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Elastomer leachates modulate haemocytes responses in *Mytilus edulis*

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ABSTRACT

Rubber pollution is widespread in aquatic environments, where hazardous additives used in manufacturing pose serious risks to aquatic life. Both natural and synthetic rubbers undergo vulcanization, incorporating stabilizers to enhance durability and elasticity, alongside additives like antioxidants, antimicrobials, cross-linking agents and pigments. These substances, not chemically bound to the rubber polymer matrix, can leach into aquatic systems and interact with biota. Thus, this study investigated the ecotoxicological effects of rubber leachates from commercial products, specifically balloons (BAL) and dishwashing gloves (DG), on *Mytilus edulis* haemocytes. After 24 h of exposure, flow cytometry revealed several toxic effects, including reduced cell viability, metabolic activity, lysosomal content, and neutral lipid levels, as well as DNA alterations indicative of apoptosis. Mitochondrial dysfunction was evidenced by altered membrane potential and reduced reactive oxygen species (ROS) formation, while cytoplasmatic effects included decreased ROS levels and membrane depolarization. Mechanistically, the observed toxicity is likely driven by metabolic and membrane disruption, as well as mitochondrial dysfunction. Chemical analysis tentatively identified several hazardous organic compounds, including cyclic amines, benzothiazoles, and elevated zinc concentrations, known disruptors of cellular homeostasis. This study highlights the sublethal impacts of rubber-derived pollutants on mussel immune cells, providing mechanistic insight into how chemical additives associated with consumer rubber products may pose a threat to the health of marine organisms.

1. Introduction

Rubber is a crucial material used across various industries, including medical, agricultural, transportation, commercial and domestic applications (Ali Shah et al., 2013). In 2022, global rubber production reached approximately 29.6 million metric tons, with Asia accounting for 90 % of the global supply (Global Synthetic Rubber Production 2022 | Statista, n.d.). Rubber is broadly classified into natural rubber (NR) and synthetic rubber (SR). While NR, poly(*cys*-1,4-isoprene), is a biopolymer synthesized by plants, SR is manufactured as monomers from petroleum-based hydrocarbons and is classified into styrene-butadiene rubber (SBR), acrylonitrile, butadiene copolymers (NBR latex), ethylene-vinyl chloride copolymers (EVCL), polybutadiene and polychloroprene (neoprene) (Ali Shah et al., 2013). Both rubber types are further classified as elastomers, due to their property to stretch and return to their original shape (Gent, 2005).

To enhance properties such as elasticity, abrasion resistance and damping ability, rubber polymers are mixed with a broad spectrum of chemical additives during manufacturing, like plastic polymers (Rodgers and Waddell, 2005). These additives include fillers, stabilizers, vulcanization agents, pigments, oils, resins and processing aids (Rodgers and Waddell, 2005). This complex composition poses significant environmental challenges, especially in marine ecosystems where rubber waste frequently ends up through stormwater runoff, maritime activities and improper waste management (Chittella et al., 2021). Inefficient recycling exacerbates this issue, contributing to microplastic and chemical pollution in aquatic environments. Once released into marine waters, elastomers degrade due to UV light exposure and biodegradation, leading to the release of potential toxic chemical additives (Xu et al., 2022). Although environmental data on rubber additives are scarce, some compounds such as benzothiazoles, a common class of vulcanization accelerators, have been detected in aquatic environments.

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For example, concentrations of 2-mercaptobenzothiazole (MBT) in Swedish surface waters have been reported between <3 ng/L and 19 ng/L (Brorström-Lundén et al., 2023), while total benzothiazoles in Chinese shelf seas surface water range from 1.7 to 13.1 ng/L (Zhao et al., 2024). In Australian urban waters, related benzothiazole derivatives have been found up to 217 ng/L, levels comparable to those in China, Germany, and Spain (BTHs detection frequencies 75–89 %) (Rauert et al., 2022). These findings indicate that rubber additives, including benzothiazoles, are present in surface waters worldwide, although data remains limited.

A well-documented example of rubber additive toxicity is 6PPD-quinone, a transformation product of a common rubber antioxidant, which has caused acute mortality in coho salmon (Tian et al., 2021). Additionally, other studies have reported the negative impacts of rubber leachates on aquatic organisms' behaviour, growth and survival (Capolupo et al., 2021; Halle et al., 2021; Kim et al., 2022; Koski et al., 2021; Page et al., 2022; Stephensen et al., 2005; Tallec et al., 2022). These findings underline the ecological risks of elastomer-derived pollutants, however knowledge on their cellular effects and toxicity mechanisms, particularly in marine invertebrates, remains limited.

Bivalve molluscs, particularly *Mytilus* species, are keystone species and are widely used in marine pollution biomonitoring due to their filter-feeding nature and resilience to environmental stress (Beyer et al., 2017; Canesi et al., 2012). Mussel haemocytes play a key role in immune responses and physiological homeostasis, making them ideal models for assessing the ecotoxicological impacts of environmental pollutants. Thus, this study aimed to investigate the modulation and the activation of toxicity pathways on *Mytilus edulis* haemocytes exposed to leachates derived from balloons (BAL) and dishwashing gloves (DG). These materials were chosen based on a previous study (Sørensen et al., 2023), which identified BL and DG as among the most toxic consumer products in a sample of 50 elastomer-based products and thermoplastics on marine bacteria and microalgae, likely due to their complex chemical composition and high leaching potential. The present study was conducted using filtered natural marine water to simulate environmentally relevant exposure conditions. To assess the cellular responses to rubber-derived leachates, this study employed a reproducible *in vivo*-like methodology using flow cytometry (FCM), a laser-based technique enabling rapid, quantitative, reproducible and statistical accurate analysis of multiple morphological and functional parameters on single cells, widely used for assessing immune and cytotoxic effects in aquatic organisms (Van Nguyen and Alfaro, 2019). FCM allowed the evaluation of a range of biological endpoints at various concentrations, including cell viability, reactive oxygen species (ROS) activity, cytoplasmatic and mitochondrial membrane potential, lipid peroxidation (LPO), neutral lipid content, metabolic activity, lysosome presence and DNA content.

2. Materials and methods

Balloons (BAL) and dishwashing gloves (DG) were purchased from a commercial store and processed by CARAT GmbH (Germany). The materials were cryomilled and dry-sieved into distinct particle fractions: <1000 μm for BAL and < 500 μm for DG. Further details on the cryomilling process are provided in Section S1 of the Supplementary Information: Test materials: Balloons and dishwashing gloves characterization. Polymer identification was conducted using pyrolysis-gas chromatography mass spectrometry (pyr-GC–MS), as detailed in Sørensen et al. (2023), confirming both polymers as natural rubber elastomers. The micromorphology of BAL and DG particles was examined using a Philips® Field Emission Gun-Scanning Electron Microscope (FEG-SEM). Detailed descriptions of the methods and results can be found in Section S1 of the Supplementary Information.

2.1. Leachates preparation, particles presence and chemical characterization

The preparation of leachates followed the methods previously described in Capolupo et al. (2020) and Michelangeli et al. (2025). Briefly, BAL (<1000 μm) and DG particles (<500 μm) were transferred to 1 L glass amber bottles and diluted in natural seawater (NSW, 34 PSU; pH 8.012) to a final concentration of 100 g/L (100 %), corresponding to a liquid solid ratio (L/S) of 10. Samples were shaken for 14 days at 125 rpm in a rotating incubator in the dark and at 20 °C to avoid degradation of the polymer-associated chemicals through light exposure. The stock concentration chosen intended to match those used in literature assessing the effects of plastic debris on marine biota (Capolupo et al., 2021; Halsband et al., 2020). Furthermore, a L/S of 10:1 was recommended by the European standards for testing the leaching of waste materials with particle sizes <4 mm (Beiras et al., 2019). After incubation, leachates were double filtered through a glass-microfiber filter (GF/F; porosity 1.2 μm Whatman™) and a sterile filter (0.22 μm Stericap™ PLUS) to eliminate coarse particles. Both leachates were divided in different subsamples and kept at −20 °C prior to experimental use and chemical analysis.

