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Impact of face masks weathering on the mussels *Mytilus galloprovincialis*

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Abstract

The COVID-19 pandemic has triggered an unprecedented need for single-use face masks, leading to an alarming increase in plastic waste globally. Consequently, the improper disposal of face masks has added to the existing burden of plastic pollution in the oceans. However, the complete environmental and marine ecotoxicological impact remains unclear. This study aims to investigate the ecotoxicological impact caused by the weathering of disposable face masks (DFMs) in the marine environment on mussels *Mytilus galloprovincialis* (*M. galloprovincialis*) by assessing biochemical, cytotoxic, and genotoxic effects. The mask leachate was analysed for the presence of nano and microplastics. Furthermore, the leachate was used in *in vivo* and *in vitro* toxicity bioassays to assess its impacts on *M. galloprovincialis*. The *in vivo* exposure of *M. galloprovincialis* to face mask leachate for 14 days induced

a significant increase in catalase (CAT) activity in mussel gills, although not enough to prevent oxidative damage to cell membranes. DNA damage was also registered in mussel haemocytes after *in vivo* exposure to mask leachate. The *in vitro* Neutral Red (NR) cytotoxicity assay indicated that leachate concentrations ≤ 0.5 g/L^{-1} pose a significant risk to the health of mussel haemocytes, which seems a reliable tool for the cytotoxicity impact assessment of face masks in the marine environment. Therefore, the leachate obtained from face masks in seawater causes oxidative stress, oxidative damage, cytotoxicity, and genotoxicity in *M. galloprovincialis*, indicating that the plastic burden generated by DFMs in the ocean and its subsequent weathering represents a ubiquitous and invisible threat to the marine biota.

Keywords: Face masks, leachate, microplastics, toxicity, marine mussels

INTRODUCTION

The severe acute respiratory syndrome of coronavirus (SARS-Cov-2), first detected in 2019, gave rise to the COVID-19 pandemic[1]. Social distance, travel restrictions, lockdowns, and sanitary measures were globally adopted to avoid airborne virus transmission and reduce its spreading. One of the most widely accepted actions was the usage of single-use plastics (SUPs)[2], and personal protective equipment (PPEs), including protection suits, surgical face masks, examination gloves, and face shields, employed by frontline health professionals and the general population[3]. As a result of the remarkable shift in the demand for disposable items, a new plastic waste boom emerged, scaling up the already existing plastic pollution crisis[3,4].

In this context, with the mandatory use of disposable face masks (DFMs) worldwide, an explosive demand for its supply at exceptional levels occurred. On the rise of the coronavirus outbreak, projections estimate that 129 billion face masks were used monthly worldwide, amounting to over 1.24 trillion discarded globally since the start of the pandemic[5,6], and in the case of Portugal, PPE usage represents an additional contribution of 4.97% to the municipal solid waste[7].

Global improper disposal of these face masks led to their ubiquitous presence in urbanised areas, lakes, beaches, and mountains worldwide[8-11]. In addition, face masks that end up in landfills or open dumps may easily leak into the surrounding environment and be flushed into rivers and coastlines by rainfall or wind[12,13], ultimately reaching the ocean. Considering the global production, it is estimated that about 1.56 billion face masks entered the marine environment in 2020[14].

Once in the marine realm, DFMs pose a physical threat to marine life through entanglement or ingestion[15]. Moreover, face masks undergo weathering (also known as ageing) by sunlight, mechanical abrasion, oxidation, and biodegradation, breaking down the textile material into microplastics (MPs) and nanoplastics (NPs) (plastic fragments less than 5 mm and 1 μm, respectively), whereby fibres are dominant (70%)[16,17]. Research revealed that a single face mask might release between 3,600 to 1.6×10^7 microfibres into the water, depending on the duration and intensity of physical and chemical disturbances[17-19]. A myriad of synthetic microfibres are dispersed in the marine environment[20,21] and DFMs have been pointed out as a critical secondary source of plastic burden in the ocean[7,16].

Polypropylene is the most used plastic polymer assembled in DFMs, but other polymers like polyurethane, polyester or polyacrylonitrile can also be incorporated into its structure[1]. The release of plastic polymers

may act as vectors of hazardous substances, such as persistent organic pollutants (POPs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides, or emerging contaminants. Moreover, chemical additives from face masks' matrices may also be released into the marine environment, including plasticisers, pigments, dyes, metals, antioxidants, stabilisers, and lubricants[13]. The plastic burden from MPs and NPs released from DFMs may enter marine biological systems through ingestion, dermal contact, or filtration[16], particularly in the case of filter-feeding bivalves.

Microfibres are known to be ingested by crustaceans, molluscs, fishes, birds, and seals[22-24]. An extensive body of evidence demonstrated that marine organisms undergo MPs and NPs ingestion, leading to inflammatory responses, oxidative stress, membrane damage, cytotoxicity, genotoxicity, cell death, and reproductive impairments, detected either through *in vivo* or *ex vivo* exposures[25-28]. Likewise, ecotoxicological assessments also indicate the extent of the biological effects following the exposure to microplastic fibres (nylon, polyester, polypropylene polymers) on marine zooplankton representatives[29,30] and mussels[31-33].

However, to the best of the authors' knowledge, the present study is the first marine ecotoxicological assessment conducted on the weathering of DFMs. Considering the high representation of DFMs in the current marine litter composition and the biological disturbances associated with MPs and NPs across several biological levels in the marine biota, there is a pressing need to investigate whether the weathering and fragmentation of DFMs in the ocean pose an additional ecotoxicological threat to the marine environment. The present study hypothesizes that DFMs ageing release plastic particles and further cause biochemical, cytotoxic, and genotoxic injuries to the marine mussel *Mytilus galloprovincialis* (*M. galloprovincialis*). The main objective is to unravel the biological responses posed by DFMs on the mussels through *in vivo* and *in vitro* assays. The use of a cell-based *in vitro* approach conducted with *M. galloprovincialis* haemocytes under DFM leachate exposure revealed a notorious advantage in reducing the considerable number of mussels needed to carry out experiments, allowing the screening of a broader spectrum of exposure conditions and rapid generation of consistent data[34], which is line with demanding regulatory needs of the European Union. The findings herein will shed light on the biological effects that result from the presence of this ubiquitous and unprecedented type of plastic litter on marine mussels.

