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1 **Graphene oxide worsens copper-mediated embryo-larval toxicity in the Pacific oyster while**
2 **reduced graphene oxide mitigates the effects**

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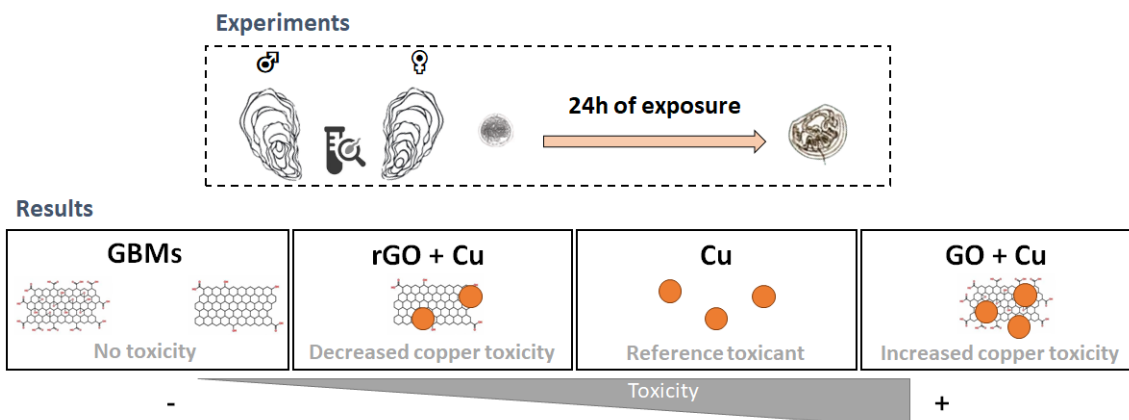
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12 **Abstract**

13 Due to their properties, graphene-based nanomaterials (GBMs) are triggering a great interest leading
14 to an increase of their global production and use in new applications. As a consequence, their release
15 into the environment is expected to increase in the next years. When considering the current
16 knowledge in the evaluation of GBMs ecotoxic potential, studies aiming to evaluate the hazard
17 associated to these nanomaterials towards marine species and particularly considering potential
18 interactions with other environmental pollutants such as metals are scarce. Here we evaluated the
19 embryotoxic potential of GBMs, which include graphene oxide (GO) and its reduced form (rGO), both
20 individually and in combination with copper (Cu) as a referent toxicant, towards early life stages of the
21 Pacific oyster through the use of a standardized method (NF ISO 17244). We found that following
22 exposure to Cu, dose-dependent decrease in the proportion of normal larvae was recorded with an
23 Effective Concentration leading to the occurrence of 50 % of abnormal larvae (EC₅₀) of 13.85 ± 1.21
24 µg/L. Interestingly, the presence of GO at a non-toxic dose of 0.1 mg/L decreased the Cu EC₅₀ to 12.04
25 ± 0.85 µg/L while it increased to 15.91 ± 1.57 µg/L in presence of rGO. Based on the measurement of
26 copper adsorption, the obtained results suggest that GO enhances Cu bioavailability, potentially
27 modifying its toxic pathways, while rGO mitigates Cu toxicity by decreasing its bioavailability. This
28 research underscores the need to characterize the risk associated to GBMs interactions with other
29 aquatic contaminants and supports the adoption of a safer-by-design strategy using rGO in marine
30 environments. This would contribute to minimize the potential adverse effects on aquatic species and
31 to reduce the risk for economic activities associated to coastal environments.

32 **Keywords:** Ecotoxicology, nanomaterials, combined toxicity, safer-by-design, marine environment



