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Altered gene expression in *Chironomus riparius* (insecta) in response to tire rubber and polystyrene microplastics☆

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ABSTRACT

The extent until which plastics are present in our surrounding environment completely exceeds our expectations. Plastic materials in the form of microplastics have been found in terrestrial, freshwater and marine environments and are transported through the atmosphere even to remote locations. However, we are still far from understanding the effects that they may have caused and are causing to biota. In the present study, we investigated the alterations in the expression of twelve genes in the aquatic insect *Chironomus riparius* after 36 h exposures to polystyrene and tire rubber microplastics at nominal concentrations of 1 and 10 mg L^{-1}. The results indicated that several genes encoding for heat shock proteins (*hsp90,* Glycoprotein 93 *(Gp93), hsc70, hsp60, hsp40*, and the small HSP *hsp17*) were overexpressed respect to the control. In addition, the genes coding for manganese superoxide dismutase (*SOD Mn*, related to alleviation of oxidative stress) and for the FK506-binding protein of 39 kDa. (*FKBP39*, related to development and pupation) showed altered expression. Most of the alterations on gene expression level occurred at a concentration of 10 mg L^{-1} of tire rubber microplastics, although specific modifications arose at other concentrations of both rubber and polystyrene. On the contrary, one *hsp* gene (*hsp10*) and genes related to biotransformation and detoxification (*Cyp9f2*, *Cyp12a2*, and *ABCB6*) did not alter their expression in any of the treatments. Overall, the results of the gene expression indicated that microplastics (especially tire rubber) or their additives caused cellular stress that led to some alterations in the normal gene expression but did not cause any mortality after 36 h. These results highlight the need for more studies that describe the alterations caused by microplastics at the molecular level. Additionally, it opens questions about the effects caused to aquatic fauna in environmental realistic situations, especially in hot spots of microplastic contamination (e.g., tire rubber released in storm water runoff discharge points).

1. Introduction

The contamination of waters with plastics and microplastics is one of the most important and distressing water risks and chemical pollution episodes that has ever existed during the history of humankind. Although the issue has produced an enormous concern among all levels of stakeholders, these risks are not yet completely defined. Partially, the main reason is that the extent until what microplastics are present in the planet is still not fully understood, as we are likely underestimating the amount of plastic in the water environment (Lindeque et al., 2020).

To date, there is not a clear consensus on whether microplastics are a real risk to the environment. Despite the myriad of articles reporting toxic effects of microplastics, the reported concentrations Worldwide are not high enough to cause toxic effects on organisms (Burns & Boxall, 2018). Therefore, there is a need to conduct more studies at all levels of biological organization to achieve results that show realistic effects for the protection of our environment.

To date, the evaluation of microplastics on aquatic organisms has mainly focused on survival or growth alterations (Lin et al., 2019; Silva et al., 2019). However, at the molecular level studies are scarce

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(Franzellitti et al., 2019; Zhang et al., 2020) but necessary to know the underlying mechanisms by which microplastics may be potentially toxic. In this line, there is a growing number of studies reporting that microplastics cause oxidative stress or an activation of the mechanisms to ameliorate it (e.g., Jeong et al., 2016; Liu et al., 2021; Silva et al., 2021).

In the present study, we used two commonly used plastic materials: polystyrene and automobile tire rubber. Polystyrene (PS) is a thermoplastic from the monomer styrene, confirmed to be carcinogenic (National Research Council, 2014) and found as plastic pollution in marine and freshwater environments (e.g.: Uurasjärvi et al., 2020). Recent studies have evaluated the effects of PS on aquatic organisms, causing behavioural alterations in *Daphnia magna* (Lin et al., 2019; Wang et al., 2020). In sediment exposures, a decrease in the growth of *Arenicola marina* (Besseling et al., 2013) and *Gammarus pulex* (Redondo-Hasselerharm et al., 2018b) was reported, but in the latter study *G. pulex* was the only freshwater invertebrate of six showing toxic effects. Furthermore, some studies have evaluated the molecular response in aquatic organisms after exposure to PS microplastics, performing a massive transcriptome analysis in shrimps or corals (Tang et al., 2018; Suman et al., 2020). In addition, oxidative stress is commonly found in exposures to PS (Jeong et al., 2016; Li et al., 2020; Liu et al., 2021; Ribeiro et al., 2017; Yu et al., 2018). In long-term field experiments, PS nano- and microplastics affected invertebrate communities (Redondo-Hasselerharm et al., 2020), although the effects were detected at concentrations which are not environmentally relevant as of today.

Regarding tire rubber, despite it may not strictly be considered as a plastic material, its synthetic origin and use as a commodity material in our modern society justify its addition to this category. Recently, Halle et al. (2020) have wisely suggested the term "microrubber" to comprise the wide terminology related to micronized rubber particles and their inclusion in the broad category of "anthropogenic polymer particulates". Although their sources are varied, it is generally accepted that automobile transit is the main cause of microrubber emissions, that are estimated to be almost 6 million tonnes per year (Kole et al., 2017). Between 0.1 and 10% of these are projected to reach surface waters (Wagner et al., 2018). The use of tire rubber is not strictly limited to transport as for example, tire rubber crumbs are used in artificial turf sport fields to increment the life cycle of these materials. This may create new scenarios for the unintentional discharge of microrubber.

The toxicity of microrubber to aquatic organisms has been the subject of few studies, examining the effects of the particles in water (Khan et al., 2019) and sediment (Panko et al., 2013; Redondo-Hasselerharm et al., 2018a). While the latter did not reveal any toxicity to a wide array of benthic invertebrates, the water experiments detected toxic effects to *Hyalella azteca* at 500 particles ml^{-1} (0.145 g L^{-1}). In addition, the EC_{50} for a range of aquatic species was established in the range of 0.01–7.45 g of tire rubber L^{-1}, considering the compounds leaching from these amounts of rubber (Wik et al., 2009).

The focus of microplastic research has been mostly performed about marine waters (Blettler et al., 2018), but soils and freshwaters eventually were found to be affected. Inland waters and their biota are a common sink to plastic particles (Bellasi et al., 2020; Xu et al., 2020) and therefore, freshwater biota may be at risk due to microplastic contamination.

Among freshwater biota, *Chironomus riparius* is an insect belonging to the family of chironomidae (~7500 species). Chironomids are a key species in freshwater ecosystems (Tokeshi, 1995) and a link to terrestrial riparian habitats (Baxter et al., 2005). As benthic dwellers during their larval stage, chironomids may be threatened by pollutants associated with water and sediments, being an ideal model animal for ecotoxicological studies (e.g., OECD, 2010). Polyethylene (PE) microplastics affected the normal life cycle of *C. riparius* (Silva et al., 2019) and caused oxidative damage due to their ingestion (Silva et al., 2021). Scherer et al. (2020), also found alterations in *C. riparius* life cycle in response to PVC, although at concentrations not environmentally relevant. However, so far the OECD guidelines have not considered studies at the molecular level, even though alterations in the gene expression patterns can be used to explore the metabolic pathways modified in response to the uptake of pollutants. To undertake this goal, a group of genes related to essential metabolic pathways were selected as first approach to enrich the knowledge of microplastics toxicity in aquatic invertebrates. The genes employed were related to stress response (heat shock proteins (HSPs): (*hsp90, Gp93, hsc70(4), hsp40, hsp60, hsp10* and *shsp17*); oxidative damage response (Manganese Superoxide dismutase (*SOD Mn*), detoxification (CYPs: *Cyp9f2, Cyp12a1*, ABC transporter (*ABCB6*) and development (FK506-binding protein 39 (*FKBP39*).

Overall, the literature on the molecular effects of microplastics covers only a few polymers (mostly PS), what hampers the comparison among different materials. Often the plastic particles used in the tests are manufactured spheres of fixed sizes, what contributes to the lack of realistic conditions.

The aim of the present study was to determine the specific molecular response of twelve genes related to crucial metabolic routes in invertebrates, such as the stress response, development, detoxification and amelioration of oxidative stress using *Chironomus riparius* as representative aquatic organism. For this, fourth instar larvae of *C. riparius* were exposed to tire rubber and polystyrene as model materials at two nominal concentrations (1 and 10 mg L^{-1}) in water during 36 h.

2. Materials and methods

2.1. Materials: origin, preparation, characterization and quantification

2.1.1. Origin & preparation

Commercially available polystyrene (PS-Styron 678-E; 1.05 g cm^{-3}) was obtained in pellets (0.018 ± 0.002 g, n = 25) and cryogrinded in a liquid nitrogen-compatible mill (Retsch MM400). Once the polystyrene pellets were inserted in the grinding jar, it was immersed in liquid nitrogen for 2 min and then ground at a frequency of 1/30 s for 10 min. These two steps were repeated until the material was a fine powder under visual inspection. The pellets were confirmed to be polystyrene by comparing its Fourier transformed infra-red (FTIR) spectra to the known PS spectra. A Thermo Nicolet iS50 instrument (Thermo Scientific, Finland) was used in ATR mode, with 16 number of scans, 4 cm^{-1} spectral resolution and a spectral range of 4000–400 cm^{-1}.

The tire rubber used in the present study was provided as a fine powder. Tires of unknown model or brand at the end-of-life stage, ready to start the recycling process, were crushed and part of the resulting material was cryogrinded to the final fine powder.

