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Chemical leachates from car tyre granulates and PET bottles induce toxic effects on *Mytilus edulis* haemocytes

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ABSTRACT

Plastic materials contain hazardous chemicals, including additives like flame retardants, antioxidants, stabilizers, and metals, which can leach into the environment and impact aquatic life. This study is the first to evaluate the toxicity of leachates derived from car tyre granulates (CTG) and PET bottles (PET) on haemocytes of the mussel *Mytilus edulis* using a flow cytometry approach close to in vitro conditions. Following 24 h exposure, CTG leachates, characterized by a complex chemical profile (13,520 features) and high metal load, reduced cell viability, metabolic activity, mitochondrial reactive oxygen species (ROS) formation, lipid peroxidation (LPO) and lysosomal content. These leachates also impaired cytoplasmatic and mitochondrial membrane potentials, increased neutral lipids and altered DNA content. PET leachates, although with fewer chemical features (5631) and metal levels, also reduced cell viability, metabolic activity, LPO, and lysosome content, while increasing cytoplasmic membrane potential, ROS levels, NL, and altering MMP and DNA content. These findings indicated that leachates from CTG and PET can impair haemocytes functions in bivalves through mechanisms such as oxidative stress, membrane depolarization, and disrupted metabolic processes, underscoring their toxic potential. This study highlighted the toxicity pathways of plastic leachates in marine organisms, linking their complex chemical composition of organic and inorganic compounds to high ecotoxicological risks in environmental conditions.

1. Introduction

Tyre wear particles (TWPs) and end-of-life-tires (ELTs) are increasingly recognized as significant environmental sources of plastic pollution. TWPs are primarily generated during abrasion of tyres on road surfaces [1,2], whereas ELTs consist of worn tyres that are processed into 'crumb rubber' for reuse in various applications, such as outdoor artificial sports fields, playgrounds, general safety surfaces, trails and walkways. Environmental weathering events, such as stormwater and road runoff, facilitate the transport of these particles into aquatic ecosystems, contributing to their accumulation in the environment [3,4]. Siegfried et al. [5] estimated that tyre-derived particles can account up to 40 % of total microplastics (MPs) present in marine and freshwater ecosystems. ELTs further exacerbate this issue, with an estimated release of approximately half million tons of particles annually [6]. Research on MPs has predominantly focused on thermoplastic polymers such as polyethylene (PE), polystyrene (PS), polyethylene terephthalate (PET), polypropylene (PP) and polyvinyl chloride (PVC), often neglecting elastomers like rubber [2]. However, styrene butadiene rubber (SBR), butadiene rubber (BR) and natural rubber (NR) are the main polymer components used in tyres. Similar to plastic polymers, these elastomers are not readily degradable once introduced into the environment and can be persistent and accumulate in different environmental matrices over time [7,8]. Additionally, TWP and ELTs contain a broad range of chemical compounds including vulcanization agents, stabilizers, antioxidants, fillers, softeners, cross-linking agents, pigments, and oils. Müller et al. [9] identified up to 214 different organic chemicals in tyres, 145 of which were classified as leachable. Many of these compounds belong to the class of persistent organic pollutants (POPs), such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls

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(PCBs) and heavy metals. These chemical additives, many of which exhibiting carcinogenic, mutagenic and reprotoxic properties, are incorporated during manufacturing to modify or enhance plastic properties. However, since they are not covalently bound to the polymer matrix, they can leach into the environment under various conditions, posing potential risks to aquatic biota [10]. Among these leachable compounds benzothiazole (BHT), diphenylguanidine (DPG) and 6PPD-quinone (6PPDQ) have gained particular attention due to their widespread occurrence in the aquatic ecosystems and potential ecotoxicity on aquatic life [11].

Bivalve molluscs such as mussels (*Mytilus* spp.) have emerged as important bioindicator species in marine pollution monitoring. As filter-feeders, they possess high filtration capacity, which can result in the bioaccumulation of xenobiotics, such as plastic particles and associated chemicals [12,13]. The immune system of bivalves is primarily composed of haemocytes which circulate within the haemolymph and migrate to other tissues, such as connective tissues and epithelia [14]. Haemocytes play a crucial role in several physiological functions, including phagocytosis, reactive oxygen species (ROS) formation and detoxification processes [14–18]. Their sensitivity to environmental contaminants makes them a valuable indicator of organismal health, as both in vivo and in vitro studies have demonstrated haemocyte dysregulation in response to various pollutants [19,20]. In this context, in vitro assays using mussel haemocytes provide a powerful tool to evaluate contaminant toxicity and mechanisms of action [21]. Given their reproducibility and standardization, these assays are particularly relevant for environmental risk assessment, offering controlled conditions to investigate the biological impacts of pollutants. The current knowledge on the hazardous effects of chemical additives present in plastic leachates on bivalve haemocytes is still limited. Accordingly, this study aimed to assess the ecotoxicological impact of leachates derived from car tyre granulates (CTG) and PET bottles on *Mytilus edulis* haemocytes. Tyre wear particles are estimated to be the largest land-based source of MPs in the Norwegian environment, potentially leaching hazardous additives, and have also been recently detected at significantly high concentrations in blue mussels from the Oslofjord area [22]. Therefore, due to their widespread environmental presence, and potential for bioaccumulation and toxicity, tyre particles were selected for use in the experiments to assess the sublethal effects of their leachates. In addition to tyre particles leachates, we choose PET, a polymer known for having a lower additive content and being potentially less toxic, to compare the potential immune effects on haemocytes. In fact, PET is generally considered a safe polymer for human health, and it is among the most widely used packaging material in the beverage and food sector [23]. Furthermore, a previous screening study with Bacterial Luminescence 168 Toxicity (BLT) and marine microalgae growth inhibition *Skeletonema pseudocostatum* indicated that PET bottles extracts exhibited less toxicity compared to extracts from CTG or other rubber polymers [24]. PET is also the third most commonly polymer group found in mussels from coastal areas of Norway, highlighting its environmental relevance and potential for biological interactions [25].

The assessment focused on several biological parameters, including cell viability, reactive oxygen species (ROS) formation, cytoplasmatic and mitochondrial membrane potential, lipid peroxidation (LPO), neutral lipid content, metabolic activity, lysosome presence and DNA content. This was achieved through a reliable and reproducible methodology using flow cytometry (FCM). FCM is a powerful method that enables rapid quantitative data acquisition analysis on single cells in suspension [26,27]. This laser-based technique provides statistical accuracy, reproducibility, and sensitivity, allowing a simultaneous multiparametric analysis at cell-to-cell basis [28,29]. FCM has been recently applied in marine research to enhance the understanding of bivalve haemocyte structure, metabolic and functional characteristics, as well as immune-related functionalities associated with pathological or environmental stress [30–34]. In addition to the quantitative measurement of metals, a comprehensive organic chemical screening and analysis of the leachates was conducted to tentatively identify compounds potentially responsible for the observed effects. To the best of our knowledge, this is the first study to evaluate the impact of plastic and rubber leachates on bivalve haemocytes close to an in vitro state using a wide battery of biological endpoints.

2. Materials and methods

2.1. Test materials and leachate preparation

Car tyre granulates (CTG) (1–2.8 mm) were purchased from RagnSells (Norway). PET water bottles were purchased from a common shop in Norway. PET water bottles were further cryomilled and dry-sieved to a fraction <500 µm by CARAT GmbH (Germany). The characterization of the particles is detailed in Section S1 of the Supplementary Information.

Prior to use, all glassware was burned at 500 °C for 30 min in a muffle furnace. Natural seawater (NSW) used for leachate preparation was collected from a 60 m depth at NIVA's marine research station at Solbergstrand in the Oslo Fjord, Norway (59°36′55.5″N 10°39′04.2″E), and subsequently filter sterilized (0.22 µm Stericap™ PLUS). Leachate preparation and test concentrations were based on previous studies using car tyre particles, with minor modifications [6,35]. Leachates were prepared by diluting CTG and PET particles in natural seawater (NSW; 34 PSU, pH 8.012) into a 1 L amber glass bottle to achieve a concentration of 100 g/L (100 %), corresponding to a liquid solid ratio (L/S) of 1:10. This L/S ratio adheres to European standards for testing the leaching of waste materials with particle sizes less than 4 mm [36]. Samples were incubated in the dark, at 20 °C and shaken at 125 rpm for 14 days in a rotating incubator (Binder GmbH, Germany) to keep particles in suspension. The incubation period of 14 days has been shown to allow sufficient time for most plastic additives reach equilibrium [37]. After incubation, CTG particles were isolated by sterile filtration (0.22 µm Stericap™ PLUS), while PET particles underwent a two-step filtration, first through a glass-microfiber filter (GF/F; porosity 1.2 µm Whatman™), followed by a 0.22 µm filter to remove coarse particles. The resulting CTG and PET leachates were then divided in various aliquots for subsequent use and stored in the dark at −20 °C. Additional aliquots of the 100 % CTG and PET stock solutions were also stored at −20 °C for chemical analysis. For the exposure experiments, leachates were further diluted in NSW to reach the final concentrations of 0.1, 0.32, 1, 3.2, 10 and 32 %. The analysis of particle presence in the leachates can be found in Section S2 of the Supplementary Information.

