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Looking beyond the obvious: The ecotoxicological impact of the leachate from fishing nets and cables in the marine mussel Mytilus galloprovincialis

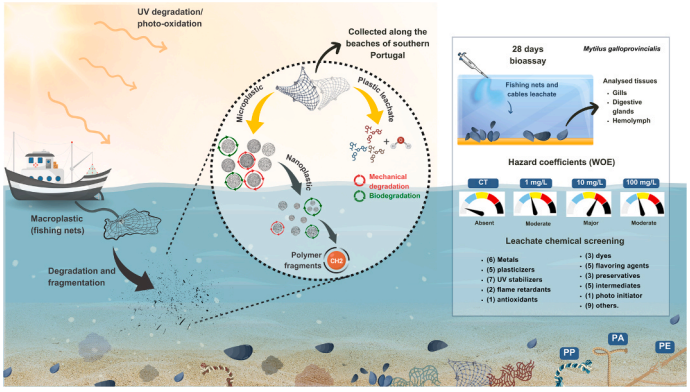
Juliano M. Vilke [a,b], Tainá G. Fonseca [a], Gilberto D. Alkimin [a], Joanna M. Gonçalves [a], Carlos Edo [a,c], Giuseppe d' Errico [d], Fredric Södergren Seilitz [f], Anna Rotander [f], Maura Benedetti [d], Francesco Regoli [d], Karim H. Lüchmann [e], Maria João Bebianno [a,*]

[a] Centre for Marine and Environmental Research – CIMA/ARNET – Infrastructure Network in Aquatic Research, University of Algarve, Campus de Gambelas, Faro 8000-139, Portugal
[b] Multicenter Program in Postgraduate in Biochemistry and Molecular Biology – PMBqBM, Santa Catarina State University, Lages 88520-000, Brazil
[c] Department of Analytical Chemistry, Physical Chemistry and Chemical Engineering, University of Alcalá, Alcalá de Henares, E-28871 Madrid, Spain
[d] Dipartimento di Scienze Della Vita e Dell'Ambiente, Università Politecnica Delle Marche, Via Brecce Bianche, Ancona, Italy
[e] Department of Scientific and Technological Education, Santa Catarina State University, Florianopolis 88035-001, Brazil
[f] Man-Technology-Environment (MTM) Research Centre, Örebro University, SE-701 82 Örebro, Sweden

HIGHLIGHTS

- Fishing nets and cables were mainly composed of PP, PE, and PA.
- Fishing nets and cables release a myriad of orgânic compounds and metals.
- Leachate impairs antioxidant defenses, leading to oxidative damage and genotoxicity.
- Biochemical biomarkers were disrupted at 1 mg/L of leachate concentration.
- PCA and WOE analysis revealed high toxicity for 10 and 100 mg/L of leachate treatments.

GRAPHICAL ABSTRACT



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ABSTRACT

Once in the marine environment, fishing nets and cables undergo weathering, breaking down into micro and nano-size particles and leaching plastic additives, which negatively affect marine biota. This study aims to unravel the ecotoxicological impact of different concentrations of leachate obtained from abandoned or lost fishing nets and cables in the mussel *Mytilus galloprovincialis* under long-term exposure (28 days). Biochemical biomarkers linked to antioxidant defense system, xenobiotic biotransformation, oxidative damage, genotoxicity, and neurotoxicity were evaluated in different mussel tissues. The chemical nature of the fishing nets and cables and the chemical composition of the leachate were assessed and metals, plasticizers, UV stabilizers, flame retardants, antioxidants, dyes, flavoring agents, preservatives, intermediates and photo initiators were detected. The

* Corresponding author.
E-mail address: mbebian@ualg.pt (M.J. Bebianno).

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leachate severely affected the antioxidant and biotransformation systems in mussels' tissues. Following exposure to 1 mg·L^{-1} of leachate, mussels' defense system was enhanced to prevent oxidative damage. In contrast, in mussels exposed to 10 and 100 mg·L^{-1} of leachate, defenses failed to overcome pro-oxidant molecules, resulting in genotoxicity and oxidative damage. Principal component analysis (PCA) and Weight of Evidence (WOE) evaluation confirmed that mussels were significantly affected by the leachate being the hazard of the leachate concentrations of 10 mg·L^{-1} ranked as major, while 1 and 100 mg·L^{-1} was moderate. These results highlighted that the leachate from fishing nets and cables can be a threat to the heath of the mussel *M. galloprovincialis*.

1. Introduction

Plastic pollution is at the center of great discussions in diverse spheres of society due to its significant environmental, social, and economic impact. Since 1950, about 400.3 million tons of plastics have been produced yearly worldwide [1], 266 -fold than in the 50 s, representing between 75% and 90% of total marine debris in the coastal zone on the surface of the water column, and on the seabed [2-4]. A large proportion (75–86%) of the plastic found in the ocean is fishing artifacts [5] abandoned, lost, or otherwise discarded fishing gear (ALDFG), cables, and other plastic-based objects used during fishing operations. These ALDFG break down into smaller plastic particles at sea, known as microplastics (i.e., particles less than 5 mm; MPs) and nanoplastics (i.e., particles smaller than 100 nm; NPs) [6], through processes such as mechanical, biological, or photodegradation. In addition to producing MPs and NPs, the breakdown of plastics causes the polymer chains to break [7], leaching the plastic additives into the seawater.

According to Andrady and Neal [8], 90% of the MPs and NPs found in the ocean are polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), and polyethylene terephthalate (PET). These plastics contain over 400 chemicals used as additives to alter the polymer properties, improving its production, stability, malleability, and durability (www.echa.europa.eu). The main classes of polymer additives are plasticizers (e.g., phthalates), flame retardants (e.g., brominated flame retardants, phosphate esters, metals), dyes (e.g., metal-based colorants), antioxidants (e.g., irganox, cyanox, irgafos), UV stabilizers (e.g., nonylphenols), and antistatic agents (e.g., metallic stearates, waxes) [9]. In addition to the release of polymer additives, the plastic itself can also be a vector for other toxic chemicals and microbial agents present in the water, potentially causing other adverse effects on marine biota [10,11].

Indeed, it is well known that plastics interact with marine organisms by ingestion or entanglement, causing adverse consequences [12]. Nonetheless, emphasis is placed herein on the impact of the chemical composition of plastic leachate since the leachate represents an unknown hazard to marine organisms' health due to its myriad of chemicals.

In fact, tire wear particles leachate caused a significant decrease in phytoplankton growth [13], a substantial increase in the mortality rate of copepods [14], and a negative impact on oyster *Crassostrea gigas* such as reduction of filtration and respiration [15]. Disposable face masks leachate triggered oxidative damage, genotoxicity, and cytotoxicity on the marine mussel *Mytilus galloprovincialis* [16]. On the other hand, PP leachate causes negative chemotaxis behaviour in mussels *Mytilus edulis* [17], while the leachate produced from food packaging causes reproductive and neurobehavioral effects in zebrafish *Danio rerio* [18]. So far, the effects of the leachate from the aging of plastic on the biological responses of marine mussels are scarce.

The present study aimed to assess the biological responses of mussels *Mytilus galloprovincialis* exposed, for 28 days, to different concentrations of leachates obtained from abandoned or lost fishing nets and cables. The hypothesis was that the leachate can affect the mussel enzymatic antioxidant defense system (catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities), its ancillary enzymes (glucose-6-phosphate dehydrogenase (G6PDH), and glutathione reductase (GR) activities) and the phase II xenobiotic biotransformation enzyme (glutathione *S*-transferases (GST)) activity, as well as, neurotoxicity (acetylcholinesterase (AChE)), oxidative damage (lipid peroxidation (LPO)), and genotoxicity (DNA damage). The chemical components of the leachate and the polymer type of the fishing and cable nets were characterized to identify possible relationships between the polymer type and the chemical composition of the leachate with biomarker responses in mussel tissues. The results will contribute to a better understanding of the environmental impact of abandoned plastic debris on the marine ecosystem.

2. Materials and methods

2.1. Plastics collection and processing

Abandoned or lost fishing nets and cables were handpicked and collected from four beaches on the southern coast of Portugal: Praia da Falésia (37°05′12″N, 8°10′12″W); Praia da Ilha de Tavira (37°5'N 7°40'O); Praia de Faro (37°00′30.6″N 7°59′39.6″W); and Praia da Fuseta (37°03'N 7°45'O). All plastic items were transported to the laboratory, counted, weighed, and separated into seven color groups (green, blue, red, orange, black, yellow, and transparent). The green plastic nets were the most abundant item (70.2%), followed by blue, red, orange, black, yellow, and transparent, representing 4.4%, 2.6%, 6.6%, 14.5%, 0.3%, and 1.5%, respectively, of the total fishing gear collected.

2.2. Polymer identification

Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy was used to identify the composition of the polymers of the fishing nets and cables collected. A subsample of each color was analyzed in a Thermo-Scientific Nicolet iS10 equipped with Smart iTR-Diamond using Attenuated Total Reflectance (ATR) to identify the chemical composition of the polymer. The conditions chosen for the analysis were 20 scans, 8 cm^{-1} of spectral resolution, a 4000–550 cm^{-1} spectral range, and transmittance mode. A minimum of three particles per color was evaluated, and their spectra were compared with the existing databases in the OMNIC 9 software (Thermo Scientific, USA). The program used Pearson correlation to compare the spectra, resulting in a positive with a minimum of 65% matching, as established by [19].

2.3. Leachate preparation

Plastic leachates were prepared according to the procedure described by [20] with minor changes. Briefly, samples of each polymer colour were cut into < 10 mm pieces. To prepare a solution of 10 g L^{-1} of leachate, 10 g of each plastic color was added into 1 L of sterilized filtered seawater (FSW). The plastics solutions were placed in a rotating incubator (~ 150 rpm) at room temperature (~ 25 ºC) for 72 h in the dark with constant agitation. Although in the marine environment plastics remain in seawater for longer periods of time, the aging period used (72 h) was in accordance with what was established by [20]. The leachate from each color was individually filtered using a glass microfiber filter (Whatman 0.8 μm) to remove the plastic particles. A blank sample was also prepared in paralled. The leachates were kept in glass flasks at − 20 ºC until further use. Finally, a mixture (10 g·L^{-1}) was prepared, mixing all colors maintaining the environmental proportion of

the plastics described in the 2.1.1 section. A detailed figure of the process is in Fig. S1.