2.1.2. Characterization

A representative sample of the microplastics used was photographed with a camera (Zeiss Axiocam ERc 5s) attached to an optical microscope (Zeiss Steni 508). The particle size distribution of both materials was determined by measuring the feret diameter of ~2000 particles from each of the materials with ImageJ software.

Microrubber was extracted under acidic conditions with modifications from the methods described in Canepari et al. (2018). Briefly, known amounts of microrubber (n = 3) were extracted in a sonicator for ~11 h with 10 ml of acetic acid 0.5 M (Fisher Scientific). Suspensions were diluted to 30 ml and filtered through gamma sterile MCE membranes (0.22 μm pore size, 47 mm Ø; Fisher Scientific). The extracts were further diluted by sampling 2 ml and mixing with 11 ml of milliQ water. Extraction blanks were also set and handled with the same method as the tire rubber samples. Metals were measured by ICP-MS as described in the Supplemental information.

2.1.3. Quantification

The number of particles per unit of mass was determined by weighing known amounts of each material and counting the number of particles under an optical microscope (Zeiss Stemi 1000).

2.2. Animals

Chironomus riparius (Insecta) were reared at the Department of Environmental and Biological Sciences of the University of Eastern Finland in Kuopio (Finland) in aquaria containing Lake Höytiäinen sediment (62° 41′ 21″ N, 29° 23′49″ E) and artificial freshwater (AFW; hardness Ca^{2+} and Mg^{2+} 0.5 mM, pH = 6–9) at 20 ± 2 °C and under a light regime of 16:8 light: dark. Tetramin® (Tetrawerke, Germany) was added three times per week as a food source and the culture was aerated permanently. This culture originated from a population that had been recently sampled from Hasselbach (Hessen, Germany, 50.167562°N, 9.083542°E; Pfenninger and Foucault, 2020).

The larvae used in the exposures were obtained from egg ropes laid by adults collected from the permanent cultures. When hatching occurred, the larvae were transferred to an aquarium containing inorganic sand (<400 μm particle size) and AFW and allowed to grow for approximately 10 d in the same conditions as described above.

2.3. Experimental design

Suspensions of tire rubber and polystyrene microplastics were made in AFW at nominal concentrations of 1 and 10 mg L^{-1} (according to our measurements, this corresponds to 1181.9 and 11819 particles L^{-1} for microrubber and 7588.4 and 75884 particles L^{-1} for PS). Stock suspensions of microrubber (66.66 mg L^{-1}) and polystyrene (69.1 mg L^{-1}) were made by adding known amounts of the materials in 0.5 L of AFW. The experimental waters were prepared by sampling the needed volumes from the stock suspensions after a vigorous shake. The experimental units (n = 3) consisted in glass flasks (5.5 cm in diameter) containing 150 ml of the experimental waters or control, clean AFW. Ten fourth instar *C. riparius* larvae were added to each experimental unit. Aeration was provided throughout the test and temperature was 20 ± 1 °C. The duration of the exposure was 36 h and larvae were not fed. Surviving larvae were sampled after 36 h, dried over paper towels and frozen individually in Eppendorf tubes at −80 °C. Mortality was assessed and compared to that of controls. The analyses of the gene expression were performed in three larvae per experimental vessel (n = 3, per replicate, total n = 9).

2.3.1. Quantification of microplastics in experimental waters

The experimental waters were stored in the dark at 6 °C until further analyses. The real concentrations of microplastics in experimental waters were measured by filtration in previously weighed paper filters and MCE gamma sterile membranes (0.22 μm pore size, 47 mm Ø Fisher Scientific). Additionally, the same measurements were performed for the stock suspensions in three replicates of 125 ml

2.3.2. Quantification of metals in experimental waters

In order to measure the metals leached to water from tire rubber strictly in 36 h, an additional experiment with the same conditions as the exposures but without larvae was settled. After 36hrs, the experimental waters were filtered (0.22 μm pore size, 47 mm Ø gamma sterile MCE membranes, Fisher Scientific) and analyzed in ICP-MS as described in the Supplemental information.

2.4. RNA extraction and cDNA synthesis

The total RNA was extracted from individual larvae (three larvae per replicate) with a guanidinium isothyocyanate-based method using TRIzol™ reagent (Invitrogen™) according to the manufacturer's instructions. Followingly, RNA was purified and isolated according to existing literature (Muñiz-González & Martínez-Guitarte, 2020a). Finally, RNA was resuspended in diethyl pyrocarbonate (DEPC)-treated water and stored at −80 °C.

The whole quantity of isolated RNA was used to synthesize cDNA. The RNA was retrotranscribed using the Moloney Murine Leukemia virus (MMLV) enzyme (Invitrogen™) as was previously described in Muñiz-González and Martínez-Guitarte (2020a).

2.5. Real-time PCR

Real time PCR (RT-PCR) was used to evaluate the mRNA levels for each gene using the cDNA as template. The reaction was carried out with 0.5 units of DNA polymerase (Biotools, Spain), 0.4 mM dNTPs (Biotools, Spain) and 0.5x Eva Green (Biotium, USA). The RT-PCR was performed employing CFX96 thermocycler (Bio-Rad, USA). The cycling conditions were previously reported (Martinez-Guitarte, 2018). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and ribosomal protein L11 (*rpL11*) genes were employed as endogenous reference controls. The efficiencies were calculated following Ozáez et al. (2016). Primer sequences and their efficiencies can be found in Table S1. Finally, the total mRNA levels of normalized gene expression ($2^{-\Delta Cq}$) were calculated using Bio Rad CFX Maestro software. Each sample was run in duplicate wells as technical replicates and additionally, two independent plates were done as replicates in each experiment.

2.6. Statistical analysis

The gene expression analyses were performed using IBM SPSS Statistics 25. The normality and homogeneity were tested by Shapiro-Wilk ($n < 50$) and Levene's tests, respectively. The analyses were performed using the values obtained from nine individual larvae, three from each replicate. The differences among treatments were evaluated by One-Way ANOVA using a two-sided Dunnett's test to assess which treatment groups showed transcriptional activity significantly different from the control group at the $p \leq 0.05$ level. The mortality % among the treatments was analyzed for significant differences by One-Way ANOVA (Sigma Plot v. 13.0).

3. Results and discussion

Overall, it is not clear whether the toxicity caused by microplastics of different types is mainly caused by the particles or the additives present in the plastic particles. Recently, Silva et al. (2021) found oxidative damage in *C. riparius* in response to the ingestion of PE microplastics of three sizes. Furthermore, plastic additives can be toxic to a range of aquatic organisms (Capolupo et al., 2020; Li et al., 2016) and have been found in natural water bodies (Schmidt et al., 2019). Moreover, zinc has been identified as one of the most toxic compounds present in tire rubber (Wik et al., 2009; Marwood et al., 2011). However, organic compounds such as aniline, N,N0-bis(1,4-dimethylpentyl)-p-phenylenediamine (77PD), benzothiazole and 1-indanone have been also identified as tire rubber-associated toxic chemicals to aquatic organisms (Marwood et al., 2011; Halle et al., 2021). Although the potential toxicity may be caused by very complex mixture of chemicals, very recently a quinone transformation product of a tire rubber antioxidant was identified as the sole chemical inducing high toxicity in coho salmon (Tian et al., 2021). This study opened a new horizon on the ecotoxicity of plastic additives to aquatic organisms.

3.1. Materials tested

The PS-styron-678 E pellets were confirmed as polystyrene material with Fourier transformed infrared spectroscopy (FTIR) (Fig. 1A). The average particle size was 38.9 ± 28.6 and 82.3 ± 40 μm for polystyrene and microrubber, respectively (Fig. 1B and C). The number of particles per unit of mass was 1181.9 ± 136.4 particles mg^{-1} for microrubber and 7588,4 ± 780 particles mg^{-1} for PS.

The concentrations of metals in microrubber are shown in Table 1. Importantly, the concentration of zinc is the highest one by far, with a concentration of ~2.3 g kg^{-1}. It is important to distinguish between the extractable and total content of metals in tire rubber. In general, the

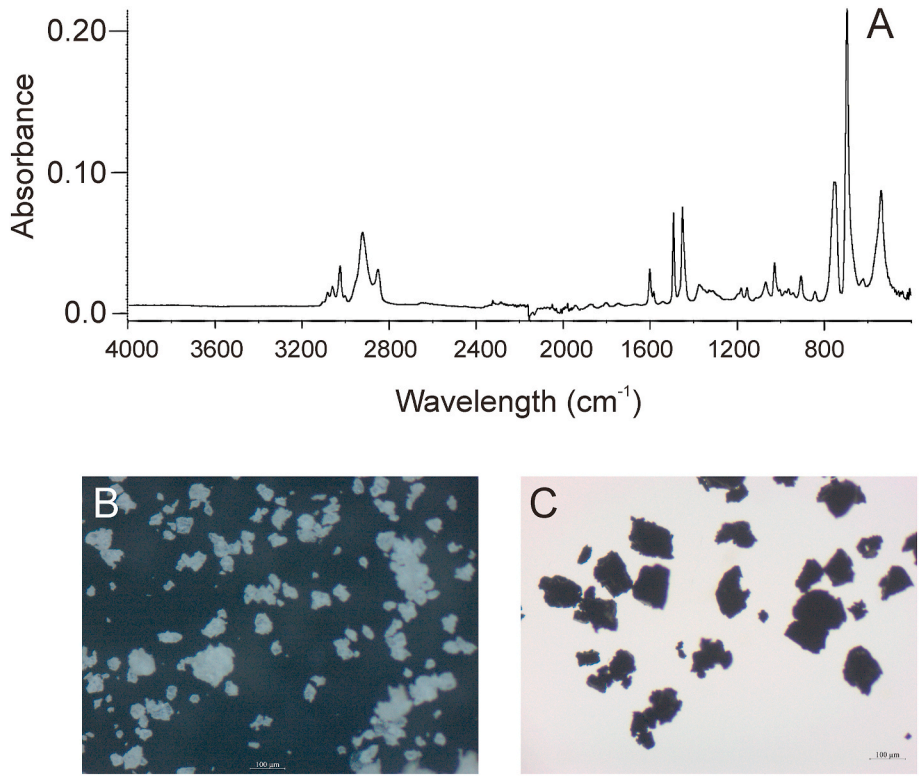

Fig. 1. Microplastic materials used in the study. In (A), the FTIR spectra of PS-Styron 678-E pellets is shown. A representative sample of polystyrene microplastics and microrubber is shown in (B) and (C), respectively.

