

1 Developmental toxicity of pre-production plastic pellets affects a large swathe of invertebrate taxa  
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24 Abstract

25 Microplastics pose risks to marine organisms through ingestion, entanglement, and as carriers of  
26 toxic additives and environmental pollutants. Plastic pre-production pellet leachates have been  
27 shown to affect the development of sea urchins and, to some extent, mussels. The extent of those  
28 developmental effects on other animal phyla remains unknown. Here, we test the toxicity of  
29 environmental mixed nurdle samples and new PVC pellets for the embryonic development or

30 asexual reproduction by regeneration of animals from all the major animal superphyla  
31 (Lophotrochozoa, Ecdysozoa, Deuterostomia and Cnidaria). Our results show diverse,  
32 concentration-dependent impacts in all the species sampled for new pellets, and for molluscs and  
33 deuterostomes for environmental samples. Embryo axial formation, cell specification and, specially,  
34 morphogenesis seem to be the main processes affected by plastic leachate exposure. Our study  
35 serves as a proof of principle for the potentially catastrophic effects that increasing plastic  
36 concentrations in the oceans and other ecosystems can have across animal populations from all  
37 major animal superphyla.

38 Keywords

39 Plastic leachates, nurdles, development, regeneration, aquatic invertebrates

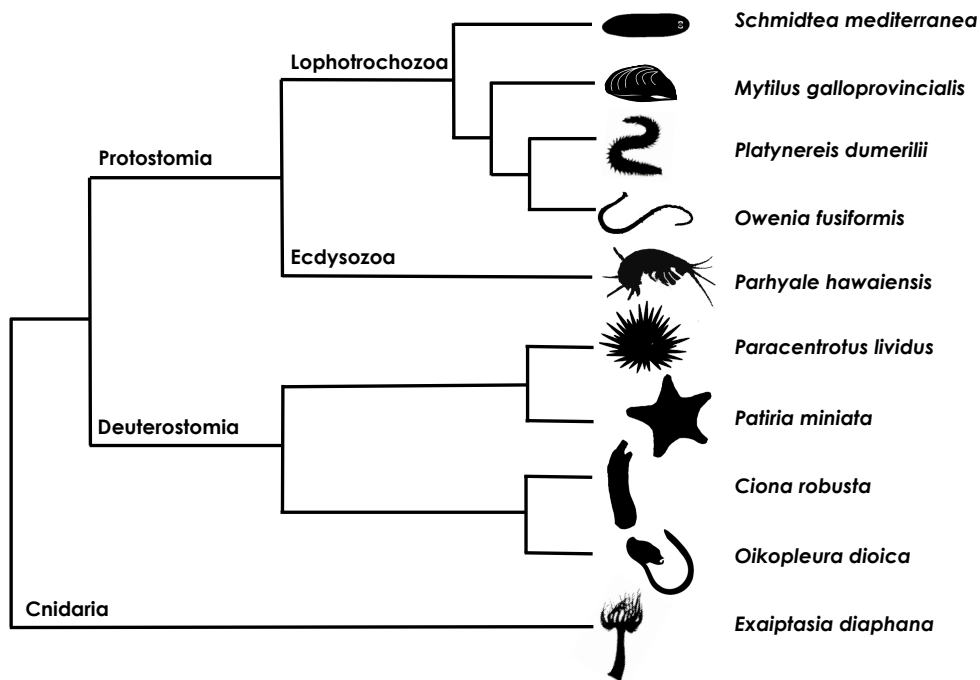
40 1. Introduction

41 Plastic contamination has emerged as a significant concern in marine ecosystems due to its  
42 pervasive presence and potential impact (Eriksen et al., 2014; Thushari & Senevirathna, 2020). Large  
43 plastic items can entangle marine animals, cause physical injuries, and alter or disrupt habitats. In  
44 time, with the physical and chemical actions of the environment, plastics can break down and  
45 produce secondary microplastics, particles smaller than 5 mm. Other microplastics already arrive as  
46 small particles to the environment, known as primary microplastics. Whichever the origin,  
47 microplastics possess certain characteristics that make them a particular concern: they have a global  
48 distribution, as they are easy to disperse because of their small size; they can be ingested by a wide  
49 range of animals, entering the food chain at any trophic level; and they have a relatively larger  
50 surface area compared to larger plastic items, allowing them to adsorb, accumulate and carry  
51 pollutants from the surrounding environment (Mato et al., 2001). These pollutants can include toxic  
52 chemicals and heavy metals, leading to potential risks when ingested by marine organisms, as well  
53 as the potential to release these harmful additives into the water, creating additional hazards to  
54 marine life and ecosystems (Teuten et al., 2009; Engler, 2012; Gauquie et al., 2015; Rendell-Bhatti  
55 et al., 2021). Among the significant contributors to primary microplastic pollution, in terms of  
56 weight, are pre-production plastic pellets commonly known as nurdles (Sherrington, 2016), the  
57 building blocks for plastic products. During manufacturing, these nurdles are combined with various  
58 chemical compounds, including plasticizers, stabilizers, and antioxidants, necessary to impart  
59 specific physical properties to the final products. These chemical compounds are readily transferred  
60 to the water (Mato et al., 2001; Rendell-Bhatti et al., 2021; Paganos et al., 2023) in the case of the  
61 nurdles being lost at sea (Sewwandi et al., 2022). Once in the water, they have the ability to

62 concentrate environmental pollutants and transport and release them to a different location, often  
63 far from the source of contamination (Teuten et al., 2009).

64 Many marine invertebrates commonly undergo embryonic development in the water column, and  
65 their larvae are usually planktonic. Their developmental strategy, combined with the absence of a  
66 protective eggshell, make them vulnerable to contamination from plastic leachates. Plastic leachate  
67 toxicity has been shown to have negative effects on the development of several marine organisms  
68 (Li et al., 2016; Oliviero et al., 2019; Gardon et al., 2020), and in particular, plastic pre-production  
69 pellet leachates can disrupt the development of sea urchins (Nobre et al., 2015; Rendell-Bhatti et  
70 al., 2021; Paganos et al., 2023) and to some extent brown mussels (Gandara e Silva et al., 2016).  
71 However, there is a lack of knowledge regarding how universal the susceptibility to this  
72 contamination is across other animal groups.

73 Here, we provide a systematic characterisation of the phenotypic abnormalities found during  
74 development and asexual reproduction by regeneration in an array of aquatic invertebrates treated  
75 with both environmental and industrial pre-production plastic pellet leachates. We select  
76 representative species of the aquatic ecosystem, including one mollusc, two annelids, one flatworm,  
77 one crustacean arthropod, two echinoderms, two tunicates and one cnidarian, to investigate  
78 microplastic-induced abnormalities in organisms across all major animal superphyla (Figure 1).



80 Figure 1. Phylogenetic tree. A schematic representation of the phylogenetic relationships among  
81 the species studied in this work. Representatives of the three major bilateria superphyla are used

82 (Protostomia (Lophotrochozoa and Ecdysozoa) and Deuterostomia), as well as a representative of  
83 the radiata (Cnidaria). *O. dioica* by Josep Martí-Solans (CC0 1.0).