To evaluate filtration success, BAL and DG leachates were evaluated for particles presence using dynamic light scattering (DLS) analysis. Detailed information on the methodology and results is provided in the Supplementary Information (Section S2). BAL and DG leachates were also chemically analysed. A suspect and non-targeted screening (SS/NTS) approach using liquid chromatography tandem high-resolution mass spectrometry (LC-HRMS) was used to identify potential organic additives in the samples. Metal concentrations were determined *via* inductively coupled plasma mass spectrometry (ICP-MS). Detailed description of the methodology used to characterize both organic and metal additive content of both leachates can be found in Sections S3 and S4 in the Supplementary Information. Before exposure experiments, both leachates were diluted in NSW to reach concentrations ranging from 0.1 to 100 %. All glassware used in the preparation and handling of leachates were burned at 500 °C for 30 min in a muffle furnace. NSW used in the preparation of leachates was collected from 60 m depth at NIVA's marine research station at Solbergstrand, located south of Drøbak in the Oslo Fjord, Norway and then sterile filtered (0.22 μm Stericap™ PLUS).

2.2. Mussels collection, haemolymph withdrawal and haemocytes exposure

Mytilus edulis (5.5 ± 0.5 cm shell length) were obtained from Sørskjell AS (Grimstad, Norway) and acclimated in a flow through system under laboratory conditions (16 °C, 16 h:8 h light/dark conditions) for two weeks. During acclimation, mussels were fed twice a week with Shellfish Diet 1800® (Reed Mariculture). After acclimation, haemolymph was aseptically extracted from the posterior adductor muscle of 60 mussels with a 23G needle, pooled together and stored on ice in sterile tubes. Cell density was measured using a flow cytometer (Novocyte Advanteon Flow Cytometer, Agilent, USA) and adjusted to $4 \cdot 10^5$ cells/mL in NSW (0.22 μm filtered, salinity 35 p.s.u).

To determinate suitable exposure concentrations to assess sublethal effects of both leachates, a preliminary test was conducted to evaluate haemocyte cell viability. Haemocytes ($2 \cdot 10^5$ cells/mL) were exposed to BAL and DG concentrations of 0.1, 0.32, 1, 3.2 10, 32 and 100 %. Based on these results, four concentrations (0.32, 1, 3.2 and 10 %) were then selected for a more comprehensive assessment of sublethal effects over a 24-h exposure period. All experiments were performed in 24-multiwell plates sealed with foil and incubated at 16 °C in a shaking incubator (80 rpm, BINDER GmbH, Germany) under dark conditions. After 24 h of exposure, different biological endpoints were assessed using flow cytometry.

2.3. Flow cytometry analysis

All flow cytometry (FCM) experimental values are presented as fold induction relative to the control (CT) based on mean fluorescence intensity. FCM analysis was conducted using a Novocyte Advanteon Flow Cytometer (Agilent, USA) equipped with a 488 nm argon-ion laser. Probe incubation was performed at room temperature in the dark. All endpoints were assessed in a final volume of 1 mL (haemolymph plus probe), with data acquired using a threshold of 100,000 events and a flow rate of 66 μL/min. Data analysis was performed using NovoExpress software version 1.6.1 (Agilent, USA).

Haemocyte subpopulations were characterized based on forward light scatter (FSC) for cell size and side light scatter (SSC) for complexity. Measurements were conducted in five replicates for all leachate concentrations and a seawater control. The following cellular parameters were assessed using specific fluorescent probes: cell viability, metabolic activity, reactive oxygen species (ROS) levels, cytoplasmic and mitochondrial membrane potential, lipid peroxidation (LPO), neutral lipid content, lysosome presence, and DNA content. Details on probe concentrations, incubation times, final concentrations, and FCM detection channels are provided in Section S5 of the Supplementary Information.

2.4. Statistical analysis

Statistical analyses were performed using XLStat2022® software (Addinsoft, Paris, France). Data were first tested for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test. Depending on these results, statistical comparisons were carried out using either the parametric one-way ANOVA or the non-parametric Kruskal-Wallis test to assess significant differences between the control and the leachate concentrations. For multiple comparisons, the Tukey test was used for parametric data, while the Dunn's test was applied for non-parametric data. A *p*-value <0.05 was considered statistically significant. Graphical representations were generated using GraphPad Prism 9 software (GraphPad Software Inc., La Jolla, CA, USA), with data displayed as box-and-whiskers plots.

3. Results

3.1. Organic and metal additive content in leachates

Different organic additives were tentatively identified in BAL and DG leachates (see Supplementary Information, Table S6, S7) using suspect-non target screening (SS/NTS). Compound identification confidence levels were assigned following the Schymanski et al. (2014) criteria. Suspected chemicals were further categorized using the PlastChem database (Wagner et al., 2024), which classifies substances based on their environmental and human health risks. The potential identified additives were categorized into five categories: (i) hazardous chemicals (red list), (ii) less hazardous chemicals (orange list), (iii) chemicals under evaluation or with inconclusive hazard data (grey list/watch list), (iv) chemicals regulated under multilateral environmental agreements (MEAs list, including the Basel, Stockholm, Minamata Conventions and the Montreal Protocol), and (v) chemicals not listed in PlastChem (Fig. 1). This classification was based on criteria such as persistence, bioaccumulation, mobility and toxicity.

In BAL leachates, 17 chemical features (levels 2 and 3) were identified, all exceeding 10 times the blank values. Only one compound, dehydroabietic acid, was categorized as hazardous (red list), while triisobutylphosphate was classified as less hazardous (orange list). However, the majority (12 compounds) were not listed in PlastChem, including *N*,*N*′-dimethyl-*N*,*N*′-diphenylurea. For DG leachates, 21 features were identified, with four classified as hazardous (*e.g.*, 2-mercaptobenzothiazole, dicyclohexylamine), one as less hazardous (linoleic acid), and one as regulated under MEAs (anthraquinone). Additionally, six compounds, including 2-hydroxybenzothiazole, were placed on the grey list due to insufficient hazard data, while nine, such as 2-aminobenzothiazole, were not listed in PlastChem. Overall, the tentatively identified compounds in both BAL and DG leachates were primarily categorized as accelerators, antioxidants, flame retardants, and plasticizers.

Metals concentrations in BAL and DG leachates are provided in the Supplementary Information (Table S8). In BAL leachates, the metals were ranked from Zn > Ti > Cu ≈ Cr ≈ Ni ≈ Co ≈ Cd whereas in DG leachates, the ranking was Zn > Cr > Sb ≈ Cu ≈ Ti ≈ Ni ≈ Co ≈ Cd. Overall, the impact of the leaching on the metal levels was significantly different between the two leachates, with BAL increasing the amount of Zn to a level almost three times higher than DG (14,305 ± 1012 μg/L and 5011 ± 236 μg/L respectively). Cr concentration was significantly higher in DG leachates compared to BAL leachates (12.34 ± 0.60 μg/L and 0.04 ± 0.04 μg/L, respectively), while Sb was only detected in DG leachates (1.24 ± 0.26 μg/L). Ni and Cu, on the other hand, were measured only in BAL leachates (0.05 ± 0.03 μg/L and 0.05 ± 0.08 μg/L, respectively).