34 1. Introduction

35 The development of technologies involving the use of graphene-based nanomaterials (GBMs) is
36 supported by their unique properties, opening up possibilities for new applications in a wide range of
37 areas (Jiříčková et al. 2022; Zhang et al. 2022). Among applications triggering interest, GBMs such as
38 graphene oxide (GO) possess a high potential for pollution removal from water systems due to their
39 sorption capacities (Adel et al. 2022; Akash et al. 2022; Kamran et al. 2022). However, while GO and its
40 reduced form (rGO) constitute the major graphene-related products in the graphene market, they are
41 likely to be mass-produced within the next years (Lin et al. 2019), conducting undoubtedly to their
42 release in the environment at multiple phases of their life cycle (Mottier et al. 2017). Among GBMs,
43 GO and rGO are the main products in the graphene market, constituting the GBMs products that are
44 most likely to be released in the environment (Lin et al. 2019). When reaching the aquatic environment
45 which act as a reservoir for nanomaterials (Scown et al. 2010), GBMs are likely to interact with other
46 pollutants and potentially modify their fate, behaviour as well as their bioavailability for aquatic
47 organisms, influencing their toxicity (Chowdhury et al. 2014; Martínez-Álvarez et al. 2021; Wang and
48 Chen 2015). While based on modelling studies, GBMs concentrations in the aquatic environment are
49 predicted to range from 0.001 to 1000 µg/L (De Marchi et al. 2018; Sun et al. 2016), to our knowledge,
50 no estimation was provided yet for estuarine or marine ecosystems. Moreover, GBMs entry in
51 estuarine areas is of particular concern as these ecosystems host an important biodiversity, provide
52 essential ecosystem services and support human activities of high economic value such as navigation,
53 fisheries and aquaculture (Barbier et al. 2011; Basset et al. 2013; Forrest et al. 2009).

54 Despite that data availability on the ecotoxic potential evaluation of GBMs is increasing (Ding et al.
55 2022; Fadeel et al. 2018), GBMs hazard evaluation on marine species has been much less investigated.
56 In addition, while considering the evaluation of interactive effects between GBMs and environmental
57 contaminants, the main body of literature available present studies performed in freshwater
58 organisms (De Marchi et al. 2018; Hu et al. 2016; Medeiros et al. 2020). Thus, knowledge gaps are
59 remaining concerning these effects in marine ecosystems. Otherwise, the available data mainly
60 focused on the effects of GBMs alone towards adult marine organisms (Khan et al. 2019a, b; Li et al.
61 2021), while in marine invertebrates, early life stages were shown to be more sensitive to pollutants
62 (Lasota et al. 2018). For this reason, there is a need to carefully evaluate their potential impact on
63 marine species and interactions with other pollutants to properly regulate the use and disposal of
64 GBMs, in order to prevent unexpected issues related to these nanomaterials (Hull 2019). We suggest
65 that GBMs are able to modulate the ecotoxic potential of environmental contaminants due to their
66 sorption capacities. For this purpose, the effects of GO alone or associated to copper as referent
67 contaminant were evaluated through the assessment of their embryotoxic potential towards early life
68 stages of the pacific oyster *Crassostrea gigas* also known as *Magallana gigas* (Bayne et al. 2017), using
69 a standardized procedure (NF ISO 17244). In addition, as graphene-metal interactions were shown to
70 depend upon the oxygen-containing surface groups (Nirmala et al. 2022), the consequences of a
71 thermal reduction of GO into rGO on the toxic potential of the nanomaterials were evaluated as the
72 loss of oxygen-containing functions might lead to decrease sorption capacities of the nanomaterial.

73 2. Material and methods

74 2.1. Tested graphene-based nanomaterials

75 Graphene oxide (GO) used in this study was provided by Antolin Group and prepared by oxidizing
76 Grupo Antolin Carbon Nanofibers (GANF®; Grupo Antolín, Burgos, Spain) using the Hummer's method
77 (Hummers and Offeman 1958; Lobato et al. 2016). The GO provided was thermally reduced at 200°C
78 under H₂ atmosphere to produce rGO. The methodology applied for the production of rGO and the

79 procedure for the characterization of the GBMs are detailed in a previous work (Evariste et al. 2019).
80 The results obtained from the characterization of the nanomaterials tested in the current study are
81 summarized in Table 1. For any of the experiment carried out, GBMs were dispersed by sonication
82 through the use of an ultrasonic bath for 10 min before contamination of the exposure medium.

83 GBMs behaviour in seawater was evaluated through the monitoring of dispersion stability using
84 Turbiscan™ LAB Stability Analyzer (Formulation SA, Toulouse, France). Dispersion stability was
85 monitored for 24 hours in seawater, corresponding to the whole embryonic development duration
86 and the exposure media used during the bioassay. To allow detection of particles by the device, GBMs
87 suspensions were prepared at 10 mg/L in seawater in presence or not of Cu at 2.5 mg/L. This condition
88 was selected as it corresponds to the same GBMs/Cu mass concentration ratio performed in the
89 embryotoxicity assessment based on the highest copper concentration to be tested according to the
90 test guidelines (ISO 2015). This test was performed to determine the potential impact of the metal on
91 the dispersion stability in the exposure media.