Table 1
Concentrations of selected elements present in the microrubber used in the present study. The values are the average concentrations (n = 3) and standard deviation (SD).

Element		Av. concentration (mg kg microrubber^{-1})	SD
Aluminum	Al	9.42	3.62
Barium	Ba	1.20	0.37
Calcium	Ca	52.83	16.65
Cobalt	Co	5.53	0.62
Chromium	Cr	0.52	0.11
Copper	Cu	24.9	6.18
Iron	Fe	311	28.7
Magnesium	Mg	36.11	14.77
Manganese	Mn	2.89	0.41
Nickel	Ni	0.53	0.22
Phosphorus	P	25.41	12.12
Lead	Pb	1.81	0.53
Rubidium	Rb	0.17	0.019
Strontium	Sr	0.40	0.13
Vanadium	V	0.044	0.008
Zinc	Zn	2309.1	139.3

metal concentrations we obtained with an enhanced acidic extraction of the microrubber are higher than in Canepari et al. (2018). However, the smaller particle size and longer extraction times (~11 h) used in our study justifies these results. In is also remarkable that the total zinc present in tire rubber is still higher, ranging from 5.65 to nearly 27 g kg^{-1} (Canepari et al., 2018; Halle et al., 2021; Redondo-Hasselerharm et al., 2018a).

3.2. Experimental waters

The measured concentrations in the 1 mg L^{-1} treatments could not be determined due to the low amount present in 150 ml of experimental waters (0.15 mg of both materials). In the high, 10 mg L^{-1} exposures, the measured concentrations of tire rubber and PS were 13 ± 1.8 (n = 3) and 12.45 ± 4.9 mg L^{-1} (n = 2), respectively. The high variability of the measurements is attributed to the difficulties to handle these small particles and quantities. To check whether the stock solutions, containing a higher concentration, were made reliably, their concentrations were also measured, yielding values of 65.25 ± 4.31 and 69.94 ± 13.1 mg L^{-1}, for microrubber and PS, respectively (n = 3). The measured concentrations for microrubber are more satisfactory (97.98 ± 6.5% of the nominal concentration) than for PS (±19.2% of SD). We hypothesize that the reason for such variability in PS is that the material tended to adhere to the glassware and that it was harder to observe than the tire rubber due to its white colour.

The levels of metals in all experimental waters were similar as those found in control waters and therefore it was concluded that metals did not leach at relevant levels during 36h from microplastics at the concentrations used.

3.3. Mortality

The mortality during the test (Fig. S1) was not statistically significant among the treatments according to One-Way ANOVA ($F_{4, 10} = 0.568$, $p = 0.692$). None of the MPs used showed any risk for the survival of the animals in the conditions tested. Microrubber may not be a threat for the survival of aquatic organisms, as a similar result were obtained with *Hyalella azteca* (LC_{50} of 1 g L^{-1}; Khan et al., 2019) which is at least 100 times higher than the concentrations used in the present study. We believe that the mortality was caused by cannibalism. We conducted exposures only with water, without any substrate and in this regard, for example sediment has been deemed as necessary to reduce cannibalism in chironomids (Choung et al., 2010). Although our short term exposures did not cause any responses, it should not be ruled out that chronic exposures produce harmful effects, since our results suggested that microplastics can alter different processes at the molecular level (see below).

3.4. Gene expression

Currently, literature about the effects of microplastics on aquatic organisms at the molecular level is scarce but growing consistently in the last years. Studies have focused on fish (e.g., Rainieri et al., 2018), coral (Tang et al., 2018) and crustacea (Suman et al., 2020; Liu et al., 2021), among others. The present study investigated the expression of several genes on the model aquatic insect *C. riparius* exposed to PS and tire rubber. The effects of these microplastics on *C. riparius* gene expression after 36 h water exposure are shown in Figs. 2–4.

3.4.1. Stress response

The role of HSPs involves maintaining and restoring cellular homeostasis in response to environmental stressors such as temperature variations, UV radiation, hypoxia, and pollutants (Sørensen et al., 2003). In *Chironomus riparius*, several *HSP* genes belonging to different families have been characterized and studied in response to diverse toxicants such as phthalates, metals or bisphenol A (BPA) (Martín-Folgar et al., 2017; Morales et al., 2011; Park and Kwak, 2008) or heat stress (Martín-Folgar et al., 2015). In this study, seven genes: *hsp90, Gp93, hsc70, hsp60, hsp40*, *hsp10*, and *hsp17* were evaluated.

First, two genes belonging to the HSP90 family, *hsp90* and *Gp93,* showed increased expression in larvae exposed to microrubber at 10 mg L^{-1} (Fig. 2). This is an important finding, considering that this is an environmental realistic concentration (Wagner et al., 2018). Hsp90 performs an essential role in the correct protein folding, combined with hsp70 (Radli & Rüdiger, 2018) so the exposure to tire rubber could modify this function. Although the expression of *hsp90* was not altered by PS, there are studies reporting an increase on its expression in *Daphnia pulex* after exposure to 75 nm PS particles (Liu et al., 2018, 2019). One explanation for this difference can be the considerably smaller particle sizes used compared to our PS.

On the other hand, Gp93 is an endoplasmic reticulum (ER) chaperone, essential for the homeostasis of the gut epithelium and related to the immune system (Maynard et al., 2010).Therefore, the alteration of *Gp93* could be related with an activation of the immune system as a response to microrubber or its chemical components. Previous studies found that the expression of *Gp93* increased in *C. riparius* exposed to copper and copper/cadmium mixtures (Martín-Folgar and Martinez-Guitarte, 2019). The presence of zinc has been associated to damage in three different aquatic organisms (Wik et al., 2009), supporting the metal release from the microrubber as a possible reason for the upregulation of this gene. However, the concentrations of metals in experimental waters were not different from the control waters, what lead us to think that there are other mechanisms involved in the overexpression of this gene (see section 3.5).

Other important genes from the HSP family are *hsp40* and the heat shock constitutive form of 70kD (*hsc70* (4), which showed an upregulation at 10 mg L^{-1} of microrubber, similarly to the members from the *hsp90* family. In addition, *hsp40* also increased its expression at 10 mg L^{-1} of PS. These two proteins belong to different families, but they are functionally related. The HSP70 family, which includes the HSP70s and the constitutive cognates HSC70s, encompasses chaperones that require the presence of a member of the HPS40 family (also called DNAJ proteins) to function. Together, they carry out the prevention and reparation of protein misfolding, aggregation or degradation (Alderson et al., 2016). The upregulation of *hsc70(4)* in response to microrubber at 10 mg L^{-1} is not in accordance with its maintained levels reported in response to other contaminants (Morales et al., 2011). In line with our results, *hsc70(3)*, increased its transcriptional activity in response to an UV filter after 8 h of exposure (Martín-Folgar et al., 2018). These results suggest a differential activation of cognates genes depending on the stress agent and such response could indicate a protective role, as was observed in moths exposed to the plasticizer bisphenol A (Michail et al., 2012).

Regarding *hsp40*, its expression was upregulated in response to microrubber and PS. These results suggest that the *hsp40* studied does not code a protein functionally related to hsc70 (4), what is plausible considering the many members present in both families. Also, it is possible that other members of the HSP70 family that were not analyzed could be affected, involving those genes responsive to stress such as *hsp70*. Supporting this idea, it has been observed that PS nanoplastics upregulated *hsp70* in *Daphnia pulex* (Liu et al., 2019). Therefore, it seems evident that microplastics (as a whole) have a relevant role in the activation of these HSPs genes, showing a different pattern of alterations depending on the polymer type, their sizes and/or additives.

