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1 **Gut microbiota impairment following graphene oxide exposure is associated to physiological**
2 **alterations in *Xenopus laevis* tadpoles**

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10 **Abstract**

11 Graphene-based nanomaterials such as graphene oxide (GO) possess unique properties triggering high
12 expectations for the development of technological applications. Thus, GO is likely to be released in
13 aquatic ecosystems. It is essential to evaluate its ecotoxicological potential to ensure a safe use of
14 these nanomaterials. In amphibians, previous studies highlighted *X. laevis* tadpole growth inhibitions
15 together with metabolic disturbances and genotoxic effects following GO exposure. As GO is known to
16 exert bactericidal effects whereas the gut microbiota constitutes a compartment involved in host
17 homeostasis regulation, it is important to determine if this microbial compartment constitutes a
18 toxicological pathway involved in known GO-induced host physiological impairments. This study
19 investigates the potential link between gut microbial communities and host physiological alterations.
20 For this purpose, *X. laevis* tadpoles were exposed during 12 days to GO. Growth rate was monitored
21 every 2 days and genotoxicity was assessed through enumeration of micronucleated erythrocytes.
22 Genomic DNA was also extracted from the whole intestine to quantify gut bacteria and to analyze the
23 community composition. GO exposure led to a dose dependent growth inhibition and genotoxic effects
24 were detected following exposure to low doses. A transient decrease of the total bacteria was noticed

25 with a persistent shift in the gut microbiota structure in exposed animals. Genotoxic effects were
26 associated to gut microbiota remodeling characterized by an increase of the relative abundance of
27 *Bacteroides fragilis*. The growth inhibitory effects would be associated to a shift in the
28 Firmicutes/Bacteroidetes ratio while metagenome inference suggested changes in metabolic
29 pathways and upregulation of detoxification processes. This work indicates that the gut microbiota
30 compartment is a biological compartment of interest as it is integrative of host physiological alterations
31 and should be considered for ecotoxicological studies as structural or functional impairments could
32 lead to later life host fitness loss.

33

34 **Keywords:** Amphibian; aquatic ecotoxicology; nanotoxicology; microbial ecology

35

36

37 **Introduction**

38 Benefiting from graphene-based nanomaterials (GBMs) properties, tremendous efforts are made for
39 the development or the improvement of applications in a wide range of fields such as energy storage
40 (Ali Tahir et al., 2016; Olabi et al., 2021), pollution remediation (Gopinath et al., 2021), medicine (Liu
41 et al., 2013) or composite reinforcement (Ahmad et al., 2018). The improvement of production
42 methods contributed to the increase of the annual production capacity of GBMs, including graphene
43 oxide (GO), that are forecasted to be produced at industrial-scale within few years (Lin et al., 2019;
44 Zhu et al., 2018). For these reasons, these nanomaterials are likely to be released in the environment
45 at any stage of their life cycle (Ding et al., 2022; Freixa et al., 2018; Mottier et al., 2017), threatening
46 ecosystems and associated wildlife, especially aquatic environments acting as a pollutant receptacle
47 (Scown et al., 2010). Although no current information regarding environmental concentration of GBMs
48 are available due to technological limitations (Goodwin et al., 2018), predicted environmental
49 concentration of GO in aquatic ecosystem was estimated to be similar to those of carbon nanotubes
50 ranging from 0.001 to 1,000 µg/L (De Marchi et al., 2018; Sun et al., 2016). Thus, carefully evaluating
51 the ecotoxicological potential of GBMs is essential to ensure a safe use and a sustainable development
52 of this nanotechnology.

53 Despite the knowledge gaps remaining concerning the evaluation of their ecotoxic potential (Fadeel et
54 al., 2018; Jastrzębska and Olszyna, 2015), the data available from the literature indicate that GBMs can
55 be accumulated through the trophic chain (Dong et al., 2018) and affect the physiology of organisms
56 from different trophic levels (Evariste et al., 2020b; Montagner et al., 2016). Indeed, various toxic
57 effects of GO towards aquatic organisms were observed, including physical membrane damages in
58 bacteria (Mohammed et al., 2020; Sengupta et al., 2019), oxidative stress and shading effects in algae
59 (Saxena et al., 2020; Yin et al., 2020), oxidative stress and motility impairment in the invertebrate
60 *Daphnia magna* (Cano et al., 2017; Fekete-Kertész et al., 2020; Lv et al., 2018) or developmental

61 impairments, DNA damages, apoptosis, oxidative stress and immunotoxicity in zebrafish (M. Chen et
62 al., 2016; Y. Chen et al., 2016; Souza et al., 2017).

63 In amphibian, previous studies highlighted that the growth of *Xenopus laevis* tadpoles was altered by
64 exposure to GBMs (Mottier et al., 2016), including GO (Lagier et al., 2017). It was also highlighted that
65 the tadpole growth inhibition was independent from a disruption of the thyroid pathway (Evariste et
66 al., 2021b), while it was associated to a decrease of the fatty acids and triglyceride metabolism (Li et
67 al., 2019). Intestinal accumulation of the nanomaterials favors direct contact with the gut microbiota
68 which may be modulated by the nanomaterials and lead to host physiological alterations (Bantun et
69 al., 2022). Indeed, while nutrients absorption and energy metabolism are known to rely on the gut
70 microbiota homeostasis, it is of importance to determine if the alteration of the gut microbiota
71 constitutes a toxicological pathway involved in GBMs-induced host physiological alterations. Emerging
72 evidences indicate that the environmental pollution constitutes a major factor negatively influencing
73 the composition and/or functioning of the host-associated microbial communities (Adamovsky et al.,
74 2018; Duperron et al., 2020; Evariste et al., 2019a). However, this biological compartment is often
75 unconsidered in ecotoxicology studies, focusing on host-centered biomarker measurements. In
76 addition of playing a crucial role in the host physiological homeostasis, it was demonstrated that early-
77 life alterations of the amphibian gut microbiome influence later life host fitness, through pathogen
78 sensitivity increases or thermal tolerance impairments (Fontaine et al., 2022; Knutie et al., 2017). Thus,
79 it is of crucial importance to consider both host and associated microbiota health in ecotoxicological
80 studies as early life alteration of the gut microbiota could lead to threat for host population dynamic.

81 For this purpose, *X. laevis* tadpoles were exposed to increasing GO concentrations under similar
82 conditions compared to the previously mentioned studies (Evariste et al., 2019b; Lagier et al., 2017),
83 to determine the consequences towards gut microbial communities and the potential existing link
84 between host physiological alterations and gut microbiota remodeling.

85

86 2. Materials and methods

87 2.1. *Xenopus laevis* breeding and exposure procedure

88 Sexually mature *Xenopus laevis* originated from our certified facilities (approval number A31113002).
89 Spawning was induced by injection of 50 IU of pregnant mare's gonadotropin (PMSG 500; Intervet,
90 France) in males and 750 IU of human chorionic gonadotropin (HCG; Organon, France) in females. The
91 obtained fecundated eggs were bred in active charcoal filtered tap water at $22 \pm 2^\circ\text{C}$ and fed ad libitum
92 with grinded aquarium fish food (TetraPhyll®, Tetra, Melle, Germany) until they reach stage 50 based
93 on the Nieuwkoop & Faber development table (Nieuwkoop and Faber, 1958).