3.2. Toxicity mechanisms of BAL and DG leachates

Flow cytometry (FSC and SSC properties) enabled the distinction of two main haemocytes subpopulations: P1 and P2, identified as granulocytes and hyalinocytes, respectively (Supplementary Information,

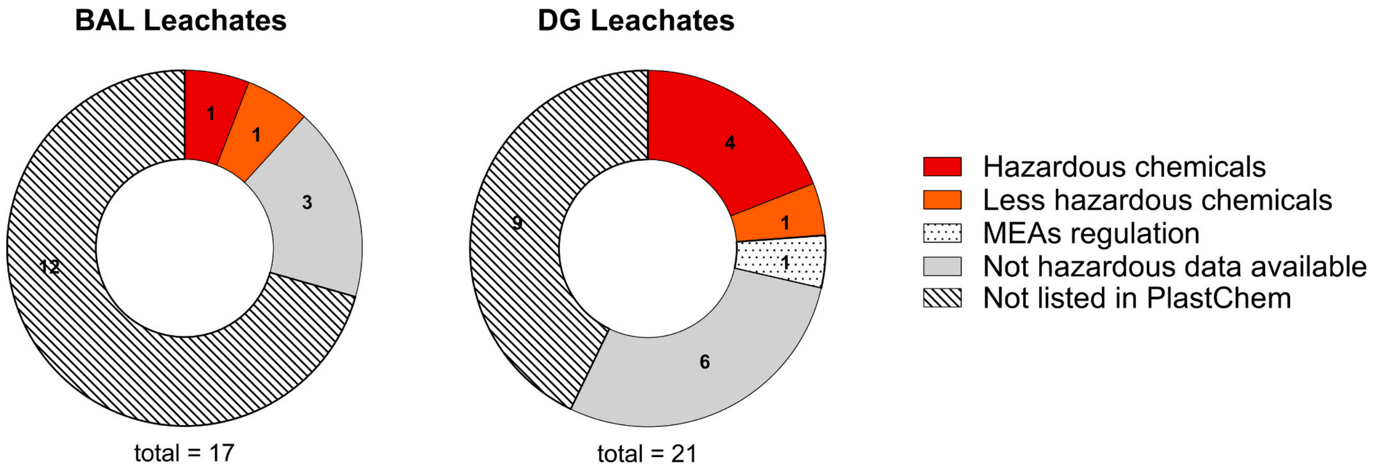

Fig. 1. Organic additives content of balloons (BAL) and dishwashing gloves (DG) leachates classified according to the PlastChem database. Hazardous chemicals (red), less hazardous chemicals (orange), plastic chemicals without hazard information (grey), regulated under existing multilateral environmental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

Fig. S5) (Barrick et al., 2018; Le Foll et al., 2010). Leachates exposure caused a clear shift in haemocytes subpopulations, with additional identification of subpopulations. In BAL-exposed haemocytes, three subpopulations (P1, P2 and P3) were detected (Supplementary Information, Fig. S5 A, B), while exposure to DG resulted in up to four subpopulations (P1, P2, P3 and P4) (Supplementary Information, Fig. S6 C, D).

3.2.1. *Cell viability*

Haemocytes viability in the total cell population was significantly affected by leachates. BAL leachates significantly reduced the number of viable haemocytes at 32 % and 100 % leachate concentration with 1.7-fold and 3.4-fold decreased compared to the control, respectively (Fig. 2A). In contrast, DG leachates had a greater toxic effect, with significant reductions in viability starting at 3.2 % (1.4-fold lower than the CT), followed by a 2.4-fold and 3.1-fold decreases at 10 and 32 %, respectively. The highest impact was observed at 100 % where viability was 3.4-fold lower than the control (Fig. 2B).

3.2.2. *Metabolic activity*

BAL leachates significantly decreased metabolic activity in P1 and P2 subpopulations at all concentrations tested, with the largest effects observed at 3.2 % and 10 % (2.0-fold and 2.4-fold reductions, respectively) (Fig. 3A). DG leachates also caused reduced metabolic activity in P1 and P2, with significant effects at 1 %, 3.2 % and 10 % in P1 (1.2-fold, 1.5-fold and 1.6-fold reductions, respectively) and at 3.2 % and 10 % in P2 (1.8-fold lower than the CT) (Fig. 3B). Moreover, the subpopulation P4, uniquely identified in DG-exposed haemocytes, was particularly affected, showing significant reductions in metabolic activity at all the concentration tested, with up to a 3.3-fold decrease at 3.2 % and, a maximum 3.5-fold reduction compared to the control at 10 % (Fig. 3B).

3.2.3. *Cytoplasmatic and mitochondrial membrane potential*

BAL leachates caused significant higher CMP values in subpopulation P1 at 3.2 % and 10 % (1.3-fold higher than the control) and in P2 at 1 %, 3.2 % and 10 % (1.2-fold increase) (Fig. 3C). DG leachates similarly increased CMP in P1 at 1 % 3.2 % and 10 % (1.2 to 1.3-fold), and in P2 at 3.2 % (higher 1.2-fold), with no effects at other concentrations (Fig. 3D). At the mitochondrial level, BAL leachates, caused a decrease in MMP in P1 at 10 % (1.4-fold reduction compared to the control) (Fig. 3E). P2 showed a different response, with a slight increase observed at 3.2 and 10 % (higher 1.0-fold and 1.1-fold, respectively). DG leachates, however, consistently reduced MMP in P1 at all concentration tested, with the lowest value (2.4-fold reduction) observed at 3.2 % (Fig. 3F). No significant effects on P2 were detected after DG exposure (Fig. 3F).

3.2.4. *Cytoplasmatic and mitochondrial ROS formation*

Cytoplasmatic ROS formation was unaffected by BAL leachates in both subpopulations, with no significant changes observed in all tested concentrations (Fig. 4A). However, DG leachates slightly reduced ROS formation in both subpopulations P1 and P2 at 10 % (1.9 and 1.4-fold lower than control, respectively) (Fig. 4B). At the mitochondrial level, BAL leachates induced significant reductions in ROS formation in both P1 and P2, with the largest decline detected at 10 % (1.3-fold and 2-fold, respectively) (Fig. 4C). Similarly, DG leachates reduced mitochondrial ROS formation in P1 and P2 at 10 % with decreases of 1.8-fold and 2.0-fold, respectively (Fig. 4D).

3.2.5. *Lipid peroxidation*

The LPO levels in subpopulation P1 were not significantly affected by any tested concentrations (0.32 %, 1 %, 3.2 %, and 10 %), with values remaining comparable to the control. In contrast, P2 showed a consistent and significant decrease in LPO levels across all tested concentrations (1.3-fold) (Fig. 4E). Upon exposure to DG leachates, LPO in P1 and P2 exhibited a significant concentration-dependent decrease, with values 3-fold lower than the control for both subpopulations at 10 % (Fig. 4F).

3.2.6. *DNA content*

A significant reduction in the DNA content of the subpopulation P1 was detected for BAL leachates at 3.2 % and 10 %, 1.1-fold and 1.2-fold compared to the control, respectively (Fig. 4G). In contrast, in P2, a significant increase was only observed at the highest tested concentration (10 %, 1.1-fold higher than CT) (Fig. 4D). Similar to BAL leachates, a reduction was observed in P1 exposed to DG leachates across all concentrations tested, with the most significant decrease at 10 % (reduction of 1.1-fold compared to the CT) (Fig. 4H). In contrast, P2 showed a significant increase in DNA content at 1 % (higher 1.1-fold). However, at the two higher concentrations (3.2 % and 10 %), the DNA content returned to values similar to the control.

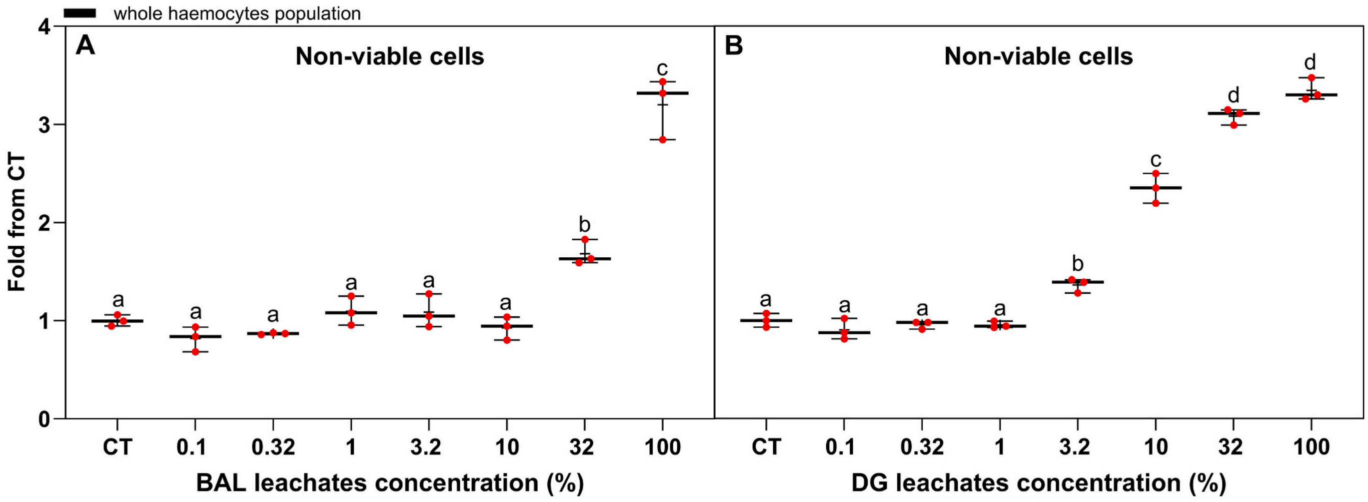

Fig. 2. Cell viability of the whole *Mytilus edulis* haemocyte population after 24-h exposure to 0.1 %, 0.32 %, 1 %, 3.2 %, 10 %, 32 %, and 100 % leachates (100 g/L) from balloons (BAL) (A) and dishwashing gloves (DG) (B). Results are expressed as fold induction from control (CT) and presented as box-and-whisker plots. The median is represented by a horizontal line (–), while the mean is indicated by a plus sign (+). Whiskers show the minimum and maximum values, and red dots represent individual replicates ($n = 3$). Different letters denote statistically significant differences between the control and leachate concentrations ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