Two genes, coding forchaperones related to the regulation and control of protein folding in the mitochondria (*hsp60*, and *hsp10*), were also analyzed (Höhfeld and Hartl, 1994; Kozlova et al., 1997). However, our results showed that the response of these genes was not correlated (Fig. 2): *hsp60* was significantly upregulated in response to 10 mg L^{-1} of microrubber and to 1 mg L^{-1} of PS, while no significant changes were observed in *hsp10*. There are no previous data about the expression of these genes in response to microplastics, although their expression did

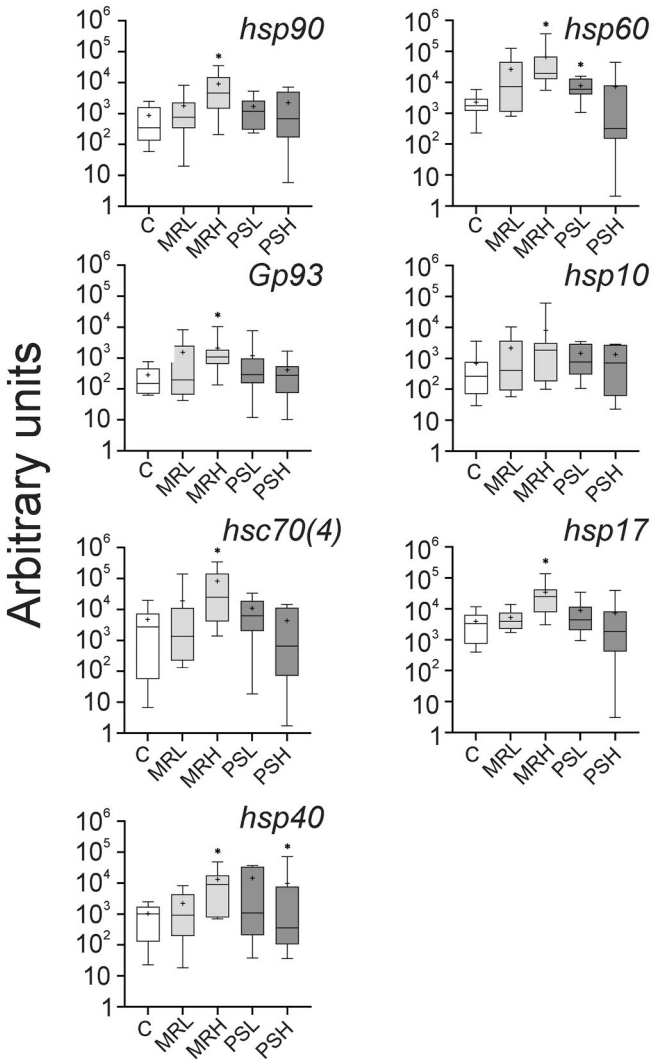

Fig. 2. Expression of the hsp genes (*hsp 90, hsp60, Gp93, hsp10, hsc70(4), hsp17* and *hsp40)*. From left to right the expression of each gene in Controls (C, white bar), microrubber (light grey) at low (MRL) and high (MRH) concentrations and polystyrene (dark grey) at low (PSL) and high (PSH) concentrations. Asterisks denote statistical differences compared to controls ($p < 0.05$). The horizontal line indicates the median, the boundaries indicate the 25th and 75th percentiles, while the whiskers denote the highest and lowest results. The mean of n = 9 values obtained is indicated by the plus sign inside the box.

not change in *C. riparius* exposed to the UV filters benzophenone 3 and 4-MBC (Martín-Folgar et al., 2018). Additionally, *hsp10* increased its transcriptional activity during recovery from exposure to the plasticizer butyl benzyl phthalate (BBP; Herrero et al., 2015). These data are interesting because both UV filters and phthalates are additives commonly used in the plastic industry. So, the differential expression could be caused by the presence of some additive(s) which activate differentially these genes, although BBP is mostly an additive in PVC plastics (Andrady and Rajapakse, 2016). Furthermore, these proteins are located mostly in the mitochondria so the alteration in the mRNA levels could indirectly suggest toxicity towards this cellular organelle, which is mainly focused on the ATP generation trough respiration.

Finally, *hsp17* a member of the small HSPs (sHSPs), was analyzed, showing upregulation by microrubber at 10 mg L^{-1}. sHSPs conform a very diverse family of proteins, related to many cell processes that react to adverse environments and diseases, control growth, or apoptosis events (Arrigo et al., 2002; Zhu & Reiser, 2018). Despite the absence of data about the expression of *hsp17* in response to microplastics, exposure to cadmium upregulated its expression in *C. riparius* (Martin-Folgar and Martinez-Guitarte, 2017). Furthermore, higher mRNA levels of other sHSPs have been detected in the oriental river prawn *Macrobrachium nipponense* and in *Musca domestica* after exposure to metals (Tian et al., 2018; Yuan et al., 2019). The lack of effect observed in larvae exposed to PS could be explained by the leaching of some metals from microrubber to the exposure waters. However, the level of metals, including zinc, in experimental waters was so low that it could not be differentiated from the levels in control waters. Other potential explanations are described in section 3.5.

3.4.2. Detoxification response

The biotransformation and detoxification of xenobiotics in the cell consist of phases I, II and III. Some of the most important enzymes involved in these phases are the cytochrome P450 family, the Glutathione-S- transferases and the ABC transporters, respectively. In our study, we evaluated the expression of the genes coding two CYPs, *Cyp9f2* and *Cyp12a2*, and one ABC transporter, *ABCB6*. None of these genes altered their expression in response to any microplastic or concentration tested (Fig. 3). This could be indirectly related to the uptake of chemicals prone to undergo biotransformation and subsequent excretion, such as polycyclic aromatic hydrocarbons (PAHs), present in microrubber. However, due to the hydrophobicity of PAHs, it is known that they minimally leach to water from tire rubber materials (Wagner et al., 2018; Redondo-Hasselerharm et al., 2018a), supporting the lack of response of genes encoding detoxification enzymes. It has been described that CYP9 family is involved in the resistance to pyrethroids in *Aedes aegypti* (Ishak et al., 2017) and CYP12 was described as a mitochondrial CYP family which can be involved in xenobiotic metabolism (Guzov et al., 1998). Although information about these enzymes is scarce in *C. riparius,* previous studies on *Cyp9f2* reported no changes in its expression in response to different UV filters (p-aminobenzoic acid and crylene derivatives) in single and/or binary mixtures (Muñiz-González and Martinez-Guitarte, 2018, 2020b) and a downregulation after exposure to another UV filter 4-methylbenzylidene camphor (4-MBC) (Martinez-Guitarte, 2018). Another member of the CYP9 family, *Cyp9AAT2*, was upregulated at 10 and 20 mg L^{-1} of Cd (Nair et al., 2013). Regarding *Cyp12a2*, its expression was upregulated by triclosan (Martinez-Paz, 2018) but downregulated by 4-MBC (Martinez-Guitarte, 2018). Although no results about the response of these families with MPs has been described, a downregulation of *Cyp1A* by PVC exposure in zebrafish larvae (Sleight et al., 2017) and modulation of members of the CYP4 family by PS MPs in crustacea (Wu et al., 2019) have been reported. These results show the variety of responses for *Cyp* genes depending on the xenobiotic or compound used and suggest that it is necessary to extend the study to other families and to other enzymes involved in the Phase I response.

Regarding *ABCB6*, it belongs to a family of ABC transporters, involved in the transport through the membranes, with the final aim of xenobiotic excretion from the cells (Xu et al., 2005). Contrarily to our results, the ingestion of PS nanoplastics by mussels produced a downregulation of two genes coding ABC transporters, what likely caused a change in the flux of the multixenobiotic resistance (MXR) system (Franzellitti et al., 2019). As we know, the size and surface of the MPs used may determine the possible effects, but the importance of putative additives in different plastic materials cannot be discarded. Similarly to our results, other ABC member (*MRP-1*) was unaltered after exposure to triclosan in *C. riparius* (Martinez-Paz, 2018). On the contrary, altered expression on diverse *ABC* members was observed in *Helicoverpa armigera* exposed to pesticides (Jin et al., 2019), showing a specific response for each compound. To sum up, the absence of response in the genes coding for detoxification proteins could be explained by the short time exposure employed comparing to previous studies. In future studies, longer exposure times could be tested with the aim of evaluating the response of these genes over time.

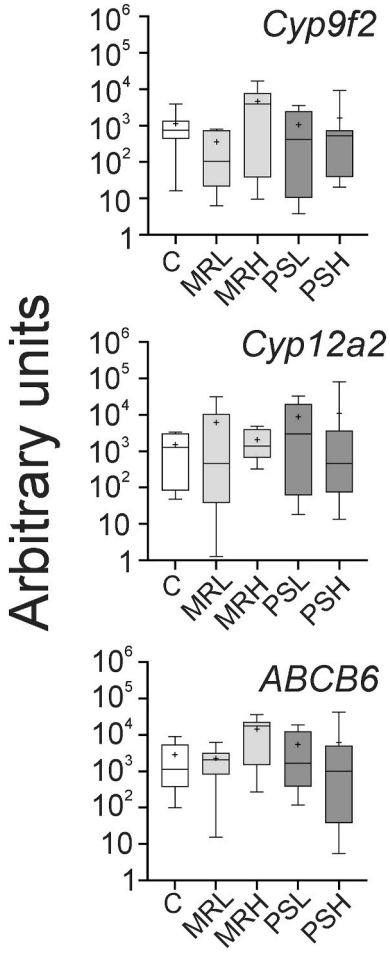

Fig. 3. Expression of the genes related to detoxification (*Cyp9f2, Cyp 12a2* and *ABCB6)*. The genes shown in each figure are written in the top of each figure for clarification. From left to right the expression of each gene in Controls (C, white bar), microrubber (light grey) at low (MRL) and high (MRH) concentrations and polystyrene (dark grey) at low (PSL) and high (PSH) concentrations. Asterisks denote statistical differences compared to controls ($p < 0.05$).The horizontal line indicates the median, the boundaries indicate the 25th and 75th percentiles, while the whiskers denote the highest and lowest results. The mean of n = 9 values obtained is indicated by the plus sign inside the box.

