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Polystyrene nanoplastics in the marine mussel *Mytilus galloprovincialis*.

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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 (LC₅₀) of *Aliivibrio fischeri* bacteria to evaluate acute toxicity. Cellular viability of mussel haemocytes decreased significantly after a 24 h exposure and nPS LC₅₀ 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

39 environment is the inevitable fate of these materials. In Europe, plastic production was equivalent
40 to 15% of GPP (57.2 Mt), with applications such as packaging and building and construction
41 composing 39.1 and 21.3% of Europe's plastic demands, respectively (PlasticEurope, 2022). In
42 2021, the European Union adopted a directive banning all single-use plastics, except for bottles, as
43 single-use plastic, and fishing gear which account for 70% of marine litter (Directive (EU)
44 2019/904).

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

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

76 *galloprovincialis*, nPS provoke oxidative stress and damage (10 µg/L; 50 nm; 21-d) (Gonçalves et
77 al., 2022), and lysosomal destabilization (1.5 – 150 ng/L; 50 nm; 21-d) (Capolupo et al., 2021).
78 Although ingestion and translocation of MPs have been evaluated, the assessment of whether nPS
79 toxicity is entirely from their ability to cross cellular boundaries, or if these nanoparticles are
80 ingested, accumulated, and translocated between tissues remains difficult. In this study, a
81 combination of *in vitro* and *in vivo* experiments was carried out to evaluate both acute toxicity and
82 chronic effects of nPS on key model species. An *in vitro* assay on *M. galloprovincialis* haemocytes
83 was performed to evaluate cellular viability after 24 h exposure to 10 µg/L of nPS (50 nm) and
84 acute toxicity was further characterized through the Microtox® bioassay (LC₅₀). An *in vivo* 28-d
85 exposure of mussels was performed at the same concentration of nPS to evaluate both ingestion of
86 nanoparticles in gills, digestive glands, and gonads, as well as the onset of neurotoxicity in gills.
87 These data were further integrated with those previously reported on a wide selection of cellular
88 biomarkers; their overall elaboration through weighted criteria was expected to provide a better
89 evaluation of the toxicological hazard of nPS.

91 **2. Materials and Methods**

92 **2.1. Polystyrene nanoplastics (nPS)**

93 Fluoresbrite® Plain YG spherical polystyrene nanoplastics of 50 nm in size (CAS 9003-53-6)
94 were purchased from Polysciences, Inc. (Germany). 50 nm particles packed as 2.5% aqueous
95 suspension, with 3.64×10^{14} particles/mL in ultrapure water (7732-18- 5) (CV = 15%, Excitation
96 max. = 441 nm, Emission max. = 486 nm). Gonçalves et al. (2022) provide a full description of the
97 characterization of nPS and demonstrate that the hydrodynamic diameter of nPS rises when
98 distributed in FSW (852 103 nm), which suggests that the high salt content in seawater causes
99 aggregation/agglomeration kinetics. A concentration of 10 µg/L was used for exposure assays.

101 **2.2. Acute toxicity assay**

102 The solid phase Microtox® bioassay (SPT, Azur Environmental, 1998) was used to evaluate
103 polystyrene nanoplastics (nPS; 50 nm) acute toxicity in the bioluminescent marine bacterium
104 *Aliivibrio fischeri* according to ISO 1134-3: 2007 and ISO 21338: 2010 according to the method
105 described in Gambardella et al., 2019). All reagents and lyophilized *A. fischeri* bacteria (NRRL B-
106 11177) were obtained from Modern Water Ltd (USA). Tests were carried out at 15°C in the
107 Microtox diluent supplied. The initial concentration of nPS used was 3724 µg/L and was
108 sequentially diluted. Acute toxicity of nPS was measured in terms of relative bioluminescence by
109 Microtox™ 500 luminometer after 5-, 15-, and 30-mins incubation. The bioluminescence inhibition
110 was determined using the Microtox®FX equipment, and data were analysed using the

111 MicrotoxOmni software. The median lethal concentration (LC₅₀) was defined as the concentration
112 that produced a 50% light reduction after 5-, 15-, and 30-mins of contact time for bacteria.

