1 Toxicity of environmental and polystyrene plastic particles on the bivalve *Corbicula*
2 *fluminea:* focus on the molecular responses

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4 Oïhana Latchere[a,*], Coraline Roman[a], Isabelle Métais[a], Hanane Perrein-Ettajani[a], Mohammed
5 Mouloud[a], Didier Georges[a], Agnès Feurtet-Mazel[b], Patrice Gonzalez[b], Cerise Daffe[b], Julien
6 Gigault[cd], Charlotte Catrouillet[de], Magalie Baudrimont[b], Amélie Châtel[a]
7
8 [a]*Université Catholique de l'Ouest, laboratoire BIOSSE, 3 place André Leroy, Angers, France*
9 [b]*Université de Bordeaux, UMR EPOC 5805, équipe Ecotoxicologie Aquatique, Station marine d'Arcachon,*
10 *Place Du Dr Peyneau, 33120, Arcachon, France*
11 [c]*Université Laval, Département de Biologie, Pavillon Alexandre-Vachon, 1045, Av. de La Médecine, Local*
12 *2064, Québec, Québec, G1V0A6, Canada*
13 [d]*Univ. Rennes, CNRS, Géosciences Rennes - UMR 6118, F-35000 Rennes, France*
14 [e]*Université de Paris, Institut de physique du globe de Paris, CNRS, F-75005 Paris, France*
15
16 *Corresponding author : Oïhana LATCHERE. Email address : oihana.latchere@uco.fr

17 ## **Abstract**

18 Microplastics (MPs) and nanoplastics (NPs) are major ecotoxicological concerns in aquatic

19 environments. Among aquatic organisms, filter feeders are particularly exposed to the ingestion

20 of MPs and NPs, filtering large quantities of water for food and having an unselective feeding

21 strategy. The present study investigates the effect of environmental microplastics (ENV MPs)

22 and nanoplastics (ENV NPs) generated from macro-sized plastic debris collected in the

23 Garonne River (France), and polystyrene NPs (PS NPs) on the freshwater bivalve *Corbicula*

24 *fluminea* (Müller 1774). The organisms were exposed to each type of plastic particle at three

25 concentrations: 0.008, 10 and 100 μg L^{-1} for 21 days. Gene expression measurements were

26 conducted in gills and visceral mass at 7 and 21 days to assess the effects of plastic particles

27 on different functions (endocytosis, detoxication, respiratory chain, oxidative stress, immunity,

28 apoptosis and neurotoxicity). Our results revealed that: i) an up-regulation of genes, mainly

29 involved in endocytosis, oxidative stress, immunity, apoptosis and neurotoxicity, was observed

30 at 7 days of exposure for almost all environmental plastic particles and at 21 days of exposure

31 for PS NPs in the gills, ii) PS NPs at the three concentrations tested and ENV MPs at 0.008 μg

32 L^{-1} induced strong down-regulation of genes involved in detoxication, oxidative stress,

33 immunity, apoptosis and neurotoxicity at 7 days of exposure in the visceral mass whereas ENV

34 MPs at 10 and 100 μg L^{-1} and all ENV NPs induced less pronounced effects, iii) overall, PS

35 NPs and ENV MPs 0.008 μg L^{-1} did not trigger the same effects as ENV MPs 10 and 100 μg

36 L^{-1} and all ENV NPs, either in the gills or the visceral mass at 7 and 21 days of exposure.

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37 This study highlighted the need to use MPs and NPs sampled in the environment for future

38 ecotoxicological studies, compared to manufactured PS NPs as their properties (composition,

39 size distribution, surface charge, additive and adsorbed contaminants) induce different effects

40 at the molecular level to living organisms.

41

<u>Introduction</u>

43 Plastics are synthetic or semi-synthetic organic materials used for a wide range of applications

44 in the industrial sector. Omnipresent in our societies, their production has continued to increase

45 in recent decades. Thus, since the middle of the 20th century, global plastic production has

46 increased from 2 million tons in 1950 to 380 million tons in 2015 (Geyer et al., 2017). However,

47 only 5% of the plastics produced are recycled, mainly into secondary products which will not

48 be further recycled and end up in landfills or the environment (Sardon and Dove, 2018). The

49 mass production of plastics, combined with high durability and low recycling rates, have led

50 to their accumulation as wastes in the terrestrial, freshwater and marine environments (de

51 Souza Machado et al., 2018; Dioses-Salinas et al., 2020; Horton et al., 2017).

52 Plastic particles ranging in size from 1 to 5 mm are defined by the term "microplastics" (MPs)

53 (Browne et al., 2007; Fendall and Sewell, 2009). MPs are considered primary or secondary

