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Research Paper

Tyre-derived ecotoxicity: Differentiating the effects from particles and chemical leachates on the blue mussel *Mytilus edulis*

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ABSTRACT

Tyre particles contain complex chemical additives that can leach out into the aquatic environment, posing potential risks to marine organisms. Despite growing evidence of adverse effects, the relative importance of particle-driven versus chemically mediated toxicity remains poorly explored, especially under environmentally relevant exposure scenarios. This study used the blue mussel (*Mytilus edulis*) as a model to differentiate these effects by exposing individuals to cryomilled tyre particles (TP), their leachates (L) and pre-leached particles (TPL) over 36 days at the environmentally relevant concentration of 0.1 g/L. Chemical analysis confirmed uptake of key organic additives such as poly(1,2-dihydro-2,2,4-trimethylquinoline) (TMQ), N-(1,3-dimethylbutyl)-*N*′-phenyl-*p*-phenylenediamine (6PPD), and 6PPD-quinone (6PPDQ), with certain compounds persisting after depuration. Particle-exposed mussels accumulated higher additive concentrations than those exposed to only leachates, indicating enhanced chemical release from particles. Biomarker responses revealed signs of oxidative stress and neurotoxicity in exposed mussels across all treatments, with earlier responses in leachate exposure and delayed responses during particle exposures. These results demonstrate that chemical additives are key toxicity drivers alongside physical particles, highlighting the importance of considering both pathways in environmental risk assessments. To our knowledge, this study is among the first to experimentally separate particle and leachate specific effects in mussels by using tyre particles before and after leaching to create contrasting chemical loads, thereby providing novel insights into their distinct and combined impacts on marine biota.

1. Introduction

The environmental impact of tyre-derived particles such as tyre road wear particles (TRWP) has emerged as a significant global environmental concern due to their widespread presence, persistence, complex chemical composition, leaching of toxic additives and potential toxicity to aquatic life [1]. TRWPs are generated through the abrasion of tyre treads against road surfaces and can account up to 40% of total microplastics present in aquatic ecosystems [2,3]. Globally, it is estimated that around 6 million tonnes of TRWP are released into the environment each year, a substantial fraction of which is transported via air movement, rainwater runoff, and watercourses [2,4,5]. Of these emissions, approximately 67% enter soils, around 12% become airborne, and about 12% are transported into surface waters. Of those entering aquatic ecosystems, it is further estimated that up to 50% may ultimately reach coastal and marine environments and accumulate in biota [6,7]. The detection of tyre-derived particles in marine organisms, including mussels along the Norwegian coastline, highlights the pervasive and bioavailable nature of these pollutants [8].

Tyres consist of a complex mixture of natural and synthetic rubbers (e.g., styrene-butadiene rubber [SBR], polybutadiene rubber [PBR], natural rubber [NR]) combined with a broad range of chemical additives such as antioxidants, plasticizers and vulcanization agents [9]. Once in the environment, tyre particles are subject to weathering processes,

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which can trigger the release of potentially hazardous substances such as zinc (Zn), benzothiazoles, and other organic additives, that can interact with aquatic biota [6]. Of particular concern are the vulcanization accelerators 2-mercaptobenzothiazole (MBT) and 1,3-diphenylguanidine (DPG), as well as the antioxidant N-(1,3-dimethylbutyl)-*N*′-phenyl-*p*-phenylenediamine quinone (6PPDQ), which occur in both particle-bound and dissolved leachate phases and have known toxic effects on fish and invertebrates [10–12]. In addition, tyre leachates have been shown to induce toxicity in *Mytilus edulis* haemocytes, causing reduced cell viability and increased formation of reactive oxygen species (ROS), effects linked to compounds such as 6PPDQ, DPG and benzothiazole [13].

Despite growing awareness of the environmental risks posed by tyre-derived particles as emerging microplastic pollutants, a critical knowledge gap remains regarding the mechanisms driving their biological effects in aquatic organisms: are the adverse biological effects observed primarily caused by the physical presence of particles, the toxicity of leached chemical additives, or their combined interaction [1]? Most existing studies have addressed these stressors separately, focusing either on particle exposure or on the toxicity of individual tyre-derived chemicals, often without parallel biological assessment under comparable exposure conditions. As a result, the relative contribution of particle-mediated versus chemically mediated effects, as well as their potential interactive or synergistic effects, remains poorly understood. Differentiating these mechanisms is essential for accurate environmental risk assessment and effective mitigation strategies. Although limiting particle release is a common management approach that often reduces both particle- and additive-driven effects, understanding the dominant driver of toxicity remains critical for determining whether such measures are sufficient or if additional controls (e.g., targeting additive leaching) are required. The main objective of this study was to address this gap by experimentally separating exposure to tyre particles and their leachates, allowing a direct comparison between physical and chemical effects on the blue mussel *Mytilus edulis*. As filter-feeding bivalves with high ecological relevance and broad distribution, mussels are particularly vulnerable to microplastic and chemical exposure in coastal environments [14–16]. Blue mussels are widely used in marine pollution monitoring and have been shown to accumulate tyre-derived particles in wild populations along the Norwegian coast, making it an appropriate sentinel species for evaluating real-world exposure risk [8]. With this in mind, it was hypothesised that particle-mediated and chemically mediated exposures can elicit distinct biological responses in mussels, reflecting differences in uptake, modes of action, and affected physiological pathways. To test this hypothesis, mussels were exposed to three different components of car tyres: (i) pristine tyre particles; (ii) tyre particles pre-leached for 14 days; (iii) chemicals leached from the tyre particles for 14 days. A multi-biomarker approach was employed to evaluate biological responses in mussels, including endpoints related to general health (condition index), cellular integrity (lysosomal membrane stability), neurotoxicity (acetylcholinesterase activity) and oxidative stress (antioxidant enzymes and lipid peroxidation). In parallel, the uptake and accumulation of tyre particles and tyre-associated additives were quantified in mussel gills and digestive glands using a combination of analytical techniques. By integrating biomarker responses with particle and chemical accumulation data, this study provides crucial insights into the distinct and overlapping roles of particles and additives in tyre pollution toxicity, thereby contributing to mechanistic understanding and supporting environmentally relevant risk assessment.

2. Materials and methods

2.1. Test materials and exposure treatments

Car tyre granulates (CTG) from end-of-life tyres (ELTs), sized 1–2.8 mm, were purchased from Ragn-Sells (Norway) and subsequently cryomilled to smaller sized particles (<300 μm) by CARAT GmbH (Germany). These cryomilled particles were then used in three distinct exposure conditions representing different components of car tyres, at a concentration of 0.1 g/L: (i) pristine tyre particles (TP) representing particles with high chemical load; (ii) tyre leachates (L) generated in natural seawater (NSW) for 14 days representing the mixture of leached tyre-associated chemical additives; and (iii) tyre particles after leaching (TPL) collected after the 14 days leaching process, representing particles expected to have reduced chemical load. Leachates were prepared following the protocol described in Michelangeli et al. [13] with minor modifications, at the standard 1:10 liquid-to-solid ratio used for tyre leachate generation [17]. Briefly, 100 g of the TP were added to 1000 mL of 0.22 μm filtered NSW (35 PSU, pH 8.0) to achieve a final concentration of 100 g/L and stirred continuously at 250 rpm for 14 days in the dark in a temperature-controlled room (20 ± 2 °C). After incubation, the resulting solution was filtered through a glass microfiber filter (GF/F; porosity 1.2 μm Whatman™) and a sterile 0.22 μm filter (STERICAP™ PLUS) to remove any residual particles. The resulting filtered leachates were diluted with NSW to reach a final working concentration 0.1 g/L (based on the initial TP-to-water ratio) and stored at −20 °C and in the dark until use in exposure experiments. The leached particles retained on the 1.2 μm and 0.22 μm filters were collected, combined, air dried, weighed in 0.1 g/L sub-samples and stored in the dark. The concentration of 0.1 g/L was selected based on prior studies investigating the impacts of tyre particles and leachates on aquatic organisms, which identified this level as environmentally relevant and sufficient to induce measurable biological responses [13,18–21]. The selected particle size (<300 μm) also reflects the range of tyre particle sizes reported in the environment, which spans from the nanoscale to several hundred micrometres [5]. The size and morphology of the TP were characterized using dynamic image analysis (DIA), while both TP and TPL were further analysed using a Philips® Field Emission Gun-Scanning Electron Microscope (FEG-SEM). A detailed description of both methodologies can be found in Michelangeli et al. [13]. The polymeric composition of TP was also previously assessed by Sørensen et al. [22], confirming a mixture of styrene-butadiene rubber (SBR) and natural rubber elastomers.

2.2. Mussel collection and acclimatisation

Mytilus edulis (5.5 ± 0.5 cm shell length) were collected from a local mussel farm, Sørskjell AS (Grimstad, Norway) and transported to NIVA's marine research station in Solbergstrand (located south of Drøbak in the Oslo fjord, Norway – 59°36′55.5″N 10°39′04.2″E). Upon arrival, mussels were transferred to a temperature-controlled laboratory and acclimatized for 2 weeks in a flow-through system with NSW to depurate background chemicals and particles previously accumulated in the field. The room temperature was maintained at 10 °C, with a16:8 h light: dark photoperiod, and the NSW used in the flow-through system was collected from 60 m depth (temperature of 16 °C and salinity of 35 ± 1 PSU) and filtered subsequentially through a combination of 100, 50 and 20 μm mesh filters. Mussels were visually inspected every 24 h and deceased individuals removed. Mussels were fed ad libitum with Shellfish Diet 1800® (Reed Mariculture).

2.3. Experimental set up

After the acclimatisation period, mussels were exposed for 4 weeks followed by a 1-week depuration period (total 36 days), to TP, TPL and L at a concentration of 0.1 g/L, alongside a seawater control. Each treatment consisted of six replicate 40 L glass tanks, each containing 45 randomly distributed mussels in 25 L of filtered NSW (1.8 mussels/L). All experimental work was carried out in the same temperature-controlled laboratory under the same light and temperature conditions as those used during the acclimatisation period. A semi-static exposure system was used throughout the experiment, with water renewal (90 to

95%) and re-dosing of particles and leachates performed twice a week. After each water change and re-dosing, mussels were fed with Shellfish Diet 1800® (concentration of 200 µL/L) and visually inspected to confirm active filtering. Tanks were continuously aerated using glass pipettes to ensure particle suspension. Water quality parameters (pH, temperature, salinity, dissolved oxygen (DO)) were monitored throughout the experiment to ensure stable conditions during the 36 days, and no alterations were detected during this period. Sixty-six mussels per treatment were randomly sampled from the replicate tanks at five time points (Days 0, 7, 14, 28 and 36), prior to water change and re-dosing. Water samples were also collected from each treatment to assess the concentration of tyre additives. After sampling, mussels were measured and gills and digestive glands rapidly dissected, snap frozen in liquid nitrogen and stored at −80 °C until further use in chemical additive accumulation and biomarker analysis. Haemolymph was also extracted and immediately processed using the neutral red retention (NRR) assay to assess lysosomal membrane stability. A subset of 5 shelled mussels was rinsed with reverse osmosis (RO) water to remove any residual loosely adhering particles and stored at −20 °C for determination of particle internalization.

2.4. Particle internalization in mussel tissues

Internalization of tyre particles in mussel gills and digestive glands was assessed following the protocol by Bråte et al. [23], with some modifications. On the day of analysis, the 5 mussels collected from each treatment at days 0, 28 and 36 were thawed on ice and gills and digestive glands carefully excised under a laminar flow hood to prevent contamination. Tissues were then placed into pre-weighed glass beakers, their wet weight recorded and then freeze-dried for at least 24 h before being re-weighed to determine the dry weight. Tissue digestion was performed using 10% potassium hydroxide (KOH) at a 1:10 biota-to-KOH ratio (*v*/v) for 24 h at 50 °C with continuous agitation (100 rpm). Following digestion, samples were sieved through a 32 µm mesh and treated with a 10% acetic acid (CH_3COOH) solution (1:10 ratio biota:CH_3COOH, v/v) and incubated for 24 h (50 °C, 100 rpm). The digested tissues were then size fractioned through a 32 µm sieve and filtered onto glass microfibre filter paper (GF-75, 13 mm, 0.4 µm pore size). Filters were dried in covered petri dishes and visually inspected using a VideometerLab before pyrolysis-GC/MS (Py-GC/MS) analysis. To ensure contamination control, all procedures were conducted in the NIVA microplastics laboratory, equipped with a HEPA-filtered positive pressure airflow system (removing 99.95% of airborne particles ≥0.3 µm), restricted personnel entry, and the use of cotton lab coats. Three laboratory procedural blanks, containing only RO water and the added chemicals, were included in each sample set and processed identically.

2.4.1. VideometerLab analysis

To visually inspect the potential internalization of tyre-derived particles in mussel gills and digestive glands, the VideometerLab, a high-precision multispectral imaging system, was employed. This system captures images at 19 distinct wavelengths, ranging from 365 nm (UV) to 970 nm (near-infrared), allowing for detailed analysis of particle surface characteristics. The process involved capturing high-resolution multispectral images, followed by particle detection and blob identification, with a detection limit of 100 µm. Further details of the methodology are provided in the Section S1 of the Supplementary Information (SI).

