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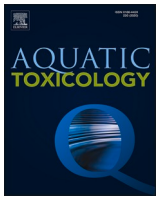

Behind conventional (micro)plastics: An ecotoxicological characterization of aqueous suspensions from End-of-Life Tire particles

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A B S T R A C T

Million tons of tires become waste every year, and the so-called End-of-Life Tires (ELTs) are ground into powder (ELT-dp; size < 0.8 mm) and granules (ELT-dg; 0.8 < size < 2.5 mm) for recycling. The aim of this study was to evaluate the sub-lethal effects of three different concentrations (0.1, 1, and 10 mg/L) of aqueous suspensions from ELT-dp and ELT-dg on *Danio rerio* (zebrafish) larvae exposed from 0 to 120 h post-fertilization (hpf). Chronic effects were assessed through biomarkers, real-time PCR, and proteomics. We observed a significant increase in swimming behavior and heart rate only in specimens exposed to ELT-dp suspensions at 1 and 10 mg/L, respectively. Conversely, the activities of detoxifying enzymes ethoxyresorufin-O-deethylase (EROD) and glutathione-S-transferase (GST) showed significant modulation only in specimens exposed to ELT-dg groups. Although no effects were observed through real-time PCR, proteomics highlighted alterations induced by the three ELT-dp concentrations in over 100 proteins involved in metabolic pathways of aromatic and nitrogen compounds. The results obtained suggest that the toxic mechanism of action (MoA) of ELT suspensions is mainly associated with the induction of effects by released chemicals in water, with a higher toxicity of ELT-dp compared to ELT-dg.

1. Introduction

Plastic materials represent one of the main inventions of the 20th Century, and their synthesis follows a positive trend worldwide, moving from 370.5 million tons (Mt) in 2018 to 400.3 Mt in 2022 (PlasticsEurope, 2023). Consequently, plastic pollution represents an emerging global issue, which affects all environmental compartments (Walker and Fequet, 2023). This phenomenon is amplified by other categories of synthetic or semi-synthetic materials that, due to their physical/chemical characteristics, are not categorized between conventional plastics. Some examples are the so-called Water-Soluble Polymers (WSPs), as polyvinyl alcohol (PVA; Magni, 2024), which, being in solution in the aqueous media, cannot be included in the definition of plastics (Hartmann et al., 2019). Also tire rubbers, containing a high percentage of both natural and synthetic materials (e.g. styrene-butadiene; SBR), are excluded from plastics by the International Organization for Standardization (ISO; Kim et al., 2023), but considered in this category by some researchers being synthetic polymers an essential ingredient (Hartmann et al., 2019). Tires are composed by different structural parts as nylon overlays, sidewalls, body piles, inner liners, steel belts, treads, and contain a plethora of chemicals as carbon black, activators and accelerator of vulcanization (Zinc - Zn and 1, 3-diphenylguanidine - DPG), vulcanizer (sulfurs), softeners (stearic acids), and antioxidants (N-(1,3-dimethylbutyl)-N'-phenyl-p-pheny lenediamine - 6PPD, an aromatic amine; Luo et al.,2021; Kim et al., 2021, 2023; Shin et al., 2022; Weyrauch et al., 2023).

Due to the high use of tires in the automotive sector (Luo et al., 2021), these objects are considered an important source of *non*-conventional (micro)plastics (Sundt et al., 2014; Leads et al., 2019). The abrasion at the tread/asphalt interface originates indeed the so-called Tire Road Wear Particles (TRWPs; Knight et al., 2020; Sieber et al., 2020; O'Brien et al., 2022). These contaminants differ from Tire Wear Particles (TWPs), which are instead obtained in controlled laboratory condition without the plethora of chemicals adsorbed by tread during the transport activity, as bituminous residues and agglomerates of exhausted fumes (Magni et al., 2022). In this context, Zn and SBR represent a marker of TRWP contamination, since the environmental detection of tire particles is difficult with conventional infrared (IR) instrumentations due to the IR absorption by carbon black (Liu et al., 2019; Magni et al., 2022; Corami et al., 2022; Sbarberi et al., 2024).

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Despite TRWPs are not found in (micro)plastic monitoring campaigns (Magni et al., 2019, 2021; Magni and Sbarberi, 2022; Sbarberi et al., 2024), it was estimated that over 70% of TRWPs is deposited in the roadside soil, while the 12–20% is dispersed in the surface waters (Baensch-Baltruschat et al., 2021).

The direct consequence of tire abrasion is the huge waste production in the form of End-of-Life Tires (ELTs). In Europe alone, approximately 3.4 Mt of tires become waste every year, and it is projected that 1.5 billion tires will be produced by 2030, with 1.2 billion discarded annually (Arulrajah et al., 2019). As reported by Forrest (2019), worldwide, each year about 3–15% of ELTs are recycled, 5–23% are reused, 25–60% are incinerated, and 20–30% are stored in landfills (Abbas-Abbadi et al., 2022). However, in Europe, tire landfill disposal is banned. Therefore, the recycling of ELTs is a pivotal step in the context of Life Cycle Assessment (LCA) of these materials. ELTs are reused in form of powder (ELT-dp) and granules (ELT-dg) after mechanical shredding of whole tire parts. This aspect represents the key difference between ELT particles and TRWPs, which are originated only by treads. Clearly, due to the analytical difficulties in the detection of tire particles in the environment, as previously explained, also the discrimination between environmental ELT particles and TRWPs is currently not feasible. ELTs are subsequently used to produce other objects such as safety floorings, road pavements, molded objects, and infills for artificial turfs (Dong et al., 2021 and citations therein). Regarding this last application, a recent regulation of the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) of the European Union imposes several restrictions from October 2031 for granular infills used loosely in synthetic sport surfaces (Commission Regulation C (2023) 6419). Consequently, the reuse of ELTs, especially when in loose form, represents a potential reintroduction of *non*-conventional (micro)plastics into the environment, with potential adverse effects on both terrestrial and aquatic ecosystems.

The ecotoxicological studies are mainly referred to TRWPs and TWPs, while only little evidence is available on the specific effects of ELTs. In addition, a wide terminological inconsistency on TRWPs, TWPs and ELTs makes difficult any comparison between different works. Some studies reported the ecotoxicity of both TRWP and TWP leachates on freshwater organisms, highlighting a plethora of effects as mortality, growth inhibition, malformations, as well as phytotoxicity, at different tested concentrations (up to g/L; Wik et al., 2009; Capolupo 2020; Cheong et al., 2023; Jiang et al., 2023; Kim et al., 2023; Roubeau Dumont et al., 2023). In some cases, the tire additives benzothiazole and Zn were identified as the main toxicants (Capolupo et al., 2020; Ding et al., 2022; Yang et al., 2022). A plethora of effects were also observed in *Danio rerio* (zebrafish), which represents an excellent biological model in freshwater ecotoxicology (Nigro et al., 2022, 2023). Zebrafish embryos were affected by tire particle leachates showing hatching delay, edemas, scoliosis and growth inhibition (Kim et al., 2023). Effects on eye development, swimming behavior and phototactic response were also observed in this model organism after exposures to tire leachate (Chang et al., 2023). Therefore, the toxicity of these contaminants seems to be high compared to those induced by conventional (micro)plastics (Kim et al., 2023), whose effects are mainly related to oxidative stress response (Magni et al., 2018, 2019, 2021). Probably, the plethora of chemicals in tires carries out a pivotal role in their toxic mechanism of action (MoA), also considering that the adsorbed substances are not covalently bound to treads and can be released in water, forming a complex pollutant mixture (Zimmermann et al., 2019).

Concerning ELT toxicity, Magni et al. (2022) characterized the effects of ELT-dp and ELT-dg suspensions at organism and population levels following the guidelines of Organization for Economic Cooperation and Development (OECD) on algae (*Raphidocelis subcapitata*, formerly known as *Pseudokirchneriella subcapitata*), crustaceans (*Daphnia magna*) and fish (zebrafish). In zebrafish, an Effect Concentration 50 (EC_{50}) > 100 mg/L (at 24 and 48 h post-fertilization; hpf) was observed for both ELT-dp and ELT-dg suspensions. Additionally, values of Lowest Observed Effect Concentration (LOEC) of 10 mg/L were recorded for survival and juvenile weight (at 30 days) in specimens exposed to ELT-dg suspensions, as well as for juvenile survival and abnormal behavior (at 30 days) in specimens exposed to ELT-dp suspensions. Based on these results, our current hypothesis is that there are some toxicity mechanisms at lowest biological levels potentially implicated in the organism and population adverse effects.

For these reasons, considering the pivotal differences in terms of composition between ELT particles and the most studied TRWPs and TWPs, as well as the great knowledge gap about the potential effects specifically referred to ELTs, the aim of this study was a wide investigation of the chronic toxicity induced by aqueous suspensions (0.1, 1 and 10 mg/L) of both ELT-dp and ELT-dg on zebrafish larvae exposed from 0 to 120 hpf. To investigate the sub-lethal effects, we performed a battery of biomarkers of cellular stress (molecular and biochemical levels), neurotoxicity (link between molecular and organism level), as well as physiological and behavioural endpoints (organism level). In addition, to better characterize the MoA of the ELT suspensions, we applied both real-time PCR (RT-PCR) and gel-free proteomics on organisms exposed to all experimental groups.

2. Materials and methods

2.1. Preparation and characterization of aqueous suspensions

The ELT-derived materials were obtained from the mechanical shredding of whole tires. The ELT-dg (0.8 < size < 2.5 mm) derived from a pool of ELT samples from 20 different ELT treatment plants in Italy (19) and Switzerland (1). To obtain the ELT-dp (size < 0.8 mm), a fraction of ELT-dg samples was gridded using a cracker mill machine. These materials were deeply characterized in our previous study, through the quantification of 152 elements/compounds on the solid fraction of ELT-dg particles (see Table S1 in Magni et al., 2022), highlighting the presence of metals, especially Zn, polycyclic aromatic hydrocarbons (PAHs), benzothiazoles, phenols, phthalates, hydrocarbon oils, and 6PPD.

We prepared 100 mg/L of both ELT-dp and ELT-dg suspensions in Milli Q water with 72 h of stirring, followed by 48 h of sedimentation (Magni et al., 2022). During this period the suspensions were kept covered with an aluminum foil and maintained at room temperature (RT). No filtration was performed on obtained matrices. This is the pivotal difference between leachates, which do not contain debris, and suspensions, which contain both chemicals and the finest fraction of ELT particles (the coarse debris was not collected during the preparation of the dilutions used in the exposures). These samples represented the stock solutions for the preparation of the exposure concentrations. As reported by Magni et al. (2022), the ELT particles released in these suspensions mainly Zn (53.4 μg/L), and the presence of other tire-related contaminants as N-cyclohexyl-cyclohexanamine and DPG was < 1 μg/L. Considering the recent attention by scientific community on the tire rubber antioxidant 6PPD, which induces a plethora of adverse effects in freshwater fish, from cardiotoxicity in zebrafish (Fang et al., 2023) to acute effects on salmonids (Tian et al., 2021), we measured its concentration in both 100 mg/L ELT-dp and ELT-dg suspensions with High Performance Liquid Chromatography Mass Spectrometry (HPLC MS) following the method EPA 8321. The observed concentration was < 0.01 μg/L in both samples.