2.4. Chemicals screening in the leachate

2.4.1. Metal content

A two-step procedure was employed to quantify the metal content in the leachate. A sample (200 mL) of the main leachate mixture (10 g·L^{-1} of plastic) was digested in duplicate using nitric acid. Subsequently, the quantification of the concentration of metals and metalloids, namely Cu, Fe, Mn, Ni, Pb, and Zn, was performed using inductively coupled plasma mass spectrometry (ICP-MS) following the guidelines outlined in the USEPA 2008 method. A stoichiometric calculation based on the measured values, including the estimate of total mineralization and the sum of Ca and Mg, was applied to determine the metal concentrations. The concentrations of the metals are expressed as μg·L^{-1} of plastic leachate.

2.4.2. Organic compounds analysis

Details on chemicals, standard solutions, and analytical procedures are presented in the supplementary information (SI). Briefly, 200 mL of leachates underwent extraction on glass HLB cartridges. Prior to elution, internal standard solutions comprising polycyclic aromatic hydrocarbons (PAHs), organophosphorus flame retardants (OPFR), benzophenone, bisphenol A (BPA), and tetrabromobisphenol A (TBBPA) were incorporated. Elution was carried out using hexane: dichloromethane and hexane: acetone, following which extracts were divided for gas chromatography (GC) and liquid chromatography (LC) analysis. GC-High Resolution Mass Spectrometry (HRMS) analysis involved batch standards preparation and data processing through MSConvert GUI and MS-DIAL software, followed by analysis on a GC Orbitrap® MS (Thermo Scientific™ Q Exactive™ GC system) operated in Electron Ionization (EI) mode. Suspect screening relied on HRMS spectral libraries and NIST14 matching criteria for identification, as proposed by Price et al. [21]. LC data underwent processing with UNIFI 1.9.4 (Waters, Milford, Massachusetts, USA), and exact mass matches were in-silico fragmented, with fragments matched within 6 mDa, following the approach described by Koelmel et al. [22]. The confidence level of GC-orbitrap data was determined according to a proposed system by Koelmel et al. [22], where Level 1 implies confirmed identification, Level 2 implies probable structure or close isomer, and Level 3 implies tentative candidate. Additional batch standards were prepared for LC analysis, including bisphenols, TBBPA, b-HBCD, PFAS, UV-328, 6 PPD quinone, and 1-phenyl-4-(1-phenylethyl)tetralin. Acetonitrile extracts were diluted and analyzed using an Acquity UPLC I-class coupled to a Xevo G2-XS Quadrupole Time-of-Flight (QTOF) mass spectrometer (both Waters Corporation, Milford, Massachusetts, USA). Comprehensive experimental and processing details are available in the supplementary information.

2.5. Experimental design

Adult mussels (*M. galloprovincialis*, 6.2 ± 0.5 cm length) were handpicked in winter from the Ria Formosa Lagoon (Faro, Southern Coast, Portugal, 37°00′30.6″N 7°59′39.6″W) in November 2021, and immediately transported alive to the Laboratory of Ecotoxicology and Environmental Chemistry at CIMA (University of Algarve), where they were first scraped to clean the fouling. The mussels that were not in the reproductive period were placed (density = 2 mussels·L^{-1}) into twelve 30 L glass aquaria filled with 25 L of filtered seawater with salinity of 35, temperature of 15 ºC, and pH of 8.0 ± 0.02, constant aeration in light: dark cycles of 12:12 h for one-week acclimation period. The seawater was renewed every 48 h, and the mussels were fed with marine microalgae *Tetraselmis chuii* cultured from the Experimental Laboratory for Aquatic Organisms of the University of Algarve once in a renovation day (150.000 cells·mussel $^{-1}$).

After the acclimation period, mussels were exposed for 28 days in a triplicate design (25 L seawater, density = 2 mussels·L^{-1}) to a leachate mixture at three different concentrations: an environmentally relevant concentration of 1 mg·L^{-1} [23] and higher concentrations of 10 and 100 mg·L^{-1}representing worse scenarios of plastic pollution, along with a control group. The light cycle was 12 h light and 12 h dark. The water was renewed every 48 h, and the leachate concentrations re-established. During the exposure period, the animals were only fed by the natural phytoplankton present in the water. Water quality was checked throughout the experiment by measuring dissolved oxygen concentration, pH, salinity, and temperature with a multimeter (Odeon, PONSEL, FR). Dissolved oxygen was 99.1 ± 2.6%, pH was 7.96 ± 0.06, and salinity and temperature were 35 and 15 ºC, respectively.

At 0, 3, 7, 14, 21, and 28 days of exposure, 16 mussels per group (5 ~ 6 per replicate) were randomly collected. Six mussels were sacrificed, and their gills and digestive glands dissected, immediately frozen in liquid nitrogen, and kept at − 80 ºC for further biochemical analysis. Another five mussels from each experimental group were randomly collected, and the hemolymph was extracted from the adductor muscle using a 3 mL sterile syringe, divided into two aliquots, and kept on ice; the first tube was filled with anti-aggregation solution (171 mM NaCl 0.2 M Tris; 0.15% v/v HCl 1 N; 24 mM EDTA; pH 7.6) for the analysis of cell viability (Trypan blue exclusion assay) and the other for the determination of DNA damage using the comet assay. The gills of the same animals were quickly dissected and immediately snap-frozen in liquid nitrogen for neurotoxic analyses. The frozen tissues were stored at − 80 ºC until biochemical analysis. Finally, five mussels were used to determine the condition index (CI) of *M. galloprovincialis* as an indicator of general mussel health.

2.6. Condition index

To calculate the condition index (CI), five mussels from each group and setup time were collected to determine their physiological status at the beginning (day 0) and after 3, 7, 14, 21, and 28 days of exposure. The CI was calculated as the proportion (%) of the ratio between the wet weight tissues (g) and the total weight (g) of each organism, according to Gomes et al. [24] and Gonçalves et al. [25].

2.7. Biochemical biomarkers

2.7.1. Antioxidant and biotransformation enzyme activities

Gills and digestive gland of mussels (n = 6 per group) were individually homogenized on ice with 3 mL of Tris-sucrose buffer (20 mM Tris, 0.5 M sucrose, 0.075 M KCl, 1 mM DTT, 1 mM EDTA, pH 7.6) using a TissueLyser Star-Beater (VWR, USA) device. The homogenate was centrifuged at 4 ºC for 15 min at 500 *g*, and the supernatant re-centrifuged at 4 ºC for 45 min at 12,000 *g*. The supernatant was then divided into six aliquots and stored at − 80 °C for subsequent enzymatic assays.

Antioxidant and biotransformation enzymes activities were determined spectrophotometrically. SOD activity was accessed by the decrease in absorbance of the substrate cytochrome *c* by competition with the xanthine oxidase/hypoxanthine system, measured at 550 nm [26]. CAT activity was determined by measuring the rate of H_2O_2 disappearance, revealing its consumption at 240 nm [27]. The Se-GPx activity was measured by the reduction of oxidized glutathione linked to the oxidation of NADPH in the presence of excess GR, using H_2O_2 as substrate according to the method described by McFarland et al. [28]. G6PDH activity was determined according to the assay described by [29], adapted by Almeida et al. [30], through the increase in absorbance at 340 nm, generated by the reduction of $NADP^+$ to NADPH. GR activity was quantified according to the protocol of Carlberg and Mannervik [31] by the oxidation of NADPH at 340 nm. GST activity was measured by increasing absorbance at 340 nm, using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate, using the method described by Habig et al. [32],

adapted for a microplate reader.

The enzymatic assays were performed in duplicate for the determination of SOD and CAT activities using a spectrophotometer (Jasco V-650 spectrophotometer), while Se-GPx, G6PDH, GR, and GST were performed in triplicate in a 96-well plate reader (Infinite M200 Pro, TECAN®). Protein concentration was measured using the **Bradford method** [33], using bovine serum albumin as a standard. Results of each biomarker are expressed in the following units: SOD (U mg^{-1} protein), CAT (μmol min^{-1} mg^{-1} protein), GPx (nmol min^{-1}mg $protein^{-1}$), GR (U mg^{-1} protein), G6PDH (U mg^{-1} protein), GST (CDNB nmol min^{-1} mg^{-1} protein).

2.7.2. Neurotoxicity

For AChE activity determination, mussels (n = 5 per treatment) were individually homogenized in 100 mM Tris-HCl buffer (pH 8.0) and 0.1% Triton using a TissueLyser (Star-Beater, VWR, USA). The homogenate was centrifuged at 12,000 g for 30 min at 4 °C and further separated into two aliquots for total protein determination [33] and AChE activity [34]. The acetylcholine (ATC) cleavage product by AChE was determined at 405 nm [34,35]. Results are expressed as nmol ATC min^{-1} mg^{-1} protein.

2.7.3. Oxidative damage

For the quantification of malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) content, the gills and digestive glands of six mussels per treatment were individually homogenized in a Tris-HCl buffer (20 mM, pH 8.6) with butylated hydroxytoluene (BHT) using a Star-Beater VWR, USA. The homogenate was centrifuged at 30,000 *g* for 45 min at 4 °C. The supernatant was separated into two aliquots for total protein quantification [33] and determination of oxidative damage [36], adapted for microplate reading. The absorbance of MDA and 4-HNE was measured at 586 nm in a microplate reader (Infinite M200Pro, TECAN®). Results are expressed as nmol MDA mg^{-1} protein.

