Polystyrene nanoplastics in the marine mussel Mytilus galloprovincialis

# Polystyrene nanoplastics in the marine mussel *Mytilus galloprovincialis*.

J.M. Gonçalves[a], M. Benedetti[b,c], G. d'Errico[b], F. Regoli[b,c], and M.J. Bebianno[a*]

[a]CIMA, Centre of Marine and Environmental Research\ARNET - Infrastructure Network in Aquatic Research, University of Algarve, Campus de Gambelas, 8000-139 Faro, Portugal.

[b]Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy

[c]National Future Biodiversity Centre (NFBC), Palermo, Italy

*Corresponding author: mbebian@ualg.pt / +351 289 244 434

## Abstract

Concerns about plastic pollution and its toxicity towards animals and people are growing. Polystyrene (PS) is a plastic polymer highly produced in Europe for packaging purposes and building insulation amongst others. The marine environment is inevitably the fate of PS products, whether originating from illegal dumping, waste mismanagement, or lack of treatment for the removal of plastic debris from wastewater treatment plants. Nanoplastics (< 1000 nm) is the new focus for plastic pollution gaining a wide array of interest. Whether primary or secondary, their relatively small size permits nanoparticles to cross cellular boundaries, and consequently lead to adverse toxic effects. An *in vitro* assay of *Mytilus galloprovincialis* haemocytes exposed to 10 µg/L of polystyrene nanoplastics (nPS; 50 nm) for 24 h was used to test cellular viability along with the median lethal concentration ($LC_{50}$) of *Aliivibrio fischeri* bacteria to evaluate acute toxicity. Cellular viability of mussel haemocytes decreased significantly after a 24 h exposure and nPS $LC_{50}$ range from 180 and 217, µg/L. Moreover, a 28-day exposure of the marine mussel *M. galloprovincialis* to nPS (10 µg/L; 50 nm) was carried out to evaluate neurotoxic effects and the ingestion of these plastic particles in three mussel tissues (gills, digestive gland, and gonads). The ingestion of nPS was time and tissue-specific, suggesting that nPS are ingested through the gills and then translocated through the mussel bloodstream, to the digestive gland and gonads where the highest amount of ingested nPS was found. Once nPS enters these plastic particles are transported to mussels tissues, with the gonads and digestive glands being the tissue deposits for these particles. Data on acetylcholinesterase inhibition and those previously obtained on a wide range of cellular biomarkers were elaborated through weighted criteria providing a synthetic assessment of cellular hazard from nPS.

## 1. Introduction

Concerns about plastic pollution and its toxicity towards animals and people are growing. Global plastic production (GPP) increased to 390.7 Mt in 2021 (PlasticEurope, 2022) and, among the consequences of illegal littering and dumping, waste mismanagement, as well as a low percentage of recycled plastics (8.3%) compared to fossil-based plastics (90.2%), the aquatic

environment is the inevitable fate of these materials. In Europe, plastic production was equivalent to 15% of GPP (57.2 Mt), with applications such as packaging and building and construction composing 39.1 and 21.3% of Europe's plastic demands, respectively (PlasticEurope, 2022). In 2021, the European Union adopted a directive banning all single-use plastics, except for bottles, as single-use plastic, and fishing gear which account for 70% of marine litter (Directive (EU) 2019/904).

Polystyrene (PS) is a plastic polymer highly produced for packaging purposes, building insulation, medical equipment, and toys as well as single-use plastics (PlasticEurope, 2022). Other than being inexpensive and readily available, PS is known for its incredible dimensional stability and water resistance, permitting it to remain consistent in size and shape, along with being certified as safe for use in food and beverages (Block et al., 2017). In day-to-day products, for example, PS can be found in disposable cutlery, food containers, plates, cups, automotive and electrical components, and in household items. However, not all plastic products are recyclable, and PS products are not (The Waste and Resources Action Programme, 2023). With PS products placed in waste bins, waste mismanagement and illegal dumping, with the addition that wastewater treatment plants (WWTP) also lack the correct technique or method for plastic debris removal, the marine environment will be the endpoint for these PS particles. Once in the ocean, plastics undergo five main processes—hydrolysis, mechanical/physical degradation, thermal-oxidative degradation, photo-degradation, and biodegradation (Andrady, 2011) — that break down and fragment plastics into smaller sizes. After much focus on microplastics (>1 and 5 mm), nanoplastics (< 1000 nm; nPS) are gradually gaining priority as nano-sized plastic particles may cross cellular boundaries, increasing their potential toxicity towards marine organisms (Peng et al., 2020). nPS in the marine environment can be either primary or secondary. Primary nPS enter the environment already in the nano-size range, and they are found in daily-use products such as cosmetics, clothing fibres, drug delivery and in 3D ink printers (Bergami et al., 2016; Bessa et al., 2018; Canesi et al., 2015; Dong et al., 2019; Tamminga et al., 2018; Wang et al., 2018). The breakdown and fragmentation of macroplastics (> 5 mm) and microplastics (< 5 mm) generates secondary nPS. Although techniques to detect and quantify nPS in the ocean remain challenging, there is evidence for the presence of plastics in the nano-size range. In the North Atlantic Gyre, a nanoplastic segment was found containing PS in the nano-size range, as well as polyethylene, polyvinyl chloride, and polyethylene terephthalate (Halle et al., 2017); after simulation of coastal activities on a PS-cup and lid, nano-sized particles were liberated after 5 minutes (Ekvall et al., 2019).