2.2. Characterization of leachates

2.2.1. Analysis of organic additives

2.2.1.1. Sample extraction. CTG and PET leachate samples (100 g/L or 100 % leachate) were spiked with 100 µL of internal standard (IS) solution in methanol (Supplementary Information, Table S1) and extracted using solid-phase extraction (SPE) according to the method reported by Qiu et al. [38] with minor modifications. Briefly, hydrophilic-lipophilic balance (HLB) cartridges (Oasis® HLB, 150 mg, 6 mL, Waters Corporation) were conditioned and equilibrated using 6 mL methanol and 6 mL Mili-Q water (MQ) respectively. Subsequently, 30–100 mL of sample were loaded. Cartridges were washed with 6 mL MQ and dried under vacuum using a freeze dryer for 2 h. Plastic additives were eluted with 6 mL of methanol and 6 mL of ethyl acetate. Eluates were combined and brought to almost dryness using a nitrogen evaporator (TurboVap®, Biotage) at 30 °C, after which extracts were resuspended in 500 µL of 20 % methanol in MQ and kept at −20 °C until characterization using liquid chromatography tandem to high resolution mass spectrometry (LC-HRMS).

2.2.1.2. Liquid chromatography tandem high-resolution mass spectrometry (LC-HRMS) analysis of plastic additives. Chemical additives present in the CTG and PET leachates were analysed using the workflow developed by Jonkers et al. [39], with minor modifications. Briefly, extracts (5 µL) were injected with a 1290 Infinity HPLC system (Agilent Technologies) and the separation of the analytes was performed using a BEH C18 column (XBridge, 100 × 2.1 mm, 2.5 µm, Waters Corporation) set at 30 °C. The mobile phases consisted of MQ (A) and methanol (B), under a constant flow of 0.5 mL/min. Formic Acid (0.1 %) was used as modifier for the analysis in positive ionization mode, while 10 mM Ammonium Fluoride was used as modifier in the negative ionization mode analysis. The gradient was increased linearly from 10 % to 99 % B over 18 min, maintained at 99 % B for 7.5 min, then decreased to 10 % B in 0.5 min and re-equilibrated at 10 % B for 4 min. HRMS data were recorded on a Bruker Daltonics Compact II QTOF mass Spectrometer. Electrospray ionization (ESI) was used to ionize compounds in positive and negative mode. Precursor ions were selected for fragmentation based on data-dependent acquisition (DDA), i.e. masses were hierarchically selected based on signal intensity. Specific Instrument settings and MS/MS data acquisition are provided in the Supplementary Tables S2, S3. Mass measurements were calibrated by injecting a tuning mix at the beginning of each sample injection. Chemical features were extracted from the calibrated HRMS raw data using Metaboscape 4.0 (Bruker Daltonics). The detailed settings for feature extraction are included in Supplementary Table S4 and are similar to those used by Jonkers et al. [39]. The resulting features were annotated with Spectral Libraries (MassBank EU and MassBank North America). The Schymanski confidence levels of identification [40] were assigned using the Total Annotation Quality code (TAQ-code) [39]. Supplementary Table S5 shows the minimum annotation quality parameters for levels 2, and 3.

2.2.2. Inductively coupled plasma mass spectrometry (ICP-MS) metal analysis

CTG and PET 100 % leachate stocks were analysed for the presence of Al, Ti, Cr, Fe, Co, Ni, Cu, Zn, As, Ag, Cd, Sb, Hg and Pb using an Agilent Q-ICP-MS 7700. Prior to analysis, all samples were filtered (0.22 µm, CA 25 mm, VWR® Sterile Syringe Filter) and diluted 10 times with MQ water and acidified with ultrapure HNO_3 (65 %) to obtain a 1 % HNO_3 solution. Filtered natural seawater was diluted and acidified as described above and used as a blank to provide a matrix-matched calibration in the range from 0.1 to 50 µg/L. ICP-MS calibration standards were made from dilutions of commercial standard mixtures from Inorganic Ventures (Christiansburg, VA). A tap water CRM (CRM-TMWD -250, 2 % HNO_3, High-Purity Standards, US) was diluted with filtered natural seawater (0.22 µm) and ultrapure HNO_3 (65 %) to match the matrix and measured between samples allowing to monitor the quality of the measurements. Sc, Ge, In and Re were used as internal standards. All samples were measured in triplicate. After each sample measurement, the instrument was rinsed with a 10 % HNO_3/HCl solution followed by a 1 % HNO_3 rinse. All presented metal concentrations are blank corrected after subtracting the mean of the measurement blank from the values obtained from the samples.

2.3. Mussel collection, haemolymph withdrawal and haemocytes exposure

Mytilus edulis (5.5 ± 0.5 cm shell length) were collected from Sørskjell AS in Grimstad (Norway) and transported to NIVA's marine research station at Solbergstrand. Mussels were transferred to the laboratory and acclimatized for 2 weeks in a flow-through system at 16 °C and fed ad libitum with Shellfish Diet 1800® (Reed Mariculture), a commercial formulation of microalgae, comprising *Isochrysis* sp., *Pavlova* sp., *Tetraselmis* sp., *Chaetoceros calcitrans*, *Thalassiosira weissflogii* and *Thalassiosira pseudonana*.

Two separate exposures with slightly different concentrations of CTG and PET leachates using *Mytilus edulis* haemocytes were used in this study, with identical exposure set up and methodology. Haemocytes exposure was adapted from Sendra et al. [21] with some modifications. Briefly, haemolymph was aseptically withdrawn from the adductor muscle of 60 mussels using syringes with a 23G needle, pooled together and stored in sterile tubes on ice until further use. Cell density was analysed using a flow cytometer (Novocyte Advanteon Flow Cytometer Systems 1–3 Lasers, Agilent, USA) and then fixed at $4{\cdot}10^5$ cells/mL in NSW (0.22 µm filtration, salinity 35 p.s.u). Haemocytes were incubated in 24-multiwell plates with CTG and PET leachates in a 1:1 dilution to have a final haemocytes concentration of $2{\cdot}10^5$ cells/mL. Microplates were covered with plate sealers to avoid evaporation and cross-contamination. All experiments were carried in the dark and under shaking movement (80 rpm) in an incubator (BINDER GmbH, Germany) at 16 °C for 24 h. This study focused on short-term toxicity, a common approach in in vitro assays using primary cell cultures due to their limited viability [21]. Five replicates per concentration plus a seawater control were tested. A preliminary test was performed with concentrations ranging from 0.1 to 100 % leachate to assess cell viability and determine the sub-lethal leachate concentrations to use for a more comprehensive assessment of leachate toxicity. Based on this preliminary test, the concentrations of 0.32 %, 1 %, 3.2 %, and 10 % were selected for both leachates. These concentrations were chosen to assess a range of potential effects and build on previous research. Specifically, they ensure a worst-case scenario approach by testing higher concentrations that might represent peak exposure conditions, which are not always fully captured in environmental monitoring due to sampling and analytical limitations [41]. These concentrations also align with previous research on tyre leachates in different marine organism, including in vitro studies with fish cell lines [4,42–45]. Overall, the selection of these concentrations was driven by the need to ensure ecological relevance, explore a broad spectrum of responses, and maintain consistency with existing research. By using these concentrations, this study also accounts for potential underestimation of environmental concentrations and ensures that measurable effects can be detected in a controlled system. After 24 h exposure metabolic activity, cytoplasmatic and mitochondrial membrane potential, cytoplasmatic and mitochondrial ROS formation, LPO, DNA content, lysosome presence and neutral lipids content were assessed in haemocytes using FCM.

2.4. Flow cytometry analysis

Flow cytometry (FCM) analysis was performed with an Novocyte Advanteon Flow Cytometer Systems 1–3 Lasers (Agilent, USA), for which a 488 nm argon-ion laser was used as excitation source. All endpoints were assessed at a final volume of 1 mL and data was acquired using a threshold of 100.000 and 66 µL/min flow rate. Haemocytes were characterized based on cell size (Forward light Scatter, FSC) and complexity (Side light Scatter, SSC) properties. FCM data was analysed with the NovoExpress software (Agilent, USA) and fluorescence values were expressed as fold induction from the control (CT) using mean fluorescent values.

2.4.1. Cell viability

Propidium Iodide (PI, Invitrogen, ThermoFisher Scientific, Eugene, OR, USA) is a fluorogenic probe that diffuses through injured or dead cell membranes that once into the cytoplasm intercalates with nucleic acids to form a bright red fluorescent complex [46]. Haemocytes (990 µL cell suspension) were incubated with 10 µL PI (15 µM final concentration) for 10 min in the dark at room temperature. Resulting fluorescence was then analysed in the FL3 channel (488 nm excitation, 670 nm emission).

2.4.2. Metabolic activity

Fluorescein diacetate (FDA, Invitrogen, ThermoFisher Scientific, Eugene, OR, USA) was used to determine metabolic activity in exposed haemocytes. The FDA non-fluorescent lipophilic substrate can be

cleaved by a wide spectrum of extracellular enzymes and membrane bounded enzymes, such as protease, lipase, and esterase, producing fluorescent fluorescein that can be measured by FCM [27,47]. Haemocyte samples (1 mL) were incubated with 2 µL FDA (12.5 mM final concentration) for 15 min in the dark at room temperature. Subsequently, fluorescent fluorescein was analysed in the FL1 channel (488 nm excitation, 533/30 emission).