94 In accordance with the international standard ISO 21427-1 guidelines, groups of 20 larvae from stage
95 50 were exposed for 12 days under semi-static conditions to GO at 0; 0.05; 0.1; 1 or 10 mg/L. For
96 comparison purpose, based on the specific surface area of the tested GO (Table 1) and according to
97 previous works (Lagier et al., 2017; Mottier et al., 2016), the mass-concentration tested are equivalent
98 to an exposure to a surface area per unit volume of 0; 0.0114; 0.0228; 0.228 and 2.28 m²/L respectively.
99 The experiment was conducted under natural dark/light cycle at $23.2 \pm 0.2^\circ\text{C}$ and feeding (TetraPhyll®,
100 Tetra, Melle, Germany) as well as exposure media (294 mg/L CaCl₂·2H₂O; 123.25 mg/L MgSO₄·7H₂O;
101 64.75 mg/L NaHCO₃; 5.75 mg/L KCl) were renewed daily to allow ad libitum feeding and water quality
102 maintaining. Following 12 days of exposure, *X. laevis* larvae were anesthetized by bathing in 200 mg/L
103 of MS222 solution in sodium bicarbonate prior samples collection. The whole experimental procedure
104 was approved by an ethic committee (CEEA-073).

105 2.1. Graphene oxide characteristics, preparation of GO suspensions and behavior in exposure

106 media

107 The graphene oxide used for this experiment was provided by Antolin Group and was prepared by
108 oxidation of Grupo Antolin Carbon Nanofibers (GANF®) (Grupo Antolín, Burgos, Spain) using the
109 Hummer's method (Hummers and Offeman, 1958; Lobato et al., 2016). The detailed characterization

110 of the GO batch used for this experiment was previously presented (Evariste et al., 2019b),
111 demonstrating a non-significant amount of byproducts within the tested nanomaterials. The main
112 characteristics of GO are summarized in Table 1.

113 For the experiment, GO was dispersed extemporaneously in deionized water to prepare a stock
114 solution at 1 mg/mL. After 15 min sonication in ultrasonic bath (Bioblock 89863, typ 570 HF Freq 35
115 kHz), the stock solution was diluted to different concentrations in 20 mL of deionized water. Working
116 solution of GO were sonicated during 2 minutes under same sonication conditions prior to exposure
117 media contamination.

118 GO dispersion stability was evaluated using the UV-absorption properties of the nanomaterial,
119 exhibiting an absorption peak at 235 nm (Johra et al., 2014). The experiment was conducted in
120 triplicate at the single GO concentration of 10 mg/L due to detection limit constraints. Absorbance
121 (235 nm) of the exposure media was measured in water column samples after 0; 10; 30; 60; 90; 120;
122 240; 420 and 1440 minutes, corresponding to the maximum duration prior media renewal, using
123 CLARIOstar® microplate reader. GO concentrations were calculated using an absorbance-based
124 calibration plot based on the optical density (OD) at 235nm, obtained after serial dilution of GO
125 dispersions (Zhang et al., 2019). GO was quantified in the exposure media over time in absence as well
126 as in presence of *X. laevis* tadpoles.

127 **2.3. Growth measurement**

128 In order to determine larval growth during the exposure, the length of the larvae was monitored since
129 day 0 until day 12 every two days using ImageJ 1.49 software. Larval growth was calculated as a
130 percentage of length increase since day 0. Normalized growth rate was determined as previously
131 described (Mottier et al., 2016), using the following formula:

$$132 \text{ Normalized size (\%)} = \left(\frac{(Ldt - MLd0)}{MLd0} \times 100 \right) \times \left(\frac{100}{MLCdt} \right)$$

133 With Ldt corresponding to the length of a larvae at the time of interest, MLd0 as the mean length of
134 the larvae at day 0 from the exposure condition and MLCdt for the mean length of larvae from the
135 negative control at the time of interest.

136 Intestinal growth was evaluated using similar calculations based on the intestinal weight instead of
137 larval length.

138 **2.4. Gut microbiota survey**

139 Following anesthesia, ventral skin was incised to collect the whole intestine. Gut microbial community
140 DNA was extracted from the whole larval intestine samples, snap frost in liquid nitrogen after 0(T0), 2
141 (T2) or 12 days (T12) of exposure (n = 5 per condition and per time). DNA extractions were performed
142 using DNeasy PowerSoil kit (QIAGEN) as previously described for *X. laevis* gut samples (Evariste et al.,
143 2020a).

144 Quantitative PCR (qPCR) targeting the universal 16S rRNA gene was used to measure the abundance
145 of total intestinal bacteria. To provide a standard curve, tenfold factor dilution series of the gene
146 ligated onto synthetic plasmid pEX-A128 (Eurofins Genomics, Belgium) was carried out. The reaction
147 mixture (total volume = 10 µL) consisted of 5 µL of SsoAdvanced Universal SYBR Green Supermix
148 (BioRad, United States), primer pairs (341-F CCTACGGGAGGCAGCAG / 534-R
149 ATTACCGCGGCTGCTGGCA) and DNA template (diluted 100 times). Each reaction was run in triplicate
150 in 96-well plates using a CFX96 Touch™ Real-Time PCR Detection System (BioRad, United States), using
151 the following PCR protocol: 95°C for 130s, 35 cycles of 95°C for 5s, 60°C for 30s, 72°C for 10 minutes.

152 For 16S rRNA amplicon sequencing, the V4-V5 region of the gene was amplified from the whole
153 intestine DNA extract using 515 F (5'-GTGYCAGCMGCCGCGGTA-3') / 928 R (5'-
154 CCCCYCAATTCMTTTRAGT-3') primer pair (Wang and Qian, 2009) and the following PCR protocol:
155 94°C for 120s, 30 cycles of 94°C for 60s, 65°C for 40s, 72°C for 10 minutes. The amplicon sequencing
156 was performed by the Get_PlaGe platform (Genotoul, Toulouse, France) using an Illumina MiSeq
157 (2x250 pb).

158 Microbiome bioinformatics were performed using QIIME 2 219.4 (Bolyen et al., 2019). Forward and
159 reverse Illumina demultiplexed sequences were joined after import into QIIME2 prior denoising using
160 DADA2 (Callahan et al., 2016). Based on the illumina primer used, trimming parameters were --p-trim-
161 left-f 18 and --p-trim-left-r 19. According to interactive quality plots, truncation length parameter of
162 DADA2 were -- p-trunc-len-f 220 and -- p-trunc-len-r 210. A total of 2,762,174 reads were obtained
163 (minimum = 19,230, maximum = 61,452 and mean = 36,828.98). All amplicon sequence variants (ASVs)
164 were aligned with mafft (Kato et al., 2002) and a phylogenetic tree was built using FastTree (Price et
165 al., 2009). Taxonomy was assigned to ASVs using the q2-feature-classifier (Bokulich et al., 2018),
166 classify-sklearn naive Bayes taxonomy classifier against the Greengenes 13.8 99% OTUs reference
167 sequences (McDonald et al., 2012).

168 To get insight at the species level of the most abundant ASV recovered, leading to a strong gut
169 microbiota shift at T12 following exposure to low GO concentrations, a BLASTn search of the amplicon
170 sequence was performed. The report suggested that the amplicon sequence produced significant
171 alignment with *Bacteroides fragilis* (Accession: MN567591.1; Query cover: 100%; E-value: 2e-174;
172 percentage identity: 97.07%).

173 PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was
174 used to predict functional gene content predictions based on 16S rRNA gene data (Douglas et al.,
175 2020). The resulting abundance of KEGG (Kyoto Encyclopedia of Genes and Genomes) genes
176 orthologues in each sample was categorized into biological pathways.

177 **2.5. Micronucleated erythrocyte count and cell cycle analysis**

178 Blood samples were collected through cardiac puncture. For micronuclei accounting, air dried blood
179 smears are then fixed in methanol for 10 minutes prior staining with hematoxylin and eosin.
180 Micronucleated erythrocytes are accounted over a total of 1000 cells (MNE %) using optical
181 microscopy. For cell cycle analysis, blood sub-samples were fixed using cold ethanol (70% v/v) prior to
182 perform analysis using Beckman Coulter Cytoflex flow cytometer (Beckman Coulter, USA). Cells were

183 stained using FxCycle™ PI/RNase Staining Solution (Life Technologies SAS) according to
184 manufacturer's recommendations.