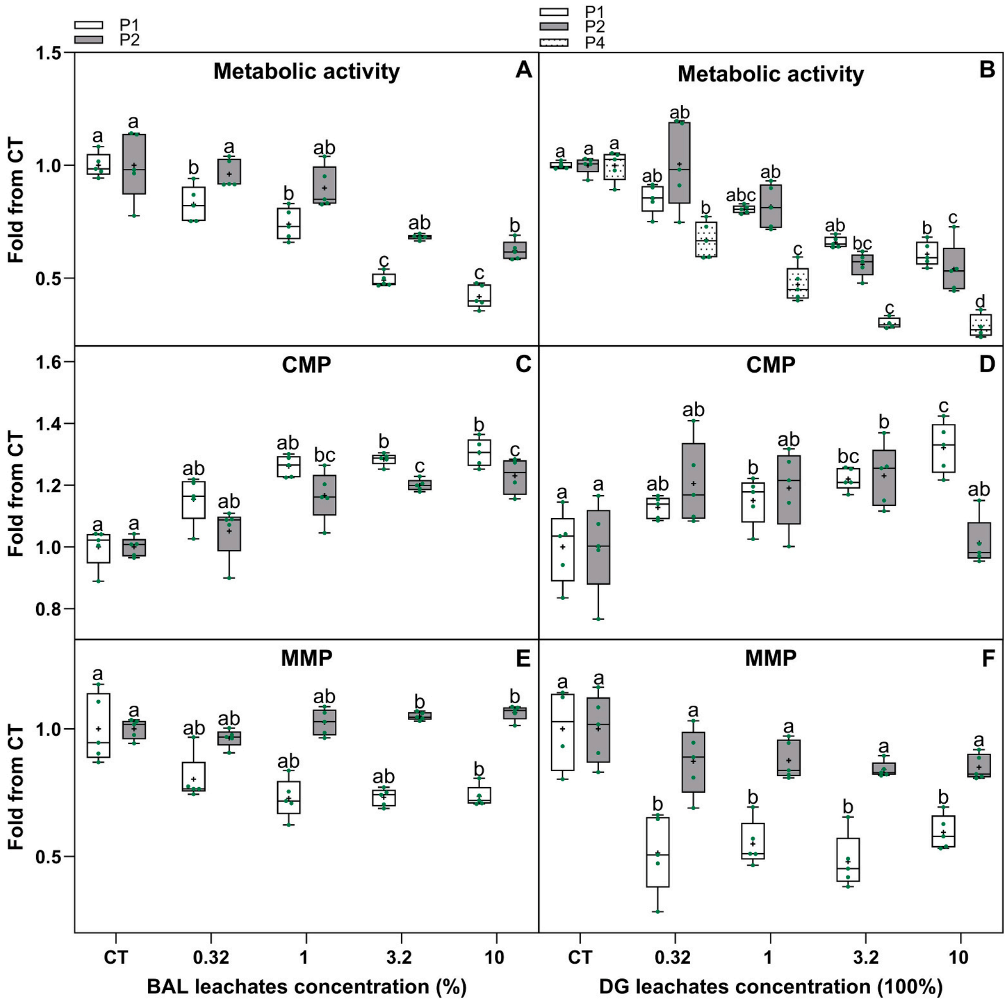

Fig. 3. Metabolic activity, cytoplasmatic membrane potential (CMP) and mitochondrial membrane potential (MMP) in *Mytilus edulis* haemocytes subpopulations (P1 and P2) after 24 h exposure to 0.32, 1, 3.2 and 10 % leachates originated from ballons (BAL) and dishwashing gloves (DG). Results are expressed as fold induction relative to the control (CT) and presented as box-and-whisker plots. The boxes represent the interquartile range (IQR), with the median shown as a horizontal line (−) and the mean as a plus sign (+). Whiskers show the minimum and maximum values, and green dots represent individual replicates ($n = 5$). Different letters denote statistically significant differences between the control and leachate concentrations ($p < 0.05$). Note: The P4 subpopulation was only observed in metabolic activity measurements of haemocytes exposed to DG leachates measurements and was not detected in haemocytes exposed to BAL leachates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2.7. Lysosomal parameters

BAL leachates significantly reduced lysosome presence in both P1 and P2 subpopulations at the highest concentration tested (10 %), with decreases of 1.6-fold and 2.6-fold, respectively (Fig. 5A). Additionally, P1 exposed to DG leachates exhibited a reduction at 1 % and 3.2 % (both 1.6-fold lower than the control). In contrast, DG leachates caused a reduction in lysosome presence in P2 at the lowest concentration (0.32 %), with a decrease of 1.8-fold (Fig. 5B).

3.2.8. Presence of neutral lipids

BAL leachates did not affect the NL content of either subpopulation (P1 or P2) across all tested concentrations (0.32 %, 1 %, 3.2 %, and 10 %), with NL levels remaining consistent with those of the control (Fig. 5C). In contrast, DG leachates caused a reduction in NL content in both subpopulations (P1 and P2), but this effect was observed only at the highest tested concentration of 10 %, with a 1.2-fold reduction compared to the control (Fig. 5D).

4. Discussion

Most studies on leachates have focused on the impacts of plastic polymers such as polyethylene (PE), polypropylene (PP), and high density polyethylene (HDPE) (Oliviero et al., 2019; Schiavo et al., 2020, 2021). However, the potential toxicity of leachates from elastomeric materials remains largely unexplored. This gap is critical to address, as elastomers play a significant role in releasing toxic chemicals into the environment. Unlike thermoplastics, elastomers are associated with higher toxicity levels for aquatic organisms, including algae and bacteria, due to their greater chemical complexity (Sørensen et al., 2023). To evaluate their potential impact on marine biota, it is essential to conduct toxicity testing, as such studies offer crucial insights into the pathways of toxicity associated with elastomers. While mortality or embryotoxicity are commonly used as endpoints (Lithner et al., 2009; Rendell-Bhatti et al., 2021), there is a substantial knowledge gap regarding their toxicity at the cellular level. This study aimed to fill this gap by employing a methodology that closely mimics *in vitro* conditions, using haemocytes, primarily immune cells from bivalves, as a biological model. The findings obtained in this study provided a valuable baseline for predicting and understanding the mechanisms through which elastomers can affect marine ecosystems.