54 depending on their sources. MPs are primary when produced during manufacture in the form

55 of small particles. They are notably present in certain cosmetic products, skin cleansers, and

56 production wastes from plastic processing plants (Wang et al., 2016). They are very stable in

57 this form, with a lifespan of over 1000 years (Cózar et al., 2014). Secondary MPs derive from

58 the fragmentation of larger pieces of plastic under the effect of different biotic and abiotic

59 factors such as photodegradation, waves, wind, microorganisms, and sediment abrasion

60 (Andrady, 2011; Kale et al., 2015). Secondary MPs represent a significant part of the MPs

61 present in the marine environment. Recently, smaller plastic particles have been identified and

62 described as nanoplastics (NPs) (Gigault et al., 2016). These particles have colloidal properties

63 in aqueous media (e.g., they do not sediment) and their size varies from 1 to 1000 nm in one

64 of the three dimensions of space (Gigault et al., 2021). NPs are also introduced in significant

65 quantities into the natural environment but their presence is difficult to estimate due to

66 methodological challenges (da Costa et al., 2016; Hernandez et al., 2017; Koelmans et al.,

67 2015). Owing to the massive use of plastics and to the additives they may contain, MPs and

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68   NPs pose environmental risks (Besseling et al., 2019; Koelmans et al., 2022). In addition,
69   chemicals can be adsorbed on plastic particles due to the surface alteration of the particles and
70   to the small size of the particles which increases their surface. When chemical conditions
71   changed (pH for example), such pollutants can be desorbed. Moreover, due to their nanoscale
72   properties, NPs can easily cross biological barriers and accumulate in tissues and organs (Chae
73   and An, 2017; Mattsson et al., 2018). In addition, they have a longer retention time than MPs
74   in bivalves (Ward and Kach, 2009). The ingestion of MPs and NPs by aquatic organisms is of
75   particular concern since numerous studies have demonstrated their harmful effects (Al-
76   Thawadi, 2020; Issac and Kandasubramanian, 2021). Indeed, plastic particles induce effects
77   from the cellular to the ecosystem levels by impairing, for example, metabolic and
78   physiological processes, morphology, food absorption and behavior (Al-Thawadi, 2020;
79   Gardon et al., 2018; Sussarellu et al., 2016; Watts et al., 2015).

80   Among aquatic organisms, filter feeders are particularly exposed to the ingestion of MPs and
81   NPs because they filter large quantities of water for food and because of their unselective
82   feeding strategy (Wesch et al., 2016). *Corbicula fluminea* is an endobenthic bivalve used as a
83   bioindicator for the assessment of environmental quality (Arini et al., 2019; Guo and Feng,
84   2018; Zhou et al., 2008). These organisms assimilate small particles from both the sediment
85   and freshwater. They can bioaccumulate chemical substances and are widely used to evaluate
86   the toxicity of freshwater and sediment (Guo and Feng, 2018). Recent studies have been
87   conducted on this species to assess the effects of plastic particles (Fu et al., 2022; Guilhermino
88   et al., 2018; Guo and Feng, 2018; Li et al., 2021). However, the plastic particles tested in most
89   of these studies are standard beads and are not representative of the particles in the
90   environment. Composed of a single type of plastic, mainly PS, perfectly spherical and uniform
91   in size, they differ from secondary MPs and NPs resulting from the degradation of plastic
92   wastes (Gigault et al., 2018, 2016; Haegerbaeumer et al., 2019). Some studies have already
93   started to demonstrate the more deleterious effects of environmental NPs compared to
94   reference ones at environmental levels of exposure in *C. fluminea* (Baudrimont et al., 2019),
95   underlining the relevancy of using this type of NPs for ecotoxicological studies.

96   Thus, this study aims to investigate the effect of both MPs and NPs resulting from the
97   degradation of macroplastics sampled in the field, and standard PS NPs on the bivalve *C.*
98   *fluminea*. To this end, bivalves were exposed to plastic particles at different environmentally
99   relevant concentrations for 21 days. Of the various analyses carried out to evaluate the effects
100   of environmental pollutants, the measurement of gene expression levels is helpful for

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101 identifying the mechanisms involved in the toxicant-specific responses and characterizing
102 stress-induced expression patterns (Piña et al., 2007; Snell et al., 2003). These molecular
103 markers can also provide early-detection of environmental stress. Therefore, we chose to study
104 the influence of different sources of plastic particles (standard and environmental) and of size
105 scale (micro and nano) on the expression level of a panel of genes involved in the responses to
106 environmental stressors.