The toxic effects of nPS in marine organisms have been evaluated (Auguste et al., 2020; Capolupo et al., 2021; Ferreira et al., 2019; Gonçalves & Bebianno, 2021; Gonçalves et al., 2022; Kihara et al., 2021), showing that nPS mediated toxicity influences cell growth, larvae development, embryo malformation, inflammation, and also possible inactivation of photosystems in algae (Gonçalves & Bebianno, 2021; Kögel et al., 2020). In the marine mussel *Mytilus*

*galloprovincialis,* nPS provoke oxidative stress and damage (10 µg/L; 50 nm; 21-d) (Gonçalves et al., 2022), and lysosomal destabilization (1.5 – 150 ng/L; 50 nm; 21-d) (Capolupo et al., 2021). Although ingestion and translocation of MPs have been evaluated, the assessment of whether nPS toxicity is entirely from their ability to cross cellular boundaries, or if these nanoparticles are ingested, accumulated, and translocated between tissues remains difficult. In this study, a combination of *in vitro* and *in vivo* experiments was carried out to evaluate both acute toxicity and chronic effects of nPS on key model species. An *in vitro* assay on *M. galloprovincialis* haemocytes was performed to evaluate cellular viability after 24 h exposure to 10 µg/L of nPS (50 nm) and acute toxicity was further characterized through the Microtox® bioassay ($LC_{50}$). An *in vivo* 28-d exposure of mussels was performed at the same concentration of nPS to evaluate both ingestion of nanoparticles in gills, digestive glands, and gonads, as well as the onset of neurotoxicity in gills. These data were further integrated with those previously reported on a wide selection of cellular biomarkers; their overall elaboration through weighted criteria was expected to provide a better evaluation of the toxicological hazard of nPS.

## 2. Materials and Methods

### 2.1. Polystyrene nanoplastics (nPS)

Fluoresbrite® Plain YG spherical polystyrene nanoplastics of 50 nm in size (CAS 9003-53-6) were purchased from Polysciences, Inc. (Germany). 50 nm particles packed as 2.5% aqueous suspension, with 3.64 × 1014 particles/mL in ultrapure water (7732-18- 5) (CV = 15%, Excitation max. = 441 nm, Emission max. = 486 nm). Gonçalves et al. (2022) provide a full description of the characterization of nPS and demonstrate that the hydrodynamic diameter of nPS rises when distributed in FSW (852 103 nm), which suggests that the high salt content in seawater causes aggregation/agglomeration kinetics. A concentration of 10 µg/L was used for exposure assays.

### 2.2. Acute toxicity assay

The solid phase Microtox® bioassay (SPT, Azur Environmental, 1998) was used to evaluate polystyrene nanoplastics (nPS; 50 nm) acute toxicity in the bioluminescent marine bacterium *Aliivibrio fischeri* according to ISO 1134-3: 2007 and ISO 21338: 2010 according to the method described in Gambardella et al., 2019). All reagents and lyophilized *A. fischeri* bacteria (NRRL B-11177) were obtained from Modern Water Ltd (USA). Tests were carried out at 15ºC in the Microtox diluent supplied. The initial concentration of nPS used was 3724 µg/L and was sequentially diluted. Acute toxicity of nPS was measured in terms of relative bioluminescence by Microtox™ 500 luminometer after 5-, 15-, and 30-mins incubation. The bioluminescence inhibition was determined using the Microtox®FX equipment**,** and data were analysed using the

MicrotoxOmni software. The median lethal concentration ($LC_{50}$) was defined as the concentration that produced a 50% light reduction after 5-, 15-, and 30-mins of contact time for bacteria.

### 2.3. *In vitro* assay

Fifteen mussels *M. galloprovincialis* (60 ± 5 mm) were collected from an offshore aquaculture site in Lagos, Southeast Portugal (A: 37º04'200" N 8º42,800" W, B: 37º04'200" N 8º41'000" W, C: 37º03'400" N 8º41'000" W, D: 37º03'400" N 8º42'800" W; Testa & Cunhas Ltd) and transported alive to the laboratory. Mussels (2 mussels/L) were placed into a 10 L tank, with 7.5 L of seawater (S: 36 ± 1) and left to acclimatise for 48 hours. Then, mussel haemolymphs were extracted from the posterior adductor muscle of mussels with a sterile hypodermic syringe (1 mL) (25 G needle), under aseptic conditions in a vertical laminar airflow cabinet, and kept on ice. Cell extraction and incubation methods were based on the modifications of protocols developed by Gómez-Mendikute & Cajaraville (2003) and Katsumiti et al. (2014). To have sufficient haemolymph for *in vitro* exposures, five pools of three mussels per pool were used. Firstly, to avoid clumping and aggregation of cells, 10 µL of pooled haemolymph was added to an anti-aggregation solution (0.2M Tris; 171mM NaCl; 24mM EDTA; 0.15% v/v HCl 1 N) (Katsumiti et al., 2014). Trypan blue dye (0.4% in physiological solution; v/v) was added for cell staining in a 1:2 proportion (cell suspension: Trypan Blue 0.4%). Using a Neubauer chamber (200 cells per specimen) and with the aid of a haemocytometer and light microscopy (Compound Light Microscopy; 400x), cell viability was determined by the following equation:

$$Concentration\ (cells\ mL^{-1}) = \frac{nº\ cells\ \times\ 10\,000}{nº\ squares} \times\ dilution\ factor$$

Following cellular viability, cell suspensions were diluted at a density of 2 x $10^5$ haemocyte cells/mL (in anti-aggregation solution), and 100 µL were seeded into 96-well microplates (6 replicated per pool) with cell culture media Dulbecco's Modified Eagle Medium (DMEM, pH 7.4) and exposed to 10 µg/L of nPS. Microplates were incubated for 24 h at 18 °C. Afterwards, culture media was discarded, and the neutral red (NR) assay was applied following an adaptation from the protocol described in Katsumiti et al. (2014). The culture medium from the wells was removed and the state of the cells was verified under light microscopy (Compound Light Microscopy; 400x). 50 µL of neutral red working solution (0.4%, pH 7.3-7.4) was added to each well, including empty wells for the negative control, and left to incubate for 1 hour in the dark. The microplate was then centrifuged at 270 *g* (4ºC, 10 mins) and the supernatant was removed and carefully washed with PBS. The dye was then extracted from the viable cells in acetic acid/ethanol solution (1:100), following samples transference to U-bottom 96-well microplates and centrifuged at 270 *g* for 10