MATERIAL AND METHODS

Weathering procedure

Due to its broad use during the COVID-19 pandemic and its wide disposal in urban and natural spots, DFM was selected to assess the release of plastic particles to seawater, simulating natural weathering conditions. Instead of applying virgin DFM, timeworn face masks were collected to mimic realistic conditions of the masks ending up in the marine environment. After their collection, the elastic ear loops were removed from the surgical masks.

Twelve DFM (10 g of masks) were immersed in three litres of natural seawater (salinity 35) from the Ria Formosa lagoon, previously UV-sterilized and filtered (FSW) through 0.8 µm glass microfibre filters (Whatman), and the leachate prepared according to the method proposed by Almeda *et al.*[35]. For this purpose, the container was vigorously agitated over 72 h to simulate wave abrasion. After that period, masks were removed and dried in an oven at 60 ºC for three days to identify the polymer composition. The mask leachate was then frozen at -20 ºC until further use. To limit the overestimation of MPs present in the leachate, glassware and cotton clothing were adopted and applied during the whole assay to avoid plastic contamination, and a blank was run in parallel to assess possible MP and NP contamination.

Plastic polymer composition of face masks

Wave-weathered DFMs were analysed using Fourier Transform Infrared Spectroscopy (ATR-FTIR, Thermo Fisher Nicolet iS10) to identify the polymers present in the masks' structure. The spectra were acquired using a resolution of 4 cm^{-1}, 16 scans, 4,000-650 cm^{-1} spectral region, and transmittance mode. The obtained spectra were compared with existing databases using the OMNIC software. This software uses Pearson correlation to give a coincidence value. After the analysis, only the results above 70% of coincidence were considered positive. The different layers from all the masks used to generate the leachate were separated and tested individually, namely the outer lyophobic non-woven layer (O layer), the middle melt-blown layer (M layer), and the inner hydrophilic non-woven layer (I layer).

Analysis of plastic particles in mask leachate

Another leachate from used masks was prepared, following the same procedures described in section "Weathering procedure" to analyse the presence of MPs in the extract. The liquid was filtered through 0.8 µm glass microfibre filters (Whatman) and the filters were dried in an oven at 60 ºC to ensure that the chemical composition of the MPs and NPs was not altered. Filters were evaluated under an Edublue stereomicroscope (Euromex) at ×4 magnification. The present particles were counted, and their colour was assessed. In addition, the particles were measured in length and width, and their equivalent diameter was calculated.

To avoid interference from other submicron materials in seawater, NPs released in mask leachate were determined using artificial seawater (ASW), salinity 35, prepared according to ASTM D1141-98 standard.

To analyse the NPs, total organic carbon (TOC) measurements were performed in a Shimadzu TOC-VCSH equipped with an autosampler. Samples were filtered through Puradisc 25 TF filters (1 µm pore size) and analysed in Non-Purgeable Organic Carbon (NPOC) mode. Moreover, the size of the NPs in the leachate was confirmed with a Malvern Zetasizer Nano ZS apparatus. This equipment uses Dynamic Light Scattering (DLS) technology to analyse the particles present in the submicron portion of the leachates. With this aim, samples were filtered through 1 µm with Puradisc 25 TF filters. Comparison between fragments and fibres was allowed by calculating an equivalent diameter , converting the two flat dimensions into comparable diameters as done elsewhere[36,37].

M. galloprovincialis in vivo bioassay

Mussels *M. galloprovincialis* (n = 90; 6.0 ± 0.34 cm shell length) were handpicked during low tide in the Ria Formosa lagoon (Faro, Southern Coast, Portugal) and transported alive to the laboratory, scrap-cleaned and distributed over six glass aquaria containing 7 L of natural seawater. Mussels were acclimated over 5 days, at 16 ± 1 °C, salinity 35 ± 1.0 and pH 8.0 ± 0.2, with continuous aeration during a 12 h light : 12 h dark photoperiod. Seawater was renewed every 48 h during the acclimation period, and organisms were fed with marine microalgae *Tetraselmis chuii.*

After the acclimation period, ninety mussels were randomly selected and exposed for 14 days to each treatment (CT and 100 $mg{\cdot}L^{-1}$ of DFM leachate) in a triplicate design in 10-L glass aquaria filled with 7 L of seawater (15 animals per aquaria, density = 2 mussels L^{-1}. The seawater was changed every 48 h, and the leachate concentration was re-established. Animals were fed with the only food present in the seawater. Throughout the 14 days of the bioassay, the system was kept under constant aeration, controlled photoperiod, salinity (36), pH (8.0 ± 0.2), temperature (16 ºC), and oxygen saturation (96% ± 4%). On the 14th day of the bioassay, mussels (n = 6 per treatment) were collected for the determination of the individual biometric parameters (length, height, and width) and for the calculation of the condition index (CI). For that purpose, the soft and drained body tissues were weighted, and the CI for each organism was

calculated according to the equation:

$$CI\ (\%) = \frac{whole\ soft\ tissue\ (wet\ weight)\ \times\ 100}{whole\ drained\ body\ tissue}$$

On the last day of the experiment, mussels from each treatment (n = 6) were collected and dissected into gills and digestive glands, which were subsequently flash-frozen in liquid nitrogen and stored at -80 ºC until further biochemical analysis to assess antioxidant and oxidative damage effects by measuring the activity of antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT) and glucose-6 phosphate dehydrogenase (G6PDH)], gllutathiona-S-transferases (GST), lipid peroxidation (LPO) and respective total protein content.

In vitro cytotoxic assessment

Adult specimens of *M. galloprovincialis* (n = 15) from the Ria Formosa coastal lagoon (5.0 ± 0.3 cm shell length) were kept in a 25 L tank filled with clean natural seawater (salinity 35 ± 1), pH 7.8, temperature 18.5 ºC and constant aeration, until haemolymph extraction. Mussels were fed every two days by incorporating ~10 mL microalgae mix of *T. chuii* into the aquaria. After the three days of mussel acclimation, individuals (n = 10) were randomly chosen, and their haemolymph was retrieved under aseptic conditions[38,39]. Briefly, haemolymph was obtained from the posterior adductor muscle using a 2-mL sterile hypodermic syringe. Haemolymph collected from the ten specimens was pulled into one 15-mL Falcon tube and mixed with anti-aggregation solution (pH 6.7; 171 mM NaCl; 0.2 M Tris; 0.15% v/v HCl 1 N; 24 mM EDTA) in a 1:3 ratio, to prevent cell clumping and agglomeration[39,40]. Aliquots of this cell suspension were then used for cell counting in the Neubauer chamber (Hirschmaan, Eberstadt, Germany) through cell staining with the addition of Trypan blue dye (0.4% in physiological solution; v/v). Cell viability was determined by the percentage of live cells in cell suspension (100 cells counted). The following equation was used to calculate cell density:

$$Viable\ cells\ per\ mL = \frac{Viable\ cells}{nº\ of\ squares\ counted} \times\ dilution\ \times\ 10{,}000$$