2.4.2. Py-GC/MS analysis

Tyre particle identification and quantification were also performed by Py-GC/MS following methods based on Rødland et al. [24] and referred as M4 in the present study. Samples were pyrolyzed at 700 °C for 12 s using a 50:1 split. All samples were spiked with 25 µg deuterated Polybutadiene (d6-PB, Polymer Source Inc., Canada) and then analysed in two separate rounds. Analysis round 1 (April 2024) used a calibration curve with points 0.1, 1, 2, 5, 20, 40, 60, 100 and 140 µg ($R^2 = 0.972$) and the second round (May 2024) used a calibration curve with points 1, 2, 5, 20, 40, 60, 100, 140 µg ($R^2 = 0.997$). Seven different pyrolysis markers were monitored: 4-vinylcyclohexene (4VCH, mz 54), benzene (Bz, mz 78), butadiene trimer (BT, mz 91), Styrene butadiene dimer (SB, mz 104 height), styrene butadiene trimer (SBB, mz 104), α-methylstyrene (α-MS, mz 118) and ethylstyrene (ES, mz 117). For the quantification of total mass of SBR and BR rubbers, the peak of the four makers Bz, α-MS, ES and BT were summarized and used as the marker combination M4. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated based on the S/N of the combined markers used for M4 [24]. LODs were 0.020 µg/cup in April 2024 and 0.129 µg/cup in May 2024, while LOQs were 0.126 µg/cup in April 2024 and 0.426 µg/cup in May 2024. More details on the Py-GC/MS method are given in Section S2 of the SI. The expected mass of SBR + BR in this specific TP material was determined by analysing 15 individual TPs from the same cryo-milled material as that used in the exposures. The measured SBR + BR concentrations in each blue mussel sample were then converted to total tyre particles (TP) using the average mass of SBR + BR in the reference TP material (more details are given in the SI, Section S2).

2.5. Organic additives analysis

2.5.1. Leachates and water samples

Prior to chromatographic analysis, organic contaminants from the leachate and water samples were extracted using solid-phase extraction as previously described in Michelangeli et al. [13]. The targeted organic additives were selected based on the suspect and non-target screening performed on tyre leachates in that same study, which identified key tyre-associated compounds of environmental relevance. The quantification of the target compounds was performed using a liquid chromatographer (Acquity UPLC, Waters) coupled to a triple quadrupole mass spectrometer (Xevo TQ-XS, Waters). The measurements were based on the analytical method previously described by Weyrauch et al. [25] with some modifications. Briefly, the analytes were separated using a XBridge BEH C18 column (100 × 2.1 mm i.d., 2.5 µm particle size, Waters) including a XBridge BEH C18 XP VanGuard Cartridge (5 × 2.1 mm i.d., 130 Å, 2.5 µm particle size, Waters) at 45 °C. *ultra*-pure Milli-Q water and methanol containing Ammonium formate (5 mM) and formic acid (0.1% *v*/v) were used as mobile phases A and B, respectively. Technical information regarding the separation specifics can be found in Supplementary Table S1. Electrospray ionization (ESI) was conducted at 150 °C and 0.15 mL/min gas flow with 7.00 Bar of nebuliser gas pressure, 500 V of capillary voltage, 30 V of source offset, and a desolvation temperature of 650 °C. The mass spectrometer was operated in positive mode and MS/MS data were acquired in Multiple Reaction Monitoring (MRM) mode. Optimised MRM conditions for the analysis of the chemicals studied using HPLC-ESI-MS/MS are presented in Supplementary Table S2. Quantification was performed by normalizing the area of the quantifying ion of each analyte with its closest related internal standard (IS). The concentrations of the targeted organic additives in leachate and water samples are reported as dissolved fractions (after 0.22 µm filtration) and expressed in µg/L. Detailed information of the chemical standards used for the analysis can be found in the supplementary material (Table S3).

2.5.2. Mussel tissues

Gills and digestive glands were analysed for additive content, following the methodology described in Ruus et al. [26] with modifications. For each treatment, 3 replicates of mussel tissues ($n = 3$) were freeze-dried overnight using a Freeze Dryer GT2 LYO (Seib Industrie GmbH, Germany). The dried tissues were homogenized using a mortar, and approximately 0.6–0.9 g aliquots were transferred into 25 mL Falcon tubes. Samples were spiked with 8 mL of a 9:1 (*v*/v) methanol/ethyl acetate mixture and vortexed thoroughly. The extraction process consisted of two 30-min cycles in an ultrasonic bath. Afterwards, the

extracts were divided into 1.5 mL aliquots and stored at −80 °C until further analysis.

Mussel tissues were screened for tyre-derived chemicals using Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MSMS), following the methodology described by Meland et al. [27]. Target compounds included: poly- (1,2-dihydro-2,2,4-trimethylquinoline) (TMQ), 2-methylthiobenzothiazole (MTBT), 2-phenylbenzothiazole (PhBT), 1,3-diphenylguanidine (DPG), 2,4,6-tris[bis (methoxymethyl)amino]-1,3,5-triazine (HMMM), N-(1,3-dimethylbutyl)-*N*′-phenyl-*p*-phenylenediamine (6PPD), N-(1,3-dimethylbutyl)-*N*′-phenyl-*p*-phenylenediamine- quinone (6PPDQ), Nisopropyl-*N*-phenyl-1,4-phenylenediamine (IPPD), N-cyclohexyl-*N*-phenyl-*p*-phenylenediamine (CPPD) and *N*,*N*-diphenyl-1,4-phenylenediamine (DPPD). The concentrations of the targeted organic additives in mussel samples are reported as total fractions and expressed in ng/g wet weight.

2.6. Metal analysis

Metal analysis of (Al), titanium (Ti), chromium (Cr), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), silver (Ag), cadmium (Cd), antimony (Sb), mercury (Hg), and lead (Pb) was conducted in mussel tissues (gills and digestive glands), tyre leachate stock, and water samples from the exposure treatments (CTRL, TP, TPL, and L), collected prior to water removal, from day 7, 14, 28 and 36, by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) using an Agilent 7700 Q-ICP-MS. The analysis was performed following the method described in Michelangeli et al. [13].

2.6.1. Leachates and water samples

Prior to analysis, the leachates stock (100 g/L) and the water samples were filtered through 0.22 µm VWR® sterile syringe filters (CA, 25 mm), diluted tenfold with Milli-Q water, and acidified with ultrapure nitric acid (69% HNO_3, Suprapur®) to achieve a 1% HNO_3 solution. Filtered natural seawater, prepared and acidified in the same manner, was utilized as a blank to generate matrix-matched calibration standards, with calibration curves spanning from 0.1 to 50 µg/L. Calibration standards were prepared using serial dilutions of commercial standard solutions from Inorganic Ventures (Christiansburg, VA). A certified reference material (CRM) for tap water (CRM-TMWD-250, 2% HNO_3, High-Purity Standards, US) was diluted with filtered natural seawater (0.22 µm) and acidified with ultrapure nitric acid to match the matrix, serving as a quality control measure during analysis. Scandium (Sc), germanium (Ge), indium (In), and rhenium (Re) were employed as internal standards. Each sample was analysed in triplicate. Between measurements, the instrument was rinsed with a solution of 10% HNO_3 and 3% HCl, followed by a final rinse with 1% HNO_3 to prevent carryover. Metal concentrations in the samples were corrected for blanks by subtracting the mean blank value from the measured concentrations. Metal concentrations in leachate and water samples are reported as dissolved fractions (after 0.22 µm filtration) and expressed in µg/L.

2.6.2. Mussel tissues

Mussel gills and digestive glands were freeze-dried, weighed, and digested overnight in concentrated trace metal-grade nitric acid (HNO_3) at 50 °C. After digestion, the samples were diluted to approximately 10% HNO_3 for analysis. For quality assurance, two certified reference materials, DORM-4 (fish protein) and DOLT-5 (dogfish liver), both from the National Research Council of Canada, were analysed alongside the samples. Their inclusion allowed the assessment of the digestion process in terms of recovery of the relevant metals, as well as verification of the accuracy and reliability of the trace metal analysis. Metal concentrations in mussel tissues are reported as total fractions and expressed in µg/g dry weight.

2.7. Biomarkers analysis

Biomarkers were assessed in mussels to evaluate various physiological responses. The Condition index (CI) was calculated by measuring the ratio of soft tissue dry weight to shell dry weight, following the method by Orban et al. [28]. Lysosomal membrane stability (LMS) was evaluated using the Neutral Red Retention (NRR) method described in Brooks et al. [29], which assesses lysosomal integrity in haemocytes as an indicator of cellular functionality. Acetylcholinesterase (AChE) activity was measured in gill tissues, as described by Bocquené & Galgani [30] to assess neurotoxicity. The antioxidant enzymes catalase (CAT), glutathione reductase (GR), selenium-independent glutathione (GPx) and the biotransformation enzyme glutathione S-transferase (GST) were also assessed in gills and digestive glands of control and exposed mussels. CAT activity was determined by measuring the decrease in absorbance due to H_2O_2 consumption [31]. GPx and GR activities were assessed based on the protocols of Flohé & Günzler [32] and Cohen & Duvel [33], respectively, modified for a 96-well format. GST activity was determined by the conjugation of reduced glutathione (GSH) with 1-chloro-2,4- dinitrobenzene (CDNB) following the method of Habig & Jakoby [34] modified for a 96-well format. Lipid peroxidation (LPO) in gills and digestive glands was evaluated by measuring malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) resulting from the breakdown of polyunsaturated fatty acid peroxides, following the protocol outlined by Erdelmeier et al. [35]. Protein concentrations were determined using the modified Lowry method [36] or the Bradford method [37]. For full assay protocols, please refer to the Section S3 of the Supplementary Information.

2.8. Statistical analysis

Statistical analyses were conducted using XLStat2024® software (Addinsoft, Paris, France). Data were first tested for normality using the Shapiro-Wilk test and homogeneity of variance using the Levene's test. When assumptions of normality and homoscedasticity were met, group differences were assessed using either one-way ANOVA or two-way ANOVA, depending on the experimental design. Two-way ANOVA was applied when complete treatment-time combinations were available, with treatment and exposure duration included as fixed factors. One-way ANOVA was used when factorial analysis was not possible due to incomplete treatment-time combinations. In both cases, Tukey's post-hoc test was applied for multiple comparisons. When necessary, data were log-transformed to improve compliance with ANOVA assumptions. In cases where normality and homogeneity of variance was not met, the non-parametric Kruskal-Wallis test was applied instead, followed by Dunn's post-hoc test. Graphical representations were created using GraphPad Prism 10® (GraphPad Software Inc., La Jolla, CA, USA), and data was displayed as box-and-whisker plots. For particle size and morphology, binary images of projection particles obtained from the QICPIC imaging system were analysed using the QICPIC Dynamic Image Analysis application software (Sympatec GmBH). The size and morphology data were exported and further processed with the R software [38]. Particle-size distribution obtained using SEM analysis was determined using the software ImageJ (National Institutes of Health (NIH), Bethesda, MD, USA). A Principal component analysis (PCA) was also carried out using XLStat2024® software (Addinsoft, Paris, France) to assess the influence of organic additives and metal concentrations on the measured biomarkers in gills and digestive glands of control and exposed mussels following exposure (Day 28) and depuration (Day 36). A Pearson's correlation analysis was also performed to evaluate the strength of association between organic and inorganic chemical accumulation and biological responses of mussels. The level of significance for all statistical analyses was set to $p < 0.05$.

3. Results and discussion

3.1. Particles (TP, TPL) characterisation

Morphological analysis of tyre particles (TP, <300 μm) was carried out using two complementary techniques: dynamic image analysis (DIA) and scanning electron microscopy (SEM) as previously described in Michelangeli et al. [13]. DIA analyses large numbers of suspended particles from optical projections, providing statistically robust size and morphology distributions with minimized selection bias [39]. In contrast, SEM, applied here to smaller manually selected subsamples, provided high-resolution images for qualitative assessment of surface features. DIA results showed that TP particles exhibited predominantly circular shapes with smooth surfaces, with aspect ratio (AR) of 0.69 ± 0.15 and solidity (SLD) of 0.96 ± 0.06 (Fig. 1A, B). Particle size (n = 558) ranged from 30.2 to 389 μm, with a mean of 135 ± 65 μm. Pixel density varied between 222 and 36,706 pxl/p (average 5457 ± 4950 pxl/p). Size distribution peaked at ~114 μm (Fig. 1C), and 75% of particles were < 179 μm (Fig. 1D). Weak correlations between pixel density and AR ($-0.22, p < 0.001$) or SLD ($-0.09, p = 0.04$) indicated no strong size-dependent morphology. Fibre content was low (1.3%). These findings suggest that TP particles are largely spherical, smooth, and morphologically consistent across size classes, characteristics that may facilitate environmental dispersal and bioavailability by reducing aerodynamic drag and surface roughness.