Materials and methods

Collection, preparation and characterization of environmental derived MPs and NPs

109 Plastic wastes were collected by hand with pliers on the right bank of the Garonne River at low
110 tide, near the Langoiran bridge (44°42'14.56"N, 0°24'3.91"W). The most oxidized plastic
111 debris was sampled, rinsed in the laboratory with ultra-pure water and dried at 45°C for 48h
112 before preparation for micro and nanoplastic solutions.

Environmental micro and nanoplastics production

114 Environmental microplastics (ENV MPs) and nanoplastics (ENV NPs) were generated from
115 macro-sized plastic debris according to the protocol described by Blancho et al. (2021). Briefly,
116 NPs and MPs were produced by coupling agitation and sonication in aquatic media. The size
117 range was between 235 ± 70 nm for ENV NPs and between 1.2 and 300 µm for ENV MPs.
118 ENV NPs and ENV MPs were characterized in terms of composition, size, shape and surface
119 properties by Pyrolysis (Pyrolyzer PY-3030 Frontier Lab) coupled to gas chromatography-
120 mass spectrometry (Py-GC-MS) (5977B, Agilent Technologies). Plastic analysis showed that
121 ENV NPs and ENV MPs were mainly composed of polyethylene (PE) (95%). They were
122 anisotropic, polydisperse in size and possessed high levels of carboxylic groups on their
123 surface. In addition to ENV NPs, carboxylated polystyrene nanobeads (PS NPs) with 200 nm
124 of size, were used as reference material (Polysciences). Contrary to the ENV MPs and NPs, PS
125 NPs did not contain additives.

Acidic digestion and ICP-MS measurements

127 To optimize the total digestion, 100 mg of microplastics and nanoplastics powder were acid-
128 digested (12 N HNO_3 subgrade) using a multi-step procedure with a microwave oven
129 (MW7000 system from Anton-Paar; increasing ramp of the temperature of 6.6°C per minute
130 until reaching 250°C, then 25 min at 250°C under 140 bar of pressure). Metal concentrations

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131   were measured by ICP-MS from Agilent Technologies (7700x Model, Agilent)

132   (Supplementary Information Table A). The solution of three tubes was mixed, evaporated at

133   90°C, and solubilized in 0.37 N HNO3 before ICP-MS measurements. The digestion and

134   analysis process were validated using reference materials (ERM-EC 680 and ERM-EC 681)

135   from the Joint Research Centre of the European Commission (JRC, Ispra, Italy).

Suspensions of microplastics

137   For each type of plastic particle (ENV MPs, ENV NPs and PS NPs), stock suspensions at 1 and

138   0.1 g L^{-1} were prepared in ultra-pure (milliQ) water at pH 7. A working solution at 0.1 mg L^{-1}

139   was obtained for each type of plastic particle by three serial dilutions of the stock suspension

140   at 0.1 g L^{-1} in ultra-pure water (milliQ) at pH 7 as performed in Revel et al. (2019). A specific

141   volume of the stock suspension (1 g L^{-1} or 0.1 g L^{-1}) or the working solution (0.1 mg L^{-1}) was

142   distributed in the aquaria to obtain the final concentrations of 0.008, 10 and 100 µg L^{-1}. Each

143   solution was well mixed before adding it to the aquaria. No surfactant was used during MPs

144   and NPs preparation to prevent any additional effect. All MPs and NPs solutions were prepared

145   and spilled in the aquaria every 3 days just after a water change to maintain the same

146   concentration during exposure.

Bivalve collection and laboratory exposure assay

148   Individuals of *Corbicula fluminea* were collected in the lake of Parentis-Biscarrosse (France).

149   Clams were transported to the laboratory in boxes with sediment from the collection site.

150   Bivalves were then transferred into aquaria (30L) containing 27L of tap water in a temperature-

151   controlled room at 15°C for an acclimatization period of 7 days. Photoperiod was maintained

152   at 12 hours:12 hours. The aquarium water was renewed entirely every three days. Clams were

153   fed once a week with microalgae of the genus *Scenedesmus* (Greensea).

154   Clams were exposed for 21 days to manufactured polystyrene nanoplastics (PS NPs, 200 nm,

155   Polysciences), field derived microplastics (ENV MPs, 1.2-300 µm) and field derived

156   nanoplastics (ENV NPs, 235 ± 70 nm) at the following concentrations: 0.008µg L^{-1}, 10µg L^{-1}

157   and 100 µg L^{-1}. These concentrations were chosen in accordance following the study of Revel

158   et al. (2020) to expose clams to concentrations of MPs and NPs which are close to the ones

159   measured in coastal regions and gyres (Goldstein et al., 2013). The experimental conditions are

160   abbreviated in the results section: for example, ENV MPs 10 is used for environmental

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161 microplastics at 10 µg L $^{-1}$. One group of individuals was used as control (no added plastic
162 particles). The use of plastic material was avoided during all the experiments.