## 84 2. Materials and methods

### 85 2.1. Microplastic leachate preparation

86 Environmentally retrieved nurdles were obtained from Watergate bay (Cornwall, UK) by Beach  
87 Guardian CIC in December 2021 and manually sorted from other plastic particles. Commercial PVC  
88 nurdles were purchased from Northern Polymers and Plastics Ltd. (UK) in January 2022. In brief, for  
89 each of the plastic particles, pellets were added to filtered seawater (0.22  $\mu\text{m}$ ) (FSW) at a  
90 concentration of 10% (v/v). Pellets were leached for 72 h on a platform shaker, with continuous  
91 shaking at room temperature (ca 18°C). Leachates were obtained by filtering through filter paper in  
92 order to remove particles. Leachates were diluted to the final concentration in FSW. Only for  
93 *Schmidtea mediterranea*, leachates were obtained and diluted in Planarian Artificial Medium (1X  
94 PAM) (Cebrià & Newmark, 2005) rather than FSW. We tested 10% nurdle leachates and 1%, 5% and  
95 10% PVC leachates (v/v), concentrations which had previously been shown to induce aberrant  
96 phenotypes in echinoderms (Rendell-Bhatti et al., 2021; Paganos et al., 2023), to produce  
97 comparable results with our previous work. Tests with lower concentrations than the ones used  
98 here did not produce evident aberrant phenotypes.

### 99 2.2. Animal husbandry, fertilisation and embryo exposure

100 Sexually mature specimens of *Mytilus galloprovincialis* were obtained from Irsvem Srl, a commercial  
101 shellfish farm (Bacoli, Napoli, Italy). Animals were mechanically stimulated to promote spawning by  
102 scraping the shells to remove adherent organisms and pulling the byssus. Approximately, 20–30  
103 mussels were placed in a tank with Mediterranean FSW at 18°C and spread to easily monitor the  
104 spawning. When mussels began to spawn, each individual was washed and then transferred into a  
105 beaker containing 200 ml of Mediterranean FSW to isolate male and female gametes. Eggs were  
106 fertilised with an egg/sperm ratio 1:15 in a volume of 50 ml. The resulting zygotes were left to grow  
107 at 18°C in treatment plates at a concentration of 250 eggs per ml until the developmental stage of  
108 interest, the D-larva at 48 hours post fertilisazion (hpf).

109 *Platynereis dumerilii* gametes were obtained from an in-house culture at the University of Exeter,  
110 (UK), with culture conditions based on (Hauenschild & Fischer, 1969). Batches of embryos were  
111 created by allowing a single male and female epitoke to freely spawn in a 100ml glass beaker with  
112 80ml 0.22  $\mu\text{m}$  filtered artificial seawater. Developing eggs were stripped of their protective jelly by

113 thorough rinsing through a 100 µm filter mesh cup one hour after fertilisation. De-jellied fertilised  
114 eggs were then added to the different treatments and left to develop in an incubator at a constant  
115 temperature of 18°C with a regular light-dark cycle (16h light, 8h dark) for 96 hours.

116 *Parhyale hawaiiensis* were housed in artificial sea water at 24°C at the University of Exeter, Penryn  
117 campus (UK). Sexually mature pairs in amplexus were transferred to beakers containing the  
118 different treatments. When the female moults, pairs separate and oocytes are fertilised and  
119 deposited in the female's ventral pouch (Rehm et al., 2009). After a few days, females were  
120 anaesthetised with clove oil (Rehm et al., 2009) and eggs removed and transferred to containers with  
121 the same treatment as the parents. This was done to avoid predation of eggs or hatched larvae by  
122 the adults. Because of the way *P. hawaiiensis* embryos are fertilised, embryos from every pairing  
123 were only assigned to one treatment, differing from the other species here studied. Embryos were  
124 left to develop at 24°C until hatching occurred.

125 *Paracentrotus lividus* were housed in circulating seawater aquaria at 18°C in the aquarium facility  
126 of the Stazione Zoologica Anton Dohrn, Naples (Italy). Gamete acquisition and fertilisations were  
127 performed as described elsewhere (Rendell-Bhatti et al., 2021). Embryos were added to treatment  
128 beakers immediately after confirmation of fertilisation at a concentration of 50 embryos per ml and  
129 left to develop at 18°C until the 48 hpf pluteus stage.

130 *Patiria miniata* were housed in circulating seawater at 14 °C, to prevent them from spontaneous  
131 spawning, in the aquarium facility of the Stazione Zoologica Anton Dohrn, Naples (Italy). Gametes  
132 were retrieved from adults by arm incision and extraction of intact gonads. Eggs were released from  
133 the ovaries through manual dissection, while male gonads were placed dry in an Eppendorf tube at  
134 4°C. Immature oocytes were incubated with 1:1000 dilution of 10mM 1-Methyladenine in FSW for  
135 1h at 15°C. Once the germinal vesicle was broken down, an indication of the oocytes' maturity, they  
136 were fertilised with a few drops of diluted sperm in FSW (1µl of dry sperm in 10ml of FSW). Embryos  
137 were then added to treatment beakers at a concentration of 50 embryos per ml and left to develop  
138 at 15 °C until 4 days post fertilisation (dpf, bipinnaria larvae stage) in 9:1 diluted FSW (9-parts  
139 Mediterranean FSW, 1-part distilled water) to obtain the appropriate salinity of approximately  
140 35ppt.

141 *Ciona robusta* were collected in Taranto (Italy) in March 2023, and left for at least seven days in an  
142 aquarium facility at 16-18°C with permanent light to promote gametogenesis at the Stazione  
143 Zoologica Anton Dohrn, Naples (Italy). Gametes were obtained as described before (Eliso et al.,  
144 2020), with modifications. In brief, oocytes and sperm were harvested from each individual by

145 dissecting the gonoducts sequentially, to avoid self fertilisation. Fertilization was performed by  
146 adding diluted sperm (1:100 in FNSW) to the eggs suspension. After 15 minutes of incubation on a  
147 rotating shaker, the fertilized eggs were rinsed in FSW to avoid polyspermy and added to treatment  
148 plates about 30 minutes post-fertilization, with a density of 10 embryos per ml and left to develop  
149 at 18°C until the desired developmental stage (hatched larva).

150 *Oikopleura dioica* specimens were cultured in the animal facility of the University of Barcelona  
151 (Spain) as previously described (Martí-Solans et al., 2015). Mature females and males were collected  
152 separately at day 5, and left to spawn naturally. For each experiment, multiple egg and sperm  
153 batches were mixed, *in vitro* fertilised and transferred before the first cell division to treatment  
154 plates at 19°C until the desired larval stage.

155 *Owenia fusiformis* collected from the coasts near the Station Biologique de Roscoff were maintained  
156 in artificial seawater at the Queen Mary University of London, London (UK) at 15°C. Animals were  
157 removed from their sand tubes as described elsewhere (Carrillo-Baltodano et al., 2021) and  
158 decapitated with a razor blade just above the first parapodia before being left to regenerate in  
159 seawater supplemented with penicillin (100U/ml) and streptomycin (200 µg/ml) at 19°C.

160 *Schmidtea mediterranea* from an asexual clonal line were housed at 20°C in 1X PAM water (Cebrià  
161 & Newmark, 2005) at the University of Barcelona (Spain). Animals were fed twice per week with  
162 organic veal liver and were starved for at least 1 week before experiments. Pre-pharyngeal  
163 amputation was performed using a razor blade. After amputation, animals were immediately  
164 transferred to treatment plates at 20°C and left to regenerate until observations at five and seven  
165 days.

