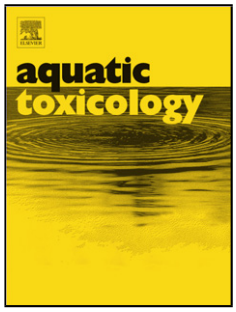

Prodiamesa olivacea: *de novo* biomarker genes in a potential sentinel organism for ecotoxicity studies in natural scenarios<!–<ForCover>Llorente L, Herrero Ó, Aquilino M, Planelló R, *Prodiamesa olivacea*: *de novo* biomarker genes in a potential sentinel organism for ecotoxicity studies in natural scenarios, *Aquatic Toxicology*, doi: 10.1016/j.aquatox.2020.105593</ForCover>–>

Lola Llorente (Investigation) (Data curation) (Writing - original draft) (Formal analysis), Óscar Herrero (Resources) (Methodology) (Writing - review and editing), Mónica Aquilino (Visualization) (Writing - review and editing), Rosario Planelló (Resources) (Supervision) (Funding acquisition) (Writing - review and editing)

PII: S0166-445X(20)30343-X

DOI: https://doi.org/10.1016/j.aquatox.2020.105593

Reference: AQTOX 105593

To appear in: *Aquatic Toxicology*

Received Date: 3 March 2020

Revised Date: 30 July 2020

Accepted Date: 3 August 2020

Please cite this article as: { doi: https://doi.org/

Prodiamesa olivacea: *de novo* biomarker genes in a potential sentinel organism for ecotoxicity studies in natural scenarios.

Lola Llorente[1], Óscar Herrero[1], Mónica Aquilino[1], Rosario Planelló[1*]

[1] Biology and Environmental Toxicology Group, Faculty of Sciences, Universidad Nacional de Educación a Distancia (UNED), Paseo de la Senda del Rey 9, 28040 Madrid, Spain.

Email or corresponding author: rplanello@ccia.uned.es

Highlights

- *P. olivacea* and *C. riparius* were used to assess the toxic effects of BBP, BPA and BP3
- New genes for detoxification and oxidative stress were characterized in *P. olivacea*
- Transcript levels of the studied genes showed alterations after xenobiotic exposure
- Genes related to the oxidative stress response presented species-dependent responses
- *P. olivacea* could be a suitable sentinel organism for aquatic toxicity tests of EDCs

Abstract

Along with traditional ecotoxicological approaches in model organisms, toxicological studies in non-model organisms are being taken into consideration in order to complement them and contribute to more robust approaches. This allows us to figure out the complexity of the exposures involved in natural ecosystems. In this context, in the present research we have used the model species *Chironomus riparius* (Chironomidae, Diptera) and the non-model species *Prodiamesa olivacea* (Chironomidae, Diptera) to assess the aquatic toxic effects of acute 4-h and 24-h exposures to 1 μgL^{-1} of three common environmental pollutants: butyl benzyl phthalate (BBP), bisphenol A (BPA), and benzophenone 3 (BP3). Individuals of both species were collected from a contaminated river (Sar) in Galicia (Spain).

Regarding *Chironomus*, there are four OECD standardized tests for the evaluation of water and sediment toxicity, in which different species in this genus can be used to assess classical toxicity parameters such as survival, immobilization, reproduction, and development. In contrast, *Prodiamesa* is rarely used in toxicity studies, even though it is an interesting toxicological species because it shares habitats with *Chironomus* but requires less extreme conditions (e.g., contamination) and higher oxygen levels. These different requirements are particularly interesting in assessing the different responses of both species to pollutant exposure.

Quantitative real-time PCR was used to evaluate the transcriptional changes caused by xenobiotics in different genes of interest. Since information about *P. olivacea* in genomic databases is scarce, its transcriptome was obtained using *de novo* RNAseq. Genes involved in biotransformation pathways and the oxidative stress response (*MnSOD*, *CAT*, *PHGPx, Cyp4g15, Cyp6a14-like* and *Cyp6a2-like*) were *de novo* identified in this species. Our results show differential toxic responses depending on the species and the xenobiotic, being *P. olivacea* the dipteran that showed the most severe effects in most of the studied biomarker genes.

This work represents a multi-species approach that allows us to deepen in the toxicity of BBP, BPA, and BP3 at the molecular level. Besides, it provides an assessment of the tolerance/sensitivity of natural populations of model and non-model insect species chronically exposed to complex mixtures of pollutants in natural scenarios. These findings may have important implications for understanding the adverse biological effects of xenobiotics on *P. olivacea*, providing new sensitive biomarkers of exposure to BBP, BPA, and BP3. It also highlights the suitability of *Prodiamesa* for ecotoxicological risk assessment, especially in aquatic ecosystems.

Keywords

Transcriptional biomarker, detoxification, oxidative stress, emergent pollutants, sentinel organism, endocrine disruptor

Introduction

Water pollution can be caused in many ways, including direct sources such as effluent outfalls (from factories, waste treatment plants, etc.), and indirect sources in which contaminants enter the aquatic systems from soils or groundwater systems and the atmosphere via rainwater. Substances such as fertilizers, pesticides, herbicides, and a wide variety of toxic chemicals enter the dynamics of the ecosystems, disrupt the physical or biological components of the aquatic environments, and can lead to shifts in the viability of their populations. Among the polluting substances, those that are most persistent in the environment often give rise to a particular concern, since the effects resulting from their exposure can be prolonged over time. In this regard, butyl benzyl phthalate (BBP), bisphenol A (BPA), and benzophenone 3 (BP3) are ubiquitous contaminants whose presence in the environment is expected for decades.

The primary use of BBP is as a softener (plasticizer) in PVC products, but it is also used in sealants, adhesives, paints, inks and lacquers, car care products, cosmetics, food conveyor belts, or vinyl gloves (CPSC, 2010; EC, 2007). Based on its widespread use worldwide, it is considered a global pollutant. According to Annex I of Directive 67/548/EEC, this substance is classified as toxic to reproduction in humans, capable of damaging the unborn child and with a possible risk of causing infertility. It is further classified as very toxic to aquatic organisms, and susceptible to cause long-term adverse effects in aquatic environments (EC, 2007; EFSA, 2019). Alterations caused by BBP in the ecdysone hormone pathway, the cellular response to stress, the energy metabolism, and several detoxification mechanisms have been described in *Chironomus riparius* larvae (Herrero et al., 2015; Planelló et al., 2011).

BPA is also considered a ubiquitous pollutant. It is mainly used in the manufacture of polycarbonate plastics, epoxy resins, or thermal papers, and its release to the environment can occur from long-life materials used in flooring, furniture, toys, construction, curtains, foot-wear, leather products, paper and cardboard products, electronic equipment (ECHA, 2020a). BPA is classified as toxic for reproduction, and

also as a skin-sensitizing product and an endocrine-disrupting chemical. Based on its toxic properties, in recent years, its use in toys, thermal paper, and materials in contact with food has been restricted (ECHA, 2020b). It is a representative endocrine disruptor in both vertebrate and invertebrate species, including chironomids (Lee and Choi, 2007; Planelló et al., 2008).

BP3 is one of the most frequently used UV filters, and it is also included in a variety of products to protect them from light damage. Due to the extensive use of sunscreens, it has been detected in natural water, sediments, and even in tap water (Kim and Choi, 2014; Ramos et al., 2015). Although the compound displays a low acute toxicity profile (EC, 2008), it is currently under assessment due to its endocrine-disrupting properties (ECHA, 2020c). In recent years, some studies tried to elucidate the molecular effects of BP3 in *C. riparius*, and alterations on relevant genes (related to the endocrine system, detoxification mechanisms, and the stress response), enzyme activities (immune system, oxidative stress), and the emergence and development rates have been described (Campos et al., 2019; Martín-Folgar et al., 2018; Muñiz-González and Martínez-Guitarte, 2020).

The selection of appropriate protocols for the study of contaminants in invertebrates is one of the priority objectives for the evaluation of their environmental impact (OECD, 2006). Species selection should be based on the ecological relevance of the group of invertebrates to which they belong, and the existence of standardized tests. Macroinvertebrates are useful organisms in water quality monitoring, and their presence or absence are indicators of the degree of contamination (Gresens et al., 2009). In this group of animals, the use of chironomids as bioindicators have significantly increased in the last decades (Arimoro et al., 2018; Ospina-Pérez et al., 2019; Rosenberg, 1992). Chironomids are critical elements for the functioning of freshwater ecosystems, as they sustain higher trophic levels and contribute to the carbon cycle in streams and rivers. The group includes relevant species in ecotoxicology and is at the base of the trophic chain of aquatic ecosystems. Besides,

their benthic nature makes them especially suitable, since some pollutants tend to accumulate in the sediments of inland waters. Due to that, certain developmental stages within their life cycle are particularly vulnerable to environmental stressors, and chronic exposures during these critical periods may have irreversible consequences (Weis, 2014).

The status of the organisms and their biological responses are used in ecotoxicology studies to determine water quality. In this regard, different internationally standardized tests using *Chironomus* species have been widely used to assess the quality of water and sediment samples (OECD, 2011, 2010, 2004a, 2004b). This benthic species is ubiquitously distributed in the Northern hemisphere at temperate latitudes, and very easy to maintain in the laboratory, usually in organically enriched water (Charles et al., 2004). Several ecotoxicity studies have been carried out with this species, using both natural populations (Im et al., 2019; Khosrovyan and Kahru, 2020; Lee and Choi, 2007), or laboratory cultures (Arambourou et al., 2020; Arambourou et al., 2019; Ding et al., 2011; Herrero et al., 2016; Lee et al., 2009; Planelló et al., 2011). Alterations in growth, fecundity, sex ratio, genotoxicity, expression levels of relevant genes (related to the stress response, the hormonal system, and the detoxication metabolism; e.g., *CypP450*, *EcR*, *GST*, *Hsp70*), and antioxidant enzymatic activities (e.g., CAT, GPx, GST, SOD) have been described in *C. riparius* after exposure to heavy metals, UV filters, BPA, or pesticides, among others (Herrero et al., 2015; Ilkova et al., 2017; Monteiro et al., 2019; Muñiz-González and Martínez-Guitarte, 2018; Planelló et al., 2008). Other species, such as *Chironomus tentans, Chironomus plumosus,* or *Chironomus prasinus,* have also been used for ecotoxicity studies (Meehan et al., 2014; Sánchez et al., 2005; Sánchez and Tarazona, 2002; Tang et al., 2018). These approaches have acquired great importance in recent years, thanks to improvements in laboratory protocols (Arambourou et al., 2019; Martínez-Paz et al., 2017; Muñiz-González and Martínez-Guitarte, 2020).