In addition, the elemental compositions of ELT-dp and ELT-dg, as well as the presence of ELT particles in the 100 mg/L suspensions, were evaluated using a Scanning Electron Microscopy (JEOL JSM-5500LV) coupled with an Energy Dispersing Spectrometry (SEM-EDS) at the UNITECH COSPECT facility of the University of Milan. This technique was applied directly on the solid samples deposited on stubs, as well as on evaporated drops of each 100 mg/L suspension, without metallization of the samples.

2.2. Zebrafish exposures

Considering the analytical problems for the tire particle detection in the environment and the lack of information about the specific monitoring of ELT particles, we chose the exposure concentrations based on the results observed in our previous study on the toxicity of ELTs at organism and population levels. Since in Magni et al. (2022) we obtained a No Observed Effects Concentration (NOEC) on zebrafish hatching of 10 mg/L (at 96 hpf) for both ELT-dp and ELT-dg suspensions, in this work we exposed the organisms to 0.1, 1 and 10 mg/L of abovementioned contaminants to both investigate the eventual effects at the lowest biological levels and the possible MoA.

The fertilized eggs of zebrafish (AB wild-type) were provided by the facility of the Department of Earth and Environmental Sciences of the University of Milano-Bicocca, according to the Italian law, rule and regulation (Legislative Decree n. 116/92; authorization n. 0020984 - 12/02/2018). The exposures were conducted in triplicate placing 20 eggs in each Petri dish, in static conditions, from 0 to 120 hpf, with 50 mL of pre-aerated ELT suspension dilutions in zebrafish water (0.1% methylene blue - $C_{16}H_{18}ClN_3S$, 0.1 g/L sodium bicarbonate - $NaHCO_3$, 0.1 g/L Instant Ocean® and 0.2 g/L calcium sulphate - $CaSO_4$, in 1 L of Milli Q water). To provide enough biological material for biomarkers, RT-PCR, and proteomics, we conducted five different exposures, all in triplicate, for a total of more than one thousand larvae. During the exposure, the specimens were maintained at 28 °C and every day acute (egg coagulation, lack of somite formation, *non*-detachment of the tail and lack of heartbeat; OECD 236, 2013) and chronic effects (scoliosis, development delay, edemas, and malformations; Schiwy et al., 2015) were registered. Since no significant acute effects were observed, at the end of the exposure, the larvae were processed for the sub-lethal effect analyses.

2.3. Evaluation of chronic toxicity: biomarkers

2.3.1. Endpoints of cellular stress

The activity of both antioxidant and detoxifying enzymes was evaluated in triplicate on the homogenate of 20 larvae *per* treatment ($n = 3$ pools of 20 larvae *per* treatment). Obtained organisms were pooled, using a potter, in 250 µL of 100 mM phosphate buffer (pH 7.4) with 100 mM potassium chloride (KCl), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT) and inhibitors of protease (1:100 *v/v*). After homogenization, the samples were centrifuged at 15,000 g for 30 min at 4 °C. The proteins in the obtained fraction (S15) were quantified through the Bradford method (1976) to normalize the biomarker results. As endpoints of oxidative stress, we measured the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) using the 6715 UV–Vis spectrophotometer (Jenway). In detail, SOD activity was evaluated through the reduction inhibition of 10 µM cytochrome C due to the superoxide anion formed by xanthine oxidase-50 µM hypoxanthine. The kinetic was read at wavelength (λ) 550 nm for 2 min. The CAT activity was determined through the consumption of 50 mM hydrogen peroxide (H_2O_2), reading the absorbance at λ 240 nm for 1 min. Concerning GPx the kinetics was measured evaluating the consumption of 120 µM dihydro-nicotinamide-adenine-dinucleotidephosphate (NADPH) using 0.2 mM H_2O_2, 2 mM glutathione (GSH), 1 mM sodium azide (NaN_3) and glutathione reductase (2 U/mL). The absorbance was read at λ 340 nm for 1 min. In addition, we quantified the Reactive Oxygen Species (ROS) levels through the EnSight™ multimode plate reader (PerkinElmer). The ROS content was measured using 10 mg/mL dichlorofluorescein-diacetate (DCFH-DA) in dimethyl sulfoxide (DMSO). In detail, 20 µL of samples were added to a 96-well plate and incubated for 5 min at 37 °C. Subsequently, we added 100 µL of phosphate buffer saline (PBS) and 8.3 µL of DCFH-DA incubating for 30 min at 37 °C. The fluorescence was read at λex 485 nm and λem 530 nm.

As endpoints of detoxification, we measured the activities of ethoxyresorufin-O-deethylase (EROD) and glutathione-S-transferase (GST) through the EnSight™ multimode plate reader (PerkinElmer). For EROD activity the S15 fraction was added in a 96-well plate with 50 mM Tris buffer (pH 7.4), 5.32 mg/mL bovine serum albumin (BSA), 6.7 mM NADPH, and 100 µM 7-ethoxyresorufin (ER) in methanol (MeOH). EROD activity was calculated from relative fluorescence units such as resorufin product, through the resorufin curve. The fluorescence was read at λex 535 nm and λem 590 nm at 37 °C. The GST activity was measured at the 6715 UV–Vis spectrophotometer (Jenway) using 20 mM GSH and 20 mM 1-Cl-2,4-dinitrobenzene (CDNB) in ethanol. The absorbance was read at λ 340 nm for 1 min (Gagné, 2014; Magni et al., 2018; Parenti et al., 2019).

2.3.2. Endpoints of neurotoxicity

As endpoints of neurotoxicity, we evaluated the activities of acetylcholinesterase (AChE) and monoamine oxidase (MAO) through the EnSight™ multimode plate reader (PerkinElmer). The activity of these neuro-enzymes was evaluated in triplicate on the homogenates of 20 larvae *per* treatment ($n = 3$ pools of 20 larvae *per* treatment). The obtained homogenates (see paragraph 2.3.1) were centrifuged for 30 min at 4 °C at 15,000 g for AChE activity and at 1000 g for MAO kinetic. The AChE activity was evaluated through the Ellman reagent, containing 1 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in 100 mM Tris-acetate at pH 7.4, and 1 mM acetylthiocholine in 50 mM Tris–HCl. The absorbance was read at λ 412 nm for 15 min at 30 °C. The kinetic of MAO was measured using 1 mM tyramine, 10 µM dichlorofluorescein diacetate in a 140 mM sodium chloride (NaCl), 10 mM 4-(2-hydroxyethyl)−1-piperazineethanesulfonic acid (HEPES) and sodium hydroxide (NaOH) buffer (pH 7.4), 1 mg/mL peroxidase and 10 mM of 3-amino-1,2,4-triazole. The activity was measured at λex 485 nm and λem 528 nm for 3 min (Gagné, 2014; Magni et al., 2018).

2.3.3. Behavioural and physiological endpoints

The behavioural alterations on the swimming activity at 120 hpf were evaluated on 9 specimens *per* treatment through the DanioVision™ video tracking system (Noldus IT, Wageningen, Netherlands). The videos were recorded from three independent experiments with 30 frames/s through an infrared camera. Each embryo was placed in a single well of a 24 multi-well with 3 mL of zebrafish water and treated with two consecutive cycles of dark/light phases of 10 min each (acclimation phase of 5 min of darkness and 5 min with light intensity at 4400 lx as stress condition; Zheng et al., 2021). Data were acquired every min, and two different behavioral parameters were analyzed by EthoVision XT 11.5 software (Noldus IT). In detail, we measured the total distance moved, as the distance traveled by the organism from the initial track to the final one, and the mobility, as the percentage changes in the animal position between two tracks (Nigro et al., 2022, 2023). Lastly, as physiological endpoint, we measured the heart rate of larvae (9 specimens *per* treatment). Each embryo was placed in a water drop on a glass slide and subsequently filmed using a video camera connected to an optical microscope (Basler acA1300–60 gm GigE camera). Videos were recorded for 10 s and heartbeats were counted (Binelli et al., 2024).

2.4. Evaluation of chronic toxicity: RT-PCR

To perform the analysis 3 specimens for each replicate were analyzed (9 specimens *per* treatment). Considering that Zn was the main chemical released by ELTs in the suspensions (Magni et al., 2022), we investigated the transcription of metallothionein (MT) genes (*mt2* and *mtb*). In addition, since aromatic and nitrogen compounds can be adsorbed by ELTs during the activity of transport mean, or used in the rubber mixture, we investigated the transcription of GST and EROD genes (*gstm, gstp, gsto* and *cyp1a*), whose activities were also assessed through biomarkers. The samples were lysed and homogenized in 1 mL of TRI reagent (Sigma). Subsequently, we added 0.2 mL of chloroform and mixed

for 15 s. Samples were incubated at RT and then centrifuged at 12,000 g at 4 °C for 15 min. The upper aqueous phase, containing the RNAs, was transferred into a new tube. To precipitate the RNA, we added 0.5 mL isopropanol and centrifuged the sample at 12,000 g at 4 °C for 10 min. The RNA pellets were washed with 0.5 mL of 75% ethanol. After centrifugation, the ethanol was removed, and the samples were dried for 10 min. The RNA pellets were resuspended by adding 100 µL of RNAse-free water and incubated at 55 °C for 10 min. We checked sample quality and quantity using a NanoDrop spectrophotometer. Then, 1 µg of each RNA sample was retrotranscribed with StoS M-MLV Supermix (Genespin) using standard protocol. cDNAs were used for RT-PCR (SYBR® Green Master Mix, Bio-rad Laboratories) analysis. RT-PCRs were performed with oligonucleotides designed to amplify 100–200 bp fragments of the coding sequence of indicated genes. The housekeeping gene Gapdh was used to normalize expression data. The relative sample enrichment was calculated with the formula 2–(ΔΔCt), where ΔΔCt = [(Ct sample – Ct Gapdh)x − (Ct sample – Ct Gapdh)y], x = sample and y = sample control. RT-PCR analyses were performed on three independent biological replicates.

