Sar ($n = 174$), and Louro ($n = 152$). Different letters indicate significant differences across groups ($P \leq 0.05$).

Stress Response: Analysis of the Expression Levels of the 70-kD Heat-Shock Gene Family

The expression of the *hsp70* and *hsc70* genes encoding for these proteins was measured by the levels of their respective mRNA using RT-PCR. As shown in Figure 2(A), a significantly higher expression of the *hsp70* gene was found in field larvae collected in the three rivers in comparison with that for larvae reared under laboratory conditions and not exposed to toxicants. Significant differences were also found among the populations from the different rivers analyzed, with the highest levels of *hsp70* in larvae sampled from the river Con, which had the highest content of metals measured in sediments (Fig. 1). In contrast, analysis of the expression of the constitutive *hsc70* gene did not show any differences, with similar *hsc70* mRNA levels in both field and laboratory samples [Fig. 2(B)].

Ecdysone-Mediated Pathway: Expression Profiles of the Ecdysone Receptor Gene

The *EcR* gene was selected as an endocrine-related biomarker and RT-PCR analysis was used to evaluate transcript levels with specific primers. As shown in Figure 2(C), no statistically significant differences were found, although a trend of increasing levels of *EcR* in field populations from the Con, Sar, and Louro rivers was observed when compared

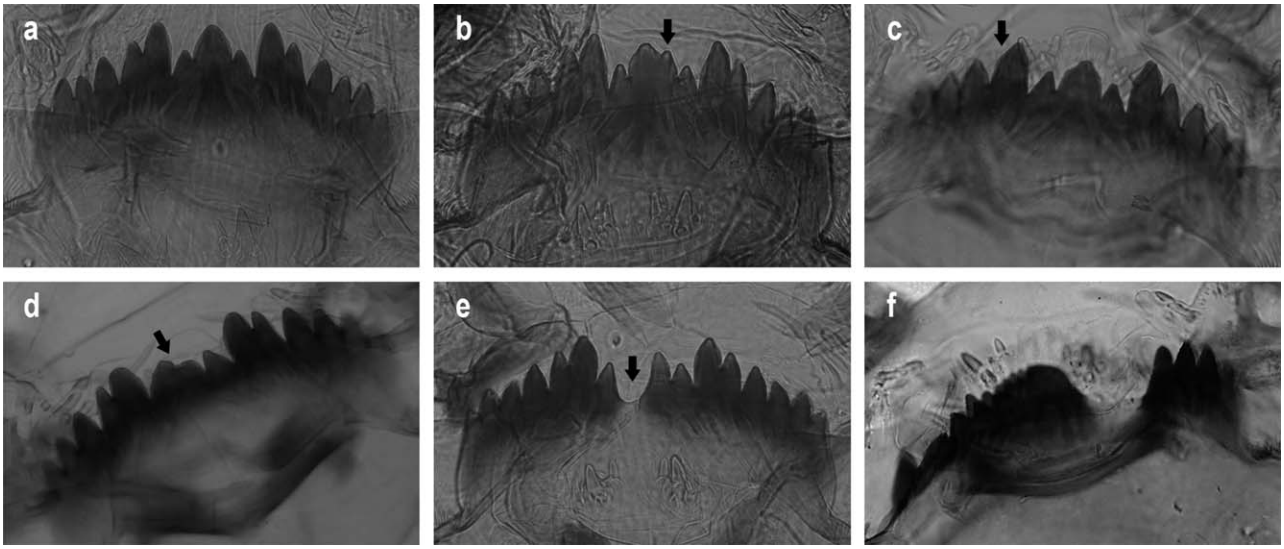

Fig. 3. Normal and deformed mentum of *Chironomus riparius*. (A) Normal mentum. (B) Extra tooth. (C) Missing tooth. (D) Fused teeth. (E) Köhn gap. (F) Massive deformities.

to those reared in the laboratory, with the highest levels found in larvae sampled from the Louro.