163 After 7 days and at the end of the experiment at 21 days, 3 individuals per condition and per
164 replicate were sampled for gene expression. Gills and visceral mass were dissected and
165 immediately frozen at -80°C for further gene expression measurements.

166 **Analysis of gene expression by quantitative PCR**

167 *Corbicula fluminea* samples were pooled by three for each condition. Triplicates were analyzed
168 by quantitative RT-PCR for each condition. Total RNA was extracted using TRIzol reagent®
169 (Life Technologies) from the gills and the visceral mass, according to the manufacturer's
170 recommendations and precipitated with propan-2-ol. RNA concentration (µg.µL^{-1}) was
171 quantified using a NanoDrop 2000 spectrophotometer (ThermoScientific®). First-strand
172 cDNA was synthesized from 5 µg of total RNA using the Invitrogen™ SuperScript™ III kit
173 (ThermoScientific® ; T100™ Thermal Cycler, BIORAD®) according to the manufacturer's
174 recommendations. The expression levels of twenty genes involved in endocytosis, oxidative
175 stress, detoxication, respiratory chain, immunity, neurotoxicity and apoptosis. They were
176 analyzed using a set of forward and reverse primers by quantitative RT-PCR. Three genes were
177 used as housekeeping genes, including *β-actin*, elongation factor 1 α (*ef1α*) and ribosomal
178 protein 7 (*rpl7*) (Table 1). Specific primers for *ache* and *acp* genes were designed using the
179 software Primer 3 V 4.0. Previously, the quality of each pair of primers was checked: cDNA
180 tests were amplified by PCR (T100™ Thermal Cycler, BIORAD®. 30 cycles: 30s à 95°C, 30s
181 à 60°C, 30s à 72°C), then the amplification products were separated on 1.5% agarose
182 electrophoresis gel. After staining with ethidium bromide, the presence and size of each
183 amplicon were verified. Quantitative PCR (qPCR) amplifications were carried out in triplicate
184 in 96-well microplates (CFX Connect™ Real-Time System, BIORAD®) using SYBR™
185 Master Mix PCR Power SYBR™ Green (Invitrogen) containing the SYBR Green dye, DNA
186 Taq Polymerase and dNTPs. For each reaction, 1µL of each primer (50ng.µL^{-1}), 6.25µL of
187 SYBR Green mix, 3.75µL of water treated with DEPC (DNase-free water) and 0.5µL of cDNA
188 were added in each well. The qPCR reactions consisted of the first step of 10 min at 95°C
189 (enzyme activation) followed by 40 cycles (95°C for 30 s, 60°C for 30 s and 72°C for 30 s) and
190 5 min at 72°C. Expression levels were estimated by evaluating the fluorescence signal emitted
191 by SYBR-Green®. This fluorescent marker binds to double-stranded DNA (dsDNA) and the
192 fluorescence emitted is proportional to the dsDNA present in the reaction mix. Calculations are

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193 based on cycle threshold (Ct) values. The relative gene expression ratio of each target gene was
194 calculated following the delta-delta method normalized with reference genes (Livak and
195 Schmittgen, 2001), which is defined as:

$$ratio = \frac{2 - \Delta\Delta Ct \ (exposed)}{2 - \Delta\Delta Ct \ (control)}$$

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198 **Statistical analysis**

199 The statistical analyzes were performed using the software XLSTAT 2019 (version
200 21.4.63762). The normality of data distribution and homogeneity of variance were tested using
201 the Shapiro-Wilk test and Bartlett test, respectively. As the assumptions for parametric tests
202 were not met for the gene expression measurements, we used the Kruskal-Wallis test to test for
203 differences between the treatments. As the overall test was significant, a Dunn procedure was
204 performed to determine which means were significantly different. p values $\leq$ 0.05 were
205 considered statistically significant,

