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# Tritiated thymidine induces developmental delay, oxidative stress and gene overexpression in developing zebrafish (*Danio rerio*)

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1 **Title: Tritiated thymidine induces developmental delay, oxidative stress and gene**  
2 **overexpression in developing zebrafish (*Danio rerio*)**

3  
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18  
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20 **rhythm, genic expression, TaqMan**

22       **Abstract**

23           Tritium is a beta emitter radionuclide. Being an isotope of hydrogen, it is easily  
24 transferred to different environmental compartments, and to human and non-human biota.  
25 Considering that tritium levels are expected to rise in the upcoming decades with the  
26 development of nuclear facilities producing tritium using fission processes, investigating  
27 the potential toxicity of tritium to human and non-human biota is necessary. Tritiated  
28 thymidine, an organic form of tritium, has been used in this study to assess its toxicity on  
29 fish embryo development. Zebrafish embryos (3.5 hpf; hours post fertilization) have been  
30 exposed to tritiated thymidine at three different activity concentrations (7.5; 40; 110  
31 kBq/mL) for four days. These experiments highlighted that zebrafish development was  
32 affected by the exposure to organic tritium, with smaller larvae at 3 dpf after exposure to  
33 the two lowest dose rates (22 and 170  $\mu$ Gy/h), a delayed hatching after exposure to the two  
34 highest dose rates (170 and 470  $\mu$ Gy/h), an increase in the spontaneous tail movement (1  
35 dpf) and a decrease in the heartbeat (3 dpf) after exposure to the highest dose rate. The  
36 results also highlighted an increase in ROS production in larvae exposed to the intermediate  
37 dose rate. A dysregulation of many genes, involved in apoptosis, DNA repair or oxidative  
38 stress, was also found after 1 day of exposure to the lowest tritium dose rate. Our results  
39 thus suggest that exposure to tritiated thymidine from a dose rate as low as 22  $\mu$ Gy/h can  
40 lead to sublethal effects, with an effect on the development, dysregulation of many genes  
41 and increase of the ROS production. This paper provides valuable information on toxic  
42 effects arising from the exposure of fish to an organic form of tritium, which was the main  
43 objective of this study.

44

45 **1. Introduction**

46 Tritium is a radioactive isotope of hydrogen<sup>1</sup>. In the environment, it comes from  
47 different sources: the interaction between the cosmic rays and atmospheric oxygen and  
48 nitrogen atoms is the only natural source of tritium, while there are more types of  
49 anthropogenic releases, including nuclear power plants, nuclear reactors, but also nuclear  
50 incidents or weapon testing<sup>1,2</sup>. Despite those numerous anthropogenic sources of tritium,  
51 those releases are still being closely monitored, those monitoring mostly focusing on the  
52 activity concentrations in the different environmental compartments. In France, for  
53 example, in non-nuclearized aquatic ecosystems, tritium levels range from 3 to 4 Bq/L,  
54 while in nuclearized areas, those levels rise and reach 2.5 to 12.85 Bq/L, with a mean value  
55 of 6.31 Bq/L<sup>3</sup>. In the environment, tritium can be found in three different forms: free in the  
56 water (HTO), atmospheric tritium (HT) or organically bound tritium (OBT)<sup>1</sup>. This last form  
57 can be separated into two different ones: the exchangeable OBT (eOBT), where tritium is  
58 weakly bound to oxygen, sulphur or nitrogen atoms, and the non-exchangeable OBT  
59 (neOBT), where tritium is strongly bound to a carbon atom<sup>3</sup>. Numerous studies have shown  
60 that tritium can be transferred to biota, accumulate in living tissues and, depending on its  
61 chemical form (free or organic), can be incorporated in the DNA during its synthesis<sup>4-10</sup>.  
62 This ability made the study of its possible toxic effects even more relevant. Considering  
63 that radiations emitted by tritium have a low penetrating power, the only exposure source  
64 considered in this study is the internal exposure (*i.e.*, the exposure to the tritium that has  
65 been translocated in the embryos and larvae).