2.5. Evaluation of chronic toxicity: gel-free proteomics

For the proteomic analysis 3 pools of 20 larvae *per* treatment were used. The larvae were homogenized in 250 µL of lysis buffer with 20 mM HEPES (pH 7.5), 320 mM sucrose, 1 mM EDTA (pH 8.5), 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (pH 8.1; EGTA), 1 mM sodium ortho-vanadate (Na_3VO_4), 10 mM β-glycerophosphate, 10 mM sodium fluoride (NaF), 10 mM sodium pyrophosphate (NaPPi), 1 mM phenylmethylsulfonyl fluoride (PMSF) in ethanol, 5 mM DTT and inhibitors of protease. Homogenates were centrifuged at 15,000 g at 4 °C for 10 min, and, as performed for biomarkers, proteins were quantified through the Bradford method (1976). Then, 200 µg of protein was precipitated with methanol/chloroform/Milli Q® water in the ratio 4:1:3 (*v/v*). The samples were centrifuged at 14,000 g for 30 min and the pellets were resuspended in 8 M urea in 50 mM Tris hydrochloride, 30 mM NaCl (pH 8.5) and inhibitors of protease and centrifuged at 14,000 g at 4 °C for 30 min. Subsequently, proteins were re-quantified in the samples and 10 µg of proteins were treated with 50 mM DTT in 50 mM ammonium bicarbonate (AMBIC) at 52 °C for 30 min under stirring to reduce the disulfide bonds. After reduction, proteins were treated with 100 mM iodoacetamide (IANH2), incubating 20 min at RT, to alkylate the sulfhydryl groups. The proteins were digested by Trypsin Sequencing Grade (Roche) in 50 mM AMBIC at 37 °C overnight under stirring (Magni et al., 2019, 2021; Binelli et al., 2024). Lastly, 25 µL of samples was processed for the purification procedure (desalting) with 5 µg C18 zip-tip. The obtained eluate was reduced in a speedvac and reconstituted with 20 µL of 0.1 % formic acid. Proteins (5 µL) were then identified by high resolution mass spectrometry (nLC-MS/MS) at the UNITECH OMICS facility of the University of Milan using a Dionex Ultimate 3000 nano-LC system (Sunnyvale CA, USA) connected to an Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Scientific) equipped with a nano-electrospray ion source. Peptide mixtures were pre-concentrated (Acclaim PepMap 100 - 100 µm x 2 cm C18, Thermo Scientific) and separated on an EASY-Spray ES802A column, 25 cm x 75 µm (Thermo Scientific Acclaim PepMap RSLC C18, 3 µm, 100 Å) using a mobile phase A (0.1 % formic acid in water) and a mobile phase B (0.1 % formic acid in acetonitrile 20/80, v/v) with a flow rate of 0.300 µL/min. The temperature was set to 35 °C and the sample was injected in triplicate. A blank was run to avoid sample carryover. All results were processed with the Proteome Discoverer 2.5 software by setting *Danio rerio* (sp_incl_isoforms) as database (Magni et al., 2019, 2021; Binelli et al., 2024).

2.6. Statistical approach

The possible presence of outliers was verified using GraphPad. For biomarkers and RT-PCR, to detect significant differences between treated and control groups, STATISTICA 7.0 Software was used. One-way Analysis of Variance (one-way ANOVA) followed by the Fisher LSD post-hoc test was subsequently performed. Regarding proteomics, only proteins with an abundance ratio (AR) between treated and control groups of at least 2-fold change (< 0.5 for down-regulated proteins and > 2.0 for up-regulated proteins), and with a p-value < 0.01, were considered significantly modulated by the treatment. To identify the main protein pathways modulated by the exposure, the STRING freeware (Gene Ontology, Biological Function) was employed.

3. Results

3.1. Material characterization

The SEM-EDS observation of both ELT-dp and ELT-dg particles allowed the elemental identification of the samples, adding further information to those reported by Magni et al. (2022). Indeed, as showed in Fig. 1A, it is possible to observe in solid samples of ELT-dg and ELT-dp the presence of Copper (Cu), Sulfur (S), Zn and Silicon (Si), in addition to carbonaceous (C) nature of the rubber. Furthermore, the presence of other particles with a possible metallic nature (as Iron; Fe), with a size between 10 and 20 µm, was found on the ELT surface (Fig. 1B).

Regarding the characterization of 100 mg/L ELT-dp and ELT-dg aqueous suspensions, the presence of dispersed particles in the aqueous medium was observed (Fig. 2A, B). These particles were represented by ELTs, due to the presence of C and Zn (Fig. 2A), and by debris with metal and glass origin, as indicated by the Si peak (Fig. 2B). Metal particles derived from structural parts of tires, while glass debris are used as stabilizers in the rubber mixture.

3.2. Evaluation of chronic toxicity induced by ELT-dp and ELT-dg suspensions

During the exposure no significant acute effects were observed and, in all treatments, the larvae viability was always over the 82%, with a value of 87% in the controls. In the same manner, no significant chronic effects on zebrafish phenotype were obtained during the exposure.

3.2.1. Biomarkers and RT-PCR

Concerning the oxidative stress biomarkers, we did not observe any significant modulation of antioxidant enzyme activities, neither alteration of the ROS level, despite an increase in the biological trends of both SOD and GPx activities in larvae exposed to ELT-dg suspensions (Figure S1). On the other hand, the suspensions of ELT-dg impacted on the detoxifying performance of exposed larvae. A significant effect of treatment ($F_{6,14} = 9.3221$; $p < 0.01$) was observed for EROD activity, with a significant decrease in larvae exposed to ELT-dg 1 mg/L ($p < 0.01$), and a significant increase compared to control in larvae exposed to ELT-dg 10 mg/L ($p < 0.01$; Fig. 3). In the same manner, the activity of GST showed a significant effect of treatment ($F_{6,14} = 4.210$; $p < 0.05$), with a significant increase only in the group exposed to 1 mg/L ELT-dg suspension (Fig. 3). Regarding the activity of the neuro-enzymes AChE and MAO, no significant effects were observed after the exposure (Figure S2). Moving to behavioral endpoints, we found a significant effect of treatment in both total distance moved ($F_{3,31} = 2.96443$; $p < 0.05$) and mobility ($F_{3,31} = 3.55080$; $p < 0.05$) parameters in the larvae exposed to ELT-dp suspensions, with a significant increase ($p < 0.05$) of the swimming activity in the organisms exposed to ELT-dp 1 mg/L under the stress condition of 4400 lx (Fig. 4). A *non*-significant increasing trend of both behavioral parameters was also observed in specimens exposed to 10 mg/L of ELT-dp suspension, with an increase compared to control of 40% for total distance and 45% for mobility (Fig. 4). On the contrary, no significant effects on the swimming performance were observed in the specimens exposed to ELT-dg suspensions (Figure S3). In this context, also the physiological endpoint of heart rate was not modulated

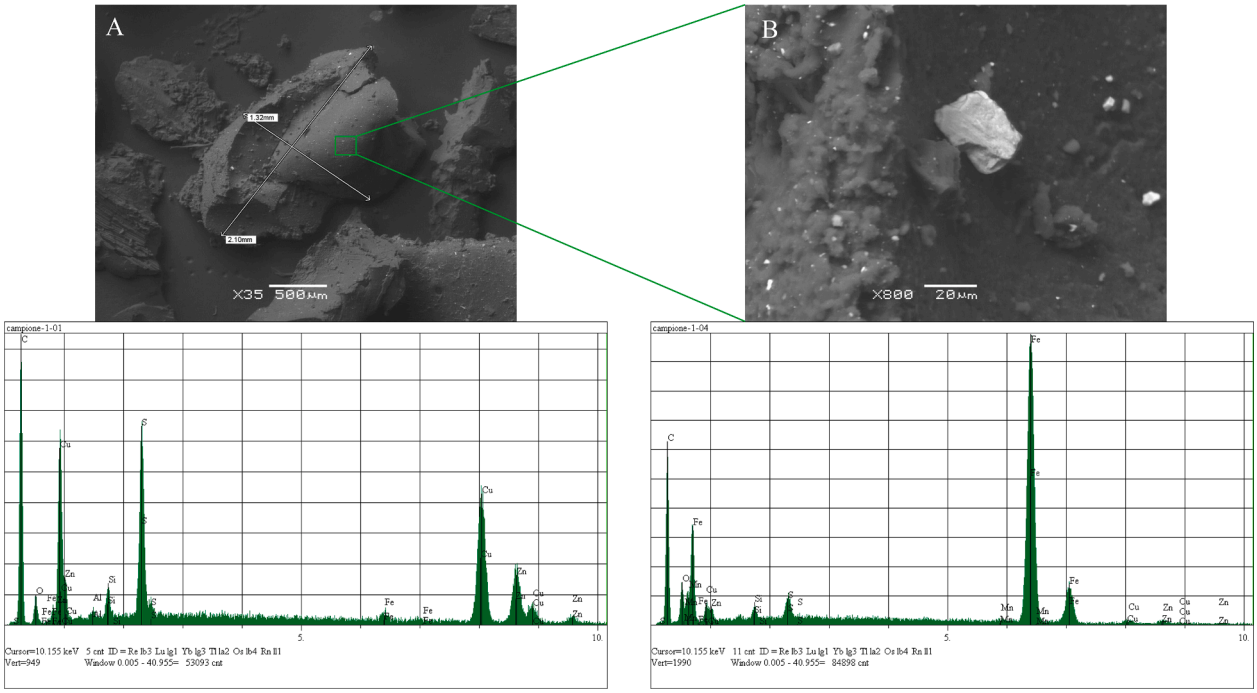

Fig. 1. SEM-EDS on solid samples of ELT-dp and ELT-dg (A), as well as on other particles of suspected metallic material detected in both ELT-dp and ELT-dg samples (B).