2.7.4. Cell viability and genotoxicity

Gomes et al. [24] described the standard procedure adopted to determine genotoxicity using the alkaline comet assay. Fonseca et al. [37] and Gonçalves et al. [25] described detailed information regarding these procedures. Briefly, for the cell viability assay, cell suspensions were stained with trypan blue in a 1:1 proportion (cell suspension: Trypan Blue 0.4%). Further, cells were counted using a Neubauer chamber (100 cells per specimen) and a manual hemocytometer under light microscopy (Olympus CH30). The average percentage of live cells for all treatments was above 80%. For the comet assay, firstly, cell suspensions were centrifuged for 3 min at 350 rpm, and further pellets were resuspended in agarose and placed in pre-cleaned slides. Afterwards, electrophoresis and lysis were followed by a neutralizing step. Finally, DAPI was used to dye the slides, and the pictures (50 cells per slide) were taken using an optical fluorescence microscope (Axiovert S100) coupled with a camera (Sony), and % of DNA tail was calculated. Scoring analysis was performed using Imaging Software Komet 7.1 (Kinetic Imaging Ltd). The comet assay is herein presented as DNA tail %.

2.8. Statistical analysis

Data obtained was first tested for normality (Shapiro-Wilk test), followed by the homogeneity of variance (Bartlett's test). Further, a two-way ANOVA was applied, followed by a pairwise post-hoc Tukey's multiple comparison tests, to identify differences among groups by comparing means of controls and means of leachate-treated groups ($p < 0.05$). Principal Component Analysis (PCA) was performed according to Durou et al. [38], correlation coefficients were significant when they were higher than $\sqrt{d/n}$ (i.e., d is the number of principal components; n is the number of variables), thus equal to 0.19 and 0.21 for the gills and digestive glands, respectively ($p < 0.05$). Statistical analysis was performed using GraphPad Prism (v. 9.0).

2.9. Weight of evidence elaboration

The biomarkers results obtained in gills and digestive glands were elaborated through the specific Line of Evidence (LOE) using a Weight of Evidence approach (WOE). In this LOE, analyzed biomarkers were assigned a weighting between 1 and 3, based on the relevance of the biological endpoint [39]. As detailed in the Flow Chart (Fig. S1), for each biomarker, the values measured in 'exposed' organisms were compared with those of the controls (n ⩾ 3) (3) and corrected for the statistical significance of the difference, according to function Z(i) (4); the 'effect' was calculated as the ratio between the measured percentage variation and the relative threshold of the response (5), and the weighted Effect (Effectw) was finally obtained considering the weighting of the biomarker (6). Based on expert judgment, variations of each biomarker were assigned to one of five classes of hazard, depending on the calculated Effects: classes A and B (Absent and Slight hazard) for Effect < 1 and < 1.5 respectively (lower, but possibly close to threshold); class C (Moderate hazard) for Effect ranging between 1.5 and 2.5 (up to two-fold compared to the threshold for statistically significant changes), class D (Major hazard) for Effect ranging between 2.5 and 4, and class E (Severe hazard) for biomarkers showing an effect > 4 (8). The elaboration of the HQBM did not consider the contribution of biomarkers in classes A and B, calculated the average of Effectw for those in class C, and added the summation (Σ) of Effectw for the responses in classes D and E (10). The class of hazard for biomarkers was obtained by multiplying the percentage of individual biomarkers within each of the five classes by a factor increasing from 1 to 6 (10). This value will range between 100 (corresponding to hazard absent/white when 100% of the analyzed biomarkers are in class A) and 600 (severe/black if 100% of the analyzed biomarkers are in class E) (11).

3. Results

3.1. Polymer identification

The FTIR spectra showed that the plastic composition varied according to the polymer color. The green, blue, red, and black nets and cables were mainly formed by PP. In contrast, the orange nets and cables were made of PP and PE, and the transparent and yellow plastics were composed of PA (Nylon 6,6). The minimum matching obtained for identification was 80% of correspondence for all the colors. All spectra graphics are presented in the Supplementary Material (Fig. S2).

3.2. Chemical screening in leachate

3.2.1. Metal content

Metal concentrations in the main mixture leachate are displayed in Table 1. Cu, Fe, Mn, Ni, Pb, and Zn were detected in the leachate. Fe showed the highest concentration (548 μg·L^{-1}) in the leachate, followed by Zn, Mn, Cu, Ni, and Pb (200, 31.20, 27, 13, and 2.50 μg·L^{-1}, respectively).

3.2.2. Organic compounds analysis

The characterization of the organic compounds was assessed in the individual color leachate samples and in the mixture. Results generated

Table 1
Metal content in the mixture of the leachate from fishing nets and cables.

Metal content in the main mixture leachate (μg·L^{-1})						
Treatment						
Metals	**Mn**	**Fe**	**Ni**	**Cu**	**Zn**	**Pb**
Blank	0.27	1.72	0.28	1.77	77.5	0.18
Leachate (10 g·L^{-1})	31.20	548	13	27	200	2.50

with confidence levels 1 and 2 (confirmed structure and probable structure, respectively) using the HRMS library are shown in Table 2. A. NIST14 LRMS library was used to identify other organic compounds with confidence levels 3 (tentative structure), as depicted in Table 2. B.

A broad range of compounds were identified (41) among the two procedures. These chemicals were further classified into 10 categories according to their functionalities and properties, being (5) plasticizers, (7) UV stabilizers, (2) flame retardants, (1) antioxidants, (3) dyes, (5) flavoring agents, (3) preservatives, (5) intermediates, (1) photo initiator, and (9) others.

Three compounds were quantified or semi-quantified, with the plasticizer diethyl phthalate concentrations ranging from 440 ng·L^{-1} in the orange leachate to 1610 ng·L^{-1} in the red leachate, while the concentration of the leachate mixture was 920 ng·L^{-1} (Table 2 A). In contrast, benzophenone concentrations were lower, ranging from 225 to 515 ng·L^{-1}, for black and orange, respectively. For the leachate mixture, the benzophenone concentration was 295 ng·L^{-1}. Furthermore, the concentrations of tris(2-chloroethyl) phosphate showed a higher variability between samples, reaching 2715 ng·L^{-1} for the orange leachate and a concentration of 1210 ng·L^{-1} in the mixture. The remaining compounds present in both Table (2. A – B) were identified and not quantified (ID) or are presented in relation to the blank signal.

3.3. General health status of mussels

No significant differences in the CI were detected among controls and leachate-treated mussels. CI levels for the controls ranged between 22.1 ± 4.1 and 30.5 ± 6.8 ($p > 0.05$). For the leachate treatments, the CI values ranged between 24.3 ± 5.9 and 30.6 ± 6.8, 24 ± 4.5 and 28.3 ± 2, and 23.2 ± 4.6 and 29.8 ± 3.8 for mussels exposed to 1, 10, and 100 mg·L^{-1} respectively and data is presented as Supplementary Material (Fig. S3).

Table 2
Classes of chemical compounds (ng·L-1) identified using in-house and external GC-HRMS libraries, confidence level 1 – confirmed structure, and confidence level 2 – probable structure (2. A), and classes of chemical compounds identified using NIST14 LRMS library, confidence level 3 – tentative structure (2. B).

Class	Organic compounds	Black	Blue	Red	Orange	White	Green	Yellow	Mix
GC-HRMS									
Plasticizers	Acetyl tributyl citrate	-	-	ID	-	-	-	-	-
	Diethyl phthalate	1095	570	1610	440	460	895	1155	920
UV stabilizers	Benzothiazole[1]	-	0.6	0.7	0.7	-	1	-	-
	2-(Methylthio) benzothiazole[1]	-	0.8	1	1	-	0.7	-	0.6
	4-tert-Octylphenol[1]	-	-	1	0.2	-	-	-	-
	Benzophenone	225	430	500	515	395	440	435	295
	Enzacamene[1]	-	-	-	-	1	-	0.3	-
Flame retardants	Tris(2-chloroethyl) phosphate	1015	1595	1325	2715	890	895	1395	1210
Antioxidant	Tris(nonylphenyl) phosphite	ID	-	-	-	-	-	-	-
Dyes	o-Toluidine	-	-	-	-	-	-	ID	-
	2-Chloroaniline	-	-	-	-	-	-	ID	-
Flavouring agents	Coumarin[1]	-	-	-	0.2	-	1	-	0.2
Others	Caffeine[1]	1	0.1	0.1	0.1	0.2	0.1	0.6	0.2
LC-HRMS[1]									
Flame retardants	Triphenylphosphine oxide	-	ID	-	-	-	-	-	-
Preservative	1,2-Benzisothiazolin-3-one	-	-	0.8	1	-	0.9	-	-
	Ethylparaben	0.1	0.1	0.2	0.1	1	-	0.2	-
	Methylparaben	0.1	0.1	0.1	-	1	-	0.1	-
Dyes	N,N-Dimethylaniline	0.2	0.5	0.3	1	-	0.3	0.3	0.3
UV stabilizers	Bis(2,2,6,6-tetramethyl-4-piperidyl) sebacate	0.2	0.2	0.5	-	-	0.2	0.3	1
Other	1,3:2,4-Bis(3,4-dimethylbenzylidene)-D-sorbitol	-	-	-	ID	-	-	-	-

All values in black and identifications (ID) are based on > 8-fold blank signal. Values in red are below 8-fold blank signal.1 Values indicate relative blank fold concentrations to the extract with the highest blank fold. – in the cells indicate not detected above 8 blank fold.