146 min, at 4 °C. The supernatants were then transferred into flat-bottom microplates and the
147 absorbance was measured at 550 nm (Infinite M200 Pro, TECAN®).
148
149 **2.4. *In vivo* assay**
150 Mussels belonging to the species *M. galloprovincialis*, with a shell size of 60 ± 5 mm were
151 procured from the Ria Formosa Lagoon, Southeast Portugal (37°00'30.6" N 7°59'39.6" W) and
152 brought alive to the laboratory. Following a four-day acclimatisation period, a total of 50 mussels
153 were placed into each 30 L tank, with 25 L of seawater in a triplicate design, to follow the ratio of
154 2 mussels per litre. Mussels were contaminated with 10 µg/L of polystyrene nanoparticles (nPS; 50
155 nm) for 28 days. Seawater was exchanged every two days and nanoparticles were re-dosed. No
156 mortality was encountered in either treatment.
157 Mussels were collected at 0 days, before exposure, and 3, 7, 14, 21 and 28 days after exposure.
158 The gills, digestive gland, and gonads of the weighted mussels were dissected and immediately
159 frozen in liquid nitrogen before being preserved at -80°C for further research on neurotoxicity
160 (AChE) in the gills, and ingestion of nPS in the three different tissues.
161
162 **2.5. Quality control and assessment**
163 Each tank was covered with glass lids to limit airborne pollution and glass pipettes were used
164 to administer aeration to avoid plastic contamination. No plastic gloves, tools, or materials were
165 utilised during tissue dissection to avoid further contamination.
166
167 **2.6. Acetylcholinesterase activity (AChE)**
168 AChE activity was only assessed in the gills of *M. galloprovincialis* (n=5 per treatment and
169 time of exposure) following a modification of Ellman's colorimetric method (Ellman et al., 1961).
170 Firstly, gills were individually homogenized in 5 mL of Tris-HCl buffer (100 mM, pH 8) and 50
171 µL of Triton – X 100 (0.1%). Following centrifugation (12 000 x *g*, 4ºC, 30 min), the supernatant
172 was collected, and stored at -80ºC until further analysis.
173 For the determination of AChE activity, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB, 0.75 mM)
174 was added to samples and left to incubate at room temperature for 5 mins. Then, acetylthiocholine
175 solution (ATC, 3 mM) was added to trigger the reaction, and the absorbance was read at 412 nm
176 using a Tecan (Infinite 200 Pro) microplate reader, for 5 mins with 30-sec intervals. AChE activity
177 is expressed as nmol ATC $min^{-1}$ mg $protein^{-1}$.
178
179 **2.7. Total protein**

AChE activity was standardized with the determination of total protein concentrations (mg protein $g^{-1}$ tissue), calculated using bovine serum albumin (BSA), as a standard, following the method described by Bradford (1976), adapted for the microplate reader.

## 2.8. Ingestion

Following the method described by Gagné (2019), ingestion of nPS was evaluated using a fluorescence-based methodology using the molecular rotor probe 9-(dicyanovinyl)-julolidine (DCVJ). First and foremost, individual tissues (gills, digestive glands, and gonads) (n=6 per treatment, per time of exposure) of mussels were homogenized at 20% (w/v) in an ice-cold buffer solution (50 mM NaCl, 10 mM Hepes – NaOH [pH 7.4], 1 mM EDTA, and 1mM DTT), using a VWR Star-Beater (5 min, 20/s shaking, with grinding balls). Samples were centrifuged for 20 mins (2ºC, 15 000 *g*) to isolate the cytosolic fraction, and the resulting supernatant was immediately frozen (-80ºC) until further analysis. Samples were then analysed using the spectrofluorometric microplate reader (Berthold Tristar 5) with excitation at 450 nm and emission spectra ranging from 400 – 800 nm. The wavelength equalling nPS was 510 nm. Results are expressed as nPS µg/g wet weight relative controls.

## 2.9. Statistical Analysis

The significance of the differences between treatments and time were determined using the Shapiro-Wilk test for data distribution and variance homogeneity and either parametric tests (ANOVA, followed by Tukey's posthoc test) or non-parametric equivalent tests (Kruskal-Wallis and a two-tailed multiple comparisons test). The results were significant if $p < 0.05$. GraphPad Prism version 9.4.1 (GraphPad Software, Inc. CA) was utilized for the statistical analysis. Additionally, the relationship between treatments (not exposed and exposed to nPS) and the examined tissues (gills, digestive gland, and gonads) was assessed using a Principal Component Analysis (PCA) (Statistica 7.0 software (Statsoft Inc., 2005; USA).

## 2.10. Weight of Evidence (WOE)

In addition to results on acetylcholinesterase, data on cellular biomarkers (antioxidant enzymes: superoxide dismutase SOD, catalase CAT, glutathione peroxidase GPx, biotransformation enzymes: glutathione S-transferases GST, oxidative damage: lipid peroxidation LPO and genotoxicity: DNA damage) were also obtained from Gonçalves et al. (2022) who exposed mussels to the same exposure to nPS as described in the present work (10 µg/L of nPS 50 nm for 21 days). The overall biomarkers results were elaborated through a quantitative Weight Of Evidence model (WOE, Sediqualsoft) that provides a synthetic hazard index based on a specifically developed

algorithm and mathematical procedure (Regoli et al., 2019). For each analysed biomarker, the magnitude of the observed variation is compared to a specific threshold, corrected for the toxicological relevance of the biological endpoint (weight) and the statistical significance of the difference concerning controls. In-depth descriptions of complete calculations, intricate flow charts, the justification for weights, thresholds, and expert opinions have previously been provided by (Regoli et al., 2019).

## 3. Results

### 3.1. nPS Acute Toxicity

The $LC_{50}$ (µg/L) of *A. fischeri* after 5, 15 and 30 minutes of exposure to nPS was 217, 196, and 180 µg/L, respectively (Table 1).

### 3.2. *In vitro* assay

In *M. galloprovincialis* haemocytes, the 24 h exposure to nPS (10 µg/L) caused a significant reduction in cell viability compared to unexposed ones ($p < 0.05$) (Fig. 1).