66 As for many other contaminants, tritium effects on early development stages have been  
67 studied on numerous species. For example, it was shown that tritium induced a delay in  
68 hatching of zebrafish embryos (*Danio rerio*), no matter what chemical form was used<sup>11,12</sup>.  
69 Literature also reports that tritium can induce a delay in development in species like

70 zebrafish, blue mussel (*Mytilus edulis*) and grass puffer (*Fugu niphobles*), while it seems  
71 to have the opposite effect on the prussian carp (*Carrasius gibelis*)<sup>3,10,13</sup>. While affecting  
72 the macroscopic development, it seems that it can also affect microscopic and molecular  
73 processes. For example, a study showed that the spontaneous tail movement in 24 hpf  
74 zebrafish embryos decreased after an exposure to HTO at 3.7 kBq/mL, while the same  
75 exposure increased the heartbeat of the individuals at 60 and 120 hpf<sup>12</sup>.

76 Other studies also highlighted that exposure to tritium leads to an increase in the  
77 reactive oxygen species (ROS) in bacterial cells, fathead minnow (*Pimephales promelas*)  
78 and zebrafish, while the GPx activity also increased with the tritium dose rate in fathead  
79 minnows<sup>7,10,14</sup>. Finally, it was shown that genes involved in detoxication and neuro-  
80 transmission in zebrafish were affected after exposure to tritium, but only in a transient  
81 way, while a RNA sequencing study carried out on zebrafish embryos and larvae showed  
82 that many genes were either over- or under-expressed after exposure<sup>11,15</sup>. Genes involved  
83 in somite differentiation, oxidative reduction processes and peripheral nervous system  
84 axonogenesis were down-regulated. In contrast, genes involved in the response to oxidative  
85 stress, circadian rhythm, response to light stimulus, and regulation of DNA methylation  
86 were up-regulated<sup>15</sup>. Genes involved in processes linked to the muscle contraction or the  
87 sarcomere assembly were up- and down-regulated depending on both the developmental  
88 stage and the dose rate used for the exposure. Thus, when studying the toxic effects of  
89 various compounds on living organisms, multiple biological scales should be used, as it  
90 could give more information on the possible modes of action of the contaminant to induce  
91 toxicity<sup>3,16,17</sup>.

92 Even though all those studies give multiple information on HTO toxic effects on  
93 various aquatic species, very few studies focused on the toxic effects that could arise from  
94 an exposure to organic tritium, which is the main objective of our study<sup>8,18</sup>. Another

95 objective was to determine and confirm toxic mechanisms specific to tritium and, more  
96 generally, radiations, by comparing the toxicity induced by tritiated thymidine to the  
97 toxicity induced by HTO or gamma irradiation. To do so, three tritiated thymidine dose  
98 rates were used (22, 170 and 470  $\mu\text{Gy/h}$ ). Zebrafish is used as a biological model because  
99 it is a convenient species, with adults being able to lay hundreds of eggs, but also because  
100 these eggs are transparent, easy to handle, and because its genome is fully sequenced<sup>19,20</sup>.  
101 Early development stages were also chosen as it is well known that those are usually  
102 considered as being more sensitive to most contaminants, but also because is it a commonly  
103 used model in toxicological studies<sup>3,10,11,15,21-26</sup>. Zebrafish embryos were exposed three  
104 activity concentrations (7.5; 40; 110 kBq/mL) to study toxicological effects than can be  
105 classified in three different categories depending on the biological scale concerned. First,  
106 the macroscopic part of the study will focus on the development of the larvae, while a  
107 second part of the study will focus on toxicity markers as the spontaneous tail movement  
108 and the heartbeat. Finally, molecular studies will focus on the ROS production index and  
109 the expression of 30 different genes. The selected genes are involved in multiple biological  
110 processes such as DNA repair, apoptosis, oxidative stress regulation and eye development.  
111 As multiple effects have already been observed after the exposure of fish to different  
112 radionuclides, with for example the deregulation of various genes involved in the response  
113 to oxidative stress and DNA damage, but also with disruption of the development, the main  
114 hypothesis behind this study is the induction of disruptions by tritiated thymidine on the  
115 same biological targets, but with different severities.