206

207 **Results**

208 *Gills*

209 As shown in Table 2, at 7 days post-exposure, genes were mainly up-regulated in gills for
210 almost all environmental plastic particles (ENV MPs 10, ENV MPs 100, ENV NPs 0.008, ENV
211 NPs 10 and ENV NPs 100). These genes are involved in endocytosis (*cltl, cav*), detoxication
212 (*gst*), oxidative stress (*sod2, sod1*), immunity (*atg12, acp, gal*), apoptosis (*bcl2*, *bax*, *gadd45*)
213 and neurotoxicity (*ache*). Downregulations were also observed for genes involved in
214 detoxication (*mdr*), the respiratory chain (*cox1*, *12s*) and immunity (*atg13*) for some of the
215 environmental plastic particles. There was no clear dose-dependent effect for ENV MPs
216 treatments and ENV NPs treatments. Results for PS NPs treatments showed different trends
217 compared to environmental plastic particles. Only a few genes were impacted in gills with both
218 up and downregulations. Up-regulated genes after PS NPs 100 treatment were involved in
219 detoxication (*gst*), immunity (*atg12*, *acp*, *gal*), apoptosis (*p53*) and neurotoxicity (*ache*). Other
220 genes were upregulated after PS NPs 0.008 *(cltl)* and after PS NPs 10 (*cav* and *gpx7*). Down-
221 regulated genes concerned detoxication (*mt*, *mdr*), the respiratory chain (*cox1*) after treatment

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with PS NP at one or two of the concentrations tested. Two genes were downregulated for the ENV MPs 0.008 treatment (*mdr* and *12S*).

At 21 days post-exposure (Table 3), a clear difference of gene expression responses in the gills was observed between two groups: 1) the PS NPs treatment whatever the tested concentration and ENV MPs 0.008, and 2) ENV MPs 10, ENV MPs 100 and all the ENV NPs. For the first group, many genes were up-regulated, particularly concerning immunity, apoptosis, detoxication and neurotoxicity functions. For the second group, only few genes were upregulated for one or two treatments.

Visceral mass

After 7 days of exposure to the different plastic conditions, two trends were observed in the visceral mass (Table 4). For the first group (all PS NPs concentrations and ENV MPs 0.008), almost all the studied genes involved in immunity (*atg13*, *atg12*, *acp*, *gal*), apoptosis (*bcl2*, *p53*, *bax*, *gadd45*), neurotoxicity (*ache*) and some of the genes involved in the oxidative stress (*cat, gpx7*) and detoxication (*mdr*, *gst*) were strongly downregulated. Only a few genes were overexpressed for some of these treatments and were involved in endocytosis (*cltl*), detoxication (*mt*) and oxidative stress (*sod1*, *sod2*). For the second group (ENV MPs 10, ENV MPs 100 and all the ENV NPs concentration), the gene' responses were relatively similar to the first group for the functions related to endocytosis, detoxification, respiratory chain and oxidative stress. However, a clear difference regarding the genes involved in oxidative stress, immunity, apoptosis and neurotoxicity was depicted, since very few of these genes were under-expressed compared to the first group.

As shown in Table 5, fewer genes were impacted after 21 days of exposure than at 7 days in the visceral mass. The PS NPs and ENV particles (MPs and NPs) did not induce the same effects. The PS NPs had little effect on the studied genes, whatever the concentration tested. Concerning the ENV MPs and NPs, some genes were under-expressed for some concentration tested and were involved mainly in these different functions: detoxication (*mdr* and *gst*), oxidative stress (*cat*), immunity (*atg13*, *atg12* and *acp*) and apoptosis (*blc2* and *gadd45*). Some genes were up-regulated for the ENV MPs and NPs treatments such as the ones involved in endocytosis (*cav*), respiratory chain (*12s*), oxidative stress (*gpx7*), immunity (*gal*) and neurotoxicity (*ache*) for some conditions and concentrations tested.

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253

<u>Discussion</u>

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The present study investigated the effects of field-derived ENV MPs and NPs and standard PS NPs on the expression of genes involved in the molecular response to toxicity in two tissues, gills and visceral mass, in *C. fluminea*. Our results first highlighted that the exposure led to changes in gene expression patterns at environmentally relevant concentrations whether the plastic source, manufactured plastics beads or environmental particles. Two main types of responses emerged from the analysis of two target tissues (gills and visceral mass) : firstly, the earlier pattern of response, after 7 days of exposure, was linked to exposure to ENV NPs and MPs in gills and to PS NPs in visceral mass; secondly, after a more prolonged exposure (21 days), the effects of PS NPs on gene expression was highlighted in gills while in the visceral mass, modifications in gene expression were instead linked to environmental plastic particles.

266

Endocytosis

Endocytosis is a main process involved in the uptake of nanoparticles in many species (Weng et al., 2022). In our study, caveolin (*cav*) and clathrin (*cltl*) gene expression varied significantly under NPs and MPs exposures, showing their role in the plastic particles uptake.