SEM imaging revealed lower average particle sizes (TP: 83 ± 59 μm, n = 71; TPL: 101 ± 64 μm, n = 64) and more irregular morphologies. Both pristine TP (Fig. 2A–C) and post-leached TPL (Fig. 2D–F) displayed sharp edges and fractured surfaces. These findings support previous observations that cryomilling produces rough and fragmented particles [40,41]. Taken together, DIA provided robust quantitative information on particle size distributions, while SEM offered complementary insights into microstructural surface characteristics.

3.2. Particles internalization

In this study, particle internalization in mussel gills (Fig. 3A) and digestive glands (Fig. 3B) was assessed using two complementary methods: visual inspection with the VideometerLab reflectance imaging system and chemical quantification via Py-GC/MS (M4, specific markers for SBR, BR, and SBS, independent of particle size but dependent on total mass). Detailed results are provided in Table S5 (visual inspection) and Table S6 (Py-GC/MS) in the supplementary information.

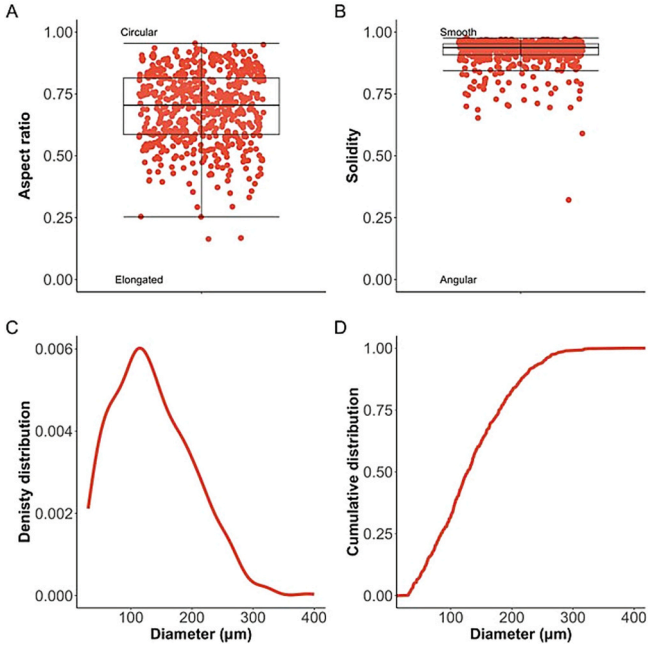

Fig. 1. Morphological characteristics of car tyre particles measured by Dynamic Image Analysis (DIA). (A) Aspect ratio, (B) Solidity, (C) Density and (D) Cumulative distribution. A total of 588 particles were analysed.

No particles were detected in the gills of control groups at D0 (initial day), D28 (end exposure), or D36 (after depuration) using either method, confirming the absence of contamination under control conditions and serving as a baseline for the treated groups (Fig. 3A). At D28, the TP treatment group showed particle presence in two samples by visual inspection (one particle per filter), but no particles were detected using Py-GC/MS. In contrast, Py-GC/MS detected particles in one TPL sample, as well as two L samples, suggesting the presence of smaller or lower-mass particles not captured visually. After the depuration period (D36), Py-GC/MS identified particles in three TP and L samples, while the VideometerLab identified particles in two TPL samples. These results highlight discrepancies between these detection methods and suggest that gills tissues retained particles even after depuration.

Similar to the gills, no particles were detected in digestive glands from the control group at any time point (Fig. 3B). At D28, both Py-GC/MS and visual inspection confirmed particle presence in all TP and TPL samples. In the L group, particle detection was inconsistent between methods, with Py-GC/MS identifying one positive sample and the VideometerLab another. Visual inspection also detected particles in TP and TPL groups after the depuration week (D36), but Py-GC/MS only detected particles in one L sample, suggesting a reduction in overall particle mass but persistence of visually detectable particles. The sporadic and method-dependent detection of particles in the L group, observed in both gills and digestive glands, was unexpected. These signals may reflect low-level contamination during handling or analysis, or the presence of smaller particles not fully retained despite filtration.

Overall, the accumulation and retention of tyre particles differed between gills and digestive glands (Fig. 3). By D28, digestive glands showed higher particle accumulation, likely due to ingestion and ciliary movement [42]. This aligns with existing literature suggesting mussels primarily ingest particles within the low micrometer range (<100 μm), overlapping with their natural phytoplankton prey size [43]. These organisms, however, have been shown to ingest particles of varying size and shape, including larger microplastic fibres [44,45]. Although the ingestion mechanisms for elongated fibres and more compact tyre particles may differ, these observations demonstrate the capacity of mussels to internalize diverse particulate forms, supporting the ingestion of TPs in this study. Following ingestion, particulate material may be internalized by digestive epithelial cells via endocytic pathways, including phagocytosis, enabling cellular uptake and subcellular processing of particles and associated chemicals [43]. By D36, digestive glands appeared to have expelled most particles, particularly those detected by Py-GC/MS. This goes in line with previous studies showing that bivalve digestive tissues can effectively eliminate ingested microplastics via faeces and pseudofaeces within a short time [43,45]. Although biodeposits were not analysed in this study, excretion is a plausible explanation. On the other hand, particles persisted in gill tissues post-depuration. While some may have translocated from the digestive system, as previously suggested by Fernández & Albentosa [43], another likely explanation is insufficient depuration time. A one-week depuration period may not be adequate for complete particle clearance, especially if particles become lodged in gill tissues or internalized by epithelial or haemolymph cells following endocytic or phagocytic uptake. Previous studies indicate that particle retention in gills can extend over longer periods depending on particle type, size, and organismal stress [42,45]. Therefore, the persistence of particles in mussel tissues may reflect a combination of biological uptake and retention mechanisms and methodological detection constraints related to the inherent limitations of Py-GC/MS and visual inspection.

The Videometer system can detect single particles down to 30 μm, but accuracy below 60 μm is limited. For this reason, a conservative 60

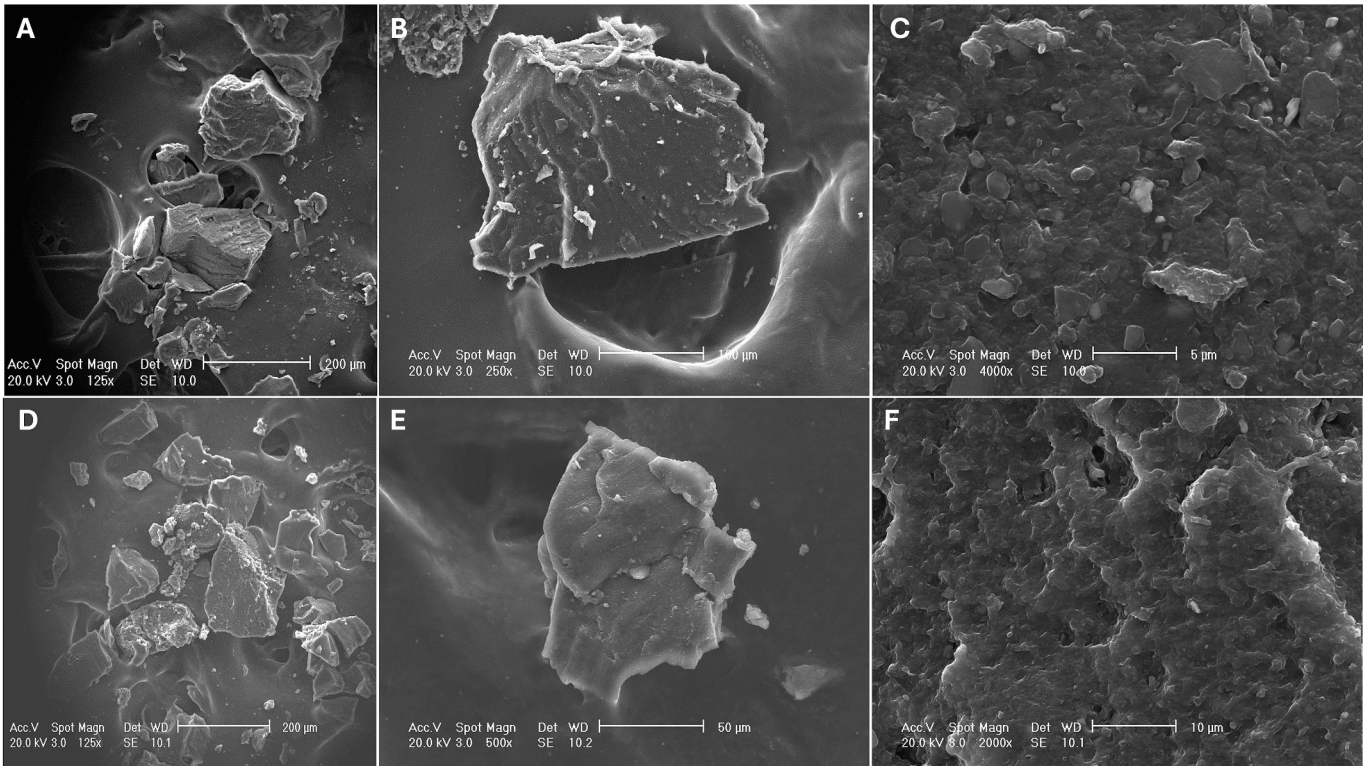

Fig. 2. Scanning electron microscopy (SEM) images of tyre particles (TP) at (A) 125×, (B) 250×, and (C) 4000× magnifications, and tyre particles after leaching (TPL) at (D) 125×, (E) 5000×, and (F) 2000× magnifications. Each batch includes three images at different magnifications to illustrate particle morphology and surface texture. Scale bars are provided at the base of each image.

µm threshold was applied in this study, which likely underestimated the presence of smaller particles, which constituted a significant portion of the sample (75% of particles were below 179 µm). This is particularly relevant as mussels can ingest particles well below this threshold, including nano-sized particles, which remain undetectable with the Videometer. As a result, particle presence in the <60 µm size range may have been underestimated by this method. Py-GC/MS, on the other hand, is a mass-based method that detects particles based on total amount of specific polymers (in this case SBR and BR), rather than particle size or count. Detection depends on whether the combined SBR + BR mass exceeds the method's sensitivity threshold, defined by signal to noise ratios of the samples (S/N), the LOD calculated as 3xS/N (0.020–0.129 µg/sample SBR, SI-X) and LOQ as 10xS/N (0.126–0.426 µg/sample SBR, SI-X). This means that even small particles can be chemically detected if their combined mass exceeds these thresholds. However, this method cannot determinate particle size or number and may miss larger particles if their SBR + BR content is low (e.g., tyre particles with higher natural rubber content). This is particularly relevant given that the tyre material used in this study is a mixture of ELTs, comprising of a heterogenous mix of rubber types and polymer concentrations, which may reduce chemical detectability for particles with low SBR + BR content. Nonetheless, Py-GC/MS improves tyre particle detection by targeting tyre-specific markers, accounting for variability in tyre composition, and minimizing interference from other environmental materials, leading to a more accurate assessment of tyre contamination [24]. In summary, the Videometer system enables size-based detection of particles ≥60 µm irrespective of their chemical composition, whereas Py-GC/MS quantifies tyre polymers (SBR, BR, SBS) based on mass rather than particle size. However, inconsistencies between methodologies emphasize the need for improved detection strategies and further validation. Apart from methodological differences, practical aspects as sample preparation may also influence detection efficiency. After Videometer analysis, filters were folded and transferred into pyrolysis cups, a process that could potentially result in particle loss, especially in samples with small-sized particles that are more easily lost during handling. This could help explain why Py-GC/MS indicated reduced tyre material in digestive glands after depuration, while visual inspection still identified particles. A small number of larger particles may remain visually detectable, while contributing less to total polymer mass to be consistently reflected in chemical quantification. Furthermore, possible contamination during filter handling, especially in the L treatment group, cannot be excluded. Despite the limitations described, this study showed that tyre particles can accumulate in mussel tissues, with clear organ-specific differences. Digestive glands appear to be the primary site of ingestion and early accumulation of tyre particles, while gills show prolonged retention. These findings highlight the importance of considering organ-specific physiology in assessing particle uptake in bivalves. Overall, the data presented here illustrated the broader challenges of identifying tyre particles in biological samples. Tyres compositions vary widely across manufacturers, tyre types, and specific applications [46–48]. Consequently, identifying reliable chemical markers for tyre particles is difficult, as no single marker can fully capture this diversity [9,47]. Environmental aging and weathering further complicate identification by altering marker properties over time [46]. These findings underscore the need for stable, specific markers for type particles that resist environmental degradation [9,47].