Detoxification Activities: Cytochrome *P450* Gene Expression Levels and GST Enzymatic Activity

CYP4G gene expression and glutathione *S*-transferase activity were evaluated as a measurement of detoxification activity in the different *Chironomus riparius* populations. *CYP* genes coding for cytochrome P450, a superfamily of major phase I detoxification enzymes are xenobiotic-responsive genes. As shown in Figure 2(D), no significant differences were found in *CYP4G* gene expression, although slightly higher activity appeared in populations from the rivers Sar and Louro. Glutathione *S*-transferase (GST), one of the major phase II detoxification enzymes, plays a central role in defense against various environmental toxicants. The results obtained showed a similar trend in both detoxification systems measured using gene and enzyme activities. GST activity was similar in laboratory and river Con larvae [Fig. 2(E)], while the values for GST activity were significantly higher in the populations from the Sar and the Louro [Fig. 2(E)], which had the two highest levels of organic pollutants in sediments.

Growth and Metabolic Rates: The RNA:DNA Ratio, Total Protein Content and Glycogen Content

The results showed that there were no significant differences in the RNA:DNA ratio or in total protein content among the different field populations analyzed, not even between field and laboratory populations [Fig. 2(F,G)]. In contrast, drastic differences were observed for glycogen content among populations; the larvae sampled from the river Con showed a significant reduction in glycogen reserves, when compared with the rest of populations analyzed [Fig. 2(H)].

Sublethal Effects During Development: Evaluation of Mouthpart Deformities

Chironomid mouthpart deformities are considered to be useful bioindicators of a variety of pollutants (Martínez et al., 2006; Park and Kwak, 2008). The normal arrangement of teeth in *C. riparius* larvae consists of three median lateral teeth and two sections of lateral teeth. Extra teeth, missing teeth, fused teeth and the presence of Köhn gaps were considered to be mouthpart deformities (Park et al., 2010). Differences in the percentage of total deformities evaluated among sampling sites are presented in Figure 2(I), and illustrated in Figure 3. A significant increase in mouthpart deformities was found in larvae populations sampled from the river Sar, when compared to the rest of samples obtained from laboratory cultures and the Con and Louro rivers.

DISCUSSION

Although the development of biomarkers requires experimental research under laboratory controlled conditions to identify the specific response to particular chemicals or group of compounds, further validation in field populations exposed to complex mixtures of pollutants in their natural habitats is required before making conclusions about their usefulness in realistic field scenarios. The comparative study of a battery of biomarkers revealed remarkable differences when analyzed in natural populations of *Chironomus* larvae collected from different polluted habitats. Most of the biomarkers have been previously tested under experimental

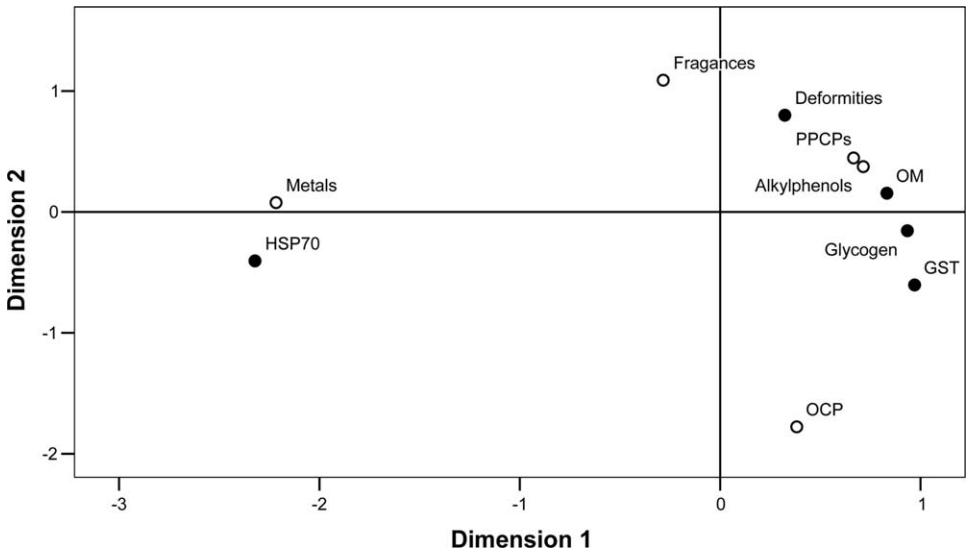

Fig. 4. Two-dimensional MDS representation of the distance values calculated on contaminants and biomarkers with significant differences between localities. Abbrev.: PPCPs: pharmaceutical products, OM: organic matter; OCP: organochlorine products.