185 **2.6. Statistical analysis**

186 Graphs were generated and statistical analysis performed using the software GraphPad Prism 9.3.1.
187 Data related to *X. laevis* physiological parameters such as growth rate or intestinal weights were
188 analyzed using one-way analysis of variance (ANOVA) followed by Tukey test after verifying that the
189 assumptions of normality and homogeneity of variance are met.

190 For microbiota survey, differences in the alpha diversity indexes and phyla relative abundances were
191 analyzed for each sampling time by one-way analysis of variance (ANOVA) followed by Tukey test.
192 Principal coordinates analysis (PCoA) plots were used to assess the variation between experimental
193 conditions based on Bray-Curtis distances. Differences in beta-diversity were assessed *via* pairwise
194 Permanova tests with 999 permutations. Intergroup taxa abundance differences were analyzed by the
195 linear discriminant analysis (LDA) effect size (LEfSe) method (Segata et al., 2011). To conduct LEfSe
196 analysis, the raw abundance of bacterial genera obtained from QIIME2 were exported on the
197 Huttenhower Lab Galaxy Server (<https://huttenhower.sph.harvard.edu/galaxy/root>). For stringency,
198 differentially abundant taxa that were statistically significant using an alpha of 0.05, and LDA score
199 (log₁₀) exceeding 3.0.

200 Differentially abundant pathways inferred using PiCRUST2 were evaluated using LEfSe analysis.
201 Differentially abundant pathways were considered statistically significant using an alpha of 0.05, and
202 LDA score (log₁₀) exceeding 2.0.

203 Results from the micronucleus assay were statistically analyzed using McGill non-parametric test
204 (McGill et al., 1978) on median values of each group of larvae as recommended in the standardized
205 procedure ISO 21427-1. For cell-cycle data, after verifying the normal distribution of data, differences
206 in cell-cycle phase distribution among conditions was evaluated using ANOVA followed by Tukey test.

207 **3. Results**

208 **3.1. Exposure to GO affect larval growth**

209 Using the calibration plot based on the optical absorbance at 235 nm of the GO dispersion used for our
210 experiment (Figure 1A), GO concentration in the water column was determined to be 9.14 ± 0.34 mg/L
211 immediately following exposure media contamination which is close to the nominal value of 10 mg/L
212 expected. GO concentration in the water column decreased over time due to GO sedimentation,
213 leading to a decreased GO concentration of $70.7 \pm 5.4\%$ after 420 minutes (Figure 1B). In presence of
214 *X. laevis*, the water column is clear from GO only after 240 minutes (Figure 1B). Thus, the significantly
215 lower area under the curve calculated from the GO concentration kinetic (T-test, $p < 0.001$) indicated
216 that the presence of larvae in the exposure media is leading to a faster clearance of GO from the water
217 column.

218 Monitoring of the larval growth parameters over the whole experiment indicated that GO exposure
219 led to a time and dose-dependent alteration of the tadpole growth (Figure 2A). Growth inhibition is
220 noticed following two days of exposure at the lowest dose of 0.05 mg/L (ANOVA, $p < 0.001$), while the
221 growth of larvae exposed to the highest dose was decreased by $51.8 \pm 17.7\%$ compared to the control
222 group (Figure 2B). This effect is maintained over the whole experiment while growth alteration is less
223 marked following 12 days of exposure as the growth of larvae exposed to 10 mg/L of GO is decreased
224 by $20.7 \pm 7.2\%$ compared to unexposed larvae (Figure 2C). Concomitant with the growth inhibition, a
225 strong intestinal accumulation of GO is noticed in the gut of larvae exposed to any GO concentration
226 after 2 days of exposure and up to the end of the experiment (Figure 2D).

227 Based on intestinal sample weights, larvae exposed to GBMs exhibited similar intestinal weight
228 following 2 days of exposure to the different GO concentrations (ANOVA, $p = 0.732$) (Figure 2E) while
229 only a significant decrease (ANOVA, $p = 0.018$) of intestinal weight is noticed after 12 days of exposure
230 to GO at 10 mg/L, leading to a mean intestinal weight $37.8 \pm 10.5\%$ lower compared to unexposed

231 larvae (Figure 2F). At the end of exposure, no developmental stage delay was observed as most of the
232 tadpoles reached the NF stage 57 in all experimental conditions (ANOVA, $p = 0.093$) (Figure S1).

233 **3.2. Exposure to GO transiently decrease the microbiota density**

234 According to the 16S rRNA quantification of intestinal DNA extracts using qPCR, the results indicate
235 that the amount of bacteria per mg of intestine remained constant over the experiment in larvae from
236 the control group (ANOVA, $p = 0.814$) (Figure 3A). However, a transient decrease of 16S gene
237 abundance is noticed following 2 days of exposure to the highest GO concentration (ANOVA, $p < 0.001$)
238 prior recovering to similar values compared to the control group at T12 (ANOVA, $p = 0.666$) (Figure
239 3B,C).

240 **3.3. Short term exposure to GO initiate a shift in the gut microbiota structure**

241 Sequencing of the 16S rRNA V4-V5 region from gut DNA extracts allowed to explore for the
242 consequences of an exposure to GO on the gut microbial communities structure and composition.
243 While the gut microbial diversity indexes were similar between conditions prior to exposure media
244 contamination (Figure S2), comparing the gut microbiome richness and diversity of unexposed
245 tadpoles to the ones exposed during 2 days to increasing concentrations of GO indicated a significant
246 increase of richness at the highest dose of exposure (ANOVA, $p = 0.035$) without influencing the
247 Shannon index (Figure 4A, B). This suggest that the dominant species relative abundances from the gut
248 microbiota are not impacted by the short duration of exposure to GO. However, based on Bray-Curtis
249 distances, a significant increase of beta-diversity is noticed between the microbiota of larvae from the
250 control group and those from larvae exposed to 0.05, 0.1 and 10 mg/L of GO while not following
251 exposure to 1 mg/L (PERMANOVA, $p = 0.001$) (Figure 4C, D). PCoA plot and pairwise comparisons
252 indicated that the microbiota shifted similarly in larvae exposed to 0.05 and 0.1 mg/L (Figure 4C).

253 At T2, the gut microbiota from control larvae is composed of three main phyla including Bacteroidetes,
254 Firmicutes and Proteobacteria which relative abundances are respectively $54 \pm 6.4\%$, $25.5 \pm 5.2\%$ and
255 $12.8 \pm 7.8\%$. In tadpoles exposed to GO, only larva exposed to 0.05 mg/L presented a microbiota

256 containing a significantly increased relative abundance of bacteria from the phylum Proteobacteria
257 compared to the control group (ANOVA, $p = 0.005$). Otherwise, only slight nonsignificant modulations
258 of others phyla relative abundances are observed (Figure 4E-I).

259 Analysis of differential taxa abundances using linear discriminant analysis (LDA) effect size (LEfSe)
260 indicated that the exposure to the lowest GO concentrations led to an increased relative abundance
261 of bacteria from the family *Cryomorphaceae* and genus *Rikenella* while the abundance of *Clostridiales*
262 (order) decreased (Figure S3A). Following exposure to GO at 0.1 mg/L, larvae harbored a gut microbiota
263 with increased proportion of bacteria from the genera *Rikenella* and *Acinetobacter* while bacteria from
264 the family *Lachnospiraceae* decreased (Figure S3B). On contrary, none of the taxa found in the gut of
265 larvae exposed to GO at 1 mg/L during 48 hours were differentially abundant compared to the control
266 group. However, exposure to the highest GO concentration altered the abundance of bacteria from
267 the family *Lachnospiraceae* and from the genus *Flectobacillus* while increased the proportion of
268 *Anaerorhabdus furcosa* and family *Neisseriaceae* (Figure S3C).