3.4.3. Antioxidant and developmental response

Finally, other genes, *SOD Mn* and *FKBP39*, related to oxidative stress and development respectively, were analyzed (Fig. 4). *SOD Mn* was

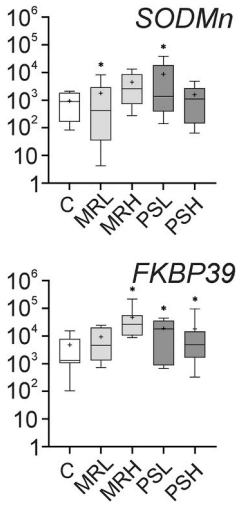

Fig. 4. Expression of *SOD Mn* and *FKBP39*. From left to right the expression of each gene in Controls (C; white bar), microrubber (light grey) at low (MRL) and high (MRH) concentrations and polystyrene (dark grey) at low (PSL) and high (PSH) concentrations. Asterisks denote statistical differences compared to controls ($p < 0.05$).The horizontal line indicates the median, the boundaries indicate the 25th and 75th percentiles, while the whiskers denote the highest and lowest results. The mean of $n = 9$ values obtained is indicated by the plus sign inside the box.

upregulated in larvae exposed to 1 mg L^{-1} of both materials tested. *SOD Mn* encodes a mitochondrial protein that deactivates superoxide radicals created during mitochondrial respiration and therefore protects the cell from oxidative stress (Wang et al., 2018). It is therefore plausible that the MPs used in the present study (at 1 mg L^{-1}) caused oxidative stress, stimulating the upregulation of *SOD Mn* to alleviate its effects. Supporting this statement, signs of oxidative stress in response to low concentrations of MPs have been found in aquatic organisms (Tlili et al., 2020; Yu et al., 2018). Furthermore, in *Daphnia pulex* PS nanoplastics caused oxidative stress and upregulated the expression of *SOD CuZn* (Liu et al., 2020), related to antioxidant response, like *SOD Mn*. Interestingly, the upregulation of *SOD CuZn* was only observed at the lowest concentrations tested (Liu et al., 2020), like in our study. The fact that the highest concentrations did not alter neither the *SOD CuZn* nor *SOD Mn* expressions respectively in *D. pulex* and *C. riparius* could be due to a disruption of the antioxidant systems that the cell is not able to compensate, and that is caused by excessive levels of reactive oxygen species (ROS). Another plausible explanation is that *SOD Mn* reacts towards the amelioration of oxidative stress during a first stage of the response. The level and speed at what oxidative stress occurs would depend on the severity of external factors and therefore it is expected that they would be higher with increasing concentrations of microplastics. Therefore, *C. riparius* would adapt to this response according to the levels of oxidative stress. At the low microplastics exposure, the uptake of chemical additives to *C. riparius* would be slower and so would be the generation of ROS. However, at the high microrubber exposure the tendency is to find overexpression of the *HSPs* but not of *SOD Mn*. It is plausible that the responses to oxidative stress caused by microplastics would be sequential, involving first the upregulation of *SOD Mn* and second, of *HSPs*. It could be hypothesized that these responses are connected or alternatively, that *HSPs* are overexpressed to help the correct folding of damaged proteins due to high oxidative stress. Along with this hypothesis, metals can trigger the overexpression of *HSPs* in aquatic organisms (Kim et al., 2014). Overall, the responses to oxidative stress caused by microrubber and PS may be different due to the specific constitution of each material (e.g.: higher number of metals present in tire rubber).

FKBP39 was upregulated at 10 mg L^{-1} of microrubber and at both concentrations of PS microplastics (Fig. 4). This gene codes an immunophilin, proteins that exhibit versatile and key biological functions, such as protein folding, receptor signalling, protein trafficking and transcription (Harikishore and Yoon, 2016). Specifically, FKBP39 has been proposed to act as a transcriptional modulator of gene expression in 20-hydroxyecdysone (20-E) and juvenile hormone (JH) signal transduction in *D. melanogaster* (Li et al., 2007) so it is related with growth and development processes. *FKBP39* was the only gene altered by both concentrations of PS, suggesting that this material could alter the development and the growth of the larvae. No previous data are available in the literature regarding the expression of *FKBP39* in response to xenobiotics on invertebrates.

In addition, FKBP39 has been identified as an inhibitor of autophagy in *D. melanogaster* larvae, process that has a direct implication for the correct pupariation and development (Juhász et al., 2007). The molecular processes that lead to metamorphosis include the induction of developmental autophagy (leading to pupariation) by the insect molting hormone ecdysone, through enhancing the expression of ecdysone-responsive genes (Li et al., 2007). In this scenario, the levels of FKBP39 are low. In our study, the overexpression of *FKBP39* in both PS and high microrubber exposures suggests that the induction of pupariation by ecdysone is not activated, indicating a potential delay in metamorphosis. In normal conditions, the high expression of *FKBP39* occurs in early development stages, as was demonstrated in *Drosophila* (Theopold et al., 1995). This potential delay in the development was previously detected in chironomids in response to polyethylene MPs (Silva et al., 2019; Ziajahromi et al., 2018). So, it appears that microplastics have the potential to act as endocrine disruptors (EDCs) in chironomids.

Summarizing, the highest molecular response was observed in the exposure to the highest concentration of microrubber, where the expression of seven of the genes studied was modified (*hsp90, Gp93, hsc70 (4), hsp40, hsp60, hsp17 and FKBP39*). The lowest concentration of microrubber altered the expression of *SOD Mn*. Furthermore, polystyrene only affected the expression of four of the genes evaluated at 1 mg L^{-1} (*hsp60, SOD Mn, FKBP39*) and at 10 mg L^{-1} (*hsp40, FKBP39*). This uneven response may be due to the ability of the microrubber to release part of its additives to the water, therefore increasing its toxic effects, as has already been observed previously in aquatic organisms (Capolupo et al., 2020).

3.5. Potential causes of the toxicity

At the lowest concentrations of both materials, the response starts with the overexpression of *SOD Mn*, that helps to alleviate oxidative stress. However, what causes the oxidative stress is still not clear. We hypothesized that the metals present in tire rubber and leached to water caused the oxidative stress, however the concentration of metals in water in the experimental conditions tested were under detection limits and additionally, it would not explain the results with PS. Similarly, in *C. riparius* PE microplastics obstructed its guts (Silva et al., 2021), what likely caused a series of toxic effects that included inflammation and oxidative damage. Furthermore, this could lead to an energy imbalance, possibly delaying other vital processes such as development and metamorphosis, as suggested by the overexpression of *FKBP39*.

Although we did not study the ingestion of particles due to the obvious destruction of the tissues during the RNA extraction, it is plausible that it occurred. Therefore, as in Silva et al. (2021), the sole ingestion may have caused the oxidative stress characterized by the overexpression of *SOD Mn* and may have contributed to the cellular stress that caused the overexpression of *HSPs*. Another possibility is that with ingestion of the microplastics, the additives present (including metals) would leach in the guts of *C. riparius* and enter its tissues. This could imply two stressors: the ingestion of the particles and the chemicals released in the gut of the animal and could also explain the clear differences in the responses to both materials. The ingestion of the

particles would cause oxidative stress as described in Silva et al. (2021). This would trigger the responses to its amelioration (overexpression of *SOD Mn*) in both materials. However, in the case of microrubber, the high presence of metals (up to 2 g Kg^{-1} of zinc) in the ingested particles could promote their uptake, as it has been observed in dietary sources with high metal content (van Hattum et al., 1989). The general overexpression of *HSPs* is in accordance with the gene expression profiles of single metals (Martin-Folgar and Martinez-Guitarte, 2017, 2019). In the case of PS, the metal content is not as abundant as in microrubber (Capolupo et al., 2020) and therefore it is logical that the responses of the *HSPs* are not as high.

4. Conclusions

The observed results indicate a strong stress response in *Chironomus riparius* at the conditions studied, involving the overexpression of several *HSPs* and *SOD Mn* and aimed at the reduction of the effects derived from oxidative stress. Additionally, the results obtained with *FKBP39* suggest that PS and microrubber can affect the development and metamorphosis of *C. riparius*. This is the first report that analyses the effects of microrubber on invertebrates at the molecular level. The comparative analysis with PS microparticles shows that there is a specific gene expression profile depending on the material and its concentration, likely due to ingestion and metal uptake. Although the stress response was altered after a short exposure time (36 h), the detoxification mechanisms seemed to be not activated.

The environmental significance of the present study lies within the relevant concentrations used, especially for tire rubber. This opens questions about the significance of tire rubber contamination in freshwaters, although the short exposure time we used does not allow us to conclude on ecosystem effects. However, it suggests for additional research with longer exposure times, multiple generations and lower concentrations. Our data support the utility of molecular tools to analyse the effect of microplastics and highlight the risk that these emergent contaminants supposed to freshwater ecosystems, mainly by their effects in the basal links of the food web.