116

## 117 **2. Material and methods**

### 118 **2.1. Fish**

119 All experimentations presented in this paper were previously approved by IRSN  
120 Animal Care Committee and followed French regulations for animal experimentation  
121 (protocol P2019-19; registration number of IRSN laboratory: C13-013-07). Three to four  
122 months old wild-type zebrafish (AB genetic background) were supplied by Paris Sorbonne  
123 University (France) and were kept in a zebrafish housing system (Zebtec Techniplast®  
124 Stand Alone). The light:dark photoperiod chosen was 12h:12h and fishes were fed three  
125 times a day with Gemma Wean 0.3 (Sketting). All water parameters were monitored daily  
126 (pH  $7.5 \pm 0.2$ ; conductivity  $450 \pm 50 \mu\text{S}\cdot\text{cm}^{-1}$ , temperature  $27.5 \pm 0.5 \text{ }^\circ\text{C}$ ). As males and  
127 females were kept in separated tanks to increase the effectiveness of the mating process,  
128 the mating was also done once every three weeks during the periods during which no  
129 experimental procedures were planned. All matings were done in the husbandry using the  
130 water from the housing system and using fishes that were between 4 and 13 months old.

131

## 132 **2.2. Embryo exposure and tritium contamination**

133 Embryo medium (pH 7.5, conductivity  $500 \mu\text{S}/\text{cm}$  in Milli-Q water) was prepared  
134 using InstantOcean® sea salt at a concentration of 60 mg/L and was used to dilute the  
135 tritiated thymidine solution and as a negative control<sup>27</sup>. Tritiated thymidine solutions (97  
136 % purity, 185 MBq, PerkinElmer, Courtaboeuf, France) were prepared in three different  
137 activity concentrations (7.5; 40 and 110 kBq/mL), chosen according to a previous study in  
138 order to obtain 3 different internal dose rates: 22; 170; 470  $\mu\text{Gy}/\text{h}^5$ . As multiple  
139 experiments were necessary to study the effects of tritiated thymidine at one dose rate, a  
140 10 % variability was accepted in the initial measured activity concentration between each  
141 experiment.

142 All animals were exposed to tritiated thymidine as presented elsewhere<sup>5</sup>. Briefly, at  
143 3.5 hpf, zebrafish embryos were placed in two to three glass crystallizers covered with

144 Parafilm at a density of two eggs per mL of working solution for four days. Each  
145 crystallizer was considered as an exposure replicate. The embryos were kept in the dark in  
146 thermo-regulated incubators (Heratherm, ThermoScientific) at  $27 \pm 1$  °C and 80 % of the  
147 working solution was replaced after 48 hours of exposure. Temperature (°C) and relative  
148 humidity (%) were monitored every 30 minutes during the exposure (HOBO data logger  
149 U12, Onset), and beakers were filled with tritium-free water (Abatilles water, France, 40  
150 mL) to monitor tritium exchanges in the incubator.

151 For both development and hatching studies, two 25-well plastic plates were added in  
152 the incubators. In the development plate, 4 wells were filled with 5 embryos each in 2.5  
153 mL of working solution, while the wells that did not contain any embryos were kept empty.  
154 In the hatching plate, 8 wells were also filled with the same amount of embryos and  
155 working solution. Both plates were covered with Parafilm® to avoid any significant  
156 evaporation of the medium.

157

### 158 **2.3. Tritium activity concentration measurements**

159 In order to monitor the evaporation of the medium from both the crystallizers and the  
160 plates, tritium activity concentration was measured in the beakers (1 replicate/crystallizer,  
161 once a day) and in the crystallizers (3 replicates/crystallizer, after 0; 24; 48 and 96 hours  
162 of exposure). The sampling and the measurement was adapted from Schiano Di Lombo et  
163 al.<sup>5</sup>. The water sampling, differing depending on the activity concentration used in the  
164 experiment, is presented in Table 1. All 1 mL samples were diluted in 19 mL of the liquid  
165 scintillation cocktail (Ultima Gold LLT, PerkinElmer). The liquid scintillation  
166 measurements were done using a Quantulus (Quantulus 1220, Perkin Elmer) monitored by  
167 WinQ (1220-307 WinQ, Perkin Elmer). Each measurement was done five times and lasted  
168 75 minutes.