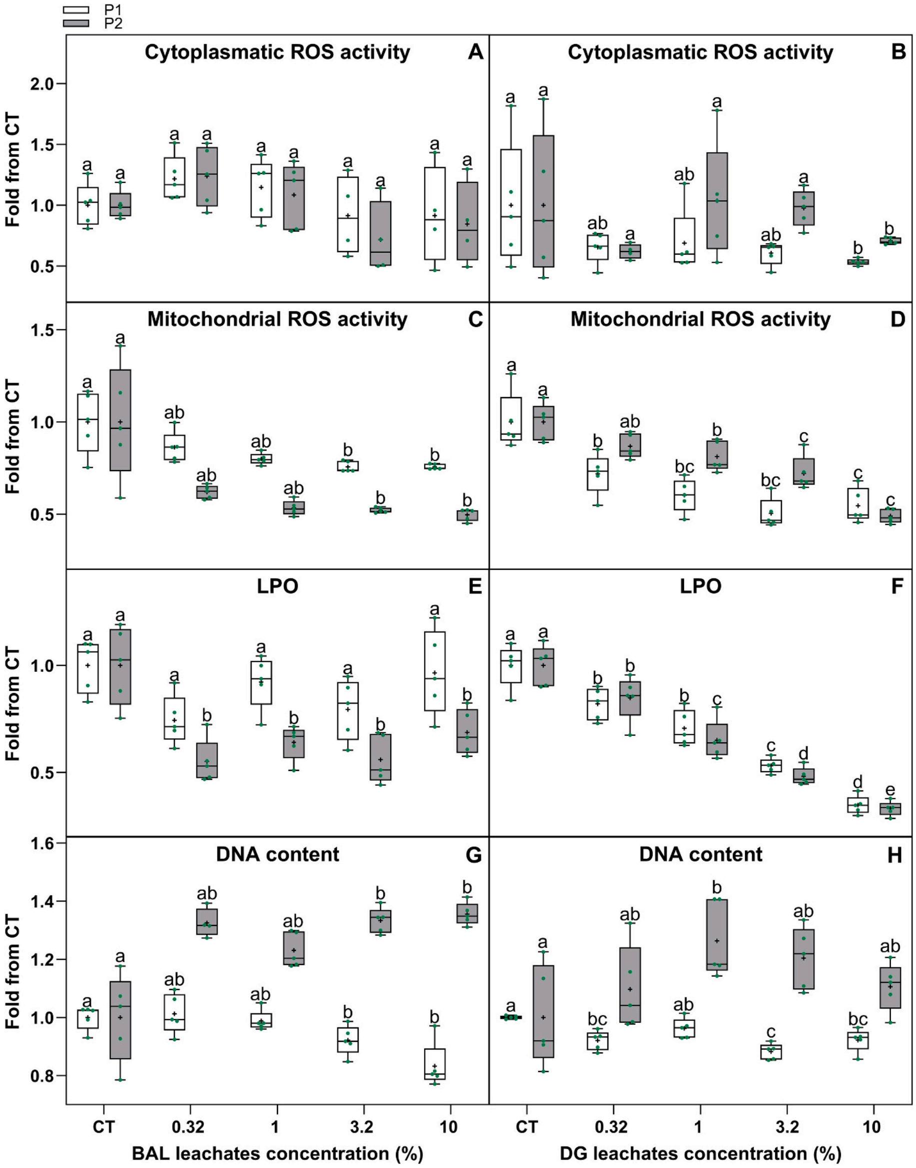

Fig. 4. Cytoplasmatic ROS activity, mitochondrial ROS activity, lipid peroxidation and DNA low content in *Mytilus edulis* haemocytes subpopulations (P1 and P2) after 24 h exposure to 0.32, 1, 3.2 and 10 % leachates originated from ballons (BAL) and dishwashing gloves (DG). Results are expressed as fold induction relative to the control (CT) and presented as box-and-whisker plots. The boxes represent the interquartile range (IQR), with the median shown as a horizontal line (−) and the mean as a plus sign (+). Whiskers show the minimum and maximum values, and green dots represent individual replicates ($n = 5$). Different letters denote statistically significant differences between the control and leachate concentrations ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.1. *Organic additives and metals*

The widespread use of elastomers in products like tyres, gloves, and toys, exacerbates their environmental impacts through the leaching of toxic substances. This issue is intensified by their complex chemical composition, enriched with organic and inorganic additives from manufacturing and vulcanization processes. Identifying all these associated compounds requires advanced analytical techniques. To address this, the present study employed SS/NTS with LC-HRMS to screen for organic chemical additives present in BAL and DG leachates. The goal was to identify hazardous substances and provide insights into the chemical profiles that might be responsible for the observed toxicity. Among the tentatively identified compounds in both leachates, only a few had available toxicity assessments, as they were listed in the PlastChem hazardous list (Wagner et al., 2024). However, most lacked hazard evaluations or were not included in the database used in this study, aligning with reports stating that only 6 % of the 16,000 chemicals used in plastic and rubber are currently subject to international regulations (Wagner et al., 2024). The occurrence of these additives in aquatic environments, some of which bioaccumulate in biota, underscores their potential for adverse effects on marine organisms. The SS/NTS revealed that the organic additives in BAL and DG leachates included vulcanization accelerators, cross-linking agents, flame retardants, antioxidants, stabilizers, surfactants and plasticizers. Hazardous chemicals in DG leachates included 2-Mercaptobenzothiazole (2-MBT) and dicyclohexylamine (DCHA), widely used as vulcanization accelerators (Chibwe et al., 2022; Eva Brorström-Lundén et al., 2023; Zhao et al., 2024), as well as N,N-dibutylformamide, a thermal stability enhancer in polymers (Diera et al., 2023). Dehydroabietic acid (hazardous), tri(isobutyl) phosphate (TiBP, less hazardous), and tramandol-n-oxide (grey list in the PlastChem database) were chemical suspects present in BAL leachates. Polyethylene glycols (PEGs), such as the tentatively identified

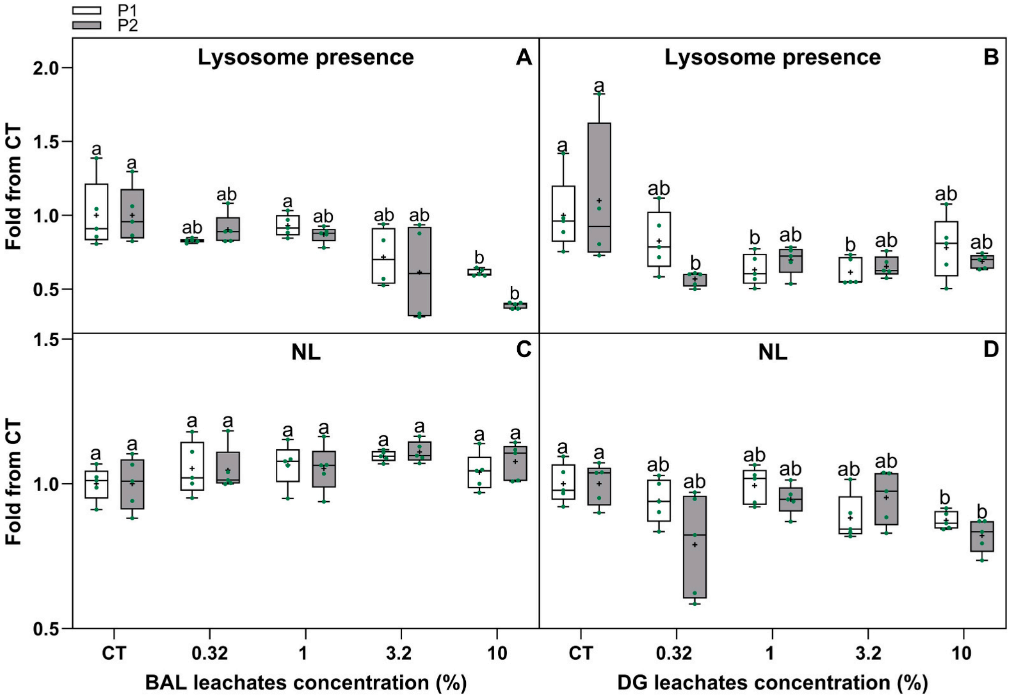

Fig. 5. Lysosome presence and neutral lipid content (NL) in *Mytilus edulis* haemocytes subpopulations (P1 and P2) after 24 h exposure to 0.32, 1, 3.2 and 10 % leachates originated from ballons (BAL) and dishwashing gloves (DG). Results are expressed as fold induction relative to the control (CT) and presented as box-and-whisker plots. The boxes represent the interquartile range (IQR), with the median shown as a horizontal line (−) and the mean as a plus sign (+). Whiskers show the minimum and maximum values, and green dots represent individual replicates ($n = 5$). Different letters denote statistically significant differences between the control and leachate concentrations ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