Prodiamesa olivacea (Chironomidae; Diptera) is an aquatic dipteran that has the same ecological niche than *C. riparius*, although *P. olivacea* usually lives in less extreme conditions and requires higher environmental oxygen levels (Servia et al., 1998). Few studies with *Prodiamesa* species describe their sensitivity to polluted environments (Ilkova et al., 2018; Michailova et al., 2003; Servia et al., 2000). Furthermore, to date, there is no information available on this species in genomic databases, so ecotoxicological studies equivalent to those mentioned in the case of *Chironomus*, where classical toxicity parameters are assessed in parallel with effects on gene expression, could not yet be carried out.

Determining water quality based on the health status of *Chironomus* species could sometimes lead to underestimating environmental alterations, since these organisms tend to have a high tolerance to the presence of contaminants or other stress factors. In fact, under natural conditions, it is sometimes difficult to find populations of this organism in unpolluted environments, since they usually require a certain degree of eutrophication for their development, generally due to the discharge of nutrient-rich effluents into water bodies (Armitage et al., 1995). In contrast, the use of more sensitive chironomids, such as *Prodiamesa* species, could be very useful to improve the accuracy of ecosystem health assessments. Therefore, the apparent lower tolerance of *P. olivacea* to changes in biotic and abiotic factors in ecosystems (Ilkova et al., 2018) could be an advantage in the detection of early-effect targets. Furthermore, the concurrent use of different taxa provides a broader perspective in the ecological assessment of water quality, since each biological entity may respond differently to certain environmental variables (Cheimonopoulou et al., 2011).

In the present study, and for the first time in *P. olivacea*, we have characterized six *de novo* genes related to biotransformation pathways and the oxidative stress response (*MnSOD*, *CAT*, *PHGPx*, *Cyp4g15*, *Cyp6a14-like*, *Cyp6a2-like*), in order to assess their usefulness as early biomarkers of ecotoxicological effect in this species. We have also studied the toxic effects of an environmentally relevant concentration of BBP, BPA, and

BP3 (1 µg/L) on the transcriptional activity of these six genes in the aquatic larvae of *P.olivacea*. Although these have been our two main objectives, we have additionally selected three of these molecular biomarkers to analyze possible differential responses between individuals of *P. olivacea* and *C. riparius* after exposure to the three xenobiotics studied.

2. Material and methods

2.1. Testing animals and sampling site

The experimental animals were the aquatic fourth larval stage of the *P. olivacea* and *C. riparius* midges. Larvae were collected the 6th and 7th of May in 2016 in the Sar River, one of the most polluted watercourses in Galicia (Spain) (Prego et al., 2008; Planelló et al., 2015). The sampling site (UTM: 29T 528728E 4744922N) is located about 4 km downstream of the Santiago de Compostela (100,000 inhabitants) sewage plant. The sampling was performed choosing the most suitable microhabitats for these species, i.e., places with fine sediment, accumulated organic matter, and slow current. The harvesting was carried out with a small hand net (15 cm diameter, 20 cm deep, and 250 µm mesh size), and the study organisms were quickly transported to the laboratory maintaining natural temperature and aeration conditions in buckets containing water and sediment from the sampling point. Once in the laboratory, the buckets were immediately emptied into trays that were kept for 2 hours with constant aeration until the collection of the larvae needed for the experiments. The fourth larval stage was determined by the size of the head capsule (USEPA, 2000).

2.2. Experimental design of the bioassay

Solutions of butyl benzyl phthalate (BBP, CAS No. 85-68-7, purity ≥ 98%), bisphenol A (BPA, CAS No. 80-05-7, purity ≥ 99%), and benzophenone-3 (BP3, CAS No. 131-57-7, purity ≥ 98%) were purchased from Sigma-Aldrich (USA) and dissolved in analytical grade ethanol to provide a stock concentration of 10 g/L. The test solutions were

constructed in culture medium with a final ethanol concentration of 0.01 v/v %, which was tested to have no effect on the biomarkers analyzed. Negative (culture medium only) and solvent (0.01v/v % ethanol) controls were included in both 4h and 24h experiments. 4th instar larvae of *P. olivacea* and *C. riparius* were exposed for 4h and 24h to 1 µg/L BBP, BPA, or BP3 in 100 mL glass beakers with 50 mL of culture medium. This nominal concentration was chosen based on its environmental relevance and previously published data (Herrero et al., 2018, 2016; Ozáez et al., 2016). Each experimental condition was tested in a separate glass beaker, in triplicate, with 45 larvae per beaker (n = 135 larvae/condition). Larval survival rate was measured after 4 or 24-hour exposures, and 5 survivors per replicate and experimental condition were randomly selected for the subsequent gene expression analyses.

2.3. Sequencing, de novo assembly and annotation of a reference transcriptome

Three cDNA libraries were constructed by the Macrogen company following the Tru-Seq Stranded mRNA (Illumina, USA) protocol and sequenced on an Illumina Hi-Seq 4000 using a 100 cycles paired-ended protocol. For each sample, total RNA was extracted from ten 4th instar larvae using TRIzol Reagent (Invitrogen), following the manufacturer's instructions. RNA was then treated with DNase I (Invitrogen) and extracted with phenol:chloroform:isoamyl alcohol (Fluka, Germany) using 5PRIME Phase Lock Gel Light tubes (Quantabio, USA). Purified RNA was resuspended in nuclease-free water, quantified by spectrophotometry at 260 nm using a BioPhotometer (Eppendorf, Germany), and stored at -80 °C.

A reference transcriptome was assembled by integrating the RNA-seq reads from the three libraries obtained. Before assembly, the quality of the sequences was checked using FastQC v0.11.7 (Andrews, 2007), and reads with low-quality (Phred value < 33) and adaptor sequences from the raw data were removed using Trimmomatic v0.32 (Bolger et al., 2014). The filtered reads were then *de novo* assembled into contigs with Trinity software (Grabherr et al., 2011) using default parameters. All unigenes > 200 bp

were searched using BLASTx with the following protein sequences databases: UNIPROT (v20170706), Kyoto Encyclopedia of Genes and Genomes (KEGG_v20170706) and GO (v20150407) (e-value < 10^{-5}), to identify proteins with high sequence similarity, and to assign putative functional annotations. Subsequently, Gene Ontology (GO) annotations of the unigenes were obtained using Blast2GO (Conesa et al., 2005).

2.4. Gene characterization and sequence alignment

The nucleotide sequences for relevant genes related to biotransformation and the oxidative stress response were obtained from our *P. olivacea de novo* transcriptome (Project reference PRJEB36742): *MnSOD* (manganese superoxide dismutase), *CAT* (catalase), *PHGPx* (phospholipid-hydroperoxide glutathione peroxidase), *Cyp4g15*, *Cyp6a14-like*, and *Cyp6a2-like*. SnapGene ® (GSL Biotech), BLAST (Agarwala et al., 2018), and DOG 2.0 (Ren et al., 2009) software were used in the identification and characterization of these genes.

Through the GenBank database, a selection of sequences from the main insect taxa was obtained for each characterized gene and analyzed using BLAST software. For the different genes studied, the alignment of the sequences was done with Clustal 2 (Larkin et al., 2007) and MAFFT version 7 (Katoh and Standley, 2013) software. GenBank Accession numbers, length of homologous amino acid sequences, and identities are shown in Table SM1.

2.5. Phylogenetic analyses

Phylogenetic trees were generated using the MEGA X version 7 software (Kumar et al., 2018) with the UPGMA method (Sokal and Michener, 1958). The bootstrap consensus tree inferred from 1,000 replicates (Felsenstein, 1985), and the evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965).

2.6. Gene expression studies

Three biological replicates with 5 larvae per replicate were used for each experimental condition (n = 15 larvae/condition) in the gene expression analyses. Total RNA of *P. olivacea* or *C. riparius* larvae was extracted using TRIzol Reagent (Invitrogen, Germany), following the manufacturer's instructions. After that, RNA was treated with RNase-free DNase (Roche, Germany) and extracted with phenol:chloroform:isoamyl alcohol (Fluka) using 5PRIME Phase Lock Gel Light tubes (Quantabio). Aliquots containing 7 µg of isolated RNA were reverse-transcribed in an MJ Mini Thermal Cycler (Bio-Rad, USA) using iScript Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad), according to kit instructions. The obtained cDNA was conserved at -20 ºC and used as a template for subsequent qPCR analyses.

The primers used for the amplification of the selected genes were designed based on the *de novo P. olivacea* transcriptome obtained in our laboratory and using Primer 3 version 0.4.0 software (Untergasser et al., 2012). The sequences of the primers and the size of the amplified fragments are shown in Table 1. The size of the PCR products was checked in a 9% acrylamide gel at 85V for 2 h in 1x TGE buffer (40 mM Tris-Cl (pH 8.5), 200mM glycine and 2.5mM EDTA), stained with ethidium bromide and visualized with Chemigenius3 (Syngene, USA). The identities of the amplified fragments were verified using BLAST (Altschul et al., 1990). Amplification efficiencies and correlation coefficients for each primer pair were calculated as described in the Real-Time PCR Applications Guide (Bio-Rad catalog #170–9799). For all genes, the efficiencies were between 90% and 105% ($R^2 > 0.980$).

The conditions for the RT-qPCR were the same as those described previously in Herrero et al. (2018). Genes encoding the 26S ribosomal subunit and actin were used as endogenous reference genes. Fragments of these genes were amplified using the same pair of primers designed for *C. riparius*, and their identities were confirmed by Sanger sequencing (STABvida company). The statistical validation of the stability of the reference genes was performed by means of CFX Manager 3.1 software (Bio-Rad),

using an iterative test for pairwise variation, according to Vandesompele et al. (2002). The $2^{-\Delta\Delta Ct}$ method was used to analyze relative changes in gene expression with CFX Manager 3.1 software. Each sample was run in duplicate wells, and for each experimental condition studied (with three biological replicates) three technical replicates were carried out. Gene expression results were normalized to the control values for subsequent analysis.

2.7. Data analysis

The statistical analyses were performed using R. 3.4.3 software (R Core Team, 2018). Mean and median were calculated respectively as the average and the middle of a data set, while the standard deviation represented the square root of the variance. Regarding the survival studies, the Student t was used to check the statistically significant differences ($p \leq 0.05$) between the different experimental conditions. For transcriptional analyses, the normality and homoscedasticity of the data were checked with the Shapiro-Wilk and Levene tests, respectively. Normal and homoscedastic data were analyzed by ANOVA followed by Bonferroni's *post hoc* test. Otherwise, differences in transcript levels were evaluated using the nonparametric Kruskal-Wallis test followed by Mann-Whitney-Wilcoxon *post hoc* test. p-values ≤ 0.05 and ≤ 0.1 were used as cutoffs for statistical significance.