### 3.3. AChE activity

Significant differences in unexposed mussels were observed on days 14, 21 and 28 compared to the beginning, while the effects of nPS were particularly evident after 3 and 7 days of the exposure ($p < 0.05$; Fig 2). AChE activity in the gills of nPS-exposed mussels, significantly decreased after 3 days of exposure ($p < 0.05$), followed by a further 2-fold decrease measured between 3 and 28 days ($p < 0.05$).

### 3.4. Ingestion

Mussel gills presented a significant ingestion of nPS (1.7 ± 0.01 µg/w.w.), equivalent to 6.75 x $10^8$ particles/mL (Fig. 3A) after 3 days of exposure compared to other exposure times ($p < 0.05$). In the digestive glands, a significant increase in nPS ingestion occurred after 14 days of exposure ($p < 0.05$), equalling 5.5 x $10^{10}$ particles/mL (Fig. 3B). Compared to gills and digestive glands, the highest levels of nPS ingestion were found in gonads after 7 and 14 days of exposure, with 2.6 ± 0.5 and 2.0 ± 0.6 µg nPS/g w.w., representing 2.9 x $10^{10}$ and 2.1 x $10^{10}$ particles/mL, respectively ($p < 0.05$), (Fig. 3C).

### 3.5. Principal Component Analysis (PCA)

The ingestion of nPS in the three mussel tissues (gills, digestive gland, and gonads) was described using a PCA, which was applied to all the data collected at each time point throughout the 28-d of exposure (Fig. 4). The two principal components accounted for 74.2% of the overall

252 variation (PC1 = 50.37 %, PC2 = 23.82 %; Fig. 3). There is a clear separation between mussel gills
253 and the other mussel tissues evaluated, as well as a distinction between unexposed and exposed to
254 polystyrene nanoplastics. The ingestion in the mussel digestive gland and gonads are positively
255 related to the principal component and support the results observed in Fig. 3B and 3C. Moreover,
256 PCA implies that although gills are the first tissue to contact with the nanoplastic, this tissue does
257 not accumulate within its tissues. This suggests that the translocation of the ingested polystyrene
258 nanoplastics in mussels is distributed, once filtered by the gills, to the other tissues, the digestive
259 gland, and gonads the destination of these nano-sized particles. PCA supports that at days 7, 14 and
260 21 of exposure, ingestions of nPS are most critical for mussels, as they are positively related to the
261 1st component. Therefore, PCA confirms that ingestion of nPS in *M. galloprovincialis* is tissue-
262 dependent and time-dependent. Furthermore, PCA corroborates the different time-point increases
263 in nPS ingestion observed in individual tissues.
264
265
266 **3.6. WOE**
267 The overall WOE elaboration of biomarker results (Table 2) indicated an increase of the cellular
268 hazard after 3 days (Moderate) and even more after 7 and 14 days (Major), while it returned to a
269 Slight level at day 21. The parameters that mostly contributed to the time-course increase of hazard
270 were the antioxidant enzymes (SOD, CAT, GPx and GST) further reflected by severe variations of
271 oxidative damages in terms of DNA and lipid peroxidation.
272
273 **4. Discussion**
274 The haemolymph of mussels reports the functional condition of the organs it perfuses as well
275 as extensive information on the animals' overall physiological status (Digilio et al., 2016). The
276 viability of haemocyte cells in *M. galloprovincialis* haemolymph was affected by the exposure to
277 nPS (10 µg/L; 50 nm; Fig. 1). Functionalized – nPS (PS-COOH and PS-$NH_2$), alter immune
278 parameters, such as phagocytosis, lysosome activity, ROS and NO production (Auguste et al., 2020;
279 Canesi et al., 2015). Additionally, an increase in DNA damage in *M. galloprovincialis* haemolymph
280 was caused by exposure to nPS [10 µg/L; 50 nm; 14-d (Gonçalves et al., 2022) and 0.05 – 50 mg/L;
281 106 ± 10 nm; 96 h (Brandts et al., 2018)], and a protein corona formation with PS-$NH_2$ was
282 noticeable in the haemolymph serum of *M. galloprovincialis* [50 nm; 50 µg/mL; 30 mins (Canesi
283 et al., 2015)]. The translocation of nPS from tissues to cells was evaluated, demonstrating that
284 internalization of nPS in haemocytes is size-dependent, being 50 nm nPS mostly internalized
285 compared to 100 nm and 1 µm polystyrene particles (Sendra et al., 2020). Therefore, nPS of 50 nm
286 in size cause cytotoxicity and can have more severe implications on the overall physiological status
287 of *M. galloprovincialis*. Regarding acute toxicity, no effects of microplastics were demonstrated at

environmentally relevant concentrations (Booth et al., 2016; Gagné, 2017; Gambardella et al., 2019), while exposure of the luminescent bacteria to nPS (50 nm) caused toxic effects after 5, 15 and 30 mins of exposure (Table 1). Gambardella et al. (2019) suggested that particles smaller than 1µm would be toxic as the pore size of cell walls prevents larger particles from entering (PE-MPs; 1 – 500 µm; 25 mg/L), thus confirming the ability of nano-sized particles to cross cellular boundaries; nano-sized plastics also affected the growth of the bacterium *Halomonas alkaliphile* [nPS; 20 mg/L; 55 nm; (Sun et al., 2018)]. With impacts on cellular viability, growth, and their possible internalization of nPS, nano-sized particles certainly pose a greater risk than micro-sized ones, with still unpredictable consequences on trophic transfer and impacts on the whole ecosystem.

In the gills of mussels, neurotoxicity occurred after 3 days of exposure to 10 µg/L of nPS (50 nm). Despite the differences observed in controls, a decrease in AChE activity was noticeable throughout the 21-d exposure. A 15 ng/L exposure of nPS (50 nm) for 21 days also caused a decrease in AChE activity in the *M. galloprovincialis* gills (Capolupo et al., 2021), while no neurotoxicity was observed in the freshwater clam *Corbicula fluminea* after exposure to nPS (80 nm; 0.1 – 5 mg/L; 96 h) (Li et al., 2020). Microfibres of polyethylene terephthalate caused a dose-dependent increase of AChE activity in the gills of *M. galloprovincialis* (PET-MFs; 100 µm; 0.0005 – 100 mg/L; Choi et al., 2021). Acetylcholine, a key neurotransmitter in the neurological and sensory systems, is broken down by AChE to convey impulses at cholinergic synapses, essential for neurotransmitter release and synaptic plasticity (Picciotto et al., 2012). Therefore, from all available data, it is deducible that the smaller the plastic particles, the more neurotoxic they are towards mussels, being more effective in seawater compared to freshwater.