113

114 **2.3. *In vitro* assay**

115 Fifteen mussels *M. galloprovincialis* (60 ± 5 mm) were collected from an offshore aquaculture
116 site in Lagos, Southeast Portugal (A: 37°04'200" N 8°42,800" W, B: 37°04'200" N 8°41'000" W,
117 C: 37°03'400" N 8°41'000" W, D: 37°03'400" N 8°42'800" W; Testa & Cunhas Ltd) and
118 transported alive to the laboratory. Mussels (2 mussels/L) were placed into a 10 L tank, with 7.5 L
119 of seawater (S: 36 ± 1) and left to acclimatise for 48 hours. Then, mussel haemolymphs were
120 extracted from the posterior adductor muscle of mussels with a sterile hypodermic syringe (1 mL)
121 (25 G needle), under aseptic conditions in a vertical laminar airflow cabinet, and kept on ice. Cell
122 extraction and incubation methods were based on the modifications of protocols developed by
123 Gómez-Mendikute & Cajaraville (2003) and Katsumiti et al. (2014). To have sufficient
124 haemolymph for *in vitro* exposures, five pools of three mussels per pool were used. Firstly, to avoid
125 clumping and aggregation of cells, 10 µL of pooled haemolymph was added to an anti-aggregation
126 solution (0.2M Tris; 171mM NaCl; 24mM EDTA; 0.15% v/v HCl 1 N) (Katsumiti et al., 2014).
127 Trypan blue dye (0.4% in physiological solution; v/v) was added for cell staining in a 1:2 proportion
128 (cell suspension: Trypan Blue 0.4%). Using a Neubauer chamber (200 cells per specimen) and with
129 the aid of a haemocytometer and light microscopy (Compound Light Microscopy; 400x), cell
130 viability was determined by the following equation:

$$131 \text{ Concentration (cells mL}^{-1}\text{)} = \frac{n^{\circ} \text{ cells} \times 10\,000}{n^{\circ} \text{ squares}} \times \text{dilution factor}$$

132

133

134 Following cellular viability, cell suspensions were diluted at a density of 2 x 10⁵ haemocyte
135 cells/mL (in anti-aggregation solution), and 100 µL were seeded into 96-well microplates (6
136 replicated per pool) with cell culture media Dulbecco's Modified Eagle Medium (DMEM, pH 7.4)
137 and exposed to 10 µg/L of nPS. Microplates were incubated for 24 h at 18 °C. Afterwards, culture
138 media was discarded, and the neutral red (NR) assay was applied following an adaptation from the
139 protocol described in Katsumiti et al. (2014). The culture medium from the wells was removed and
140 the state of the cells was verified under light microscopy (Compound Light Microscopy; 400x). 50
141 µL of neutral red working solution (0.4%, pH 7.3-7.4) was added to each well, including empty
142 wells for the negative control, and left to incubate for 1 hour in the dark. The microplate was then
143 centrifuged at 270 g (4°C, 10 mins) and the supernatant was removed and carefully washed with
144 PBS. The dye was then extracted from the viable cells in acetic acid/ethanol solution (1:100),
145 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, 4°C, 30 min), the supernatant
172 was collected, and stored at -80°C 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**

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

184 **2.8. Ingestion**

185 Following the method described by Gagné (2019), ingestion of nPS was evaluated using a
186 fluorescence-based methodology using the molecular rotor probe 9-(dicyanovinyl)-julolidine
187 (DCVJ). First and foremost, individual tissues (gills, digestive glands, and gonads) (n=6 per
188 treatment, per time of exposure) of mussels were homogenized at 20% (w/v) in an ice-cold buffer
189 solution (50 mM NaCl, 10 mM Hepes – NaOH [pH 7.4], 1 mM EDTA, and 1mM DTT), using a
190 VWR Star-Beater (5 min, 20/s shaking, with grinding balls). Samples were centrifuged for 20 mins
191 (2°C, 15 000 g) to isolate the cytosolic fraction, and the resulting supernatant was immediately
192 frozen (-80°C) until further analysis. Samples were then analysed using the spectrofluorometric
193 microplate reader (Berthold Tristar 5) with excitation at 450 nm and emission spectra ranging from
194 400 – 800 nm. The wavelength equalling nPS was 510 nm. Results are expressed as nPS µg/g wet
195 weight relative controls.

197 **2.9. Statistical Analysis**

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

207 **2.10. Weight of Evidence (WOE)**

208 In addition to results on acetylcholinesterase, data on cellular biomarkers (antioxidant enzymes:
209 superoxide dismutase SOD, catalase CAT, glutathione peroxidase GPx, biotransformation
210 enzymes: glutathione S-transferases GST, oxidative damage: lipid peroxidation LPO and
211 genotoxicity: DNA damage) were also obtained from Gonçalves et al. (2022) who exposed mussels
212 to the same exposure to nPS as described in the present work (10 µg/L of nPS 50 nm for 21 days).
213 The overall biomarkers results were elaborated through a quantitative Weight Of Evidence model
214 (WOE, Sediqualsoft) that provides a synthetic hazard index based on a specifically developed
215

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

222

223 **3. Results**

224 **3.1. nPS Acute Toxicity**

225 The LC₅₀ (µg/L) of *A. fischeri* after 5, 15 and 30 minutes of exposure to nPS was 217, 196, and
226 180 µg/L, respectively (Table 1).