92 Table 1: Summary of the physico-chemical characteristics of the graphene oxide (GO) and reduced
93 Graphene oxide (rGO) tested in the study. BET: Brunauer-Emett-Teller

	GO	rGO
Carbon content (at. %)	68.62	83.01
Oxygen content (at. %)	30.37	16.81
Number of layers	1-5	1-5
Lateral size (µm)	0.2-8	0.2-8
Specific surface area (BET; m ² .g ⁻¹)	153.8	155.7

94 2.2. GBMs adsorption capacity

95 Copper adsorption capacity of the tested GBMs was evaluated. For this purpose, GO and rGO
96 dispersions were prepared in deionized water at 10 mg/L and with Cu at 2.5 mg/L (added as copper
97 sulfate) without agitation. Following 24 hours of incubation at room temperature, 10 mL of the solution
98 were sampled prior filtration at 0.22 µm to remove GBMs from the solution.

99 Copper levels were analysed in samples using Inductively Coupled Plasma Emission Spectroscopy (ICP-
100 OES, quantification limit: 2–10000 µg kg⁻¹, precision measure: 1–5%; AMETEK Spectro ARCOS FHX22,
101 Kleve, Germany). Sample solutions were diluted with a HNO₃ 2% solution to reduce the spectral
102 interference and viscosity effects. With this method, the amount of copper in a quality control sample
103 was recovered at 96% (nominal concentration: 50 µg L⁻¹, actual concentration: 48 µg L⁻¹). The solution
104 was acidified at 2% of HNO₃ with ultra-pure 69% HNO₃ to avoid precipitation or adsorption in
105 containers. Cu adsorption was determined based on the difference between the Cu concentration
106 recovered in solutions devoid of GBMs compared to the concentration measured in the supernatant
107 of GBMs-containing solutions.

108 2.3. Embryotoxicity assessment

109 The embryo-larval toxic potential of GBMs and its influence on copper toxicity was evaluated using the
110 standardised NF ISO 17244 procedure (ISO 2015), previously AFNOR XP-T90-382. For this purpose,
111 males (68.3 ± 13 g) and females (82.8 ± 10.8 g) conditioned genitor oysters were purchased from
112 Guernesey Sea Farm Ltd. hatchery (Guernsey, UK). Gamete emissions were induced by thermal
113 stimulation through successive baths at 16 °C and 28 °C for 30 minutes. Male gametes were mixed with
114 oocytes at a ratio of 6:1. 20-minutes post-fertilization embryos are then distributed at a density of
115 50,000 oocytes/L in wells of 6-wells microplates (300/wells) containing 6 mL of exposure media

116 composed of natural sterile seawater (Agua de mar, Vizmaragua grupo, Spain), pH = 7.97, with salinity
 117 adjusted to 35 ‰ to fit with the salinity of the site of origin of the genitors. Embryo were exposed to a
 118 positive control composed of Cu (CuSO₄·5H₂O) at concentrations ranging from 2.5 to 25 µg/L as
 119 recommended by the test guidelines. While copper was shown to mainly be associated to carbonate
 120 ions in seawater (Millero 2005), the term Cu will be used for the different forms of dissolved copper.