2.4.3. Cytoplasmatic and mitochondrial membrane potential

Mitochondrial and cytoplasmatic membrane potentials were evaluated using the fluorescent probes tetramethylrhodamine, methyl ester, perchlorate (TMRM; Invitrogen, ThermoFisher Scientific, Eugene, OR, USA) and Bis-(1,3-Dibutylbarbituric Acid)Trimethine Oxonol ($DiBAC_4(3)$; Invitrogen, ThermoFisher Scientific, Eugene, OR, USA), respectively. TMRM is a cell-permeant probe that accumulates in active mitochondria with intact membrane potential [29]. $DiBAC_4(3)$, on the other hand, can enter depolarized cells and bind to intracellular membranes [48]. 2 µL of TMRM (0.2 µM final concentration) and $DiBAC_4(3)$ (0.97 µM final concentration) were used to incubate haemocytes for 30 min in the dark at room temperature. After incubation, TMRM and $DiBAC_4(3)$ fluorescence were analysed in the FL2 (488 nm excitation, 585/40 emission) and FL1 (488 nm excitation, 533/30 emission) channels, respectively.

2.4.4. Lysosome presence and neutral lipids content

Lysosome presence was measured using the LysoTracker® Green DND-26 fluorescent probe (LT, Invitrogen, ThermoFisher Scientific, Eugene, OR, USA). LT permeates the cell membrane and accumulates within lysosomal compartments [49]. 1 mL of haemocytes suspension was incubated with 2 µL of this probe (75 nM final concentration) for 30 min, in the dark and at room temperature. Fluorescence was detected in the FL1 channel. BODIPY® 493/503(4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene Invitrogen, ThermoFisher Scientific, Eugene, OR, USA) is a cell-permeable lipophilic probe that stains neutral lipids. 2 µL of BODIPY 493/503 (5 mM final concentration) were used to stain haemocytes (1 mL). After 30 min incubation, in dark and at room temperature, fluorescence was recorded in the FL1 channel (488 nm excitation, 533/30 emission).

2.4.5. Cytoplasmatic and mitochondrial ROS activity

Cytoplasmatic and mitochondrial ROS formation were quantified by the fluorescent probes carboxy-2-,7-difluorodihydrofluorescein diacetate (H_2DFCDA, Invitrogen, Molecular Probes Inc., Eugene, OR, USA) and dihydrorhodamine123 (DHR 123, Invitrogen, Molecular Probes Inc., Eugene, OR, USA), respectively. H_2DFCDA and DHR 123 have structural similarities and are triggered by several oxidants such as H_2O_2, nitric oxide (NO), peroxynitrite ($ONOO^-$), hydroxyl ($\bullet OH$), O_2 and OH radicals [27,50]. Haemocyte samples (1 mL) were incubated with 2 µL of H_2DCFDA and DHR123 (5 µM, final concentration) for 1 h at room temperature in the dark. Fluorescence for both probes was recorded in the FL1 channel (488 nm excitation, 533/30 emission).

2.4.6. Lipid peroxidation

LPO was analysed by the 4,4- difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene- 3-undecanoic acid fluorescent probe, commonly known as C11-BODIPY581/591 probe (Invitrogen, ThermoFisher Scientific, Eugene, OR, USA). Due to its lipophilic properties, C11-BODIPY581/591 readily enters cellular membranes. Upon oxidation, this fluorescent probe remains lipophilic, ensuring that it remains embedded within the lipid bilayer [51]. Haemocytes (1 mL) were stained with 4 µL of C11-BODIPY581/591 (2.5 mM, final concentration), followed by incubation for 30 min at room temperature in the dark. Fluorescence was then detected using the FL1 channel (488 nm excitation, 533/30 emission).

2.4.7. DNA content

SYTO 13 (Invitrogen, ThermoFisher Scientific, Eugene, OR, USA) is a cell permeable probe with a high fluorescent yield when bound to DNA or RNA [52]. 2 µL of SYTO 13 (1 µM, final concentration) were used to stain haemocytes (1 mL) for 10 min in the dark at room temperature and the resulting fluorescence was analysed using the FL1 channel (488 nm excitation, 533/30 emission).

2.5. Data analysis

All FCM experimental data from both experiments are expressed as fold induction from the control (CT) from mean fluorescence values. Statistical analyses were performed using XLStat2022® software (Addinsoft, Paris, France). Data was first tested for normality (Shapiro-Wilk) and homogeneity of variances (Levene's test), after which the parametric one-way ANOVA or the non-parametric Kruskall-Wallis test were used to check for significant differences between the control and the CTG and PET leachates concentrations. Multiple comparisons were then performed using the Tukey or the Dunn's tests for parametric and non-parametric analyses, respectively. Statistical significance was set at $p < 0.05$. GraphPad Prism 9 software (GraphPad Software Inc., La Jolla, CA, USA) was used fror graphical representations, with data presented using box-and-whiskers plots. For particle size and morphology data, all comp images of projection particles from the QICPIC imaging system were processed with the QICPIC Dynamic Image Analysis application software (Sympatec GmBH), and size and morphology measurements were exported and further analysed in R software (version 4.4.2) [53].

3. Results and discussion

3.1. Leachates characterization

3.1.1. Organic additives content

Using suspect and non-target screening (SS/NTS) with LC-HMRS, a total of 13,520 and 5631 chemical features were tentatively identified in CTG and PET leachates (100 % or 100 g/L), respectively. Considering that the minimum peak intensity was set at 10000 for CTG and 3000 for PET, the differences would be even more pronounced when applying the same peak intensity threshold. In total, 62 organic additives were tentatively identified in CTG, compared to only 7 in PET, with confidence levels of 2a or 3 (Supplementary Tables S6 and S7). These compounds were classified according to the PlastChem database [54], based on criteria such as persistence, bioaccumulation, mobility and toxicity (Fig. 1). Chemicals were classified as: hazardous, less hazardous, not hazardous, regulated under existing multilateral environmental agreements (MEAs), or no hazardous information available.

As expected, the SS/NTS results highlighted the significant differences in the chemical complexity of the two leachates. A higher number of chemical features and tentatively identified compounds (62) were detected in CTG leachates compared to PET leachates (7), suggesting a more complex composition in terms of organic additive content. Several hazardous chemicals tentatively identified in the CTG leachates are listed in the PlastChem database, including benzothiazole, hexa (methoxymethyl)melamine (HMMM), N-(1,3-Dimethylbutyl)-N'-phenyl-*p*-phenylenediamine (6PPD), 6PPD-quinone, 1,3-Diphenylguanidine (DGP), ditolylguanidine tributylamine, diphenylamine and cyclohexylamine, N,N-dibutylformamide. These chemicals have been previously detected in tyre particles and rubber materials, highlighting the presence of potentially harmful chemicals in these leachates. Benzothiazoles (BTHs) represent a class of heterocyclic aromatic compounds extensively used as vulcanization accelerators in tyre industry [55]. Designated as high production volume chemicals (HPVC) [56] these compounds have emerged as contaminants of environmental concern with limited understanding of their ecological impact [57]. Studies have shown BTHs to be neurotoxic towards e.g. sheepshead minnow larvae [55] as well as to induce cytotoxicity, oxidative stress,