This is the first data set on the ingestion of polystyrene nanoplastics (50 nm) in three tissues of *M. galloprovincialis* after a 10 µg/L exposure for 28 days, that we know of. Considering the pathways of NPs intake for a filter-feeder organism like *M. galloprovincialis*, due to their small size these particles can directly enter the organism through the gills, or their ability in crossing cellular boundaries. With biological reactivity increasing with decreasing particle size (Peng et al., 2020), the relation between NPs ingestion and toxicity towards organisms is a crucial issue. After 28 days of exposure to 10 µg/L of nPS (50 nm), *M. galloprovincialis* gills, digestive gland and gonads presented time-specific ingestion rates. As filter-feeding organisms, the increase in nPS ingestion by the gills after 3 days of exposure is expected, as this is the first tissue to encounter contaminants in the surrounding environment. Looking at the same experimental conditions (10 µg/L nPS; 50 nm; 21-d), the gills of *M. galloprovincialis* after 3 days of exposure led to a significant decrease of antioxidant enzymes activity until the 14$^{th}$ day, leading to oxidative damage (Joanna M. Gonçalves et al., 2022). Results suggested that the internalization of nPS in the gills has a prolonged toxic effect, although an adaptive response was observed after 21 days. In another study, the presence of nPS (50 nm) was also found in *M. galloprovincialis* gills, although larger nPS (100 nm and 1µm) were more abundant after 24h of exposure (Sendra et al., 2020); this was partly expected

as larger particles have similar sizes to food sources and are often mistaken as non or low nutritional food (nPS; 30 nm; 0.1 – 0.3 g/L; 8 h) (Wegner et al., 2012).

The digestive gland of mussels is known as being responsible for key metabolic functions, such as food intracellular uptake/digestion and as a storage for reserve substances (Faggio et al., 2018). Here, the mussel's digestive gland shows a significant intake of nPS after 14 days of exposure ($p < 0.05$). This suggests that after initial ingestion of the gills at day 3, nPS translocate to the digestive gland, and remains stored until the end of the experiment. Observing toxicity of nPS in the digestive gland after a 21-d exposure to 10 µg/L (50 nm), the 7$^{th}$ day was the most critical, causing inhibition of CAT and GPx activity and consequently leading to oxidative damage (Joanna M. Gonçalves et al., 2022). In this case, results imply that although not significant, the presence of nPS at 7-d is most critical for this tissue and that the increase in ingestion after this time-point truly overwhelm the mussels antioxidant defence system. Moreover, Sendra et al. (2020) found the digestive gland of *M. galloprovincialis* as the common destination for nPS (50 nm; 3 and 24h), despite the difficulties in observing these particles using histological assessments. Also, glitter particles (62.5 particles/L; 7-d) ingested by *M. galloprovincialis* accumulated in the digestive tract of mussels, whereby ingested glitter increased with the decrease in particle size (Provenza et al., 2022). Findings confirm that the smaller the size of the nanoplastic, the higher the accumulation of these particles in the digestive system of mussels.

Gonads of mussels showed the highest levels of nPS among those measured in the three tissues, particularly after 7 and 14 days of exposure (2.6 ± 0.5 and 2.0 ± 0.6 µg nPS/g w.w., respectively; $p < 0.05$). Considering the anatomy of mussels, a hypothesis is that nPS, due to their small size and ability to cross cellular boundaries, may 'diffuse' across tissues besides being transported to different tissues via the haemolymph. A translocation study from cells to tissue showed that internalization of nPS (50 nm, 100 nm and 1µm; 3 – 24 h) into haemocytes was size–dependent, with nPS of 50 nm being the most internalized (Sendra et al., 2020). Therefore, with the same size of particles, the observed ingestion of nPS in gonads may be due to their translocation in the haemocytes, despite their ability to cross cellular boundaries should not be excluded. Looking at the toxic effects of 10 µg/L of nPS pursue in *M. galloprovincialis* gonads after 21 days of exposure, a similar pattern to the digestive gland is clear **(**Gonçalves & Bebianno, 2023). The most critical time-point for gonads is at 3 days of exposure, as nPS exposure leads to an 18.6-fold increase in oxidative damage, although the highest ingestion is seen on days 7 and 14. Considering the antioxidant enzyme activities at both 7 and 14 days of exposure, CAT and GPx activity increased, whereas GST activity decreased **(**Gonçalves & Bebianno, 2023), suggesting that with the increase of ingested nPS in gonads of mussels, the organism's antioxidant defence mechanisms are hindered. The gonads of mussels and their development are crucial for reproductive success and during the gametogenesis cycle, lipid content has been shown to enhance with the ripening of female gonads, as well as been used as an energy source during gametogenesis and embryo-larval development

(Martínez-Pita et al., 2012). More concerning, as nPS of 50 nm internalizes haemocytes, spermatozoa and oocytes may also be at risk. In *Crassostrea gigas*, fertilization rate decreased after gametes were exposed to nPS (50 nm; 1.5 h) (Tallec et al., 2018), and sperm mobility and fertilization success were impaired, as agglomerates of nanoparticles were found attached to spermatozoa and the jelly coating of oocytes (PS-COOH and PS-$NH_2$; 0.1 to 100 mg/L; 1, 3 and 5 h) (González-Fernández et al., 2018). In *M. galloprovincialis,* malformations of D-larvae were found after embryos were exposed to nPS (50 nm; 10 µg/L; 48 hpf) (Auguste et al., 2021), suggesting the high content of ingested nPS in mussel gonads may pose a serious threat for the reproduction of these organisms.