Subsequently, the cell suspension was seeded into 96-well flat microplates (2×10^5 cells·mL^{-1}; 50 µL per well) and exposed, over 24 h in the dark, to a range of leachate concentrations prepared from a stock solution of mask leachate (10 g·L^{-1}) sequentially diluted in Dulbecco's Modified Eagle Medium (hereafter DMEM, pH 7.4) to obtain the following tested concentrations of the leachate: 1, 2.5, 5 and 7.5 g·L^{-1}. These solutions were prepared on the day of the bioassay and maintained at 4 ºC, in the dark, until incubation of mussel haemocytes to the respective exposure conditions. Blanks containing only DMEM cell culture media and anti-aggregation solution, absent of cells, were prepared as a reference, jointly with a negative control group (CT-; cells jointly with an anti-aggregation solution and DMEM) and a positive control group (CT+) prepared with sodium dodecyl surfactant (5 mM SDS, in DMEM), known to cause cytotoxic effects in the endpoint measurement. Eight replicates were prepared per treatment and control conditions. After the 24-h incubation, centrifugation at 1,200 rpm (10 min, at 4 ºC) was carried out to promote cell adhesion to the bottom. The supernatant (medium) was discarded, and cell viability assessed through the NR assay described in section "*NR cytotoxicity assay*".

NR cytotoxicity assay

The NR assay was applied to reveal the viability of mussel haemocytes through the capacity of live cells to incorporate the dye in lysosomes via non-ionic passive[41], according to the protocols of Katsumiti *et al.*[39]

and Fonseca *et al.*[42], with slight adaptations. Subsequently, 50 µL of filtered (Sartorius, 0.22 µm cellulose acetate filters) NR working solution was added to each microplate well and left in the dark for incubation over 1 h. Afterwards, to remove the excess dye from the medium, microplates were again centrifuged and gently washed with PBS until complete removal of the dye from the blanks. An acetic acid and ethanol solution (1:100 v/v) was seeded into a microplate and left over 20 min in the dark at 18 ºC for dye extraction from viable cells. Then, the cell suspension was transferred into a new V-bottom 96 well microplate and centrifuged. The supernatant was carefully placed into a new flat bottom microplate to measure the absorbance obtained from the neutral red extracts of the viable cells (550 nm, Infinite M200 Pro, TECAN®). Live and viable cells present higher absorbance, given the higher embodiment of dye into the lysosomes.

Cell viability and genotoxicity

Haemolymph was extracted as previously described (Section "*In vitro* cytotoxic assessment") and divided into two aliquots: one for the Trypan blue exclusion assay to measure cell viability, and the other was used for the Comet assay to assess genotoxicity.

Cell staining was performed with Trypan blue dye (0.4% in physiological solution; v/v) in a proportion of 1:1 (cell suspension: Trypan Blue 0.4%), whereby the percentage of live cells was determined. Cell viability was obtained through the relative number of viable and non-viable cells by counting blue-stained cells as dead and the translucid ones as alive. Results were expressed as a percentage of viable cells over total cells.

DNA damage was estimated using the alkaline Comet assay, adapted for marine mussels by Gomes *et al.*[43]. Microscope slides were pre-cleaned with ethanol and cast with normal melting point agarose (NMA) in Tris-acetate EDTA. Individual haemolymph aliquots were centrifuged at 3,000 rpm over 3 min (4 ºC), and the pellets were suspended in 0.65% low melting point agarose (LMA, in Kenny's salt solution) and cast over the microscope slides. Cells in the slides were then submitted to a lysis step over 1 h, and electrophoresis was carried out for 5 min at 25 V and 300 mA, followed by immersion in a neutralising solution (0.4 mM Tris, pH 7.5) over 15 min.

For the evaluation of the DNA in the comet tail (tail DNA %), slides were stained with DAPI, and pictures were taken from 50 random cells from each slide under a magnification of ×400 in an optical fluorescence microscope (Axiovert S100) coupled with a camera (Sony). Scoring analysis was performed using Imaging Software Komet 7.1 (Kinetic Imaging Ltd). Results are expressed as mean tail DNA % ± STD.

Biochemical analysis in *M. galloprovincialis*

Antioxidant and biotransformation enzyme activities

Antioxidant (SOD, CAT, G6PDH) and biotransformation (GST) enzyme activities were determined in gills and digestive glands from unexposed and leachate-exposed mussels. For that purpose, tissues of organisms (n = 6 per treatment) were individually homogenised in 5 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). The homogenate was centrifuged at 500 g, under 4 ºC, and the supernatant re-centrifuged at 12,000 g (45 min, 4 ºC). Cytosolic fraction was isolated and stored at -80 ºC to determine enzymatic activities and total protein content.

SOD activity was determined through the method described by McCord and Fridovich[44], whereby the decrease in the absorbance of the substrate cytochrome-*c*, by competition with the xanthine oxidase/hypoxanthine system, is measured spectrophotometrically at 550 nm. Results are expressed as U·mg^{-1} protein. To evaluate CAT activity, the decrease in the absorbance of the hydrogen peroxide (H_2O_2) was measured, revealing its consumption at 240 nm. CAT activity is herein presented in nmol·min^{-1}·mg^{-1}

protein.

The activity of the housekeeping enzyme G6PDH was indirectly determined through the method described by Glock and McLean[45], adapted by Almeida *et al.*[46], through which the reduction of nicotinamide adenine dinucleotide phosphate (NADP) to NADPH is measured spectrophotometrically at 340 nm. Results are expressed as U·mg^{-1} protein. The metabolism of biotransformation mediated by GST activity was quantified according to the method of Habig *et al.*, adapted for microplate reader, by the conjugation of 0.2 mM reduced form of glutathione (GSH) with 0.2 mM 1-chloro 2,4 dinitrobenzene (CDNB), in a reaction mixture of 0.2 M KH_2PO_4/K_2HPO_4 buffer (pH 7.9), at 340 nm[47]. The respective enzymatic results are expressed as CDNB nmol·min^{-1}·mg^{-1} protein.