3. Results

3.1. Survival rates

Overall, *P. olivacea* was more sensitive than *C. riparius* in 4 or 24-hour exposures to 1 µg/L BBP, BPA, or BP3 (Table 2). After 24 hours, a significant ($p \leq 0.05$) reduction in survival compared to controls was observed in *P. olivacea* larvae exposed to 1 µg/L BBP (11.7%), BPA (15%), or BP3 (15%). Meanwhile, *C. riparius* larvae showed significant mortality only in the case of BBP (13.3%) and BP3 (4.2%). For BP3 24h, the response was also significantly different between the two species. On the other hand,

while *C. riparius* showed no mortality in the 4-hour studies, the survival of *P. olivacea* was again affected in the case of BBP (3.2%) or BPA (3.3%), although without statistical significance.

3.2. Reference transcriptome

The cDNA of the reference transcriptome was assembled by pooling the data from three libraries, built from the whole body-RNA of *P. olivacea* larvae collected in the Sar River. After trimming, a total of 60,247,802 reads were used to construct the reference transcriptome of this species, with a GC content of 42.13% and a ratio of reads with Phred quality score over 30 (Q30) of 92.15%. Data was deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under the accession number PRJEB36742. The assembly of the high-quality reads reported a total of 34,900 contigs with an N50 length of 808 bp. The number of successfully annotated contigs was 22,413. Among them, the GO categorization identified 62 GO Terms, within three categories: biological process (22), cellular component (19), and molecular function (21).

3.3. Characterization of P. olivacea genes involved in detoxification or oxidative stress pathways

We identified genes related to the oxidative stress response and several detoxification pathways: *MnSOD*, *CAT*, *PHGPx, Cyp4g15, Cyp6a14-like,* and *Cyp6a2-like*. Their sequences were recorded in the NCBI database as: MN862061 (*MnSOD*), MN862062 (*CAT*), MN862064 (*PHGPx*), MN862063 (*Cyp4g15*), MN862066 (*Cyp6a14-like*), and MN862065 (*Cyp6a2-like*). A systematic search in our *P. olivacea de novo* transcriptome rendered sequences with open reading frames (ORFs) for their corresponding proteins. Five sequences with the complete ORF (*MnSOD*, *CAT*, *PHGPx*, *Cyp4g15* and *Cyp6a14-like*) and one incomplete (*Cyp6a2-like*) were obtained (Table 3). Relevant domains of each ORF are presented in Fig. 1. To confirm the identity of each *de novo* characterized protein in *P. olivacea*, a phylogenetic study was

conducted to evaluate the relationship between each identified protein and homologue proteins in other insects and arachnids (Fig. 1; Table SM1).

The complete ORF for *MnSOD* was 654 bp in length and encoded a 217 aa protein (Fig. 1A), sharing 87% sequence identity with *C. riparius*, 69% with *Aedes aegypti*, and 64% with *Papilio xuthus* (Table SM1). The ORF for *CAT* was 1,506 bp in length, encoding a protein with 501 aa residues and a highly conserved catalase domain at the N-terminal end (Fig. 1B); it shared 91% identity with *C. riparius* and more than 70% identity with other selected dipteran and lepidopteran species (Table SM1). The ORF for *PHGPx* was 603 bp in length and encoded a protein with 200 aa residues and a glutathione peroxidase family domain at the C-terminal end (Fig. 1C); it shared 88% identity with *C. riparius* and more than 60% identity with representative dipteran, lepidopteran and hymenopteran organisms (Table SM1).

Three nucleotide sequences encoding different P450 cytochromes were identified, all of them with a *Cyp450* domain; two ORFs were complete (Fig. 1D, E) and one was incomplete (Fig. 1F). The first *Cyp450* complete ORF was a 1,707 bp DNA encoding a 568 aa protein, with more than 70% identity with representative species such as *Clunio marinus*, *Aedes aegypti*, A*nopheles darlingi*, *Drosophila eugracilis*, *Papilio xuthus*, or *Nasonia vitripennis* (Table SM1). The second Cyp450 complete ORF was a 1,491 bp sequence encoding a protein with 496 aa residues and sharing 65% and 48% identities with *C. marinus* and *A. aegypti,* respectively (Table SM1). Finally, the incomplete ORF had 1,117 bp and encoded a 371 aa sequence corresponding to the 3' end of the protein Cyp6a2, sharing more than 50% identity with other dipterans and around 40% identity with the hymenopterans *Apis mellifera* and *N. vitripennis* (Table SM1).

3.4. Alterations in transcriptional activity of P. olivacea larvae exposed to BBP, BPA, or BP3

In general, larval exposure to BPA, BBP, or BP3 showed a time-dependent differential response of selected genes (*MnSOD*, *CAT*, *PHGPx*, *Cyp4g15*, *Cyp6a14-like*, and *Cyp6a2-like*) in the non-model species *P. olivacea* (Fig. 2).

Compared to controls, *MnSOD* reduced its transcriptional activity under all the experimental conditions tested (Fig. 2A), especially in BBP 4h (60% below control values; $p \leq 0.05$), BBP 24h (54%; $p \leq 0.05$), and BPA 4h (50%; $p \leq 0.1$). The smallest decrease was observed for BP3 24h (14%).

CAT (Fig. 2B) was downregulated in 4-hour exposures to BBP (76% below control values; $p \leq 0.05$), BPA, and BP3 (38%; $p \leq 0.1$). In 24-hour studies, BBP and BPA led to significant drop in the transcriptional activity (46% and 73%; $p \leq 0.05$), while BP3 induced a strong overexpression (338%; $p \leq 0.05$).

BBP caused the downregulation of *PHGPx* (Fig. 2C), significantly only in 24-hour experiments (79%; $p \leq 0.05$). 4-hour exposures to BPA also led to a decrease in the transcriptional activity (53%, $p \leq 0.05$), although 24-hour experiments showed no effects (Fig. 2C). On the other hand, while not statistically significant, 24-hour exposure to BP3 triggered transcriptional activity to 206% (Fig. 2C).

Cyp4g15 (Fig. 2D) showed similar responses to those observed for *CAT*. Except for the strong overexpression detected for BP3 24h (201%; $p \leq 0.05$), all other experimental conditions led to a decrease in its transcriptional activity, especially significant ($p \leq 0.05$) for BBP 24h (54% below control values), BPA 4h (66%), and BPA 24h (42%).

No statistically significant responses could be detected for the other two studied P450 cytochromes. In the case of *Cyp6a14-like* (Fig. 2E), 4-hour exposures showed values close to the controls, while in 24-hours treatments there was a general trend towards downregulation, remarkable for BP3 (80% below control values). Data for *Cyp6a2-like* (Fig. 2F) showed high dispersion, and no differences were found with respect to the controls.

3.5. Comparative analysis of the transcriptional responses of antioxidant genes between the aquatic dipterans P. olivacea and C. riparius after exposure to xenobiotics

Among all the genes analyzed in *P. olivacea*, those involved in the cellular response to oxidative stress (*MnSOD*, *CAT*, and *PHGPx*) were selected to assess possible differences in their transcriptional responses with respect to *C. riparius* larvae after exposure to BBP, BPA, or BP3 (Fig. 3).

Regarding *MnSOD*, while *P. olivacea* showed clear downregulation at 4 and 24-hour exposures to BBP (60% and 54%; $p \leq 0.05$) and for BPA 4h (50%; $p \leq 0.1$), *C. riparius* larvae were unaffected at 4 hours and triggered overexpression at 24 hours (168% for BBP, and 243% for BPA; $p \leq 0.05$) (Fig. 3A, 3D). This effect on *C. riparius* was also observed in BP3 24h (177%; $p \leq 0.05$) (Fig. 3G), while *P. olivacea* showed no transcriptional alterations. In all cases, statistical analyses detected differences between the performance of both species ($p \leq 0.05$).

CAT showed similar results to those observed for *MnSOD*. On the one hand, the significant changes already described in *P. olivacea* after 4 and 24 hours of exposure to xenobiotics were attenuated in the case of *C. riparius* larvae exposed 4 hours to BBP (34% inhibition, compared to 76% in *P. olivacea*) (Fig. 3B), or disappeared for BPA or BP3 (Fig. 3E, 3H). Furthermore, in the 24-hour experiments, the downregulation detected in *P. olivacea* for BBP and BPA became over-expression in *C. riparius* (107% for BBP; $p \leq 0.1$) (155% for BPA, no significance), while the increase observed in *P. olivacea* for BP3 was not reflected in the other species. As observed for *MnSOD*, differences were found ($p \leq 0.05$) in the *CAT* response between the two study species after exposures to BBP and BPA.

Finally, the differential effects between species regarding *PHGPx* mRNA levels were assessed and were significant ($p \leq 0.05$) after BBP exposures (Fig. 3C). BBP induced a differential expression at 4 hours, with a tendency towards downregulation in *P. olivacea* (52% below control values) that was turned into over-expression in *C. riparius* (142% above control), although without statistical significance. On the other hand,

while in *P. olivacea* a decrease in transcriptional levels of *PHGPx* was observed in BBP 24h (79%; $p \leq 0.05$), *C. riparius* maintained the levels close to control values. This effect was similar in BPA 4h, with inhibition in *P. olivacea* (53%; $p \leq 0.05$) and no effect in *C. riparius* (Fig. 3F). No differences were detected between the two species under BP3 exposures (Fig. 3I).

4. Discussion

The assessment of ecosystem health is a complex task that requires not only a multidisciplinary approach but the use of a variety of biological indicators that can provide a global and detailed overview of the alterations that may be taking place. To this end, model species are very valuable tools, since they have a broad scientific consensus and allow the assessment of environmental disturbances from molecular mechanisms to population dynamics. However, many other species could be considered as good bio-indicators, but for which there is not enough knowledge or research tools.

Sometimes the difficulty lies in the lack of information on a specific aspect of the species of interest, which is more frequent when we move away from the classic ecological targets and move on to newer endpoints, such as molecular biology. For this reason, these newer approaches often require previous work to fine-tune the methodology to be used and to confirm its usefulness. From this perspective, in the present work, we intended to use in the non-model species *P. olivacea* gene expression studies usually carried out in the model species *C. riparius*. Both chironomids have some similar physiological and developmental characteristics (Armitage et al., 1995), coexist in many aquatic ecosystems, and may be useful to assess the health of those environments through molecular biomarkers. However, to date, there is a significant lack of genetic information in the case of *P. olivacea*, which has impeded its use in this type of study.