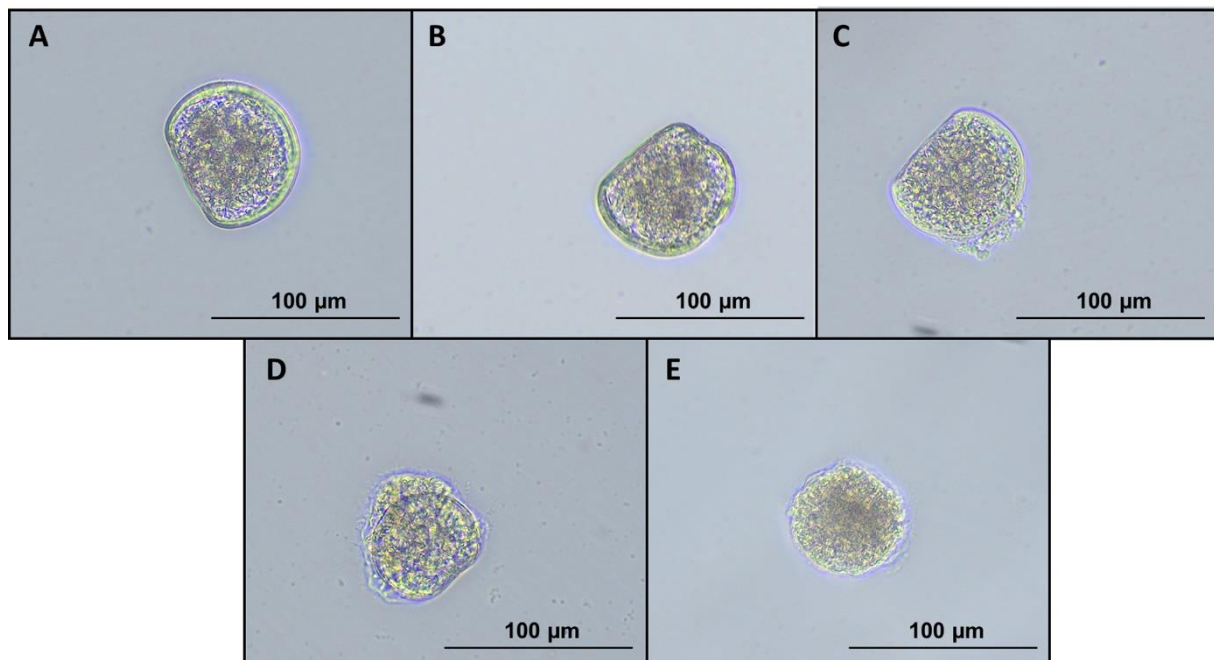
121 The effects of GO and rGO were tested using the GBMs alone at concentrations of 0.1, 1 or 10 mg/L or
 122 at 0.1 mg/L associated with the tested Cu doses. The table 2 summarize the experimental conditions
 123 tested. GBMs-Cu stock solutions were prepared 24 hours prior to the experiment and each
 124 experimental condition was assessed in triplicate for each couple. After 24 h at 23.5 ± 0.5 °C, embryos
 125 reached the D-shaped larvae stage in the negative control and were fixed using 100 µL of an 8%
 126 formalin solution. Compared to normal larvae, anomalies were categorized as previously described as
 127 shell abnormality, mantle abnormality, shell plus mantle abnormalities and development arrest (Figure
 128 1) (Mottier et al. 2013).

129 Table 2: Summary of the experimental conditions tested, indicating the nominal concentrations of Cu
 130 and GBMs tested in each experimental group.

Ctrl		GO		rGO		Cu		Cu+GO		Cu+rGO	
[Cu]	[GBM]	[Cu]	[GBM]	[Cu]	[GBM]	[Cu]	[GBM]	[Cu]	[GBM]	[Cu]	[GBM]
-	-	-	0.1 mg/L	-	0.1 mg/L	2,5 µg/L	-	2,5 µg/L	0.1 mg/L	2,5 µg/L	0.1 mg/L
		-	1 mg/L	-	1 mg/L	6.25 µg/L	-	6.25 µg/L	0.1 mg/L	6.25 µg/L	0.1 mg/L
		-	10 mg/L	-	10 mg/L	12.5 µg/L	-	12.5 µg/L	0.1 mg/L	12.5 µg/L	0.1 mg/L
						18.75 µg/L	-	18.75 µg/L	0.1 mg/L	18.75 µg/L	0.1 mg/L
						25 µg/L	-	25 µg/L	0.1 mg/L	25 µg/L	0.1 mg/L

131

132



133

134 Figure 1: Photomicrographs of D-shaped larvae of *Crassostrea gigas* following 24 hours of
 135 development showing the anomalies considered for the analysis. A: normal larvae; B: shell anomaly;
 136 C: mantle anomaly; D: shell + mantle anomaly; E: embryo

137

138

139 **2.4. Statistical analysis**

140 Statistical analysis was performed using Graphpad Prism 9 software. The results of embryo-larval
141 development of exposed larvae are expressed as net percentages of normal larvae from the control
142 condition. Non-linear four parameters regression was applied on the obtained data to calculate the
143 EC₅₀ values, corresponding to the Effective Concentration leading to the occurrence of 50 % of larvae
144 with development anomaly. Data were statistically tested by one-way ANOVAs after ensuring that the
145 assumption of normal distribution and variance homogeneity are met.