Credit author statement

Victor Carrasco-Navarro: Conceptualization, Methodology, formal analyses, Investigation, Writing – original draft preparation, Writing – review & editing Resources, Visualization, Validation and Funding acquisition. Ana-Belén Muñiz-González: Conceptualization, Methodology, formal analyses, Investigation, Writing – original draft preparation, Writing – review & editing Resources, Validation and Visualization. Jouni Sorvari: Methodology, Writing – review & editing Resources, Supervision. Jose-Luis Martínez-Guitarte: Conceptualization, Writing – review & editing Resources, Funding acquisition and Supervision.

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Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.117462

References

Alderson, T.R., Kim, J.H., Markley, J.L., 2016. Dynamical structures of Hsp70 and hsp70-hsp40 complexes. Structure 24, 1014–1030. https://doi.org/10.1016/j.str.2016.05.011.

Andrady, A.L., Rajapakse, N., 2016. Additives and chemicals in plastics. In: Takada, H., Karapanagioti, H. (Eds.), Hazardous Chemicals Associated with Plastics in the Marine Environment, The Handbook of Environmental Chemistry, vol. 78. Springer, Cham. https://doi.org/10.1007/698_2016_124.

Arrigo, A.P., Paul, C., Ducasse, C., Manero, F., Kretz-Remy, C., Virot, S., Javouhey, E., Mounier, N., Diaz- Latoud, C., 2002. Small stress proteins: novel negative modulators of apoptosis induced independently of reactive oxygen species. In: Arrigo, A.P., Müller, W.E.G. (Eds.), Small Stress Proteins, Progress in Molecular and Subcellular Biology, vol. 28. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-56348-5_10.

Baxter, C.V., Fausch, K.D., Carl Saunders, W., 2005. Tangled webs: reciprocal flows of invertebrate prey link streams and riparian zones. Freshw. Biol. 50, 201–220. https://doi.org/10.1111/j.1365-2427.2004.01328.x.

Bellasi, A., Binda, G., Pozzi, A., Galafassi, S., Volta, P., Bettinetti, R., 2020. Microplastic contamination in freshwater environments: a review, focusing on interactions with sediments and benthic organisms. Environments - MDPI 7 (4). https://doi.org/10.3390/environments7040030.

Besseling, E., Wegner, A., Foekema, E.M., van den Heuvel-Greve, Martine, J., Koelmans, A.A., 2013. Effects of microplastic on fitness and PCB bioaccumulation by the lugworm Arenicola marina (L.). Environ. Sci. Technol. 47, 593–600. https://doi.org/10.1021/es302763x.

Blettler, M.C.M., Abrial, E., Khan, F.R., Sivri, N., Espinola, L.A., 2018. Freshwater plastic pollution: recognizing research biases and identifying knowledge gaps. Water Res. 143, 416–424. https://doi.org/10.1016/j.watres.2018.06.015.

Burns, E.E., Boxall, A.B., 2018. Microplastics in the aquatic environment: evidence for or against adverse impacts and major knowledge gaps. Environ. Toxicol. Chem. 37, 2776–2796. https://doi.org/10.1002/etc.4268.

Canepari, S., Castellano, P., Astolfi, M.L., et al., 2018. Release of particles, organic compounds, and metals from crumb rubber used in synthetic turf under chemical and physical stress. Environ. Sci. Pollut. Control Ser. 25, 1448–1459. https://doi.org/10.1007/s11356-017-0377-4.

Capolupo, M., Sorensen, L., Jayasena, K.D.R., Booth, A.M., Fabbri, E., 2020. Chemical composition and ecotoxicity of plastic and car tire rubber leachates to aquatic organisms. Water Res. 169, 115270 doi: S0043-1354(19)31044-9.

Choung, C.B., Hyne, R.V., Stevens, M.M., Hose, G.C., 2010. A low concentration of atrazine does not influence the acute toxicity of the insecticide terbufos or its breakdown products to Chironomus tepperi. Ecotoxicology 19, 1536–1544. https://doi.org/10.1007/s10646-010-0538-4.

Franzellitti, S., Capolupo, M., Wathsala, Rajapaksha H.G. R., Valbonesi, P., Fabbri, E., 2019. The Multixenobiotic resistance system as a possible protective response triggered by microplastic ingestion in Mediterranean mussels (Mytilus galloprovincialis): larvae and adult stages. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 219, 50–58. https://doi.org/10.1016/j.cbpc.2019.02.005.

Guzov, V.M., Unnithan, G.C., Chernogolov, A.A., Feyereisen, R., 1998. CYP12A1, a mitochondrial cytochrome P450 from the house fly. Arch. Biochem. Biophys. 359, 231–240 doi: S0003-9861(98)90901-8.

Halle, L.L., Palmqvist, A., Kampmann, K., Khan, F.R., 2020. Ecotoxicology of micronized tire rubber: past, present and future considerations. Sci. Total Environ. 706, 135694. https://doi.org/10.1016/j.scitotenv.2019.135694.

Halle, L.L., Palmqvist, A., Kampmann, K., Jensen, A., Hansen, T., Khan, F.R., 2021. Tire wear particle and leachate exposures from a pristine and road-worn tire to Hyalella azteca: comparison of chemical content and biological effects. Aquat. Toxicol. https://doi.org/10.1016/j.aquatox.2021.105769, 105769.

Harikishore, A., Yoon, H.S., 2016. Immunophilins: structures, mechanisms and ligands. Curr. Mol. Pharmacol. 9, 37–47. https://doi.org/10.2174/1874467208666150519113427.