In the gills, endocytosis seems to be an entry pathway for MPs and NPs since the caveolin (*cav*) and clathrin (*cltl*) genes were over-expressed for specific concentrations in the three plastic conditions (both ENV MPs and NPs, and PS NPs) at 7 days of exposure. Indeed, the internalization rate of 50 nm PS NPs is lower when caveolae and clathrin endocytosis pathways were inhibited in the mussel *Mytilus galloprovincialis* (Sendra et al., 2020). These mechanisms were also already observed in oysters exposed to environmental NPs, attesting of an easy uptake of these particles in bivalves (Arini et al., 2022b). But endocytosis by caveolin or clathrin pathways is limited to sizes of particles below 500nm or 200nm respectively (Rejman et al, 2004), suggesting in the case of exposure to ENV MPs the presence of NPs in solution.

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Oxidative stress and detoxication

In the gills, the genes related to oxidative stress (*gpx*, *sod1 and sod2*) were overexpressed for ENV MPs and NPs at 7 days of exposure. The *cat*, *sod2* and *gpx* genes were overexpressed

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284 after 21 days of exposure to PS NPs for some concentrations. Conversely, *cat* and *gst* genes
285 were under-expressed in the visceral mass after exposure to PS NPs for 7 days. Catalase is an
286 enzyme that acts as a defense mechanism against reactive oxygen species, allowing the
287 disproportionation of hydrogen peroxide into water and dioxygen. The GST enzyme protects
288 cells against toxicants by conjugating the glutathione as substrate to xenobiotics. The increased
289 expression of both *cat* and *gst* genes observed in the gills of *C. fluminea* is a sign of cellular
290 oxidative stress. A previous study also showed an increase in the activity of the catalase in the
291 gills of *C.fluminea* after an exposure to PS MPs (200 µm) at a concentration of 2 mg L^{-1} for 7
292 days (Parra et al., 2021), while in our study, this is observed for PS NPs and ENV MPs at
293 considerably lower concentrations. The gene relating to the detoxification system *mdr* was
294 overexpressed in the gills after 21 days for the different plastic conditions and specific
295 concentrations. This may be related to the increased expression of the *gst* gene. Indeed, GSTs
296 are enzymes that catalyze the conjugation of reduced glutathione (GSH) with metabolites and
297 reactive electrophiles, representing an essential chemical detoxification route. This suggests
298 the presence of additives and/or some chemical compounds adsorbed on the surface of the
299 plastic particles. This is consistent with the high metal concentrations measured in the ENV
300 MPs and NPs used in this study (SI Table A).

301

Respiratory chain

303 12S ribosomal RNA refers to the mitochondrial metabolism. Thus, an overexpression of the
304 12S gene represents an increasing number of mitochondria necessary to respond to oxidative
305 stress in the bivalves. In our study, an under-expression was observed in both tissues after 7
306 days of exposure. The same observation was followed by a decrease in the activity of isocitrate
307 dehydrogenase, involved in the Krebs cycle and therefore mitochondrial activity, in the fish
308 *Pomatoschistus microps* after exposure to 0, 18.4 and 184 µg L^{-1} of PE MPs (1-5 µm) for 96h
309 (Oliveira et al., 2013). At the opposite, an over-expression of the 12S gene was demonstrated
310 in the visceral mass of oysters *Isognomon alatus* after 7 days of exposure to PS NPs and
311 derived-field NPs at 7.5 µg L^{-1} (Arini et al., 2022a). In their study, the authors suggest that the
312 overexpression of the 12S gene was linked to the repression of the *cox1* gene and would be
313 involved in a compensatory mechanism aimed at maintaining mitochondrial metabolism (Arini
314 et al., 2022a). In our study, we observed both the *cox1* and *12S* genes repression after 7 days

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315 of exposure to ENV MPs 100 and all ENV NPs in the visceral mass, suggesting an excessive

316 oxidative stress which the mitochondria cannot support.

317

318 **Immunity**

319 The responses of the organisms to the environmental and polystyrene particles exposure were

320 different in the gills and the visceral mass. In the gills, ENV MPs and NPs induced an over-

321 expression of 3 of the 4 genes involved in immunity after 7 days of exposure (*atg12*, *AcP*, *gal*).

322 After 21 days of exposure, the organisms exposed to the PS NPs showed an over-expression

323 of the 4 genes studied (*atg13*, *atg12*, *AcP* and *gal*). This indicates an important immune system

324 activity even at low concentrations of plastic particles. Such a shift in the immune response has

325 already been reported for bivalves exposed to MPs and NPs (Auguste et al., 2020; Mkuye et

326 al., 2022).