In this work, we have built a *de novo* transcriptome (Ref. PRJEB36742) of *P. olivacea* from whole 4th instar larvae collected from the Sar River (Galicia, Spain). In addition, in order to assess their response as biomarkers of exposure to xenobiotics, we have identified and characterized for the first time in this species genes related to biotransformation pathways and response to oxidative stress (Table 3): *MnSOD*, *CAT*, *PHGPx*, *Cyp4g15*, *Cyp6a14-like*, and *Cyp6a2-like*. We have also exposed *P. olivacea* larvae to an environmentally relevant concentration (1 µg/L) of BBP, BPA or BP3, in order to test the response of these genetic biomarkers in 4-hour and 24-hour laboratory exposures. And finally, we have compared the transcriptional responses induced by xenobiotics in the non-model species *P. olivacea* with those obtained under the same experimental conditions in the model species *C. riparius*.

When organisms are exposed to a variety of toxic chemicals, reactive oxygen species (ROS) are formed and play an important role in the occurrence of oxidative stress (Macherey and Dansette, 2008). ROS accumulation in cells damages a variety of macromolecules such as nucleic acids, lipids, and proteins. Evolution of an antioxidant defense system let organisms to prevent oxidative damage caused by toxic substances and to maintain body homeostasis (Ighodaro and Akinloye, 2018). Detoxification enzymes are important components of the antioxidant defense system, and genes related to this system are expected to respond to exposure to toxic chemicals (Liu et al., 2019).

The genes manganese superoxide dismutase (*MnSOD*), catalase (*CAT*), and phospholipid-hydroperoxide glutathione peroxidase (*PHGPx*) are part of the first line of defense in the antioxidant response (Ighodaro and Akinloye, 2018). Their role is essential in maintaining the normal metabolism of organisms together with the cytochrome P450 monooxygenases, which are involved in xenobiotic transformation as a detoxification mechanism (Martínez-Paz et al., 2012). *MnSOD* gene encodes for an enzyme that participates in the oxidative stress response; since it catalyzes at the mitochondrial matrix the transformation of superoxide anion radical (O_2^{-}•) to hydrogen

peroxide (H_2O_2) (Sierra et al., 2015). *CAT* catalyzes the degradation or reduction of hydrogen peroxide (H_2O_2) to water and molecular oxygen, as well as *PHGPx*, which breakdowns H_2O_2 into water and lipid peroxides (Ighodaro and Akinloye, 2018).

There are no previous data regarding the effects of BBP, BPA, or BP3 on genes related to detoxification in *P. olivacea.* In the present work, BBP showed the most toxic effects on most of the analyzed genes, with significant repression after 24 hours of exposure, observable even after only 4 hours in some cases (*MnSOD* and *CAT*). Our results are consistent with those observed with laboratory larvae of *C. riparius* exposed up to 96 hours to BBP (Herrero et al., 2015), in which the compound induced time-dependent downregulation of genes related to critical cellular processes, such as stress response, hormonal pathways, energy metabolism, and detoxication activities. However, using larvae from natural populations of *C. riparius*, a previous study with BBP (Herrero et al., 2014) induced a significant overexpression of the genes studied, in line with the observations for this species in the present work and contrary to what we have detected in *P. olivacea*. Taken together, the results of these studies seem to indicate that 1) the genetic history and pressures of each population modify their response to xenobiotic exposure, 2) responses observed in laboratory populations of model organisms may not be extrapolated to natural scenarios, and 3) nearby species may show different sensitivity to the same study conditions, as observed with *P. olivaea*. Also regarding BBP, a previous work with earthworms (Song et al., 2019) showed the activation of CAT enzymatic activity, contrary to the transcriptional inhibition observed in this work for our two study species.

Similar responses were detected in *P. olivacea* under BPA exposure, with general repression on the activity of detoxification genes, especially *CAT* and *Cyp4g15*. Previous results in other organisms reported the BPA- induced downregulation of *Cyp4g15* in *C. riparius* (Martínez-Paz et al., 2012), or *CAT* in the fish *Oryzias javanicus* (Woo et al., 2014). BPA also inhibited the expression of *SOD* in Atlantic salmon kidney cells (Yazdani et al., 2016) and the mollusk *Mytilus galloprovincialis* (Balbi et al., 2016).

In contrast to the repression observed in the *CAT* gene in *P. olivacea*, *C. riparius* exposed 24 hours to BPA triggered an increase in transcript levels. In addition, previous works showed the activation of the catalase enzyme (Lee and Choi, 2007), and the upregulation of this gene in *M. galloprovincialis* after 24 and 48-hour exposures (Balbi et al., 2016). These results might reflect that the transcriptional activity of *CAT* could be species-specific. Considering the significant drop observed in *cyp4g15*, *CAT* and *MnSOD* gene expression in *P.olivacea* for BBP or BPA, the Phase I and II biotransformation processes might be compromised in this species as a consequence of exposure to both xenobiotics.

Finally, BP3 showed completely different effects on the studied genes in *P. olivacea*. *CAT*, *PHGPx*, and *Cyp4g15* showed a time-dependent upregulation in 24-hour exposures, which contrasts with their drop in transcriptional levels induced by BPA and BBP. This effect could be related to a prooxidative state of the larvae. These findings suggest that 1 µg/L BP3 is enough to activate the Phase I and Phase II detoxification responses, and also the oxidative stress response at the genetic level. A previous study reported no effect on genes encoding different P450 cytochromes, in contrast to the activation of Phase II-related genes in *C. riparius* larvae exposed to BP3 (Martínez-Guitarte, 2018).

These novel results suggest that BBP, BPA, and BP3 may compromise the detoxification and the oxidative stress responses in different ways. They provide valuable information about detoxification mechanisms in the non-model species *P. olivacea*, and also about its ability to metabolize these compounds and trigger an adaptive response against toxic exposures in natural scenarios. Additionally, this study reveals new potential biomarkers of toxicity in *P. olivacea* that could be useful in water quality monitoring and complements *C. riparius* data obtained in natural scenarios. Our observations provide a broader view of possible molecular effects of natural stressors in these organisms, as well as novel information about crucial detoxification mechanisms.

Time-dependent alterations were observed in the comparative study of the effects of BBP, BPA, and BP3 on exposed 4th instar larvae. The response of the studied genes differed depending on the species and the pollutant. In general, *C. riparius* showed an upregulation after 24-hour exposure to any of the xenobiotics tested. In contrast, BBP and BPA caused a time-dependent inhibition of *P. olivacea* gene activity. These differences might reflect a prooxidative state in *C. riparius*, which responds to pollutants by activating Phase II reactions, whereas these compounds could compromised the detoxification capacity in *P. olivacea* under the same conditions. A critical aspect of aerobic life is the potentially dangerous effects derived from the imbalance between antioxidant defences and oxidative forces, leading to a prooxidative state. DNA damage, protein and enzymatic inactivation and peroxidation of cells components are some examples of deleterious effects reported in aquatic organisms as a consequence of prooxidative state (Winston and Di Giulio, 1991). The induction observed in *C. riparius* in detoxification genes may be the first step towards to response against oxidant effects of BPA and BBP. This could be translated into a higher capacity to respond to possible harmful effects derived from a prooxidative state. On the other hand, the gene repression observed in *P. olivacea* could point out that these larvae do not activate the detoxification machinery, suggesting a lower capacity to respond to a potentially harmful prooxidative state provoked by xenobiotic exposures. Nevertheless, this new hypothesis makes necessary further studies related to MnSOD, CAT and PHGPx enzymatic variations in both species. The relation between detoxification capacity and adaptation has been previously described, specially related to insects-host plant interaction but also after exposure to xenobiotics. Adaptation to insecticides by insects is given by a more refined detoxification mechanism (Alyokhin and Chen, 2017). As an example, it has been reported that insects that are resistant to host plants (*Helicoverpa armigera*), natural insecticides (*Leptinotarsa decemlineata*) or chemical insecticides (*Drosophila melanogaster*), have upregulated levels of cytochromes

compared to non-exposed and more sensitive individuals (Jin et al., 2019; Zhu et al., 2016; Perry et al., 2011).

This study provides for the first time valuable information about the effects of BBP, BPA, and BP3 in the non-model aquatic dipteran *P. olivacea* at the molecular level. It offers new putative biomarker genes related to detoxification and oxidative stress that could be useful in ecotoxicological studies and risk assessment. The next step in our study will be to investigate new biomarker genes related to relevant metabolic pathways (cell response to stress, immune system, hormonal pathways, etc.) to deepen the physiological response of *P. olivacea* to different xenobiotics. Since BBP, BPA and BP3 have been previously described as endocrine disrupting compounds in *C. riparius* (Herrero et al., 2015; Ozáez et al., 2014; Planelló et al., 2008), ecdysone-responsive genes and genes related to hormone synthesis/degradation are of special interest.

The possible influence of the origin of the larvae must be taken into account in the results obtained. We have previously reported differences among data obtained between laboratory cultures and natural population of *C. riparius* in both the toxicity of BBP and the behavior of some targets (Herrero el al., 2015; Herrero el al., 2014). This emphasizes the need of carrying out studies with different populations to get a more realistic approach to the effects of contaminants. We aim to implement laboratory cultures of *P. olivacea*, to carry out studies in different populations in order to define more precisely the effect targets that are altered by exposure to these compounds, as well as the type of response that they trigger depending on the particular circumstances of a real scenario, where larvae are chronically exposed to complex mixtures of contaminants.

5. Conclusions

The assembled transcriptome of *P. olivacea* is a valuable resource for various derivative applications, such as the identification of potential molecular biomarkers to

deepen the knowledge of the physiological effects on this species caused by exposure to xenobiotics. Besides, the *de novo* genomic information obtained in this non-model species will complement the toxicological studies carried out on model species providing a more global vision of the effects that pollutants cause in aquatic ecosystems.

The differential responses detected between *P. olivacea* and *C. riparius* provide novel information on the harmful effects of BBP, BPA, and BP3 on aquatic midges. In addition, they reflect possible differences in the ability of each species to adapt to altered environments through metabolic pathways related to detoxification and response to oxidative stress. This work illustrates the potential of the analyzed genes to be used as sensitive molecular biomarkers in *P. olivacea*, which could be considered as a suitable sentinel organism for ecotoxicity studies in natural scenarios. The combination of molecular tools and multi-organism testing, with both tolerant and sensitive species, could lead to more robust environmental risk assessments and a better understanding of the risks that emerging contaminants may bring to aquatic biota.

Author statements

Lola Llorente: Investigation, Data Curation, Writing - Original Draft, Formal analysis.

Óscar Herrero: Resources, Methodology, Writing - Review & Editing

Mónica Aquilino: Visualization, Writing - Review & Editing

Rosario Planelló: Resources, Supervision, Funding acquisition, Writing - Review & Editing

Funding: This work was financed by the projects of Universidad Nacional de Educación a Distancia (2018I/PPRO/008) and the Spanish Ministry of Economy and Competitiveness (AGL2015-67733-R). Lola Llorente were co-financed by the Government of the Community of Madrid and the European Social Fund with a research assistant contract (PEJ-2017-AI/BIO-6834) and a post-doctoral contract (PEJD-2019-POST/AMB-16425), respectively. Lola Llorente is currently financed by a UNED-Santander pre-doctoral contract.