269 **3.4. Longer exposure duration to low or high GO concentrations differentially impair the gut** 270 **microbiota**

271 At the end of exposure (T12), a significant decrease of species richness is noticed in the gut microbiota
272 of larvae exposed to 0.1 mg/L (ANOVA, $p = 0.0011$), while not in other conditions compared to the
273 control group (Figure 5A). Considering the Shannon index, a significant and marked decrease of the
274 index value is measured following 12 days of exposure to GO at 0.05 and 0.1 mg/L (ANOVA, $p < 0.001$)
275 (Figure 5B). Based on Bray-Curtis dissimilarities analysis (Figure 5C, D), the results indicated that the
276 gut bacterial communities of larvae exposed to GO at 0.05 and 0.1 mg/L were more dissimilar to the
277 control group compared to larva exposed to the highest GO concentration, while larvae exposed to 1
278 mg/L exhibited a gut microbiota similar to the control group (PERMANOVA, $p = 0.001$).

279 As previously determined at T2, bacteria composing the gut microbiota of larvae from the control
280 group at T12 belong to the phyla Bacteroidetes, Firmicutes and Proteobacteria with different relative

281 abundances, accounting respectively for $67.1 \pm 5.2\%$, $11.6 \pm 4.7\%$ and $17.3 \pm 8.2\%$ of the whole
282 bacterial populations (Figure 5E). At the phylum level, 12 days of exposure to GO led to a decrease of
283 Bacteroidetes relative abundance following exposure to GO at 10 mg/L (ANOVA, $p < 0.001$), while
284 bacteria from the phylum Firmicutes significantly increased in the gut of larvae following exposure to
285 GO at 1 and 10 mg/L compared to larvae exposed to low GO concentrations (ANOVA, $p < 0.001$) (Figure
286 5F, G). Concomitantly, a significant increase of the relative abundance of bacteria from the phyla
287 Proteobacteria is noticed following exposure to GO at 0.1 and 10 mg/L while the relative abundance
288 of bacteria from the phyla Fusobacteria significantly decreased in the gut of larvae exposed to GO at
289 10 mg/L (Figure 5H, I). As a consequence of these changes, a dose-dependent increase of the
290 Firmicutes/Bacteroidetes ratio is noticed (ANOVA, $p < 0.001$) (Figure 5J). Interestingly, at the genus
291 level, the bacteria *Bacteroides fragilis* from the phylum Bacteroidetes became dominant following 12
292 days of exposure to GO at 0.05 and 0.1 mg/L, reaching up to 68.3% of the whole microbial community
293 (ANOVA, $p < 0.001$). This increase of relative abundance benefited from the significant decrease of a
294 bacteria from the genera Parabacteroides, belonging to the same phylum, after exposure to GO at low
295 concentrations (Figure 5K). Such change in the relative abundance of these phyla can explain the
296 previously described decrease of the Shannon index.

297 Determination of differential taxa abundances using linear discriminant analysis effect size (LEfSe)
298 indicated that the phylum Proteobacteria was discriminant in the group of larvae exposed to GO at
299 0.05, 0.1 and 10 mg/L compared to the control group (Figure 6A-C). As for the LEfSe analysis performed
300 at T2, none of the taxa allowed to discriminate the microbiome from the control larvae to the one of
301 tadpoles exposed to GO at 1 mg/L for 12 days. However, more differentially abundant taxa were
302 determined in animals exposed to GO at 10 mg/L compared to the control group (Figure 6C). Thus, the
303 microbiome of larvae from the control group harbored a higher prevalence in bacteria from the genera
304 *Bacteroides* and *Cetobacterium* (phylum Fusobacteria), while the abundance of bacteria from the
305 family *Neisseriaceae* and *Lachnospiraceae* as well as genus *Rheinheimera* increased in larvae exposed
306 to GO at 10 mg/L (LDA score $[\log_{10}] > 4$) (Figure 6 C, D).

307 **3.5. Marked gut microbiota alterations following exposure to low GO concentrations are linked to**
308 **genotoxic effects.**

309 Exposure to low GO concentrations are associated to an increase in the relative abundance of the
310 bacteria *Bacteroides fragilis* which is becoming dominant in the gut of tadpoles exposed to GO at 0.05
311 and 0.1 mg/L (Figure 7A). Under these conditions, the micronucleus assay revealed a significant
312 increase of micronucleated erythrocytes following 12 days of exposure (Figure 7B). The absence of
313 genotoxic effects in larvae exposed to the higher GO concentrations of 1 or 10 mg/L would be due to
314 the decreased proportion of mitotic erythrocytes circulating in the tadpole bloodstream as indicated
315 by the decreased proportion of cell populations in G2 phase (Table 2), decreasing the probability of
316 micronuclei formation occurrence.

317 Interestingly, a significant linear correlation between the MNE occurrence and the relative abundance
318 of *Bacteroides fragilis* can be found (Figure 7C) (Pearson, $r = 0.701$; $p < 0.001$). However, PCR analysis
319 indicated no amplification of the targeted gene coding for the Bft from the gut microbiota samples,
320 indicating that the *B. fragilis* strain recovered are not able to produce the enterotoxin.

321 **3.6. Changes in gut microbiota structure following GO exposure predict functional alterations**

322 PiCrust2 analysis showed evidences of predicted functions (KEGG genes collapsed at level 3) that are
323 differentially abundant between the control and exposed groups, except after exposure to 1 mg/L of
324 GO which is in accordance with the previously presented results indicating minimal gut microbiota
325 modulations following exposure to this dose. Thus, LEfSe analysis identified 59, 42 and 65 pathways
326 that differentiate the predicted functional profiles of the larvae exposed respectively to GO at 0.05,
327 0.1 and 10 mg/L from the control group. Among the tested conditions, 30 differentially abundant
328 pathways compared to the control were shared following exposure to GO at 0.05 and 0.1 mg/L,
329 suggesting closely-related toxicological effects (Figure S4A).

330 Under these conditions (0.05 and 0.1 mg/L), PICRUST predicted functions indicated an increase of
331 carbohydrate metabolism involving oxidative phosphorylation while glycolysis-related pathways are

332 downregulated as indicated by the decrease of pyruvate metabolism, pentose phosphate or glycolysis
333 gluconeogenesis pathways compared to the control group (Figure S4B, C). The highest number of
334 unique altered predicted function was found following exposure to the highest GO dose (Figure S4A).
335 Under this exposure condition, the gut microbiota of exposed tadpoles exhibited upregulated
336 pathways involved into bacterial motility and chemotaxis (Figure S4D). In addition, pathways related
337 to the secretion system including ABC transporters, detoxification process involving cytochrome P450
338 and phosphotransferase system as well as naphthalene degradation are increased.

339 **4. Discussion**

340 **4.1. Effects on larval growth**

341 The bioavailability of GO, which conditions organism exposure in the environment, is influenced by its
342 behavior in aquatic ecosystems (Ren et al., 2018). Thus, monitoring of GO behavior in the exposure
343 media indicated a sedimentation of the nanomaterial over the time which was previously shown to be
344 influenced by the interactions between GO surface functions and ions from the exposure media
345 (Chowdhury et al., 2013). In the presence of larvae in the exposure media, the faster clearance of GO
346 from the water column is due to larval filtration activity, leading to accumulation of the nanomaterial
347 in the digestive tract as previously observed in amphibians (Lagier et al., 2017), zebrafish (Chen et al.,
348 2021) and daphnids (Souza et al., 2018). Such exposure to GO is associated to larval growth inhibition
349 within a similar magnitude compared to a previous study (Lagier et al., 2017). As the larvae from the
350 group exposed to the highest dose exposed to GO during 12 days reached the size of unexposed larvae
351 at day 8, the obtained results suggest a marked growth delay associated to high GO concentrations.

352 Over the metamorphosis process, the intestine of premetamorphic tadpoles consist in a simple thin
353 tube which undergoes elongation, looping and rotation events (Bloom et al., 2013), as visible in
354 unexposed larvae between T2 and T12. At the same time, gut complexity increases through crypt and
355 villi structuration and shortening at climax (from stage 60) under the action of T3 hormone (Chalmers
356 and Slack, 1998; Heimeier et al., 2010; Schreiber et al., 2005; Shi et al., 2001; Sterling et al., 2012).