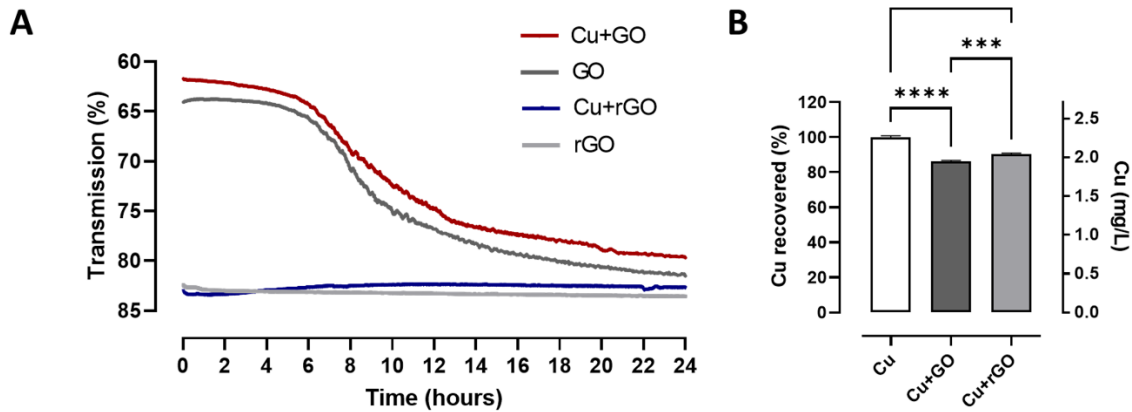
146 **3. Results and discussion**

147 **3.1. GBMs behaviour in the exposure media and copper adsorption capacities**

148 The dispersion stability of GBMs monitored over 24 hours in seawater is presented in Fig. 2A. While
149 rGO nanoparticles in the seawater are almost not detected at a concentration of 10 mg/L based on the
150 high and constant transmission value ($83.2 \pm 0.2\%$), GO dispersion showed sedimentation starting after
151 6 hours of incubation as indicated by the transmission percentage increase over time (Figure 2A). The
152 presence of copper ions did not significantly influence the GBMs stability in seawater.

153 Bioavailability of GBMs in the environment is driven by their behaviour in aqueous media. Multiple
154 parameters including nanomaterial concentration, size, physicochemical properties, natural organic
155 matter or salt composition were previously shown to influence GBMs colloidal properties (Castro et al.
156 2018; Chowdhury et al. 2013, 2014, 2015; Ren et al. 2018; Su et al. 2017). Aggregation and
157 sedimentation processes were previously shown to occur in seawater (Ding et al. 2018) which is
158 consistent with the present data. Interestingly, the GO sedimentation pattern over time observed in
159 this study is close to the data previously obtained using mineral water containing a much lower amount
160 of salts while GO dispersion is stable in deionised water (Evariste et al. 2020). This, suggest that the
161 critical amount of salt/ions leading to GO sedimentation is low and interactions with ions from
162 seawater might be responsible of the GBMs sedimentation. However, as GBMs concentration was
163 shown to influence its colloidal properties (Su et al. 2017), we cannot exclude that the stability of the
164 tested materials would be higher at lower concentrations, including at 0.1 mg/L used in the
165 embryotoxicity assessment.