Declaration of interests

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

Acknowledgements

The authors thank Dr. Pablo Gómez Sande (Universidad de Santiago de Compostela) for his inestimable assistance during the sampling.

References

Agarwala, R., Barrett, T., Beck, J., Benson, D.A., Bollin, C., Bolton, E., Bourexis, D., Brister, J.R., Bryant, S.H., Canese, K., Cavanaugh, M., Charowhas, C., Clark, K., Dondoshansky, I., Feolo, M., Fitzpatrick, L., Funk, K., Geer, L.Y., Gorelenkov, V., Graeff, A., Hlavina, W., Holmes, B., Johnson, M., Kattman, B., Khotomlianski, V., Kimchi, A., Kimelman, M., Kimura, M., Kitts, P., Klimke, W., Kotliarov, A., Krasnov, S., Kuznetsov, A., Landrum, M.J., Landsman, D., Lathrop, S., Lee, J.M., Leubsdorf, C., Lu, Z., Madden, T.L., Marchler-Bauer, A., Malheiro, A., Meric, P., Karsch-Mizrachi, I., Mnev, A., Murphy, T., Orris, R., Ostell, J., O'Sullivan, C., Palanigobu, V., Panchenko, A.R., Phan, L., Pierov, B., Pruitt, K.D., Rodarmer, K., Sayers, E.W., Schneider, V., Schoch, C.L., Schuler, G.D., Sherry, S.T., Siyan, K., Soboleva, A., Soussov, V., Starchenko, G., Tatusova, T.A., Thibaud-Nissen, F., Todorov, K., Trawick, B.W., Vakatov, D., Ward, M., Yaschenko, E., Zasypkin, A., Zbicz, K., 2018. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res. 46, D8–D13. https://doi.org/10.1093/nar/gkx1095

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–10. https://doi.org/10.1016/S0022-2836(05)80360-2

Alyokhin, A., Chen, Y.H., 2017. Adaptation to toxic hosts as a factor in the evolution of insecticide resistance. Curr. Opin. Insect Sci. 21, 33–38. doi:10.1016/j.cois.2017.04.006.

Andrews, S., 2007. FastQC A Quality Control tool for High Throughput Sequence Data [WWW Document]. URL http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (accessed 7.2.20).

Arambourou, H., Llorente, L., Moreno-Ocio, I., Herrero, Ó., Barata, C., Fuertes, I., Delorme, N., Méndez-Fernández, L., Planelló, R., 2020. Exposure to heavy metal-contaminated sediments disrupts gene expression, lipid profile, and life history

traits in the midge *Chironomus riparius*. Water Res. 168, 115165. https://doi.org/10.1016/j.watres.2019.115165

Arambourou, H., Planelló, R., Llorente, L., Fuertes, I., Barata, C., Delorme, N., Noury, P., Herrero, Ó., Villeneuve, A., Bonnineau, C., 2019. *Chironomus riparius* exposure to field-collected contaminated sediments: From subcellular effect to whole-organism response. Sci. Total Environ. 671, 874–882. https://doi.org/10.1016/J.SCITOTENV.2019.03.384

Arimoro, F.O., Auta, Y.I., Odume, O.N., Keke, U.N., Mohammed, A.Z., 2018. Mouthpart deformities in Chironomidae (Diptera) as bioindicators of heavy metals pollution in Shiroro Lake, Niger State, Nigeria. Ecotoxicol. Environ. Saf. 149, 96–100. https://doi.org/10.1016/j.ecoenv.2017.10.074

Armitage, P.D., Cranston, P.S., Pinder, L.C. V. (Eds.), 1995. The Chironomidae: Biology and ecology of non-biting midges. Springer Netherlands, Dordrecht. https://doi.org/10.1007/978-94-011-0715-0

Balbi, T., Franzellitti, S., Fabbri, R., Montagna, M., Fabbri, E., Canesi, L., 2016. Impact of bisphenol A (BPA) on early embryo development in the marine mussel *Mytilus galloprovincialis*: Effects on gene transcription. Environ. Pollut. 218, 996–1004. https://doi.org/10.1016/j.envpol.2016.08.050

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: A flexible read trimming tool for Illumina NGS data [WWW Document]. URL http://www.usadellab.org/cms/?page=trimmomatic (accessed 7.2.20).

Campos, D., Silva, A.R.R., Loureiro, S., Grabicová, K., Staňová, A.V., Soares, A.M.V.M., Pestana, J.L.T., 2019. Two-generational effects of Benzophenone-3 on the aquatic midge *Chironomus riparius*. Sci. Total Environ. 669, 983–990. https://doi.org/10.1016/j.scitotenv.2019.03.023

Charles, S., Ferreol, M., Chaumot, A., Péry, A.R.R., 2004. Food availability effect on population dynamics of the midge *Chironomus riparius*: a Leslie modeling approach. Ecol. Modell. 175, 217–229.

https://doi.org/10.1016/j.ecolmodel.2003.10.016

Cheimonopoulou, M.T., Bobori, D.C., Theocharopoulos, I., Lazaridou, M., 2011. Assessing ecological water quality with macroinvertebrates and fish: a case study from a small Mediterranean river. Environ. Manage. 47, 279–90. https://doi.org/10.1007/s00267-010-9598-8

Conesa, A., Götz, S., García-Gómez, J.M., Terol, J., Talón, M., Robles, M., 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21, 3674–3676. https://doi.org/10.1093/bioinformatics/bti610

CPSC, 2010. Toxicity Review for Benzyl-n-butyl Phthalate (Benzyl Butyl Phthalate or BBP). U.S. Consumer Product Safety Commission, Bethesda.

Ding, Y., Weston, D.P., You, J., Rothert, A.K., Lydy, M.J., 2011. Toxicity of Sediment-Associated Pesticides to *Chironomus dilutus* and *Hyalella azteca*. Arch. Environ. Contam. Toxicol. 61, 83–92. https://doi.org/10.1007/s00244-010-9614-2

EC, 2008. Scientific Committee on Consumer Products opinion on Benzophenone-3 [SCCP/1201/08]. European Commission, Brussels.

EC, 2007. European Union Risk Assessment Report - benzyl butyl phthalate (BBP). European Commission, Luxembourg.

ECHA, 2020a. 4,4'-isopropylidenediphenol - Substance Information [WWW Document]. InfoCard. URL https://echa.europa.eu/es/substance-information/-/substanceinfo/100.001.133 (accessed 6.8.20).

ECHA, 2020b. Bisphenol A [WWW Document]. InfoCard. URL https://echa.europa.eu/hot-topics/bisphenol-a (accessed 6.9.20).

ECHA, 2020c. Oxybenzone - Substance Information [WWW Document]. InfoCard. URL https://echa.europa.eu/substance-information/-/substanceinfo/100.004.575 (accessed 6.8.20).

EFSA, 2019. Update of the risk assessment of di-butylphthalate (DBP), butyl-benzyl-phthalate (BBP), bis(2-ethylhexyl)phthalate (DEHP), di-isononylphthalate (DINP)

and di-isodecylphthalate (DIDP) for use in food contact materials. EFSA J. 17, 5838. https://doi.org/10.2903/j.efsa.2019.5838

Felsenstein, J., 1985. Confidence Limits on Phylogenies: An Approach Using the Bootstrap. Evolution (N. Y). 39, 783. https://doi.org/10.2307/2408678

Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B.W., Nusbaum, C., Lindblad-Toh, K., Friedman, N., Regev, A., 2011. Trinity: reconstructing a full-length transcriptome without a genome from RNA-Seq data. Nat. Biotechnol. 29, 644–652. https://doi.org/10.1038/nbt.1883

Gresens, S., Smith, R., Sutton-Grier, A., Kenney, M., 2009. Benthic macroinvertebrates as indicators of water quality: The intersection of science and policy. Terr. Arthropod Rev. 2, 99–128. https://doi.org/10.1163/187498209X12525675906077

Herrero, Ó., Aquilino, M., Sánchez-Argüello, P., Planelló, R., 2018. The BPA-substitute bisphenol S alters the transcription of genes related to endocrine, stress response and biotransformation pathways in the aquatic midge *Chironomus riparius* (Diptera, Chironomidae). PLoS One 13, e0193387. https://doi.org/10.1371/journal.pone.0193387

Herrero, Ó., Planelló, R., Morcillo, G., 2016. The ribosome biogenesis pathway as an early target of benzyl butyl phthalate (BBP) toxicity in *Chironomus riparius* larvae. Chemosphere 144, 1874–84. https://doi.org/10.1016/j.chemosphere.2015.10.051

Herrero, Ó., Planelló, R., Morcillo, G., 2015. The plasticizer benzyl butyl phthalate (BBP) alters the ecdysone hormone pathway, the cellular response to stress, the energy metabolism, and several detoxication mechanisms in *Chironomus riparius* larvae. Chemosphere 128, 266–77. https://doi.org/10.1016/j.chemosphere.2015.01.059

Herrero, O., Planelló, R., Gómez-Sande, P., Aquilino, M., Morcillo, G., 2014. Evaluación de los efectos tóxicos de ftalatos sobre poblaciones naturales de

Chironomus riparius (Diptera): implicaciones en estudios de ecotoxicidad [Evaluation of the toxic effects of phthalates on natural populations of *Chironomus riparius* (Diptera): implications for ecotoxicity studies]. *Revista de Toxicología* 31(2): 176-186. http://rev.aetox.es/wp/index.php/3122-10

Ighodaro, O.M., Akinloye, O.A., 2018. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. Alexandria J. Med. 54, 287–293. https://doi.org/10.1016/j.ajme.2017.09.001

Ilkova, J., Michailova, P., Szarek-Gwiazda, E., Kownacki, A., Ciszewski, D., 2018. *Prodiamesa olivacea* Meigen and *Prodiamesa bureshi* Michailova (Diptera, Chironomidae, Prodiamesinae) as a candidate for assessing the genotoxicity of trace metals in fluvial sediments. Environ. Monit. Assess. 190, 542. https://doi.org/10.1007/s10661-018-6928-4

Ilkova, J., Michailova, P., Szarek-Gwiazda, E., Kownacki, A., Ciszewski, D., 2017. The Response of Genome of the Chironomidae (Diptera) to Heavy Metal Pollution in Two Rivers of Southern Poland. Acta Zool. Bulg. 8, 9–15.