In the present study, the biological significance of the results observed in this and a previous study (Gonçalves et al., 2022) was summarized in specific hazard indices allowing an easier qualitative and quantitative comparison of the effects of nPS at different exposure times. The applied elaboration contemplates the measured endpoints' toxicological importance (weight), as well as the extent and magnitude of shifts, normalised to predetermined thresholds. This model has been validated in several case studies aimed to evaluate biological and environmental risk assessment providing the possibility to integrate large datasets of heterogeneous data and to better interpret asynchronous variations of complex pathways (Mezzelani et al., 2021; Nardi et al., 2022; Pittura et al., 2018; Regoli et al., 2014). The weighted elaboration of biomarker results confirmed the involvement of oxidative stress in modulating the toxicity of nanoparticles with a clear time-dependent trend as already outlined by the discussion of individual results (Table 2). The cellular hazard was raised to Moderate after 3 days further increasing on days 7 and 14 when the observed variations were summarized in a Major hazard. After this peak, a counteracting response to oxidative challenge is evidenced by the return to a Slight hazard condition.

## 5. Conclusion

This dataset provides evidence for the ingestion of polystyrene nanoplastics in the marine mussel *M. galloprovincialis*, as well as neurotoxicity and *in vitro* effects on mussel haemolymph. nPS of 50 nm cause neurotoxicity in mussel gills and reduce viable haemocytes in mussels' haemolymph. Ingestion in mussels is tissue and time specific, whereby gonads present the highest ingested nPS. Biochemical alterations of mussel tissues may be explained by the quantity of ingested nPS, however further investigation is necessary. Moreover, the effects of ingested nPS in the gonads of mussels need more insight into how it can affect gametogenesis, fertilization success, and embryo-larval development. Lastly, the impacts of eating nPS-contaminated shellfish batches can cause adverse effects on human health and should be considered for future analysis.

## 6. Acknowledgements

A PhD grant (UI/BD/150758/2020), the JPI Oceans project RESPONSE (FCT JPIOCEANS/0005/2020), which was funded by FCT and the European Regional Development Fund (ERDF) (ALG-01-0145-FEDER-030922) through Portugal 2020 and Portuguese national funds, were all used to support this research. We also value the funding provided by FCT for projects LA/P/0069/2020 awarded to the Associate Laboratory ARNET and UID/00350/2020 awarded to CIMA of the University of Algarve.

## 7. References

Andrady, A. L. (2011). Microplastics in the marine environment. Marine Pollution Bulletin, 62(8), 1596–1605. https://doi.org/10.1016/j.marpolbul.2011.05.030

Auguste, M., Balbi, T., Miglioli, A., Alberti, S., Prandi, S., Narizzano, R., Salis, A., Damonte, G., & Canesi, L. (2021). Comparison of different commercial nanopolystyrenes: Behavior in exposure media, effects on immune function and early larval development in the model bivalve *Mytilus galloprovincialis*. Nanomaterials, 11(12). https://doi.org/10.3390/nano11123291

Auguste, M., Lasa, A., Balbi, T., Pallavicini, A., Vezzulli, L., & Canesi, L. (2020). Impact of nanoplastics on hemolymph immune parameters and microbiota composition in *Mytilus galloprovincialis*. Marine Environmental Research, 159(May), 105017. https://doi.org/10.1016/j.marenvres.2020.105017

Azur Environmental. (1998). *Microtox Acute Toxicity Test*.

Bergami, E., Bocci, E., Vannuccini, M. L., Monopoli, M., Salvati, A., Dawson, K. A., & Corsi, I. (2016). Nano-sized polystyrene affects feeding, behavior and physiology of brine shrimp *Artemia franciscana* larvae. Ecotoxicology and Environmental Safety, 123(2016), 18–25. https://doi.org/10.1016/j.ecoenv.2015.09.021

Bessa, F., Barría, P., Neto, J. M., Frias, J. P. G. L., Otero, V., Sobral, P., & Marques, J. C. (2018). Occurrence of microplastics in commercial fish from a natural estuarine environment. Marine Pollution Bulletin, 128(February), 575–584. https://doi.org/10.1016/j.marpolbul.2018.01.044

Block, C., Brands, B., & Gude, T. (2017). Packaging Materials: Polystyrene for food Packaging applications. In ILSI Europe Report Series. http://europe.ilsi.org/publications

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72(1), 248–254. https://doi.org/https://doi.org/10.1016/0003-2697(76)90527-3

Brandts, I., Teles, M., Gonçalves, A. P., Barreto, A., Franco-Martinez, L., Tvarijonaviciute, A., Martins, M. A., Soares, A. M. V. M., Tort, L., & Oliveira, M. (2018). Effects of nanoplastics on *Mytilus galloprovincialis* after individual and combined exposure with carbamazepine. Science of the Total Environment, 643, 775–784. https://doi.org/10.1016/j.scitotenv.2018.06.257

Canesi, L., Ciacci, C., Bergami, E., Monopoli, M. P., Dawson, K. A., Papa, S., Canonico, B., & Corsi, I. (2015). Evidence for immunomodulation and apoptotic processes induced by cationic polystyrene nanoparticles in the hemocytes of the marine bivalve *Mytilus*. Marine Environmental Research, 111, 34–40. https://doi.org/10.1016/j.marenvres.2015.06.008

Capolupo, M., Valbonesi, P., & Fabbri, E. (2021). A comparative assessment of the chronic effects of micro-and nano-plastics on the physiology of the mediterranean mussel *Mytilus galloprovincialis*. Nanomaterials, 11(3), 1–17. https://doi.org/10.3390/nano11030649

Choi, J. S., Kim, K., Hong, S. H., Park, K. Il, & Park, J. W. (2021). Impact of polyethylene terephthalate microfiber length on cellular responses in the Mediterranean mussel *Mytilus galloprovincialis*. Marine Environmental Research, 168(March), 105320. https://doi.org/10.1016/j.marenvres.2021.105320