166 *Exaiptasia diaphana* (formerly *Aiptasia pallida*) polyp specimens were housed in circulating  
167 seawater aquarium facility at the Stazione Zoologica Anton Dohrn, Naples (Italy). For our  
168 experimental purposes, they were kept starved in crystallizing dishes at 24°C in a light/dark cycle of  
169 12/12 hours. Pedal lacerations were collected manually and placed in a 12-well plate, one laceration  
170 per well. They were allowed to regenerate in FSW for one week. After that time, FSW was replaced  
171 with leachate solution at the defined concentration, and regenerating fragments were incubated at  
172 18°C for one week.

### 173 2.3. Phenotypical observations

174 Larvae of the species object of the study were arrested by fixing them in 4% PFA and imaged using  
175 either a Leica M165C with a Leica DFC295 camera, or a Leica DMI8 with a Leica flexacam C3

176 microscope. *O. dioica*, *S. mediterranea* and *E. diaphana* were imaged alive using an Olympus SZX16  
177 stereomicroscope, an sCM EX-3 high end digital microscope camera (DC.3000s, Visual Inspection  
178 Technology) and a Zeiss AXIO Zoom V16 microscope equipped with Axiocam 208 colour camera,  
179 respectively. Larvae were classified into two groups: normal developed larvae and aberrant larvae,  
180 including phenotypes ranging from delayed to totally aberrant. However, notes were made in the  
181 phenotypes that, despite looking normal, were not quite like the controls, as well as for delayed  
182 larvae that looked otherwise correct. Statistical differences were analysed by performing One-Way  
183 ANOVA followed by Post Hoc Tukey HSD. Mussel area size differences between controls and treated  
184 animals for each individual spawning (n=6 spawning events) were calculated with ImageJ and  
185 differences were analysed by performing unpaired t-tests.

#### 186 2.4 *S. mediterranea* immunohistochemistry and cell proliferation counts.

187 Whole-mount immunohistochemistry in planarians was performed as previously described (Ross et  
188 al., 2015; Fraguas et al., 2021). The following antibodies were used: mouse anti-SYNAPSIN, used as  
189 pan-neural marker (anti- SYNORF1, Developmental Studies Hybridoma Bank, Iowa City, IA, USA)  
190 diluted 1:50; mouse anti-VC1(Sakai et al., 2000), specific for planarian photosensitive cells (anti-  
191 arrestin, kindly provided by H. Orii and Professor K. Watanabe) diluted 1:15000; rabbit anti-  
192 phospho-histone H3 (Ser10) to detect cells at the G2/M phase of cell cycle (H3P, Cell Signaling  
193 Technology) diluted 1:300. The secondary antibodies Alexa 488-conjugated goat anti-mouse and  
194 Alexa-568-conjugated goat anti-rabbit (Molecular Probes, Waltham, MA, USA) were diluted 1:400  
195 and 1:1000, respectively. Samples were mounted in 70% glycerol before imaging. Fixed and stained  
196 animals were observed with a Leica MZ16F stereomicroscope and imaged with a ProgRes C3 camera  
197 (Jenoptik, Jena, TH, Germany). Confocal images were obtained with a Zeiss LSM 880 confocal  
198 microscope (Zeiss, Oberkochen, Germany). Image processing and quantifications were performed  
199 with Adobe Photoshop and ImageJ2. Counting of the H3P-positive cells was carried out manually  
200 and normalized by the total body area. Statistical analyses and graphical representations were  
201 performed using GraphPad Prism 9. A box plot displaying the minimum, lower first quartile, median,  
202 upper third quartile, and maximum values was used to represent the data. Kruskal-Wallis test was  
203 performed to compare the means between conditions after discarding data normality and  
204 homogeneity for some samples using the Shapiro-Wilk test.

### 205 3. Results

206 Plastic pellet leachates affect a large swathe of animal phyla

207 We tested the effects of leachates of high concentrations of new and beach plastic pellets on the  
208 development of *Mytilus galloprovincialis* (mollusc), *Platynereis dumerilii* (annelid), *Parhyale*  
209 *hawaiiensis* (arthropod), *Paracentrotus lividus* and *Pariria miniata* (echinoderms), and *Ciona robusta*  
210 and *Oikopleura dioica* (tunicates) (Figure 2), and on the regeneration capacity of *Owenia fusiformis*  
211 (annelid), *Schmidtea mediterranea* (platyhelminth) and *Exaiptasia diaphana* (cnidaria) (Figure 3).  
212 Our results showed that the effects were treatment and dose-dependent, as well as species-specific  
213 (Figure 4).

214 We observed *M. galloprovincialis* larvae at 48 hpf. At this stage, the controls displayed normal D-  
215 larvae phenotype, with a well-formed early shell that covered the mantle of the larvae (Marin, Le  
216 Roy & Marie, 2012) (Figure 2. A1; Supplementary figure 1. A). Nurdle-leachate-treated larvae had a  
217 very similar phenotype to the controls, but they were slightly smaller in size ( $p < 0.05$ ) (Figure 2. A2;  
218 Supplementary figure 1. A, B, F). No differences were detected between 1% PVC-leachate treated  
219 embryos and controls (Supplementary Figure 1. C), but at 5% there was a significant reduction in  
220 size, with a misshaped shell in what we classified as an aberrant larva with protruding mantle (Figure  
221 2. A3; Supplementary figure 1. D). At 10% PVC-leachate, the larvae did not develop properly and  
222 remained arrested around the trochophore stage, barely forming, in some instances, a very  
223 rudimentary incipient shell (Figure 2. A4; Supplementary figure 1. E).

224 *Platynereis dumerilii* develops first into a trochophore larvae, followed by a nectochaete larval stage  
225 (Özpolat et al., 2021). We assessed morphological changes at 4 dpf, the nectochaete larva. At this  
226 point, control and nurdle-treated larvae looked the same, with normal segmentation, chaetae,  
227 digestive system and lipid droplet distribution (Figure 2. B1, B2). However, larvae treated with PVC  
228 leachates did not develop properly. For 1% PVC leachate-treated larvae, despite looking otherwise  
229 normal, a few of the animals showed a deformed gut phenotype (not shown). This gut phenotype  
230 was more pronounced and common in 5% PVC-treated animals (Figure 2. B3). While the rest of the  
231 larvae could be considered normal, the developing digestive system showed a probable over-  
232 extension of the foregut tissue, and the lipid droplet distribution was also aberrant. In the 10% PVC  
233 leachate treatment, many larvae failed to complete the trochophore-to-nectochaete transition  
234 (Figure 2. B4). Where these larvae had chaetae, they showed that segmentation was not properly  
235 completed, and they resembled a truncated malformed nectochaete. The lipid droplet distribution  
236 of these larvae was also abnormal. A normal nectochaete should have four prominent lipid droplets,  
237 with two larger and two smaller droplets, sitting just below the foregut-mouth region, but these  
238 larvae showed more droplets, and oddly distributed. A normal trochophore displays four big lipid



239 droplets (Fischer, Henrich & Arendt, 2010), and the higher number of droplets seen in the truncated  
240 nectochaete could be a remnant of the failed transition between the two larval stages. The larvae  
241 that became more like nectochaete also showed apparent problems with the developing gut similar  
242 to what is seen at 5% PVC, potentially due to an inability to differentiate the foregut from the rest  
243 of the gut, or over-proliferation of the foregut.