Im, J., Chatterjee, N., Choi, J., 2019. Genetic, epigenetic, and developmental toxicity of *Chironomus riparius* raised in metal-contaminated field sediments: A multi-generational study with arsenic as a second challenge. Sci. Total Environ. 672, 789–797. https://doi.org/10.1016/j.scitotenv.2019.04.013

Jin, M., Liao, C., Fu, X., Holdbrook, R., Wu, K., Xiao, Y., Adaptive regulation of detoxification enzymes in *Helicoverpa armigera* to different host plants. Insect Mol. Biol. 28(5), 628-636. doi:10.1111/imb.12578

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772–80. https://doi.org/10.1093/molbev/mst010

Khosrovyan, A., Kahru, A., 2020. Evaluation of the hazard of irregularly-shaped co-polyamide microplastics on the freshwater non-biting midge *Chironomus riparius*

through its life cycle. Chemosphere 244, 125487. https://doi.org/10.1016/j.chemosphere.2019.125487

Kim, S., Choi, K., 2014. Occurrences, toxicities, and ecological risks of benzophenone-3, a common component of organic sunscreen products: A mini-review. Environ. Int. 70, 143–157. https://doi.org/10.1016/j.envint.2014.05.015

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol. Biol. Evol. 35, 1547–1549. https://doi.org/10.1093/molbev/msy096

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–8. https://doi.org/10.1093/bioinformatics/btm404

Lee, S.-B., Choi, J., 2007. Effects of bisphenol A and ethynyl estradiol exposure on enzyme activities, growth and development in the fourth instar larvae of *Chironomus riparius* (Diptera, Chironomidae). Ecotoxicol. Environ. Saf. 68, 84–90. https://doi.org/10.1016/j.ecoenv.2006.07.003

Lee, S.-W., Kim, S.-M., Choi, J., 2009. Genotoxicity and ecotoxicity assays using the freshwater crustacean *Daphnia magna* and the larva of the aquatic midge *Chironomus riparius* to screen the ecological risks of nanoparticle exposure. Environ. Toxicol. Pharmacol. 28, 86–91. https://doi.org/10.1016/j.etap.2009.03.001

Liu, H., Zhang, J., Yuan, J., Jiang, X., Jiang, L., Zhao, G., Huang, D., Liu, B., 2019. Omics-based analyses revealed metabolic responses of *Clostridium acetobutylicum* to lignocellulose-derived inhibitors furfural, formic acid and phenol stress for butanol fermentation. Biotechnol. Biofuels 12, 101. https://doi.org/10.1186/s13068-019-1440-9

Macherey, A.C., Dansette, P.M., (2008).Biotransformations Leading to Toxic Metabolites: Chemical Aspects, in: Wermuth, C. (Ed.), The Practice of Medicinal

Chemistry: Third Edition. Academic Press, pp. 674–696. doi:10.1016/B978-0-12-417205-0.00025-0.

Martín-Folgar, R., Aquilino, M., Ozáez, I., Martínez-Guitarte, J.-L., 2018. Ultraviolet filters and heat shock proteins: effects in *Chironomus riparius* by benzophenone-3 and 4-methylbenzylidene camphor. Environ. Sci. Pollut. Res. 25, 333–344. https://doi.org/10.1007/s11356-017-0416-1

Martínez-Guitarte, J.-L., 2018. Transcriptional activity of detoxification genes is altered by ultraviolet filters in Chironomus riparius. Ecotoxicol. Environ. Saf. 149, 64–71. https://doi.org/10.1016/j.ecoenv.2017.11.017

Martínez-Guitarte, J.L., Planelló, R., Morcillo, G., 2007. Characterization and expression during development and under environmental stress of the genes encoding ribosomal proteins L11 and L13 in *Chironomus riparius*. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 147, 590–6. https://doi.org/10.1016/j.cbpb.2007.03.015

Martínez-Paz, P., Morales, M., Martínez-Guitarte, J.L., Morcillo, G., 2012. Characterization of a cytochrome P450 gene (CYP4G) and modulation under different exposures to xenobiotics (tributyltin, nonylphenol, bisphenol A) in *Chironomus riparius* aquatic larvae. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 155, 333–43. https://doi.org/10.1016/j.cbpc.2011.10.001

Martínez-Paz, P., Morales, M., Urien, J., Morcillo, G., Martínez-Guitarte, J.L., 2017. Endocrine-related genes are altered by antibacterial agent triclosan in *Chironomus riparius* aquatic larvae. Ecotoxicol. Environ. Saf. 140, 185–190. https://doi.org/10.1016/j.ecoenv.2017.02.047

Meehan, S., Shannon, A., Gruber, B., M. Rackl, S., E. Lucy, F., 2014. Ecotoxicological impact of Zequanox®, a novel biocide, on selected non-target Irish aquatic species. Ecotoxicol. Environ. Saf. 107, 148–153. https://doi.org/10.1016/j.ecoenv.2014.05.017

Michailova, P. V, Ilkova, J., White, K., 2003. Cytogenetic alterations in Prodiamesinae

species (Diptera, Chironomidae) from different polluted regions. Folia Biol. (Praha). 51, 69–79.

Monteiro, H.R., Pestana, J.L.T., Novais, S.C., Soares, A.M.V.M., Lemos, M.F.L., 2019. Toxicity of the insecticides spinosad and indoxacarb to the non-target aquatic midge *Chironomus riparius*. Sci. Total Environ. 666, 1283–1291. https://doi.org/10.1016/j.scitotenv.2019.02.303

Muñiz-González, A.-B., Martínez-Guitarte, J.-L., 2020. Combined effects of benzophenone-3 and temperature on gene expression and enzymatic activity in the aquatic larvae *Chironomus riparius*. Sci. Total Environ. 698, 134292. https://doi.org/10.1016/j.scitotenv.2019.134292

Muñiz-González, A.-B., Martínez-Guitarte, J.-L., 2018. Effects of single exposure and binary mixtures of ultraviolet filters octocrylene and 2-ethylhexyl 4-(dimethylamino) benzoate on gene expression in the freshwater insect *Chironomus riparius*. Environ. Sci. Pollut. Res. 25, 35501–35514. https://doi.org/10.1007/s11356-018-3516-7

Nair, P.M.G., Park, S.Y., Choi, J., 2012. Characterization and expression analysis of phospholipid hydroperoxide glutathione peroxidase cDNA from *Chironomus riparius* on exposure to cadmium. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 163, 37–42. https://doi.org/10.1016/j.cbpb.2012.04.004

Nair, P.M.G., Park, S.Y., Choi, J., 2011. Expression of catalase and glutathione S-transferase genes in *Chironomus riparius* on exposure to cadmium and nonylphenol. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 154, 399–408. https://doi.org/10.1016/j.cbpc.2011.07.008

OECD, 2011. Test No. 235: *Chironomus* sp., Acute Immobilisation Test. Organisation for Economic Co-operation and Development, Paris.

OECD, 2010. Test No. 233: Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment. Organisation for Economic Co-operation and Development, Paris.

OECD, 2006. Detailed review paper on aquatic arthropods in life cycle toxicity tests with an empashis on developmental, reproductive and endocrine disruptive effects. OECD Series on Testing and Assessment. Number 55. Organisation for Economic Co-operation and Development, Paris.

OECD, 2004a. Test No. 218: Sediment-Water Chironomid Toxicity Test Using Spiked Sediment. Organisation for Economic Co-operation and Development, Paris.

OECD, 2004b. Test No. 219: Sediment-Water Chironomid Toxicity Test Using Spiked Water. Organisation for Economic Co-operation and Development, Paris.

Ospina-Pérez, E.M., Campeón-Morales, O.I., Richardi, V.S., Rivera-Páez, F.A., 2019. Histological description of immature *Chironomus columbiensis* (Diptera: Chironomidae): A potential contribution to environmental monitoring. Microsc. Res. Tech. 82, 1277–1289. https://doi.org/10.1002/jemt.23278

Ozáez, I., Aquilino, M., Morcillo, G., Martínez-Guitarte, J.-L., 2016. UV filters induce transcriptional changes of different hormonal receptors in *Chironomus riparius* embryos and larvae. Environ. Pollut. 214, 239–247. https://doi.org/10.1016/j.envpol.2016.04.023

Ozáez, I., Martínez-Guitarte, J.L., Morcillo, G., 2014. The UV filter benzophenone 3 (BP-3) activates hormonal genes mimicking the action of ecdysone and alters embryo development in the insect *Chironomus riparius* (Diptera). Environ Pollut. 192:19–26. doi:10.1016/j.envpol.2014.04.038

Park, S.-Y., Nair, P.M.G., Choi, J., 2012. Characterization and expression of superoxide dismutase genes in *Chironomus riparius* (Diptera, Chironomidae) larvae as a potential biomarker of ecotoxicity. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 156, 187–94. https://doi.org/10.1016/j.cbpc.2012.06.003

Perry, T., Batterham, P., Daborn, P.J., 2011.The biology of insecticidal activity and resistance. Insect Biochem. Mol. Biol. 41, 411–422. doi:10.1016/j.ibmb.2011.03.003.

Planelló, R., Servia, M.J., Gómez-Sande, P., Herrero, Ó., Cobo, F., Morcillo, G., 2015.

Transcriptional responses, metabolic activity and mouthpart deformities in natural populations of *Chironomus riparius* larvae exposed to environmental pollutants. Environ. Toxicol. 30, 383–95. https://doi.org/10.1002/tox.21893

Planelló, R., Herrero, O., Martínez-Guitarte, J.L., Morcillo, G., 2011. Comparative effects of butyl benzyl phthalate (BBP) and di(2-ethylhexyl) phthalate (DEHP) on the aquatic larvae of *Chironomus riparius* based on gene expression assays related to the endocrine system, the stress response and ribosomes. Aquat. Toxicol. 105, 62–70. https://doi.org/10.1016/j.aquatox.2011.05.011

Planelló, R., Martínez-Guitarte, J.L., Morcillo, G., 2008. The endocrine disruptor bisphenol A increases the expression of HSP70 and ecdysone receptor genes in the aquatic larvae of *Chironomus riparius*. Chemosphere 71, 1870–6. https://doi.org/10.1016/j.chemosphere.2008.01.033

Prego, R., Belzunce Segarra, M.J., Cobelo-García, A., Helios-Rybicka, E., 2008. Particulate metal in the Ulla River estuary: State and sources of contamination (Arosa Ria, NW Iberian Peninsula). Ciencias Mar. 34, 381–388.