penthaethylene glycol used in rubber vulcanization, were detected in both leachates. Overall, these chemicals, detected in various ecosystems, raise concerns due to their potential for interaction and possible toxicity towards biota. For example, 2-hydroxybenzothiazole, a by-product of 2-MBT, was detected in urban surface waters (Johannessen et al., 2021, 2022; Rauert et al., 2022; Seiwert et al., 2020), while benzothiazoles (BTHs) are categorized as high production volume chemicals (HPVC) and contaminants of emerging concern (Liao et al., 2018; Zhao et al., 2024). DCHA, easily leachable from tyre rubber materials (Capolupo et al., 2020; Chibwe et al., 2022; Foscari et al., 2023) was identified in urban creeks, wastewater treatment plants (WWTP), sludge, stormwater, groundwater, surface water, soil, and sediment (Lindfors et al., 2008; Seiwert et al., 2020). N,N-dibutylformamide has also raised an environmental concern due to its detection in water distribution systems and groundwater in Denmark and Northen Greece, respectively (Diera et al., 2023; Iordanidis et al., 2016). TiBP, an organophosphate flame retardant (Choi et al., 2020), has been detected in marine species such as *Mytilus galloprovincialis* and *Salmo salar* (Castro et al., 2023), indicating its potential to bioaccumulate within the food chain. Tramadol-n-oxide, used as an analgesic, is also known for its potential persistency in the aquatic environment (Bergheim et al., 2012), while PEGs have recently been recognized as urban pollutants (Peter et al., 2018).

Metals are integral to many elastomers, serving as additives, catalyst, pigments or stabilizers, enhancing properties like durability and flexibility (Sendra et al., 2021). While trace amounts of some metals, like zinc (Zn), are essential for biological processes, elevated concentrations pose risks to aquatic life (Sharma et al., 2024). Zn, widely used in vulcanization processes in rubber manufacturing as zinc oxide (ZnO), has raised environmental concern due to the release of Zn^{2+} ions, toxic at high levels towards aquatic life (Bragato et al., 2022). In this study, Zn was the predominant trace element in both BAL (14,305 ± 1013 μg/L) and DG (5011 ± 236 μg/L) leachates, with concentrations comparable to those previously reported in tyre granulates leachates in (Halsband et al., 2020) (8.4 mg/L). These findings suggest that in addition to tyre wear, other elastomers products may serve as significant sources of Zn to the environment, potentially posing toxic risks to marine biota. Titanium (Ti) was detected in BAL leachates, likely originated from titanium dioxide (TiO_2), one of the most widely used pigments in plastic industry (Turner and Filella, 2023). Although Ti levels were below toxic thresholds for aquatic organisms (Hou et al., 2019), its environmental impact warrants further investigations. Trace levels of Ni, Cr and Co, commonly used as catalysts in rubber production (O'Loughlin et al., 2023), as well as Cd, employed as heat and light stabilizer and antioxidant in plastics (Sendra et al., 2021), were also detected in both leachates. Ni and Cd levels were below the safe limits established for coastal waters in Norway (Ni, 0.5 to 8.6 μg/L) (Miljødirektoratet of Norway, 2020) and the environmental quality standard for Cd in groundwater in Denmark (0.5 μg/L) (Kubier et al., 2019), while Cr in DG leachates fell within the range of moderate environmental hazard according to Norwegian guidelines (3.4–35.8 μg/L) (Miljødirektoratet of Norway, 2020). Antinomy (Sb), detected in DG leachates, likely originated from antimony trioxide (Sb_2O_3), used as flame-retardant and smoke-suppression in synthetic rubber elastomer production, (Liu et al., 2024) did not exceed the EU's maximum admissible concentration for drinking water (5 μg/L) (Obiakor et al., 2017).

While the present study provides a detailed characterization of leachates from specific commercial gloves and balloons, there is limited comparative data available in literature, particularly for balloons. To date, no other studies have specifically investigated the chemical composition or leachates of balloons in detail. In contrast, rubber gloves have been more extensively studied due to their widespread use in medical, industrial, and domestic applications. A recent study by Sørensen et al. 2024 examined chemical leaching from the same dishwashing gloves (DG) and balloon (BAL) materials under different experimental and analytical conditions. Several compounds identified in DG leachates overlapped with those detected in the present study, including the vulcanization accelerator 2-Mercaptobenzothiazole (MBT; CAS 149–30-4), the heat stabilizer N,N-dibutylformamide (CAS 761–65-

9), the precursor 2-Hydroxybenzothiazole (CAS 934–34-9), and the plasticizer N-Ethyl-o-toluenesulfonamide (CAS 1077-56-1), confirming that these are commonly used additives in various types of rubber gloves. In contrast, no overlapping compounds were found in BAL leachates, highlighting a strong influence of methodological settings, including leaching duration and loading concentration. In addition, distinct analytical platforms were used, pyrolysis-GC/MS in Sørensen et al. 2024 *versus* LC-HRMS in the present study, as well as different spectral libraries (NIST17 *vs.* MassBank Norman suspect list). These methodological choices likely influenced the compounds detection outcomes and underscore the need for analytical transparency and standardization when comparing leachate compositions across studies.

The use of MBT in gloves is well documented. For instance, Bergendorff et al. (2006) reported the presence of MBT in 2 out of 19 rubber gloves used in healthcare settings, with concentrations ranging from 0.005 to 0.008 mg/g. Additionally, alternative vulcanization agents, such as zinc oxide or UV-light curing, are employed in accelerator-free gloves, such as those used by hairdressers (Havmose et al., 2020), reflecting product-specific formulations. While BAL and DG leachates contained a similar number of tentatively identified compounds, their composition varied significantly, reflecting differences in the properties and specific use of balloons and dishwashing gloves. These variations suggest that different elastomers may pose different environmental risks based on their complex organic and inorganic content. These interactions could amplify harmful impacts on aquatic organisms, underlining the need for further research into the combined toxicity of these mixtures from plastic and rubber leachates. Additionally, this supports the importance of identifying compounds that are either common across products or unique to specific brands in order to better assess environmental risk and prioritize monitoring efforts.

4.2. *Toxicity mechanisms on BAL and DG leachates on M. edulis haemocytes*

Flow cytometry has been extensively employed to characterize molluscan haemocyte sub-populations and their immune-related functions (Evariste et al., 2016). Haemocytes classification remains a topic of debate due to methodological differences that result in inconsistencies in criteria and outcomes for haemocytes subtype identification, complicating the development of a standardized classification system (Allam et al., 2002; Evariste et al., 2016; Le Foll et al., 2010). Despite this variability, granulocytes (GR) and hyalinocytes (HY) are consistently recognized as primary immune cells in marine bivalves (Barrick et al., 2018; Cajaraville et al., 1995; Hégaret et al., 2003; Rolton et al., 2020; Wang et al., 2012). In this study, FCM allowed the identification of subpopulations P1 (GR) and P2 (HY), based on forward and side scatter characteristics, consistent with Sendra et al. (2020). The higher scatter of P1 may reflect the complexity of GR, which contain granules, lipid droplets, endoplasmic reticulum, phagosomes, lysosomes, mitochondria and Golgi apparatus (Le Foll et al., 2010; Wootton and Pipe, 2003). In contrast, P2, with lower scatter and forward, likely represent HY, smaller cells which have a higher nucleus to cytoplasm ratio, and fewer or no granules in their cytoplasm but still contain organelles such as mitochondria, endoplasmic reticulum, lysosomes, free ribosomes and Golgi apparatus (Cajaraville et al., 1995; Le Foll et al., 2010; Travers et al., 2008). Haemocytes play critical roles in bivalve homeostasis and immune defence, and their modulation is linked to environmental stress responses (de la Ballina et al., 2022).