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, 266–277.
Höhfeld, J., Hartl, F.U., 1994. Role of the chaperonin cofactor Hsp10 in protein-folding and sorting in yeast mitochondria. J. Cell Biol. 126, 305–315. https://doi.org/10.1083/jcb.126.2.305.
Ishak, I.H., Kamgang, B., Ibrahim, S.S., Riveron, J.M., Irving, H., Wondji, C.S., 2017. Pyrethroid resistance in Malaysian populations of dengue vector Aedes aegypti is mediated by CYP9 family of cytochrome P450 genes. PLoS Negl. Trop. Dis. 11, e0005302 https://doi.org/10.1371/journal.pntd.0005302.
Jeong, C., Won, E., Kang, H., Lee, M., Hwang, D., Hwang, U., Zhou, B., Souissi, S., Lee, S., Lee, J., 2016. Microplastic size-dependent toxicity, oxidative stress induction, and p-JNK and p-p38 activation in the monogonont rotifer (Brachionus koreanus). Environ. Sci. Technol. 50, 8849–8857. https://doi.org/10.1021/acs.est.6b01441.
Jin, M., Liao, C., Chakrabarty, S., Zheng, W., Wu, K., Xiao, Y., 2019. Transcriptional response of ATP-binding cassette (ABC) transporters to insecticides in the cotton bollworm, Helicoverpa armigera. Pestic. Biochem. Physiol. 154, 46–59. https://doi.org/10.1016/j.pestbp.2018.12.007.
Juhsz, G., Puskas, L.G., Komonyi, O., Erdi, B., Maroy, P., Neufeld, T.P., Sass, M., 2007. Gene expression profiling identifies FKBP39 as an inhibitor of autophagy in larval Drosophila fat body. Cell Death Differ. 14, 1181–1190. https://doi.org/10.1038/sj.cdd.4402123.
Khan, F.R., Halle, L.L., Palmqvist, A., 2019. Acute and long-term toxicity of micronized car tire wear particles to Hyalella azteca. Aquat. Toxicol. 213, 105216. https://doi.org/10.1016/j.aquatox.2019.05.018.
Kim, B., Rhee, J., Jeong, C., Seo, J.S., Park, G.S., Lee, Y., Lee, J., 2014. Heavy metals induce oxidative stress and trigger oxidative stress-mediated heat shock protein (hsp) modulation in the intertidal copepod Tigriopus japonicus. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 166, 65–74. https://doi.org/10.1016/j.cbpc.2014.07.005.
Kole, P.J., Löhr, A.J., Van Belleghem, Frank, G.A.J., Ragas, A.M.J., 2017. Wear and tear of tyres: a stealthy source of microplastics in the environment. Int. J. Environ. Res. Publ. Health 14 (10), 1265. https://doi.org/10.3390/ijerph14101265.
Kozlova, T., Perezgasga, L., Reynaud, E., Zurita, M., 1997. The Drosophila melanogaster homologue of the hsp60 gene is encoded by the essential locus l(1)10Ac and is differentially expressed during fly development. Dev. Gene. Evol. 207, 253–263. https://doi.org/10.1007/s004270050113.
Li, H., Getzinger, G.J., Ferguson, P.L., Orihuela, B., Zhu, M., Rittschof, D., 2016. Effects of toxic leachate from commercial plastics on larval survival and settlement of the barnacle amphibalanus amphitrite. Environ. Sci. Technol. 50, 924–931. https://doi.org/10.1021/acs.est.5b02781.
Li, Y., Liu, Z., Li, M., Jiang, Q., Wu, D., Huang, Y., Jiao, Y., Zhang, M., Zhao, Y., 2020. Effects of nanoplastics on antioxidant and immune enzyme activities and related gene expression in juvenile Macrobrachium nipponense. J. Hazard Mater. 398, 122990. https://doi.org/10.1016/j.jhazmat.2020.122990.
Li, Y., Zhang, Z., Robinson, G.E., Palli, S.R., 2007. Identification and characterization of a juvenile hormone response element and its binding proteins. J. Biol. Chem. 282, 37605–37617. https://doi.org/10.1074/jbc.M704595200.
Lin, W., Jiang, R., Hu, S., Xiao, X., Wu, J., Wei, S., Xiong, Y., Ouyang, G., 2019. Investigating the toxicities of different functionalized polystyrene nanoplastics on Daphnia magna. Ecotoxicol. Environ. Saf. 180, 509–516. https://doi.org/10.1016/j.ecoenv.2019.05.036.
Lindeque, P.K., Cole, M., Coppock, R.L., Lewis, C.N., Miller, R.Z., Watts, A.J.R., Wilson-McNeal, A., Wright, S.L., Galloway, T.S., 2020. Are we underestimating microplastic abundance in the marine environment? A comparison of microplastic capture with nets of different mesh-size. Environ. Pollut. 265, 114721. https://doi.org/10.1016/j.envpol.2020.114721.
Liu, Z., Cai, M., Wu, D., Yu, P., Jiao, Y., Jiang, Q., Zhao, Y., 2020. Effects of nanoplastics at predicted environmental concentration on Daphnia pulex after exposure through multiple generations. Environ. Pollut. 256, 113506. https://doi.org/10.1016/j.envpol.2019.113506.
Liu, Z., Cai, M., Yu, P., Chen, M., Wu, D., Zhang, M., Zhao, Y., 2018. Age-dependent survival, stress defense, and AMPK in Daphnia pulex after short-term exposure to a polystyrene nanoplastic. Aquat. Toxicol. 204, 1–8. https://doi.org/10.1016/j.aquatox.2018.08.017.
Liu, Z., Li, Y., Perez, E., Jiang, Q., Chen, Q., Jiao, Y., Huang, Y., Yang, Y., Zhao, Y., 2021. Polystyrene nanoplastic induces oxidative stress, immune defense, and glycometabolism change in Daphnia pulex: application of transcriptome profiling in risk assessment of nanoplastics. J. Hazard Mater. 402, 123778. https://doi.org/10.1016/j.jhazmat.2020.123778.
Liu, Z., Yu, P., Cai, M., Wu, D., Zhang, M., Huang, Y., Zhao, Y., 2019. Polystyrene nanoplastic exposure induces immobilization, reproduction, and stress defense in the freshwater cladoceran Daphnia pulex. Chemosphere 215, 74–81. https://doi.org/10.1016/j.chemosphere.2018.09.176.
Martinez-Guitarte, J., 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.
Martinez-Paz, P., 2018. Response of detoxification system genes on *Chironomus riparius* aquatic larvae after antibacterial agent triclosan exposures. Sci. Total Environ. 624, 1–8. https://doi.org/10.1016/j.scitotenv.2017.12.107.
Martín-Folgar, R., Aquilino, M., Ozaez, I., Martinez-Guitarte, J., 2018. Ultraviolet filters and heat shock proteins: effects in *Chironomus riparius* by benzophenone-3 and 4-methylbenzylidene camphor. Environ. Sci. Pollut. Res. Int . 25, 333–344. https://doi.org/10.1007/s11356-017-0416-1.
Martín-Folgar, R., de la Fuente, M., Morcillo, G., Martinez-Guitarte, J., 2015. Characterization of six small HSP genes from *Chironomus riparius* (Diptera, Chironomidae): differential expression under conditions of normal growth and heat-induced stress. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 188, 76–86. https://doi.org/10.1016/j.cbpa.2015.06.023.
Martin-Folgar, R., Martinez-Guitarte, J., 2017. Cadmium alters the expression of small heat shock protein genes in the aquatic midge *Chironomus riparius*. Chemosphere 169, 485–492. https://doi.org/10.1016/j.chemosphere.2016.11.067.
Martín-Folgar, R., Martinez-Guitarte, J., 2019. Effects of single and mixture exposure of cadmium and copper in apoptosis and immune related genes at transcriptional level on the midge *Chironomus riparius* Meigen (Diptera, Chironomidae). Sci. Total Environ. 677, 590–598. https://doi.org/10.1016/j.scitotenv.2019.04.364.
Marwood, C., McAtee, B., Kreider, M., Ogle, R.S., Finley, B., Sweet, L., Panko, J., 2011. Acute aquatic toxicity of tire and road wear particles to alga, daphnid, and fish. Ecotoxicology 20, 2079–2089. https://doi.org/10.1007/s10646-011-0750-x.
Maynard, J.C., Pham, T., Zheng, T., Jockheck-Clark, A., Rankin, H.B., Newgard, C.B., Spana, E.P., Nicchitta, C.V., 2010. Gp93, the Drosophila GRP94 ortholog, is required for gut epithelial homeostasis and nutrient assimilation-coupled growth control. Dev. Biol. 339, 295–306. https://doi.org/10.1016/j.ydbio.2009.12.023.
Michail, X., Kontogiannatos, D., Syriou, V., Kourti, A., 2012. Bisphenol-A affects the developmental progression and expression of heat-shock protein genes in the moth Sesamia nonagrioides. Ecotoxicology 21, 2244–2253. https://doi.org/10.1007/s10646-012-0980-6.
Morales, M., Planelló, R., Martínez-Paz, P., Herrero, O., Cortés, E., Martínez-Guitarte, J. L., Morcillo, G., 2011. Characterization of Hsp70 gene in *Chironomus riparius*: expression in response to endocrine disrupting pollutants as a marker of ecotoxicological stress. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 153, 150–158. https://doi.org/10.1016/j.cbpc.2010.10.003.
Muñiz-González, A., Martinez-Guitarte, J., 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. Int . 25, 35501–35514. https://doi.org/10.1007/s11356-018-3516-7.
Muñiz-González, A., Martínez-Guitarte, J., 2020a. 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., 2020b. Unveiling complex responses at the molecular level: transcriptional alterations by mixtures of bisphenol A, octocrylene, and 2′-ethylhexyl 4- (dimethylamino)benzoate on *Chironomus riparius*. Ecotoxicol. Environ. Saf. 206 (September) https://doi.org/10.1016/j.ecoenv.2020.111199.
Nair, P.M.G., Park, S.Y., Choi, J., 2013. Characterization and expression of cytochrome p450 cDNA (CYP9AT2) in *Chironomus riparius* fourth instar larvae exposed to multiple xenobiotics. Environ. Toxicol. Pharmacol. 36, 1133–1140. https://doi.org/10.1016/j.etap.2013.08.011.
National Research Council, 2014. Review of the Styrene Profile in the National Toxicology Program 12th Report on Carcinogens. National Academies Press (US).
OECD, 2010. In: Test No. 233: Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment, OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publishing, Paris. https://doi.org/10.1787/9789264090910-en.
Ozáez, I., Morcillo, G., Martínez-Guitarte, J., 2016. Ultraviolet filters differentially impact the expression of key endocrine and stress genes in embryos and larvae of *Chironomus riparius*. Sci. Total Environ. 557–558, 240–247. https://doi.org/10.1016/j.scitotenv.2016.03.078.
Panko, J.M., Kreider, M.L., McAtee, B.L., Marwood, C., 2013. Chronic toxicity of tire and road wear particles to water- and sediment-dwelling organisms. Ecotoxicology 22, 13–21. https://doi.org/10.1007/s10646-012-0998-9.
Park, K., Kwak, I., 2008. Characterization of heat shock protein 40 and 90 in *Chironomus riparius* larvae: effects of di(2-ethylhexyl) phthalate exposure on gene expressions and mouthpart deformities. Chemosphere 74, 89–95. https://doi.org/10.1016/j.chemosphere.2008.09.041.
Pfenninger, M., Foucault, Q., 2020. Genomic processes underlying rapid adaptation of a natural *Chironomus riparius* population to unintendedly applied experimental selection pressures. Mol. Ecol. 29, 536–548. https://doi.org/10.1111/mec.15347.
Radli, M., Rüdiger, S.G.D., 2018. Dancing with the diva: hsp90–client interactions. J. Mol. Biol. 430, 3029–3040. https://doi.org/10.1016/j.jmb.2018.05.026.
Rainieri, S., Conlledo, N., Larsen, B.K., Granby, K., Barranco, A., 2018. Combined effects of microplastics and chemical contaminants on the organ toxicity of zebrafish (Danio rerio). Environ. Res. 162, 135–143. https://doi.org/10.1016/j.envres.2017.12.019.
Redondo-Hasselerharm, P., de Ruijter, V.N., Mintenig, S.M., Verschoor, A., Koelmans, A. A., 2018a. Ingestion and chronic effects of car tire tread particles on freshwater benthic macroinvertebrates. Environ. Sci. Technol. 52, 13986–13994. https://doi.org/10.1021/acs.est.8b05035.
Redondo-Hasselerharm, P.E., Falahudin, D., Peeters, Edwin T.H. M., Koelmans, A.A., 2018b. Microplastic effect thresholds for freshwater benthic macroinvertebrates. Environ. Sci. Technol. 52, 2278–2286. https://doi.org/10.1021/acs.est.7b05367.
Redondo-Hasselerharm, P.E., Gort, G., Peeters, E.T.H.M., Koelmans, A.A., 2020. Nano- and microplastics affect the composition of freshwater benthic communities in the long term. Sci. Adv. 6, eaay4054 https://doi.org/10.1126/sciadv.aay4054.
Ribeiro, F., Garcia, A.R., Pereira, B.P., Fonseca, M., Mestre, N.C., Fonseca, T.G., Ilharco, L.M., Bebianno, M.J., 2017. Microplastics effects in Scrobicularia plana. Mar. Pollut. Bull. 122, 379–391. https://doi.org/10.1016/j.marpolbul.2017.06.078.
Scherer, C., Wolf, R., Völker, J., Stock, F., Brennhold, N., Reifferscheid, G., Wagner, M., 2020. Toxicity of microplastics and natural particles in the freshwater dipteran *Chironomus riparius*: same same but different? Sci. Total Environ. 711, 134604. https://doi.org/10.1016/j.scitotenv.2019.134604.