327 In the visceral mass, we observed an opposite trend. An intense repression was depicted after

328 7 days of exposure to PS NPs and ENV MPs 0.008 whereas little effect was observed for ENV

329 MP 10 and 100 and ENV NPs. After 21 days of exposure, almost no effect of PS NPs and a

330 down-regulation of some genes were observed for the ENV MPs and NPs. Our results

331 suggested that PS NPs induced a stronger response in the short term than ENV MPs and NPs.

332 Due to their small size (200 nm) and potentially their carboxyl groups, PS NPs may reach the

333 visceral mass faster while ENV MPs and NPs may tend to be retained in the gills explaining

334 the responses observed at 7 days of exposure. These results are in agreement with two studies

335 which demonstrated a more significant accumulation of PS plastic particles in the digestive

336 gland tissues than in the gills of the mussel *Mytilus galloprovincialis* (Fabbri et al., 2020; Wei

337 et al., 2021).

338

339 **Apoptosis**

340 Apoptosis is the process of programmed cell death which plays a significant role in the immune

341 response triggered by various factors including virus, diseases and toxic agents (Ekert and

342 Vaux, 1997; Romero et al., 2015). Our results on apoptosis were consistent with those obtained

343 for immunity genes and demonstrated significant differences in the response to the two types

344 of plastics (PS NPs vs ENV MPs and NPs), both in the gills and in visceral mass. At 7 days of

345 exposure, the ENV MPs and NPs induced an up-regulation of 3 genes involved in apoptosis

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346 processes (*bcl2*, *bax* and *gadd45*) in gills. In contrast, only one gene (*p53*) was up-regulated

347 for the highest concentration of PS NPs. The apoptosis response induced by environmental

348 plastic particles can be related to eliminating damaged cells to maintain the tissue's integrity

349 and to preserve the physiological activity of gill filaments (Romero et al., 2015). In the visceral

350 mass, after 7 days of exposure, organisms exposed to PS NPs and ENV MPs 0.008 µg L^{-1}

351 showed an intense repression of the 4 genes involved in apoptosis.

352 In contrast, the ones exposed to environmental particles showed little or no effect for MPs 10

353 and 100 µg L^{-1} and NPs. MPs have been shown to induce apoptosis in bivalves, particularly via

354 caspase-related genes (Mkuye et al., 2022; Shi et al., 2020; Sun et al., 2021). However, very

355 few studies described the effects of NPs on apoptosis processes in bivalves. One study related

356 to direct exposure to environmental NPs derived from plastic macro-wastes reported effect on

357 apoptotic genes in gills and visceral mass in the oyster *I. alatus* (Arini et al., 2022b). Our

358 divergent results from those on the oyster *I. alatus* could be partly explained by the different

359 environmental plastics tested, specifically by differences in plastic characteristics (i.e.,

360 composition, surface charge, size, shape, additives and adsorbed chemicals).

361

Neurotoxicity

363 Acetylcholinesterase (AChE) is the primary enzyme responsible for the hydrolytic metabolism

364 of the neurotransmitter acetylcholine (ACh) into choline and acetate to remove the neurotoxic

365 effects of pollutants. The exposure to plastic particles induced an inhibition of the AChE

366 activity in different bivalves (Avio et al., 2015; Oliveira et al., 2013; Ribeiro et al., 2017). This

367 is consistent with the inhibition of *ache* gene expression we observed in the visceral mass after

368 an exposure of 7 days to PS NPs (for all tested concentrations) and ENV MPs (0.008 µg L^{-1}).

369 This inhibition may reflect a possible disturbance of nerve impulse transmission and could be

370 due to the toxicity of plastic particles and the chemical compounds they carry.

371 At the opposite, our results indicated an induction of the *ache* gene in the gills after a 7 days

372 exposure to ENV MPs and NPs and after a 21 days exposure to PS NPs and ENV MPs (0.008

373 µg L^{-1}). In the same way, an increase of the AChE activity has been reported in barnacle nauplii

374 exposed to PS MPs for 48h at 0.001, 0.01 and 1 mg L^{-1} (Gambardella et al., 2017) and in the

375 freshwater insect larvae *Culex quinquefasciatus* exposed to PE at 4.24 x 10^6 particles m^{-3} for 5

376 days (Malafaia et al., 2020). This increase in *ache* gene expression in our study may be related

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377 to the inflammation of the visceral mass since it has been reported that inflammatory conditions
378 can trigger the up-regulation of *ache* gene expression (Oliveira et al., 2012).