R Core Team, 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Ramos, S., Homem, V., Alves, A., Santos, L., 2015. Advances in analytical methods and occurrence of organic UV-filters in the environment — A review. Sci. Total Environ. 526, 278–311. https://doi.org/10.1016/j.scitotenv.2015.04.055

Ren, J., Wen, L., Gao, X., Jin, C., Xue, Y., Yao, X., 2009. DOG 1.0: illustrator of protein domain structures. Cell Res. 19, 271–273. https://doi.org/10.1038/cr.2009.6

Rosenberg, D.M., 1992. Freshwater biomonitoring and Chironomidae. Netherlands J. Aquat. Ecol. 26, 101–122. https://doi.org/10.1007/BF02255231

Sánchez, P., Alonso, C., Fernández, C., Vega, M.M., García, M.P., Tarazona, J. V., 2005. Evaluation of a Multi-species Test System for Assessing Acute and Chronic Toxicity of Sediments and Water to Aquatic Invertebrates: Effects of Pentachlorophenol on *Daphnia magna* and *Chironomus prasinus*. J. Soils

Sediments 5, 53–58. https://doi.org/10.1065/jss2004.10.114

Sánchez, P., Tarazona, J. V, 2002. Development of a multispecies system for testing reproductive effects on aquatic invertebrates. Experience with *Daphnia magna*, *Chironomus prasinus* and *Lymnaea peregra*. Aquat. Toxicol. 60, 249–56. https://doi.org/10.1016/S0166-445X(02)00014-0

Servia, M.J., Cobo, F., González, M.A., 2000. Seasonal and Interannual Variations in the Frequency and Severity of Deformities in Larvae of *Chironomus riparius* (Meigen, 1804) and *Prodiamesa olivacea* (Meigen, 1818) (Diptera, Chironomidae) collected in a polluted site. Environ. Monit. Assess. 64, 617–626. https://doi.org/10.1023/A:1006333808107

Servia, M.J., Cobo, F., González, M.A., 1998. Deformities in larval *Prodiamesa olivacea* (Meigen, 1818) (Diptera, Chironomidae) and their use as bioindicators of toxic sediment stress. Hydrobiologia 385, 153–162. https://doi.org/10.1023/A:1003466012110

Sierra, H., Cordova, M., Chen, C.-S.J., Rajadhyaksha, M., 2015. Confocal imaging-guided laser ablation of basal cell carcinomas: an ex vivo study. J. Invest. Dermatol. 135, 612–615. https://doi.org/10.1038/jid.2014.371

Sokal, R.R., Michener, C.D., 1958. A statistical method for evaluating systematic relationships. Univ. Kansas Sci. Bull. 38, 1409–1438.

Song, P., Gao, J., Li, X., Zhang, C., Zhu, L., Wang, Jinhua, Wang, Jun, 2019. Phthalate induced oxidative stress and DNA damage in earthworms (*Eisenia fetida*). Environ. Int. 129, 10–17. https://doi.org/10.1016/j.envint.2019.04.074

Tang, G., Yao, J., Zhang, X., Lu, N., Zhu, K.Y., 2018. Comparison of gene expression profiles in the aquatic midge (*Chironomus tentans*) larvae exposed to two major agricultural pesticides. Chemosphere 194, 745–754. https://doi.org/10.1016/j.chemosphere.2017.12.040

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S.G., 2012. Primer3—new capabilities and interfaces. Nucleic Acids Res.

40, e115–e115. https://doi.org/10.1093/nar/gks596

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, research0034.1. https://doi.org/10.1186/gb-2002-3-7-research0034

Weis, J.S., 2014. Physiological, Developmental and Behavioral Effects of Marine Pollution, Physiological, Developmental and Behavioral Effects of Marine Pollution. Springer Netherlands, Dordrecht. https://doi.org/10.1007/978-94-007-6949-6

Winston, G.W., Di Giulio, R.T., 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. Aquat. Toxicol. 19, 137–161. https://doi.org/10.1016/0166-445X(91)90033-6

Woo, S., Denis, V., Yum, S., 2014. Transcriptional Changes Caused by Bisphenol A in *Oryzias javanicus*, a Fish Species Highly Adaptable to Environmental Salinity. Mar. Drugs 12, 983–998. https://doi.org/10.3390/md12020983

Yazdani, M., Andresen, A.M.S., Gjøen, T., 2016. Short-term effect of bisphenol-a on oxidative stress responses in Atlantic salmon kidney cell line: a transcriptional study. Toxicol. Mech. Methods 26, 295–300. https://doi.org/10.1080/15376516.2016.1177864

Zhu, F., Moural, T.W., Nelson, D.R., Palli, S.R., 2016. A specialist herbivore pest adaptation to xenobiotics through up-regulation of multiple Cytochrome P450s. Sci. Rep. 6, 1–10. doi:10.1038/srep20421.

Zuckerkandl, E., Pauling, L., 1965. Evolutionary divergence and convergence in proteins, in: Bryson, V., Vogel, H.J. (Eds.), Evolving Genes and Proteins. Academic Press, New York, pp. 97–166.

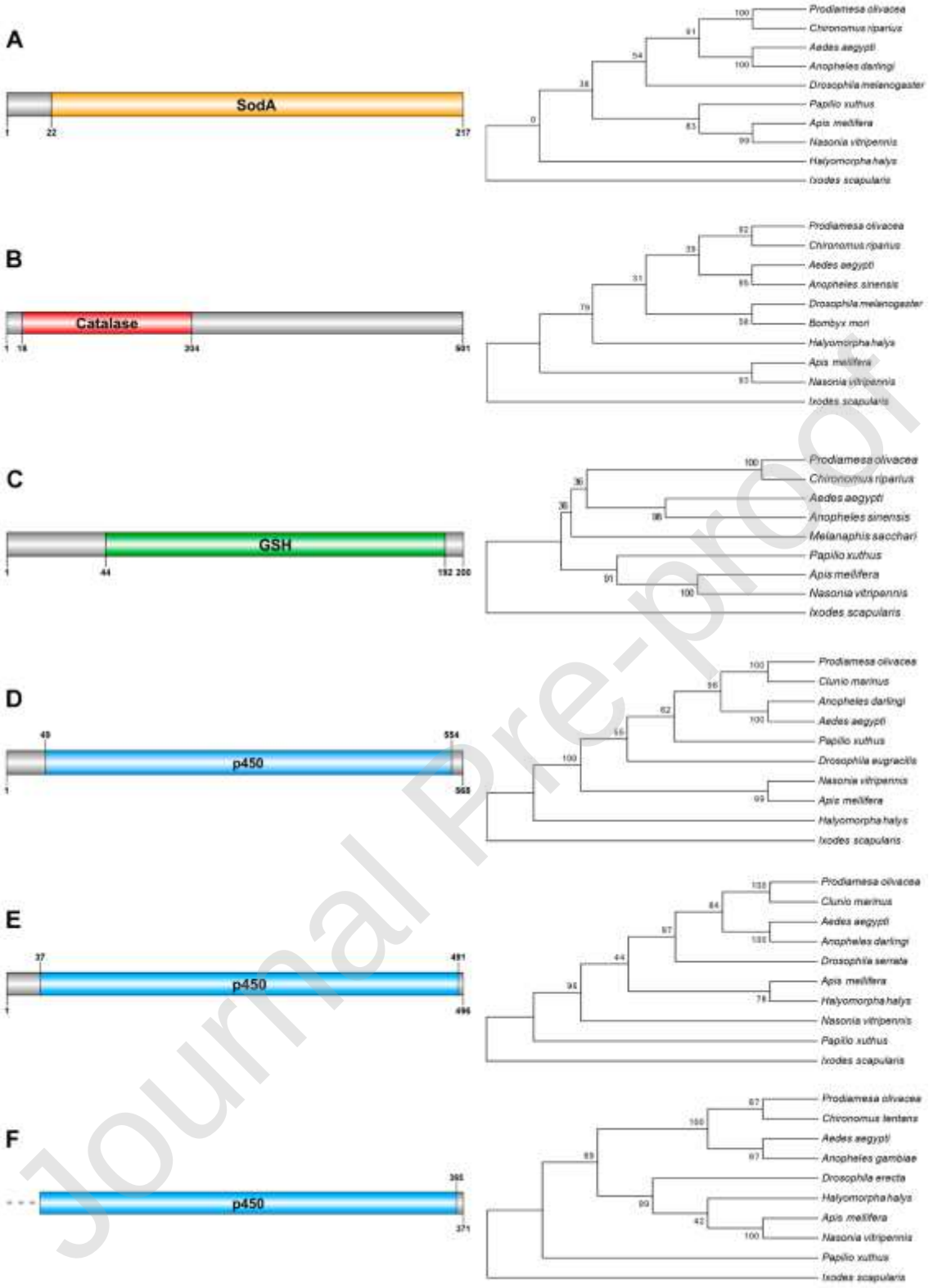

Figure 1. Detoxification and oxidative stress proteins of *P. olivacea* identified from its *de novo* transcriptome. Left) Diagram of the proteins identified as putative mRNAs, and their conserved domains: *MnSOD (A)*, *CAT (B)*, *PHGPx (C)*, *Cyp4g15*

(D), *Cyp6a14-like (E)*, *Cyp6a2-like* (F). SodA: Superoxide dismutase conserved protein domain, GSH: Glutathione peroxidase protein domain family, p450: Cytochrome p450 protein domain. Right) Bootstrap consensus tree inferred from 1000 replicates. Branches corresponding to partitions reproduced in less than 40% bootstrap replicates are collapsed. The evolutionary distances are in units of number of amino acid substitutions per site.