FCM results revealed distinct alterations in P1 and P2 in response to increasing concentrations of BAL and DG leachates over 24 h, indicating cellular alterations in mussel haemocytes. Significant reductions in cell viability, metabolic activity, mitochondrial ROS activity and DNA content, along with alterations in cellular and mitochondrial membrane potentials, were observed. Cell viability in *M. edulis* whole haemocytes population was evaluated using the fluorescent probe PI, which selectively enters cells with compromised membranes (Huang et al., 2016). After 24 h of exposure to BAL and DG leachates, this method was used to assess haemocyte health and determine leachate concentrations eliciting sublethal responses for use in subsequent toxicity testing. Cytotoxicity was evidenced by the reduced cell viability in haemocytes exposed to both leachates and attributed to the complex mixture of organic and inorganic additives in the leachates. Similar cytotoxicity was previously seen for *in vitro* testing with rainbow trout cell lines (RTgill-W1 and RTL-W1), where 2-MBT, one of the additives present in DG leachates, led to a decrease in cell viability with EC_{50} ranging from 35 to 105 mg/L (Zeng et al., 2016). Organophosphorus flame retardants (OPFRs), as the one tentatively identified in BAL leachates, were reported to similarly reduce cell viability in human models (An et al., 2016). Additionally, *in vitro* studies using bivalves' haemocytes showed that metals such as Zn, suppress viability through various mechanisms, including oxidative stress, membrane damage, and disruption of cellular processes such as phagocytosis (Caza et al., 2015; Gómez-Mendikute and Cajaraville, 2003; Luo and Wang, 2022).

Metabolic activity assessed using the fluorescent probe FDA, which is cleaved by non-specific esterases in metabolically active cells (Wanandy et al., 2005) was reduced in haemocytes exposed to BAL and DG leachates. These decreases, reflected by lower FDA uptake, suggests alterations in cell membrane permeability/integrity likely resulting in esterase inhibition (Cossarizza et al., 2017; Gomes et al., 2020). The reduced activity in BAL-exposed haemocytes may be associated with the presence of TiBP, an organophosphate known to inhibit esterases by phosphorylating serine residues in the esterase active sites, as shown in *in vitro* human cell models (Makhaeva et al., 2016). Numerous studies have also reported that OPFRs causes esterase inhibition in several species, including *Daphnia magna, Hyalella azteca* and *Oreochromis niloticus* (Kinareikina et al., 2024). Benzothiazoles such as 2-MBT, tentatively identified in DG leachates, also decrease metabolic activity, as shown in rainbow trout cell lines (Dudefoi et al., 2024). Interestingly, FDA staining revealed the presence of additional subpopulations alongside P1 and P2, in both BAL and DG exposures. In BAL and DG leachates, control samples exhibited the presence of subpopulation P3, which was characterized by lower internal complexity compared to P1 and P2 but similar in size compared to P2. This subpopulation potentially represented blast-like cells (BL), considered to be haemocytes precursors, thus suggesting that haemocytes were undergoing differentiation (Evariste et al., 2016). Although P3 was only detected in control samples and was therefore not included in the analysis of haemocytes responses to higher leachates concentrations, its presence indicates a certain level of metabolic activity. Despite BL cells typically having low organelle content, previous studies on haemocytes functionalities have shown that they may still exhibit some level of metabolic activity, including reduced oxidative and mitochondrial activities, as well as lower lysosomal content, supporting their classification as precursor cells (Evariste et al., 2016). In DG leachates, a fourth subpopulation (P4), likely consisted of small granulocytes with a higher decrease in metabolic activity. This finding is supported by previous studies on bivalves, which demonstrated that degradative enzymes such as esterase are primarily associated with haemocytes granules (Pipe, 1990; Wootton and Pipe, 2003). The high internal complexity observed in P4 in this study further supports the hypothesis of functional specialization and differentiation of haemocyte subpopulations in response to stressors.

The potential loss of membrane integrity reflected by the alterations in metabolic activity of exposed haemocytes was further evidenced by changes in cytoplasmic membrane potential in both subpopulations. Interferences within the CMP are critical, as membrane functional integrity is essential for energy storage, signalling, compartmentalization and structural support (Glazachev et al., 2012). The observed increases in CMP for GR and HY exposed to BAL and DG leachates suggest membrane depolarization, indicative of cellular damage and interference within membrane transport proteins. Such disruptions could involve channel blockage or the generation of ionic leaks (Wiederschain, 2011). Membrane depolarization has been previously observed in microalgae exposed to Bisphenol A, a compound widely used in plastic production as an antioxidant, flame retardant and plasticizer (Esperanza et al., 2020). The complex chemical mixtures in BAL and DG leachates, including additives such as cyclic amines like N`, N`-diphenylurea, may have contributed to membrane disruption. This compound in particular has previously been shown to degrade plasma membranes in *S. obliquus* (Jiang et al., 2023). Furthermore, an increase in CMP may also be linked to a rise in intracellular Ca^{2+}, as previously observed in green algae exposed to antibacterial agents. These antibacterial chemicals are commonly found in consumer plastic products and are known to activate intracellular Ca^{2+} levels, leading to membrane depolarization (González-Pleiter et al., 2017). This mechanism also suggests that the lower lysosomal content observed in this study could indicate lysosomal release, potentially driven by lysosomal endocytosis through a Ca^{2+}-regulated process. This process may serve as a compensatory response to the Ca^{2+} influx, playing a critical role in physiological functions such as membrane repair (Buratta et al., 2020).

Lysosomes are acidic organelles containing various enzymes (*e.g.*, hydrolases) that play a central role in cellular defence and detoxification (Kim et al., 2020). The reduced lysosomal content observed in this study supports the hypothesis that haemocytes were actively mobilizing their energy reserves to cope with cellular stress. Under such conditions, neutral lipids, which include triacylglycerol (TAG), cholesteryl esters (CE), and wax esters (WE), can be mobilized to support cellular defence mechanisms and energy metabolism through the action of lipases and hydrolases (Athenstaedt and Daum, 2006; Rolton and Ragg, 2020). The reduced NL content observed in both GR and HY exposed to high concentration of DG leachates in this study highlights the interconnected roles of lysosomal release and NL mobilization in cellular defence mechanisms and energy regulation. This finding is consistent with previous studies, where a decrease in NL content was observed in digestive glands of *M. edulis* after 1 day exposure to Cd (500 μg/L) (Fokina et al., 2013).

Mitochondria, essential for ATP production, metabolic processes, apoptosis, and ion homeostasis, are key targets of pollutant-induced toxicity (Meyer et al., 2013). In this study, exposure to BAL and DG leachates caused significant mitochondrial dysfunction in haemocytes, evidence by a reduction in MMP in the P1 subpopulation. A similar decrease in MMP was previously observed in *M. galloprovincialis* haemocytes exposed to ZnO (Canesi et al., 2012). One possible explanation is that Zn ions present in the leachates may interact with membrane thiol groups (-SH), disrupting intracellular ionic balance and compromising ion homeostasis, which could lead to bioenergetic stress (Auguste et al., 2018; Ji et al., 2019; Perry et al., 2011). Additionally, TiBP tentatively detected in BAL leachates, has been shown to reduce MMP in human cells *in vitro* by activating ROS pathways (Yuan et al., 2020). OPs like TiBP are also known to interact with cholinergic receptors in human cells, causing a reduction in MMP and suppression of NADH levels (Chan et al., 2006). Conversely, the increase in MMP observed in the subpopulation P2 exposed to BAL may also be linked to TiBP, as well as other compounds like dehydroabietic acid. TiBP has been reported to also increase MMP in the rotifer *B. plicatilis*, although the exact mechanism remains unclear. Either way, this increase was associated with mitochondrial damaged and reduced ATP content, suggesting mitochondrial impairment (Zhang et al., 2023). Similarly, in human cell lines (HEK 293) dehydroabietic acid and its derivatives were shown to induce membrane hyperpolarization by promoting K^+ efflux in response to initial depolarization triggered by Ca^{2+} influx (Ohwada et al., 2003).