169

## 170 **2.4. Monitoring of the embryo-larval development**

### 171 **2.4.1. Hatching and morphometric measurements**

172 After 24, 48, 50, 52.5, 69, 71, 73, 75, 77, 93 and 96 hours of exposure, embryos and  
173 larvae were counted in each well of the plate. Hatching time 50 % (HT50, time at which  
174 50 % of the individuals have hatched for a same replicate) has been calculated using  
175 REGTOX® (n = 500 simulations, <http://www.normalesup.org/~vindimian/fr.index.html>)  
176 and was used in the statistical analyses.

177 Morphometric measurements were made by following the procedure already  
178 presented<sup>28</sup>. Briefly, 1 to 4 dpf individuals were pictured every day at the same hour using  
179 a camera (Nikon D5000) connected to a binocular microscope (ZEISS SteREO Discovery  
180 V20, magnification 93x). Pictures were first analysed to qualify if the individual was  
181 normal or malformed, and the egg and yolk sac diameters (1 to 2 dpf) were measured using  
182 ImageJ, as well as the total length of the larvae, the yolk sac and the eye diameters (3-4  
183 dpf).

184

### 185 **2.4.2. Spontaneous tail movements of 1 dpf embryos**

186 The spontaneous tail movement was measured in 1 dpf embryos using a protocol  
187 adapted from the one presented by Murat et al. in 2019<sup>29</sup>. Six pools of 10 embryos per  
188 condition were used and, in a room set at 28 °C, 10 embryos were immobilized in 2 % w/v  
189 methylcellulose in PBS and set under the light on a stereomicroscope (Nikon SMZ800)  
190 connected to a high-resolution camera (acA1300-60gm, Basler, Germany). After two min  
191 of acclimatation, a 5 min-video was recorded using EthoVision XT16 (Noldus,  
192 Netherlands). The burst activity, the burst duration, and the burst count (%) was determined  
193 using DanioScope (Noldus, Germany).

194

### 195 **2.4.3. Heart rate of 3 dpf larva**

196 Measurement of the heart rate was measured in 3 dpf larvae using a protocol adapted  
197 from Murat et al.<sup>29</sup>. Six pools of 5 larvae per condition were used. In a room set at 28 °C,  
198 5 larvae were immobilized in 2 % w/v methylcellulose with 0.02 % MS-222 (Sigma-  
199 Aldrich, France) in a 55 mm petri dish. After an acclimatation period of 2 min under the  
200 light on the stereomicroscope (Nikon SMZ800) connected to the high-resolution camera  
201 (acA1300-60gm, Basler, Germany), a 25 seconds video was recorded using EthoVision  
202 XT16 (Noldus, Germany). Heart rate (BPM) was measured using DanioScope (Noldus,  
203 Germany).

204

## 205 **2.5. Gene expression analysis**

### 206 **2.5.1. RNA extraction and quantification**

207 RNAs from the samples were extracted following the protocol published previously<sup>29</sup>.  
208 Briefly, 3 pools of 25 to 30 embryos (1 dpf) and 3 pools of 15 larvae (4 dpf) were grinded  
209 using 300 µL of Trizol in ceramic beads tubes (CK14 tubes, Bertin Instruments, France)  
210 with the Precellys 24 grinding machine (Bertin Instruments, France). After  
211 homogenisation, all samples were stored at – 80 °C. For RNA extraction, samples were  
212 thawed, and all sample handling and centrifugations were done at 4 °C. Chloroform (60  
213 µL) was added to the homogenized samples in Trizol, followed by vortexing and  
214 centrifugation at 13000g for 15 min at 4 °C. The upper aqueous phase was collected in a  
215 new tube and 60 µL of chloroform was added before centrifuging in the same conditions  
216 as before. The upper aqueous phase was collected in a new tube and 150 µL of isopropyl  
217 alcohol was added before the samples were vortexed and stored at – 80 °C for 30 min. The  
218 samples were then centrifuged during 30 min at 13000 g. The supernatant was removed,

219 and the RNA sample was washed using 150  $\mu$ L of ethanol 80 % (v/v) by centrifuging the  
220 sample for 5 min at 13000 g. The supernatant was then removed and once the RNA sample  
221 had been air-dried, it was diluted in 15  $\mu$ L of RNase and DNase free water. All RNA  
222 concentrations (ng/ $\mu$ L) and quality (RNA Integrity Number, RIN) were assessed using  
223 RNA Nano Chips (BioAnalyzer 2011, Agilent Technologies). All samples were used as all  
224 RINs were above 9.