3.3. Organic additives and metals in leachates and water analysis

Targeted chemical analyses by HPLC-ESI-MS/MS of the 100% tyre leachate stock and water from the exposure tanks identified 19 different compounds (Table S7 in the SI), confirming that various organic additives leached from the tyre particles into the surrounding water. The selection of the additives to use in this targeted analysis was based on findings from our previous study [13] and were mainly key tyre associated chemicals of known environmental relevance. Among the

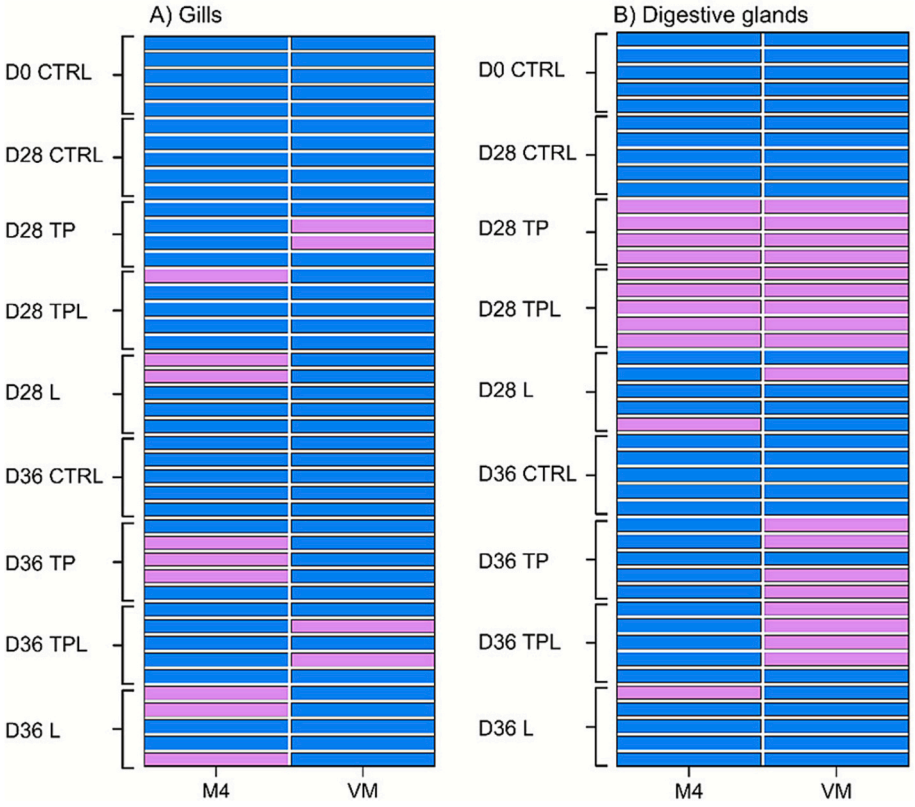

Fig. 3. Heatmap showing the presence of particles internalized into gills (A) and digestive glands (B) from 5 individual mussels across different treatments and time points. The treatments include control mussels (CTRL) at Day 0 (D0), 28 (D28), and 36 (D36), as well as mussels exposed to tyre particles (TP), tyre particles after leaching (TPL), and tyre leachates (L) at Day 28 (D28) and 36 (D36). The heatmap is based on data generated using pyrolysis-GC/MS marker (M4) and visual inspection with VideometerLab (VM). The colour represents particle detection levels, with blue indicating no particles detected and pink indicating particles detected. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

compounds detected in the leachate stock, the highest concentrations were observed for 1,3-Diphenylguanidine (1,3-DPG; vulcanization accelerator), 2-Hydroxybenzothiazole (antioxidant), dicyclohexylamine (vulcanization accelerator derivative), and cyclohexylamine (vulcanization accelerator) with concentrations ranging from 1276 to 4065 μg/L. Additionally, the antioxidant N(1,3-dimethyl-butyl)-*N*′-phenyl-*p*-phenylenediamine (6PPD) and its derivative 6PPD-quinone (6PPDQ) were also detected. Particularly, the concentrations of cyclohexylamine, 6PPD, and 1,3-DPG were reported as indicative or semi-quantitative values for not meeting all the quality control requirements for accurate quantification. These results confirm that tyre particles release a complex mixture of potentially toxic organic additives into the aquatic environment, some of which at very high concentrations. The detection of transformation products like 6PPDQ also indicates that tyre-derived chemicals undergo transformation, which may occur both during tyre aging on the particle surface and during the leaching process. Such compounds are of particular concern due to their known toxicity to aquatic organisms, indicating a potential risk associated with tyre particle leachates. In water samples collected from the exposure tanks, several additives including cyclohexylamine, N-cyclohexylformamide, *N*-phenylurea, 2-hydroxybenzothiazole, dicyclohexylamine (DCH), and DPG were consistently detected in all treatment groups (TP, TPL, and L) at days 7, 14, and 28. However, their concentrations were relatively low, ranging from 0.1 to 7.2 μg/L, which were between 500 and 10,000 times lower than the concentrations found in the leachates stock. While these compounds were generally below the limit of detection (0.01 μg/L) in control samples, they were marginally elevated in exposure treatments, indicating minor leaching from the particles into the surrounding water. In a similar study where mussels were exposed for 7 days to model TRWP (0.05 and 0.5 g/L), vulcanization accelerators were predominant in water samples, with 1,3-DPG comprising over 80% of the detected compounds [49]. DCH was also identified by the authors at substantial concentrations, noting it as a transformation product of the vulcanization accelerator N, N′-dicyclohexyl-2-benzothiazole sulfenamide (DCBS). In the current study, both DPG and DCH were detected at higher concentrations than other additives in the leachate stock and exposure tanks, highlighting their significant leaching potential and environmental relevance. Additionally, other compounds such as 6PPD and 6PPD-quinone, were only detected in the TP and TPL treatments during the exposure period, suggesting their predominance in tyre composition. Following depuration, most of the additives were not detected in the water samples, indicating effective removal or degradation over time. The detection of these compounds in water samples from all treatments during the uptake phase confirms the hypothesis that additives from tyre particles, whether from the TP or TPL, can be released into the surrounding environment during exposure. However, even though these additives do leach into the surrounding water, their concentrations decrease to undetectable levels after depuration. This finding is ecologically relevant, as it demonstrates that mussels were exposed not only to the physical particles (as evidenced by tissue internalization), but also to soluble organic chemicals leached from these particles. This combined exposure has implications for mussel health, as it may increase the risk of chemical uptake through gills or other ingestion pathways. The extent to which additives leach from tyre materials is governed by a combination of intrinsic polymer material properties (e.g., polymer type, porosity, glass transition temperature), extrinsic environmental parameters (e.g., temperature, salinity, turbulence, contact time, particle loading, pH), and intrinsic chemical properties such as solubility and hydrophobicity (log K_{OW}) [50]. The experimental conditions used in this study, i.e. moderate salinity (~34 PSU), stable

temperature of 16 °C, and constant aeration, likely supported leaching by maintaining particles in suspension, ensuring prolonged contact with water, and enhancing additive diffusion. As for the properties of specific chemical additives, 6PPDQ for example, is known to have low aqueous solubility (38 ± 10 mg/L) [51], yet this was sufficient for migration from tyre rubber into the surrounding environment. Despite its moderate-high hydrophobicity (log K_{OW} of 4.30 ± 0.02) [51], 6PPDQ can still partition into aqueous systems, especially under continuous water agitation and particle suspension, as in this study. These physicochemical characteristics are consistent with its detection in water samples, albeit at low concentrations. It should be mentioned, however, that the exact mechanism underlying its occurrence remains uncertain: 6PPDQ may leach directly from tyre and road wear particles (TRWP), or alternatively, it may form in situ via oxidation of 6PPD within the aqueous phase. Both pathways are plausible, and further investigation is required to elucidate their relative contributions. Similarly, while the exact solubility of DPG is not well-established, Foscari et al. [49] identified DPG as a major leaching component from cryo-milled TRWP. Its relatively small molecular size, compared to the larger polymeric components of rubber or other antioxidant additives used in tyres, increases its mobility and diffusion within the rubber matrix, facilitating its release into water [50,52]. This aligns with its frequent detection in all exposure tanks in the present study, despite overall low concentrations.

Metal analysis revealed that only Co and Zn were detectable in water samples, while all the other metals were below the LOQ (Table S8 in the SI). Co was detected at low levels, with concentrations not exceeding 0.1 μg/L, whereas Zn was consistently present at higher concentrations. Zn is a key additive in tyre manufacturing, commonly used in the form of zinc oxide (ZnO) as a vulcanization activator. It is also environmentally relevant due to its known toxicity to aquatic organisms at elevated concentrations and its potential to accumulate in sediments and biota. Zn was consistently detected across treatments during the exposure period. At day 7, Zn was found in TP (10.19 ± 0.87 μg/L), TPL (16.09 ± 1.17 μg/L), and L (8.00 ± 0.39 μg/L), with no significant differences between groups. By day 14, Zn concentrations only increased in TP (18.16 ± 1.74 μg/L), while for TPL (12.02 ± 1.01 μg/L) and L (9.02 ± 0.12 μg/L) the values remained comparable to day 7. The highest Zn concentrations were recorded on day 28 in all exposure groups, with TP exhibiting the highest concentration (31.56 ± 0.04 μg/L), followed by TPL (21.51 ± 0.80 μg/L) and L (11.56 ± 0.57 μg/L). After the depuration period (day 36), Zn concentrations reduced significantly across all groups: with levels in TP decreasing to 4.74 ± 0.50 μg/L, 18.37 ± 2.48 μg/L in TPL, and below the LOQ for L. These results showed progressive Zn leaching from all groups over time, with TP consistently yielding the highest levels, followed by TPL. This pattern suggests a more persistent release mechanism from the particle treatments, confirming that Zn is highly soluble and readily leachable when tyre materials are exposed to water, particularly over time. Although Zn concentrations remained below the Norwegian Environmental Agency's toxicity threshold of 60 μg/L for coastal waters [53], it might still pose a risk under environmentally realistic scenarios.

Overall, chemical analysis of the water samples from the mussel exposure demonstrated that TP, TPL and their leachates acted as sources of organic and inorganic chemical additives, with particles being the primary contributor to the chemical load. Mussels were thus exposed not only through particle uptake but also via the continuous release of soluble chemical additives into the surrounding water, including organics and metals like Zn. While this study was conducted under controlled laboratory conditions, it does not fully reflect the complexity of natural marine environments, where leaching, long-term exposure, bioaccumulation of organic and inorganic additives in sediments or organisms and interactions with other stressors could amplify ecological risk posed by tyre-associated chemicals. Nonetheless, these findings underscore the potential chemical exposure risks posed by tyre particle pollution, emphasizing the importance of considering both organic additives and metals such as Zn in addition to particle exposure in environmental assessments.

3.4. Accumulation of organic additives and metals in gills and digestive glands

The accumulation of tyre-derived chemicals in mussel tissues (Table S9 in the SI) after 28 days of exposure to TP, TPL and L supports the findings from water analyses, which showed that tyre particles released chemical additives into the surrounding water. This parallel accumulation suggests that, in addition to waterborne exposure, chemical additives can also be released from the polymer matrix of ingested particles directly ending up in mussel tissues, leading to internal exposure. In the gills, uptake was most pronounced in the TP treatment, with the highest concentrations observed for TMQ (881 ± 96.1 ng/g ww), DPG (123 ± 61.7 ng/g ww), 6PPDQ (65.3 ± 4.48 ng/g ww), DPPD (45.7 ± 9.65 ng/g ww), and 6PPD (40.3 ± 14.37 ng/g ww). Several of these additives, such as DPG, 6PPD and its transformation product 6PPDQ, were also detected in the corresponding water samples, underscoring their potential for bioavailability and uptake. When comparing concentrations across both matrices, DPG, present at 1.66 μg/L in water, showed the second highest gill concentration, indicating efficient uptake. 6PPDQ, despite a low water concentration (0.17 μg/L), highly accumulated in the gills, suggesting a strong affinity for this tissue. Conversely, 6PPD had a slightly higher water concentration (0.90 μg/L) but lower tissue levels (40.3 ± 14.37 ng/g ww). These patterns indicate that beyond exposure levels, certain additives, particularly 6PPDQ, may have a higher tendency to partition into or bind with biological tissues, reflecting compound-specific uptake and retention characteristics. Route-dependent effects have been demonstrated for 6PPDQ in the mussel *M. coruscus*, where waterborne and dietary exposures caused distinct biological responses, highlighting the importance of exposure route when interpreting additive uptake and toxicity [54]. Mussels exposed to TPL showed additive levels comparable to the TP treatment, with TMQ concentrations reaching 470 ± 214.52 ng/g ww, confirming that these chemicals continued to leach from the particles even after the initial leaching process. After the depuration period (day 36), residual levels of additives such as TMQ, DPG, 6PPD, and 6PPDQ persisted in the gills of mussels exposed to the TP and TPL treatments, indicating limited elimination. In the L treatment, only DPG and HMMM accumulated in the gills, with DPG concentrations reaching levels similar to those of the TP treatment (83.5 ± 28.08 ng/g ww). After the depuration period, DPG concentrations decreased but remained above control levels. HMMM levels returned to values comparable to controls, indicating a more effective elimination.