227

228 **3.2. *In vitro* assay**

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

231

232 **3.3. AChE activity**

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

238

239 **3.4. Ingestion**

240 Mussel gills presented a significant ingestion of nPS (1.7 ± 0.01 µg/w.w.), equivalent to $6.75 \times$
241 10^8 particles/mL (Fig. 3A) after 3 days of exposure compared to other exposure times ($p < 0.05$).
242 In the digestive glands, a significant increase in nPS ingestion occurred after 14 days of exposure
243 ($p < 0.05$), equalling 5.5×10^{10} particles/mL (Fig. 3B). Compared to gills and digestive glands, the
244 highest levels of nPS ingestion were found in gonads after 7 and 14 days of exposure, with $2.6 \pm$
245 0.5 and 2.0 ± 0.6 µg nPS/g w.w., representing 2.9×10^{10} and 2.1×10^{10} particles/mL, respectively
246 ($p < 0.05$), (Fig. 3C).

247

248 **3.5. Principal Component Analysis (PCA)**

249 The ingestion of nPS in the three mussel tissues (gills, digestive gland, and gonads) was
250 described using a PCA, which was applied to all the data collected at each time point throughout
251 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-NH₂), 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-NH₂ 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

288 environmentally relevant concentrations (Booth et al., 2016; Gagné, 2017; Gambardella et al.,
289 2019), while exposure of the luminescent bacteria to nPS (50 nm) caused toxic effects after 5, 15
290 and 30 mins of exposure (Table 1). Gambardella et al. (2019) suggested that particles smaller than
291 1 µm would be toxic as the pore size of cell walls prevents larger particles from entering (PE-MPs;
292 1 – 500 µm; 25 mg/L), thus confirming the ability of nano-sized particles to cross cellular
293 boundaries; nano-sized plastics also affected the growth of the bacterium *Halomonas alkaliphile*
294 [nPS; 20 mg/L; 55 nm; (Sun et al., 2018)]. With impacts on cellular viability, growth, and their
295 possible internalization of nPS, nano-sized particles certainly pose a greater risk than micro-sized
296 ones, with still unpredictable consequences on trophic transfer and impacts on the whole ecosystem.

297 In the gills of mussels, neurotoxicity occurred after 3 days of exposure to 10 µg/L of nPS (50
298 nm). Despite the differences observed in controls, a decrease in AChE activity was noticeable
299 throughout the 21-d exposure. A 15 ng/L exposure of nPS (50 nm) for 21 days also caused a
300 decrease in AChE activity in the *M. galloprovincialis* gills (Capolupo et al., 2021), while no
301 neurotoxicity was observed in the freshwater clam *Corbicula fluminea* after exposure to nPS (80
302 nm; 0.1 – 5 mg/L; 96 h) (Li et al., 2020). Microfibres of polyethylene terephthalate caused a dose-
303 dependent increase of AChE activity in the gills of *M. galloprovincialis* (PET-MFs; 100 µm; 0.0005
304 – 100 mg/L; Choi et al., 2021). Acetylcholine, a key neurotransmitter in the neurological and
305 sensory systems, is broken down by AChE to convey impulses at cholinergic synapses, essential
306 for neurotransmitter release and synaptic plasticity (Picciotto et al., 2012). Therefore, from all
307 available data, it is deducible that the smaller the plastic particles, the more neurotoxic they are
308 towards mussels, being more effective in seawater compared to freshwater.

309 This is the first data set on the ingestion of polystyrene nanoplastics (50 nm) in three tissues of
310 *M. galloprovincialis* after a 10 µg/L exposure for 28 days, that we know of. Considering the
311 pathways of NPs intake for a filter-feeder organism like *M. galloprovincialis*, due to their small
312 size these particles can directly enter the organism through the gills, or their ability in crossing
313 cellular boundaries. With biological reactivity increasing with decreasing particle size (Peng et al.,
314 2020), the relation between NPs ingestion and toxicity towards organisms is a crucial issue. After
315 28 days of exposure to 10 µg/L of nPS (50 nm), *M. galloprovincialis* gills, digestive gland and
316 gonads presented time-specific ingestion rates. As filter-feeding organisms, the increase in nPS
317 ingestion by the gills after 3 days of exposure is expected, as this is the first tissue to encounter
318 contaminants in the surrounding environment. Looking at the same experimental conditions (10
319 µg/L nPS; 50 nm; 21-d), the gills of *M. galloprovincialis* after 3 days of exposure led to a significant
320 decrease of antioxidant enzymes activity until the 14th day, leading to oxidative damage (Joanna M.
321 Gonçalves et al., 2022). Results suggested that the internalization of nPS in the gills has a prolonged
322 toxic effect, although an adaptive response was observed after 21 days. In another study, the
323 presence of nPS (50 nm) was also found in *M. galloprovincialis* gills, although larger nPS (100 nm
324 and 1 µm) were more abundant after 24h of exposure (Sendra et al., 2020); this was partly expected