Lipid peroxidation

Gills (n = 6) and digestive glands (n = 6) of *M. galloprovincialis* were individually homogenised in Tris-HCl buffer (20 mM, pH 8.6) with butylated hydroxytoluene (BHT) and centrifuged over 45 min (30,000 g, at 4 ºC). The resulting supernatant was stored under -80 ºC for further measurement of total protein content[48] and the determination of lipid peroxidation by-products, namely malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE), both products of the peroxidation of polyunsaturated fatty acids. The levels of MDA + 4-HNE were determined according to the method described by Erdelmeier *et al.*[49]. For that purpose, malondialdehyde bis-(dimethyl acetal) (Sigma-Aldrich) was used as standard, and the absorbance of the samples was measured at 586 nm in a microplate reader (Infinite M200Pro, TECAN®). Results are expressed as nmol MDA + 4-HNE mg^{-1} protein.

Determination of total protein content

Total protein concentration was determined in the cytosolic fraction of the aliquots regarding the analysis of antioxidant enzyme activity, GST activity and LPO levels, using Bovine Serum Albumin (BSA) as a standard[48]. Absorbance was read at 595 nm, and total protein concentrations were expressed as mg protein g^{-1} wet-weight tissue.

Statistical analysis

Results regarding biomarker responses were first checked for normality and homogeneity by the Kolmogorov-Smirnov and Bartlett's tests using GraphPrism 9 (GraphPad Software, Inc.). Student's t-test was applied to determine significant statistical differences between the effects addressed in paired samples from mask-leachate and control treatments. The critical value for statistical significance was $P < 0.05$.

RESULTS

FTIR analysis

The FTIR analysis of the surgical masks confirmed that the composition of the three protective layers was polypropylene. All the layers showed the same typical bands of this polymer with the CH_3 and CH_2 stretches (asymmetric and symmetric) in the region 3,000-2,850 cm^{-1}, as well as the methyl group present near 1,380 cm^{-1} and the aromatic ring in 1,450 cm^{-1} [Figure 1].

Particle size distribution in the mask leachate

Face mask leachate revealed the presence of 126 microparticles·m^{-3}. The main morphology was fibres (97%) of different colours, except for some coloured fragments, at a density of 3.4 fragments·m^{-3} [Figure 2]. The average size for fibres was 66.5 ± 24.4 µm and 34.6 ± 15.9 µm for fragments, showing the bigger size of the fibres in these samples.

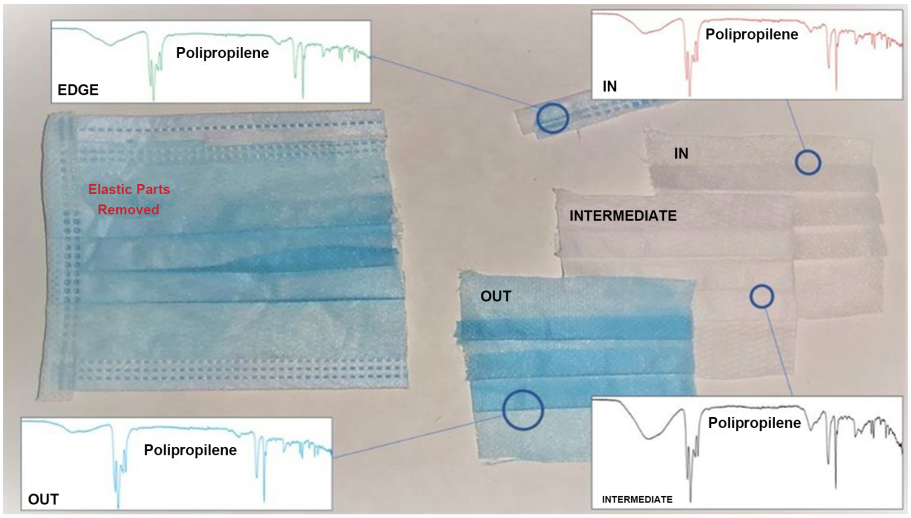


Figure 1. FTIR spectra and visual appearance of the different layers that form the facial masks. Polypropylene was the only polymer present in all layers. FTIR: Fourier Transform Infrared Spectroscopy.

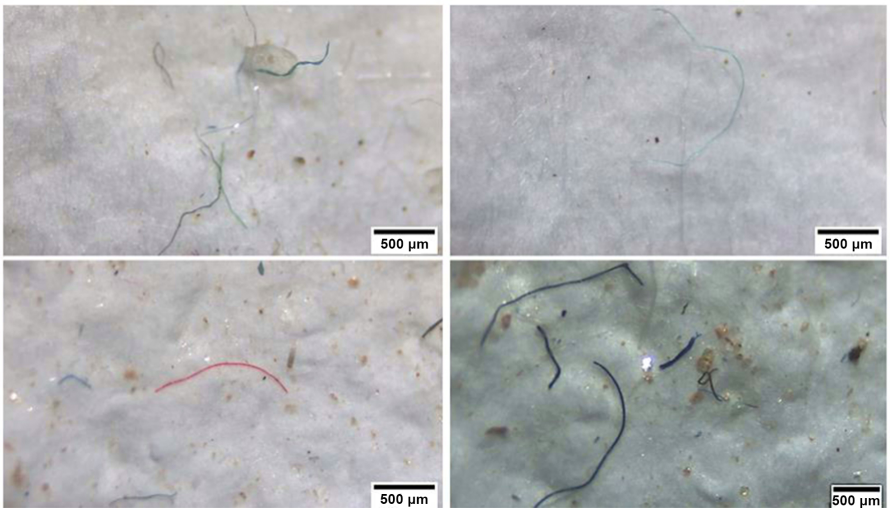


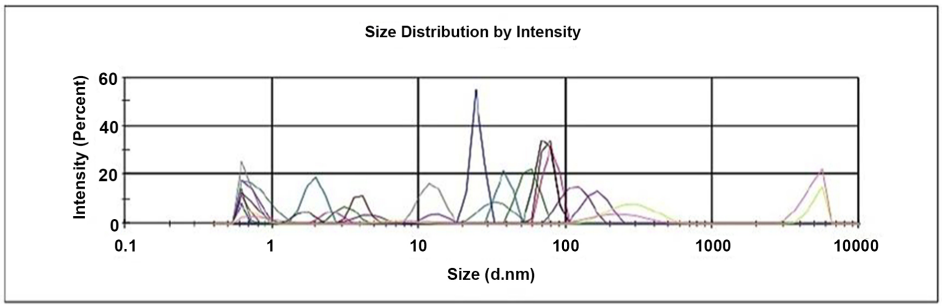
Figure 2. Images from stereomicroscope (×4 magnification) of the microparticles found in the leachate from used masks. Fibres and fragments of different colours were observed.