Digilio, G., Sforzini, S., Cassino, C., Robotti, E., Oliveri, C., Marengo, E., Musso, D., Osella, D., & Viarengo, A. (2016). Haemolymph from *Mytilus galloprovincialis*: Response to copper and temperature challenges studied by 1H-NMR metabonomics. Comparative Biochemistry and Physiology Part - C: Toxicology and Pharmacology, 183–184, 61–71. https://doi.org/10.1016/j.cbpc.2016.02.003

Dong, Z., Zhang, W., Qiu, Y., Yang, Z., Wang, J., & Zhang, Y. (2019). Cotransport of nanoplastics (NPs) with fullerene (C60) in saturated sand: Effect of NPs/C60 ratio and seawater salinity. Water Research, 148, 469–478. https://doi.org/10.1016/j.watres.2018.10.071

Ekvall, M. T., Lundqvist, M., Kelpsiene, E., Šileikis, E., Gunnarsson, S. B., & Cedervall, T. (2019). Nanoplastics formed during the mechanical breakdown of daily-use polystyrene products. Nanoscale Advances, 1(3), 1055–1061. https://doi.org/10.1039/c8na00210j

Ellman, G. L., Courtney, K. D., Andres, V., & Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology, 7(2), 88–95. https://doi.org/10.1016/0006-2952(61)90145-9

Faggio, C., Tsarpali, V., & Dailianis, S. (2018). Mussel digestive gland as a model tissue for assessing xenobiotics: An overview. Science of the Total Environment, 636, 220–229. https://doi.org/10.1016/j.scitotenv.2018.04.264

Ferreira, I., Venâncio, C., Lopes, I., & Oliveira, M. (2019). Nanoplastics and marine organisms: What has been studied? Environmental Toxicology and Pharmacology, 67(January), 1–7. https://doi.org/10.1016/j.etap.2019.01.006

Gagné, F. (2019). Detection of polystyrene nanoplastics in biological tissues with a fluorescent molecular rotor probe. Journal of Xenobiotics, 9, 7–9. https://doi.org/10.4081/xeno.2019.8147

Gambardella, C., Piazza, V., Albentosa, M., Bebianno, M. J., Cardoso, C., Faimali, M., Garaventa, F., Garrido, S., González, S., Pérez, S., Sendra, M., & Beiras, R. (2019). Microplastics do not affect standard ecotoxicological endpoints in marine unicellular organisms. Marine Pollution Bulletin, 143(February), 140–143. https://doi.org/10.1016/j.marpolbul.2019.04.055

471 Gómez-Mendikute, A., & Cajaraville, M. P. (2003). Comparative effects of cadmium, copper,
472 paraquat and benzo[a]pyrene on the actin cytoskeleton and production of reactive oxygen
473 species (ROS) in mussel haemocytes. Toxicology in Vitro, 17(5–6), 539–546.
474 https://doi.org/10.1016/S0887-2333(03)00093-6
475 Gonçalves, J. M., & Bebianno, M. J. (2023). Ecotoxicity of emerging contaminants in the
476 reproductive organ of marine mussels *Mytilus galloprovincialis*. Science of The Total
477 Environment, 163486. https://doi.org/https://doi.org/10.1016/j.scitotenv.2023.163486
478 Gonçalves, J. M., & Bebianno, M. J. (2021). Nanoplastics impact on marine biota: A review.
479 Environmental Pollution, 273, 116426. https://doi.org/10.1016/j.envpol.2021.116426
480 Gonçalves, J. M., Sousa, V. S., Teixeira, M. R., & Bebianno, M. J. (2022). Chronic toxicity of
481 polystyrene nanoparticles in the marine mussel *Mytilus galloprovincialis*. Chemosphere,
482 287(January 2022), 132356. https://doi.org/10.1016/j.chemosphere.2021.132356
483 González-Fernández, C., Tallec, K., Le Goïc, N., Lambert, C., Soudant, P., Huvet, A., Suquet, M.,
484 Berchel, M., & Paul-Pont, I. (2018). Cellular responses of Pacific oyster (*Crassostrea gigas*)
485 gametes exposed in vitro to polystyrene nanoparticles. Chemosphere, 208, 764–772.
486 https://doi.org/10.1016/j.chemosphere.2018.06.039
487 Halle, A. Ter, Jeanneau, L., Martignac, M., Jardé, E., Pedrono, B., Brach, L., & Gigault, J. (2017).
488 Nanoplastic in the North Atlantic Subtropical Gyre. Environmental Science and Technology,
489 51(23), 13689–13697. https://doi.org/10.1021/acs.est.7b03667
490 Katsumiti, A., Gilliland, D., Arostegui, I., & Cajaraville, M. P. (2014). Cytotoxicity and cellular
491 mechanisms involved in the toxicity of CdS quantum dots in hemocytes and gill cells of the
492 mussel *Mytilus galloprovincialis*. Aquatic Toxicology, 153, 39–52.
493 https://doi.org/10.1016/j.aquatox.2014.02.003
494 Kihara, S., Köper, I., Mata, J. P., & McGillivray, D. J. (2021). Reviewing nanoplastic toxicology: It's
495 an interface problem. Advances in Colloid and Interface Science, *288*, 102337.
496 https://doi.org/10.1016/j.cis.2020.102337
497 Kögel, T., Bjorøy, Ø., Toto, B., Bienfait, A. M., & Sanden, M. (2020). Micro- and nanoplastic
498 toxicity on aquatic life: Determining factors. Science of the Total Environment, 709(5817),
499 136050. https://doi.org/10.1016/j.scitotenv.2019.136050
500 Li, Z., Feng, C., Wu, Y., & Guo, X. (2020). Impacts of nanoplastics on bivalve: Fluorescence tracing
501 of organ accumulation, oxidative stress and damage. Journal of Hazardous Materials,
502 392(February), 122418. https://doi.org/10.1016/j.jhazmat.2020.122418
503 Martínez-Pita, I., Sánchez-Lazo, C., Ruíz-Jarabo, I., Herrera, M., & Mancera, J. M. (2012).
504 Biochemical composition, lipid classes, fatty acids and sexual hormones in the mussel *Mytilus*
505 *galloprovincialis* from cultivated populations in south Spain. Aquaculture, 358–359, 274–283.
506 https://doi.org/10.1016/j.aquaculture.2012.06.003
507 Mezzelani, M., Nardi, A., Bernardini, I., Milan, M., Peruzza, L., d'Errico, G., Fattorini, D., Gorbi, S.,