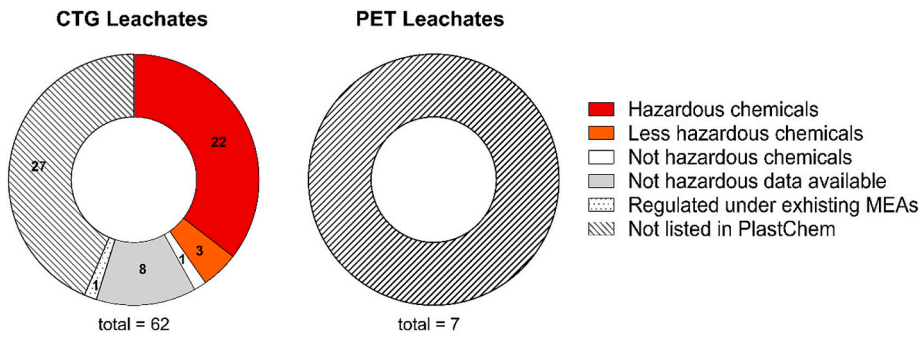

Fig. 1. Organic additives content of car tyre granulates (CTG) and PET bottles (PET) leachates classified according to the PlastChem database. Hazardous chemicals (red), less hazardous chemicals (orange), not hazardous chemicals (white), plastic chemicals without hazard information (grey), regulated under existing multilateral environmental agreements (dotted white), chemicals not listed in PubChem (white stripped). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and activation of cytochrome P4501A in rainbow trout cell lines [58]. Hexa(methoxymethyl)melamine (HMMM) is a tyre bonding and cross-linking agent that was identified in urban runoff, rivers, surface waters, creek water samples, tyre and road dust, tyre particles and tyre leachates [59–63]. Amines are among the most common type of antioxidants used in tyre industry to prevent cracking and degradation of rubber during wear [64]. Among the several cyclic amines tentatively detected in CTG leachates that are within the hazardous list, *N*-phenyl-N'-(1,3-dimethylbutyl)-*p*-phenylenediamine (6PPD), 6PPD-quinone (6PPDQ), tributylamine (TBA) and cyclohexylamine, dicyclohexylamine have been previously reported in crumb rubber and road dust, urban creek, wastewater treatment plants (WWTPs), surface water, tyre particles and their leachates [9,44,61,63,65,66]. From these amines, 6PPD is a common antioxidant agent used in synthetic rubber production that under environmental conditions is oxidized into his by-product 6PPD-quinone (6PPDQ) [67]. 6PPD and 6PPDQ have recently gained attention as emerging pollutants due to their widespread presence in various ecosystems and their harmful effects on organisms [68]. Recent studies have identified 6PPDQ to be among the tyre compounds associated with mortality in coho salmon [69,70], mostly due to disruptions in the osmotic balance of fish blood cells [69]. DPG is commonly incorporated in rubber and other polymers during manufacturing processes as an accelerator [71]. DPG is a polar compound, reported to have the potential to bioaccumulate within organism's tissues and interact with cellular components [64]. Another hazardous amine tentatively identified in CTG leachates is 2-Napthylamine (2-NA). Although widely used in the rubber industry as an antioxidant for several decades, its large-scale production has been banned in the European Union since 1998. 2-NA is classified as carcinogenic and hazardous to the aquatic environment, and it is listed in Annex XVII of the REACH Regulation, which restricts the use of certain hazardous substances [72]. Many of the additives tentatively detected in the CTG leachates are currently under evaluation for their hazardous potential. Some examples are dicyclohexylurea, 2-Hydroxybenzothiazole, 1-Phehylurea, phthalic acid and 3,5-Ditert-butyl-4-hydroxybenzaldehyde. Dicycohexylurea was recently recognized as a tyre-derived related contaminant [9] and has been detected in surface water, agricultural watersheds, urban creeks and WWTPs [61–63]. 1-Phenylurea plays a crucial role as an intermediate compound in the production of herbicides [73] and has been listed as a contaminant of concern due to its high toxicity and potential carcinogenicity in the USA, UK, Australia, and Brazil [74].

As expected, PET leachates contained a lower number of chemical features than CTG. PET is the most commonly used polymer for food and water packaging due to its high chemical inertness and low additive-content [75]. While the European Commission regulation No. 10/2011 (consolidated version: 31/08/2023) restricted or banned the use of certain compounds in food-contact materials based on their toxicity, potentially hazardous chemicals can still be introduced during manufacturing and recycling processes [23]. These potential chemicals include polymer additives, catalyst residues, degradation products, polymerization by-products, or residual monomers [76]. Therefore, the compounds identified in PET leachates in this study may have originated from these sources. Previous studies have detected endocrine-disrupting chemicals such as phthalates and bisphenol A, as well as carbonyl compounds such as formaldehyde, acetaldehyde and benzaldehyde in PET bottles [23]. In this study, steroid hormones have been tentatively identified in these samples with a confidence level of identification 3 [40]. However, since these chemicals have not been confirmed with reference analytical standards, it cannot be discarded that these features belong to other chemical families (which could have a hormone-like structure).

3.1.2. Metal analysis

Metal-based additives have a wide range of applications in polymer manufacturing, such as fillers, inorganic pigments for coloration, stabilizers, biocides, antimicrobial agents, flame retardants, and lubricants [77,78]. Trace metal concentrations obtained for CTG and PET leachates (100 %, 100 g/L) are reported in Table S8. All the metals quantified in the leachates had higher concentrations compared to the SW control (see Supplementary Table S8). Metal concentrations in CTG leachates ranked from Zn > Cu > Co > Ni > Cd ≈ Sb, while for PET leachates ranked from Zn > Sb > Cu > Ni > Co ≈ Cd. Overall, CTG leachates had higher metal concentrations, except for Sb and Ni, which were higher in the PET leachates. Zn was the most abundant metal in both leachates, with concentrations of 10,968. ± 372 μg/L (~11 mg/L) and 171 ± 67 μg/L for CTG and PET, respectively. Zn is widely used as a catalyst in the vulcanization process of tyre rubber and represents approximately 1–2 % of tyre weight [79]. Zn compounds also function as stabilizers to prevent discoloration, degradation and as lubricants [78]. According to the Norwegian Environmental Agency (Miljødirektoratet of Norway, 2020), Zn levels >60 μg/L in coastal waters are considered to cause toxic effects. Zn concentrations observed in leachates were substantially higher than this toxicity threshold, indicating a strong potential for adverse effects towards haemocytes. Additionally, the Zn concentration in CTG leachates observed in this study is consistent with that found by Capolupo et al. [6] (5.14 ± 1128 mg/L) and by Halsband et al. [4] (8.4 mg/L). Both studies used the same tyre granulate particles as those in this study, but with slightly differences in the methodology used to produce the leachates, such as rpm and temperature. Furthermore, the presence of Zn in PET leachates is in accordance with Capolupo et al. [42] which reported Zn levels of 51 ± 3 μg/L in leachates originated from recycled PET materials (< 1000 μm). This concentration is about half of what was observed in the current study, suggesting that smaller PET particles may release more chemicals due to their higher surface area. Overall, these findings highlight the importance of using standardized leaching protocols to ensure comparability between studies. In

addition, differences in metal detection were also seen when comparing the results obtained in all studies, such as the absence of lead (Pb) in the present study, further highlighting potential variability in chemical additive profiles of leachates due to measurement techniques.

Another prevalent metal in both leachates was Cu, which is commonly employed as a biocide in plastic materials [78]. Cu levels found in CTG and PET leachates were 48 µg/L and 10 µg/L, respectively. These values exceed the tolerance range for Cu in coastal waters in Norway (0.3 and 2.6 µg/L), being also considered hazardous to organisms [80]. In contrast, Ni levels (2.0 ± 0.2 µg/L and 8.5 ± 0.5 µg/L, for CTG and PET leachates, respectively) were within safe limits of 0.5 to 8.6 µg/L [80]. The Sb concentration found in PET leachates (27 µg/L) was 20 times higher to that of CTG (0.7 µg/L). Sb presence in PET leachates was not surprising, as antimony trioxide (Sb2O3) is employed as a polycondensation catalyst in PET production [76]. Additionally, the release of Sb from PET bottles/packaging has been previously reported in several studies [76,81–83] No tolerance levels exist for this metal in coastal or marine ecosystems, however, Sb concentrations in PET leachates exceed the interim freshwater quality guideline for Sb(III) (9 µg/L) set by Australia and New Zealand and the maximum admissible concentration for drinking water (5 µg/L) set by the European Union [84]. Even though these quality criteria are not directly applicable to marine waters, it shows that the levels obtained for this metal have the potential to be hazardous towards organisms. The presence of other trace elements such as Cd was also not surprising, as various Cd soaps are used as heat, light stabilizers and antioxidants in plastic materials [78]. Nonetheless, this was one of the metals found in lower concentrations in both leachates.

3.2. Identification of haemocytes populations by flow cytometry

Mussels' haemocytes are one of the most debated topics encountered by researchers, mainly due to the different criteria adopted in their classification [17,85]. Based on FCM analysis, two main distinct haemocyte subpopulations were identified and designed as P1 and P2 (Supplementary Information, Figure S6 A, B) according to size (FSC) and forward (SSC) scatter. P1 appeared to be the largest population with a higher internal complexity and therefore designated as granulocytes (GR). P2 was characterized by its smaller size and intermediate internal complexity and identified as hyalinocytes (HY). GR cells are characterized by the presence of granules in their cytoplasm and typically exhibit a low nucleus-to-cytoplasm (N/C) ratio. In contrast, HY, also known as agranulocytes, have few or no granules in their cytoplasm and display a higher N/C ratio [86]. The haemocyte classification used in this study goes in line with what can be found in literature, where several studies have identified and validated the presence of these two haemocyte types in *Mytilus* spp. [15,87,88].

3.3. Leachates effects on cell viability

Cell viability was assessed using the fluorescent probe PI on *M. edulis* haemocytes. Since PI selectively enters cells with damaged membrane [89], a first assessment was performed to check the health status of the overall haemocytes' population after 24 h exposure to CTG and PET leachates. The goal of this initial approach was to identify the concentrations at which these leachates induced sublethal cellular responses for use in a more comprehensive toxicity testing. Exposure to CTG leachates for 24 h increased the number of non-viable cells at the two highest concentrations tested, 32 and 100 % (higher 2.2-fold and 2.6-fold compared to control, respectively) while no significant differences were seen for the other concentrations (Fig. 2A). On the other hand, no significant differences in viability were observed in haemocytes exposed to PET leachates comparatively to the control (Fig. 2B). These findings suggest that cell viability is a sensitive parameter in response to chemical additive mixtures, indicating that the compounds present in CTG leachates, such as heterocyclic aromatic compounds and heavy metals, are toxic to haemocytes and can led to a decrease in cell viability. This is in accordance with previous in vitro studies, that demonstrated that heavy metals such as Cd and Zn, as well as organic aromatic compounds (e.g. benzo(*a*)pyrene), can decrease the cell viability of bivalves' haemocytes through various mechanisms, such as oxidative stress, membrane damage, and interference with cellular processes such as phagocytosis [90–92]. Overall, the higher leachates concentrations (32 % and 100 %) significantly reduced cell survival, therefore, lower concentrations (0.32, 1, 3.2 and 10 %) were selected to assess sublethal effects on different haemocytes subpopulations.