Class	Organic compounds	Black	Blue	Red	Orange	White	Green	Yellow	Mix
GC-HRMS									
Plasticizer	Phthalic acid, di(oct-3-yl) ester	1.0	-	-	-	-	-	-	0.4
	Phthalic acid, di(2-propylpentyl) ester	1.0	-	-	-	-	-	-	0.4
Intermediates	2-Cyclohexen-1-one, 4-hydroxy-3,5,6-trimethyl-4-(3-oxo-1-butenyl)-	0.2	-	-	0.2	0.3	1.0	0.4	0.3
	2(3 H)-Benzothiazolone	0.2	0.3	0.8	0.5	-	1.0	0.2	0.7
	Benzenesulfonanilide	0.7	1.0	0.6	0.6	0.3	-	0.4	0.7
Flavouring agents	Cyclopentaneacetic acid, 3-oxo-2-pentyl-, methyl ester	0.4	-	0.4	-	-	0.5	1.0	0.5
	2,6,6-Trimethyl-2-cyclohexene-1,4-dione	0.1	0.2	0.6	0.2	0.9	1.0	0.5	0.2
	Cyclohexanol, 4-methyl-1-(1-methylethyl)-	-	0.3	1.0	0.1	-	0.9	0.1	0.1
	2-Butanone, 3-phenyl-	-	-	-	-	0.4	0.4	1.0	-
Photo initiator	2'-Hydroxy-4',5'-dimethylacetophenone	0.2	0.2	0.8	0.2	-	0.6	1.0	0.3
Others	Cholesterol	-	-	1.0	0.5	-	-	-	1.0
	Pyridine, 2-ethyl-4,6-dimethyl-	0.4	0.3	0.4	1.0	-	0.3	0.4	0.3
	Pyridine, 2,4,6-trimethyl-	0.4	0.3	0.4	1.0	-	0.3	0.4	0.3
	Glycine, N-(2-ethoxy-2-oxoethyl)-N-phenyl-, ethyl ester	1.0	-	-	-	-	-	-	-
LC-HRMS									
Plasticizer	Dibenzyl ether	-	-	-	-	ID	-	-	-
UV stabilizers	Bisoctrizole	-	1	0.6	-	-	-	-	-
Intermediate	N,N-Dimethyl-1-octadecanamine	-	-	-	-	-	-	ID	-
	N,N-Dimethyl-1-hexadecanamine	-	-	1	-	-	-	-	0.6
Other	Isobornyl acrylate	-	0.5	0.9	0.6	-	1	0.8	-
	2,2-Dimethoxy-1,2-diphenylethanone	-	-	-	-	ID	-	-	-
	26-(4-Nonylphenoxy)− 3,6,9,12,15,18,21,24-octaoxahexacosan-1-ol	-	-	-	-	-	-	1	0.3

The numbers indicate relative blank fold concentrations to the extract with the highest blank fold (1.0). – in the cells indicate below 8 blank fold concentrations.

3.4. Antioxidant and biotransformation enzyme activities

Overall, there were no significant changes in the activities of all antioxidant and biotransformation enzymes within the control groups (CT) over the exposure time in the gills and digestive gland ($p > 0.05$, Fig. 1 and Fig. 2). In contrast in the exposed mussels, two-way ANOVA analysis revealed a significant effect of time and concentration for all analyzed enzymes ($p < 0.0001$).

Regarding the comparison of SOD activity along the time of exposure, in the gills of mussels exposed to 1 mg·L^{-1} of leachate, SOD activity increased after 21 days of exposure ($p = 0.0002$), while mussels exposed to 10 mg/L showed a significant increase after 3 days of exposure ($p = 0.0004$) (Fig. 1A). In contrast, in the digestive gland, SOD activity increased following 21 days of exposure to the concentrations of 1 and 10 mg·L^{-1} of leachate ($p = 0.0056$ and $p = 0.0002$, respectively), with its activity remaining significantly higher after 28 days of exposure to 10 mg·L^{-1} of leachate ($p = 0.0108$) (Fig. 1B).

CAT activity in the gills of mussels exposed to 1 mg·L^{-1} of leachate

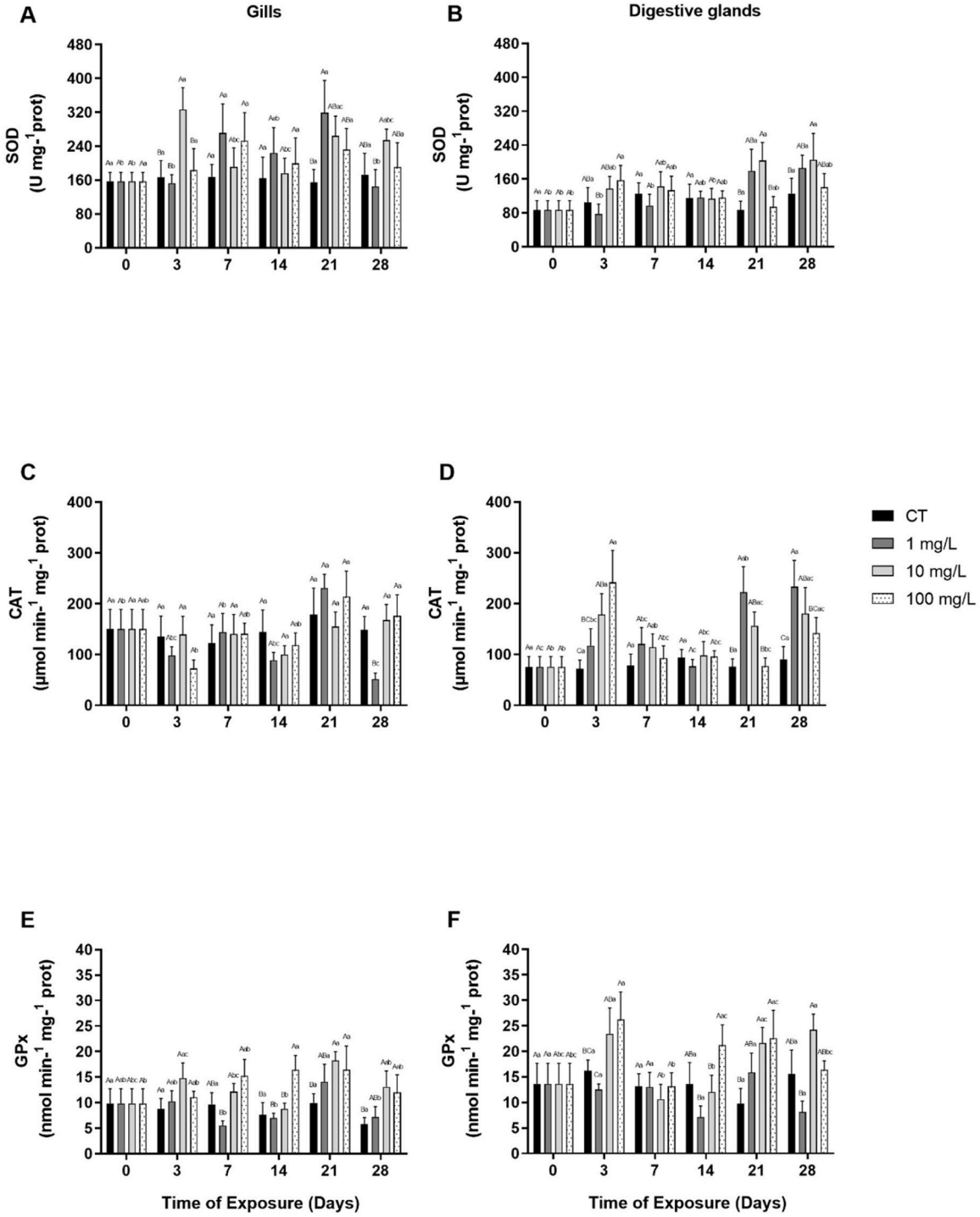

Fig. 1. Biochemical biomarkers SOD, CAT, GPx activities in gills (A, C, E) and digestive glands (B, D, F) of *Mytilus galloprovincialis*, unexposed (CT) and exposed to three concentrations of leachate (1, 10, and 100 mg·L^{-1}) for 28 days. Different capital letters indicate a significant difference among treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment among times (ANOVA; $p < 0.05$).