508 Patarnello, T., & Regoli, F. (2021). Environmental pharmaceuticals and climate change: The
509 case study of carbamazepine in *M. galloprovincialis* under ocean acidification scenario.
510 Environment International, 146, 106269. https://doi.org/10.1016/j.envint.2020.106269
511 Nardi, A., Mezzelani, M., Costa, S., d'Errico, G., Benedetti, M., Gorbi, S., Freitas, R., & Regoli, F.
512 (2022). Marine heatwaves hamper neuro-immune and oxidative tolerance toward carbamazepine
513 in *Mytilus galloprovincialis*. Environmental Pollution, 300(February), 118970.
514 https://doi.org/10.1016/j.envpol.2022.118970
515 Peng, L., Fu, D., Qi, H., Lan, C. Q., Yu, H., & Ge, C. (2020). Micro- and nano-plastics in marine
516 environment: Source, distribution and threats — A review. Science of the Total Environment,
517 698, 134254. https://doi.org/10.1016/j.scitotenv.2019.134254
518 Picciotto, M. R., Higley, M. J., & Mineur, Y. S. (2012). Acetylcholine as a Neuromodulator:
519 Cholinergic Signaling Shapes Nervous System Function and Behavior. Neuron, 76(1), 116–129.
520 https://doi.org/10.1016/j.neuron.2012.08.036
521 Pittura, L., Avio, C. G., Giuliani, M. E., d'Errico, G., Keiter, S. H., Cormier, B., Gorbi, S., & Regoli,
522 F. (2018). Microplastics as vehicles of environmental PAHs to marine organisms: Combined
523 chemical and physical hazards to the mediterranean mussels, *Mytilus galloprovincialis*. Frontiers
524 in Marine Science, 5(APR). https://doi.org/10.3389/fmars.2018.00103
525 PlasticEurope: Plastics – The Facts 2022, H. org/knowledge-hub/plastics-the-fact.-2022/. (2022).
526 *Plastics – the Facts 2022*. *October*.
527 Provenza, F., Anselmi, S., Specchiulli, A., Piccardo, M., Barceló, D., Prearo, M., Pastorino, P., &
528 Renzi, M. (2022). Sparkling plastic: Effects of exposure to glitter on the Mediterranean mussel
529 *Mytilus galloprovincialis.* Environmental Toxicology and Pharmacology, 96(September).
530 https://doi.org/10.1016/j.etap.2022.103994
531 Regoli, F., d'Errico, G., Nardi, A., Mezzelani, M., Fattorini, D., Benedetti, M., Di Carlo, M.,
532 Pellegrini, D., & Gorbi, S. (2019). Application of a weight of evidence approach for monitoring
533 complex environmental scenarios: The case-study of off-shore platforms. Frontiers in Marine
534 Science, 6(JUL), 1–15. https://doi.org/10.3389/fmars.2019.00377
535 Regoli, F., Pellegrini, D., Cicero, A. M., Nigro, M., Benedetti, M., Gorbi, S., Fattorini, D., D'Errico,
536 G., Di Carlo, M., Nardi, A., Gaion, A., Scuderi, A., Giuliani, S., Romanelli, G., Berto, D.,
537 Trabucco, B., Guidi, P., Bernardeschi, M., Scarcelli, V., & Frenzilli, G. (2014). A
538 multidisciplinary weight of evidence approach for environmental risk assessment at the Costa
539 Concordia wreck: Integrative indices from Mussel Watch. Marine Environmental Research, 96,
540 92–104. https://doi.org/10.1016/j.marenvres.2013.09.016
541 Sendra, M., Saco, A., Yeste, M. P., Romero, A., Novoa, B., & Figueras, A. (2020). Nanoplastics:
542 From tissue accumulation to cell translocation into *Mytilus galloprovincialis* hemocytes.
543 resilience of immune cells exposed to nanoplastics and nanoplastics plus *Vibrio splendidus*
544 combination. Journal of Hazardous Materials, 388(November 2019), 121788.

https://doi.org/10.1016/j.jhazmat.2019.121788

Sun, X., Chen, B., Li, Q., Liu, N., Xia, B., Zhu, L., & Qu, K. (2018). Toxicities of polystyrene nano- and microplastics toward marine bacterium *Halomonas alkaliphila*. Science of the Total Environment, 642, 1378–1385. https://doi.org/10.1016/j.scitotenv.2018.06.141

Tallec, K., Huvet, A., Di Poi, C., González-Fernández, C., Lambert, C., Petton, B., Le Goïc, N., Berchel, M., Soudant, P., & Paul-Pont, I. (2018). Nanoplastics impaired oyster free living stages, gametes and embryos. Environmental Pollution, 242, 1226–1235. https://doi.org/10.1016/j.envpol.2018.08.020

Tamminga, M., Hengstmann, E., & Fischer, E. K. (2018). Microplastic analysis in the South Funen Archipelago, Baltic Sea, implementing manta trawling and bulk sampling. Marine Pollution Bulletin, 128(October 2017), 601–608. https://doi.org/10.1016/j.marpolbul.2018.01.066

The Waste and Resources Action Programme. (2023). Recycle. https://www.recyclenow.com/recycle-an-item/polystyrene

Wang, W., Yuan, W., Chen, Y., & Wang, J. (2018). Microplastics in surface waters of Dongting Lake and Hong Lake, China. Science of the Total Environment, 633(41907352), 539–545. https://doi.org/10.1016/j.scitotenv.2018.03.211

Wegner, A., Besseling, E., Foekema, E. M., Kamermans, P., & Koelmans, A. A. (2012). Effects of nanopolystyrene on the feeding behavior of the blue mussel (*Mytilus edulis* L.). Environmental Toxicology and Chemistry, 31(11), 2490–2497. https://doi.org/10.1002/etc.1984