The TOC calculation determined the presence of NPs (below 1 µm). In all cases, the amount of carbon in leachate was higher than in negative controls. The concentration of carbon after 72 h was 3.21 $mg \cdot m^{-3}$ in leachate, in contrast to the 0.72 $mg \cdot m^{-3}$ found in the ASW control, indicating that NPs have been leached from the facial masks. DLS analysis confirmed the presence of submicron particles in the leachate in the 195.6 ± 96.6 nm range [Figure 3] that were not present in the controls. In addition, few NPs in the 10-100 nm size range were present.

Antioxidant and biotransformation enzyme activities

After 14 days of exposure to the mask leachate, mussels revealed an increasing trend in SOD activity in gills, although not significant compared to controls ($P > 0.05$) [Figure 4A], whereas digestive glands experienced a significant increase in SOD activity compared to unexposed mussels ($P < 0.05$) [Figure 4B]. In contrast, the mechanism that H_2O_2 scavenging exerted by CAT activity increased significantly in leachate-exposed

Size distribution (<1 µm): ASW Control



Size Distribution (<1 µm): Mask Leachate in ASW

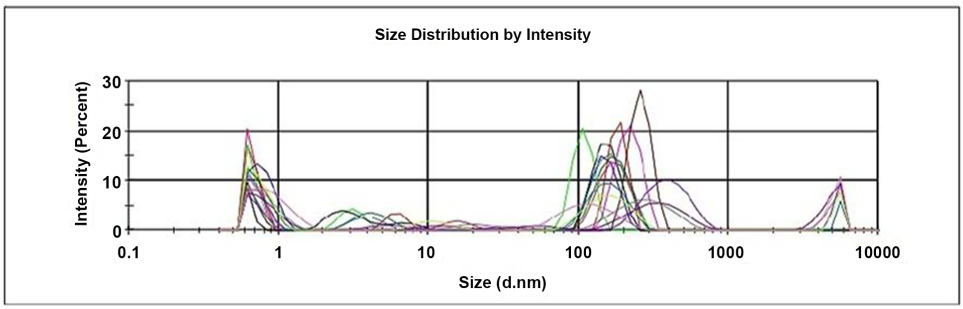


Figure 3. Size distribution of nanosize particles (< 1 µm) in ASW control and in mask leachate measured with DLS in artificial seawater medium (ASTM D1141-98 standard). ASW: Artificial seawater; DLS: Dynamic Light Scattering.

mussels' gills [Figure 4C], while in digestive glands, levels were comparable to the controls ($P > 0.05$) [Figure 4D]. Regarding G6PDH activity, although there was a decreasing trend in the gills exposed to the mask leachate, this decrease was not significant compared to unexposed mussels ($P > 0.05$) [Figure 4E] while in digestive glands, G6PDH activity from leachate exposed mussels significantly decreased in comparison to control levels ($P < 0.05$) [Figure 4F]. The results of the biotransformation metabolism showed that GST activity decreased in the gills of mussels exposed to mask leachate, while there was a slight increase in GST activity in the digestive glands. However, this trend was not significant in either tissue compared to the control group ($P > 0.05$) [Figure 5].

Lipid peroxidation

Levels of LPO by-products detected in gills from mussels exposed to mask leachate were significantly higher (2.7-fold) than those from the controls ($P < 0.05$) [Figure 6A], whereas in digestive glands, no significant differences were detected ($P > 0.05$) [Figure 6B].

Genotoxicity

Haemocytes retrieved from mussels exposed over 14 days to mask leachate experienced a significant increase of 150% of DNA tail compared to the control treatment ($P < 0.05$) [Figure 7].

In vitro cell viability

As observed in Figure 8, haemocytes revealed a significant and monotonic dose-responses relationship with a decrease in cell viability from the concentration of 0.5 $g{\cdot}L^{-1}$ and onwards ($P < 0.0001$). This significant change in cell viability indicates that concentrations of the leachate > 0.25 $g{\cdot}L^{-1}$ led to the mussel's haemolymph cell death.