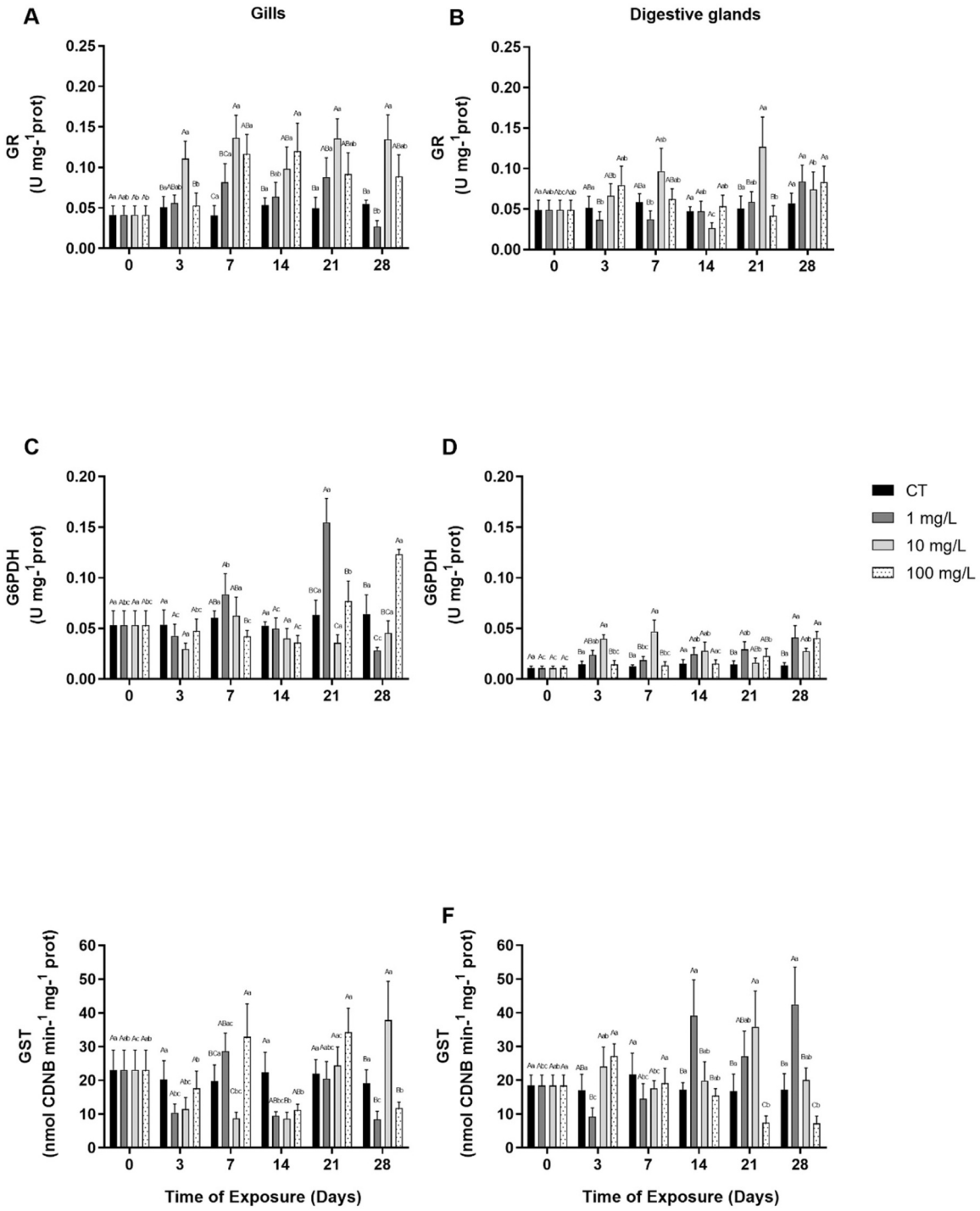

Fig. 2. Biochemical biomarkers GR, G6PDH, and GST activities in gills (A, C, E) and digestive glands (B, D, F) of *Mytilus galloprovincialis*, unexposed (CT) and exposed to three concentrations of leachate (1, 10, and 100 mg·L^{-1}) for 28 days. Different capital letters indicate a significant difference among treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment among times (ANOVA; $p < 0.05$).

decreased after 28 days ($p = 0.0044$) (Fig. 1C). On the other hand, in the digestive gland, CAT activity (Fig. 1D) was significantly higher in mussels exposed to 1 mg·L^{-1} of leachate after 21 and 28 days of exposure ($p = 0.0002$ and $p < 0.0001$, respectively), while in those exposed to the intermediate concentration (10 mg·L^{-1}), the enzymatic activity increased earlier (after 3, and 28 days of exposure) ($p < 0.0001$ and $p = 0.0058$). Similarly, in the highest concentration (100 mg·L^{-1}), CAT activity increased after 3 days of exposure ($p < 0.0001$).

Concerning the GPx activity in gills (Fig. 1E), significant differences were detected in the 10 mg·L^{-1} treatment after 21 and 28 days of exposure, with an increased GPx activity ($p < 0.001$ and $p = 0.0013$). Likewise, mussels exposed to the highest concentration of the leachate (100 mg·L^{-1}) showed an increased GPx activity after 14, until the end of exposure ($p < 0.0001$, $p = 0019$, and $p = 0.03$, respectively). On the other hand, a significantly higher GPx activity was detected in the digestive glands (Fig. 1F) of 10 mg·L^{-1} treatment after 21 days of exposure ($p = 0.0056$). In contrast, in those exposed to the highest leachate concentration (100 mg·L^{-1}), significant differences were found after 3 and 21 days of exposure ($p = 0.0042$ and $p = 0.0048$, respectively).

Regarding GR activity (Fig. 2A), the gills of mussels submitted to the 10 mg·L^{-1} treatment presented a significant increase in GR activity during the whole exposure period ($p < 0.0001$), except on the 14th day of exposure ($p > 0.05$). In contrast, GR activity only increased significantly in the gills exposed to the highest concentration between 7 and 14 days of exposure ($p < 0.0001$ and $p = 0.0015$, respectively). However, in the digestive glands of mussels, a significantly higher GR activity was only found in mussels exposed to the 10 mg·L^{-1} treatment after 21 days of exposure (Fig. 2B) ($p < 0.0001$).

G6PDH activity (Fig. 2C) in mussels gills exposed to the lowest concentration (1 mg·L^{-1}) of leachate significantly increased after 21 days of exposure ($p < 0.0001$), whereas, after 28 days of exposure, G6PDH activity treatment decreased to levels lower than the controls ($p = 0.0203$), while for the highest concentration (100 mg·L^{-1}) G6PDH activity increased only after 28 days of exposure ($p < 0.0001$). G6PDH activity in the digestive gland was significantly higher for the lowest concentration (1 mg·L^{-1}) after 21 and 28 days of exposure (Fig. 2D) ($p = 0.0095$ and $p < 0.0001$, respectively), while the 10 mg·L^{-1} treatment induced a significant increase in G6PDH activity after 3, 7, and 28 days ($p < 0.0001$, $p < 0.0001$, $p = 0.0156$, respectively). In the highest concentration (100 mg·L^{-1}), increased G6PDH activity was significantly induced in the digestive gland only at the end of the exposure period ($p < 0.0001$).

The activity of GST in the gills of mussels significantly decreased after 14 days of exposure at the intermediate concentration (10 mg·L^{-1}) (Fig. 2E) ($p = 0.0132$). Conversely, GST activity significantly increased after 28 days of exposure to the intermediate concentration ($p = 0.0001$). In addition, GST activity increased after 7 days of exposure for the highest concentration (100 mg·L^{-1}) ($p = 0.0394$). On the other hand, in digestive glands (Fig. 2F), a significant increase in GST activity was detected in mussels exposed to 1 mg·L^{-1} of leachate after 14 and 28 days ($p = 0.0087$ and $p = 0.0001$, respectively). Similarly, GST activity significantly increased in the 10 mg·L^{-1} treatment after 21 days ($p = 0.0304$). In contrast, GST enzymatic activity significantly decreased in the digestive glands exposed to the highest concentration of leachate (100 mg·L^{-1}) after 21 until the end of exposure ($p = 0.0123$ and $p = 0.0004$).

3.5. Genotoxicity

The comet assay was used to evaluate DNA damage in the hemocytes of mussels exposed to the leachates, and the results are presented in Fig. 3A. There were no significant variations between controls over time ($p > 0.05$). Conversely, in the exposed mussels, two-way ANOVA analysis revealed a significant effect of time and concentration on the DNA damage levels ($p < 0.0001$). Moreover, in mussels exposed to 10 mg·L^{-1} of concentration, a significant increase in DNA was found after 7 and 14 days of exposure ($p < 0.0001$ and $p = 0.0002$, respectively). A more pronounced increase was also found in mussels exposed to the highest concentration (100 mg·L^{-1}) between three and 21 days of exposure ($p < 0.01$). At the end of the period (28 days), the DNA levels of all treatments were similar to those of the control group (Fig. 3 A) ($p > 0.05$).

3.6. Neurotoxicity

No significant differences were detected in AChE activity between the controls ($p > 0.05$) (Fig. 3 B). In addition, no significant differences were found in the mussels exposed to the different leachate concentrations (1, 10, 100 mg·L^{-1}) compared with the respective controls ($p > 0.05$). In contrast, differences were detected between treatments; in the intermediate concentration (10 mg·L^{-1}), AChE activity was significantly inhibited after 7 days of exposure when compared with the highest concentration (100 mg·L^{-1}) ($p < 0.0001$) (Fig. 3 B).

3.7. Oxidative damage

On one hand, LPO levels in mussels did not change between controls for both gills and digestive glands ($p > 0.05$) (Fig. 3C – D). On the other hand, in exposed mussels, two-way ANOVA analysis revealed a

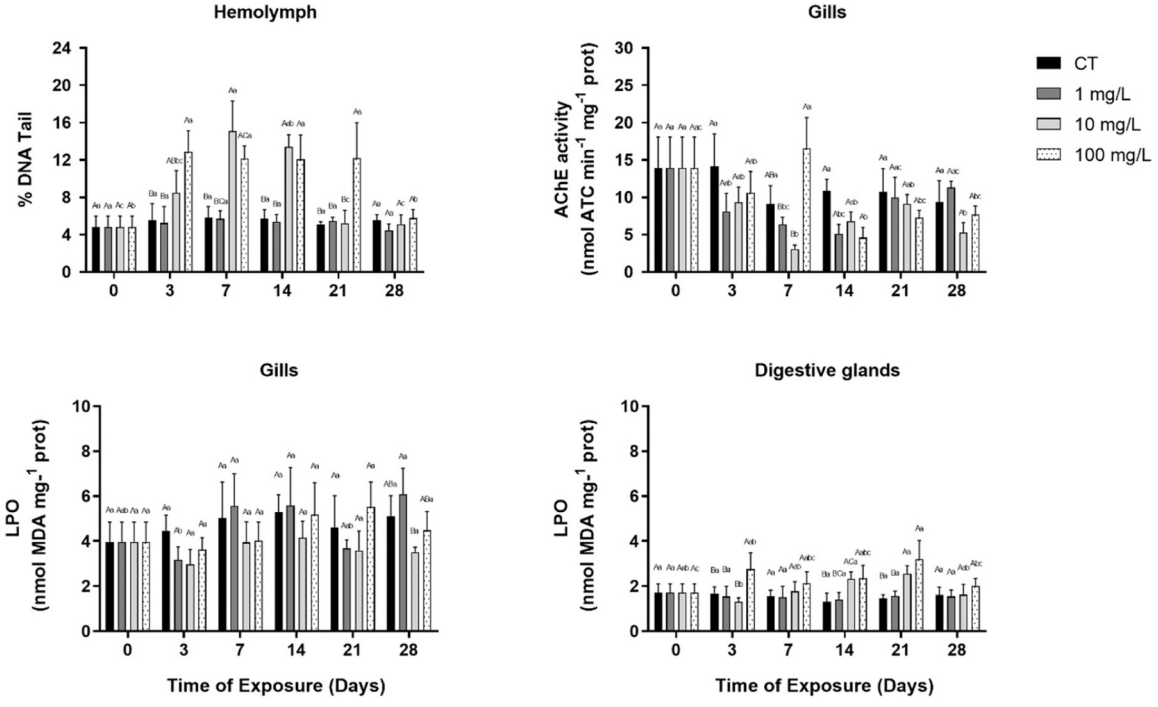

Fig. 3. DNA damage measured as % DNA tail (A) in the hemolymph, AChE activity (B) in gills, and LPO levels in gills and digestive glands (C and D, respectively) of mussels *Mytilus galloprovincialis* unexposed (CT) and exposed to three concentrations of leachate (1, 10, and 100 mg·L^{-1}) for 28 days. Different capital letters indicate a significant difference among treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment among times (ANOVA; $p < 0.05$).

significant effect of time and concentration on the oxidative damage content ($p < 0.0001$). Moreover, no significant differences were found in the gills of mussels exposed to the different leachate concentrations compared with the controls, except at the end of the exposure period (28 days) ($p > 0.05$) (Fig. 3 C). On the other hand, levels of LPO in the digestive gland significantly increased after 14 and 21 days for the 10 mg·L^{-1} treatment ($p = 0.024$ and $p = 0.0253$, respectively) and after 3, 14, and 21 days for the 100 mg·L^{-1} treatment ($p = 0.0341$, $p = 0.0227$, and $p < 0.0001$, respectively). At the end of the experiment (28 days), LPO levels were similar to those of the control group for all treatments (Fig. 3 D) ($p > 0.05$).