exposures to particular toxicants but, to our knowledge, few had been tested to date in field assays. Four out of nine parameters analyzed (*hsp70* gene, GST enzyme, glycogen content and mouthpart deformities) showed significant differences in populations submitted chronically to complex mixtures of toxicants. Other parameters (*hsc70* gene, *EcR* gene, *CYP4G* gene, RNA:DNA ratio, total protein content) appeared to be constant even when comparing field and non-exposed laboratory populations. The rivers differed in the levels and types of contaminants in sediments, although none of the sampling sites showed elevated levels of contamination according to the compounds analyzed in sediments and the freshwater sediment guidelines of MacDonald et al. (2000). Figure 4 summarizes the results of a multivariate analysis combining statistically significant endpoints and groups of contaminants, as well as identifying associations between them. Some potential correlations between sensitive biomarkers and physical or chemical characteristics measured at each site are discussed below. It is worth mentioning the following associations: *hsp70* with metals, mouthpart deformities with PPCPs/alkylphenols, and glycogen content with organic matter. Although this information constitutes a first approach in the search of cause-effect relationships in natural scenarios, more and detailed research is needed to establish clear correlations between exposure to a group of contaminants and differences observed on biomarkers. It is important to bear in mind that, under field conditions, it is difficult to attribute observed changes in the endpoints analyzed to a single group of chemicals, because synergistic and antagonistic interactions may take place, and their inputs into the natural environment not only encompass their pure state, but also that of other products as a result of degradation (Hassan et al., 2005).

One of the most remarkable differences was found in the *hsp70* gene in the four different *Chironomus riparius* populations analyzed. Heat-shock proteins function to maintain protein integrity, and are commonly considered to be an indicator of cellular stress. The 70-kD heat-shock protein family is an ancient and conserved group of proteins, present in all species and every cell type analyzed to date, which plays a basic role in cells as molecular chaperones. The family includes the cognate proteins (HSC70), highly abundant in normal cellular conditions, as well as inducible members (HSP70) present in stressed conditions provoked by a broad spectrum of physical and chemical insults. A significant overexpression of the *hsp70* gene was observed in the three wildlife populations sampled in the rivers Sar, Con, and Louro, as compared to the laboratory cultures that were never exposed to toxicants. Because temperature is one of the stronger inducers of this gene and HSP70 is known to respond to different stressors, to avoid differences in *hsp70* levels linked to temperature fluctuations, we minimized larvae stress controlling this parameter during their transport to the laboratory. Furthermore, this parameter was similar in the three rivers analyzed (about 16°C) and even higher (20°C) in laboratory cultures. Therefore, high *hsp70* levels were most likely related to the presence of a huge range of organic and metal contaminants in the sediments of the rivers, as shown the strong association between *hsp70* gene activity and metals (Fig. 4), whereas the laboratory cultures were never exposed to toxicants.