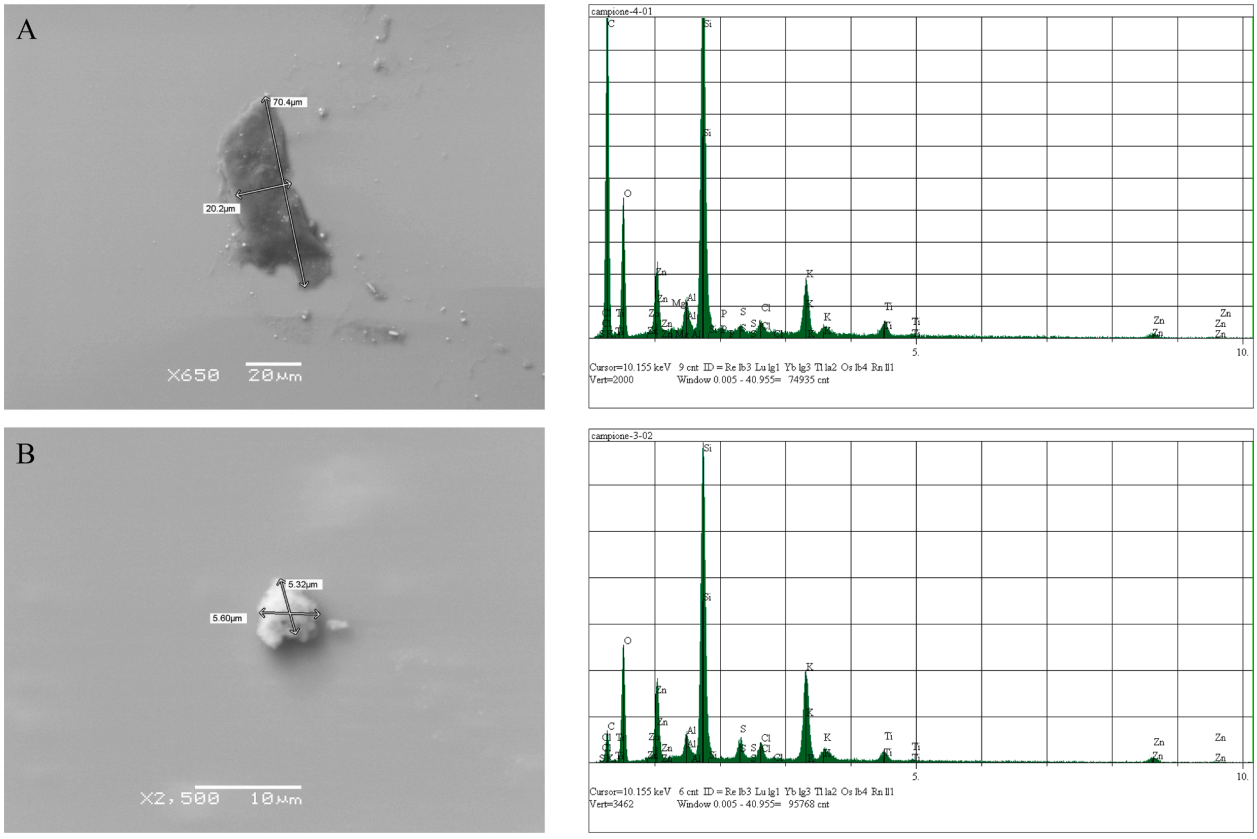

Fig. 2. SEM-EDS on 100 mg/L of ELT-dp and ELT-dg aqueous suspensions (stock solutions). We detected suspected ELT particles (A) as well as other debris with a glass or metal origin (B).

by ELT-dg suspensions, resulting significantly ($p < 0.05$) impacted only by ELT-dp ($F_{3,31} = 5.314$; $p < 0.01$) with an increase in larvae exposed to ELT-dp 10 mg/L (Fig. 5).

The results of RT-PCR showed no significant effects on the transcription of the GST (*gstm, gstp, gsto*), EROD (*cyp1a*) and MT (*mtb, mt2*) genes, despite some increasing trends compared to control were

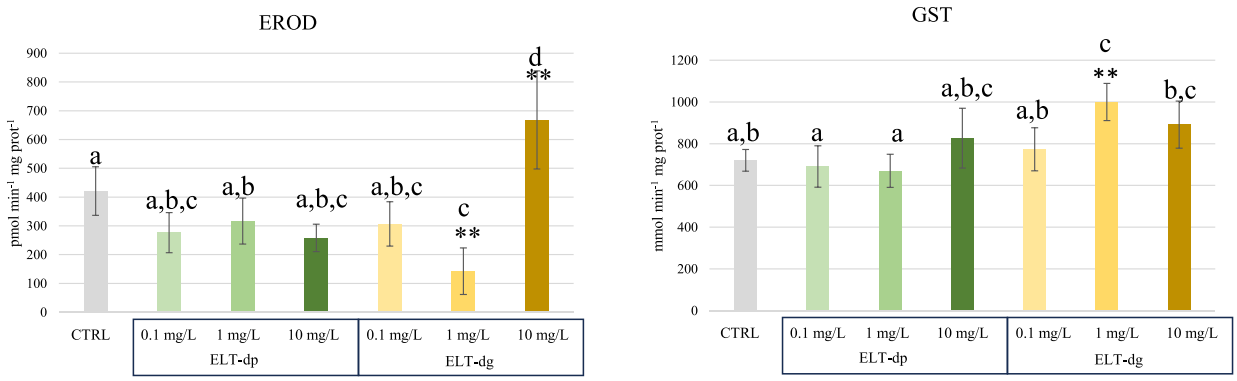

Fig. 3. Activity of detoxifying enzymes EROD and GST (mean ± SD; $n = 3$ pools of 20 specimens *per* treatment; one-way ANOVA, Fisher LSD post-hoc test). The asterisks indicate the significant differences ($^{**}p < 0.01$) between treated and control, while letters indicate the significant differences between treated.

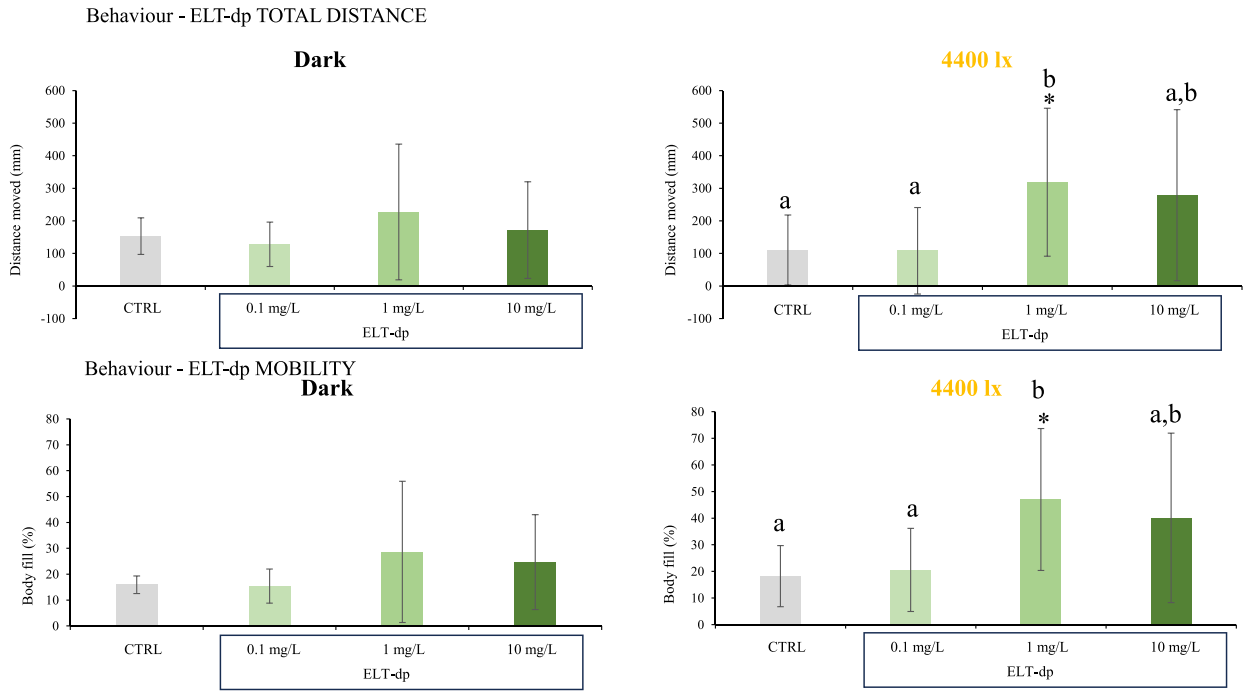

Fig. 4. Behavioural parameters of specimens exposed to ELT-dp (mean ± SD; $n = 9$ specimens *per* treatment; one-way ANOVA, Fisher LSD post-hoc test). The asterisks indicate the significant differences ($^{*}p < 0.05$) between treated and control, while letters indicate the significant differences between treated.

observed for *cyp1a, gsto* and *mtb* in specimens exposed to ELT-dg suspensions (Figure S4).

3.2.2. *Omic techniques*

Regarding proteomics, we detected 167 proteins in common between the different groups (treated and control), most of these significantly modulated by the treatments. In detail, using the selected double cut-offs (2-fold changes and significant differences), we observed a comparable amount of proteins modulated by the three different ELT-dp suspensions. Indeed, 108 proteins were modulated by both 0.1 mg/L and 1 mg/L ELT-dp (16 down- and 92 up-regulated and 21 down- and 87 up-regulated, respectively), and 102 proteins modulated by 10 mg/L ELT-dp (15 down- and 87 up-regulated). On the other hand, we obtained a concentration-dependent modulation of proteins by ELT-dg suspensions, with 37 proteins modulated by 0.1 mg/L ELT-dg (3 down- and 34 up-regulated), 87 proteins modulated by 1 mg/L ELT-dg (6 down- and 81 up-regulated) and 106 proteins in the group exposed to 10 mg/L ELT-dg suspension (14 down- and 92 up-regulated; Fig. 6). The amount of proteins modulated by the highest ELT-dg concentration was comparable to those impacted by the lowest ELT-dp concentration. Grouping the proteins in the Venn Charts, it is possible to note that many of these macromolecules were commonly modulated by the different groups (Fig. 7). This aspect is not surprising since ELT-dp and ELT-dg derive from the same ELT pool. In particular, 93 proteins were modulated in common by the three concentrations of ELT-dp (Fig. 7A), while only 22 proteins were modulated in common by the three concentrations of ELT-dg, with 61 proteins shared between the two highest concentrations (1 and 10 mg/L of ELT-dg suspensions; Fig. 7B). Integrating all treatment groups, it is possible to note that 57 proteins were modulated in common by ELT-dp and ELT-dg suspensions, excluding the lowest ELT-dg concentration that impacted only 37 proteins (Fig. 7C).

4. Discussion

Since the exposed zebrafish larvae to both ELT-dp and ELT-dg suspensions did not show significant alteration of the oxidative status (Figure S1), this result highlights the first difference of MoA between ELT particles and conventional (micro)plastics, whose main effect seem

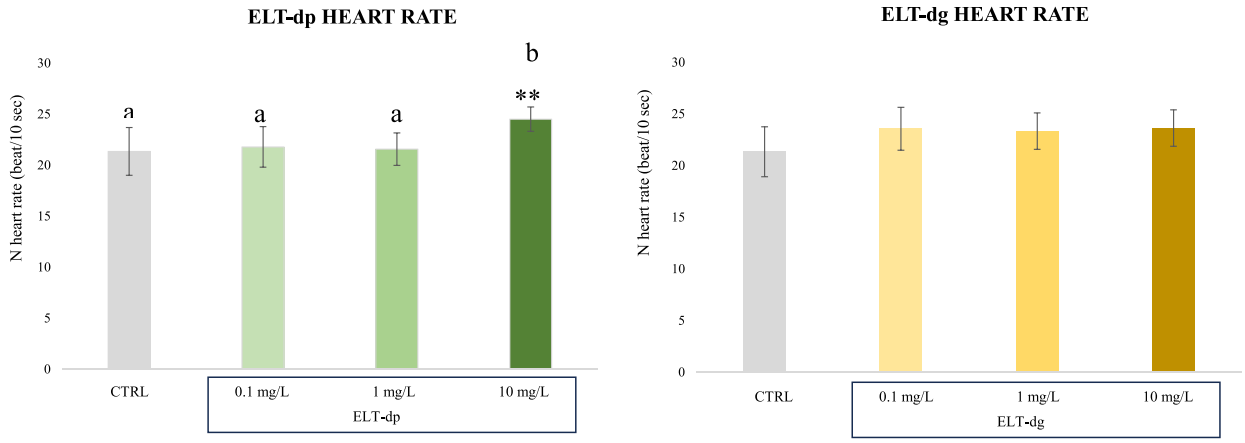

Fig. 5. Heart rate of specimens exposed to ELT-dp and ELT-dg suspensions (mean ± SD; $n = 9$ specimens *per* treatment; one-way ANOVA, Fisher LSD post-hoc test). The asterisks indicate the significant differences ($^{**}p < 0.01$) between treated and control, while letters indicate the significant differences between treated.