3.8. Principal component analysis (PCA) and weight of evidence (WOE) analysis

To integrate the biological effects detected in the gills and digestive glands of mussels exposed to the different leachate concentrations, PCA was performed with all the biochemical data (SOD, CAT, GPx, GR, G6PDH, GST, AChE activities, DNA damage, and LPO levels) (Fig. 4 A – B, and Fig. S4 A – B). The results revealed that for the mussel gills (Figs. 4A, and S4 A), the three principal components (PCs) hold 71.01% of the variance, in which PC1, PC2, and PC3 corresponded to 35.0%, 21.8%, and 14.2% of the variance, respectively. In PC1, SOD, CAT, GPx, GR, G6PDH, GST activities, and DNA damage were directly related, while LPO levels and AChE activity were negatively related. In PC2, CAT, G6PDH, GST, AChE, and LPO were positively related, whereas GPx, GR activities, and DNA damage were negatively related. Regarding the PC3 (Fig. S4 A), only G6PDH activity and LPO levels were positively related, and negative relationships were found in GPx, GST, and AChE activities.

On the other hand, for the digestive glands (Figs. 4B, and S4 B), the three principal components represent 77.4% of the variance, in which PC1, PC2, and PC3 corresponded to 38.4%, 25.6% and 13.4% of the variance, respectively. In PC1, SOD, CAT, GPx, GR, G6PDH, and GST activities were positively related, while in PC2, only GST activity was positively associated. Meanwhile, GPx activity, LPO levels, and genotoxicity were negatively related. Moreover, PC3 (Fig. S4 B) has a positive relationship between G6PDH activity and genotoxicity. In contrast, a negative relationship was found for GPx activity.

Overall, PCAs exhibited different responses among tissues regarding leachate concentrations and exposure time. For the gills, PC1 clearly shows a cluster of unexposed mussels (CTR) that were clearly separated from those exposed to lower leachate concentration (1 mg·L^{-1}) in the negative part of PC1 (Fig. 4A) which was not so evident for the digestive glands (Fig. 4B). This outcome indicates a possible chronic effect in the gills, whereas 10 and 100 mg·L^{-1} treatments displayed a different pattern on the opposite (positive) side of PC1. Regarding the 10 and 100 mg·L^{-1} groups, a time-specific response was found in PC2 with the longer exposure time on its positive side. The digestive glands clearly separate the 1 mg·L^{-1} treatment and the other two in the PC2.

The results of the overall WOE model are presented in Table 3. Results indicate a hazard quotient of "MODERATE" almost for all times and exposure doses except for the controls (ABSENT) and exposure at 10 mg·L^{-1} after 21 and 28 days of exposure (MAJOR). These elaborations were based on the magnitude and biological relevance of the responding biomarkers. The WOE results obtained for both tissues from organisms exposed to the lower concentration (1 mg·L^{-1}) was primarily induced by the effects of antioxidant and biotransformation enzymes. On the other hand, the results for DNA damage and lipid peroxidation product showed to be determinant on the WOE elaboration for mussels exposed to 10 mg·L^{-1} and 100 mg·L^{-1}, respectively.

4. Discussion

4.1. Chemicals present in the leachate

Metals (Cu, Fe, Pb, Mn, Ni, and Zn) were present in the leachate (Table 1). These metals are well known as part of chemical additives used in manufacturing plastics [9,40]. Plastic additive formulations exhibit considerable variation in quantity and quality and significantly influence product quality [9]. Moreover, the characteristics of each polymer depend on the production method used, as well as the additives and their respective quantities incorporated [41]. According to Lithner et al. [42], most plastics lack a composition declaration, including information about the polymer type.

Although determining the individual composition, the percentage of each additive in plastics is challenging. It is known that plasticizers and flame retardants make up approximately three-quarters of the total additives in plastics [9], and metals are also utilized for these purpose. Zn, Mn, Fe, Ni, and Cu are known as flame retardants in polymers such as PE, PP, and PA [43] and were found in the PE, PP, and PA-based leachates produced in the present study. Metals are widely employed as additives, with one of their primary applications being as colorants such as Zn and Pb, often at higher concentrations [44]. Pb is used for coloring within the yellow-to-red spectrum and serves as a stabilizer against thermal, UV, and oxygen degradation [45,43,46]. The presence of these metals in the plastic leachate represents a hazard to the marine biota since metals when accumulated by marine organisms can induce several impairments such as imbalance the antioxidant defense system as well as induce metallothionein levels that can put its health at risk

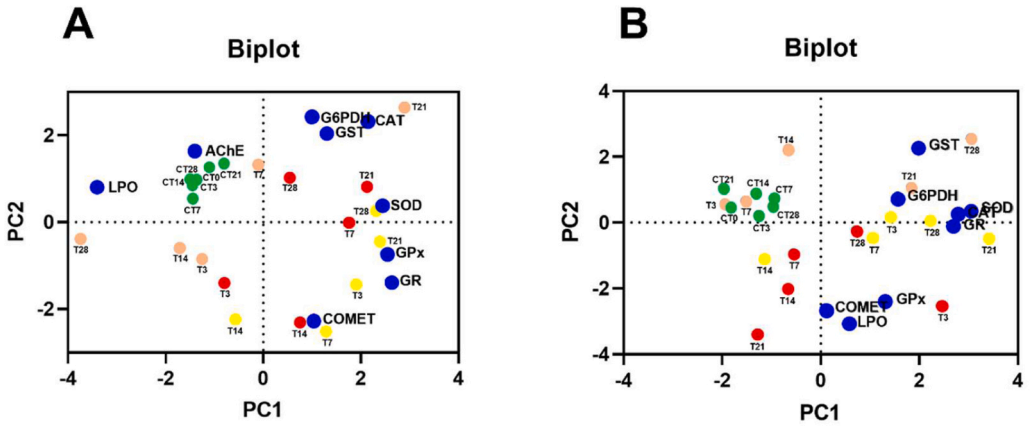

Fig. 4. Principal component analysis (PCA) of a battery of biomarkers (SOD, CAT, GPx, GR, G6PDH, and GST activities, and LPO levels) in gills (A), and digestive glands (B); AChE activity in gills, and DNA damage (Comet assay) in the hemolymph of mussels *Mytilus galloprovincialis* from controls (CT) and exposed groups exposed to fishing nets and cables leachate for 28 days ($p < 0.05$). Green, beige, yellow, and red circles represent the control groups, 1 mg·L^{-1}, 10 mg·L^{-1}, and 100 mg·L^{-1}, respectively.

Table 3
Weight of Evidence (WOE) integration of biomarkers data for the whole dataset of analyzed endpoints for each exposed condition. The assigned class of hazard is given.

Treatments	N. parameters in each class					Level of hazard for biomarkers	
	ABSENT	SLIGHT	MODERATE	MAJOR	SEVERE		
1mg_3	10	1	5	0	0	MODERATE	
1mg_7	9	2	4	1	0	MODERATE	
1mg_14	11	1	3	0	1	MODERATE	
1mg_21	8	2	0	4	2	MODERATE	
1mg_28	7	0	5	1	3	MODERATE	
10mg_3	6	3	3	2	2	MODERATE	
10mg_7	10	0	2	1	3	MODERATE	
10mg_14	9	0	4	2	1	MODERATE	
10mg_21	6	0	3	3	4	MAJOR	
10mg_28	6	1	4	3	2	MAJOR	
100mg_3	10	1	3	0	2	MODERATE	
100mg_7	9	2	3	0	2	MODERATE	
100mg_14	9	0	4	1	2	MODERATE	
100mg_21	7	2	4	2	1	MODERATE	
100mg_28	9	0	4	2	1	MODERATE	

[47].

Besides these six metals, other metals might also be present, but these were the only metals analysed in the main mixture and not in the leachate from the individual colors. Moreover, these plastics were collected from the environment, and the aging period of these nets on the beaches is uncertain. However, due to the aging process, these nets probably underwent chemical compounds adsorption/sorption processes, which could affect the potential leaching power of those items. Although data on the migration rates of plastic additives into seawater are scarce [48], a positive relationship was detected between the molecular weight of the additive and the leaching time, and metals were substances with relatively slow migration [49,50]. Therefore, longer time than the leaching time used in the present study might be required to detect other metals in the leachate.

The screening of chemical compounds also revealed an array of organic compounds in the leachates produced from the different colors and from the mixture (Table 2), which included plasticizers, UV stabilizers, flame retardants, antioxidants, dyes, flavoring agents, preservatives, intermediates, photo initiators, and others. Plasticizers represent a large proportion of the plastic additives, although their contribution depends on the chemical compound and type of polymer. In this study, five plasticizers were detected (Table 2): acetyl tributyl citrate was only one found in the leachate from the red plastic and represents between 10% and 35% of the additives [9] while diethyl phthalate represents 10 to 70% of the added additives [9] and was detected in all colors and in the main mixture of leachate, but the higher concentrations were in the red, yellow, and black leachate. Two phthalic acids (di(oct-3-yl) ester, di(2-propylpentyl) ester) were identified in the black leachate and in the main mixture. Conversely, dibenzyl ether was only detected in the leachate from the white PA plastic, and dibenzyl

ether is a common plasticizer used in PA manufacturing [51].