It has been previously shown that the *hsp70* gene from *Chironomus* is activated by experimental exposures to different toxicants (Morales et al., 2011), pesticides (Yoshimi et al., 2002), bisphenol A (Planelló et al., 2008), and phthalates (Park and Kwak, 2008; Planelló et al., 2011). Here we

have shown, for the first time, the presence of high levels of *hsp70* gene expression in natural populations collected from aquatic polluted sites, which indicates that the level of expression of *hsp70* could have a relevant protective role in long-term adaptation for survival in chemically adverse environments. Previous studies have reported that organisms living in a thermally polluted habitat experience warm acclimation also resulting in higher levels of *hsp70* expression (Lund et al., 2006). It is interesting to note that the highest levels of *hsp70* were found in larvae sampled from the river Con, which has the highest levels of metals in sediments, especially Fe and Mn. Metals are considered to be potent inducers of heat-shock proteins (Fredj et al., 2010; Guo and Ki, 2012), and exposure to copper, cadmium, lead, and chromium has been shown to activate the *hsp70* gene in *Chironomus* (Karouna-Renier and Zehr, 2003; Lee et al., 2006; Planelló et al., 2010). It would be interesting to analyze the effect of Fe and Mn as inductors of the *hsp70* gene, because to our knowledge there is no information concerning these metals in invertebrates. Currently, the potential use of heat-shock genes and proteins in pollution monitoring is being actively investigated (Gupta et al., 2010). In addition to the growing body of literature that connects the HSP70 protein to different classes of chemical inducers, our results demonstrate that the stress gene *hsp70* could be a sensitive molecular biomarker in wildlife populations permanently exposed to a complex mixture of pollutants. In contrast to the notable differences found in *hsp70*, it is worth pointing out that the expression of the constitutive gene *hsc70* remained constant, suggesting that the *hsc70* seems to be a relatively stable gene in natural populations submitted to the effects of different mixtures of toxicants. The stability of the *hsc70* gene concurs with previous results obtained following experimental exposures to a wide variety of chemicals (Morales et al., 2011). On the basis of our results, it is tempting to propose the ratio of transcripts from both gene members of the 70 kD family (*hsp70*/*hsc70*) as a consistent indicator for assessing toxicity, when comparing wildlife populations living in polluted environments.

Another interesting result was related to energy reserves. A significant decrease in glycogen content was observed in larvae from the river Con when compared to the rest of the populations analyzed, with a relevant association between glycogen content and organic matter as shown in Figure 4. This data correlates with the highest level of *hsp70* gene expression in the larvae, with the highest content of metals, as well as with the lowest organic matter content in the sediments of that river. These results are in accordance with the assumption that chronic stress causes an energetic cost and, by extension, a decrease in glycogen storage (Bischof, 1995; Choi et al., 2001). One interesting problem in ecotoxicology is the cost of tolerance to toxic chemicals, since presumably detoxification mechanisms are energy-dependent. Nevertheless, the larvae sampled from the rivers Sar and Louro, also exposed to chemical stressors, did not show significant variations in glycogen reserves; these results concur with those of previous studies in this species, which found no alterations in sugar contents, suggesting that other factors, such as food availability, could compensate for the energy expenditure devoted to counteracting the effects of toxicants (Stuijfzand et al., 2000; Servia et al., 2006). In contrast to glycogen content, other metabolic parameters, such as the RNA:DNA ratio and the total protein content, remained constant with similar values for all the field and laboratory populations assayed. The RNA:DNA ratio is considered to be a biochemical indicator of the physiological and nutritional state of aquatic organisms in natural environments, and a useful indicator of anthropogenic impacts in invertebrates and fishes (Okumura et al., 2002; Dahlhoff, 2004; Chícharo and Chícharo, 2008). While DNA content remains constant, RNA concentration, directly related to translation activity, may change depending on age, developmental stage, size, nutritional activity or environmental conditions (Chícharo and Chícharo, 2008). The protein content faithfully reflects the physiological condition of an organism (Villarroel et al., 2009), and glycogen levels are related to energy reserves. The levels of pollution in the rivers studied were probably not sufficiently high to alter these parameters, or to thereby compromise the vitality and survival of the species.