Mitochondrial dysfunction is closely associated with ROS imbalance (Yu et al., 2013). ROS are byproducts of the aerobic metabolism and play a crucial role in maintaining cellular homeostasis through redox signalling at basal concentrations. However, an imbalance between ROS production and antioxidant defences can lead to oxidative stress (Regoli and Giuliani, 2014). In this study, a decrease in mitochondrial ROS levels was observed in both haemocytes' subpopulations exposed to BAL and DG leachates. This reduction may be a result of mitochondrial membrane dysfunction such as the depolarization observed in exposed haemocytes reflected by the loss of MMP. This suggests a loss of membrane integrity, which could potentially disrupt oxygen availability and impair NADPH-oxidase activity, thereby preventing ROS formation (Ayala et al., 2014). Indeed, mitochondria and the membrane-bound NADPH-oxidase are major source of ROS production (Zhang et al., 2022). ROS suppression was previously detected in oyster haemocytes exposed to pesticides due to NADPH inhibition (Baier-Anderson and Anderson, 2000). Furthermore, 2-MBT, tentatively detected in DG leachates, has been shown to inhibit NADH oxidation in bacteria (De Wever et al., 1994), suggesting 2-MBT potentially being one of the compounds responsible for the reduced ROS production observed in this study.

At the cytosol level, a reduction of ROS levels was only recorded in haemocytes exposed to DG leachates at the highest concentration of 10 %, while no ROS were formed for BAL leachates. The absence of cytosolic ROS formation in mussel haemocytes may be due to the chemical composition of the BAL and DG leachates, which might lack pro-oxidant compounds required to induce oxidative stress. The low concentrations of the leachates or the short exposure duration (24 h) used in this study might also not been sufficient to trigger a measurable oxidative response. In addition, mussels possess robust antioxidant defence mechanisms (*e.g.* enzymatic antioxidants such as superoxide dismutase and non-enzymatic antioxidants like glutathione) that can neutralize ROS, potentially preventing their accumulation even in response to a complex chemical mixture, as that present in both leachates.

A decrease and/or absence of ROS formation in cells is likely linked to a lack of oxidative damage to cellular components, as lipid peroxidation. Free radicals and ROS are known to oxidize lipids, crucial components of cell membranes that play key roles in various physiological processes, including tissue protection and resistance to exogenous substances (Chen et al., 2024; Li et al., 2024). This oxidation leads to LPO, a process involving the formation of lipid radicals and lipid hydroperoxides, which can damage membranes, disrupting signal transduction pathways and potentially causing cell death (Gaschler and Stockwell, 2016). In this study, a decrease in the formation of oxylradicals responsible for lipid peroxidation was observed in haemocytes exposed to both BAL and DG leachates, which goes in line with the decrease and absence of ROS formation observed in the mitochondria and cytosol, respectively. This decrease in LPO could result from either insufficient ROS formation or a rapid detoxification of ROS by the antioxidant defence system before they interact with polyunsaturated fatty acids in membrane lipids (Prokić et al., 2019). These findings highlight the interplay between antioxidant systems and the chemical composition of leachates in modulating oxidative stress responses in mussel haemocytes.

DNA is essential for regulating cellular processes that maintain balance and function within cells (Kim et al., 2024). Quantifying nucleic acids, such as DNA, is a reliable method for understanding cellular mechanisms, including apoptosis, where DNA fragmentation is a key marker (Ullal et al., 2010). Accordingly, measuring DNA content is considered a reliable indicator of primary DNA damage (Yuan et al., 2020). In this study, the higher DNA content observed in P2 exposed to both leachates may be associated with apoptosis due to the presence of fragmented DNA (Almeida et al., 2019). Similarity, elevated DNA content was reported in human cells exposed to TiBP (Yuan et al., 2020). However, apoptotic pathways may also result from MMP loss, which could lead to a reduction in DNA content, as observed in the subpopulation P1 (Nguyen et al., 2018). This mechanism aligns with established apoptotic pathways seen in *Mytlus spp.* haemocytes exposed to Cu, where loss of MMP was associated with the release of pro-apoptotic factors such as caspase activators (Nguyen et al., 2018), ultimately leading to lower DNA content (Plesca et al., 2008). Furthermore, as Michelangeli et al. (2025) previously suggested, complex chemical mixtures in tyre rubber and plastic leachates can modulate DNA-related apoptotic pathways in mussel haemocytes. This aligns with the findings in this study, where leachates containing diverse organic compounds may have similarly influenced DNA-related apoptotic mechanisms in *M. edulis* haemocytes.

4.3. Implications for the marine environment

Consumer products, including those of elastomeric composition, are a significant source of microplastic and chemical pollution through degradation and leaching processes in coastal waters (Andrady, 2022; Jacobs et al., 2015; Patrawoot et al., 2021). While the ecological impacts of conventional plastics have been widely studied, the specific effects of elastomer leachates on marine organisms remain poorly understood (Sørensen et al., 2023). This represents a critical knowledge gap, especially as the global production and disposal of plastic and elastomer-based products continues to rise. The present study addresses this gap by demonstrating that even short-term exposure to elastomer leachates can trigger measurable physiological disruptions in mussel haemocytes, indicative of potential sublethal toxicity.

These findings are consistent with previous work by Michelangeli et al. (2025), which reported similar immunotoxic effects following exposure to leachates from car tyre granulates, further supporting the sensitivity of haemocyte-based endpoints in detecting cellular stress induced by complex leachate mixtures. Most published studies investigating rubber leachates have focused on *in vivo* developmental endpoints (*e.g.*, embryo mortality or malformations) or biomarker responses (*e.g.* oxidative stress, neurotoxicity) in different invertebrate species (*e.g.* Capolupo et al., 2021; Rendell-Bhatti et al., 2021; Tallec et al., 2022). While these endpoints differ from our immune-focused approach using haemocytes, similar mechanistic responses, such as metabolic stress, lysosomal and membrane destabilization and cytotoxicity have been reported, suggesting a common toxicity profile across biological levels.

Together with the present study, these findings suggest that elastomer leachates can elicit consistent and biologically meaningful effects across different levels of biological organization and species. If such effects occur in marine environments, particularly under chronic or repeated exposures, they could impair immune function and overall fitness in marine invertebrates. Over time, this could translate into reduced resilience at population level, especially considering the complexity of additive mixtures leaching from rubber-based materials, with significant ecological implications. By applying *in vitro* assays, this study was able to detect sensitive biomarkers of toxicity, allowing the distinction of a clear link between elastomer leachates exposure and early sign of cellular stress, suggesting that elastomer pollution like other forms of plastic contamination, poses a real threat to marine ecosystems.

5. Conclusion

This study provides new evidence into how chemicals leaching from common rubber products, such as balloons (BAL) and dishwashing gloves (DG), can negatively affect the immune cells of the marine mussel *Mytilus edulis*. This study found that both types of leachates contained complex mixtures of substances, including trace metals and organic compounds, like benzothiazoles and cyclic amines. Despite compositional differences, both leachates induced comparable adverse effects on haemocyte subpopulations, including reduced viability, disrupted mitochondrial function, altered energy metabolism, and DNA content changes. These results suggest that the chemical mixtures may activate common toxicity pathways, particularly those related to mitochondrial and metabolic stress. Although identifying the most toxic individual compounds remains challenging, the study underscores the ecological risk posed by everyday rubber products in marine organisms. Importantly, this research demonstrates the potential utility of using mussel haemocytes as sensitive *in vitro* toxicity screening models for marine biomonitoring. However *in vitro* systems do not capture the full complexity of natural environments, but allow for sensitive, mechanistic assessment of toxic effects under controlled conditions. This makes them valuable early warning signals, helping to detect environmental stress before effects become visible at the population or ecosystem level. Combining *in vitro* data, such as that in the present study, with results from *in vivo* studies, field-based environmental monitoring and advanced chemical profiling, will be critical for capturing real-world implications of pollution associated with rubber polymers. This integrated approach can help identify high-risk chemicals, evaluate their persistence and determine their impacts across multiple species and trophic levels. Such a comprehensive strategy is essential for improving risk assessment and informing evidence-based policies aimed at protecting marine ecosystems from the growing threat of polymer-associated contaminants.

CRediT authorship contribution statement

M. Elisabetta Michelangeli: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sicco H. Brandsma:** Writing – review & editing, Validation, Investigation. **Maria Margalef:** Writing – review & editing, Validation, Investigation. **Sebastian Kuehr:** Writing – review & editing, Validation, Investigation. **Davide Spanu:** Writing – review & editing, Validation, Investigation. **Tânia Gomes:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

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Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2025.118298.

Data availability

Data will be made available on request.

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