On the other hand, seven UV stabilizers were detected, in which benzothiazole, 2-(methylthio) benzothiazole, and benzophenone were present in all leachates. These compounds are UV stabilizers, and their concentrations in the plastic are between 0.05% and 3% of the weight [9]. In contrast, 4-tert-octyphenol was only found in the red and orange leachates. This compound was also present in the leachates from plastic debris detected by Staniszewska et al. [52]. Another UV stabilizer detected was bis(2,2,6,6-tetramethyl-4-piperidyl), frequently used as a radical scavenger in polymer manufacturing [53]. Interestingly, enzacamene was only encountered in the white and yellow leachates. Enzacamene is a neurotoxic compound present in sunscreens [54]. Similarly, bisoctrizole is a broad-spectrum UV filter used in also in sunscreens [55], and was present in the red and blue leachates. As these plastics were collected on tourist beaches, its presence in seawater may be the result of these compounds have been adsorbed to the drifting plastics.

Many flame retardants were also detected in the leachates (Tab, 2). An example is the tris(2-chloroethyl) phosphate, which represents up to 15% of the amount of the additives [9], and higher concentrations were encountered in the blue, yellow, red, and mainly in the orange leachate, reaching a concentration of 2715 ng/L. These findings are consistent with the results obtained by Qiu et al. [56], who found tris (2-chloroethyl) phosphate in thirteen samples of plastic bags made of PE and Nylon. In contrast, the antioxidant tris(nonyl phenyl) phosphite was only identified in the black leachate. This antioxidant is widely used in PE polymers [57] and PE bioplastics [58].

The dyes o-toluidine, 2-chloroaniline, and N,N-dimethylaniline are widely used as yellow colorants in plastic production [59,60], and these two dyes were only present in the yellow leachate. Overall, the present results reinforce the idea that the predominant additives in plastic are plasticizers, UV stabilizers, antioxidants, and flame retardants, as already demonstrated by Capolupo et al. [61], Qiu et al. [56], and Li et al. [62].

In addition to these aforementioned compounds, photoinitiators (2'-hydroxy-4',5'-dimethylacetophenone), intermediates (2-cyclohexen-1-one, 4-hydroxy-3,5,6-trimethyl-4-(3-oxo-1-butenyl), 2(3 H)-benzothiazolone, benzenesulfonanilide, N,N-dimethyl-1-octadecanamine, and N, N-dimethyl-1-hexadecanamine), preservatives (1,2-benzisothiazolin-3-one, ethylparaben, and methylparaben) were also identified, which may be related to the production of plastics [48] or due to its presence in seawater as contaminants. In fact, more than 10,000 substances can be added during the manufacturing process of plastics [63]. Interestingly, several flavoring agents (coumarin, cyclopentaneacetic acid, 3-oxo-2--pentyl-, methyl ester, 2,6,6-trimethyl-2-cyclohexene-1,4-dione, cyclohexanol, 4-methyl-1-(1-methyl ethyl)-, and 2-butanone, 3-phenyl-) and others (caffeine, cholesterol, isobornyl acrylate, pyridine, 2-ethyl-4, 6-dimethyl-, pyridine, 2,4,6-trimethyl-, 1,3:2,4-bis(3,4-dimethylbenzylidene)-D-sorbitol, glycine, N-(2-ethoxy-2-oxoethyl)-N-phenyl-, ethyl ester, 2,2-dimethoxy-1,2-diphenylethanone, and 26-(4-nonylphenoxy)− 3,6,9,12,15,18,21,24-octaoxahexacosan-1-ol) were detected in the leachates, which their functions are not well defined in the plastic composition. According to Fauser et al. [48], several unexpected compounds can be detected as potential additives in leachates with or without obvious specific purposes, and their widespread and more common use than previously thought.

4.2. Biomarkers responses

Despite the increasing data on plastic leachates (*e.g.*, [64]), very few focused on the ecotoxicological effects triggered by the chemicals present in the leachate, particularly on marine mollusks such as mussels. To our knowledge, this is the first study that investigates the ecotoxicological effects posed by long-term exposure to three different leachate concentrations (1, 10, and 100 mg·L^{-1}) produced from abandoned or lost fishing nets and cables, employing a multi-biomarker approach to assess their biological effects on mussels' health.

Organisms respond to xenobiotic insults through cellular defense systems, including the antioxidant defense and the xenobiotic biotransformation system. The antioxidant defense system plays a crucial role in maintaining the balance between pro-oxidant and antioxidant, thereby preventing oxidative and cellular damage [25]. A key component in the antioxidant defense system is the enzyme SOD which dismutates $^{\cdot}O_2^-$ to hydrogen peroxide (H_2O_2), providing adequate protection against the deleterious effects of reactive oxygen species (ROS) [65]. In the present study, mussels exposed to 1 and 10 mg·L^{-1} of leachate exhibited significant increases in SOD activity in both gills and digestive glands after 3 and 21 days of exposure (Fig. 1A – B). Interestingly, in the digestive gland of mussels exposed to 10 mg·L^{-1} of leachate, the increase in SOD activity was observed only after 21 days, which remained elevated until the end of the exposure. Similarly, Détrée and Gallardo-Escárate [66] found increased SOD transcript levels in the gills and digestive glands of *M. galloprovincialis* after exposure to PE microbeads. Thus, the findings suggest a higher ROS production induced by the chemicals present in the leachate and, consequently, a greater demand for $^{\cdot}O_2^-$ reduction over time.

CAT acts by decomposing H_2O_2 into water and oxygen [47], and in the present study, CAT activity displayed a clear tissue-specific response. The leachate effect on CAT activity was modest in the gills, and a decrease was observed in mussels exposed for 28 days to 1 mg·L^{-1} leachate (Fig. 1C). The decreased activity may be attributed to the increased CAT activity in the digestive glands at the same exposure period, possibly acting as a compensatory mechanism between tissues to protect the cells from ROS attacks. On the other hand, CAT activity in digestive glands was elevated following exposure to 100 mg·L^{-1} for 3 days, presenting a linear dose-response relationship, suggesting a growing production of hydrogen peroxide as a response to different levels of exposure. On the other hand, from day 21 onward, a non-monotonic response was addressed, whereby CAT activity significantly increased at the lowest concentration (1 mg·L^{-1}) while decreasing to levels similar to those of unexposed mussels (Fig. 1D). It may be hypothesized that long-term exposures of mussels to plastic leachates are accountable for a compensation mechanism of response in CAT and SOD activities, suggesting an overcompensation to an alteration in homeostasis.

Jointly with catalase, GPx also plays a crucial role in reducing H_2O_2 and other peroxides. GPx activity increased in gills and digestive glands when mussels were exposed to leachates at 10 and 100 mg·L^{-1} concentrations. (Fig. 1E – F). In addition, GPx activity increased in the gills following 14 days of exposure. It is essential to note that GPx also reduces other organic hydroperoxides [67], which could be generated by metals and organic compounds present in the leachates and possibly accumulated in mussel tissues. These results agree with O'Donovan et al. [68], who observed increased GPx activity in *Scrobicularia plana* gills after benzophenone exposure, a UV- filter present in the leachates in high concentrations. Moreover, Cravo et al. [69] also found an increase in GPx activity in *M. galloprovincialis* under Cu and Zn exposure.

Similar to the CAT response, GPx activity was significantly increased in the digestive glands after 3 days of exposure to 100 mg·L^{-1}, suggesting a high ROS production, possibly generated by metals or other organic compounds present in the leachates (Fig. 1F). In fact, Tsangaris et al. [67] observed that exposure to metals such as Ni in *M. galloprovincialis* increased ROS levels, resulting in an increase in GPx activity. Likewise, our data showed that Ni was present in the leachate used for mussel exposure. Furthermore, Wang et al. [70] observed an increase in GPx activities in *Danio rerio* larvae exposed to the flame-retardant tris-(2--chloroethyl)-phosphate ubiquitously present in the leachates. Although a significant increase in GPx activity was observed after 3 days of exposure to the highest leachate concentration, on the 7th and 14th day, the enzymatic activity was similar to the control levels. On the 21st day, however, the GPx activity was induced again at the 10 and 100 mg·L^{-1} treatments. Moreover, GPx activity is closely related to the GR activity,

which operates in the redox cycle of glutathione (GSH), regenerating GSH from oxidized glutathione (GSSG), which GPx uses in the hydroperoxide reduction processes.

A significant increase in GR activity was observed in the 10 and 100 mg·L^{-1} treatments after 3 and 7 days, respectively (Fig. 2A), indicating an increase demand for antioxidant molecules such as reduced glutathione (GSH) to support the activities of GSH-dependent enzymes. Notably, no increase in GPx activity was observed during the early stages of the experiment (days 3 and 7). Therefore, the increased demand for GSH may be associated with other processes involved in reducing pro-oxidant agents once GSH acts as a direct scavenger of ROS [71] On the other hand, in digestive glands, GR activity was induced only at the 10 mg·L^{-1} treatment after 21 days of exposure (Fig. 2B). A possible explanation is a high demand for GSH required to fulfill the cellular needs generated by the elevated GPx activity at the corresponding exposure time.

G6PDH is an auxiliary antioxidant enzyme that plays a crucial role in reducing $NADP^+$ to NADPH. G6PDH is thus essential for the metabolism of GSH, and its activity is vital for providing the necessary reducing power to support cellular growth and maintain redox homeostasis, thereby protecting cells against oxidative damage [72,73]. In the gills of mussels exposed to a leachate of 1 mg·L^{-1}, G6PDH activity was induced after 21 days (Fig. 2C). However, towards the end of the experiment, the enzyme activity decreased, suggesting a high demand for NADPH to counteract the cofactor's requirements on the 21st day. This observation coincided with the increase in GR activity but was not significant. Afterwards, both G6PDH and GR activities were inhibited on the 28th day of exposure, indicating an overwhelming antioxidant defense to the challenges caused by ROS.