Chemical additive uptake was more pronounced in the digestive glands than in the gills, particularly in the TP and TPL treatments (Table S9 in the SI). By day 28, TMQ concentrations in the digestive glands of TP-treated mussels reached 4087 ± 902.63 ng/g ww, followed by 6PPD (429 ± 145.45 ng/g ww), 6PPDQ (197 ± 67.36 ng/g ww), DPG (175 ± 17.35 ng/g ww), and DPPD (97 ± 43.82 ng/g ww). TQM accumulated to levels nearly ten times higher than 6PPD, despite 6PPD being widely regarded as one of the most hazardous tyre-derived compounds. This highlights that TMQ, though less discussed in the context of ecological risk, may have a strong affinity for biological tissues and warrants greater attention in future bioaccumulation and hazard assessments. Following the depuration period, TMQ (671 ± 557.52 ng/g ww), 6PPD (63 ± 58.81 ng/g ww), and 6PPDQ (26.5 ± 23.67 ng/g ww) concentrations were still higher than the controls, indicating persistence in digestive tissues. Even though these values decreased approximately 84–92% compared to those at day 28, the residual presence of TMQ and 6PPD suggests limited elimination and a higher affinity for tissue retention. Similar trends were observed in the digestive glands of the TPL treatment, with high concentrations of TMQ (2616 ± 377.51 ng/g ww), 6PPD (527 ± 122.54 ng/g ww), and DPG (205 ± 35.8 ng/g ww) by day 28. TMQ accumulation was lower in TPL than TP, while 6PPD and DPG levels were slightly higher. After the depuration period,

concentrations declined by approximately 66% for TMQ, 77% for 6PPD, and 90% for DPG, yet residual levels remained above control values. Furthermore, post-depuration concentrations in TPL remained higher than those in the TP group for all three compounds, suggesting limited elimination under depuration conditions. In the L treatment, uptake in the digestive glands was limited to DPG and HMMM, with DPG concentrations (171 ± 9.29 ng/g ww) significantly higher than in the control. However, depuration effectively reduced these concentrations to control values. These patterns align with findings by Foscari et al. [49], who reported that DPG was the predominant compound in mussel tissues exposed to model TRWP for 7 days, followed by DCH, 2-MTBT, PTPD, DPPD, and 6PPDQ. Similarly, in our study, DPG, 6PPDQ, DPPD, and 6PPD were dominant in the gills, while the digestive glands showed the highest levels of TMQ, 6PPD, 6PPDQ, DPG, and DPPD. This cross-study consistency reinforces the relevance of DPG, 6PPD, and 6PPDQ as bioavailable, tyre-derived contaminants. The observed differences in accumulation, such as the elevated TMQ levels reported in the present study, likely reflect variations in tyre composition, additive content, or particle preparation methods. Overall, these results demonstrate that tyre particles act as a significant source of additive release and subsequent accumulation in mussels, while leachates contribute to lower but still measurable contamination.

The frequent detection of additives as DPG, 6PPD, and 6PPDQ in water, leachates, and mussel tissues underscores their high leaching potential, bioavailability, and suitability as biomarkers of tyre particle pollution in aquatic organisms. Accumulation levels were consistently higher in the digestive glands compared to the gills, reflecting their role as the primary site for bioaccumulation of hydrophobic compounds [55]. This pattern aligns with the compounds' physio-chemical properties such as the octanol-water partition coefficient (K_{OW}), which is a key factor driving bioaccumulation [56]. Compounds with higher log K_{OW} are more lipophilic and likely to bioaccumulate in organisms, with the European REACH regulation classifying substances with log $K_{OW} \geq 4.5$ as having a high bioaccumulation potential [56]. While depuration reduced chemical levels in the gills and partially in the digestive glands, the persistence of some compounds highlights the potential risks of long-term exposure to tyre-derived additives in aquatic environments. For example, 6PPDQ, which showed high accumulation despite low concentrations in water, has a moderate log K_{OW} of 4.30 ± 0.02 [51], indicating significant potential for tissue retention. TMQ, which accumulated to the highest levels in the digestive glands, particularly in TP- and TPL- treated mussels, lacks published log K_{OW} data, but its pronounced presence suggests strong lipophilicity, potentially exceeding that of its precursor quinoline (log K_{OW} of 2.06) [57]. This may explain its persistence even after the depuration period, underscoring its potential for long-term tissue retention. Similarly, DPG which has a lower log K_{OW} of 2.4, still showed substantial accumulation, likely due to its moderate known bioaccumulation potential [58]. In contrast, HMMM, which was detected in L-exposed mussels, resembles other triazine compounds [59] characterized by lower log K_{OW} values (1.7–3) [60], a property that may explain its relatively limited accumulation and more efficient depuration.

Metal concentrations in mussel tissues (Table S10 in the SI) showed comparable distribution patterns between the digestive glands and gills. Two-way ANOVA identified only a small number of statistically significant effects, with no consistent treatment- or time-dependent patterns across tissues (Table S11 in the SI). Metal concentrations followed a consistent order of accumulation across treatments and exposure duration, with concentrations in the gills ranking from Zn ≫ As > Sr ≈ Se > Cu > Pb > Cd > Ni ≈ Cr ≈ Co > Ti > Sb > Al and the digestive gland from Zn ≫ As > Sr > Al ≈ Cu > Se > Ni > Cr > Cd > Co ≈ Pb > Sb > Ti. Zn was the most abundant metal in both tissues, occurring at concentrations one to two orders of magnitude higher than the remaining elements. This is not surprising, given the key role of Zn in tyre rubber formulations, where zinc-containing additives are essential for the vulcanization of rubber and contribute to maintaining material stability during use [61]. Overall, while the relative ranking of metals was similar between tissues, Cr, Co, Ni, Cu, and Cd concentrations were primarily higher in the digestive glands compared to the gills, suggesting a higher accumulation in this organ. This aligns with previous studies indicating that the digestive gland is the main site for metal accumulation and detoxification in mussels. When comparing Zn concentrations in the digestive gland at day 28, levels were significantly higher in the TP (300.4 ± 114.0 μg/g dw), TPL (247.4 ± 119.6 μg/g dw), and L (416.5 ± 562.5 μg/g dw) treatment groups compared to the control. However, by day 36, Zn levels in the TP (121.7 ± 59.3 μg/g dw) and TPL (133.1 ± 45.3 μg/g dw) treatments decreased to levels comparable to the control, while the L treatment (261.6 ± 47.6 μg/g dw) remained higher. This suggests a differential rate of metal processing and excretion depending on the treatment conditions. Additionally, Cr, Ni, and Cu concentrations were significantly higher in the digestive glands at day 28 compared to day 36, indicating a possible reduction in metal accumulation over time. This decline could be attributed to physiological detoxification mechanisms or reduced metal bioavailability at later stages of exposure. Similar to the digestive gland, metal concentrations in gills showed predominantly transient responses. Several metals, including Zn, Cu, Cd, and Pb, were elevated at day 28, particularly in the leachate treatment; however, by day 36, concentrations decreased to levels comparable to the control. This pattern indicates limited retention of metals in gill tissues following depuration and supports the role of gills as a dynamic exchange surface, with metals being more readily eliminated compared to digestive tissues. The presence of several metals in mussel tissues (e.g. Cr, Ni, Cd, and Cu), despite only Zn and Co being detected in water samples, highlights the strong metal-accumulation capacity of mussels over time. This suggests that the occurrence of these metals in the TP and TPL treatment groups at day 28 may be attributed to potential leaching from internalized particles rather than direct uptake from the surrounding water. On the other hand, tissue concentrations in the L treatment may reflect uptake from waterborne exposure and baseline body burdens, as also observed for organic additive chemicals. When compared to environmental baseline levels, the metal content in the gills and digestive glands of mussels exceeded in most cases the Norwegian provisional high reference contaminant concentrations (PROREF). PROREF values represent Norwegian provisional high reference concentrations rather than regulatory thresholds and are intended to contextualise biomonitoring data [62,63]. Elevated concentrations were observed for Pb (195 μg/kg ww), Cd (180 μg/kg ww), As (2503 μg/kg ww), Zn (17,660 μg/kg ww) and Cu (1400 μg/kg ww), whereas Co and Cr concentrations remained below their PROREF values (1400 μg/kg ww and 361 μg/kg ww, respectively). These findings indicate that tyre-associated metals accumulate in mussel tissues above reference environmental concentrations, suggesting a potential for adverse effects under prolonged exposure conditions.

Overall, these findings clearly show that mussels exposed to tyre particles accumulated higher levels of additives compared to those exposed to leachates, highlighting particle exposure as a dominant pathway for chemical accumulation. These findings suggest that bioaccumulation is not primarily driven by the presence of additives in the surrounding water, but rather by the internalization of tyre particles via ingestion and subsequent tissue retention, which act as a sustained source of chemicals. Although several additives were detected in the exposure water, their concentrations were insufficient to explain the levels observed in mussel tissues, particularly in the TP and TPL treatments, supporting the hypothesis that additives are gradually released from particles directly into the tissues. The accumulation of additives was compound-specific and appeared to be governed by factors such as particle internalization, leaching potential, and chemical properties such as lipophilicity and tissue affinity. Importantly, the presence of additives in mussels following exposure to the 14-day pre-leached particles (TPL treatment) suggests that this leaching duration was insufficient to deplete the chemical content of the tyre particles. Additives continued to leach from the particles and accumulated in tissues,

indicating that tyre materials retain substantial internal reservoirs of these compounds and may serve as long-term sources of contamination. Together, these findings provide critical insight into the mechanisms of additive accumulation in marine organisms and underscore the role of ingested particles as mobile vectors for chemical exposure. They also emphasize the need to consider particle-mediated transport alongside waterborne exposure in risk assessments of microplastic and tyre-derived pollution. Finally, the persistence of additives after depuration highlights the potential for long-term ecological impacts and underscores the importance of further research into their toxicological effects, persistence in food webs, and capacity for trophic transfer.

3.5. Biomarker responses in mussel tissues

3.5.1. Condition index (CI)

The condition index (CI) serves as a measure of the general health status of an organism, reflecting physiological processes such as growth, reproduction, secretion, and other activities influenced by environmental factors with low values indicative of poor health status [29]. Two-way ANOVA indicated that exposure duration significantly contributed to the overall variability of the CI in exposed mussels (Table S11 in the SI). However, no significant differences were detected in exposed mussels (TP, TPL and L) when compared to the controls (CTRL), with CI values ranging from 5.9% to 12.5% (Fig. S6 in the SI). This indicates that exposure to tyre particles and leachates did not affect the overall health status of the mussels during the experimental period.

3.5.2. Lysosomal membrane stability (LMS)

Lysosomal membrane stability (LMS), a highly sensitive biomarker for general stress in mussels, decreases when lysosomal integrity and functionality are compromised, serving as an early indicator of cellular and tissue damage in *Mytilus* species [64]. No significant changes in lysosomal membrane stability were detected in exposed mussels compared with controls at any sampling point, even though treatment contributed to the variability in NRR time (Fig. 4A, Table S11). To assess the biological relevance of LMS values, the background assessment criteria (BAC) and environmental assessment criteria (EAC) established for the NRRT assay, set at 120 min and 50 min, respectively [65], were considered. Mussels with NRR values between these thresholds are classified as stressed but still capable of compensatory responses [66]. Most mussels exhibited values within this range, indicating sub-lethal stress in response to TP, TPL and L exposure. However, values close to or below the EAC threshold indicate that mussels may be experiencing physiological damage, especially under prolonged or repeated exposure scenarios. In contrast with the present study, lysosomal membrane impairment has been documented in *M. galloprovincialis* haemocytes exposed to car tyre leachates at a comparable concentration (0.08 g/L) [64]. Additional evidence from another study using tyre leachates (prepared from the same source particles as those used in this study) at concentrations of 0.32–10% also showed decreased lysosome content in *M. edulis* haemocytes [13]. The authors linked these alterations to the presence of heterocyclic compounds such as benzothiazoles and aromatic amines in the leachates, which can disrupt membrane integrity and interfere with lysosomal enzymes [13]. In the present study, even though chemical and particle analyses conducted at day 28 and after depuration (day 36) confirmed the accumulation of tyre-associated particles and additives in gill and digestive gland tissues, no significant lysosomal destabilization was detected in haemocytes.