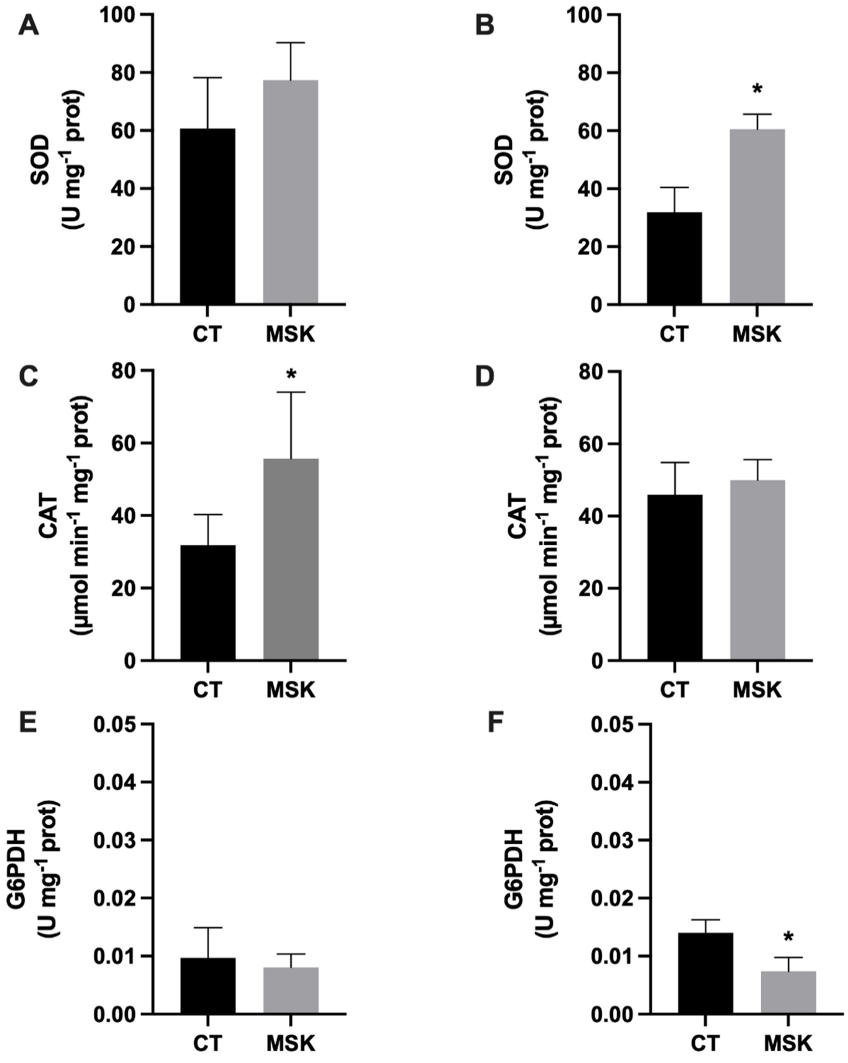


Figure 4. Antioxidant enzymes activity (mean ± STD) of: (A) and (B) SOD; (C) and (D) CAT; and (E) and (F) G6PDH, respectively in gills and digestive glands from unexposed (CT) and mask leachate-exposed mussels, after 14 days of bioassay. Asterisks indicate significant differences between control and mask leachate-exposed mussels (*t*-test; $P < 0.05$). CAT: Catalase; G6PDH: glucose-6 phosphate dehydrogenase; SOD: superoxide dismutase.

DISCUSSION

Findings from the present study are the first data unravelling the biochemical, cytotoxic, and genotoxic disturbances caused by weathering of DFM that releases MPs and NPs into seawater in the relevant marine sentinel species *M. galloprovincialis*.

Simulation of face mask weathering carried out in the present investigation was accountable for generating a total of 126.4 microparticles·m^{-3} in the aquatic system, most of which are fibres (95%). The number of fibres released depicts a high disparity with other weathering assessments with tri-layer masks[17]. Variations in the number of fibres released by non-woven face masks can be noted based on the weathering duration and exposure conditions to which they are submitted[17]. Current challenges were enumerated and emphasised regarding the realistic simulation of fibres pollution due to the lack of harmonisation of techniques applied across studies for analytical detection and quantification of fibres[50,51].

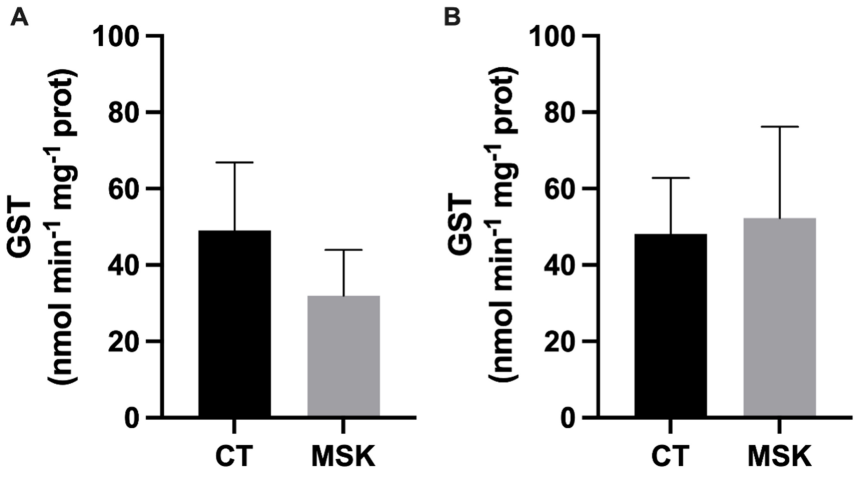


Figure 5. GST activity (mean ± STD) in gills (A) and digestive glands (B) from unexposed (CT) and mask leachate-exposed mussels, after 14 days of bioassay. GST: Gllutathiona-S-transferases.

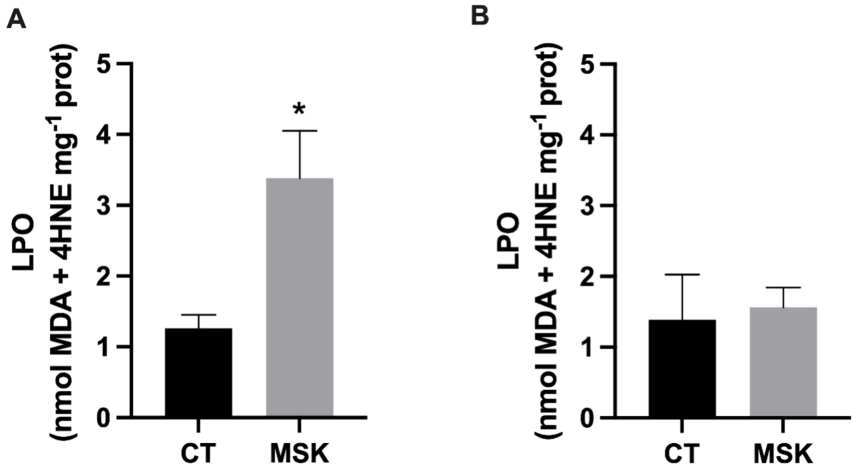


Figure 6. LPO levels by-products (mean ± STD) (nmol MDA+4-HNE mg^{-1} protein) in gills (A) and digestive glands (B) of unexposed (CT) and mask leachate-exposed mussels, after 14 days of bioassay. Asterisks indicate significant differences between control and mask leachate-treated mussels (*t*-test; $P < 0.05$). LPO: Lipid peroxidation; MDA: malondialdehyde; 4-HNE: 4-hydroxyalkenals.