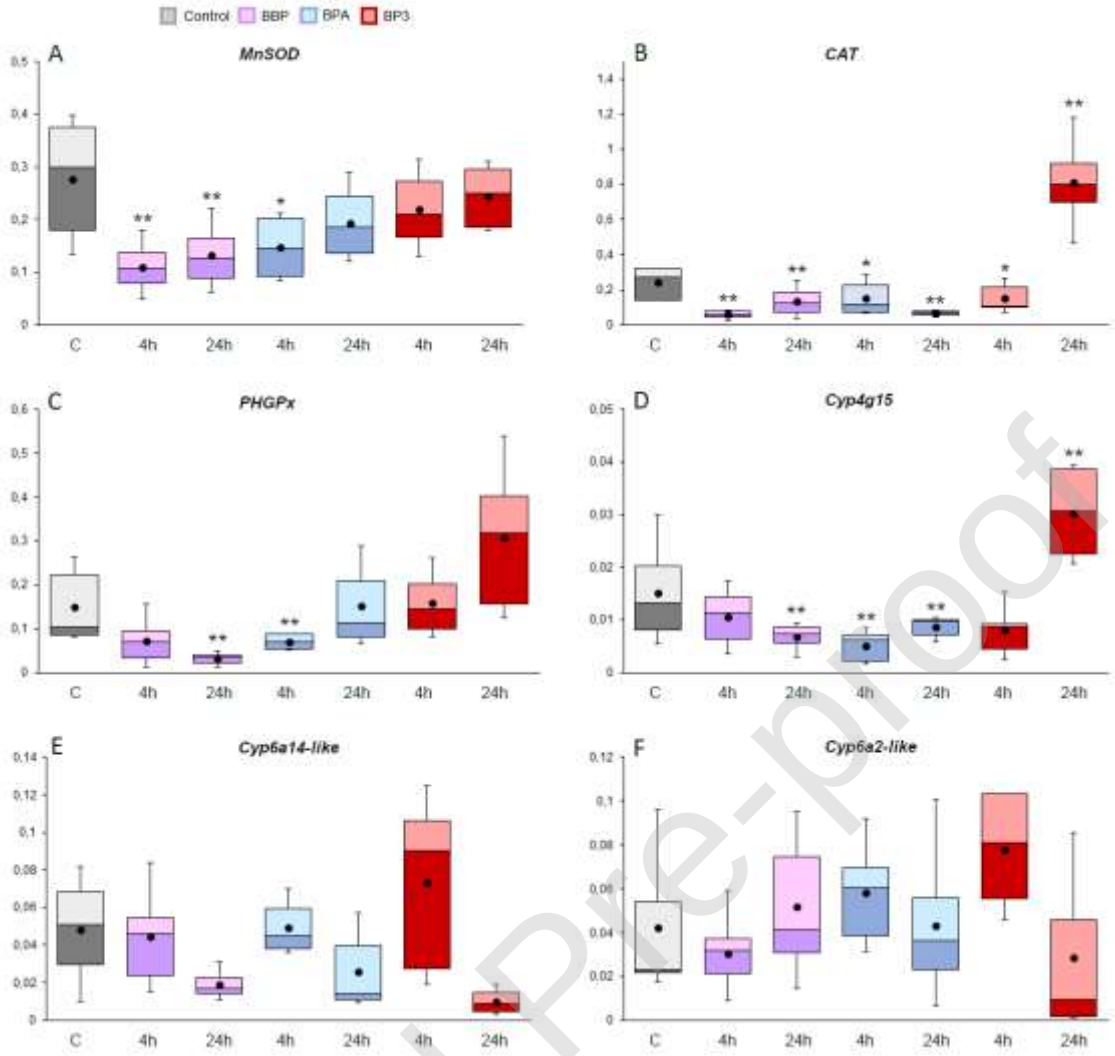

Figure 2. Transcriptional activity of *MnSOD*, *CAT*, *PHGPx*, *Cyp4g15*, *Cyp6a14-like*, and *Cyp6a2-like* in *P. olivacea* exposed to BBP, BPA, and BP3. Box and whisker plots represent the expression patterns of *MnSOD* (A), *CAT* (B), *PHGPx* (C), *Cyp4g15* (D), *Cyp6a14-like* (E) and *Cyp6a2-like* (F), measured by real-time RT-PCR. Box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; mean is represented by a dot; the horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control values: $p \leq 0.1$ (*) or $p \leq 0.05$ (**).

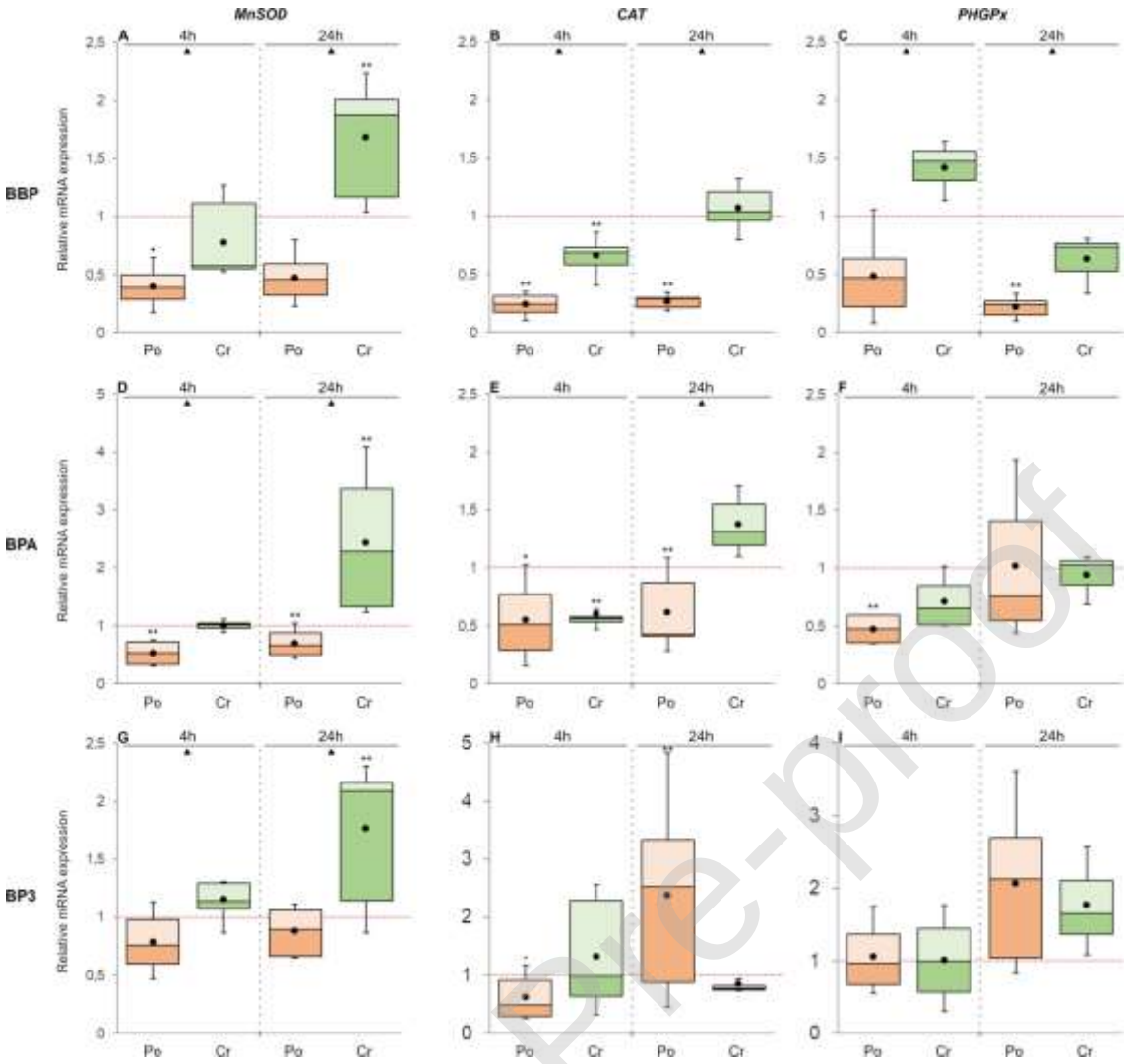

Figure 3. *P. olivacea* and *C. riparius* transcriptional activity of *MnSOD*, *CAT,* and *PHGPx* genes after exposure to BBP, BPA, and BP3. Box and whisker plots represent the expression patterns of *MnSOD*, *CAT*, and *PHGPx* measured by real-time RT-PCR. Results were normalized to the control values. A horizontal red dotted line represents the control values. Box and whiskers represent the 25-75 percentile and the minimum/maximum measured values; mean is represented by a dot; horizontal line separating the lower (dark) and the upper (light) area represents the median. Asterisks indicate significant differences with respect to control values: $p \leq 0.1$ (*) or $p \leq 0.05$ (**). Triangles (▲) indicate significant differences ($p \leq 0.05$) between species.

Gene	Primer sequence (5'-3')	Fragment size
CAT-Po	**F** CGCCAAGGGTGAAGAAGTTTT **R** CCAACAGGAATCAACGGGAAT	270 bp
MnSOD-Po	**F** GTCACCCGAGAAGTCAAATCCTT **R** TGCCAAACAATGGAACGAGA	227 bp
PHGPx-Po	**F** TGCATGACCAGGATTTCCCTA **R** TTCTGAGCCTTCCCGTTCTG	203 bp
Cyp6a2-Po	**F** CCTCCTGCAACAATTATCAATCG **R** CGCCGAATGGGAGTAAGACA	217 bp
Cyp6a14-Po	**F** TTGTTGGCGTTGGAATTGTTC **R** TCGGATCATGCTTTTCGTTAAAG	213 bp
Cyp4g15-Po	**F** GACATTGATGAGAATGATGTTGGTG **R** TAAGTGGAACTGGTGGGTACAT	232 bp
CAT-Cr	**F** CGTGATCTTCGTGGTTTTGCTG **R** GGATTGGATCGCGGATGAAG	100 bp (Nair et al., 2011)
MnSOD-Cr	**F** CTGATGCACTCCAAAAAGCA **R** AACTCCAACAGCAGCGACTT	86 bp (Park et al., 2012)
PHGPx-Cr	**F** AAGTGTGGTTACACAGCTAAGCATT **R** GATATCCAAATTGATTACACGGAAA	112 bp (Nair et al., 2012)
ACTIN-Po/Cr	**F** GATGAAGATCCTCACCGAACG **R** CGGAAACGTTCATTACCG	201 bp (Martínez-Guitarte et al., 2007)
26S-Po/Cr	**F** TTCGCGACCTCAACTCATGT **R** CCGCATTCAAGCTGGACTTA	220 bp (Planelló et al., 2011)

Table 1. Primers used for cDNA sequencing and qPCR analyses in *P. olivacea* (Po) and *C. riparius* (Cr). Forward (F) and reverse (R) sequences, length of the amplified fragments, and origin of primers (when corresponds) are detailed.

	Species	Exposure time (h)	Control	BBP	BPA	BP3
Survival rate (mean ± sd)	***P. olivacea***	4	99.3 ± 0	96.8 ± 1.6	96.7 ± 3.3	100 ± 0
		24	100 ± 0	**88.3 ± 1.67***	**85 ± 4.47***	**85 ± 0*+**
	C. riparius	4	100 ± 0	100 ± 0	100 ± 0	100 ± 0
		24	100 ± 0	**86.7 ± 1.6***	93.3 ± 4.4	**95.8 ± 3.3*+**

Table 2. Larval survival rates in *P. olivacea* and *C. riparius* 4th instar larvae exposed during 4h or 24h to 1 µg/L BBP, BPA, and BP3. Results are expressed as the mean of three biological replicates ± standard deviation (n = 135 larvae/condition). (*) Significant differences with respect to the corresponding control ($p \leq 0.05$). (+) Significant differences for the same condition between the two species ($p \leq 0.05$).

Gene	ORF length	Protein length	Accession number
MnSOD	654 bp	217 aa	MN862061
CAT	1506 bp	501 aa	MN862062
PHGPx	603 bp	200 aa	MN862064
Cyp4g15	1707 bp	568 aa	MN862063
Cyp6a14-like	1491 bp	496 aa	MN862066
Cyp6a2-like	1117 bp (incomplete)	371 aa (incomplete)	MN862065

Table 3. *De novo* characterized *P. olivacea* genes. Gene name, ORF and protein lengths of the *de novo* characterized *P. olivacea* genes as well as their database accession number.