166 Based on ICP-OES analysis, stock solution of copper used for serial dilutions, prepared at 10 mg/L of
167 Cu, were dosed at 9.5 ± 0.06 mg/L which is consistent with the nominal concentration. Monitoring of
168 the water Cu concentration over time indicated a stable concentration of copper in absence of GBMs
169 as indicated by the recovery of $95.4 \pm 0.7\%$ of the initial copper concentration after 24 hours of
170 incubation. However, after 24 hours in presence of GBMs, Cu concentration decreased by $13.69 \pm 0.5\%$
171 or $9.52 \pm 0.5\%$ in presence of GO or rGO respectively (Figure 2B). As a consequence, the adsorption
172 capacity of Cu on the tested materials was 31 mg/g of GO and 21.6 mg/g of rGO. Previous study
173 indicated Cu adsorption process was controlled by chemical adsorption involving complexation
174 between copper ions with oxygen-containing groups of the GBMs surface, which is consistent with the
175 current results (De Beni et al. 2022; Liu et al. 2018). However, these results are a bit lower compared
176 to the available data ranging from 55.47 to 294 mg/g for pristine nanomaterials and higher for
177 modified composites (Adel et al. 2022; Sitko et al. 2013; White et al. 2018). Such discrepancies between
178 studies may be due to the variability of the material characteristics in term of size, the types of oxygen-
179 containing functions or the atomic percentage of oxygen in the materials, the specific surface area
180 which are influenced by the synthesis and reduction process applied, influencing reciprocally their
181 adsorption capacities (Bai et al. 2016; Botas et al. 2013; Leão et al. 2023; Liu and Speranza 2021).



182

183 Figure 2: GBMs behaviour in seawater and copper adsorption capacity. A: monitoring of the stability
 184 of GO and rGO dispersion in seawater over 24 hours, expressed by the % of transmission detected
 185 after the light goes through the sample. B: adsorption of Cu over 24 hours onto GBMs at 10 mg/L. The
 186 values are given as the mean \pm SD (n = 3). Statistical differences are evaluated using ANOVA followed
 187 by post-hoc Tukey test as $p < 0.05$. ***: $p < 0.001$; ****: $p < 0.0001$.

188 3.2. Embryotoxic potential of GBMs alone or associated with copper

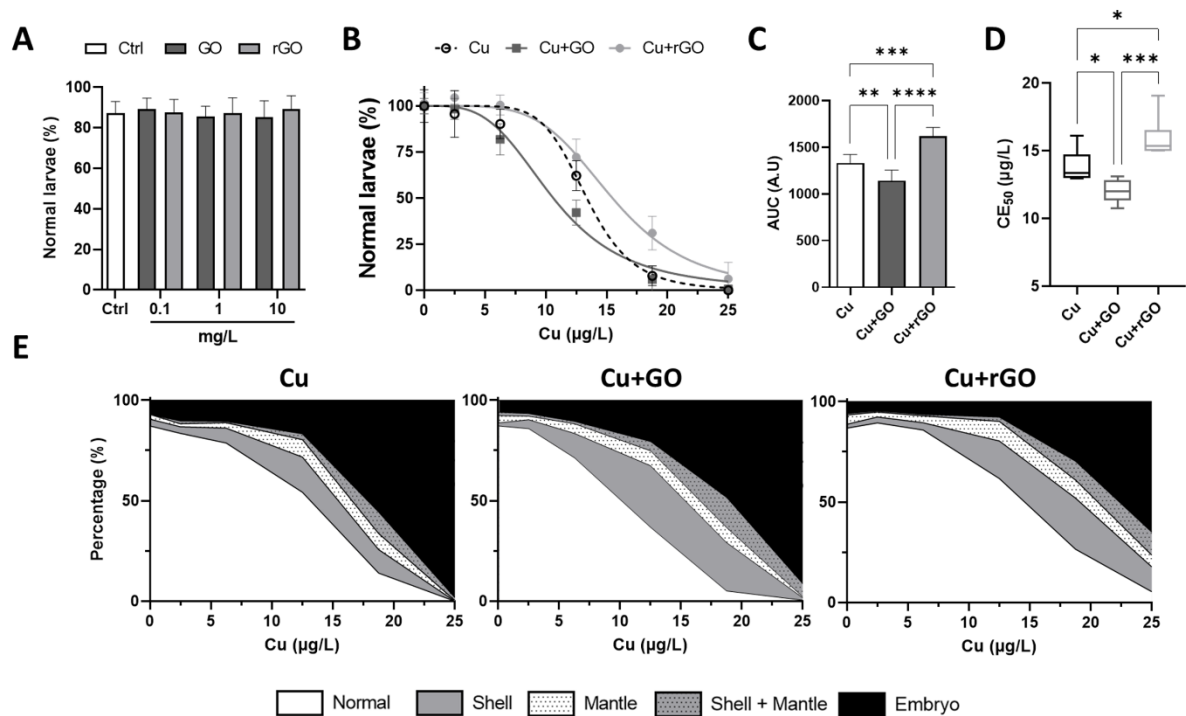
189 Following exposure to GO or rGO at concentrations up to 10 mg/L, no significant induction of larval
 190 development anomaly was observed (ANOVA, $p = 0.642$) (Figure 3A). In previous studies, sperm
 191 exposure from 0.0001 to 1 mg/L of GO induced fecundity defects and led to an increased occurrence
 192 of early development abnormalities in the sea urchin (Mesarič et al. 2015). On the contrary, others
 193 carbon-based nanomaterials such as carbon nanotubes (CNT) or nanofibers (CNF) were show to
 194 possess a weak potential of egg fertilization inhibition in sea urchin embryos while it induced mortality
 195 after 24 hours or exposure, leading to a LC_{50} of ~ 90 and ~ 30 mg/L respectively, (Pikula et al. 2020).
 196 These data suggest that the reprotoxic potential of GO towards sea urchin embryo, is higher than other
 197 carbon-based materials. In the present study, based on the test guidelines, the experimental design
 198 used allow to determine the effects of GBMs specifically on early-life development as fecundations are
 199 performed in uncontaminated water prior exposure to the contaminants. Thus, based on the literature
 200 and the current results, it is suggested that exposure to GO during the fecundation constitute a critical
 201 period for the induction of development anomalies as no developmental defects are observed in *C.*
 202 *gigas* when embryos are exposed post-fecundation to the GBMs alone.