3.5.3. Acetylcholinesterase (AChE)

AChE, a well-established biomarker for neurotoxic exposure in aquatic organisms, regulates neuro-muscular function by breaking down acetylcholine at synapses. Inhibition of AChE leads to acetylcholine accumulation, disrupting neural signalling and causing effects such as behavioural changes, paralysis, or mortality in aquatic organisms [30,64]. In this study, AChE activity was significantly affected by the

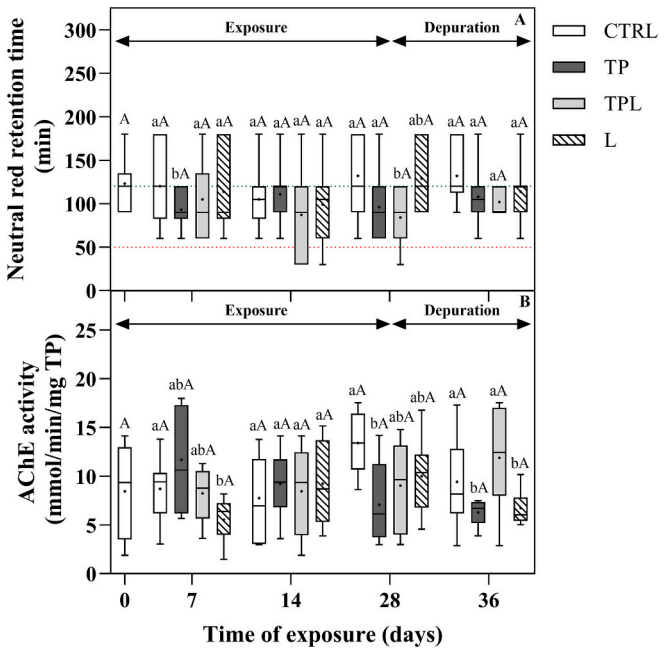

Fig. 4. Biomarkers in mussels unexposed (CTRL – control) and exposed to tyre particles (TP, tyre particles; TPL, tyre particles after leaching) and corresponding leachates (L) for 0, 7, 14, 28 and 36 days. (A) Lysosomal membrane stability in mussel haemolymph measured as neutral red retention time (NRRT); (B) – Acetylcholinesterase activity (AChE) in mussel gills. Results are presented as box-and-whisker plots ($n = 8$). The box represents the inter-quartile range (IQR), the median values are indicated with a central horizontal bar (−), the mean values are indicated with a plus (+) and the whiskers represent the 5–95 percentile. Lower case and capital letters represent statistical differences between treatments in each exposure day and for each treatment during the exposure duration, respectively ($p < 0.05$). In lysosomal membrane stability (A), the green line represents the Background Assessment Criteria (BAC) and the red line represents the Environmental Assessment Criteria (EAC) [66]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

interaction between treatments and time (Table S11). AChE activity in gills was significantly inhibited on day 7 in mussels exposed to L, indicating an early neurotoxic effect. AChE values returned to baseline by days 14 and 28 but decreased again after depuration (day 36) (Fig. 4B). In TP-exposed mussels, a significant inhibition was observed on day 28 that persisted through the depuration period. No significant changes were observed in the TPL treatment. The observed inhibition in both L and TP exposures is likely linked to the presence of tyre-derived chemical additives. In the L treatment, these compounds were immediately bioavailable, which may explain the earlier onset of neurotoxic effects. In the TP treatment, chemical release from the particles likely occurred more gradually, with neurotoxic concentrations reached later in the exposure period. Several of these additives, including 6PPDQ and other PPDs, have been previously identified as neurotoxic in aquatic species [10,67]. For example, 6PPDQ induced neurotoxicity in juvenile Coho Salmon [68] and altered neurotransmitter profile in zebrafish larvae [69]. In this study, 6PPD and 6PPDQ were detected in gills of TP exposed mussels at day 28, consistent with the observed AChE inhibition. Although 6PPDQ was not detected in the gills of mussels exposed to L, other compounds such as DPG, have also been reported as potentially neurotoxic [70] and may explain the effects seen at earlier time points. In addition to chemical exposure, the presence of particles in the TP treatment may have contributed to neurotoxicity by acting as vectors or inducing localized stress in gill tissues. Previous studies have shown that plastic particles of various sizes can interfere with cholinergic pathways in bivalves and fish [64,71,72]. This combined particle-chemical interaction may help explain the persistent AChE inhibition observed in TP-

exposed mussels. Since chemical accumulation was only measured at days 28 and 36, it was not possible to confirm the presence of neurotoxic compounds at earlier stages. However, the early AChE inhibition observed in the L treatment strongly suggests that chemicals such as DPG were already present in the gills during the first week of exposure.

3.5.4. Antioxidant defences

The antioxidant system is crucial for maintaining redox homeostasis and preventing cellular damage [73]. This system consists of enzymatic and non-enzymatic components, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), glutathione reductase (GR), and the antioxidant glutathione (GSH), which collectively contribute to antioxidant capacity and the organism's response to oxidative stress [74]. CAT plays a key role in cellular defence against oxidative stress by detoxifying reactive oxygen species (ROS), specifically by converting hydrogen peroxide (H_2O_2) into water and oxygen [74]. In this study, no significant effects of treatment, exposure duration or their interaction were detected for CAT in mussel gills, whose activity remained stable across all treatments and time points (Fig. 5A; Table S11). This suggests that even though some chemical additives accumulated in the gills, their concentrations were not high enough to trigger substantial ROS formation, or that upstream antioxidant activity, such as SOD, effectively prevented excessive H_2O_2 accumulation. This aligns with previous studies, where exposure to tyre leachates (0.08 g/L) did not affect CAT activity in the gills of mussels [64]. On the other hand, a significant effect of treatment and its interaction with time was observed for CAT activity in the digestive glands, indicating that treatment-related responses varied over the exposure period (Fig. 5B, Table S11). Mussels exposed to L showed a significant decrease in CAT activity at day 14, followed by a recovery by day 28 (Fig. 5B), suggesting a transient oxidative stress event likely driven by early chemical exposure and ROS formation. In particle treatments (TP and TPL), CAT activity significantly decreased only at day 36, indicating a delayed but persistent oxidative challenge (Fig. 5B). A decrease in CAT activity has also been reported in *M. galloprovincialis* exposed to PE and PS MPs [71,75]. However, while these studies generally associated a decrease in CAT activity with enhanced ROS production following particle exposure, the inhibition of CAT seen in this study is likely linked to the continued release of additives from internalized particles within digestive tissues. In addition to oxidative stress, certain tyre-derived additives such as PPDs (e.g., IPPD) have been shown to inhibit CAT activity in zebrafish embryos [76], suggesting that both ROS-induced enzyme degradation and direct chemical interference may contribute to this suppression. Furthermore, prolonged exposure could disrupt protein synthesis or trigger metabolic shifts that downregulate CAT expression as part of a broader stress response. Although gills are generally more sensitive to oxidative stress due to their lower baseline antioxidant defences, they likely experienced less chemical and particle accumulation in this study, which may explain the limited oxidative response observed. In contrast, the digestive glands, while typically better equipped to manage oxidative challenges thanks to their higher antioxidant capacity, are also the primary sites of chemical and particle accumulation [77]. This increased exposure likely led to elevated ROS production that exceeded its detoxification capacity, ultimately resulting in oxidative damage and enzymatic inactivation.

GPx plays a crucial role in removing H_2O_2 and other peroxides from the cell, thereby protecting against oxidative damage and maintaining redox homeostasis [78]. Two-way ANOVA indicated significant effects of treatment and exposure duration on GPx activity in the gills (Table S11), reflecting distinct temporal response patterns among treatments. Mussels exposed to L showed a significant decrease in GPx after one week of exposure (Fig. 5C), despite no corresponding changes in CAT activity. This suggests that the inhibition of GPx may have been driven by a rapid accumulation of reactive peroxides, including both H_2O_2 and organic hydroperoxides, resulting from elevated ROS production induced by the complex chemical mixture in the leachates. In contrast, TPL exposure led to a significant increase in GPx activity at day 14 and day 28, which returned to baseline levels post-depuration (Fig. 5C). This increase may reflect a response to continued ROS formation, consistent with previous studies. Elevated GPx levels were observed in the gills of *M. galloprovincialis* after 21 and 28 days of exposure to leachates prepared from fish net containing benzothiazoles at a concentration of 10 mg/L [78], as well as in zebrafish embryos exposed to a suspension from ELTs, where transcriptional levels of GPx

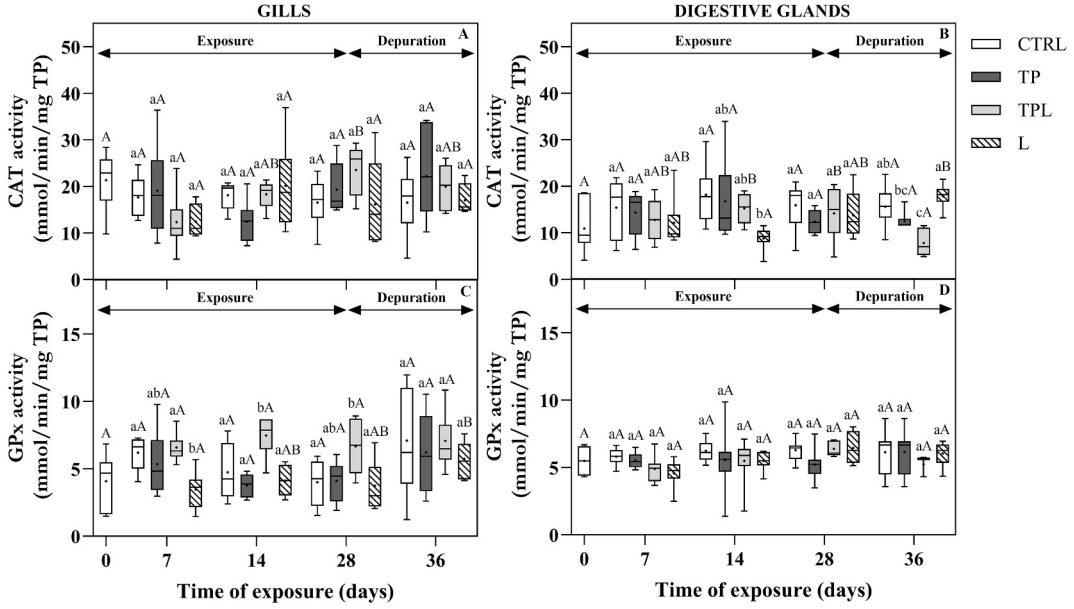

Fig. 5. Catalase (CAT) in gills (A) and digestive glands (B) and glutathione peroxidase (GPx) in gills (C) and digestive glands (D) of mussels unexposed (CTRL – control) and exposed to tyre particles (TP, tyre particles; TPL, tyre particles after leaching) and corresponding leachates (L) for 0, 7, 14, 28 and 36 days. Results are presented as box-and-whisker plots ($n = 8$). The box represents the inter-quartile range (IQR), the median values are indicated with a central horizontal bar (–), the mean values are indicated with a plus (+) and the and whiskers represent the 5–95 percentile. Lower and capital letters represent statistical differences between treatments in each exposure day and for each treatment during the exposure duration, respectively ($p < 0.05$).

were also upregulated [79]. The early inhibition of GPx observed in the L treatment likely reflects the immediate bioavailability of dissolved chemicals, leading to rapid ROS formation and oxidative stress. In contrast, the delayed upregulation of GPx in the TPL treatment suggests that the presence of particles may have modulated the release kinetics and bioavailability of associated chemicals, resulting in a more gradual accumulation and a temporally shifted oxidative response. In the digestive glands, GPx levels remained stable across treatments and timepoints (Fig. 5D, Table S11). These findings indicate that baseline GPx activity may have been sufficient to maintain redox balance despite CAT inhibition in this tissue, similar to antioxidant defence patterns observed in crabs exposed to tyre wear particles and leachates [80]. Overall, this data highlights a differential temporal and tissue-specific GPx response: gills exhibited earlier and more variable changes, while the digestive gland showed a stable response over time. These findings emphasize the need to assess GPx activity in multiple tissues to better understand how tyre-derived contaminants and their complex mixtures affect redox homeostasis over time.

Glutathione is a key non-enzymatic component of the antioxidant defence system mitigating oxidative stress directly by scavenging a wide range of free radicals, and indirectly through its involvement in the glutathione-dependent enzymatic system, including GR and GST [73,74]. GR plays a vital role in maintaining glutathione homeostasis by regenerating reduced glutathione (GSH) from its oxidized form (GSSG) [74]. A significant interaction between treatment and exposure duration was observed for GR activity in the gills, indicating that treatment-related responses varied over time (Fig. 6A, Table S11). In TPL-exposed mussels, a reduction of GR activity was observed compared to the other groups at day 28. This reduction may be attributed to oxidative stress resulting from chemical accumulation, which could impair GR function and disrupt the GSH:GSSG ratio, limiting the regeneration of reduced glutathione. This is further supported by the detection of tyre-associated compounds such as TMQ and 6PPDQ in this tissue, pointing to combined stress from chemical uptake and particle retention. The observed GR inhibition may indicate a threshold at which detoxification mechanisms are overwhelmed or GSSG accumulates excessively, as previously reported in earthworms and fish exposed to photoaged tyre wear particles and PAHs, respectively [81,82]. After the depuration period, GR activity in the TP treatment again showed a significant increase compared to the CTRL and TPL treatments (Fig. 6A), which may reflect continued oxidative pressure potentially due to residual chemical exposure. These results align with those reported by Stephensen et al. [83], where an increased GST activity was observed in rainbow trout exposed to ELTs particles (1 mg/L) and associated with the leaching of aromatic amines (e.g., TMQ) and polycyclic aromatic hydrocarbons (PAHs). Similarly, LaPlaca et al. [84] found elevated GSH:GSSG ratio in the liver of *Fundulus heteroclitus* exposed to crumb rubber (0.05 to 0.25 g/L), suggesting enhanced GR activity to maintain redox stability and prevent oxidative damage. In the digestive glands, GR activity showed a different temporal dynamic, with significant effects of treatment, exposure duration and their interaction (Fig. 6B, Table S11). GR activity in this tissue exhibited minimal variation across treatments and time points, with no significant differences between exposure groups and the control. While some fluctuations were seen, they likely reflect natural variability rather than treatment-specific effects. This suggests that the baseline GR activity in the digestive gland was sufficient to maintain redox homeostasis and neutralize reactive oxygen species (ROS) generated during both the exposure and depuration periods. The lack of pronounced GR activation in this tissue contrasts with the responses observed in the gills and indicates that the digestive gland may have a more robust inherent antioxidant capacity or experienced lower oxidative pressure from the tyre-derived exposures.