244 *Parhyale hawaiiensis* hatches into a juvenile larva after about 10 dpf. Control larvae showed a  
245 normal phenotype, with a normal head with two antennae segments, a thorax with two claws and  
246 five legs and an abdomen (Figure 2. C1). No changes were seen in the nurdle or 1% PVC treatments  
247 (Figure 2. C2; not shown). For 5% PVC leachate-treated animals, about half of the larvae showed  
248 malformed appendages, both at the level of the head, where the antennae formed but were  
249 deviated toward the posterior of the head, and the thorax, where claws and legs were malformed  
250 and twisted (Figure 2. C3). None of the 10% PVC leachate-treated embryos developed properly and  
251 all died *in ovo* (Figure 2. C4).

252 *Paracentrotus lividus* were imaged at 48 hpf, at pluteus larva stage. Control larvae are bilaterally  
253 symmetrical four-arm pluteus, with the typical tripartite gut, ciliary band and skeletal rods (Figure  
254 2. D1). Nurdle leachate-treated larvae are either delayed or malformed (Figure 2. D2; Figure 4. D).  
255 These malformations include shorter or absent arms, probably due to skeletogenic impairment and  
256 a bell-shaped morphology consistent with a radialisation problem. This phenotype agrees with the  
257 one observed before in this species (Rendell-Bhatti et al., 2021), if slightly milder, probably due to  
258 different plastic particles used (see discussion below). All PVC leachate treatment concentrations  
259 show developmental abnormalities in *P. lividus*. 1% PVC leachate-treated larvae are delayed, with  
260 no other clear phenotypic abnormality (not shown; Figure 4. D). 5% PVC leachate-treated embryos  
261 display a bell-shape, being clearly radialised. Malformation of the skeleton and of the distribution  
262 of the pigment cells is also evident (Figure 2. D3). 10% PVC-treated larvae have a very extreme  
263 phenotype. In some cases, they exhibit a radialised phenotype with no elongation of the arms and  
264 a lack of pigment cells. In other cases, the archenteron has not even elongated and, in most cases,  
265 the embryo has not proceeded post-gastrulation (Figure 2. D4).

266 *Patiria miniata* produce a bipinnaria larvae (Yankura et al., 2010). Control larvae at 4 dpf show a  
267 typical young bipinnarial larva phenotype, including a partitioned digestive tract, elongated coeloms  
268 that will give rise to the hydrovascular organ (Perillo et al., 2023) and a well-formed ciliary band  
269 (Figure 2. E1). All treated larvae show some delay or aberrant phenotypes. For nurdle-leachate-  
270 treated embryos, larvae resemble late gastrulae (Figure 2. E2), delayed more than 24 hours from

271 the normal developmental milestone. In many cases the gut appears like a non-partitioned tube,  
272 accompanied by ectodermal deficits. For instance, ectodermal regions such as the ciliary band and  
273 oral hood are missing. Last but not least, the elongation of the coelom appears to be delayed. For  
274 PVC-leachate treated embryos, a concentration-dependent delay in development is seen, with  
275 animals looking like late gastrula when treated with 1% PVC-leachates (Figure 2. E3), to mid gastrula,  
276 with some misshaping of the elongating archenteron at 10% (Figure 2. E4).

277 *Ciona robusta* tadpole larvae have two main structures: the trunk, which contains the adhesive  
278 organ (palps), the brain vesicle with two pigmented sensory organs (otolith and ocellus), endoderm  
279 and mesenchyme; and a straight tail for locomotion, bearing the neural tube, notochord,  
280 endodermal strand and muscles, all covered by the larval tunic. Control larvae showed a normal  
281 trunk and straight tail containing vacuolated notochord cells (Figure 2. F1), while all the treatments  
282 compromised the normal embryo development. Nurdle-leachate-treated larva showed a shorter,  
283 kinked or coiled tail, often disorganized in its internal structure. In most cases, the trunk shape was  
284 abnormal, the sensory vesicle was deformed, although carrying both pigmented organs, and the  
285 adhesive organ was misshaped. (Figure 2. F2). The phenotype for 1% PVC leachate-treated larvae  
286 was very similar to the observed for nurdle leachates (Figure 2. F3), although a higher percentage  
287 of embryos did not hatch and were still in the chorion. However, at 5% PVC leachate treatments,  
288 the larvae were not formed, but instead, unhatched round individuals were obtained. There were,  
289 however, two pigmented spots, probably a hint of structures corresponding to the otolith and the  
290 ocellus, demonstrating development had proceeded, but morphogenesis had not been successful,  
291 thus producing aberrant embryos (Figure 2. F4).

292 *Oikopleura dioica* develops extremely fast, with hatchling larvae appearing at 3.6 hpf at 19°C and  
293 larval development lasting a further 7 hours only, when the juvenile form is ready to make the first  
294 house (Ferrández-Roldán et al., 2019). The larvae, as in *C. robusta*, consist of a trunk that will house  
295 all the organs in the adult, and the tail with the notochord, the muscle cells and the nervous system.  
296 We only observed a significant shift from the controls at the highest concentration of PVC  
297 leachates (10%) when the percentage of malformed larvae increased (Figure 2. G4, Figure 4. G).  
298 These malformations affected the tail, which appeared shorter or kinked, and the trunk, which was  
299 misshaped. We also saw a higher proportion of animals that arrested their development at a pre-  
300 tailbud stage, previously described as a golf ball phenotype (Torres-Águila et al., 2018).

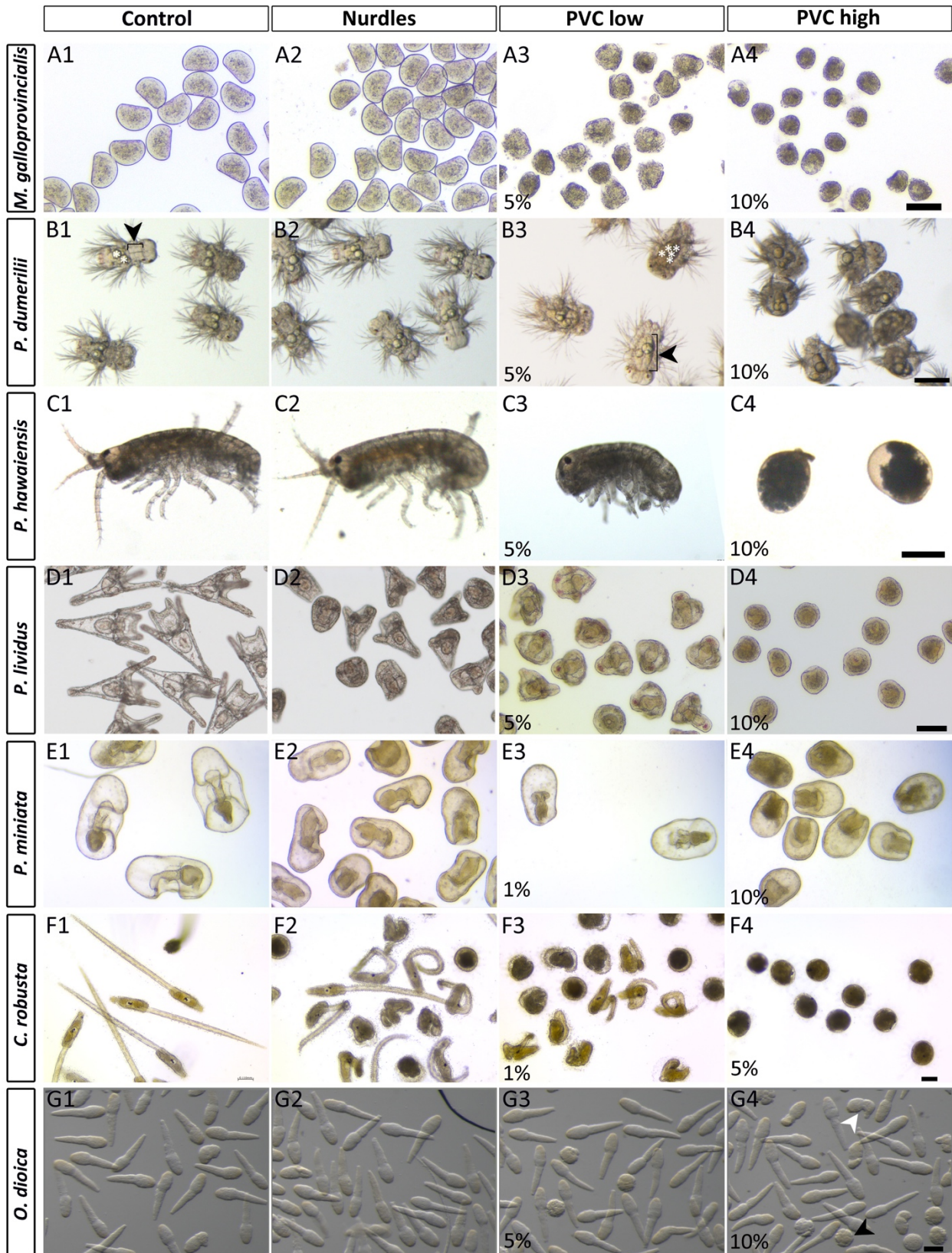
301 *Owenia fusiformis* is capable of anterior regeneration after traumatic injury. Three days after  
302 amputation, the blastema is formed, and differentiation and regeneration are complete seven days