225

### 226 **2.5.2. cDNA synthesis and preamplification and TaqMan assay**

227 Reverse transcription was done on 1  $\mu$ g of extracted RNA using the High Capacity  
228 cDNA Reverse Transcription Kit (Applied Biosystems), following the manufacturer's  
229 instructions. After the reverse transcription was completed, 100 of cDNA were used for the  
230 rest of the experiment. The cDNA samples were preamplified using Custom TaqMan  
231 PreAmp Pool and TaqMan PreAmp Master Mix (2X; Applied Biosystems) while following  
232 the manufacturer's instructions. Gene expression was quantified using the preamplified  
233 cDNA samples in a 384-well microfluidic TaqMan Array Card in which the expression of  
234 31 genes was measured for 4 samples with 3 analytical replicates (32 format). The array  
235 cards were processed in the QuantStudio 7 Flex Real-Time PCR System (Applied  
236 Biosystems). All Ct results were combined on the ExpressionSuite Software for  
237 QuantStudio 6 & 7 Flex Real Time PCR System and exported to DataAssist v3.01 (Applied  
238 Biosystems) for analysis while using the 18S RNA gene expression as an endogenous  
239 control. Ct values were used to obtain  $\Delta$ Ct values, each value corresponding to the gene  
240 expression of each biological replicate, normalized using the reference gene.  $\Delta$ Ct were then  
241 used to calculate  $2^{(-\Delta Ct)}$ , which was in turn used to determine the fold change. All genes  
242 studied in this experiment are presented in Supplementary Information (Table S1).

243

## 244 **2.6. Oxidative stress measurements**

245 Oxidative stress was studied by measuring ROS production. The protocol used was  
246 the one presented elsewhere<sup>30</sup>. Briefly, for each condition, 20 larvae were sampled after 96  
247 hours of exposure. All larvae were individually placed in 96-well plates. All of them  
248 received 100  $\mu$ L of HBSS buffer (ThermoFisher) and 50  $\mu$ L H<sub>2</sub>DCFDA (2 mg/L in DMSO  
249 0.4 %). The first ten larvae received 50  $\mu$ L DMSO at 0.4 % while the other ten larvae  
250 received 50  $\mu$ L Phorbol myristate acetate (PMA, 0.8 mg/L in DMSO 0.4 %). Fluorescence  
251 emitted by the reaction between H<sub>2</sub>DCFDA and ROS was measured over 300 min (TECAN  
252 Infinite M1000). Basal and stimulated levels were calculated on the linear part of the curves  
253 for larvae incubated with DMSO and PMA, respectively. ROS production index was  
254 calculated as the ratio PMA/DMSO.

255

## 256 **2.7. Statistical analyses**

257 All statistical analyses and figures were made in R environment using RStudio using  
258 the *PMCMR*, *PMCMRplus*, *car*, *lmtest*, *scales*, *SciViews*, *ggplot2*, *ggpubr*, *ggpmisc*, *plotly*,  
259 *plot3D*, *FSA*, *rcompanion*, *multcomp*, *drc* and *lme4* packages. The threshold chosen for  
260 significant results was 0.05.