Detoxification systems of organisms play a central role in defense against various environmental toxicants, and can lead organisms to adaptation to chronic exposures in polluted environments. Accordingly, a significantly higher GST activity was detected in larval samples from the rivers Sar and Louro than in the controls. These rivers contained the highest levels of alkylphenols, pharmaceutical products and organochlorine pesticides. GST activity in samples from the river Con, which had the lowest organic pollutant content in sediments, was similar to those of laboratory controls. In insects, increased levels of GST have been associated with organochlorine and organophosphate insecticide resistance (Vontas et al., 2000). We have previously reported that GST levels respond selectively to different xenobiotics. While tributyltin upregulated both the GST enzyme and the *CYP* gene, nonylphenol and bisphenol A exerted the opposite effect in *Chironomus riparius* (Martínez-Paz et al., 2012). P450 cytochromes constitute one of the major phase I-type classes of detoxification enzymes, and glutathione *S*-transferases are major phase II detoxification enzymes. The results of our simultaneous study of these two detoxification systems in field-sampled larvae, suggest a higher detoxification activity, as measured by GST levels, in larvae from rivers with a higher content and variety of organic pollutants (the Sar and Louro rivers).

Morphological mouthpart deformities of *Chironomus* represent sublethal response to pollutants in aquatic ecosystems, and are considered early warning indicators for deterioration of water quality (Janssens de Bisthoven and Gerhardt, 2003; Ochieng et al., 2008). Deformities are important morphological endpoints that have been widely used in toxicity studies,

and have been suggested as a biomarker for biomonitoring of contamination. In our study, mouthpart deformities were significantly higher in the population of larvae sampled in the river Sar, while larvae from the rivers Con and Louro showed similar levels to the unexposed population. Interestingly, the most notable difference between these three rivers is the presence of higher levels of alkylphenols, particularly bisphenol A, and pharmaceuticals such as ibuprofen and atenolol in the sediments of the river Sar. These results are in accordance with the strong association observed in Figure 4 between deformities, alkylphenols and PPCPs, with minimum Euclidean distances between these variables, as shown in Table IV (Supporting Information). Previous reports have demonstrated a relationship between pollutants and deformities (Hämäläinen, 1999; Di Veroli et al., 2012; Odume et al., 2012) and our results further support the high prevalence of deformities previously found at this site (Servia et al., 2000). Although it is still unknown how pollutants may induce mouthpart deformities, there is an ongoing discussion about whether or not they develop at the endocrine-regulated molting stage, and if a disruption of this process is at the base of their ontogeny (Meregalli and Ollivier, 2001). Some toxic compounds with endocrine disrupting activity have been described as inducers of mouthpart deformities such as 4-nonylphenol, DDT or heavy metals (Meregalli et al., 2001; Martínez et al., 2003).

Finally, no significant differences among populations were found for the ecdysone receptor gene, as an endocrine marker. Although the *EcR* gene appears to be a promising biomarker for testing endocrine disrupting chemicals in insects (Soin and Smagghe, 2007; Planelló et al., 2008, 2010, 2011), further studies are needed to validate this hormonal endpoint in field studies. It is important to point out that natural populations living in polluted environments may be exposed to chemicals that act as hormonal agonists and antagonists, so that compensatory effects could take place with the resulting physiological response being different in complex natural scenarios than in controlled laboratory assays.

In conclusion, this study demonstrates for the first time that differential patterns in gene expression, enzymatic and metabolic activities and morphological parameters can be detected in natural populations of *Chironomus riparius* submitted to chronic exposure to toxicants in their natural habitats, with notable specificities depending on the toxicological characteristics of the sediments. The results suggest new sensitive endpoints, including genes, as potential biomarkers in field studies that, complementing other ecotoxicological parameters, may help to improve the methodologies of freshwater monitoring. Moreover, the changes in specific parameters help to provide a better understanding of the physiological responses in real scenarios, which imply adaptation by organisms to multiple-stressor environments. Although chemical analysis of the sediments suggested some possible relationships for the observed differences, further research is needed to identify causative relationships between biomarkers and exposure to particular contaminants, as well as the influence of adaptive responses. These data provide the initial insights into the feasibility of using new molecular endpoints to better characterize the metabolic properties of natural populations under environmentally relevant scenarios (low concentrations and chronic exposures, as well as combined exposures to mixtures of toxicants).

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