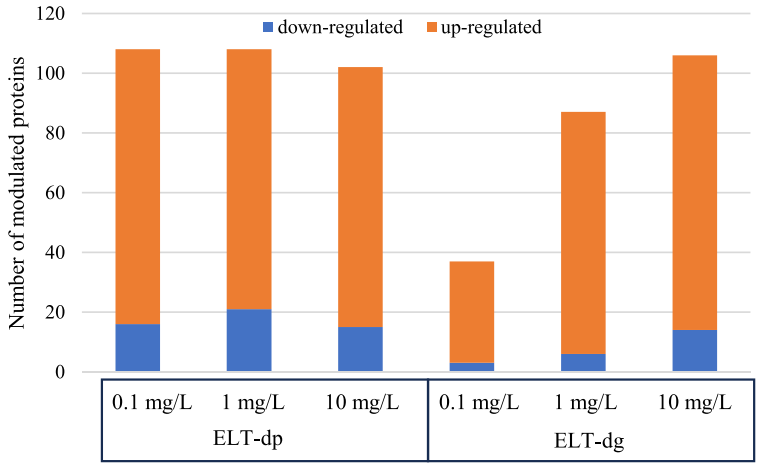

Fig. 6. Total number of modulated proteins (up- or down-) in zebrafish larvae ($n = 3$ pools of 20 specimens *per* treatment).

to be the oxidative stress, as a consequence of mechanical abrasion and inflammation, as well as a reduction in lipid storage (Wang et al., 2021 and citations therein). Indeed, some our previous studies reported the significant modulation of antioxidant enzymes in organisms exposed to conventional (micro)plastics at both laboratory and environmental-like concentrations (Magni et al., 2018, 2021, 2022), in addition to an up-/down-regulation of proteins involved in the oxidative stress response (Magni et al., 2019). An increase of ROS in the algae *Dunaliella tertiolecta* exposed to leachates from polystyrene (PS) and polyethylene (PE) particles was also described (Schiavo et al., 2021). Besides the different composition among ELT particles and conventional (micro) plastics, the lack of significant effects on the antioxidant machinery could be associated to the low concentrations of dispersed ELT-dp and ELT-dg debris in the exposure media, which can induce a low abrasion on exposed organisms. Indeed, despite the presence of ELT particles, as well as of metal and glass debris, was confirmed in the 100 mg/L suspensions by SEM (Fig. 2), their concentrations were too low in the exposure dilutions to perform the size distribution measurement with Dynamic Light Scattering (DLS), as reported by Magni et al. (2022). In any case, it is necessary to highlight how some studies on TWP leachates reported an increase of oxidative stress on treated organisms (Shin et al., 2022; Liu et al., 2023), suggesting that other investigations are needed to clarify this aspect.

Concerning the neurotoxicity, no effects were observed on AChE and MAO activity (Figure S2) that represents another difference in terms of MoA between ELTs and (micro)plastics, since a meta-analysis study indicated that the neurotoxic effect of conventional polymers is a consequence of oxidative stress, nerve damage, and metabolic disorders, with a decrease of AChE in exposed specimens by 16.2% (Xiong et al., 2023).

On the other hand, we observed effects at the organism level, with a significant increase in the swimming behavior for both distance moved and mobility in specimens exposed to 1 mg/L ELT-dp during the stressing treatment with 4400 lx (Fig. 4), while no effects were observed during the dark phase. This is in line with Magni et al. (2022), who reported a LOEC of 10 mg/L (at 30 days) for abnormal behaviour in zebrafish exposed to ELT-dp suspensions. In this context, Yu et al. (2023) observed an increase in swimming behaviour in adult female zebrafish exposed to Zn, the main chemical released by ELTs in water (Magni et al., 2022), altering in the brain and intestine the levels of some neurotransmitters as glutamate, γ-aminobutyric acid (GABA) and serotonin. In turn, the serotoninergic system, together with adrenergic and cholinergic ones, seems to control the heart rate in zebrafish (Stoyek et al., 2017). Coherently, a significant increase in the heart rate in specimens exposed to 10 mg/L ELT-dp was observed (Fig. 5). In this context, Zhang et al. (2023) reported a reduction of heart rate in zebrafish after an exposure of 48 h to 0.025, 0.1, 0.2, 0.4, 0.8 and 1.2 mg/L to both 6PPD and 6PPD-quinone, the main 6PPD metabolite, while

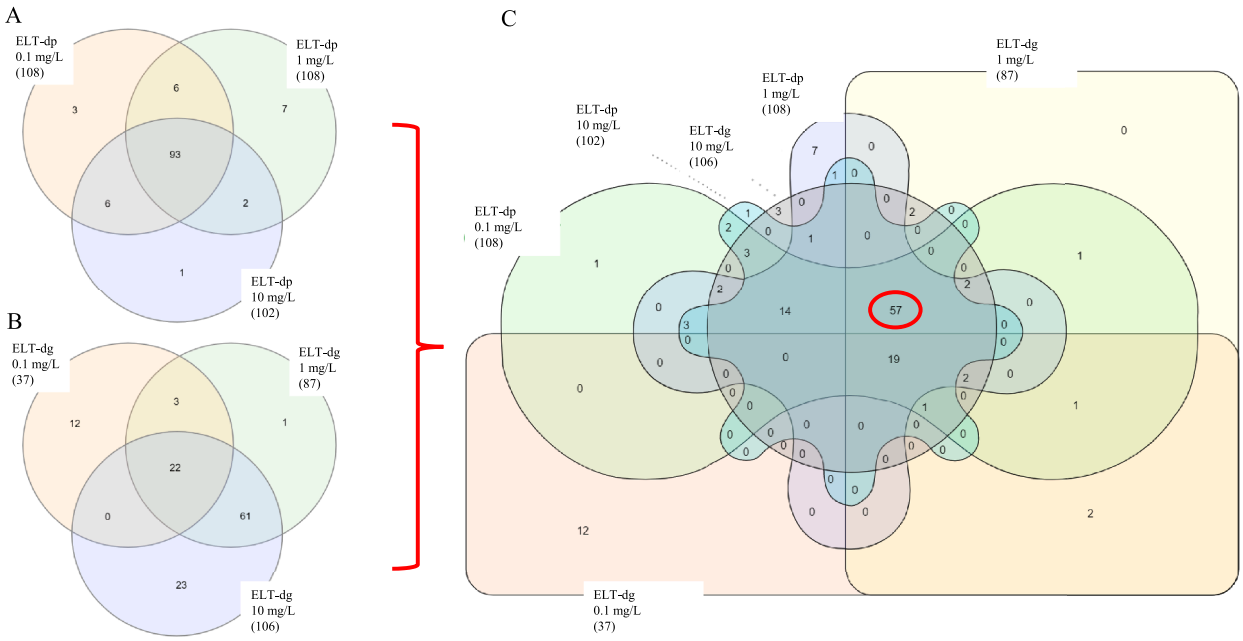

Fig. 7. Venn charts with the proteins modulated in common or in independent manner by the three ELT-dp suspensions (A), ELT-dg suspensions (B) and by all treatments (C). The red circle highlights the common proteins between all treatments with the exception of the lowest ELT-dg concentration. These charts were created using the web-based tool of Heberle et al.(2015).

Ricarte et al. (2023) observed an increase in the heart rate of embryos exposed to 6PPD-quinone at 20, 200 and 2000 ng/L after 24 h. In the same way, Kolomijeca et al. (2020) evaluated an increase in the heart rate in fathead minnow *Pimephales promelas* embryos exposed to 10,000 mg/L TRWP leachate. This evidence could suggest a possible impact by ELT-dp suspensions on the neuro-endocrine system of zebrafish, despite MAO, which are involved in the serotonin degradation, did not show any significant alterations. Perhaps, the conveyed contaminants by ELTs play an important role in the toxicity at organism level, also considering the low concentration of ELT particles in the exposure suspensions, as previously explained. In line with this hypothesis, a significant modulation of detoxifying enzymes EROD and GST was observed (Fig. 3), although only in organisms exposed to ELT-dg suspensions. However, a decreasing, but not significant, trend for EROD was observed also in specimens exposed to ELT-dp. In this context, the lack of significant effects could be associated to the fact that the kinetics were measured only at the end of the exposure ($t = 120$ hpf), but it is well known that the enzyme activity can follow a bell-shaped curve, with a wave response, during the contaminant exposures (Gagné, 2006, 2018; Colas and Le Faucheur, 2024). Therefore, in future studies it will be important to assess the enzyme kinetics at different times of exposure period to detect the eventual oscillatory response of selected endpoints. In any case, despite it not being possible to attribute the observed effects to specific chemicals in complex mixtures, the significant inhibition of EROD activity in specimens exposed to 1 mg/L ELT-dg could be associated with Zn, as suggested by Oliveira et al. (2004) in a study on the sea bass *Dicentrarchus labrax* L. treated with heavy metals. On the other hand, other substances present at lowest concentration than Zn in the suspensions, as PAHs, phthalates and nitrogen compounds (Magni et al., 2022), could induce additive and synergic toxic effects on exposed larvae, justifying the significant increase of EROD in specimens exposed to 10 mg/L ELT-dg, as observed by Stephensen et al. (2003) in the rainbow trout *Oncorhynchus mykiss* treated with tires with high aromatic oils, and of GST in 1 mg/L ELT-dg group.

Based on these results, to better investigate the potential role of released chemicals by both ELT-dp and ELT-dg, we performed the RT-PCR and proteomics. No significant alterations were observed on the transcripts of GST, EROD and MT genes (Figure S4), despite the modulation of EROD and GST kinetics was observed. Probably, the lack of *ex-novo* synthesis of these detoxifying enzymes caused the effects found at organism levels in zebrafish larvae. In line with these results, proteomics did not show the modulation of GST, EROD and MT (Table S1).