203 As expected based on the NF ISO 17244 procedure, a dose-dependent decrease in the proportion of
 204 normal larvae was recorded following exposure to Cu with an EC_{50} of 13.85 ± 1.21 $\mu\text{g/L}$ (Figure 3, B-D).
 205 *C. gigas* larvae were shown to be sensitive to a wide range of environmental pollutants including trace
 206 metal elements (Xie et al. 2017), pesticides (Mottier et al. 2014), PAHs (Nogueira et al. 2017),
 207 pharmaceuticals (Di Poi et al. 2014), nanoparticles (Ringwood et al. 2010) as well as microplastics
 208 (Bringer et al. 2020). Copper is used as a positive control to evaluate the sensitivity of the test
 209 organisms as bivalve embryos are known to be sensitive to this model contaminant (Fitzpatrick et al.
 210 2008; Gamain et al. 2016), exerting embryotoxic and genotoxic effects. In addition, *C. gigas* embryos
 211 from hatchery-raised animals or from the field were previously shown to display the same sensitivity
 212 to pollutants (Gamain et al. 2017). Thus, as the obtained results following copper exposure are in
 213 accordance with the guideline validity requirements indicating that the Cu EC_{50} must fall between 6
 214 and 16 $\mu\text{g/L}$ (ISO 2015), the data from other experimental conditions cannot be associated to a
 215 particular sensitivity or resistance of test organisms to contaminants.

216 Interestingly, when Cu is associated to GO at the non-toxic dose of 0.1 mg/L, the calculated EC₅₀
217 significantly decrease to 12.04 ± 0.85 µg/L while on contrary it significantly increases to 15.91 ± 1.57
218 µg/L in presence of rGO at the same dose (ANOVA, p < 0.001) (Figure 3 B-D). The lower EC₅₀ of Cu
219 measured in presence of GO at 0.1 mg/L while the nanomaterial alone had no effect on embryo larval
220 development suggest that GO increase Cu bioavailability while not for rGO. This hypothesis is
221 supported by the data obtained with the assessment of GBMs copper adsorption capacity. Indeed, we
222 demonstrated on the one hand that the Cu concentration in the water decreased by 13.69 ± 0.5% or
223 9.52 ± 0.5% in the presence of GO or rGO respectively. On the other hand, the toxicity of Cu increased
224 by 13.04 ± 6.15% in the presence of GO and decreased by 10.36 ± 2.43% in presence of rGO. This
225 suggests that following adsorption, the bioavailability *i.e.* toxicity of Cu ions adsorbed on GO increases
226 while it decreases in the case of rGO which possesses lower dispersion capacities in aqueous media.

227 Focusing on the type of anomalies recorded, the association of Cu with GO led to an earlier occurrence
228 of shell anomalies while in presence of rGO, the proportion of the more severe anomalies are
229 decreased following exposure to high copper concentrations (Figure 3E). The increase of shell
230 anomalies specifically occurring following exposure to low Cu concentrations and the decreased
231 proportion of development arrest following exposure to the highest copper dose suggest that the
232 copper-associated toxic pathways are modified in presence of GO.