357 Exposure to increasing GO concentrations did not led to the alteration of larval development as
358 indicated by the similar NF stage 57 reached following 12 days of exposure to any GO concentration.
359 While the stage 60 was not reached by any larvae at the end of the exposure, the gut shortening event
360 taking place at this stage is unlikely to occur and the observed impairment of the intestinal maturation
361 would be associated to GO exposure rather than due to a physiological process. Alteration of intestinal
362 development were previously observed in zebrafish following GO exposure with no impact on fish
363 growth (Zheng et al., 2019). However, in our study this effect is less marked compared to the larval
364 growth inhibitory effects determined based on the larval length.

365 The larval growth inhibitory effects following exposure to increasing concentrations to GO are in line
366 with previous studies performed using *X. laevis* as biological model (Lagier et al., 2017; Li et al., 2019).
367 Such effects were shown to be associated to a decreased fatty acid and triglyceride metabolism as also
368 observed in zebrafish exposed to GO, impairing their development as well as disturbing offspring
369 metabolism (Hu et al., 2017; Li et al., 2019; Zhang et al., 2017). As metabolic disturbances were
370 previously described in *X. laevis* tadpoles following exposure to GO at 1 mg/L, it is likely that the growth
371 inhibition measured in our study imply similar pathways. The tadpole energy metabolism mainly relies
372 on the gut microbiota metabolic capacities, providing energy substrates such as fatty acids for the host.
373 Among fatty acids, short-chain fatty acids (SCFAs) including acetate, butyrate or propionate are
374 metabolites produced through bacterial fermentation of dietary fibers in *X. laevis* intestine and were
375 suggested to account for up to 20% of larval daily energy requirements (Koh et al., 2016; Pryor and
376 Bjorndal, 2005; Scalvenzi et al., 2021). While energy metabolism is essential to ensure a proper larval
377 development (Scott et al., 2007; Zhu et al., 2021), it is of importance to determine if the growth
378 inhibition observed is associated to disturbances of the gut microbial communities, as many pollutants
379 were previously described to alter intestinal microbial communities in aquatic organisms, impairing
380 host fitness (Adamovsky et al., 2018; Evariste et al., 2019a; Sehnal et al., 2021).

381

382 **4.2. Exposure to GO leads to alterations of the gut microbial community structure**

383 It is now widely accepted that the host homeostasis depends on the gut microbiota health status (Lee
384 and Hase, 2014; Sehnal et al., 2021). Thus, gut microbiota alteration following exposure to
385 environmental pollution may lead to a loss of host fitness and affect the ability of a population to
386 maintain (Evariste et al., 2019a). In addition, GO was previously described as a bactericidal
387 nanomaterial and numerous studies described antibacterial activities through membrane impairment
388 due to physical interactions, resulting in bacterial viability loss (Dizaj et al., 2015; Kumar et al., 2019;
389 Tashan et al., 2019). Using the model bacteria *E. coli*, Liu and collaborators (Liu et al., 2011)
390 demonstrated a decreased viability in a dose and time-dependent manner following exposure to GO
391 at concentrations ranging from 5 to 80 µg/mL. Local intestinal accumulation of GO in the *X. laevis*
392 intestine favor direct interactions with bacteria, leading to the observed effects. However, differential
393 sensitivity to GBMs were shown depending on bacterial strains considered. Thereby, it was highlighted
394 that membrane composition or bacterial shape also influences GBMs antimicrobial properties (Al-
395 Thani et al., 2014; Sengupta et al., 2019; Tashan et al., 2019). As some studies indicated that GO may
396 act as a promoter of bacterial biofilm formation (Guo et al., 2017; Ruiz et al., 2011), it is possible that
397 the observed recovery in gut microbial density is associated to the presence of the nanomaterials in
398 the gut that benefit to more tolerant bacteria colonizing this new ecological niche. Otherwise, one
399 possible hypothesis is that GO accumulation in the intestine conduct to excretion of intestinal bacteria,
400 explaining the transient abundance decrease. We then monitored the consequences of the exposure
401 to GO on the gut microbial structures of the tadpoles.

402 The gut microbiota survey performed indicated that the tadpole intestine was mainly colonized by
403 bacteria from the phyla Bacteroidetes, Proteobacteria and Firmicutes which is consistent with other
404 studies in *Xenopus tropicalis* (Scalvenzi et al., 2021) or other vertebrates including mice or human
405 (Colombo et al., 2015). In amphibians, gut microbiota remodeling is occurring over the metamorphosis
406 process with food as one of the main driving factors (Kohl et al., 2014, 2013; Scalvenzi et al., 2021).

407 Indeed, dietary strategy changes within the metamorphosis switching from mainly herbivorous
408 tadpoles to insectivorous froglets. Thus, the adaptative changes within the gut microbial communities
409 allow the host to maintain energy intake in order to meet host metabolic requirements depending on
410 the food available in different ecological niches (Bletz et al., 2016). Under our experimental conditions,
411 tadpoles were maintained under constant abiotic parameters and artificial diets over a period which
412 is not influenced by changes of feeding behavior. Thus, the effects observed towards microbial
413 communities could be attributed to the GO exposure independently from host factors. The effects of
414 GO exposure towards the gut microbiome was previously monitored using mice models (Chen et al.,
415 2018; Li et al., 2018; Liu et al., 2021) or fish (Jia et al., 2019; Ma et al., 2016; Zheng et al., 2019), but to
416 our knowledge, none using amphibians which physiology was previously shown to be affected by GO
417 exposure (Evariste et al., 2019b; Lagier et al., 2017; Li et al., 2019).

418 The results from our study clearly indicated that short term exposure to GO (T2) initiates a shift of the
419 gut microbial communities. Longer exposure duration is associated to strong gut microbiota disruption
420 following exposure to low GO concentrations as indicated by the decrease of alpha diversity indexes
421 and increase of Bray-Curtis distances, while the gut microbiota shifted differently and to a lower extent
422 following exposure to higher GO concentrations. Nonlinear toxicological effects of nanoparticles were
423 previously reported in the literature (Bell et al., 2013). Interestingly, a study performed in mice orally
424 exposed to pristine graphene indicated that an exposure to a dose of 1 μ g/d exerted stronger
425 deleterious influence on the gut microbiota of mice compared to higher concentration of 10 or 100
426 μ g/d (Xie et al., 2016). The authors suggested that the differential effects could be associated to
427 differences of aggregation states of the nanomaterials between high and low graphene doses. Indeed,
428 it was previously described that an increased aggregation state reduces GBMs antibacterial activities
429 (Hegab et al., 2016; Liu et al., 2011). In addition, it was indicated that GBMs aggregation increases at
430 high concentration and that physicochemical parameters of the environment such as pH or ion
431 concentrations, which are also likely to fluctuate within the digestive tract microenvironment, may
432 also influence aggregation states (Gao et al., 2022; Suter and Coveney, 2021). Thus, it is likely that the

433 more marked adverse effects on microbial communities occurring in the present study would be due
434 to the differences of aggregation states in the gut of tadpoles exposed to low concentrations compared
435 to higher concentrations.