The main modulated proteins, in the various experimental groups, appear to be involved in both structural processes, as cytoskeleton and ribosome formation, as well as in functional processes, as translation and stress response (Table S1). Interesting is the modulation of three key protein groups represented by Calmodulin, Heat shock proteins and Crystallins. A down-regulation of Calmodulin (CALM) was induced in all treatment groups. This evolutionarily conserved and ubiquitously protein is a regulator of Calcium ion (Ca^{++}) related functions, participating in heart cell coagulation, in various cardiovascular defects as well as in the regulation of cardiac function and hypertrophy (Yang et al., 2024 and citations therein). Therefore, its regulation could be related to the significant modulation of heart rate observed in specimens exposed to ELT-dp 10 mg/L (Fig. 5), as previously explained. Except for larvae exposed to the lowest concentration of ELT-dg suspension, in all other groups we observed the modulation of Heat shock protein HSP 90-beta (HS90B) and Heat shock protein HSP 90-alpha 1 (H90A1), with levels of up-regulation among the highest of the impacted proteins by ELT suspension exposure (Table S1). These proteins are related to a general stress response induced, e.g., by metals (Martín-Folgar et al., 2017). Coherently with our results, Carrasco-Navarro et al. (2021) reported an up-regulation of Heat shock protein genes *hsp90, Gp93, hsc70, hsp60* and *hsp40* in *Chironomus riparius* exposed to tire particles.

The up-regulation of Gamma-crystallin N-B (CRGNB) in specimens exposed to ELT-dg 10 mg/L and ELT-dp 0.1 and 10 mg/L needs mention because Crystallins are the major structural proteins in mouse, human and zebrafish lens (Goishi et al., 2006) and CRGNB is involved in the cataract formation (Wang et al., 2008). The up-regulation of this protein, but also of other Crystallins, was observed in zebrafish exposed to different emerging pollutants as illicit drugs and PS (micro)plastics doped with triclosan (Parolini et al., 2018; Parenti et al., 2019). An up regulation of *crygn* gene, encoding for Gamma-crystallin, was also observed in embryos exposed to benzo(α)pyrene (BαP), while a

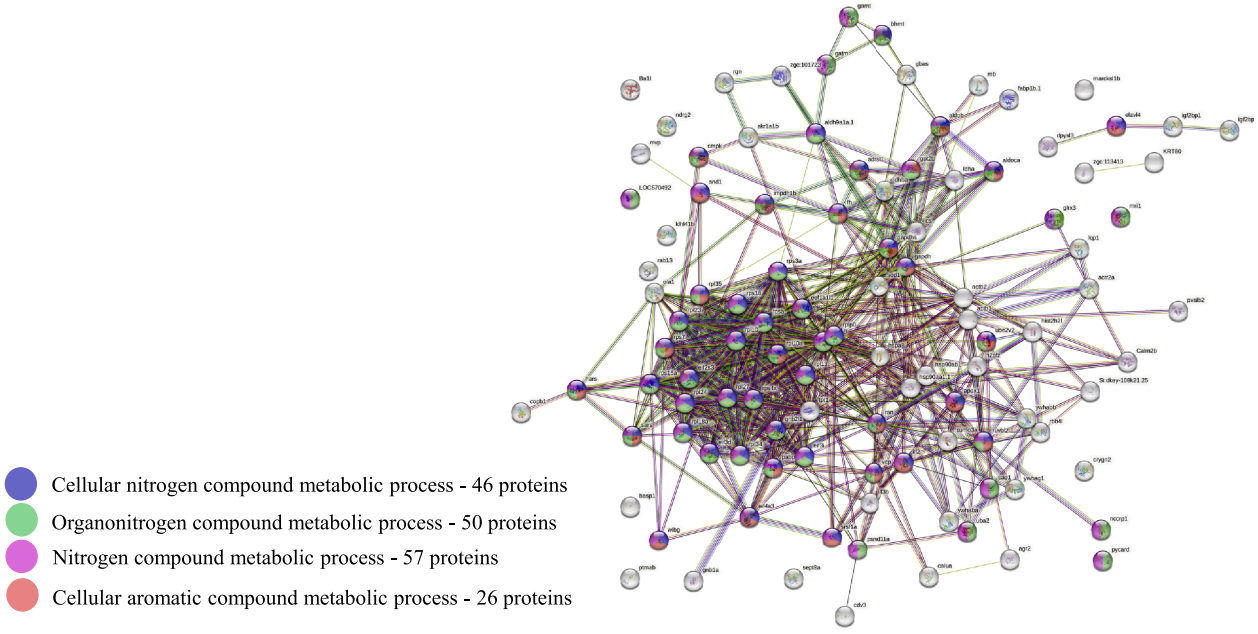

Fig. 8. Main protein pathways modulated by 0.1 mg/L ELT-dp suspension. These pathways were obtained from STRING.

down-regulation of *crybb*, encoding for beta-crystallin, was observed in zebrafish embryos exposed to carbon black doped with BαP (Binelli et al., 2017). This evidence confirms that the zebrafish eye is a sensible target to some environmental contaminants, as carbon black and PAHs, potentially conveyed by ELT-derived material. In line with these results, a myopia-like phenotype with a convex eyeball and fusion vessels in zebrafish larvae exposed to 6PPD was detected by Zhang et al. (2023), while a heavy impact in zebrafish eye development, with a down-regulated phototransduction by retina and a decreased thickness of the retinal outer nuclear layer and retinal pigmented epithelium was reported by Chang et al. (2023) after a TRWP leachate exposure.

Lastly, we observed the down-regulation of SOD [Cu-Zn] in specimens exposed to ELT-dg 10 mg/L and in all ELT-dp groups. This aspect could be in discordance with biomarker result since no significant effects on SOD activity were evaluated (Figure S1). However, it is important to consider that proteomics measures the protein expression, while the SOD biomarker evaluates the kinetics of this enzyme. Therefore, this result confirms that ELT particle suspensions do not impact on the oxidative status of exposed organisms, and the *ex-novo* SOD synthesis is not probably required. In addition, although it may seem like excessive speculation, it would be worth noting that the presence in tires of antioxidant additives, such as 6PPD, could potentially reduce the oxidative status in exposed organisms, thereby justifying the down-regulation of this enzyme.

Considering that more than 100 proteins were modulated by ELT-dp suspensions, a single description of each protein does not have sense in a holistic context of MoA identification. For this reason, we integrated all proteomic results in the STRING freeware to highlight the main impacted metabolic pathways by the ELT suspension exposure. As reported in Fig. 8, referred to specimens exposed to 0.1 mg/L ELT-dp, the recognized proteins seem to be mainly involved in the metabolism of aromatic and both organic and inorganic nitrogen cellular compounds. The same pathways were observed in the other groups (Figures S5-S9). These results could suggest that the chemicals released in water by ELT particles, which contains aromatic and nitrogen groups, affect mainly proteins involved in the metabolism of similar substances. This speculation could confirm the results obtained through the biomarkers, highlighting how the toxicity of ELT suspensions is primarily associated to the released chemicals. The potential insufficient activity of the detoxifying pathways (Fig. 8) could then be associated to an induction of adverse effects at the apical level, as observed in the organisms exposed to ELT-dp groups (Figs. 4 and 5). Based on our previous study (Magni et al., 2022), our initial hypothesis seems to be demonstrated, since the effects at molecular and biochemical levels, also observed at the lowest tested concentrations of 0.1 mg/L, exert toxicity at highest and ecological relevant functions, as indicated by the alterations in swimming behavior and heart rate.

The toxicity induced by ELT-dp suspensions resulted higher compared to those of ELT-dg suspensions, as suggested by the total number of modulated proteins by the three ELT-dp concentrations, as well as by the apical effects observed only in specimens exposed to ELT-dp. The lower size of ELT-dp, compared to ELT-dg, allows a higher chemical release in water due to the increase of surface/volume ratio, with a consequent increase of adverse effects on exposed organisms, as reported by Gong and Xie (2020) for conventional plastics.

Lastly, despite the pivotal differences in terms of composition between TRWPs, TWPs and ELT particles, the comparison between the induced effects by these three different materials is difficult considering that, being physical contaminants, the toxicity is not only concentration dependent, but also affected by size, shape and polymer composition (Zhao et al., 2024). To highlight the eventual differences/similarities of toxic effects it will be necessary to expose the same biological model at representative samples of these three materials in the same experimental conditions and applying the same endpoints. In any case, as observed for ELTs, some studies reported that the smaller size of TWPs induced the main adverse effects in zebrafish (Cunningham et al., 2022; Zhao et al. 2024). In addition, as summarized by Zhao et al. (2024), the chemical released by TRWPs impacted on some biological processes as reproduction, growth and survival in aquatic organisms.

Therefore, the results obtained in this research represent an important starting point for future studies on ELT particles, which are a particular category of tire (micro)plastics currently not considered in the ecotoxicological field.

5. Conclusions

The toxicity of suspensions from ELT particles is mainly associated to the chemical release into the aqueous matrix since a significant

modulation of the detoxifying enzymes EROD and GST was observed. In line with these results, the main modulated proteins, especially by ELT-dp suspensions, were involved in metabolic pathways of aromatic and nitrogen compounds, in turns associated to those conveyed by ELT particles. In addition, the resulted effects at the organism level only in specimens exposed to ELT-dp highlight that these suspensions were more toxic than those produced by ELT-dg.

Although the ELT recycling is a fundamental and essential aspect to find a sustainable destination for Mt of special wastes, this process must be carried out avoiding, or limiting, the re-entry into the environment of both ELT particles and adsorbed chemicals. This study provides the following food for thought from a management point of view:

1) The preference of using ELT-dg, compared to ELT-dp, should be taken into consideration in the context of LCA;
2) It is crucial to design products minimizing the potential dispersion of ELT particles into the environment, and to mitigate direct interactions between these materials and organisms. Such design considerations should encompass the entire product lifecycle, from production to final use.

CRediT authorship contribution statement

Stefano Magni: Writing – original draft, Visualization, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Riccardo Sbarberi:** Validation, Methodology, Formal analysis. **Diletta Dolfini:** Validation, Methodology, Formal analysis. **Lara Nigro:** Validation, Methodology, Formal analysis. **Andrea Binelli:** Writing – review & editing, Investigation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Stefano Magni reports financial support was provided by Ecopneus scpa. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2024.107032.

References

Abbas-Abadi, M.S., Kusenberg, M., Shirazi, H.M., Goshayeshi, B., Van Geem, K.M., 2022. Towards full recyclability of end-of-life tires: challenges and opportunities. 374, 134036. https://doi.org/10.1016/j.jclepro.2022.134036.

Arulrajah, A., Mohammadinia, A., Maghool, F., Horpibulsuk, S., 2019. Tire derived aggregates as a supplementary material with recycled demolition concrete for pavement applications. 230, 129–136. https://doi.org/10.1016/j.jclepro.2019.05.084.

Baensch-Baltruschat, B., Kocher, B., Kochleus, C., Stock, F., Reifferscheid, G., 2021. Tyre and road wear particles - A calculation of generation, transport and release to water and soil with special regard to German roads. Sci. Total. Environ. 752, 141939 https://doi.org/10.1016/j.scitotenv.2020.141939.