3.4. Leachates sublethal effects on haemocytes subpopulations

3.4.1. Metabolic activity

The metabolic activity of haemocytes exposed to CTG and PET leachates was detected using the fluorescent probe FDA, which is cleaved by non-specific esterases produced by metabolically active cells [93]. Esterases, which are hydrolytic enzymes, play a crucial role in defence functions. Consequently, alterations in their activity have been widely used as a biomarker to assess the effects of a wide range of contaminants in bivalves (e.g. organic pollutants, heavy metals, MPs and nanoparticles) [89,94–96]. In particular, a reduction in esterase activity is recognized as a sign of compromised immune functionality, further emphasizing its relevance in ecotoxicological studies [97]. In this study, CTG leachates significantly reduced the metabolic activity of P1 at the highest concentrations tested of 3.2 and 10 % (maximum 1.7-fold lower than the control). On the other hand, only small fluctuations in metabolic activity were recorded for P2, with 1 % of leachate causing a significant small increase (1.1-fold higher than control) (Fig. 3A). For

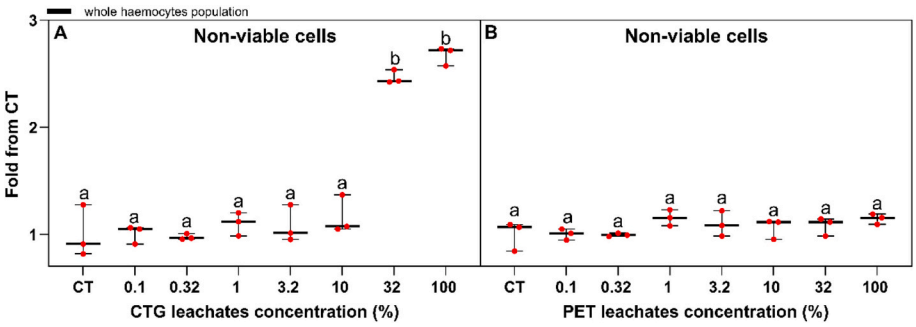

Fig. 2. Cell viability of total *Mytilus edulis* haemocyte population after 24 h exposure to 0.1, 0.32, 1, 3.2, 10, 32 and 100 % leachate originated from car tyre granulates (CTG) and PET bottles (PET). Results are expressed as fold induction from control (CT) and are presented as box-and-whiskers plots ($n = 3$). The median values are indicated with a central horizontal bar (−), and the mean values are indicated with a plus (+). Whiskers represent the minimum and maximum values, and dots indicate all individual data points. Letters represent statistical differences between the control and leachate concentrations, with different letters indicating significant differences ($p < 0.05$).

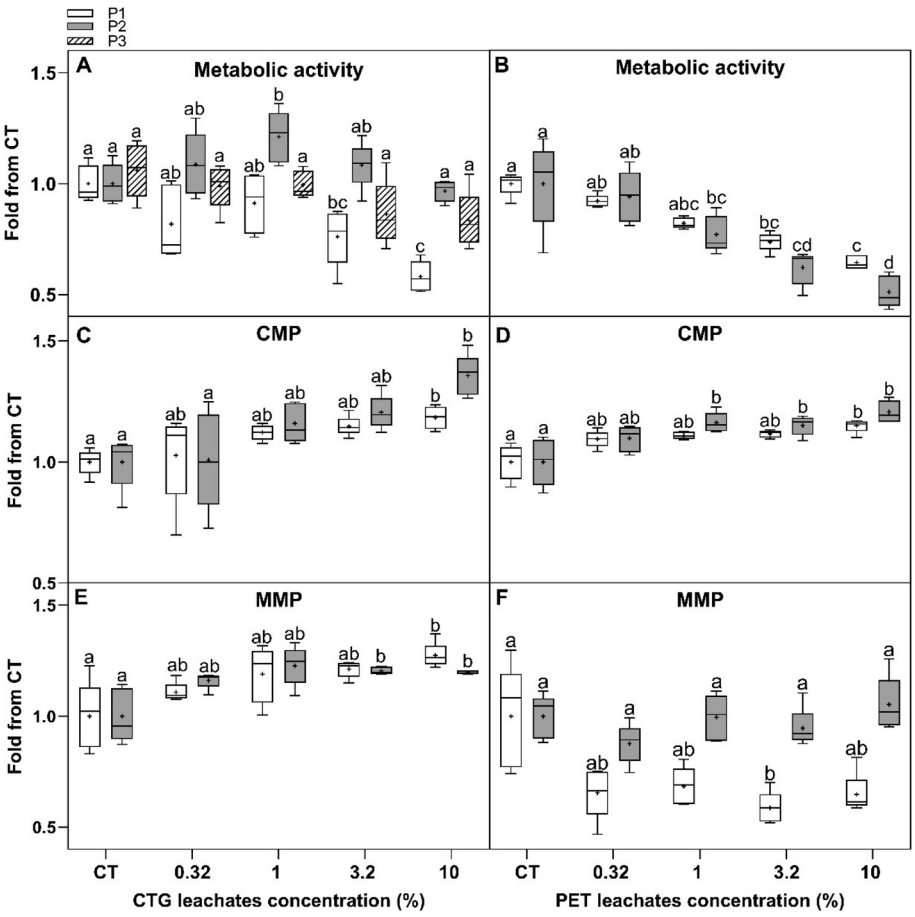

Fig. 3. Metabolic activity, cytoplasmatic membrane potential (CMP) and mitochondrial membrane potential (MMP) of *Mytilus edulis* haemocytes P1, P2 and P3 subpopulations after 24 h exposure to 0.32, 1, 3.2 and 10 % leachates originated from car tyre granulates (CTG) and PET bottles. Results are expressed as fold induction from control (CT) and are presented as box-and-whisker plots ($n = 5$). 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. Letters represent statistical differences between the control and the different leachates concentrations, with different letters indicating significant differences ($p < 0.05$). Note: The P3 subpopulation was not considered for PET leachates because it did not appear in the haemocyte subpopulations exposed to PET.

haemocytes exposed to PET leachates, a significant reduction in the metabolic activity on both P1 and P2 was recorded at concentrations higher than 1 % (maximum 1.6-fold and 2-fold lower than the control, respectively) (Fig. 3B). The general decrease in metabolic activity recorded for haemocytes exposed to CTG and PET leachates seems to reflect a lower FDA uptake in cells. A decrease in FDA fluorescence typically indicates reduced cell activity, decreased membrane permeability and esterase activity, as the fluorescence product generated by this probe can only be enzymatically processed by esterases in living cells [98]. These findings align with observations of esterase inhibition in *Crassostrea gigas* haemocytes in the presence of organic compounds [94]. Particularly, cyclic amines such as DPG and benzothiazoles were previously reported to reduce the metabolic activity of rainbow trout cell lines exposed to tyre leachates [44]. Similarly, esterase activity was also inhibited by Cd and Cu in soft tissues of *Littorina littorea* [99]. On the other hand, the increased metabolic activity observed in P2 exposed to CTG leachates is consistent with the findings by Mottin et al. [100], which demonstrated that in vitro exposure of *Haliotis tuberculata* haemocytes to Zn enhanced metabolic activity. It has been suggested that Zn, as an essential metal, is necessary for the function of numerous enzymes and may stimulate the metabolic activity of haemocytes [100]. In this study, the mixture of organic chemicals and other non-essential metals found in CTG and PET leachates might have contributed to the toxicity observed for both subpopulations. Additionally, the difference responses observed between P1 and P2 suggest that their metabolic activity varied, potentially due to the distinct complexity and functions of these cells. This is in accordance with Wang et al. [101], that observed different metabolic basal activity in *Perna viridis* granulocytes and hyalinocytes. This differentiation was further supported by de la Ballina et al. [85] which reported different proteomic profiles related to metabolism in GR and HY of *Ostrea edulis*.

Surprisingly, a third subpopulation (P3) with lower complexity compared to P1 and P2, but a similar size to P2, was detected in the control and in haemocytes exposed to CTG leachates (Supplementary Information, Fig. S8). This sub-population, however, did not show alterations in metabolic activity at any concentration tested (Fig. 3A). P3 presented characteristics similar to blast-like cells (BL), which generally have lower biological activity and are not directly involved in cellular immune responses [33,102]. These features are typical of undifferentiated cells, suggesting that P3 might be haemocytes precursors [14]. The presence of this subpopulation in control samples suggests that these cells were possibly in a differentiation state, potentially transitioning into more complex cells, such as hyalinocytes, given their similar size. In addition, the fact that P3 was only detected using FDA staining suggests that these cells initiated some form of metabolic activity, further supporting their involvement in a differentiation process. The presence of blast-like cells has been previously detected in the haemolymph of other bivalve species as *M. coruscus* [103], *Saccostrea glomerate* [104], *Saccostrea kegaki*, *Ostrea circumpicta*, *Hyotissa hyotis* [105], *Dreissena polymorpha* [33] and *Tapes philippinarum* [106]. When comparing the flow cytometry cytograms of *Crassostrea ariakensis* [32] and *Perna canaliculus* [107] with those from the present study, the population identified as BL

appears to be the smallest and less complex population. In those two studies, this population exhibited lower SSC values and some overlap with HY in terms of FSC. Based on these similarities, it is then plausible to suggest that the P3 population in this study corresponds to BL cells.