GST is an essential antioxidant enzyme family that utilizes GSH to neutralize xenobiotics and oxidized lipids [73]. GST activity in the gills of unexposed and exposed mussels remained stable across treatments and time points, with no significant effects of treatment, exposure duration or their interaction (Fig. 6C, Table S11). In the digestive gland, a significant effect of treatment was observed (Fig. 6D; Table S11), although GST activity showed no variation over time. These results suggest that the GSH-dependent detoxification system was not significantly disrupted by tyre-derived exposure. These findings are consistent with previous research on *M. galloprovincialis* exposed to tyre leachates

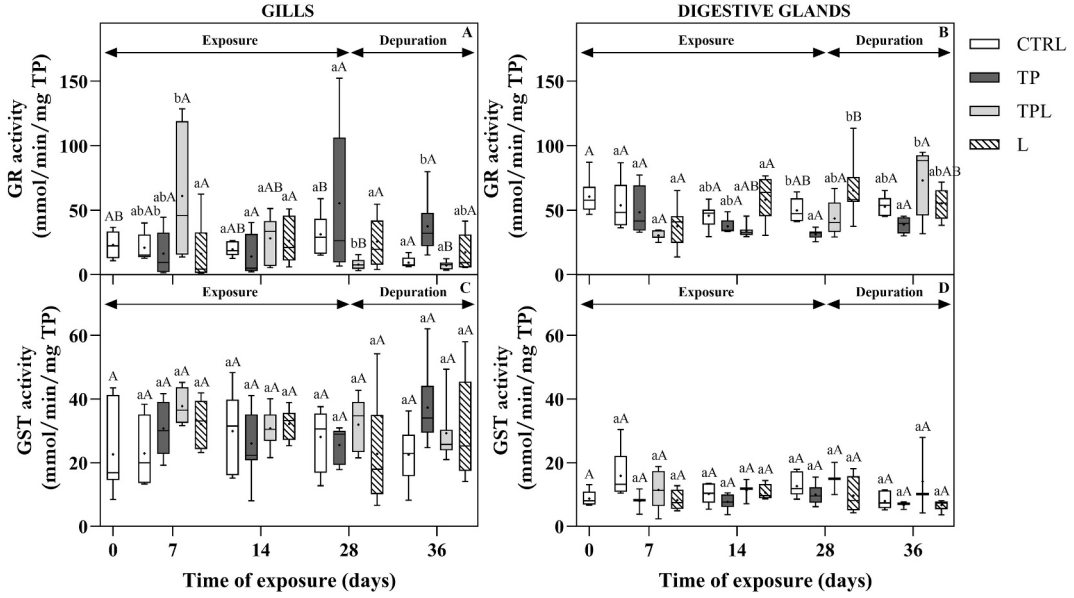

Fig. 6. Glutathione reductase (GR) in gills (A) and digestive glands (B) and glutathione-s-transferase (GST) in gills (C) and digestive glands (D) of mussels unexposed (CTRL – control) and exposed to tyre particles (TP, tyre particles; TPL, tyre particles after leaching) and corresponding leachates (L) for 0, 7, 14, 28 and 36 days. Results are presented as box-and-whisker plots ($n = 8$). The box represents the inter-quartile range (IQR), the median values are indicated with a central horizontal bar (−), the mean values are indicated with a plus (+) and the and whiskers represent the 5–95 percentile. Lower and capital letters represent statistical differences between treatments in each exposure day and for each treatment during the exposure duration, respectively ($p < 0.05$).

(0.08 g/L), where no significant changes in GST activity were observed in either tissue [64]. In addition, basal GST levels were lower in the digestive glands compared to the gills, a pattern also reported in *M. galloprovincialis* [64]. This tissue-specific difference may reflect that in the gills, GST has a more prominent role in detoxification processes, as they are directly exposed to fluctuations in environmental physico-chemical conditions and potential oxidative stressors [85]. Additionally, it is important to consider that other components of the antioxidant defence system that were not assessed in this study, such as metallothioneins (MTs), superoxide dismutase (SOD), or non-enzymatic antioxidants, may have played a more central role in mitigating oxidative stress in certain tissues or under specific treatments. These unmeasured responses could help explain the limited changes observed in some of the enzymes determined, particularly in the digestive gland.

3.5.5. Lipid peroxidation (LPO)

Commonly used as a biomarker of oxidative stress, LPO refers to the oxidative degradation of lipids, primarily polyunsaturated fatty acids within cellular membranes, caused by free radicals and the formation of ROS. This process generates lipid radicals and lipid hydroperoxides, which can compromise membrane integrity by altering its fluidity and permeability [81]. No significant changes in malondialdehyde and 4-hydroxyalkenals (MDA and 4-HNE, respectively), common end-products of LPO [73,74], were detected in the gills across treatments or sampling times, even though a significant effect of time was detected by two-way ANOVA (Fig. 7A, Table S11). LPO in the digestive gland exhibited a significant temporal effect (Fig. 7B; Table S11), with only a minor increase observed in the TP group at day 28. These findings suggest that the antioxidant defence system in *M. edulis* was sufficiently effective to prevent the onset of lipid oxidative damage under the tested conditions. In particular, the glutathione (GSH)-dependent antioxidant system may have played a key role in maintaining redox homeostasis, counteracting ROS before they could propagate LPO. This is supported by the stable glutathione-related enzymatic activity observed across most treatments, as well as the limited oxidative damage to lipids. The minor elevation in MDA and 4-HNE levels observed in the digestive glands of TP-treated mussels may point to localized or transient oxidative challenges. Compounds such as 6PPDQ, that have accumulated within this tissue, have been shown to induce LPO in other organisms like *C. elegans* [86], supporting this hypothesis. Nonetheless, the absence of widespread LPO across tissues and treatments reinforces the notion that ROS generation was either not sufficiently elevated to induce oxidative damage or was efficiently neutralized by the antioxidant system. Moreover, similar studies have reported no significant alterations in LPO levels in the digestive glands of *M. galloprovincialis* exposed to tyre leachates originated from the same original material as that used in this study [64]. Likewise, no changes in LPO were observed in the gills of *S. plana* exposed to tyre particles (250–500 µm) [87]. Together, these findings align with the present results and further supporting the limited oxidative stress induced by these tyre-exposures.

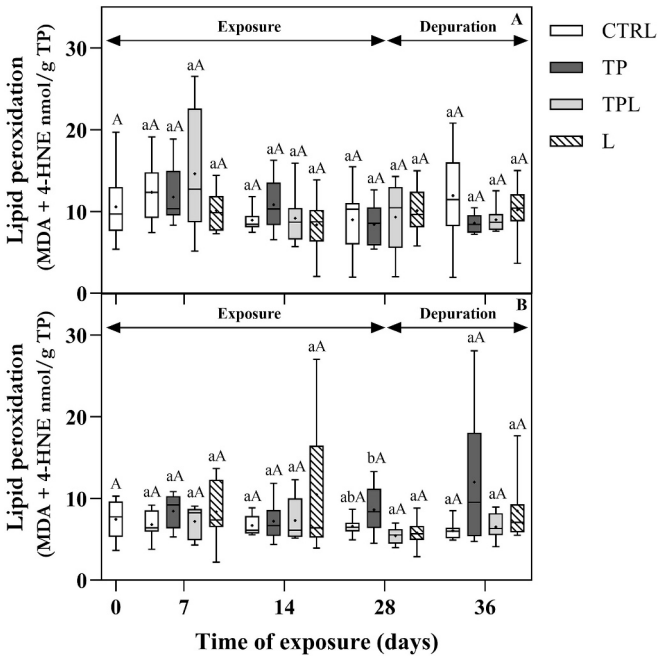

Fig. 7. Lipid peroxidation in gills (A) and digestive glands (B), expressed as malondialdehyde and 4-hydroxyalkenals (MDA and 4-HNE, respectively), in gills (A) and digestive glands (B) of mussels unexposed (CTRL – control) and exposed to tyre particles (TP, tyre particles; TPL, tyre particles after leaching) and corresponding leachates (L) for 0, 7, 14, 28 and 36 days. Results are presented as box-and-whisker plots ($n = 8$). The box represents the inter-quartile range (IQR), the median values are indicated with a central horizontal bar (−), the mean values are indicated with a plus (+) and the and whiskers represent the 5–95 percentile. Lower and capital letters represent statistical differences between treatments in each exposure day and for each treatment during the exposure duration, respectively ($p < 0.05$).

3.6. Principal component analysis (PCA)

A Principal Component Analysis (PCA) was conducted to explore the potential relationships between biochemical markers, trace metals, and organic additives in mussel gills (Fig. 8A) and digestive glands (Fig. 8B) following exposure to the various treatments (TP, TPL and L). In the gills, PC1 accounted for 47.5% of the variance and showed a clear separation between the exposure groups with higher (day 28) and lower chemical concentrations. On the other hand, PC2 explained 27.1% of the variance and showed a separation between the type of biomarker responses in the different treatments. In the digestive gland, PC1 accounted for 39.3% of the variance and differentiated groups based on chemical load, particularly separating the TP and TPL exposure groups at day 28 from all other treatments and timepoints. PC2 explained 26.8% of the variance and captured variation in biomarker responses, particularly the oxidative stress enzymes. Together, the PCA analyses of both tissues provided a comprehensive overview of the biological effects of the tested treatments and validated the patterns observed in the individual biochemical and chemical analyses. The PCA confirms that organic additives in the TP and TPL treatments at day 28 are the primary drivers of oxidative stress and neurotoxic responses, particularly in the gills, where elevated CAT, GR, and AChE inhibition were most strongly associated with these contaminants. In contrast, trace metal accumulation in the L treatment (especially at day 28) was more clearly linked to GPx and CAT responses, particularly in the digestive gland. The distinct clustering of depuration groups (especially L36) closer to controls suggests partial recovery, whereas TP and TPL depuration groups remained biochemically distinct, implying a longer-lasting impact of organic additives. The close clustering of control groups (CTRL D0, D28, and D36) underscored the stability of biochemical responses in the absence of contaminant exposure.

The multivariate patterns revealed by the PCA were further supported by specific statistically significant correlations that provided additional mechanistic insights into the different toxic modes of organic additives versus trace metals (Table S12 in the SI). In the gills, GR showed a positive correlation with HMMM, but a negative correlation with LPO. Zn was positively correlated with several elements, including Cu, As, Ni, Cr, Co, Cd, Pb, and the compound HMMM. DPG exhibited significant correlations with several of the other additives determined: it was positively associated with MBT, TMQ, HMMM, 6PPD, 6PPDQ, Cu, As, Ni, Cr, Co, and Pb. Additionally, DPPD showed positive correlations with TMQ, PhBT, MTBT and DPG. In the digestive glands, CAT was positively correlated with GPx, while GR was negatively correlated with