Binelli, A., Del Giacco, L., Santo, N., Bini, L., Magni, S., Parolini, M., Madaschi, L., Ghilardi, A., Maggioni, D., Ascagni, M., Armini, A., Prosperi, L., Landi, C., La Porta, C., Della Torre, C., 2017. Carbon nanopowder acts as a Trojan-horse for benzo (α)pyrene in *Danio rerio* embryos. Nanotoxicology 11 (3), 371–381. https://doi.org/10.1080/17435390.2017.1306130.

Binelli, A., Nigro, L., Sbarberi, R., Della Torre, C., Magni, S., 2024. To be or not to be plastics? Protein modulation and biochemical effects in zebrafish embryos exposed to three water-soluble polymers. Sci. Total Environ. 906, 167699 https://doi.org/10.1016/j.scitotenv.2023.167699.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Capolupo, M., Sørensen, L., Don Ranil Jayasena, K., Booth, A.M., Fabbri, E., 2020. Chemical composition and ecotoxicity of plastic and car tire rubber leachates to aquatic organisms. Water Res 169, 115270. https://doi.org/10.1016/j.watres.2019.115270.

Carrasco-Navarro, V., Muñiz-González, A.B., Sorvari, J., Martínez-Guitarte, J.L., 2021. Altered gene expression in *Chironomus riparius* (insecta) in response to tire rubber and polystyrene microplastics. Environ. Pollut. 285, 117462 https://doi.org/10.1016/j.envpol.2021.117462.

Chang, J., Jiao, M., Zhang, Z., Liu, W., Li, W., Xu, P., Wan, B., 2023. Mechanistic insight into the adverse outcome of tire wear and road particle leachate exposure in zebrafish (*Danio rerio*) larvae. Environ. Int. 178, 108053 https://doi.org/10.1016/j.envint.2023.108053.

Cheong, R.S., Roubeau Dumont, E., Thomson, P.E., Castañeda-Cortés, D.C., Hernandez, L.M., Gao, X., Zheng, J., Baesu, A., Macairan, J.R., Smith, A.J., Bui, H.N. N., Larsson, H.C.E., Ghoshal, S., Bayen, S., Langlois, V.S., Robinson, S.A., Tufenkji, N., 2023. Nanoparticle-specific and chemical-specific effects of tire wear particle leachate on amphibian early life stages. J. Hazard. Mater. Adv. 12, 100357 https://doi.org/10.1016/j.hazadv.2023.100357.

C(2023) 6419 - Commission Regulation (EU) …/… amending Annex XVII to Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) as regards synthetic polymer microparticles. https://single-market-economy.ec.europa.eu/system/files/2023-09/C_2023_6419_F1_COMMISSION_REGULATION_UNDER_ECT_EN_V5_P1_2620969.PDF.

Colas, S., Le Faucheur, S., 2024. How do biomarkers dance? Specific moves of defense and damage biomarkers for biological interpretation of dose-response model trends. J. Hazard. Mat 465, 133180. https://doi.org/10.1016/j.jhazmat.2023.133180.

Corami, F., Rosso, B., Sfriso, A.A., Gambaro, A., Mistri, M., Munari, C., Barbante, C., 2022. Additives, plasticizers, small microplastics (< 100 μm), and other microlitter components in the gastrointestinal tract of commercial teleost fish: method of extraction, purification, quantification, and characterization using Micro-FTIR. Mar. Pollut. Bull. 177, 113477 https://doi.org/10.1016/j.marpolbul.2022.113477.

Cunningham, B., Harper, B., Brander, S., Harper, S., 2022. Toxicity of micro and nano tire particles and leachate for model freshwater organisms. J. Hazard. Mater. 429, 128319.

Ding, J., Liu, C., Chen, Q., Zhang, Z., Han, J., Liang, A., Zhu, D., Wang, H., Lv, M., Chen, L., 2022. Extractable additives in microplastics: a hidden threat to soil fauna. Environ. Pollut. 294, 118647 https://doi.org/10.1016/j.envpol.2021.118647.

Dong, Y., Zhao, Y., Hossain, M.U., He, Y., Liu, P., 2021. Life cycle assessment of vehicle tires: a systematic review. Clean. Environ. Syst. 2, 100033 https://doi.org/10.1016/j.cesys.2021.100033.

Fang, C., Fang, L., Di, S., Yu, Y., Wang, X., Wang, C., Jin, Y., 2023. Characterization of N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine (6PPD)-induced cardiotoxicity in larval zebrafish (*Danio rerio*). Sci. Total Environ. 882, 163595 https://doi.org/10.1016/j.scitotenv.2023.163595.

Forrest, M.J., 2019. 3. Overview of the world rubber recycling market, recycling and *Re*-use of waste Rubber. De Gruyter 13–20. https://doi.org/10.1515/9783110644142-003.

Gagné, F., 2006. The wave nature of molecular responses in ecotoxicology. Cur Top Toxicol 1.

Gagné, F., 2018. The gruesome dance of contaminants and toxicity in organisms: the emerging science of chronoecotoxicology or wave ecotoxicology. Cur Top Toxicol 14, 1–20.

Gagné, F., 2014. Biochemical Ecotoxicology: Principles and Methods, 1st Edition. Elsevier, London. doi.org/978-0-12-0411604-7.

Goishi, K., Shimizu, A., Najarro, G., Watanabe, S., Rogers, R., Zon, L.I., Klagsbrun, M., 2006. αA-crystallin expression prevents γ-crystallin insolubility and cataract formation in the zebrafish cloche mutant lens. Development 144 (13), 2585–2593. https://doi.org/10.1242/dev.02424.

Gong, J., Xie, P., 2020. Research progress in sources, analytical methods, eco-environmental effects, and control measures of microplastics. Chemosphere 254, 126790. https://doi.org/10.1016/j.chemosphere.2020.126790.

Hartmann, N.B., Huffer, T., Thompson, R.C., Hasselov, M., Verschoor, A., Daugaard, A.E., Rist, S., Karlsson, T., Brennholt, N., Cole, M., 2019. Are we speaking the same language? Recommendations for a definition and categorization framework for plastic debris. Environ. Sci. Technol. 53, 1039–1047. https://doi.org/10.1021/acs.est.8b05297.

Heberle, H., Meirelles, G.V., da Silva, F.R., Telles, G.P., Minghim, R., 2015. InteractiVenn: a web-based tool for the analysis of sets through Venn diagrams. BMC Bioinform. 16 (169) https://doi.org/10.1186/s12859-015-0611-3.

Jiang, J.R., Chen, Z.F., Liao, X.L., Liu, Q.Y., Zhou, J.M., Ou, S.P., Cai, Z., 2023. Identifying potential toxic organic substances in leachates from tire wear particles and their mechanisms of toxicity to *Scenedesmus obliquus*. J. Hazard. Mater. 458, 132022 https://doi.org/10.1016/j.jhazmat.2023.132022.

Kim, J., Yang, S.I., Moon, H., Hong, Jaehwan, Hong, Junghyun, Choi, W., Son, H., Lee, B. C., Kim, G.B., Kim, Y., 2021. Potential release of nano-carbon black from tirewear particles through the weathering effect. J. Ind. Eng. Chem. 96, 322–329. https://doi.org/10.1016/j.jiec.2021.01.036.

Kim, L., Kim, H., Lee, T.Y., An, Y.J., 2023. Chemical toxicity screening of tire particle leachates from vehicles and their effects on organisms across three trophic levels. Mar. Pollut. Bull. 192, 114999 https://doi.org/10.1016/j.marpolbul.2023.114999.

Knight, L.J., Parker-Jurd, F.N., Al-Sid-Cheikh, M., Thompson, R.C., 2020. Tyre wear particles: an abundant yet widely unreportedmicroplastic? Environ. Sci. Pollut. Res. 27, 18345–18354. https://doi.org/10.1007/s11356-020-08187-4.

Kolomijeca, A., Parrott, J., Khan, H., Shires, K., Clarence, S., Sullivan, C., Chibwe, L., Sinton, D., Rochman, C.M., 2020. Increased temperature and turbulence alter the effects of leachates from tire particles on fathead minnow (*Pimephales promelas*). Environ. Sci. Technol. 54, 1750–1759. https://doi.org/10.1021/acs.est.9b05994.

Leads, R.R., Weinstein, J.E., 2019. Occurrence of tire wear particles and other microplastics within the tributaries of the Charleston Harbor Estuary. . 145, 569–582. https://doi.org/10.1016/j.marpolbul.2019.06.061.

Liu, J., Feng, Q., Yang, H., Fan, X., Jiang, Y., Wu, T., 2023. Acute toxicity of tire wear particles and leachate to *Daphnia magna*. Comp. Biochem. Physiol. C-Toxicol. Pharmacol. 272, 109713 https://doi.org/10.1016/j.cbpc.2023.109713.

Liu, X., Huang, F., Yu, Y., Zhao, P., Zhou, Y., He, Y., Xu, Y., Zhang, Y., 2019. Ofloxacin degradation over Cu–Ce tyre carbon catalysts by the microwave assisted persulfate process. Appl. Catal. B. 253, 149–159. https://doi.org/10.1016/j.apcatb.2019.04.047.

Luo, Z., Zhou, X., Su, Y., Wang, H., Yu, R., Zhou, S., Xu, E.G., Xing, B., 2021. Environmental occurrence, fate, impact, and potential solution of tire microplastics: similarities and differences with tire wear particles. Sci. Total. Environ. 795, 148902 https://doi.org/10.1016/j.scitotenv.2021.148902.

Magni, S., 2024. Polyvinyl alcohol. In: Encyclopedia of Toxicology, 4. Elsevier, pp. 859–863. https://doi.org/10.1016/B978-0-12-824315-2.00752-1.

Magni, S., Binelli, A., Pittura, L., Avio, C.G., Della Torre, C., Parenti, C.C., Gorbi, S., Regoli, F., 2019. The fate of microplastics in an Italian Wastewater Treatment Plant. Sci. Total. Environ. 652, 602–610. https://doi.org/10.1016/j.scitotenv.2018.10.269.

Magni, S., Gagné, F., André, C., Della Torre, C., Auclair, J., Hanana, H., Parenti, C.C., Bonasoro, F., Binelli, A., 2018. Evaluation of uptake and chronic toxicity of virgin polystyrene microbeads in freshwater zebra mussel *Dreissena polymorpha* (Mollusca: bivalvia). Sci. Total Environ. 631-632, 778–788. https://doi.org/10.1016/j.scitotenv.2018.03.075.

Magni, S., Nigro, L., Della Torre, C., Binelli, A., 2021. Characterization of plastics and their ecotoxicological effects in the Lambro River (N. Italy). J. Hazard. Mater. 412, 125204 https://doi.org/10.1016/j.jhazmat.2021.125204.

Magni, S., Sbarberi, R., 2022. Plastic detection in the sediments of Lambro River (North Italy): a preliminary study. Rend. Acc. *Sc*. Fis. Mat. Napoli 2022, 15–24. LXXXIX10.32092/1093Gianni EditoreISBN 978-88-6906-272-8. http://www.societanazionalescienzelettererarti.it/pdf/Rendiconto_SFM_2022.