436 Despite that other physicochemical characteristics of the GBMs such as the oxidation degree or lateral
437 size dimension were previously shown to influence their antimicrobial properties (Perreault et al.,
438 2015; Zou et al., 2016), a similar trend is noticed between several studies focusing on the effects of GO
439 on the gut microbiota of other species (Jia et al., 2019; Liu et al., 2021; Zheng et al., 2019), leading to
440 a decrease of the relative abundance of bacteria from the phylum Bacteroidetes that is consistent with
441 the present results. Similar effects were also observed in various microbial consortia exposed to GBMs
442 such as in soil (Du et al., 2015), biofilms (Evariste et al., 2021a) or swine manure (Zhang et al., 2017),
443 suggesting an overall higher susceptibility of most of bacteria from this phylum to GO. In amphibians,
444 tadpole's energy metabolism during the pro-metamorphosis stages mainly relies on carbohydrate and
445 lipid metabolism which are essential to ensure metamorphosis events (Zhu et al., 2020, 2019). While
446 gut bacteria from the phylum Bacteroidetes were shown to be involved in the polysaccharides and
447 carbohydrate degradation (Gibiino et al., 2018), the decreased Bacteroidetes relative abundance in
448 the gut of exposed larvae might be associated to an impairment of these metabolic pathways as
449 previously observed in *X. laevis* tadpoles exposed to GO at concentrations up to 1 mg/L (Li et al., 2019),
450 leading to larval growth inhibition. This hypothesis could be suggested by the significant linear
451 correlation found between the larval growth rate and the relative abundance of bacteria from the
452 phylum Bacteroidetes (Pearson correlation, $r = 0.496$, $p = 0.011$).

453 It is worth to note that the exposure to GO also influence the relative abundance of gut bacteria from
454 the phylum *Fusobacteria*, leading to a decreased proportion compared to the control group following
455 exposure to GO at the highest concentration. Previous studies suggested that this phylum was involved
456 in priming the immune system during amphibian early-life stages. Indeed, a lower *Fusobacteria*
457 abundance or gut richness during early-life stages predicted an increased host susceptibility to

458 pathogens during later life at adulthood (Knutie et al., 2018, 2017). Thus, even if it is remaining to be
459 evaluated, early-life exposure to GO might be likely to have deleterious consequences for the host
460 through an alteration of its immune system maturation.

461 Concomitant with the decrease of Bacteroidetes relative abundance, an increase of the Firmicutes
462 proportion is noticed. Thus, exposure to GO led to changes in niche partitioning between these two
463 major phyla leading to an increase of the F/B ratio. An increase of Firmicutes/Bacteroidetes ratio was
464 previously observed in mice exposed to GO (Chen et al., 2018; Liu et al., 2021). These phyla were
465 suggested to possess distinct and complementary metabolic capacities within the gut microbial
466 communities and imbalance in this ratio is generally associated to metabolic disorders in humans such
467 as obesity (Ley et al., 2006a, 2006b). In mice model, the increase of F/B ratio can be induced by energy-
468 rich diet consumption, leading to disturbances of the gut metabolism through alteration of the SCFA
469 production profile which is associated to the occurrence of metabolic syndromes (Woting and Blaut,
470 2016). Consistent with this, it was observed that rice frogs, *Fejervarya limnocharis*, living in farmland
471 environments characterized by an altered diet composition exhibited a higher F/B ratio and increased
472 Proteobacteria compared to populations from natural environments benefiting from more diverse
473 food sources (Chang et al., 2016). Otherwise, exposure of *X. tropicalis* to antibiotics was indicated to
474 alter the composition of adult frog gut flora, leading to an increase in the F/B ratio without establishing
475 link with host-related endpoints (Lin et al., 2022). As Firmicutes are suggested to be involved in energy
476 harvesting (Krajmalnik-Brown et al., 2012; Ley et al., 2006b), changes in their relative abundance may
477 alter the production of gut microbial metabolites involved in multiple host metabolic pathways,
478 unbalancing energy intake and storage, affecting larval metamorphosis and later life fitness (Crespi
479 and Warne, 2013; Zhu et al., 2019). Indeed, even if the tadpoles complete their metamorphosis, size
480 at metamorphosis is correlated to a better survival of the adults and locomotive performances (Székely
481 et al., 2020).

482

483 **4.3. Genotoxic effects detected in the host do not seem to be mediated by the gut microbiota**

484 Consistent with our results, it was previously indicated that exposure to low GO concentrations induce
485 genotoxic effects in amphibians (Evariste et al., 2020b, 2019b). The later study was performed using
486 similar nanomaterials and deciphered the involvement of oxygen-containing surface functions of GO
487 in genotoxic effects towards *X. laevis*. (Evariste et al., 2019b). Importantly, the bacteria *Bacteroides*
488 *fragilis* which is becoming dominant in the gut of tadpoles exposed to GO at 0.05 and 0.1 mg/L.
489 Enterotoxigenic strains of *B. fragilis* are known to causes colitis and promote colon tumorigenesis
490 through the induction of genotoxic effects associated to the production of the bacteroides fragilis toxin
491 (bft) (Chung et al., 2018; Goodwin et al., 2011). Thus, a particular attention was paid to the occurrence
492 of micronucleated erythrocytes in tadpoles following 12 days of exposure, in order to allow micronuclei
493 formation (Fernandez et al., 1993), in connection with the increased relative abundance of *Bacteroides*
494 *fragilis*. The presence of *bft* gene in the gut of larvae exposed to the different GO concentrations was
495 evaluated using PCR (Pantosti et al., 1997) to determine if the production of toxins within the gut could
496 contribute to the occurrence of genotoxic effects in the host. However, no amplification of the targeted
497 gene coding for the Bft resulted from PCR analysis of the gut microbiota samples, indicating that the
498 *B. fragilis* strain are not able to produce the enterotoxin. Thus, even if the link between diversity loss
499 and host physiological impairment is being remaining to be understood, it is likely that the genotoxic
500 effects are mediated by oxidative stress induced by GO as previously suggested, with no involvement
501 of gut pathobionts (Evariste et al., 2019b).

502 **4.4. Functional predictions suggest that changes in gut microbiota structure are associated to** 503 **metabolic alterations**

504 To gain insight into the potential functional adverse effects of GO exposure associated to the
505 alterations of the microbial communities, functional pathways were predicted using PiCrust2 analysis
506 on 16S amplicon sequencing data. The algorithm predictions suggested that a shift in energy
507 metabolism occurred in tadpoles exposed to low GO concentrations through an increase of

508 carbohydrate metabolism involving oxidative phosphorylation while glycolysis-related pathways are
509 downregulated as indicated by the decrease of pyruvate metabolism, pentose phosphate or glycolysis
510 gluconeogenesis pathways compared to the control group. Glycolysis and oxidative phosphorylation
511 are two major metabolic pathways to provide energy. However, the oxidative phosphorylation process
512 inevitably results in the generation of free radicals that can lead to DNA damages compared to
513 glycolysis which limit oxidative damages (Brace et al., 2016). This is consistent with the previous studies
514 revealing that *X. laevis* tadpoles exposure to similar GO dose (0.1 mg/L) was leading to the induction
515 of oxidative stress and genotoxic effects (Evariste et al., 2019b).

516 On contrary, exposure to high GO doses led to different effects as indicated by the upregulation of
517 pathways involved into bacterial motility and chemotaxis. Even though we were not able to confirm
518 an increase of gut microbiota flagellin levels using human embryonic kidney (HEK)-Blue-mTLR5 cells as
519 previously described (Chassaing et al., 2014) (data not shown) probably due to graphene capacities to
520 adsorb bacterial flagellar components (Tegou et al., 2016; Zhang et al., 2020), bacterial motility
521 increase bacteria-host epithelium interactions, leading to deleterious effects for the host through the
522 promotion of pro-inflammatory effects mediated by toll-like receptors 5 (TLR5) (Miclote et al., 2020;
523 Wiles et al., 2020). In addition, increased xenobiotic detoxification capacities are suggested under this
524 condition by the increase of pathways related to secretion system including ABC transporters which
525 are involved in efflux of products across the cell membrane (Davidson and Chen, 2004) and
526 detoxification process as indicated by the increase of cytochrome P450 activity and
527 phosphotransferase system which are involved in anti-stress responses (Cryle et al., 2003; Peng et al.,
528 2017). Interestingly, the pathway related to naphthalene degradation is also increased. As
529 naphthalene-degrading bacteria isolated from graphite mine were previously shown to degrade GO
530 (Liu et al., 2015), this suggest that GO-induced selective pressure, drive a shift in the gut microbiota
531 which is associated to improved detoxification and GO degradation capacities. However, this seems to
532 be leading to decreased metabolic capacities as indicated by the decrease of glucose-related metabolic
533 pathways, amino acid metabolism, fatty acid biosynthesis which together, contribute to the host

534 energy metabolism. Such alteration of metabolic pathways were previously shown to be induced by
535 GO exposure in *X. laevis* while essential for larval development and might lead to the observed larval
536 growth inhibition (Li et al., 2019; Zhu et al., 2021). Even if the Picrust analysis display more accurate
537 results from gut microbiota data compared to other microbial compartment, there is a need to be
538 cautious with the results from our study based on non-human biological models, which shown lower
539 performances due to the intrinsic limits of the method (Sun et al., 2020). Thus, even if the obtained
540 results propose interesting pathways which might be involved in host physiological alterations, there
541 is a need to confirm these results in further studies using shotgun sequencing analysis (Ranjan et al.,
542 2016).