303 after injury (Marilley & Thouveny, 1978). At this time point, we found no differences between  
304 controls and nudle leachate-treated animals (Figure 3. A1, A2). Heads regenerated to create fully  
305 formed tentacles and eyes. Animals treated with 5% PVC leachates looked normal (Figure 3. A3),  
306 but showed a less elaborate branching in the crown of tentacles, and two out of six did not develop  
307 the eyes properly. Likewise, five out of six of the 10% PVC leachate-treated animals regenerated  
308 properly but the branching pattern of the tentacles looked delayed. One animal failed to undergo  
309 morphogenesis at this concentration after creating an elongated blastema (Figure 3. A4).

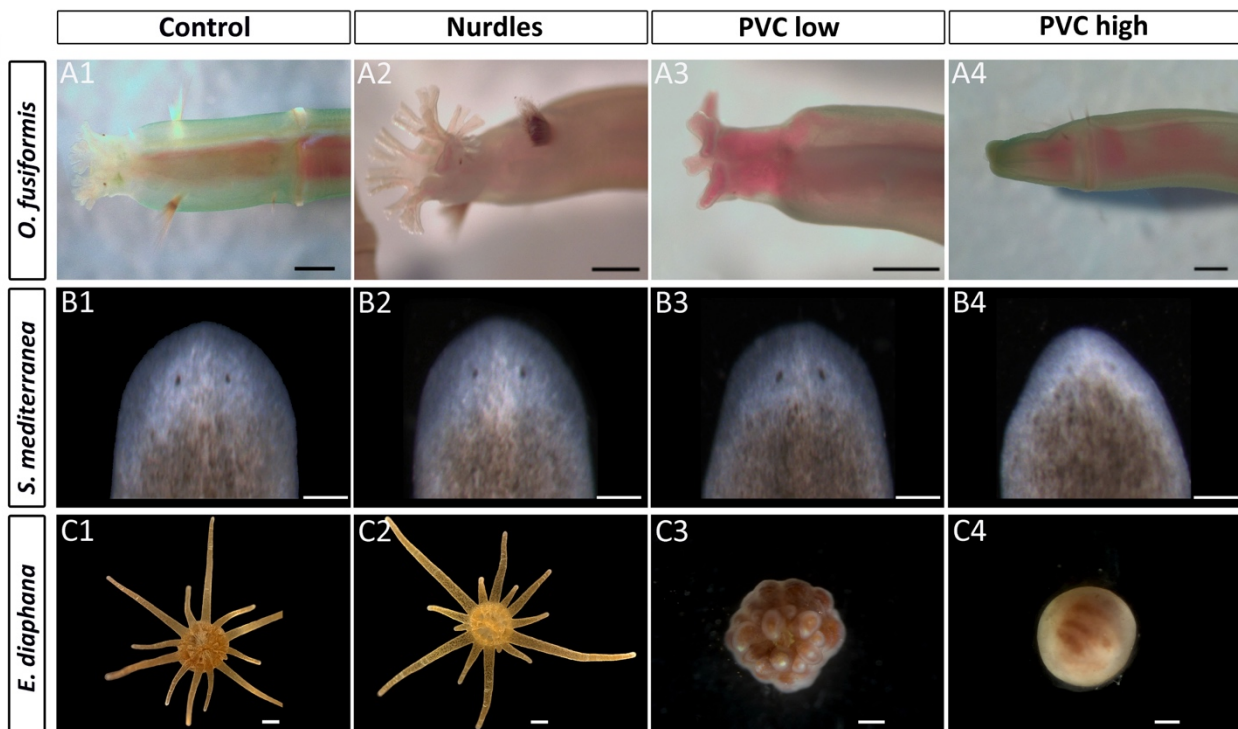
310 The asexual strain of *Schmidtea mediterranea* uses stem-cell based regeneration as its main  
311 reproductive strategy. and can regenerate new heads, tails, sides, or entire organisms from small  
312 body fragments in a process taking days to weeks (reviewed in (Reddien, 2018)). We found no  
313 differences in regeneration between controls and nudle or 5% PVC leachate-treated animals  
314 (Figure 3. B1-B3; Supplementary Figure 2). However, animals treated with 10% PVC leachates  
315 developed a smaller blastema than the controls and, at 7 days after amputation, they have  
316 regenerated smaller eyes and brains (Figure 3. B4; Supplementary Figure 2)). To investigate the  
317 proliferative rate of stem cells, we identified the G2/M stage of the cell cycle by performing  
318 immunostaining against a phosphorylated form of Histone-3. We quantified the total number of  
319 mitoses in amputated planarians regenerating anterior wounds at 5 days after amputation and  
320 observed a significant decrease in the number of mitotic stem cells in 10% PVC leachate-treated  
321 animals, but not in any other treatment (Supplementary Figure 2).