3.4.2. Cytoplasmatic and mitochondrial membrane potential

The cell membrane act as a barrier from the outside cellular environment and plays multiple functions in energy storage, signalling and compartmentalization. The balance between extra- and intra-cellular ionic concentrations is denominated as membrane potential, which reflects the living status of cells and is responsible for the maintenance of cell integrity and function [108]. Membrane-level responses are a critical component of immunotoxicity in bivalve haemocytes. These responses involve changes to the cell membrane and associated processes, which can influence cellular function, signalling pathways, and overall immune responses [109,110]. The accumulation of the fluorescent probe $DiBAC_4(3)$ is a sensitive marker of alterations in membrane potential which might be due to an inhibition of pump/leak balance, blockage of channels or generation of ionic leaks [111]. In this study, a significant increase in the CMP measured using $DiBAC_4(3)$ in both P1 and P2 exposed to CTG leachates was observed only at the highest concentration tested of 10 % (1.2-fold, and 1.4-fold, respectively) (Fig. 3C). A similar trend was detected in P1 exposed to PET leachates, with 1.1-fold higher CMP values at 10 % compared with the control. For P2, increased CMP was reported at concentrations higher than 1 % (1.2-fold) (Fig. 3D) suggesting a greater sensitivity of HY to PET leachates. Since hyalinocytes are less complex, they may be more vulnerable to external stressors. Additionally, the less complex chemical composition of PET leachates compared to CTG leachates may allow trace elements, such as metals, to interact more directly with cellular components, including membrane pumps [112]. The overall increased $DiBAC_4(3)$ uptake seen in exposed haemocyte subpopulations, indicated by higher fluorescence intensity, seems to point to membrane disturbance due to depolarization [113]. Similar effects have been previously reported in microalgae exposed to Bisphenol A, a common antioxidant, flame retardant and plasticizer used in plastic production [111]. Furthermore, the increase in membrane potential observed in this study is in accordance with the reduction in cell viability and metabolic activity observed in exposed haemocytes. Since PI staining depends on membrane integrity, the decrease in cell viability observed suggests membrane damage, which may contribute to increased membrane depolarization and subsequent metabolic disturbances [114].

Mitochondria are responsible for ATP production and are involved in metabolic processes, apoptosis, and ion homeostasis. Due to their critical function, they are often considered targets for pollutant-induced toxicity [115,116]. In the present study, TMRM, which is sequestered to active mitochondria, was used to assess the MMP in *M. edulis* haemocytes. CTG leachates caused a significant increase in MMP of both P1 and P2 at 10 % and 3.2 % leachate concentration (1.3-fold and 1.2-fold higher than CT, respectively) (Fig. 3E). On the other hand, a decreasing trend was detected in subpopulation P1 exposed to PET leachates with a significant difference only at 3.2 % (1.7-fold lower than the control). No significant differences were reported for P2 (Fig. 3F). The different mitochondrial membrane potential profiles observed for haemocytes exposed to CTG and PET leachates may be related to the varying profiles and concentrations of additives present in the two leachates. The increased MMP in haemocytes exposed to CTG leachates aligns with studies reporting increasing MMP values in Perna canaliculus haemocytes exposed to Cu^{2+} [117]. Furthermore, high concentration of 6PPDQ (400 μg/L) induced high MMP levels in the marine algae *Chlorella vulgaris* [118]. A possible mechanism for the MMP increase observed in haemocytes exposed to CTG leachates is consistent with previous studies, which reported that a rise in MMP levels typically precedes the activation of caspases, phosphatidylserine externalization, and subsequent disruption of the mitochondrial membrane potential, indicating the activation of the mitochondrial apoptotic pathway [119]. Conversely, the decreasing trend in MMP observed for PET exposure might resemble the effects of heavy metals such as Zn, which has shown to reduce MMP in *Drosophila melanogaster* haemocytes [120] and plasticizers as Di(2-ethylhexyl) phthalate (DEHP) in mice oocytes [121]. Additionally, it was previously observed that a decrease in MMP and an increase in ROS, as seen in this study, were associated with mitochondrial disorders linked to dysfunctions in the respiratory chain components [122]. In general, a reduction in mitochondrial membrane potential can trigger the release of pro-apoptotic factors, leading to the initiation of cellular apoptosis [118], suggesting that the complex mixture of organics and metals of both CTG and PET bottles are potentially involved in apoptosis pathways activation mechanisms.

3.4.3. Lysosome presence and neutral lipid content

Lysosomes are intra-cytoplasmatic acidic organelles that play a key role in cellular detoxification and defence [102]. These intracellular compartments contain various hydrolases which are involved in the intracellular degradation of ingested foreign material. In addition, lysosomes are known to accumulate a wide range of chemicals, including aromatic hydrocarbons and aminoazobenzene derived compounds [123]. In bivalve molluscs, haemocytes play a crucial role in cell-mediated immunity by engaging in various cytotoxic responses, including the release of lysosomal enzymes [124]. In the present study, haemocytes subpopulations P1 and P2 exposed to CTG leachates had lower lysosome content compared to the controls at all concentrations tested (lower 2.3-fold compared to CT at 0.32 %) (Fig. 4A). A similar trend was also detected for PET leachates, with higher effects at 3.2 and 10 % (1.6-fold lower than the CT at 3.2 %) (Fig. 4B). The reduced lysosome content observed in exposed haemocytes points to potential lysosomal membrane damage. This is supported by other studies, where lysosomal dysfunction was reported in mussel haemocytes exposed to increasing concentrations of tyre leachates (0.6–100 %) [6]. Additionally, certain chemicals tentatively identified in CTG leachates such as benzothiazoles, are known to possess metal-chelating properties that can disrupt membrane-associated processes and impair the activity of lysosomal hydrolases [125]. A reduction in lysosome content was also reported in *Crassostrea gigas* in response to organic contaminants such as polycyclic aromatic hydrocarbons (PAHs) [94] while metals such as Zn and Pb induced lysosomal alterations in haemocytes of *Dreissena polymorpha* [126]. Overall, these findings suggest that the presence of a various range of heterocyclic compounds (e.g., benzothiazoles and aromatic amines) and heavy metals in CTG leachates may have accounted for the observed toxicity in haemocytes. However, there is no specific information available regarding the toxicity of the organic compounds detected in PET, however we cannot exclude the presence of hazardous organic compounds in these samples. These findings, along with the high presence of metals, may provide a possible explanation for the reduced lysosomal content observed in the haemocytes exposed to PET leachates in the current study.

NL content is recognized as a biomarker of general stress, as neutral lipids represent an important intracellular energy storage which can be mobilized by cells to support immune responses during cellular defence [107]. In this study, a significant but small increase in the NL content was recorded for P1 at the highest CTG leachate concentration (10 %, 1.2-fold above the control), while no differences were seen for P2 (Fig. 4C). A fourth population (P4) with similar size, but higher complexity compared to P2 and smaller size and some degree of overlapping complexity with P1 was detected in haemocytes exposed to CTG leachates (Figure S9). P4 exhibited significantly higher NL values at 3.2 and 10 % compared to the control (1.4-fold and 1.6-fold, respectively) (Fig. 4C). As this fourth subpopulation showed high internal complexity, it is probably composed of smaller granulocytes with higher NL content. It has previously observed that *S. plana* granulocytes can differ in the content of lipid droplets, which are the specialized compartments where NL are stored [127,128]. Furthermore, Rolton et al. [34] using the same probe as in the current study, observed a higher basal content of NL in

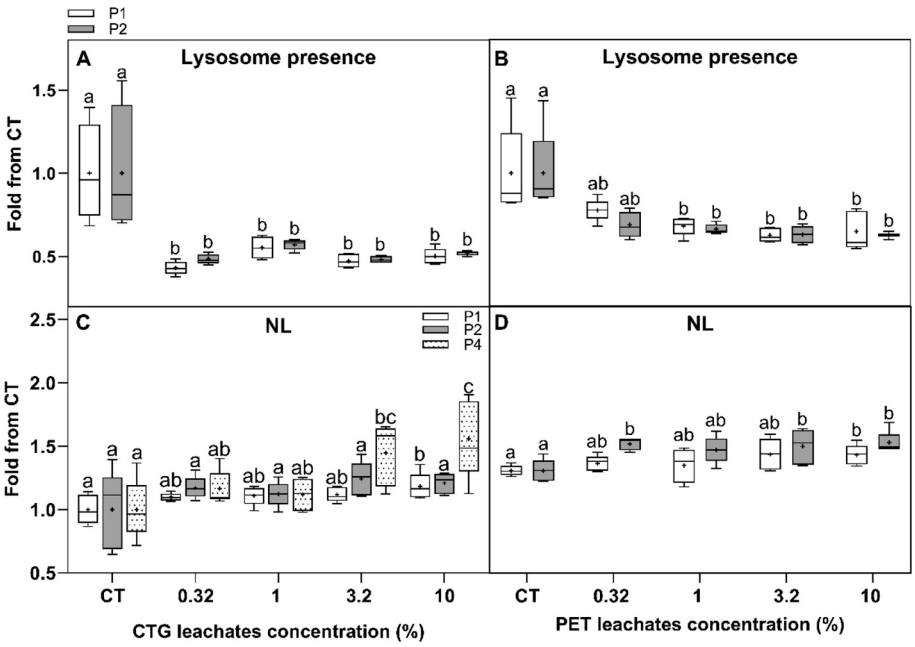

Fig. 4. Lysosome presence and neutral lipid content (NL) of *Mytilus edulis* haemocytes P1, P2 and P4 subpopulations after 24 h exposure to 0.32, 1, 3.2 and 10 % leachates originated from car tyre granulates (CTG) and PET bottles (PET). Results are expressed as fold induction from control (CT) and are presented as box-and-whisker plots ($n = 5$). 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. Letters represent statistical differences between the control and the different leachates concentrations, with different letters indicating significant differences ($p < 0.05$).