Magni, S., Tediosi, E., Maggioni, D., Sbarberi, R., Noé, F., Rossetti, F., Fornai, D., Persici, V., Neri, M.C., 2022. Ecological Impact of End-of Life Tire (ELT)-Derived Rubbers: acute and Chronic Effects at Organism and Population Levels. Toxics 10, 201. https://doi.org/10.3390/toxics10050201.

Martín-Folgar, R., Martínez-Guitarte, J.L., 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.

Nigro, L., Magni, S., Ortenzi, M.A., Gazzotti, S., Della Torre, C., Binelli, A., 2022. Are "liquid plastics" a new environmental threat? The case of polyvinyl alcohol. Aquat. Toxicol. 248, 106200 https://doi.org/10.1016/j.aquatox.2022.106200.

Nigro, L., Magni, S., Ortenzi, M.A., Gazzotti, S., Signorini, S.G., Sbarberi, R., Binelli, A., 2023. Assessment of behavioural effects of three water-soluble polymers in zebrafish embryos. Sci. Total Environ. 893, 164843 https://doi.org/10.1016/j.scitotenv.2023.164843.

O'Brien, A.M., Lins, T.F., Yang, Y., Frederickson, M.E., Sinton, D., Rochman, C., 2022. Microplastics shift impacts of climate change on a plant-microbe mutualism: temperature, CO2, and tire wear particles. Environ. Res. 203, 111727 https://doi.org/10.1016/j.envres.2021.111727.

OECD (2013), Test No. 236: fish Embryo Acute Toxicity (FET) test, OECD guidelines for the testing of chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264203709-en.

Oliveira, M., Santos, M.A., Pacheco, M., 2004. Glutathione protects heavy metal-induced inhibition of hepatic microsomal ethoxyresorufin O-deethylase activity in *Dicentrarchus labrax* L. Ecotoxicol. Environ. Saf. 58, 379–385. https://doi.org/10.1016/j.ecoenv.2004.03.003.

Parenti, C.C., Ghilardi, A., Della Torre, C., Magni, S., Del Giacco, L., Binelli, A., 2019. Evaluation of the infiltration of polystyrene nanobeads in zebrafish embryo tissues after short-term exposure and the related biochemical and behavioural effects. Environ. Pollut. 254, 112947 https://doi.org/10.1016/j.envpol.2019.07.115.

Parolini, M., Bini, L., Magni, S., Rizzo, A., Ghilardi, A., Landi, C., Armini, A., Del Giacco, L., Binelli, A., 2018. Exposure to cocaine and its main metabolites altered the protein profile of zebrafish embryos. Environ. Pollut. 232, 603–614. https://doi.org/10.1016/j.envpol.2017.09.097.

Plastics Europe, 2023. Plastics-the Facts 2023. https://plasticseurope.org/knowledge-hub/plastics-the-fast-facts-2023/.

Ricarte, M., Prats, E., Montemurro, N., Bedrossiantz, J., Bellot, M., Gómez-Canela, C., Raldúa, D., 2023. Environmental concentrations of tire rubber-derived 6PPD-quinone alter CNS function in zebrafish larvae. Sci. Total Environ. 896, 165240 https://doi.org/10.1016/j.scitotenv.2023.165240.

Roubeau Dumont, E., Gao, X., Zheng, J., Macairan, J., Hernandez, L.M., Baesu, A., Bayen, S., Robinson, S.A., Ghoshal, S., Tufenkji, N., 2023. Unraveling the toxicity of tire wear contamination in three freshwater species: from chemical mixture to nanoparticles. J. Hazard. Mater. 453, 131402 https://doi.org/10.1016/j.jhazmat.2023.131402.

Sbarberi, R., Magni, S., Boggero, A., Della Torre, C., Nigro, L., Binelli, A., 2024. Comparison of plastic pollution between waters and sediments in four Po River tributaries (Northern Italy). Sci. Total. Environ. 912, 168884 https://doi.org/10.1016/j.scitotenv.2023.168884.

Schiavo, S., Oliviero, M., Chiavarini, S., Dumontet, S., Manzo, S., 2021. Polyethylene, Polystyrene, and Polypropylene leachate impact upon marine microalgae *Dunaliella tertiolecta*. J. Toxicol. Environ. Health Part A. 84, 249–260. https://doi.org/10.1080/15287394.2020.1860173.

Schiwy, S., Bräunig, J., Alert, H., Hollert, H., Keiter, S.H., 2015. A novel contact assay for testing aryl hydrocarbon receptor (AhR)-mediated toxicity of chemicals and whole sediments in zebrafish (*Danio rerio*) embryos. Environ. Sci. Pollut. Res. 22, 16305–16318. https://doi.org/10.1007/s11356-014-3185-0.

Shin, H., Sukumaran, V., Yeo, I.C., Shim, K.Y., Lee, S., Choi, H.K., Ha, S.Y., Kim, M., Jung, J.H., Lee, J.S., Jeong, C.B., 2022. Phenotypic toxicity, oxidative response, and transcriptomic deregulation of the rotifer *Brachionus plicatilis* exposed to a toxic cocktail of tire-wear particle leachate. J. Hazard. Mater. 438, 129417 https://doi.org/10.1016/j.jhazmat.2022.129417.

Sieber, R., Kawecki, D., Nowack, B., 2020. Dynamic probabilistic material flow analysis of rubber release from tires into the environment. Environ. Pollut. 258, 113573 https://doi.org/10.1016/j.envpol.2019.113573.

Stephensen, E., Adolfsson-Erici, M., Celander, M., Hulander, M., Parkkonen, J., Hegelund, T., Sturve, J., Hasselberg, L., Bengtsson, M., Förlin, L., 2003. Biomarker responses and chemical analyses in fish indicate leakage of polycyclic aromatic hydrocarbons and other compounds from car tire rubber. Environ. Toxicol. Chem. 22, 2926–2931. https://doi.org/10.1897/02-444.

Stoyek, M.R., Jonz, M.G., Smith, F.M., Croll, R.P., 2017. Distribution and chronotropic effects of serotonin in the zebrafish heart. Auton. Neurosci. 206, 43–50. https://doi.org/10.1016/j.autneu.2017.07.004.

Sundt, P., Schulze, P.-E., Syversen, F., 2014. Sources of Microplastics-pollution to the Marine Environment. Nor. Environ. Agency Miljødirektoaret. 86 (20). https://www.miljodirektoratet.no/globalassets/publikasjoner/M321/M321.pdf.

Tian, Z., Zhao, H., Peter, K.T., Gonzalez, M., Wetzel, J., Wu, C., Hu, X., Prat, J., Mudrock, E., Hettinger, R., et al., 2021. A ubiquitous tire rubber-derived chemical induces acute mortality in coho salmon. Science 371, 185–189. https://doi.org/10.1126/science.abd6951.

Walker, T.R., Fequet, L., 2023. Current trends of unsustainable plastic production and micro(nano)plastic pollution. Trends Analyt Chem 160, 116984. https://doi.org/10.1016/j.trac.2023.116984.

Wang, C., Zhao, J., Xing, B., 2021. Environmental source, fate, and toxicity of microplastics. J. Hazard. Mater 407, 124357. https://doi.org/10.1016/j.jhazmat.2020.124357.

Wang, H., Kesinger, J.W., Zhou, Q., Wren, J.D., Martin, G., Turner, S., Tang, Y., Frank, M. B., Centola, M., 2008. Identification and characterization of zebrafish ocular formation genes. Genome 5, 222–235.

Weyrauch, S., Seiwert, B., Voll, M., Wagner, S., Reemtsma, T., 2023. Accelerated aging of tire and road wear particles by elevated temperature, artificial sunlight and mechanical stress — A laboratory study on particle properties, extractables and leachables. Sci. Total. Environ. 904, 166679 https://doi.org/10.1016/j.scitotenv.2023.166679.

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://10.1016/j.chemosphere.2009.08.034.

Xiong, F., Liu, J., Xu, K., Huang, J., Wang, D., Li, F., Wang, S., Zhang, J., Pu, Y., Sun, R., 2023. Microplastics induce neurotoxicity in aquatic animals at environmentally realistic concentrations: a meta-analysis. Environ. Pollut. 318, 120939 https://doi.org/10.1016/j.envpol.2022.120939.

Yang, H., Liu, Y.R., Song, Z.X., Tang, Z.S., Jia, A.L., Wang, M.G., Duan, J.A., 2024. Study on the underlying mechanism of Poria in intervention of arrhythmia zebrafish by integrating metabolomics and network pharmacology. Phytomedicine 122, 155143. https://doi.org/10.1016/j.phymed.2023.155143.

Yang, K., Jing, S., Liu, Y., Zhou, H., Liu, Y., Yan, M., Yi, X., Liu, R., 2022. Acute toxicity of tire wear particles, leachates and toxicity identification evaluation of leachates to the marine copepod, *Tigriopus japonicus*. Chemosphere 297, 134099. https://doi.org/10.1016/j.chemosphere.2022.134099.

Yu, F., Hou, Z.S., Luo, H.R., Li, H.X., Cui, X.F., Li, J.L., Feng, W.R., Tang, Y.K., Su, S.Y., Gao, Q.F., Xiao, J., Xu, P., 2023. Neurobehavioral disorders induced by environmental zinc in female zebrafish (*Danio rerio*): insights from brain and intestine transcriptional and metabolic signatures. Chemosphere 335, 138962. https://doi.org/10.1016/j.chemosphere.2023.138962.

Zhang, S.Y., Gan, X., Shen, B., Jiang, J., Shen, H., Lei, Y., Liang, Q., Bai, C., Huang, C., Wu, W., Guo, Y., Song, Y., Chen, J., 2023. 6PPD and its metabolite 6PPDQ induce different developmental toxicities and phenotypes in embryonic zebrafish. J. Hazard. Mater. 455, 131601 https://doi.org/10.1016/j.jhazmat.2023.131601.

Zhao, T., Zhang, Y., Song, Q., Meng, Q., Zhou, S., Cong, J., 2024. Tire and road wear particles in the aquatic organisms–a review of source, properties, exposure routes, and biological effects. Aquat. Toxicol., 107010 https://doi.org/10.1016/j.aquatox.2024.107010.

Zheng, S., Huang, W., Liu, C., Xiao, J., Wu, R., Wang, X., Cai, Z., Wu, K., 2021. Behavioral change and transcriptomics reveal the effects of 2, 2′, 4, 4′-tetrabromodiphenyl ether exposure on neurodevelopmental toxicity to zebrafish (*Danio rerio*) in early life stage. Sci. Total Environ. 752, 141783 https://doi.org/10.1016/j.scitotenv.2020.141783.

Zimmermann, L., Dierkes, G., Ternes, T.A., Volker, C., Wagner, M., 2019. Benchmarking the in vitro toxicity and chemical composition of plastic consumer products. Environ. Sci. Tech. 53 (19), 11467–11477. https://doi.org/10.1021/acs.est.9b022.