Schmidt, N., Fauvelle, V., Ody, A., Castro-Jiménez, J., Jouanno, J., Changeux, T., Thibaut, T., Sempéré, R., 2019. The amazon river: a major source of organic plastic additives to the tropical north atlantic? Environ. Sci. Technol. 53, 7513–7521. https://doi.org/10.1021/acs.est.9b01585.

Silva, C.J.M., Silva, A.L.P., Gravato, C., Pestana, J.L.T., 2019. Ingestion of small-sized and irregularly shaped polyethylene microplastics affect *Chironomus riparius* life-history traits. Sci. Total Environ. 672, 862–868. https://doi.org/10.1016/j.scitotenv.2019.04.017.

Silva, C.J.M., Silva, A.L.P., Campos, D., Machado, A.L., Pestana, J.L.T., Gravato, C., 2021. Oxidative damage and decreased aerobic energy production due to ingestion of polyethylene microplastics by *Chironomus riparius* (Diptera) larvae. J. Hazard Mater. 402, 123775. https://doi.org/10.1016/j.jhazmat.2020.123775.

Sleight, V.A., Bakir, A., Thompson, R.C., Henry, T.B., 2017. Assessment of microplastic-sorbed contaminant bioavailability through analysis of biomarker gene expression in larval zebrafish. Mar. Pollut. Bull. 116, 291–297. https://doi.org/10.1016/j.marpolbul.2016.12.055.

Suman, T.Y., Jia, P., Li, W., Junaid, M., Xin, G., Wang, Y., Pei, D., 2020. Acute and chronic effects of polystyrene microplastics on brine shrimp: first evidence highlighting the molecular mechanism through transcriptome analysis. J. Hazard Mater. 400, 123220. https://doi.org/10.1016/j.jhazmat.2020.123220.

Sørensen, J.G., Kristensen, T.N., Loeschcke, V., 2003. The evolutionary and ecological role of heat shock proteins. Ecol. Lett. 6, 1025–1037. https://doi.org/10.1046/j.1461-0248.2003.00528.x.

Tang, J., Ni, X., Zhou, Z., Wang, L., Lin, S., 2018. Acute microplastic exposure raises stress response and suppresses detoxification and immune capacities in the scleractinian coral Pocillopora damicornis. Environ. Pollut. 243, 66–74. https://doi.org/10.1016/j.envpol.2018.08.045.

Theopold, U., Dalzotto, L., Hultmark, D., 1995. Fkbp39, a Drosophila member of a family of proteins that bind the immunosuppressive drug Fk506. Gene 156, 247–251. https://doi.org/10.1016/0378-1119(95)00019-3.

Tian, L., Wang, X., Wang, X., Lei, C., Zhu, F., 2018. Starvation-, thermal- and heavy metal- associated expression of four small heat shock protein genes in Musca domestica. Gene 642, 268–276. S0378-1119(17)31005-3.

Tian, Z., Zhao, H., Peter, K.T., Gonzalez, M., Wetzel, J., Wu, C., Hu, X., Prat, J., Mudrock, E., Hettinger, R., Cortina, A.E., Biswas, R.G., Vinicius, F., Kock, C., Soong, R., Jenne, A., Du, B., Hou, F., He, H., Lundeen, R., Gilbreath, A., Sutton, R., Scholz, N.L., Davis, J.W., Dodd, M.C., Simpson, A., Mcintyre, J.K., Kolodziej, E.P., 2021. A ubiquitous tire rubber–derived chemical induces acute mortality in coho salmon. Science 371, 185–189. https://doi.org/10.1126/science.abd6951.

Tlili, S., Jemai, D., Brinis, S., Regaya, I., 2020. Microplastics mixture exposure at environmentally relevant conditions induce oxidative stress and neurotoxicity in the wedge clam Donax trunculus. Chemosphere 258, 127344. https://doi.org/10.1016/j.chemosphere.2020.127344.

Tokeshi, M., 1995. Species interaction and community structure. In: Armitage, P., Cranston, P.S., Pinder, L.C.V. (Eds.), Armitage, Patrick D.; - Cranston, Peter S.; - Pinder. Springer Netherlands, p. 297.

Uurasjärvi, E., Hartikainen, S., Setälä, O., Lehtiniemi, M., Koistinen, A., 2020. Microplastic concentrations, size distribution, and polymer types in the surface waters of a northern European lake. Water Environ. Res. 92, 149–156. https://doi.org/10.1002/wer.1229.

van Hattum, B., de Voogt, P., van den Bosch, L., van Straalen, N.M., Joosse, E.N.G., Govers, H., 1989. Bioaccumulation of cadmium by the freshwater isopod Asellus aquaticus (L.) from aqueous and dietary sources. Environ. Pollut. 62, 129–151. https://doi.org/10.1016/0269-7491(89)90183-8.

Wagner, S., Hüffer, T., Klöckner, P., Wehrhahn, M., Hofmann, T., Reemtsma, T., 2018. Tire wear particles in the aquatic environment - a review on generation, analysis, occurrence, fate and effects. Water Res. 139, 83–100. https://doi.org/10.1016/j.watres.2018.03.051.

Wang, X., Liu, L., Zheng, H., Wang, M., Fu, Y., Luo, X., Li, F., Wang, Z., 2020. Polystyrene microplastics impaired the feeding and swimming behavior of mysid shrimp Neomysis japonica. Mar. Pollut. Bull. 150, 110660. https://doi.org/10.1016/j.marpolbul.2019.110660.

Wang, Y., Branicky, R., Noe, A., Hekimi, S., 2018. Superoxide dismutases: dual roles in controlling ROS damage and regulating ROS signaling. J. Cell Biol. 217, 1915–1928. https://doi.org/10.1083/jcb.201708007.

Wik, A., Nilsson, E., Källqvist, T., Tobiesen, A., Dave, G., 2009. Toxicity assessment of sequential leachates of tire powder using a battery of toxicity tests and toxicity identification evaluations. Chemosphere 77, 922–927. https://doi.org/10.1016/j.chemosphere.2009.08.034.

Wu, D., Liu, Z., Cai, M., Jiao, Y., Li, Y., Chen, Q., Zhao, Y., 2019. Molecular characterisation of cytochrome P450 enzymes in waterflea (Daphnia pulex) and their expression regulation by polystyrene nanoplastics. Aquat. Toxicol. 217, 105350. https://doi.org/10.1016/j.aquatox.2019.105350.

Xu, C.J., Li, C., Kong, A., 2005. Induction of phase I, II and III drug metabolism/transport by xenobiotics. Arch. Pharm. Res. 28, 249–268. https://doi.org/10.1007/BF02977789.

Xu, S., Ma, J., Ji, R., Pan, K., Miao, A.J., 2020. Microplastics in aquatic environments: occurrence, accumulation, and biological effects. Sci. Total Environ. 703, 134699. https://doi.org/10.1016/j.scitotenv.2019.134699.

Yu, P., Liu, Z., Wu, D., Chen, M., Lv, W., Zhao, Y., 2018. Accumulation of polystyrene microplastics in juvenile Eriocheir sinensis and oxidative stress effects in the liver. Aquat. Toxicol. 200, 28–36. https://doi.org/10.1016/j.aquatox.2018.04.015.

Yuan, F., Yang, Z., Tang, T., Xie, S., Liu, F., 2019. A 28.6-kD small heat shock protein (MnHSP28.6) protects Macrobrachium nipponense against heavy metal toxicity and oxidative stress by virtue of its anti-aggregation activity. Fish Shellfish Immunol. 95, 635–643. S1050-4648(19)31022-8.

Ziajahromi, S., Kumar, A., Neale, P.A., Leusch, F.D.L., 2018. Environmentally relevant concentrations of polyethylene microplastics negatively impact the survival, growth and emergence of sediment-dwelling invertebrates. Environ. Pollut. 236, 425–431. https://doi.org/10.1016/j.envpol.2018.01.094.

Zhang, W., Liu, Z., Tang, S., Li, D., Jiang, Q., Zhang, T., 2020. Transcriptional response provides insights into the effect of chronic polystyrene nanoplastic exposure on Daphnia pulex. Chemosphere 238, 124563. https://doi.org/10.1016/j.chemosphere.2019.124563.

Zhu, Z., Reiser, G., 2018. The small heat shock proteins, especially HspB4 and HspB5 are promising protectants in neurodegenerative diseases. Neurochem. Int. 115, 69–79. https://doi.org/10.1016/j.neuint.2018.02.006.