261 For all development and oxidative stress analyses, linear mixed models  
262 (GLMM/LMM) were used. The treatment was set as the fixed variable, while variables as  
263 the crystallizer was used as a random variable. In more specific experiments as the  
264 measurement of the spontaneous tail movement and the heartbeat, the trial was also added  
265 as a random variable, which was also the case for the larval position when performing  
266 statistical analyses on the larval yolk measurement. For other developmental and hatching  
267 analyses, basic GLM were used as there was no variables identified as random. When the  
268 normality of the residuals and the homogeneity of the variance were not met ( $p < 0.05$ ),

269 data was transformed using either Log10, square root or boxcox transformations. The  
270 treatment effect was then checked and, when necessary, the Tukey-HSD post-hoc test was  
271 used. For the gene expression data, all analyses were made using Data Assist 3.01, where  
272 Ct were used to obtain the fold change by calculating  $\Delta\text{Ct}$  and  $2^{(-\Delta\text{Ct})}$ . All  $\Delta\text{Ct}$  were  
273 compared to the control values with the use of a t-test, and all p-values were adjusted using  
274 the Benjamini-Hochberg False Discovery Rate.

275 Except for the gene expression, all results have been represented as boxplots.  
276 Regarding the horizontal lines, the lowest one represents the first quartile, while the middle  
277 one represents the median value, and the highest one represents the third quartile. When  
278 data points are outside of the boxplot lines, they are considered as being outliers, but were  
279 not removed from the statistical analysis when there was no experimental reason to do so.  
280 In the boxplots, the dots correspond the data points obtained during the experiments. When  
281 different graphs are presented for the same analysis, it means the results come from  
282 different experiments, and different batches of embryos.

283

### 284 **3. Results**

285 All exposure experimentations led to cumulative mortality rates below 20 % in the  
286 control condition. Exposure to tritiated thymidine at all dose rates tested did not induce any  
287 significant induction of mortality (data not shown).

288

#### 289 **3.1. Hatching**

290 The hatching results are presented in Figure 1. Even if a complete hatching was  
291 observed after 96 hours of exposure, the hatching was delayed when the larvae were  
292 exposed to the intermediate and the greater tritiated thymidine activity concentrations  
293 ( $4 \times 10^4$  and  $1 \times 10^5$  Bq/mL; 170 and 470  $\mu\text{Gy/h}$ ) when compared to the control ( $p = 0.02$  and

294 p < 0.01, respectively). Even if the larvae exposed to the lowest activity concentration  
295 ( $7.5 \times 10^3$  Bq/mL; 22  $\mu$ Gy/h) did not show a significantly longer hatching time compared to  
296 controls, it also did not show any significant differences with the hatching time of the larvae  
297 exposed to the intermediate activity concentration.

298

## 299 **3.2. Developmental defects**

### 300 **3.2.1. Development measurements**

301 The exposure of the embryos and larvae to tritiated thymidine at all dose rates tested  
302 did not induce any skeletal malformations, nor peri-cardiac or peri-vitelline oedema (data  
303 not shown).

304 The egg and yolk sac diameters measured on controls and embryos exposed to 22  
305  $\mu$ Gy/h and 170  $\mu$ Gy/h for 1 and 2 days are presented in Table 2, alongside the yolk sac and  
306 eye diameters and total length of the larvae exposed to those same dose rates for 3 and 4  
307 days. First, it is seen that the embryos exposed to the lowest activity concentration for 1  
308 day had a larger yolk sac than the control embryos ( $p = 0.02$ ). This effect was only transient  
309 as it is not observed after 4 days of exposure. Most of the morphometric differences were  
310 observed for the 3 dpf larvae. Indeed, larvae exposed to both the lowest and the  
311 intermediate activity concentrations were shorter than the control larvae ( $p = 0.03$  and  $p <$   
312  $0.001$ , respectively). The yolk sac and eye diameters were also smaller for the larvae  
313 exposed to the intermediate activity concentration when compared to the control ( $p = 0.006$   
314 and  $0.004$ , respectively). All those differences were only temporary as no significant  
315 differences were observed for the 4 days old larvae. Through the whole experiment, no  
316 significant differences were observed between both thymidine activity dose rates (22 and  
317 170  $\mu$ Gy/h).

318           The egg and yolk sac diameters measured on embryos exposed to 0 and 470  $\mu\text{Gy/h}$  for  
319           1 and 2 days are presented in Table 3, alongside the yolk sac and eye diameter and lengths  
320           of the larvae exposed to those same dose rates for 3 and 4 days. When larvae were exposed  
321           to tritiated thymidine at this high dose rate, no significant morphometric changes were  
322           observed ( $p > 0.05$ ).