543 **5. Conclusion**

544 The results obtained in this study demonstrate that *X. laevis* larvae exposure to GO leads to gut
545 microbiota alterations which are linked to host physiological impairments including DNA damages and
546 growth inhibition. Based on the obtained results and the data available from the literature, the larval
547 growth impairment associated to gut microbiota structure disruption constitute two risk factors which
548 could conduct to later-life host fitness loss through potentially decreased survival of individuals,
549 impairment of immune performances or decrease of thermal tolerance. Thus, even if this is remaining
550 to be determined in future study, the marked effects of GO towards gut microbial communities, even
551 at environmentally relevant concentrations could be deleterious for amphibian population dynamic.
552 Overall, this study highlights that the monitoring of the gut microbiota status in response to GBMs
553 constitute a good integrative marker of the overall host physiological alterations.

554 **Declaration of competing interest**

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

557

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991

Table 1: Physico-chemical characteristics of GO used for the experiment. at. %: atomic %; TEM: transmission electron microscope; HRTEM: high resolution TEM; BET: Brunauer-Emett-Teller.

Graphene Oxide	
Carbon content	69.1 ± 0.4 at. %
Oxygen content	31.1 ± 0.4 at. %
Number of layers (HRTEM)	1–5
Lateral size (TEM)	0.2 to 8 μm
Specific surface area (BET)	228 ± 6.8 $\text{m}^2\cdot\text{g}^{-1}$

Table 2: *X. laevis* erythrocyte cell cycle distribution analyzed using flow cytometry after 12 days of exposure to increasing GO concentrations. Letters indicate significant differences between groups.

	GO/G1 (% cells)	S (% cells)	G2/M (% cells)
Control	77.69 ± 0.86 A	11.11 ± 0.27 AB	11,2 ± 0.86 A
0.05 mg/L	84.72 ± 0.83 B	6.49 ± 0.15 C	8.79 ± 0.80 AB
0.1 mg/L	80.02 ± 0.45 C	10.43 ± 0.35 B	9.55 ± 0.32 AB
1 mg/L	83.92 ± 0.42 B	8.60 ± 0.25 D	7.47 ± 0.33 B
10 mg/L	79.81 ± 0.56 AC	11.57 ± 0.25 A	8.62 ± 0.48 B

Figure 1: Monitoring of the GO dispersion stability at 10 mg/L in the exposure media. Calibration plot based on optical density at 235 nm of increasing GO concentrations in exposure media (A). Dispersion stability kinetic in presence and absence of *X. laevis* tadpoles in the media (B). Area under the curve (AUC) in arbitrary unit was used to evaluate statistical differences of dispersion stability. *** indicate significant differences following T-test when p-value < 0.001.

Figure 2: Effects of GO exposure on *X. laevis* tadpole growth and intestinal developments. Larval growth rate monitored over the 12 days of exposure (A). Normalized growth rate determined after 2 (B) or 12 (C) days of exposure to increasing GO concentrations. Pictures of GO intestinal accumulation after 2 or 12 days of exposure (D). Intestinal weight measured after 2 (E) or 12 days of exposure. Letters indicate significant differences between concentrations determined by the post-hoc Tukey test when ANOVA p-value < 0.05.

Figure 3: Effects of an exposure to increasing GO concentrations on the abundance of intestinal bacteria as detected by qPCR. Evolution of intestinal bacterial load in the control group over time (A). Gut bacteria abundance measured after 2 (B) or 12 (C) days of exposure to increasing GO concentrations. Differences across groups were tested using ANOVA followed by post-hoc Tukey test when p-value < 0.05. Letters indicate significant differences between groups.

Figure 4: Effects of two days of exposure to increasing GO concentration on *X. laevis* tadpole's gut microbiota structure. Gut Microbiota richness (A) and evenness (B). PCoA plot of the bacterial communities based on Bray-Curtis dissimilarities (C). Comparison of the Bray-Curtis distances among experimental groups (D). Relative abundances of the phyla composing the gut microbiota of control or exposed larvae (E), including Bacteroidetes (F), Firmicutes (G), Proteobacteria (H) and Fusobacteria (I).

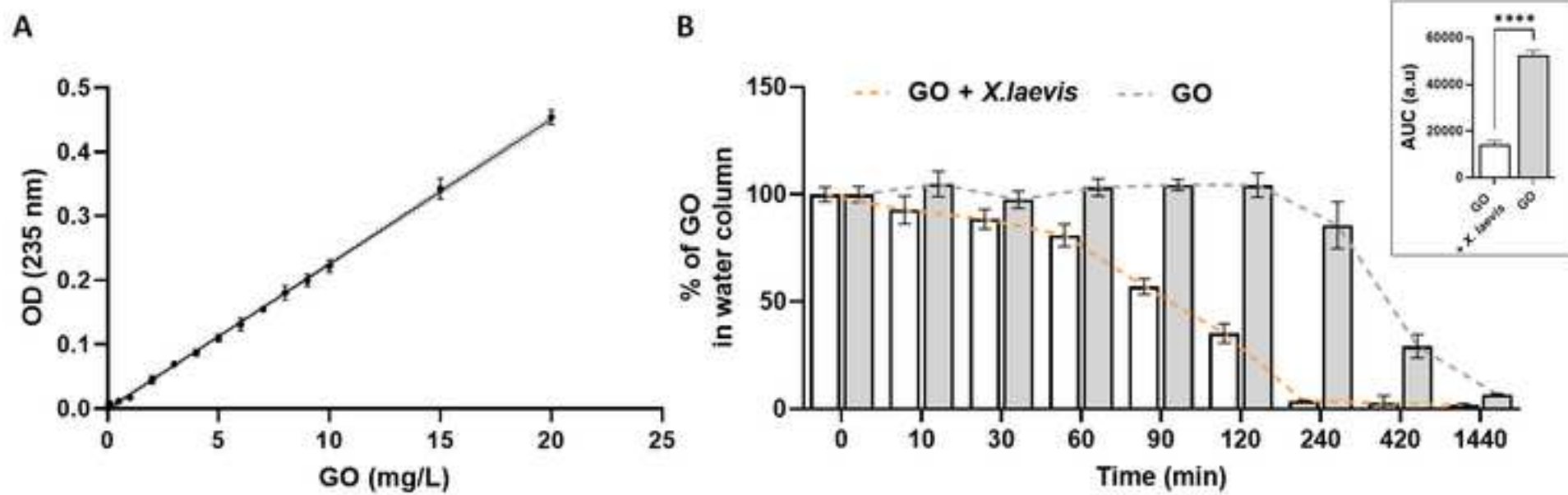
Differences across groups were tested using ANOVA followed by post-hoc Tukey test when p-value < 0.05. Letters indicate significant differences between groups (n = 5 per group).