In contrast, G6PDH activity in both gills and digestive glands was induced after 28 days of exposure to 100 mg·L^{-1} of leachate (Fig. 2C – D). According to Xu et al. [74], G6PDH induction can be explained by the involvement of pentose phosphates in the acclimation of metal metabolism to chronic metal exposure. Conversely, the G6PDH activity in digestive glands was induced after 3 and 7 days of exposure to the 10 mg·L^{-1} treatment. This indicates a higher demand for NADPH, possibly in response to pro-oxidant challenges due to the leachate exposure. At the lowest concentration of 1 mg·L^{-1}, G6PDH activity was induced only after 21 and 28 days, suggesting a chronic effect of cumulative compound exposure on the potential generation of ROS at low leachate concentrations.

GSTs are multifunctional enzymes involved in the detoxification processes of both endogenous and exogenous substances by catalyzing their conjugation with endogenous GSH, facilitating their elimination from cells [75]. In the present study, GST activity did not exhibit a clear response pattern to leachate exposure. GST activity was inhibited in the gills after 7 and 14 days of exposure, while GR activity was induced during the same period (Fig. 2E). This induction of GR activity may lead to an increase in the GSH available. However, even with the putative increase of GSH levels, they appear insufficient. A possible explanation is the burdensome organic compounds present in the leachates that overwhelmed the GSH available and further the biotransformation system. Indeed, [76] reported that flame retardants brominated diphenyl ethers (BDE), which were also present in the leachates (Table 2), limited the metabolism of organic xenobiotics in mussels *M. galloprovincialis* through a full downregulated of phase I and II enzymes' genes (CYP's and GST's) after long-term exposure.

Additionally, GST activity was induced after 7 days to 100 mg·L^{-1} and after 28 days to 10 mg·L^{-1} leachate treatment. On the other hand, the GST activity of digestive glands was induced after 14 and 28 days of exposure to 1 mg·L^{-1} treatment and after 21 days of 10 mg·L^{-1} of leachate (Fig. 2F). These results reveal the activation of detoxification processes in this tissue, potentially due to the presence of organic contaminants present in the leachate. In contrast, this enzyme activity decreases at the highest leachate concentration after 21 and 28 days of exposure, possibly due to the increasing challenge posed by chronic exposure to excessive chemicals in the leachate. This corroborates with Binelli et al. [77], who reported a decrease in GST activity in zebra mussels after 7 days of exposure to environmental plastics.

When ROS production exceeds the cells' antioxidant capacity, oxidative damage, such as lipoperoxidation (LPO) and DNA damage, might occur [39]. In this study, no oxidative damage was observed in the gills of mussels exposed to any of the leachate concentrations (Fig. 3C), indicating the effectiveness of the antioxidant and biotransformation system in reducing the potential ROS generated by the leachate exposure. However, the digestive glands of mussels exposed to the highest concentrations (10 and 100 mg·L^{-1}) showed oxidative damage (Fig. 3D). The lipid peroxidation started after 3 days of exposure to the highest leachate concentration and after 14 days to the intermediate concentration (10 mg·L^{-1}). These findings suggest an overwhelming antioxidant and biotransformation system, which could not be sufficient to reduce the pro-oxidants present in mussel tissues, thus damaging the lipid barriers of the cells. In addition, an increase in ROS production, coupled with the inefficiency of the antioxidant defense system, also led to elevated levels of MDA in mussels [78], clams [79], microalgae [80, 81], and rotifers [82] after exposure to myriad-compound leachate.

In unexposed mussels, low levels of DNA damage in hemocytes were observed, ranging from 4.82% to 5.94% fragmented DNA (Fig. 3A), which is considered typical for *Mytilus* species in pristine conditions [25, 83]. However, a high genotoxic potential was detected in individuals exposed to 10 and 100 mg·L^{-1} (Fig. 3A), which might affect mussel's defense system. This genotoxic damage was likely caused by the increase of ROS production under leachate exposure and the inability of the mussels' antioxidant defense and biotransformation systems to adequately reduce and eliminate them, particularly in digestive glands where higher LPO levels were observed. Schiavo et al. [84] also reported an increase in ROS production and DNA damage in marine microalgae exposed to PE, PP, and PS-based leachate, the same polymers present in this study. Moreover, at the end of the exposure period, DNA damage were similar to the controls ($p < 0.05$). Two hypotheses might explain these findings. Firstly, the increase in DNA damage might be related to the possible ROS overproduction, which was very explicit for the two highest concentrations (10 and 100 mg·L^{-1} of leachate). The antioxidant defense system was also activated, which could deal with the ROS production and ultimately control the DNA damage pathway. On the other hand, this behavior can be related to DNA repair systems such as the base excision repair pathway (BER), which is a well-known basis repair pathway against ROS-produced DNA damage [85]. However, more studies are needed to evaluate the efficiency of the key enzymes of this repair system against the damage generated by possible ROS induced by plastic leachates. The authors associated ROS production with the chemical contents in the leachate. However, explaining the exact mode of action of several chemicals simultaneously is challenging in such a complex mixture of contaminants as in the present case.

To investigate possible neurotransmission effects, the AChE activity, a crucial enzyme involved in the hydrolysis of acetylcholine (ACh), was measured [86]. Although the present results do not show significant differences between the leachate treatment groups and their respective controls (Fig. 3B), a consistent inhibition pattern was suggested across all concentrations when comparing results over time. This observation indicates the presence of neurotoxic compounds in the leachate. Indeed, it is well-established that hydrophobic chemicals, including neurotoxic pesticides, can be adsorbed to the surface of plastics found in the ocean [10].

To confirm these assumptions, PCA analysis clearly distinguished between exposed groups and controls (Fig. 4A – B). In addition, there are apparent differences between tissues, time, and concentrations-specific responses. In the gills, antioxidant defense and biotransformation biomarkers played a crucial role in protecting cells against oxidative damage to lipoperoxidation, evidenced by the positive correlation between SOD, CAT, GPx, GR, G6PDH, and GST activities. In contrast, AChE activity and LPO levels were negatively related, reinforcing endogenous

defense systems' fundamental role in protecting cells against oxidative attack and lacking neurotoxicity under leachate exposure. Despite the success of the defense system in protecting mussels from oxidative damage to the cell membranes, this mechanism could not combat the attack of possible ROS on DNA, which induced damage after exposure to the highest's leachate concentrations (10 and 100 mg·L^{-1}). This hypothesis is corroborated by the PCA, where the activities of SOD, CAT, GPx, GR, G6PDH, and GST were directly related to the highest % of DNA damage. This aspect was also confirmed through the weight of evidence obtained from the biomarker results (Table 3). The magnitude of observed variations and the biological relevance of analyzed parameters highlighted a general hazard level of "Moderate" to "Major". This confirmed the involvement of oxidative stress parameters and an imbalance of oxidative status, especially after 3–4 weeks of leachate exposure at 10 mg·L^{-1} (e.g., SOD, CAT, GPx, and GR activities), which presented the exposure condition with the most toxicological impact (Major). It may be hypothesized that the decrease of hazard quotient from Major to Moderate observed in mussels exposed to 100 mg·L^{-1} of leachate could be associated with a decrease in mussel filtration rates, as an adaptative long-term strategic defense due to higher chemical concentration present in this leachate [87]. It may have led to a lower uptake of chemicals present in the leachate and, subsequently, lower impacts on the sublethal responses addressed by the multi-biomarker approach. However, further investigation on the accumulation of the chemical compounds present in the leachates is needed to support this hypothesis.

5. Conclusion and final remarks

The present study provides a novel and comprehensive analysis of the ecotoxicological effects of fishing nets and cable leachates in mussels *M. galloprovincialis* under long-term exposure. It is the first insight regarding the potential leaching power of the chemical additives used in manufacturing plastic polymers (PP, PE, and PA) and other elements potentially adsorbed on the abandoned and lost fishing nets and cables, showing the release of a myriad of metals and organic compounds.

Using a multi-biomarker approach, the effects caused by leachates were strictly related to an imbalance of oxidative status, an increase in oxidative damage, and DNA damage. The data integration in a WOE procedure showed the widespread toxicity of fishing nets and cable leachates in mussels already at the lowest environmental concentration, highlighting the sensitivity of bivalves to this type of contamination.

Environmental implication

Nowadays, plastics are ubiquitous in the marine environment, and fishing activities have contributed up to 90% of the plastics found in the coast zone. Despite the well-known negative impact of plastics on marine biota, their weathering process and the potential chemical releases to seawater (i.e., leachate) that will ultimately impair ocean health are still poorly understood. The present work provides an ecotoxicological approach to the impact caused by fishing net and cable leachate on the marine mussel *Mytilus galloprovincialis* under long-term exposure through an exhaustive biomarker analysis and chemical screening of the chemical compounds present in the leachate. An innovative and comprehensive Weigh of Evidence (WOE) analysis was further applied to classify the hazards of the leachate.

CRediT authorship contribution statement

Francesco Regoli: Writing – original draft, Formal analysis. **Karim Lüchmann:** Writing – review & editing, Writing – original draft, Conceptualization. **Anna Rotander:** Writing – original draft, Investigation, Formal analysis. **Maura Benedetti:** Writing – original draft, Investigation, Formal analysis. **Fredric Seilitz:** Investigation, Formal analysis. **Carlos Edo:** Investigation, Formal analysis. **Giuseppe d'Errico:** Formal analysis, Data curation. **Gilberto Alkimin:** Methodology, Investigation. **Joanna Gonçalves:** Investigation. **Juliano Marcelo Vilke:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **M. J. Bebianno:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Tainá Fonseca:** Writing – review & editing, Methodology, Investigation, Formal analysis.

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.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.134479.

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