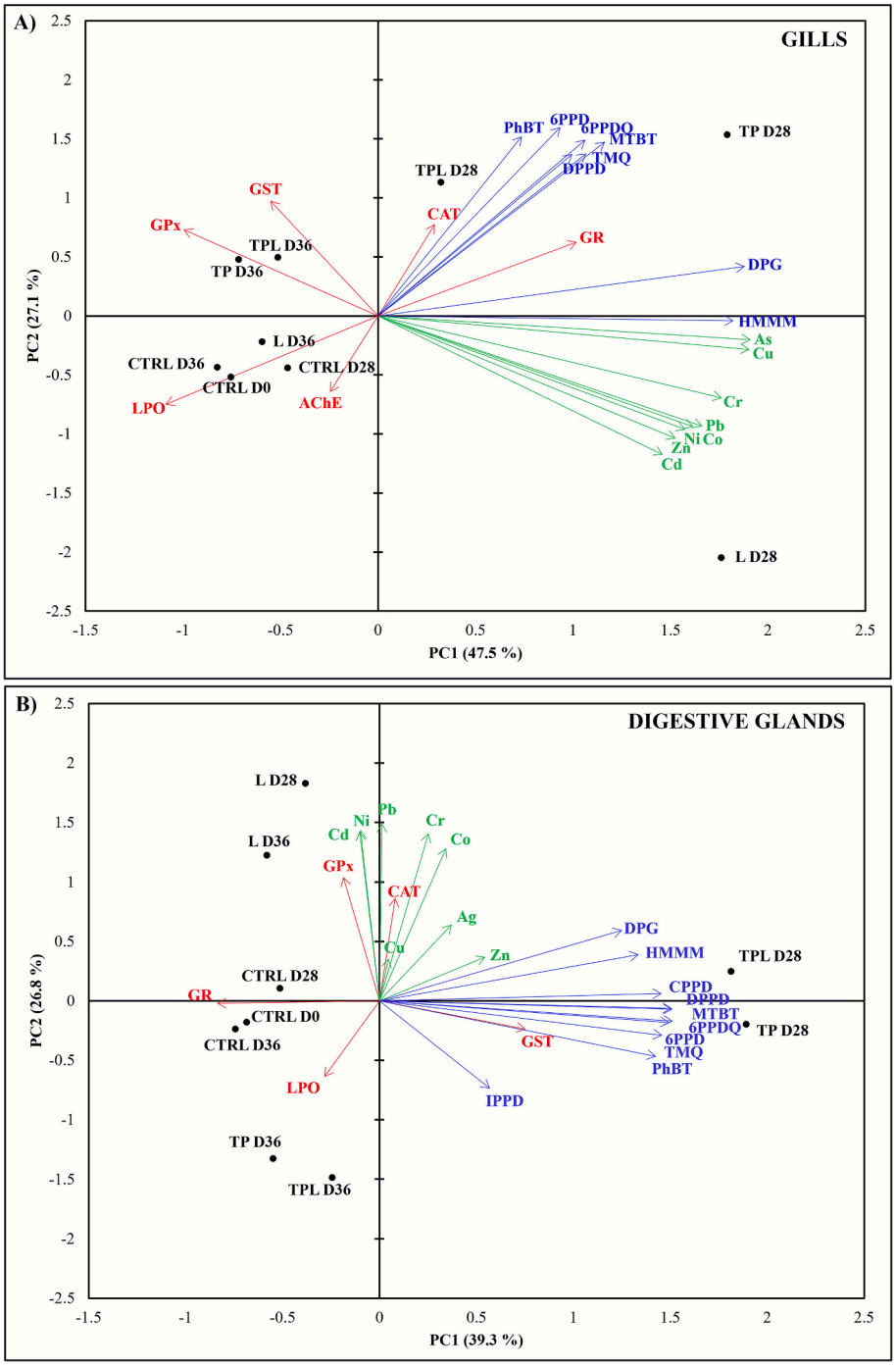

Fig. 8. Principal Component Analysis in gills (A) and digestive glands (B) of organic additives (blue) and metals (green) measurements and biomarkers (red) in gills in control mussels (CTRL) at day 0 (D0), 28 (D28) and 36 (D36) and in mussels exposed to tyre particles (TP), tyre particles after leaching (TPL) and tyre leachates (L) at day 28 and 36 (black). AChE – acetylcholinesterase activity; LPO – lipid peroxidation; CAT – catalase; GR – glutathione reductase; GPx – glutathione peroxidase; GST – glutathione-s-transferase; MTBT – metamercaptobenzothiazole; 6PPD – N-(1,3-dimethylbutyl)-*N*′-phenyl-*p*-phenylenediamine; 6PPDQ – N-(1,3-dimethylbutyl)-*N*′-phenyl-*p*-phenylenediamine- quinone; TMQ – poly(1,2-dihydro-2,2,4-trimethyl-quinoline); DPPD – diphenyl-*p*-phenylenediamine; DPG – 1,3-diphenylguanidine; PhBT – 2-(2-hydroxyphenyl)benzothiazole; HMMM – Hexa(methoxymethyl)melamine; As – Arsenic; Cd – Cadmium; Cr – Chromium; Cu – Copper; Pb – Lead; Ni – Nickel; Zn – Zinc; Co – Cobalt. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Zn. GPx showed a negative correlation with IPPD, and GST was negatively correlated with LPO. Zn was positively correlated with Cu and As, while Ni showed positive correlations with Cu, Co, Cr, and Pb. 6PPDQ was also positively correlated with a range of compounds, including TMQ, MTBT, PhBT, DPG, HMMM, DPPD, CPPD, and 6PPD. The correlations observed between organic additives and antioxidant enzymes in both tissues suggest that these chemicals trigger a coordinated oxidative stress response in mussels. In addition, several of these additives were also correlated, indicating shared distribution pathways or behaviour in mussel tissues. Overall, the PCA and correlation analysis showed that organic additives pose a greater and more persistent biochemical impact in mussels than metals, with tissue-specific profiles, reflecting their different physiological roles and exposure pathways. These insights underscore the importance of using a multi-biomarker and multi-tissue

approach when assessing the toxicity of complex contaminant mixtures in aquatic organisms.

3.7. Environmental relevance of Tyre pollution

A key challenge in ecotoxicological research related to tyre pollution is the lack of standardized, environmentally representative TRWPs. These particles are highly heterogeneous due to the incorporation of road dust, minerals and other environmental contaminants, altering their physical and chemical properties. Obtaining enough field-representative TRWPs for laboratory studies is difficult, leading researchers to use cryomilled tyre particles from ELTs as substitutes [79]. While these lack some of the environmental aging characteristics of TRWPs, they offer advantages in terms of reproducibility, size uniformity and dispersibility in aquatic solutions, enabling controlled studies on particle-chemical interactions and organismal responses [40,41]. The development of more standardized TRWP materials should remain a priority to improve environmental relevance and comparability across studies.

In this study, a key aspect of the experimental design was the deliberate separation of physical and chemical exposure pathways to allow for a mechanistic understanding of their respective roles in the overall observed toxicity. While tyre leachate exposure alone caused minimal accumulation of chemical additives in mussels, exposure to tyre particles significantly increased the concentrations of 6PPD, 6PPDQ and Zn in tissues. These results demonstrated that TPs not only act as contaminants but also serve as vectors that facilitate the internalization and bioavailability of associated chemicals. This dual exposure pathway, via both dissolved and particle-bound transport, is particularly relevant for benthic and filter-feeding organisms, which are in constant contact with suspended particles and sediments where TRWPs tend to accumulate. Biomarker responses provided additional insight into early biological effects in mussels, including inhibition of AChE activity, and altered antioxidant enzyme activities. These effects point to potential disruptions in immune function, neural activity, and redox balance. From an ecological perspective, such biochemical disturbances may translate into increased energetic costs associated with detoxification processes, altered filtration activity, and impaired physiological performance. Although the exposure in this study was short-term and sub-lethal, these changes could compromise growth, reproduction, survival, and ultimately population stability under chronic or repeated exposure scenarios that are more likely to occur in natural environments with continuous tyre particle input. In all, these results provided novel insight into the biological and ecological risks of tyre-derived pollutants and underscored the importance of considering the cumulative impacts of tyres and their associated chemical additives in ecological risk assessments. The occurrence of tyre particles in biota across geographically and anthropogenically diverse sites points to a widespread risk that could have broader ecosystem-level consequences, particularly in species-rich and ecologically sensitive coastal zones.

To accurately reflect environmentally relevant exposure scenarios, it is essential to consider both dissolved and particulate fractions in environmental monitoring, particularly in high-risk areas such as urban coastal areas and road runoff zones where TRWP levels are elevated. Recent Norwegian environmental monitoring has shown the presence of TP in blue mussels not only in urbanized areas like the inner Oslofjord, but also in remote locations such as Skallneset in the Varangerfjord, where the sources of contamination remain unclear [8]. This highlights the diffuse and persistent nature of tyre-derived pollution and reinforces the environmental relevance of laboratory studies using blue mussels. The exposure concentration of 0.1 g/L used in this study exceeds the typical levels of tyre additives in marine surface waters (e.g., benzothiazoles at 3–19 ng/L) but reflects environmentally relevant conditions observed in Norwegian coastal sediments and biota. For example, tyre particle concentrations up to 20 mg/g dry weight have been reported in sediments from the inner Oslofjord, and up to 60 mg/g in blue mussels from high-traffic or industrialized coastal areas [8,47]. While direct comparisons between solid-phase and waterborne concentrations are limited by partitioning behaviour, such data support the use of higher concentrations to model short-term peak exposure events near stormwater outfalls or harbours. Given that tyre particles readily settle in sediments, benthic organisms are particularly vulnerable to long-term exposure through both suspension feeding and direct particle uptake. The chosen concentration therefore ensured the detection of sub-lethal biological responses and provided further insights into toxicity pathways of tyre particles versus their chemical additives, while remaining consistent with exposure ranges applied in comparable experimental studies.

Nonetheless, this study also had some limitations. Only a single exposure concentration and short-term exposure were tested, and therefore additional dose-response and longer-term experiments would help contextualise the observed effects and identify potential thresholds, adaptation, or recovery patterns. Moreover, the chemical profiles and biomarker responses observed in this study have not yet been validated under field conditions. Extrapolation from controlled laboratory experiments to natural environments remains challenging due to the complexity of coastal systems, where mussels are exposed to diverse contaminant mixtures and tyre-derived particles and additives represent only one component of the overall environmental chemical cocktail. Approaches such as effect-directed analysis (EDA), targeted fractionation, and the testing of key transformation products would therefore be valuable next steps to identify compounds with higher toxic potential within tyre-derived mixtures. Molecular-level biomarkers, such as transcriptomics or metabolomics, were also not included in this study, which could further elucidate the mechanistic pathways underlying the observed biological responses in mussels. Integrating molecular approaches in future studies would improve understanding of upstream regulatory processes and strengthen causal links between chemical exposure, oxidative stress, and neurotoxicity. Future work should therefore aim to combine laboratory experiments with field monitoring of tyre markers and internal additive loads in mussel tissues, alongside biochemical and molecular endpoints, to assess how these laboratory-based patterns manifest under environmentally realistic exposure scenarios.

Overall, this study showed that both chemical and physical components of tyre pollution contributed to biological effects in marine organisms and must be jointly considered in environmental assessments. The mechanistic insights into exposure pathways, tissue-specific responses, and subcellular biomarkers contributed to a growing body of evidence useful for developing regulatory and mitigation strategies. As tyre wear pollution continues to increase globally, and with growing recognition of its environmental footprint in Norway and beyond, it is critical to improve our understanding of how these complex mixtures interact with marine life and ecosystems. Incorporating tyre-associated particles and additives into routine monitoring frameworks, as well as addressing current regulatory gaps for several chemical additives present in these particles, will be essential steps toward mitigating their long-term ecological impact.

4. Conclusions

This study demonstrates that mussels can internalize tyre particles and accumulate associated chemicals additives, confirming their role as effective sentinel species for monitoring micropollutants in coastal environments. Tissue-specific accumulation patterns revealed that particle and chemical uptake and retention are influenced by organ-level physiology. Tyre-derived additives such as TMQ, 6PPD and 6PPDQ accumulated at higher levels in mussels exposed to pristine and pre-leached particles compared to leachates alone, demonstrating clear differences between particle- and leachate-mediated exposure pathways. Although certain additives were efficiently removed during depuration, others persisted, indicating that some tyre-derived additives have the potential

for long-term bioaccumulation in marine organisms. Biomarker responses indicated cellular stress and neurotoxicity in mussels, likely driven by the combined effects of particles and associated chemical additives such as 6PPDQ. Linking internal chemical concentrations with biological responses in mussels helped clarify differences between particle- and leachate-mediated effects, with particle exposure resulting in stronger and more persistent responses than leachate exposure alone. These findings highlight the ecological relevance of particle-associated exposure pathways for benthic and filter-feeding organisms. Nevertheless, uncertainties remain regarding long-term organismal and ecosystem-level consequences, highlighting the importance of integrating laboratory experiments with field-based monitoring approaches. Future research should therefore focus on environmentally relevant exposure scenarios, including the complexity of tyre-associated chemical mixtures, longer-term or repeated exposures, and field validation, to better assess how laboratory-observed responses translate to organism-level performance and population-level consequences. Tyre particles may have greater biological effects than leachates alone, acting as concentrated and continuous sources of chemical additives. The continued leaching of chemical additives from retained particles, combined with their intrinsic properties and persistence in tissues, calls for more comprehensive environmental risk assessments and regulatory measures to improve understanding and management of tyre-derived pollution in coastal ecosystems.

CRediT authorship contribution statement

M. Elisabetta Michelangeli: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Steven Brooks:** Writing – review & editing, Validation, Investigation, Formal analysis. **Sebastian Kuehr:** Writing – review & editing, Validation, Investigation. **Emelie Forsman:** Writing – review & editing, Validation, Investigation. **Elisabeth S. Rødland:** Writing – review & editing, Validation, Investigation. **Sicco H. Brandsma:** Writing – review & editing, Validation, Investigation. **Maria Margalef:** Writing – review & editing, Validation, Investigation. **Manuel Heinzelmann:** Writing – review & editing, Validation, Investigation. **Davide Spanu:** Writing – review & editing, Validation, Investigation. **Jan Thomas Rundberget:** Writing – review & editing, Validation, Investigation. **Tânia Gomes:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

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.enceco.2026.01.003.

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