233 It was previously indicated that copper exposure induces genotoxic effects and alteration of the
234 homeotic gene transcriptome leading to development anomalies (Sussarellu et al. 2018). Based on
235 studies focusing on the effects of ocean acidification on oyster larvae development, it was indicated
236 that shell development alterations occurred in *C. gigas* when carbonate ions availability decreased
237 (Gazeau et al. 2011). Even if it was previously suggested that carbonate ions (CO₃²⁻) strongly interact
238 with GO surface (Reynosa-Martínez et al. 2020), the increased occurrence of shell anomalies observed
239 in presence of copper is unlikely to be associated to a decreased CO₃²⁻ availability due to GBMs
240 adsorption as those effects would also been noticed when embryos are exposed to GBMs alone.
241 Further studies would be needed to determine which toxic pathways are associated with the co-
242 exposure to GO and copper. Also, while technologies allowing an accurate detection and quantification
243 of GBMs in complex environmental matrices at low concentrations are not available yet (Goodwin et
244 al. 2018), their future development would help to decipher the understanding of interactions between
245 GBMs, environmental contaminants and biota.



246

247 Figure 3: Effects of increasing concentrations of GBMs alone or at 0.1 mg/L associated with copper on the embryo-larval development of the oyster *Crassostrea gigas*. A: histogram presenting the
 248 percentage of normal larvae following 24 hours of exposure to GBMs; B: dose-response curves of
 249 exposure to copper alone or associated to GO or rGO; C: resulting area under the curve calculated; D:
 250 effective concentration inducing induces 50% of abnormal larvae; E: Occurrence of the abnormality
 251 types determined after exposure to the contaminants. ANOVA followed by Tukey test. *: p < 0.05; **:
 252 p < 0.01 and ***: p < 0.001.

254

255 In the case of GO, previous studies described a similar trend in toxicological responses in the shrimp
 256 *Palaemon pandaliformis* (Batista de Melo et al. 2019), indicating that GO at 1 mg/L potentiates the
 257 ecotoxicity of Cd and Zn, leading to an increased acute lethal toxicity. Similar effects to those measured
 258 in presence of rGO were observed in presence of chelators which led to an increased larvae survival
 259 (Knezovich et al. 1981). This suggest that on contrary to GO which is suggested to increase metals
 260 bioavailability following adsorption, rGO behaviour in seawater leads to a decreased copper
 261 bioavailability and protects marine organisms from copper toxicity. Of all the contaminants
 262 transported to marine areas via the river-estuary continuum, copper occupies a predominant position
 263 as a strong marker of human contamination linked to industrial activities (metallurgy, electronics,
 264 surface industry), agriculture (wine growing, arboriculture), aquaculture (shrimp production) and
 265 urban activities (wastewater treatment plant discharges) (Lyle-Fritch et al. 2006; Panagos et al. 2018;
 266 Richardson 2014). Because of the worldwide growing development of GBMs, mixed GO/rGO and
 267 metals contaminations in the aquatic environment have to be considered in the future.

268 Based on the results presented in the present study, we suggest that the thermal treatment of GO to
 269 produce rGO constitutes a strategy promoting a safer by design approach in marine environment (Corsi
 270 et al. 2021), through mitigation of toxicity associated to the decreased bioavailability of adsorbed
 271 environmental pollutants. In this way, the reduction of GO to rGO could contribute to a better control
 272 of toxic risks in coastal waters while helping to maintain economic activities such as shellfish farming.

273 **Author statement**

274 Antoine Mottier: Formal analysis, Investigation, Methodology; Morgan Légnani: Formal analysis;
275 Frédéric Candaudap: Formal analysis; Emmanuel Flahaut: Funding acquisition, Resources; Florence
276 Mouchet: Funding acquisition, Project administration; Laury Gauthier: Funding acquisition, Writing –
277 review & editing; Lauris Evariste: Writing – original draft, Conceptualization, Methodology

278 **Declaration of competing interest**

279 The authors declare that they have no known competing financial interests or personal relationships
280 that could have appeared to influence the work reported in this paper.

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