322 *Exaiptasia diaphana* exhibits asexual reproduction capability, growing from pedal lacerations, a  
323 small portion of tissue deriving from the margin of the pedal disk and the body column. This  
324 produces of crescent-shaped fragments that successfully regenerate into fully formed polyps within  
325 a few weeks (Clayton & Lasker, 1985; Presnell, Wirsching & Weis, 2022) (Figure 3. C1). Fourteen  
326 days post-laceration, the morphology is adult-like, differing only for the smaller size. Treatment with  
327 nudle leachate at a concentration of 10% did not seem to affect the external morphology, which  
328 remained comparable to the control (Figure 3. C2). At a concentration of 1% PVC, the morphology  
329 of the polyps was also equivalent to the control group (not shown). However, higher concentrations  
330 of PVC leachate displayed concentration-dependent effects on normal development. Specifically,  
331 treatment with 5% PVC leachate caused developmental delays and reduced tentacle length without  
332 affecting the overall number of tentacles (Figure 3. C3). This result was consistent with the  
333 development period (Presnell, Wirsching & Weis, 2022). Furthermore, at a concentration of 10%  
334 PVC leachate, the regeneration of *E. diaphana* was severely compromised. Despite remaining alive,

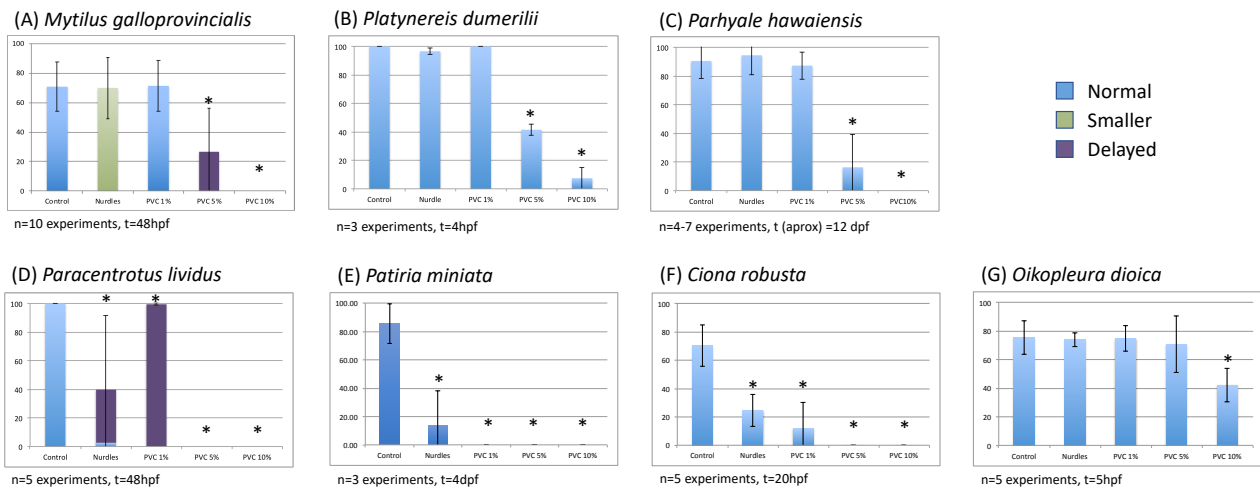
335 the laceration was covered with a protective mucous membrane (typical of new pedal lacerations),  
 336 and the animal maintained its initial state without any signs of tentacle or gut formation (Figure 3.  
 337 C4).



339 Figure 2. Morphological effects on larval development. Representative pictures of the phenotypes  
340 observed for every studied species as they undergo each treatment. See text for details. Columns  
341 are different treatments: (1) Control; (2) Nurdle; (3) PVC low, represents the lowest concentration  
342 tested (between 1% and 5% PVC) resulting in a significant phenotype; (4) PVC high, represents the  
343 concentration at which the most aberrant phenotype was obtained (10% PVC for all species except  
344 for *C. robusta*, which was already at 5%). Lines are different species, as shown in left panel. (B1)  
345 White asterisks show an example of normal number of lipid droplets; black bracket and arrowhead  
346 show an example of normal foregut. (B3) White asterisks show an example of abnormal number of  
347 lipid droplets; black bracket and arrowhead show an example of an enlarged foregut (G4) White  
348 arrowhead points to a kinked-tail phenotype, black arrowhead points to a golf ball phenotype. Scale  
349 bars are 110  $\mu\text{m}$  for all animals except *P. hawaiiensis* which represents 200  $\mu\text{m}$ .



350  
351 Figure 3. Morphological effects on regeneration. Representative pictures of the phenotypes  
352 observed for every studied species as they undergo each treatment. See text for details. Columns  
353 are different treatments: (1) Control; (2) Nurdle, 10% nurdle leachates; (3) PVC low, 5% PVC  
354 leachates; (4) PVC high, 10% PVC. Lines are different species, as shown in left panel. A and B show  
355 only the anterior regenerating heads of the animal. A, anterior to the left; B, anterior to the top.  
356 Scale bars represent 500  $\mu\text{m}$  for *O. fusiformis* and 200  $\mu\text{m}$  for *S. mediterranea* and *E. diaphana*.



357

358 Figure 4. Percentage of normal developmental phenotypes obtained for each species studied in  
 359 each treatment. Percentage of normal embryos is depicted in blue. When embryos are delayed but  
 360 otherwise normal they are depicted in purple. Smaller than normal but otherwise normal embryos  
 361 are shown in green. Number of biological replicas and time of observation are stated under each  
 362 species graph. Error bars show standard deviations. Asterisks indicate differences from the controls  
 363 have statistic significance (One-Way ANOVA followed by Post Hoc Tukey HSD).

#### 364 4. Discussion

##### 365 Developmental susceptibility across animal superphyla

366 The species employed in this study showed different developmental susceptibility to the treatments  
 367 (Figure 4). In summary, all species were affected by new PVC particle leachates, but only some  
 368 deuterostomes and the mussels had a clear response to environmental nurdle leachates. *P.*  
 369 *dumerilii*, *O. dioica* or *P. hawaiiensis* are unaffected by nurdle leachate treatment. For PVC  
 370 treatments, some species are affected already at low concentrations, like *C. robusta* and *P. miniata*,  
 371 which show severe phenotypes already at 1%, while the rest show clear aberrations at 5%, except  
 372 for *O. dioica* which seems to be more resistant and is only affected by the 10% treatment.

373 For deuterostomes, in the case of the echinoderms *P. lividus* and *P. miniata* the effect seems to start  
 374 early, with problems with gastrulation and archenteron elongation, as well as embryonic axis  
 375 formation, as seen in other studies for *P. lividus* and *S. purpuratus* (Rendell-Bhatti et al., 2021;  
 376 Paganos et al., 2023). In agreement with the phenotypes we observe, molecular data for *S.*  
 377 *purpuratus* treated with 10% PVC leachates had previously shown downregulation of genes involved  
 378 in the secondary axis formation as well as in the cell specification of certain cell types (Paganos et  
 379 al., 2023). Looking into the tunicates, *C. robusta* is affected by all treatments while *O. dioica* is more  
 380 resilient, only showing aberrant phenotypes at the higher concentration of PVC. In both cases, we

381 believe axial patterning and cell specification are correct, but that morphogenesis is altered. This is  
382 clear for the treatments that display weaker phenotypes, where structures like the trunk and the  
383 tail have formed, but the morphology of these structures is aberrant (*C. robusta*, Figure 2. F2, F3; *O.*  
384 *dioica*, Figure 2. G4). In the most extreme phenotypes, both species display a golf ball phenotype.  
385 In *C. robusta* it is still possible to identify the two pigmented spots within the otherwise amorphous  
386 ball phenotype. For *O. dioica*, both aberrant phenotypes observed here are also seen when this  
387 animal is exposed to diatom bloom-derived biotoxins (Torres-Águila et al., 2018). Molecular analysis  
388 of the golf ball phenotype in *O. dioica* has previously showed that cell and tissue specification in this  
389 phenotype are correct, but that it is morphogenesis that is at fault (Torres-Águila et al., 2018), and  
390 we believe this is also the case here. All protostomes tested, *M. galloprovincialis*, *P. dumerilii* and *P.*  
391 *hawaiiensis*, display a later effect at lower PVC concentrations, since they show correct axis  
392 specification and early morphogenesis. The appendage malformation in *P. hawaiiensis* and the  
393 aberrant gut formation in *P. dumerilii* nectochaete larvae and the failure to produce a shell in *M.*  
394 *galloprovincialis* point to later gene regulatory pathways being affected in these cases. However,  
395 high PVC concentrations affect larval metamorphosis in *P. dumerilii*, as seen with the inability to  
396 properly transit from trochophore to nectochaete larva, and *P. hawaiiensis* embryos fail to complete  
397 their developmental program.