granulocytes compared to hyalinocytes in the bivalve *O. chilensis*. This finding further supports the classification of P1 as GR, as NL are more prevalent in this cell type. When looking at the results for PET leachates, no alterations in NL were recorded for P1 (Fig. 4D). On the other hand, slightly higher NL levels were recorded for P2 at all concentrations except for 1 % leachate concentration (Fig. 4D). NL accumulation is considered a biomarker of lipidosis which is commonly associated with exposure to metals (e.g., Zn, Cu, Cd and Pb) and organic contaminants (e.g., bisphenol A) [42,129,130]. In fact, Capolupo et al. [42] showed a significant NL increasing trend in the digestive glands of *M. galloprovincialis* exposed to leachates from car tyres and PET plastics. Even though haemocytes and digestive glands have different cellular functions, the chemical content of the leachates (e.g., Zn and carboxylic acid derivate for CTG and phenyl derivate and Sb in PET) might have contributed to the activation of NL accumulation in both tissues. Additionally, 6PPD-quinone exposure induced alterations of metabolic genes which lead to increase in triglyceride content, enhancement in lipid accumulation, and increase in size of lipid droplets in the nematode *Caenorhabditis elegans* [131]. Overall, as previously suggested, the observed increasing content of NL was possibly a result of lysosomal autophagy [132]. NL, in response to cellular stress, can be mobilized by the action of lipases and hydrolases [128], which correlates with the lower lysosomal content observed in haemocytes exposed to CTG and PET leachates. Furthermore, the alterations in lipid content observed may also result from altered expression of metabolic genes [131], which might be connected with the altered metabolic activity seen in exposed haemocytes.

3.4.4. Cytoplasmatic and mitochondrial ROS activity

ROS are naturally generated during cellular pathways within the aerobic metabolism, but their overproduction can lead to oxidative damage to biomolecules such as protein, lipids, and DNA [133]. In this study, cytoplasmatic ROS levels in P1 increased with increasing CTG leachate concentrations, even though a statistical difference was only significant at 3.2 and 10 % (1.9-fold and 2.3-fold higher than the control, respectively) (Fig. 5A). A similar trend was seen for P2, with haemocytes exposed to 1, 3.2 and 10 % presenting 1.6-, 1.8- and 1.9-fold higher values than the control, respectively. An increase in ROS levels was also recorded following exposure to PET leachates, with P1 and P2 presenting statistical differences compared to the control from 3.2 % and 1 %, respectively. P1 presented maximum values of 2.8-fold at 10 % while for P2 a 2.3-fold increase was detected at 3.2 % (Fig. 5B). This increase in ROS production is in agreement with other studies, namely in *M. galloprovincialis* haemocytes exposed to Cd [134], flame retardants [135] heavy metals (e.g., Zn, Cd), PAHs and pesticides (lindane) [136], as well as PET microfibres [137]. Furthermore, compounds such as benzothiazoles and diphenylamine enhanced ROS production in rainbow trout liver and epithelial cell lines by inducing subtle cellular dysregulation [58,138]. These findings are consistent with the increased ROS production observed in the current study in response to the same tyre-derived chemicals, supporting the hypothesis that these compounds can disrupt redox homeostasis and lead to immunotoxicity in exposed cells. It is well recognized that in bivalves haemocytes, the increase in ROS production serves as a potent immunological response, especially when combined with changes in membrane potential and metabolic activity [14]. One possible pathway for the overproduction of ROS seen in this study involves the binding of metals present in the leachates with the thiol groups (-SH) in membrane proteins. This interaction can then affect electron flow, potentially amplifying ROS formation [139] and leading to the observed alterations in membrane potential and reductions in cell viability.

Haemocytes showed different responses in mitochondrial ROS activity. A decreasing trend was observed in P1 and P2 exposed to CTG leachates (Fig. 5C), with lowest values recorded at 1 % (1.6-fold lower than CT) and 10 % (2.1-fold lower than CT), respectively. In opposite, an increase in mitochondrial ROS levels was recorded for both haemocytes' subpopulations exposed to PET leachates, with a maximum response at 10 % (1.3-fold compared to the control) (Fig. 5D). Decreased ROS levels in CTG leachates aligns with the changes seen at the mitochondrial membrane level. Since ROS are commonly generated by NADPH oxidase, mitochondrial dysfunction, along with an oxygen imbalance, may have triggered alterations in the NADPH-oxidase activity, leading to

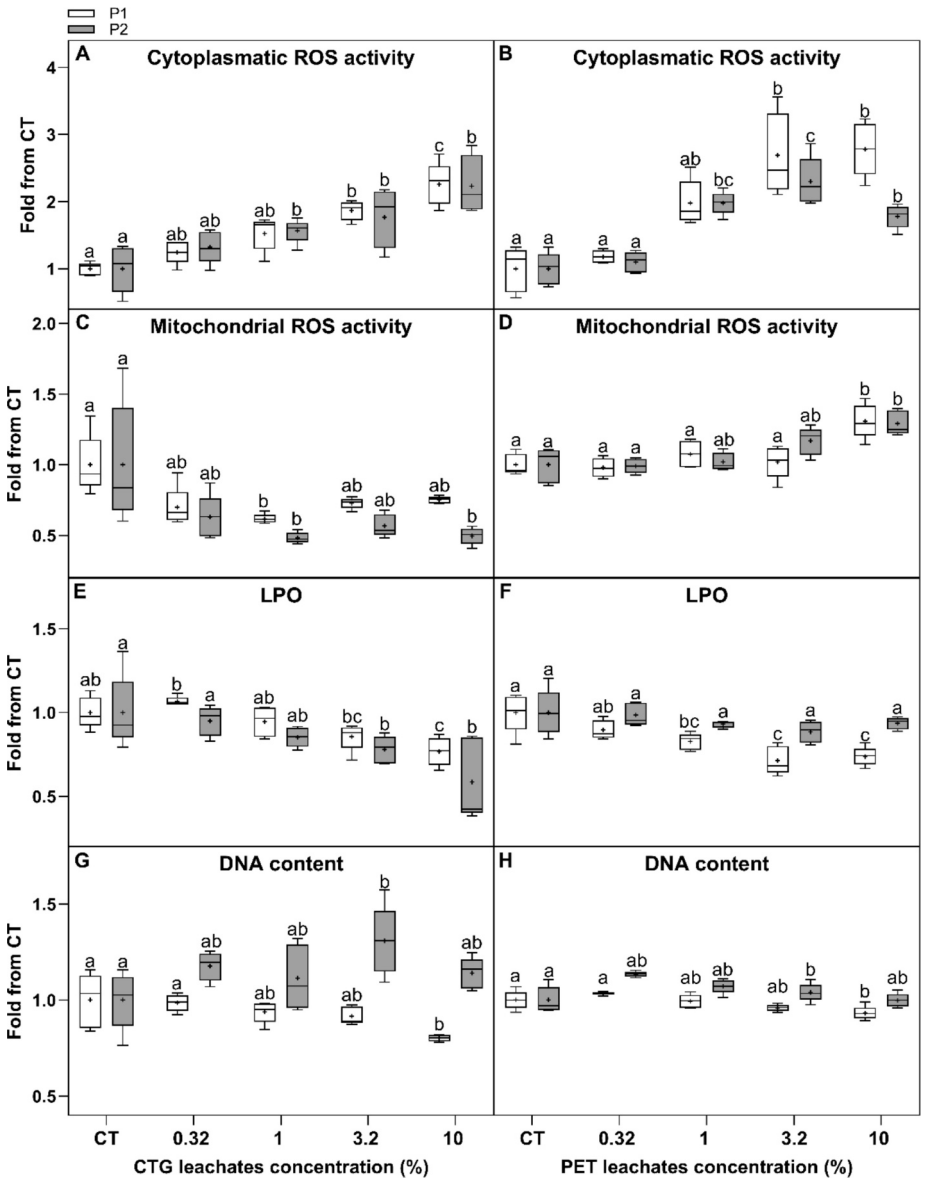

Fig. 5. Cytoplasmatic ROS activity, mitochondrial ROS activity, lipid peroxidation and DNA low content of *Mytilus edulis* haemocytes P1 and P2 subpopulations after 24 h exposure to 0.32, 1, 32 and 10 % leachates originated from car tyre granulates (CTG) and PET bottles (PET). Results are expressed as fold induction from control (CT) and are presented as box-and-whisker plots ($n = 5$). 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. Letters represent statistical differences between the control and the different leachates concentrations, with different letters indicating significant differences ($p < 0.05$).

decreased ROS production [140]. Supporting this, compounds like 6PPD-quinone, present in CTG leachates, were suggested to disrupt mitochondrial respiration in in vitro fish cell lines [141]. Additionally, DHR 123 is oxidized to rhodamine 123 which accumulates in functional mitochondria [142]. In the presence of a compromised mitochondrial membrane, this accumulation does not occur, further explaining the lower observed mitochondrial ROS levels. For PET exposure, the slight increase in mitochondrial ROS production was also inversely correlated with decrease of the MMP. A decrease in MMP is indicative of uncoupling of oxidative phosphorylation and increase in oxygen consumption rate [143], which can lead to additional generation of reactive oxygen species (ROS) due to electron leak [144].