In previous studies, face masks were submitted to mechanical and chemical external forces under laboratory conditions (rotating blender, treatment with alcohol/detergents) that are not similar to those experienced in the open environment[51,52]. Methodologies based on unrealistic simulations of shear stress forces are prone to generate a substantially higher amount of submicron fragments and particles, and the calculation of MPs and NPs generated from mask leachates may be overestimated[6]. The impact of face masks in the marine environment is in its infancy, and therefore, there are currently no standardised methods for analytical procedures and ecotoxicological assessment on this topic. As a consequence, there is a lack of calibrated procedures to ensure the realistic estimation of the impact and weathering of face masks under marine environmental conditions[51,52]. The vibration of functional groups [Figure 2] confirmed the polypropylene composition of the disposable surgical face masks, whose breakdown mainly occurs in the marine environment through photo- and thermo-oxidative degradation[53]. It is hypothesised that the application of used face masks in the present assessment, with different times of utilisation, contributed to contamination of the mask leachate with coloured fibres other than blue and transparent-white[54], potentially from the

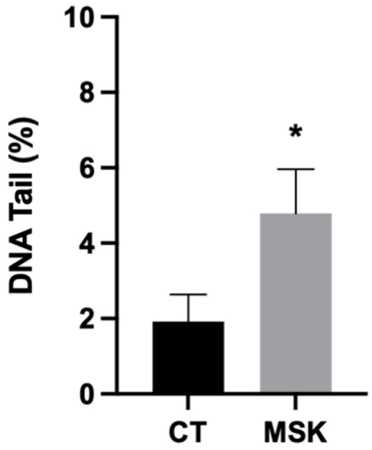


Figure 7. % of DNA tail (mean ± standard deviation) in mussel haemocytes unexposed (CT) and mask leachate-exposed, after 14 days of bioassay. Asterisks indicate significant differences between control and mask leachate-treated mussels (*t*-test; $P < 0.05$).

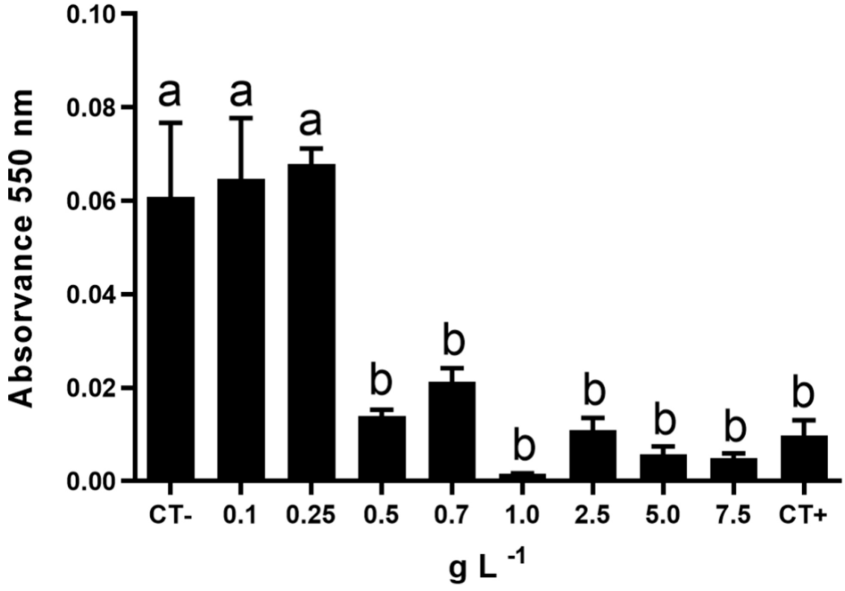


Figure 8. NR assay absorbances levels. Viability of *M. galloprovincialis* cells exposed to leachates, as well as a negative (CT-, unexposed) and positive controls (CT+, exposed to 5 mM SDS). Significant differences among treatments were labelled with different letters (one-way ANOVA test; $P < 0.0001$). NR: Neutral Red.

entrapment of MPs and NPs suspended in the air, or from tissues or clothing which may be accountable for the broader burden of MPs and NPs amount released in the leachate[13,16]. Furthermore, when MPs and NPs are present in seawater, they tend to aggregate[55]. This aggregation might significantly affect the bioavailability of the mixtures of these particles in the marine environment[55,56].

To date, scarce ecotoxicological investigation regarding face mask weathering has been conducted using aquatic species, with few approaches focused on marine biological models[57]. In the present ecotoxicological assessment, the gills of mussels submitted to the mask leachate presented a SOD activity similar to unexposed mussels. However, a significant increase in CAT activity was addressed as a hydrogen peroxide scavenging mechanism [Figure 4C] to counteract the harm generated by the physical stress promoted by

MPs and NPs ingested. Such a trend was also reported in marine mussels *M. galloprovincialis* under exposure to NPs and to emerging chemical contaminants[33,40,55,58], revealing that sources of hydrogen peroxide generation other than upon superoxide anion dismutated by SOD could be operating for CAT activation[59,60]. In contrast, SOD, the first line of defence in protecting tissues against oxidative stress[61], demonstrated to be an efficient response in the digestive glands of *M. galloprovincialis* to overcome the harm caused by the accumulation of the micro and nanoparticles ingested by the mussels[31], potentially jointly with the accumulation of other chemical additives released from the face masks[16,62,63] that were not analysed in the present work. Although CAT activity works in coordination with the activity of SOD, catalysing the reduction of hydrogen peroxide into water, the activity of such enzyme was not significantly altered in digestive glands, possibly due to the H_2O_2 clearance carried by peroxidases present in various subcellular compartments, such as glutathione peroxidases (GPx), which have a critical role in protecting cells against oxidative stress[60,61,64]. The decrease in the activity of G6PDH in gills, although significant only in the digestive gland, hypothesises the interference of the mask leachate on the activity of the glutathione-dependent system. Such an enzyme consists of an additional component of the antioxidant system accountable for catalysing the regeneration of the reduced NADPH. This essential cofactor operates jointly with glutathione reductase (GR) in the regulation of the intracellular supplies of the GSH, a potent *in vivo* antioxidant agent against oxidative damage caused by reactive oxygen species[65]. G6PDH-deficient cells experience a decrease in the GSH recycling mechanism that promptly compromises the ability of the antioxidant systems to detoxify hydrogen peroxide, thus being unable to withstand oxidative stress[66,67].