#### 398 Effects on regeneration

399 Regeneration is a type of asexual reproduction strategy widely adopted among aquatic  
400 invertebrates by which an animal can regrow certain body parts from just a part of the original  
401 organism. Studying regeneration is important to understand healing and repair mechanisms,  
402 including those happening in humans (Mehta & Singh, 2019). Regeneration involves three main  
403 events: wound healing, cell population mobilisation (of stem cells, dedifferentiated or  
404 transdifferentiated cells) and tissue morphogenesis. *E. diaphana* can regenerate a whole polyp from  
405 a small part of the peduncle (pedal laceration) (Clayton, 1985). In the planarian *S. mediterranea*,  
406 residing pluripotent cells called neoblasts are recruited in the wound site to generate a blastema,  
407 which will then differentiate, and morphogenetic processes will assure that the correct pattern is  
408 formed to create the missing body parts (Reddien, 2018). Following anterior amputation in *O.*  
409 *fusiformis*, several tissue rearrangements are in place to generate a blastema where epidermal and  
410 muscle cells proliferate (Fontés et al., 1983). In our study, we see a clear hindering of the  
411 regenerative process in *E. diaphana* treated with PVC leachates (Figure 3. A3, A4). In the case of this  
412 species, wound healing takes place before we expose the animals to the treatment. Later, we

413 cannot discern if the regeneration is obstructed at the level of cell mobilisation or tissue  
414 morphogenesis. The regeneration defects of PVC leachates in *S. mediterranea* and *O. fusiformis* are  
415 much milder (Figure 3. B1-B4, C1-C4). While *O. fusiformis* mostly shows only a delay of the  
416 regeneration process at the highest concentration of PVC leachates, the planarian displays aberrant  
417 regeneration with the formation of a smaller blastema and regenerating smaller eyes. In this case,  
418 we were able to pinpoint a reduction in the proliferation of the stem cells as well as in the  
419 differentiation of the nervous system and the new eyes (Supplementary Figure 2). This points to a  
420 correct wound healing and cell mobilisation in the planarians, and alteration in cell proliferation  
421 being the main cause of the failure in proper regeneration in this species. Whichever the  
422 mechanisms impeded by plastic leachate treatment in each of these species, there is a hinderance  
423 in regeneration because of these treatments, showing that asexual reproduction can also be  
424 affected by microplastic contamination. Indeed, nanoplastics have been found to delay  
425 regeneration in *Girardia tigrina* (Cesarini et al., 2023a) and *Hydra vulgaris* (Cesarini et al., 2023b),  
426 but to the best of our knowledge, no report has shown effects of plastic leachates on regeneration  
427 prior to our work. However, with the species sampled here, our results suggest that regeneration is  
428 more resilient to plastic contamination than development is. Further analysis of the cellular types  
429 and gene expression after infliction of the wound will be necessary to determine if pluripotent-cell  
430 recruitment and proliferation are happening correctly and if tissue morphogenesis is affected.  
431 Knowing the effects that plastic contamination can have in the regenerative processes will be  
432 informative both for the effects in asexual reproduction in marine invertebrates and for the study  
433 of possible consequences for tissue healing and regeneration in other species, potentially including  
434 vertebrates and humans subjected to plastic exposure.

#### 435 Chemical pollutants in the water

436 We have previously determined the content of persistent organic pollutants (POPs) and other  
437 contaminants in the leachates of these pellets (Rendell-Bhatti et al., 2021; Paganos et al., 2023). In  
438 no case were any phthalates found in the leachates of either plastic particles (Rendell-Bhatti et al.,  
439 2021). In 10% nurdle leachates, we had previously found high concentrations of polychlorinated  
440 biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs), 21 and 5 times higher than normal  
441 sea water content (Rendell-Bhatti et al., 2021), which could explain the developmental effects found  
442 in *P. lividus* treated with nurdle leachates (Rendell-Bhatti et al., 2021). These chemicals had also  
443 been found in similar concentrations in leachates from farming fishing gear in French Polynesia,  
444 which also induced developmental defects in the pearl oyster (Gardon et al., 2020). However, the



445 content of POPs in 10% PVC leachates were significantly lower, and the presence of these  
446 compounds alone could not explain the stronger developmental abnormalities seen in PVC leachate  
447 treated *P. lividus* (Rendell-Bhatti et al., 2021). Analysis of the elemental content of the water  
448 leachates by inductively coupled plasma – optical emission spectrometry revealed that 10% PVC  
449 leachates contained high amounts of zinc ( $1 \mu\text{g g}^{-1}$ ), but nurdle leachates did not have higher  
450 concentrations of zinc than sea water (Paganos et al., 2023). The zinc present in the PVC leachates  
451 could explain the phenotypes observed with this treatment, which were consistent with classical  
452 developmental experiments exposing sea urchin embryos to heavy metals (Mitsunaga & Yasumasu,  
453 1984; Hardin et al., 1992; Kobayashi & Okamura, 2004; Cunningham et al., 2020; Paganos et al.,  
454 2023). Since using the same plastic particles, these same chemicals are expected to be the ones  
455 responsible for the phenotypes observed in the present study.

#### 456 Intraspecific variability of the response

457 We observed that the impact of each leachate treatment can differ in severity across batches of  
458 animals of the same species. Indeed, experiments conducted with animals collected on different  
459 days showed more variation compared to those conducted with animals collected on the same day.  
460 These differences were particularly noticeable for the nurdle and low PVC concentration leachate  
461 treatments, which generally resulted in a lower percentage of aberrant embryos than in the higher  
462 PVC leachate treatments (see, for instance, standard deviations for each species in Figure 4).  
463 Oxidative stress is highly involved in the effects of plastic and plastic-leachate treatments in marine  
464 larvae ((Paganos et al., 2023); also reviewed in (Hu & Palić, 2020)) and adults (Jeong et al., 2017;  
465 Pérez-Albaladejo, Solé & Porte, 2020; Milito et al., 2020; Murano et al., 2023). Recently, our lab and  
466 others have shown that adult sea urchins subjected to higher oxidative stress produce less  
467 successful embryos than animals in normal physiological conditions (Masullo et al., 2021; Jimenez-  
468 Guri et al., 2023). We believe that the intraspecific differences we see here are due, probably  
469 amongst other reasons, to the state of the parents before obtaining the gametes for the  
470 experiments as well as to the influence of the different genetic backgrounds of the various parents  
471 used.

472 We also detected intraspecific differences due to a batch effect in the particles used. For beach-  
473 collected nurdles, we found the phenotypes observed are not always equivalent when different  
474 batches of environmental nurdles are used for one species. This is inherent to the sample type, as  
475 every environmental nurdle can have a different history from when they were lost at sea and,  
476 therefore, can have gathered different types of contaminants in their travel (Teuten et al., 2009).