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

327 The digestive gland of mussels is known as being responsible for key metabolic functions, such
328 as food intracellular uptake/digestion and as a storage for reserve substances (Faggio et al., 2018).
329 Here, the mussel's digestive gland shows a significant intake of nPS after 14 days of exposure ($p <$
330 0.05). This suggests that after initial ingestion of the gills at day 3, nPS translocate to the digestive
331 gland, and remains stored until the end of the experiment. Observing toxicity of nPS in the digestive
332 gland after a 21-d exposure to 10 $\mu\text{g/L}$ (50 nm), the 7th day was the most critical, causing inhibition
333 of CAT and GPx activity and consequently leading to oxidative damage (Joanna M. Gonçalves et
334 al., 2022). In this case, results imply that although not significant, the presence of nPS at 7-d is most
335 critical for this tissue and that the increase in ingestion after this time-point truly overwhelm the
336 mussels antioxidant defence system. Moreover, Sendra et al. (2020) found the digestive gland of
337 *M. galloprovincialis* as the common destination for nPS (50 nm; 3 and 24h), despite the difficulties
338 in observing these particles using histological assessments. Also, glitter particles (62.5 particles/L;
339 7-d) ingested by *M. galloprovincialis* accumulated in the digestive tract of mussels, whereby
340 ingested glitter increased with the decrease in particle size (Provenza et al., 2022). Findings confirm
341 that the smaller the size of the nanoplastic, the higher the accumulation of these particles in the
342 digestive system of mussels.

343 Gonads of mussels showed the highest levels of nPS among those measured in the three tissues,
344 particularly after 7 and 14 days of exposure (2.6 ± 0.5 and 2.0 ± 0.6 $\mu\text{g nPS/g w.w.}$, respectively; p
345 < 0.05). Considering the anatomy of mussels, a hypothesis is that nPS, due to their small size and
346 ability to cross cellular boundaries, may 'diffuse' across tissues besides being transported to
347 different tissues via the haemolymph. A translocation study from cells to tissue showed that
348 internalization of nPS (50 nm, 100 nm and 1 μm ; 3 – 24 h) into haemocytes was size-dependent,
349 with nPS of 50 nm being the most internalized (Sendra et al., 2020). Therefore, with the same size
350 of particles, the observed ingestion of nPS in gonads may be due to their translocation in the
351 haemocytes, despite their ability to cross cellular boundaries should not be excluded. Looking at
352 the toxic effects of 10 $\mu\text{g/L}$ of nPS pursue in *M. galloprovincialis* gonads after 21 days of exposure,
353 a similar pattern to the digestive gland is clear (Gonçalves & Bebianno, 2023). The most critical
354 time-point for gonads is at 3 days of exposure, as nPS exposure leads to an 18.6-fold increase in
355 oxidative damage, although the highest ingestion is seen on days 7 and 14. Considering the
356 antioxidant enzyme activities at both 7 and 14 days of exposure, CAT and GPx activity increased,
357 whereas GST activity decreased (Gonçalves & Bebianno, 2023), suggesting that with the increase
358 of ingested nPS in gonads of mussels, the organism's antioxidant defence mechanisms are hindered.
359 The gonads of mussels and their development are crucial for reproductive success and during the
360 gametogenesis cycle, lipid content has been shown to enhance with the ripening of female gonads,
361 as well as been used as an energy source during gametogenesis and embryo-larval development

362 (Martínez-Pita et al., 2012). More concerning, as nPS of 50 nm internalizes haemocytes,
363 spermatozoa and oocytes may also be at risk. In *Crassostrea gigas*, fertilization rate decreased after
364 gametes were exposed to nPS (50 nm; 1.5 h) (Tallec et al., 2018), and sperm mobility and
365 fertilization success were impaired, as agglomerates of nanoparticles were found attached to
366 spermatozoa and the jelly coating of oocytes (PS-COOH and PS-NH₂; 0.1 to 100 mg/L; 1, 3 and 5
367 h) (González-Fernández et al., 2018). In *M. galloprovincialis*, malformations of D-larvae were
368 found after embryos were exposed to nPS (50 nm; 10 µg/L; 48 hpf) (Auguste et al., 2021),
369 suggesting the high content of ingested nPS in mussel gonads may pose a serious threat for the
370 reproduction of these organisms.

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

385

386 **5. Conclusion**

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

396

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404

405

406 7. References

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