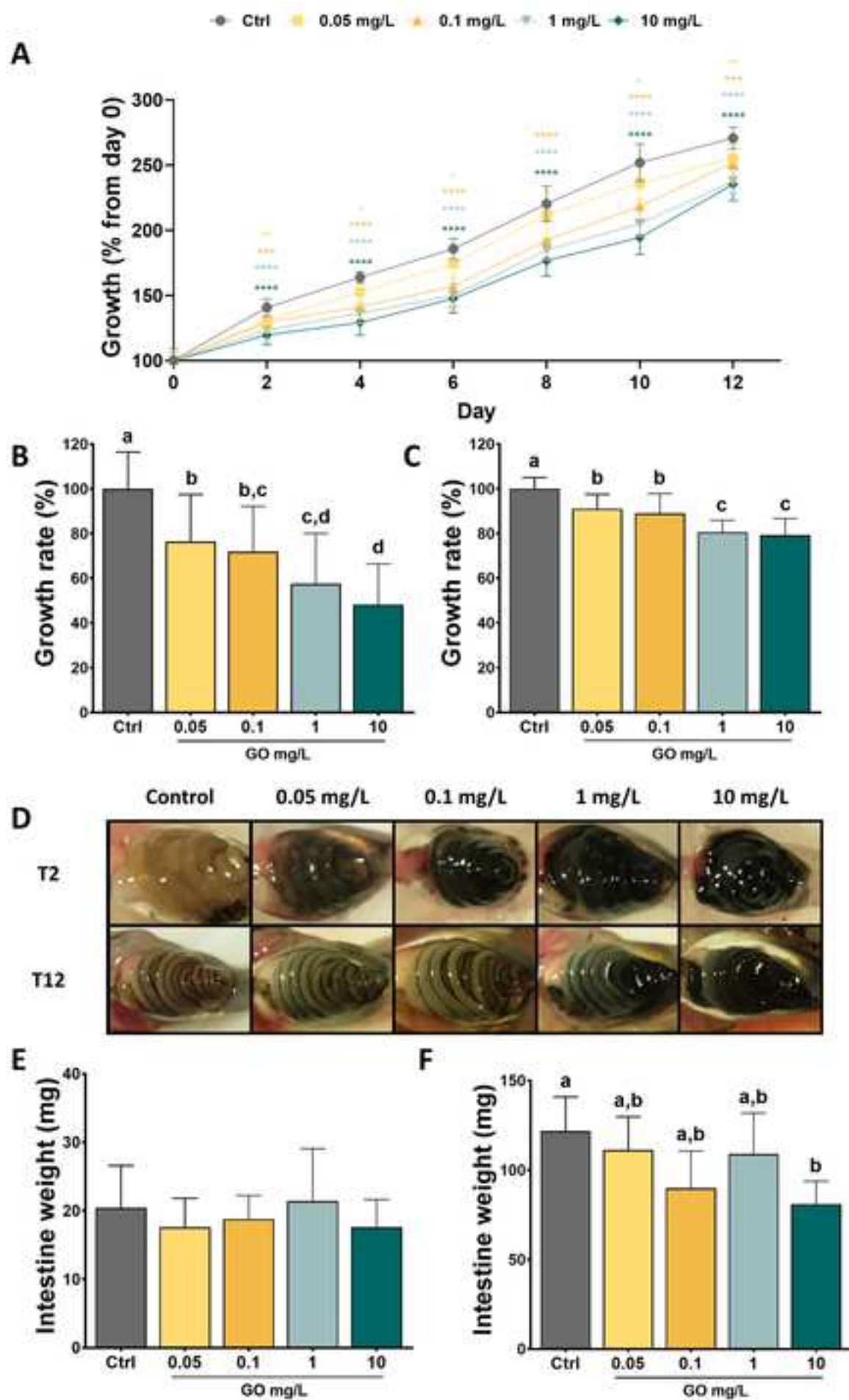
Figure 5: Effects of 12 days of exposure to increasing GO towards gut bacterial communities of *X. laevis* tadpoles. Effects on alpha-diversity parameters measured using Richness (A) and Shannon Index (B). PCoA plot of the bacterial communities based on Bray-Curtis dissimilarities (C). Comparison of the Bray-Curtis distances among experimental groups (D). Effects of GO exposure on the main phyla relative abundances (E), including Bacteroidetes (F), Firmicutes (G), Proteobacteria (H) and Fusobacteria (I) and resulting Firmicutes/Bacteroidetes ratio (J). Relative abundances of the bacteria *Bacteroides fragilis* and bacteria from the genus Parabacteroides are represented based on the results from the LEfSe analysis (K). Differences across groups were tested using ANOVA followed by post-hoc Tukey test when p-value < 0.05. Letters indicate significant differences between groups (n = 5 per group).

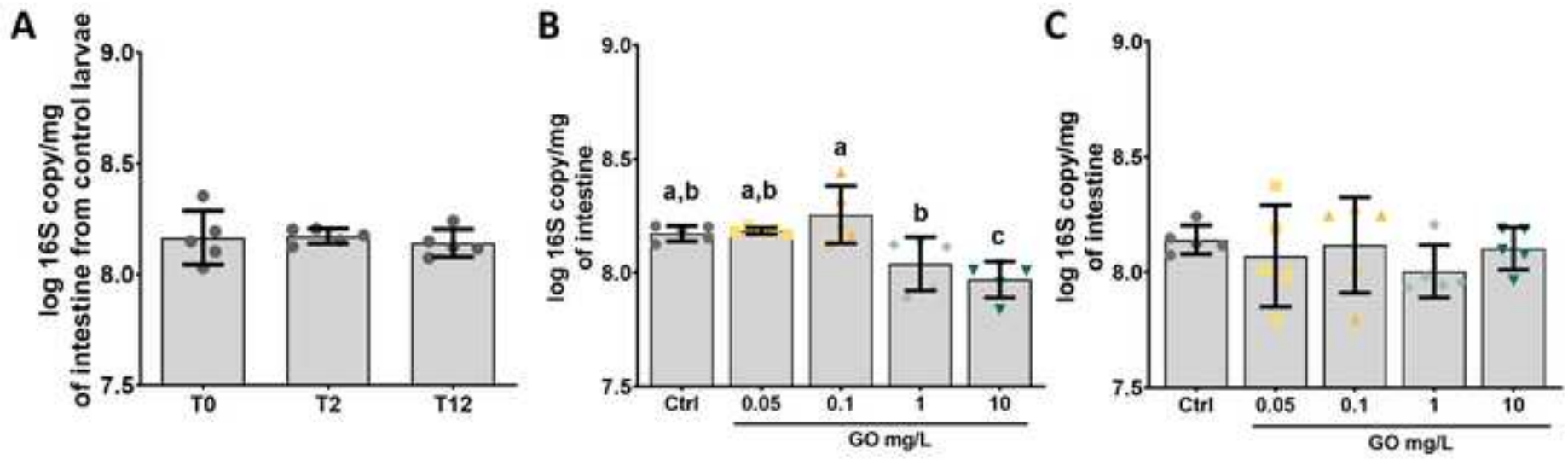
Figure 6: Determination of taxa differentially abundant in the gut microbiome of larvae exposed during 12 days to GO compared to the control group characterized by LEfSe analysis. LDA score of taxa differentially abundant in larvae exposed to GO at 0.05 mg/L (A), 0.1 mg/L (B) and 10 mg/L (C). Cladogram representation of results from the LEfSe analysis following exposure to GO at 10 mg/L compared to the control.

Figure 7: Relation between gut microbiota alterations following 12 days of exposure to increasing GO concentration and genotoxic effects towards the host. Relative abundance of the bacteria *Bacteroides fragilis* in the gut at T12 (A). Micronucleated cells accounted in the host circulating erythrocytes (B). Correlation between micronucleus frequency and *B. fragilis* relative abundance (C). Letters indicate

significant differences between groups using post-hoc Tukey test. * indicate significant differences compared to the control group.







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