477 Likewise, different types of new plastic pellets will be supplemented with, and therefore be able to  
478 leach, different chemicals at production. Therefore, it is necessary to stress again that different  
479 types of nurdles will produce different phenotypes, and that the ones shown here are only examples  
480 of what can happen. Moreover, the concentrations of pre-production plastic pellets used in this  
481 study to create the leachates are higher than those found in the sea, except maybe in the event of  
482 a nurdle spill (like the one that occurred in Sri Lanka in 2020 (Sewwandi 22) amongst others). Still,  
483 this study constitutes a proof of principle to describe that aquatic animals from different and diverse  
484 phyla are susceptible to plastic leachates during development, metamorphosis and regeneration.

485 Interspecific variability of the response

486 Plastic contamination has been previously shown to affect development in a variety of animals, and  
487 plastic leachates are sufficient to cause these effects (Nobre et al., 2015; Li et al., 2016; Gandara e  
488 Silva et al., 2016; Oliviero et al., 2019; Gardon et al., 2020; Rendell-Bhatti et al., 2021; Paganos et  
489 al., 2023). Micro- and nano-plastics activate oxidative damage and inflammatory responses, leading  
490 to adverse outcome pathways in a variety of organisms (Hu & Palić, 2020), while plastic additives  
491 have also been shown to induce oxidative stress in aquatic organisms (reviewed in (Pérez-  
492 Albaladejo, Solé & Porte, 2020)). Previous studies have shown that adult and developing sea urchins  
493 exposed to plastic leachates have increased oxidative stress and lower production or maintenance  
494 of immune cells (Jimenez-Guri et al., 2023; Paganos et al., 2023). Furthermore, the gene regulatory  
495 networks acting early in the development and necessary for proper embryo formation are affected  
496 in these animals too (Paganos et al., 2023). It would be of great interest to look into the mechanisms  
497 of action of these toxicants to learn how leachates affect the animals studied here. One would  
498 expect that detoxification genes can be important in protecting some of the more resilient species.  
499 In *O. dioica*, response genes against environmental stress (also known as the defensome (Goldstone  
500 et al., 2006)), particularly those dealing with aldehyde detoxification, are upregulated very early in  
501 development as a response to biotoxins, where phenocopies of the larval shapes seen in our study  
502 are obtained (Torres-Águila et al., 2018). However, *C. robusta* is a species that typically lives in  
503 contaminated environments, since it thrives in ports where oil and plastic pollution, amongst  
504 others, is high, and should therefore have a very developed detoxification system. Indeed, exposure  
505 to metals in *Ciona* is known to upregulate glutathione biosynthesis (Franchi et al., 2012) and  
506 upregulate transcription of Cu,Zn superoxide dismutases (Ferro et al., 2013) and metallothionein  
507 genes (Franchi et al., 2011), all of which processes are linked to exposure and protection against  
508 oxidative stress. However, this particular species is one of the most affected by our treatments.

509 Oxidative stress coping mechanisms, including increasing reduced glutathione, have also been  
510 shown to be in place in *M. galloprovincialis* reared in aquaculture environments, which have  
511 increased intake of microplastics (Capo et al., 2021), and increased metallothionein expression has  
512 also been shown in *M. galloprovincialis* and *S. purpuratus* exposed to microplastics (Paganos et al.,  
513 2023; Impellitteri et al., 2023). It would, therefore, be very interesting to study the transcriptomic  
514 state of the defensome of the species studied here to correlate the different phenotypes with  
515 alterations in these specific genes.

516 We hypothesised that another possible reason for the interspecific differences in the severity of the  
517 phenotypes, besides specific physiological, developmental and metabolic responses to the  
518 treatments, would be the speed of development of the different species, as well as the presence of  
519 a longer-lasting, stronger chorion, as a physical barrier to the exterior. If an animal is faster at  
520 developing, it will make sense that chemicals in the water may have less time to affect their  
521 development, as they still need to pass through the chorion before it can generate stress or affect  
522 the transcription of genes. On the contrary, it could be that a slow-developer could have more time  
523 to react activating the stress-defence response. Chorions are indeed protective barriers against  
524 polymer microsphere toxicity in zebrafish embryos (Feng et al., 2013). Here, we have a mix of  
525 species that develop extremely fast (*O. dioica* hatches just 4 hpf) to relatively slow (*P. hawaiiensis*  
526 hatches around 12 dpf). We do not see any emerging pattern that would allow us to determine if  
527 developing fast or slow is advantageous against plastic leachate contamination. However, the  
528 protective function of the chorion seems not to influence the effect of our treatments: despite *C.*  
529 *robusta* having a very strong chorion (not only composed of a surrounding membrane but also test  
530 cells, a group of maternal cells placed between the egg and the membrane, and maternal follicle  
531 cells outside the membrane (Kourakis et al., 2021)), it showed a clear and visible impairment in the  
532 proper development of the embryos. Notwithstanding the mechanism of each species to cope or  
533 react to the plastic leachates, we believe that transcriptomic profiling and determination of the  
534 oxidative stress state of these species after treatment could shed light on the mechanisms behind  
535 the differences and similarities between them.

### 536 Ecological consequences

537 The potential biological impacts of plastic pollution have been extensively discussed, including the  
538 chemical toxicity from plastic leachates (Oliviero et al., 2019; Ke et al., 2019; Shi et al., 2019; Rendell-  
539 Bhatti et al., 2021; Jimenez-Guri et al., 2023; Paganos et al., 2023). Chemical stressors from plastics  
540 have been identified as a possible contributor to biodiversity loss (MacLeod et al., 2021). Embryo

541 development is an extremely robust process that has evolved cellular processes and regulatory  
542 pathways to respond to environmental stressors. However, some anthropogenic stressors can elude  
543 the mechanisms that act as developmental defences to ensure the right developmental decisions,  
544 overwhelming the developmental robustness (Hamdoun & Epel, 2007). Problems in the correct  
545 development of embryos can lead to declines in the success of subsequent generations for a  
546 particular species. Developmental effects with no detectable phenotypes, which may be happening  
547 in the lower concentrations tested here, could also mediate potential transgenerational changes,  
548 which in turn may be detrimental. Given that many species have a spawning season, sporadic  
549 environmental stressors such as surges in local plastic contamination could impact a particular  
550 population's reproductive success. Here, we demonstrate that plastic leachate contamination can  
551 affect the development and regeneration of many aquatic species with potentially catastrophic  
552 effects that can result in the loss of communities and disruption of ecosystems. Our study provides  
553 data showing that microplastic-derived chemical pollution can affect major lineages of marine  
554 diversity, pointing to consequences for the health and functioning of marine ecosystems.

## 555 5. Conclusions

556 Leachates of industrial PVC pellets affect all animals tested in a concentration-dependent and  
557 species-specific manner. Leachates of environmentally retrieved nurdles at the same high  
558 concentrations have a phenotypical effect in fewer species, but still in four out of the ten species  
559 tested. Deuterostomes, excluding *O. dioica*, show the highest sensitivity to all leachates. This work  
560 constitutes proof of principle that both new and environmentally retrieved pre-production plastic  
561 pellets, at high concentrations, can release enough chemicals to affect the development and  
562 regeneration of a wide group of animals. Whether this can be generalised to other types of plastic  
563 leachates is plausible but needs to be studied. Moreover, there may be effects that do not show an  
564 evident phenotype but may be affecting development, physiology or robustness at a lower level,  
565 and cumulative effects may eventually be hindering optimal development, probably including  
566 transgenerational effects. Follow-up mechanistic studies to understand the reason for the failure of  
567 the developmental and regeneration process and any effects with no detectable phenotypes at  
568 lower concentrations would be extremely interesting to address the potential ecological  
569 consequences of plastic pollution on the marine ecosystem.

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