GST activity is associated with the biotransformation metabolism of organic compounds by catalysing the conjugation of the GSH to non-polar compounds that contain an electrophilic carbon, nitrogen, or sulphur atom[68,69], leading to the generation of less reactive products, with an ultimate protective role against oxidative stress[68,70]. The present findings revealed the absence of a biotransformation mechanism carried out by GST activity after mussels' exposure to mask leachate. This denotes low levels of organic chemicals taken up by mussels, possibly due to the low levels of organic chemicals present in the leachate or on the masks[71,72]. Beyond the physical stress carried out by micro and nano-sized fibres and particles, weathering and deterioration of the face masks are also accountable for contributing to the input of chemical additives in the environment, such as dye compounds, fragrances, and antiviral and antibacterial agents[73]. Sullivan *et al.* addressed the release of leachable inorganic and organic substances from the blue DFMs (like those used herein), namely metals, plastic additives, polyamide-66 monomer and oligomers (nylon-66 synthesis), surfactant molecules, dye-like molecules and polyethylene glycol[54]. These chemicals could have been released and then taken up by mussels in the present case, although further chemical confirmation is required. No significant alterations in GST activity were also registered in the digestive glands of mussels *M. galloprovincialis* exposed to 50 nm NPs (10 $\mu g \cdot L^{-1}$) compared to unexposed individuals, in contrast to a significant suppression in GST activity in the gills of respective animals[28]. Paul-Pont *et al.* also addressed that the biotransformation mechanism was not altered in the digestive glands of *Mytilus* spp. when exposed to polystyrene (PS) MPs (2-6 µm) at 32 $mg \cdot L^{-1}$ over seven days[26]. However, at the end of the depuration period of seven days, following PS-MPs exposure, GST activity significantly increased compared to unexposed individuals. Accordingly, Li *et al.* emphasised that wide variability in GST activity response was also reported in bivalves exposed to MPs and NPs[13].

Likewise, as addressed by Prokić *et al.*, organisms exposed to MPs and NPs exhibited varied responses in their overall antioxidant systems, ranging from no significant changes to a decrease or induction in enzymatic activities[74]. The diversity in ecotoxicological outcomes is influenced by a massive variability between features and parameters, namely the form of the plastic material (e.g., fibres, particle, bead, and powder), polymer composition, size of the particle, time of exposure, and acclimation conditions. These

factors are collectively accountable for generating diverse ecotoxicological profiles of responses, which further vary across species and tissues analysed.

Although the reactive oxygen species(ROS)-scavenging antioxidant system was herein activated due to exposure to the mask leachate, the high levels of LPO by-products in the mussels' gills evidenced that the protective mechanisms could not efficiently neutralise ROS to prevent cellular lipids from oxidative damage in this tissue[75,76]; and that micro and nanoparticles induce oxidative damage in the gills. Results from the meta-analysis conducted by Li *et al.*, aiming to elucidate the role of oxidative stress in toxicity elicited by MPs and NPs in marine species, evidenced that end products of LPO are a reliable index of membrane damage when the ability of the cells to maintain redox balance declines, and the antioxidant system is suppressed, leading to cellular damage of the tissues and potentially fitness costs[13].

Accordingly, it is noteworthy that DNA damage was registered in haemocytes under *in vivo* exposure to the mask leachate, indicating genotoxicity, which may be mainly linked to oxidative damage. These findings collectively corroborate with a vast body of research revealing significant disturbances caused by polypropylene microfibres and fragments and NPs on antioxidant systems from *Mytilus* spp.[26,34,77]. Considering the stress depicted by microfibres, Choi *et al.* observed a disruption in SOD and CAT activities, both in the gills and digestive glands of *M. galloprovincialis* exposed to 1 mg·L^{-1} of PET microfibre[33]. In addition, a monotonic dose-response pattern was verified for apoptotic mechanisms and the induction of DNA damage in haemocytes, registered from the concentration of 0.1 mg·L^{-1}[33].

Herein, mussel haemocytes in the *in vitro* NR assay confirmed their sensitivity and reliability in assessing the cytotoxicity posed by weathering and degradation of DFMs in the marine environment. A concentration of 0.5 g·L^{-1} of weathered face masks showed cellular disturbances leading to cytotoxicity and cell death. Sendra *et al.* addressed that after a 3-h exposure of *M. galloprovincialis* haemocytes to PS MPs (1 µm - 10 mg·L^{-1}), a subpopulation of large granular cells exhibited significant cytotoxicity compared to the control group[78]. Additionally, these cells reached values higher than 58% of apoptotic cells when individually exposed to PS NPs of 50 and 100 nm at 10 mg·L^{-1}. Chang and Wang[79] assessed the cytotoxicity of filtered face masks' leachate (300 g·mL^{-1}) on human alveolar basal epithelial cells (A549), revealing significant inhibition in cell proliferation and induction of DNA damage, ultimately leading to cell death with enhanced exposure time, as a result of exposure to multiple phthalate acid esters. However, to date, no studies have been conducted on the cellular disturbances caused by face masks in the innate immune system of marine mussels.

The global problem of plastic pollution caused by MPs and NPs significantly impacts various biological levels in marine species. Given the biological disturbances observed and the subsequent effects on representative marine species it is imperative to incorporate the study of face masks in plastic pollution research, which will facilitate a more accurate projection of the global plastic budget[51]. In addition, the available research evidence suggests that exposure to MPs and NPs can induce more pronounced ecotoxicological effects than microbeads or powdered plastic[80]. Bearing in mind the uncertainties regarding future sanitary crises, there is an urgent need to reduce the environmental impact of the face mask legacy. This necessitates constraints on the amount of these items and the implementation of all measures aimed at preventing their entry into coastal and marine ecosystems. In this sense, the manufacturing use of plasticisers in disposable masks needs strict control and regulation to minimize environmental and public health concerns related to exposure to substances such as phthalates, metals, MPs, and NPs[79,81]. Strong cooperation among scientists, healthcare professionals, industries, and policymakers is essential. Together, they can work towards transitioning to a circular economy that enables the repurposing of products at the

end of their life cycle, either for reuse or as raw materials[82].

CONCLUSIONS

Despite the growing scientific evidence on the mechanistic ageing and weathering of single-use face masks and subsequent interactions with aquatic biota, there are still many uncertainties regarding the overall toxicity caused by the multitude of chemical compounds and types of particles that leach from single-use face masks into the marine environment. The present study brings novel findings of the harmful legacy of global face masks in the marine environment by unravelling the oxidative, cytotoxic, and genotoxic disturbances caused by disposable face mask weathering in the mussel *M. galloprovincialis*. Herein, mussel haemocytes arise as a reliable tool for the *in vitro* cytotoxicity assessment regarding the impact of face masks in the marine environment.

DECLARATIONS

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Experimental work and biomarkers analysis: Fonseca T, Edo C, Vilke JM, Astudillo-Pascual M
Analysis and interpretation of results: Fonseca T, Edo C, Vilke JM, Astudillo-Pascual M, Bebianno MJ
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Availability of data and materials

Data will be available at the website of RESPONSE the project.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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