3.4.5. *Lipid peroxidation*

Lipid peroxidation is the oxidative degradation of lipids containing carbon-carbon double bounds, such as phospholipids and poly-unsaturated, by free radicals and ROS. This oxidative damage can lead to the formation of lipid radicals and lipid hydroperoxides which can cause damage to cell membranes, resulting in changes to signal transduction pathways and even death [145]. In the present study, LPO was assessed with C11-BODIPY581/591, which reacts to oxyl-radicals such as HO•, ROO•, RO• and peroxynitrite [27]. To the best of our knowledge this was the first time that this probe was used in bivalves' haemocytes. CTG leachates induced a significant decrease in the LPO in both haemocytes' subpopulations P1 and P2 at the higher concentrations (1.3-fold and 1.7-fold at 10 %, respectively) (Fig. 5E). A similar pattern was also observed in P1 exposed to PET leachates, with 1.4-fold lower values compared to the control, while P2 was not affected at any of the exposure concentrations (Fig. 5F). This decrease in LPO was accompanied by an increase in ROS formation, possibly indicating the presence of other radical species associated with the formation of LPO end-products (e.g. malondialdehyde), which cannot be detected by this fluorescent probe. This goes in line with previous findings which reported that PET leachates can induce the formation of LPO end-products as

malondialdehyde and lipofuscin in mussel digestive glands [42]. In addition, this reduction in LPO levels can also be explained by a possible activation of the antioxidant defence system (e.g. catalase, superoxide dismutase and glutathione-dependent enzymes) in exposed haemocytes, which can efficiently control the formation of oxyradicals and prevent the propagation of LPO [146].

3.4.6. DNA content

DNA plays a key role in regulating essential cellular processes that ensure proper balance and functionality [147]. Quantifying nucleic acids, such as DNA, is fundamental for understanding cellular mechanisms, including apoptosis, where DNA degradation is a key marker [148]. Exposure to CTG leachates caused a slight reduction in the DNA content of subpopulation P1 at 10 % (1.2-fold), while for P2 a small increase was seen only at 3.2 % (1.3-fold compared to the control) (Fig. 5G). A similar pattern was observed for haemocytes exposed to PET leachates, where a decrease in the DNA content of P1 was only detected at 10 % (1.1-lower fold compared to the control). In P2, slightly higher value was only observed at 3.2 % (1.1-fold compared to the CT) (Fig. 5H). The reduced DNA content observed for P1 in this study, indicated by the lower fluorescent signal from the SYTO probe, suggests DNA and RNA degradation, potentially linked to chromatin structural changes [148]. This is in line with Sendra et al. [21] that showed a significant decrease in DNA content of *Mytilus* spp. haemocytes exposed to PS nanoparticles. Furthermore, the potential activation of caspases by mitochondrial dysfunction may potentially led to the reduction in the DNA content, as apoptotic cells are known to exhibit lower DNA content due to DNA degradation [149]. Additionally, 6PPD was shown to induce the activation of pro-apoptotic genes such as p53 and caspase-3 in zebrafish cardiomyocytes [150] further supporting the presence of apoptotic cells. On the other hand, the activation of apoptosis can also be associated with higher DNA content due to the presence of fractional DNA content [27]. This suggests that the complex mixtures of additives released from CTG and PET particles may influence DNA content by modulating the regulation of apoptosis-related mechanisms in *Mytilus edulis* haemocytes. Overall, changes in DNA content are indicative of cellular dysfunction, as it often reflects alterations in cell membrane integrity and ROS formation [147]. So, the variations in DNA content seen for exposed haemocytes may be linked to stress-induced changes in cellular homeostasis and oxidative stress, which is consistent with the alterations observed in mitochondrial functions and ROS formation. Furthermore, GR and HY exposed to CTG leachates exhibited distinct responses, suggesting the activation of different stress response pathways. This goes in line with what has been seen previously, in which granulocytes and hyalinocytes demonstrated differential proliferation and apoptotic dynamics in response to external stressors [151].

3.5. Implications for in vivo exposure

While this study focused on the effects of CTG and PET leachates on haemocytes *in vitro*, it is important to consider how cellular alterations can correlate with responses at the organism level. To the best of our knowledge, only a limited number of studies have investigated the effects of tyre and PET leachates on mussels (*Mytilus* spp.) *in vivo*. Thomsen et al. [152] reported a significant reduction in mussel filtration rate following exposure to tyre leachates, likely due to the presence of both organic and inorganic additives in the leachates, that induced a stress response in mussels. Haemocytes, as key immune cells, play a central role in these responses. Alterations in haemocyte activity (e.g. phagocytosis or oxidative stress) can redirect energy from essential functions like filtration. Therefore, the observed decline in filtration rate may reflect a direct toxic effect of the leachates but also be associated to a disruption in the immune system of mussels via haemocyte dysregulation [153]. However, it is important to note that filtration is a complex process involving multiple physiological systems, making it difficult to directly correlate with the specific cellular endpoints assessed in the present study.

Capolupo et al. [42] exposed adult *M. galloprovincialis* to tyre and PET leachates, reporting that tyre leachates (but not PET) reduced lysosomal membrane stability in haemocytes and induced neutral lipid accumulation (NL) in the digestive glands. Similarly, in the present study, the lower lysosomal content observed may suggest lysosomal dysfunction in vivo. Since NL can be mobilized under cellular stress through lysosomal autophagy and the action of lipases and hydrolases, its accumulation in both studies may be linked to lysosomal alterations. Additionally, since NL accumulation occurred both in the digestive glands [42] and haemocytes (present study), this suggests that chemical additives in leachates may trigger similar toxicity pathways regardless of the affected tissue. Capolupo et al. [42] also reported increased LPO in digestive glands of mussels exposed to PET leachates, while no alterations were observed for tyre leachates. In contrast, the present study found a decrease in LPO levels in haemocytes exposed to both tyre and PET leachates, suggesting the activation of antioxidant defence mechanisms. However, Capolupo et al. [42] did not report changes in antioxidant responses, which may reflect tissue-specific differences, variations in experimental design (e.g. duration of exposure), the complexity of whole tissue/organism responses, and potential species or sensitivity differences.

Given the limited data on the effects of tyre leachates on mussels, additional *in vivo* studies using juvenile/adult organisms are needed to determine whether the cellular effects observed *in vitro* are reflected at the organism level. However, *in vitro* studies on this topic are also scarce, highlighting the need for further investigation at both cellular and organism levels. Assessing haemocyte responses *in vitro* provides valuable insights into toxicity mechanisms of chemicals and helps identify early biomarkers that could predictive broader physiological impacts in organisms.

4. Conclusion

This study provides critical insight into the cytotoxic effects of CTG and PET leachates on *M. edulis* haemocytes, revealing significant impacts on cell viability, metabolic activity, lysosomal integrity and ROS formation. While both leachates triggered key immune responses, differences in mitochondrial activity suggest distinct toxicity pathways linked to their additive composition. These findings emphasize the complexity of polymer-derived chemicals and the need for a deeper understanding of the unidentified substances present in plastic and rubber materials. By employing an *in vitro* haemocyte assay, this study offers a controlled approach to assessing cellular toxicity, allowing for the isolation of direct effects without systemic influences such as metabolism, digestion, and excretion. While whole-organism responses may differ due to these physiological processes, haemocytes are central to bivalve immunity and serve as a relevant proxy for evaluating sublethal effects. This reinforces the value of *in vitro* assays in complementing in vivo studies, contributing to a more comprehensive assessment of environmental risks. Ultimately, these findings highlight the urgent need for further research integrating both cellular and organism-level evaluations to fully elucidate toxicity mechanisms and ecological implications of these complex chemical mixture.

CRediT authorship contribution statement

M. Elisabetta Michelangeli: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Sicco H. Brandsma:** Writing – review & editing, Writing – original draft, Visualization, Investigation. **Maria Margalef:** Writing – review & editing, Writing – original draft, Visualization, Investigation. **Emelie Forsman:** Writing – review & editing, Writing – original draft, Visualization, Investigation. **Sebastian Kuehr:** Writing – review & editing, Writing – original draft, Visualization, Investigation. **Davide Spanu:** Writing – review & editing, Visualization, 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 influenced 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.2025.03.010.

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