379 It would be interesting to compare the *ache* gene expression levels with animal behavioral
380 responses such as valve movement activity or filtration capacity. Indeed, inhibition of AChE
381 activity was reported combined with a decrease of the filtration capacity of *C. fluminea* exposed
382 to 10 mg mL^{-1} of PS NPs (Guo et al., 2021). Moreover, disturbances in the behaviour of
383 zebrafish at the larval stage were measured together with an inhibition of AChE activity after
384 an exposition to 2 mg L^{-1} of MPs (Santos et al., 2021).

385

386 **Testing environmental particles for a realistic assessment of environmental risk**

387 In this study, we tested the effects of manufactured PS NPs and environmental MPs and NPs
388 derived from macroplastics sampled in the environment on *C. fluminea*. These particles differ
389 in plastic composition, size, shape, additives, adsorbed pollutants than the commercial ones.
390 We showed that the two types of particles (manufactured *vs.* environmental) induced
391 differential responses, whatever the sampling time or the tissue studied. Therefore, in the gills,
392 the genes involved in immunity, apoptosis and neurotoxicity are overexpressed after 7 days of
393 exposure to the ENV MPs and NPs. In contrast, it was not the case for the PS NPs. At 21 days,
394 these results are reversed with an overexpression of these genes for the PS NPs and only the
395 ENV MPs 0.008. In the visceral mass, the organisms exposed to the PS NPs and ENV MPs
396 0.008 showed an intense repression of the genes involved in immunity, apoptosis and
397 neurotoxicity after 7 days of exposure whereas little effect was observed for the organisms
398 exposed to the ENV MPs 10 and 100 and all the ENV NPs. Such differences between the
399 effects of manufactured and environmental particles have already been demonstrated in oysters
400 *Isognomon alatus* (Arini et al., 2022a,b; Lebordais et al, 2021). In these studies, the authors
401 showed notably that nanoplastic particles derived from microplastics sampled in the
402 environment triggered more effects on gene expression than PS NPs.

403 As part of this study, the effects of environmental particles of different sizes: ENV MPs (1.2-
404 300 μm) and ENV NPs (235 ± 70 nm) were tested. The results did not demonstrate differences
405 in molecular responses between the organisms exposed to the two particle sizes. In contrast, it
406 has been found that the small size of NPs and their high surface area make them more toxic to
407 the organisms than MPs (Zhang et al., 2021). However, the studies investigating the effects of

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408 NPs on aquatic organisms have emerged in recent years and there is still a knowledge gap on
409 this topic (Ferreira et al., 2019).

410 Indirect toxicity of MPs and NPs can also be due to the additives they contain and/or the
411 pollutants adsorbed on their surface. These chemical compounds can be transferred to the
412 organisms (Avio et al., 2015; Gomiero et al., 2018) and can lead to joint toxicity (synergistic,
413 additive, antagonistic, independent) (Ding et al., 2022). But studies on the effects of MPs and
414 NPs from the environment and whose pollutants have been characterized are currently very
415 scarce. In our study, the concentrations of different metals and metalloids in the environmental
416 MPs and NPs were measured. They were found to be very high and could be related to the
417 immune responses, apoptosis and neurotoxicity observed in the different tissues in *Corbicula*
418 *fluminea*. Another study revealed a higher growth inhibition of a freshwater algae with
419 environmental NPs compared to manufactured NPs (Baudrimont et al, 2019). The
420 concentration of different trace metals was shown to be higher in the environmental NPs than
421 in the manufactured ones which could explain the toxicity differences between the two types
422 of plastic particles (Baudrimont et al., 2019). Moreover, a mixture of MPs and mercury has
423 been shown to cause oxidative stress and lipid peroxidation damage in *C. fluminea* (Oliveira et
424 al., 2018). However, the authors also pointed out antagonistic effects between MPs and
425 mercury on filtration rate and the enzymatic activities of ChE and GST (Oliveira et al., 2018).
426 The interactions between the MPs, NPs and the chemicals are complex and far from
427 understood. Additional research is needed to better understand the mechanisms of toxicity of
428 MPs and NPs from the environment.

429

430 Conclusion

431

432 The present study evaluated the effects of PS NPs and environmental MPs and NPs in the
433 bivalve *C. fluminea* at environmentally relevant concentrations and under the same laboratory
434 conditions. We have evidenced major differences in the bivalve molecular responses between
435 manufactured NPs composed of polystyrene and field-derived MPs and NPs especially in the
436 oxidative stress, immunity, apoptosis and neurotoxicity. These results highlight the importance
437 of conducting further investigations including plastic particles from the environment, from
438 nano to micro size and to fully characterize these particles (composition, shape, size,
439 chemicals…) for a realistic assessment of environmental risk.

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