323

### 324           **3.2.2. Spontaneous tail movement**

325           After exposure to the two lowest activity concentrations (22  $\mu\text{Gy/h}$  and 170  $\mu\text{Gy/h}$ ),  
326           no significant changes in the spontaneous tail movement were observed compared to the  
327           control individuals (data not shown). After exposure to the highest activity concentration  
328           (470  $\mu\text{Gy/h}$ , Figure 2), a significant increase was observed in the burst activity, the total  
329           burst duration, the burst count, and the burst count per minute. The mean burst duration  
330           was the only spontaneous tail movement measurement that was not affected by the  
331           exposure to tritiated thymidine (data not shown).

332

### 333           **3.2.3. Heart rate**

334           Heart rate was measured in 3 dpf larvae exposed to 0, 170  $\mu\text{Gy/h}$  and 470  $\mu\text{Gy/h}$  of  
335           tritiated thymidine (Figure 3). After exposure to tritiated thymidine at the lowest dose rate  
336           (22  $\mu\text{Gy/h}$ , data not shown), no significant change was observed in the heart rate compared  
337           to the control. After exposure to the intermediate dose rate (170  $\mu\text{Gy/h}$ ), there were no  
338           significant changes in the heart rate, but a marginal increase was still observed ( $p = 0.07$ ).  
339           Finally, after exposure to the highest dose rate (470  $\mu\text{Gy/h}$ ), a significant decrease in the  
340           heart rate was observed ( $p < 0.01$ ).

341

## 342           **3.3. Gene expression analysis**

343 The gene expression analysis results are presented in Figure 4. The number of  
344 dysregulated genes decreased when the tritiated thymidine dose rate increased. When  
345 embryos were exposed to the highest dose rate of tritiated thymidine (470  $\mu$ Gy/h), the  
346 changes in gene expression were not significant. At 170  $\mu$ Gy/h, a marginal of over-  
347 expression was observed for *casp9*, *cyp1a* and *sod2* ( $p < 0.1$ , 1.5 to 2.5-fold increase).  
348 Finally, embryos exposed to the lowest dose rate (22  $\mu$ Gy/h) showed genes that were  
349 dysregulated, with all genes being overexpressed. Genes as *aanat2*, *baxa*, *bbc3*, *bcl2*,  
350 *cryba*, *ddb2*, *h2ax*, *mmp13*, *mpx* and *xpc* showed a marginal overexpression ( $p < 0.1$ , fold  
351 changes varying between 1.4 and 4.4), while genes as *ache*, *casp9*, *crx*, *cry5*, *cyp1a*, *efl1a*,  
352 *gstp1*, *hspb9*, *lyz*, *saga*, *sod2*, *tbpl1* and *xrcc1* were all significantly overexpressed ( $p <$   
353  $0.05$ , fold changes between 1.4 and 4.4). Other genes as *atp1a11*, *efl1a2*, *gadd45bb*, *oxt*,  
354 *per2* and *tnni2b.2* were also studied, but no significant variations were observed ( $p > 0.1$ ,  
355 data not shown).

356 The same genes were also studied after 4 days of exposure, but no significant  
357 dysregulation was observed in the gene expression, for all three dose rates ( $p > 0.1$ , data  
358 not shown).

359

#### 360 **3.4. Oxidative stress measurements**

361 The ROS production index measured in larvae after exposure to tritiated thymidine is  
362 presented in Figure 5, as well as the fluorescence levels measured after exposure of the  
363 larvae to PMA. The ROS production index only significantly rose after the exposure to the  
364 intermediate dose rate (170  $\mu$ Gy/h,  $p = 0.001$ ). The basal fluorescence level (larvae only  
365 exposed to DMSO) and the stimulated fluorescence level (larvae exposed to PMA) were  
366 also analysed to obtain more information. The basal fluorescence level did not change with  
367 any of the tritiated thymidine dose rates ( $p > 0.05$ , data not shown), while the stimulated



368 fluorescence level increased significantly after exposure to all three dose rates ( $p < 0.01$ ).  
369 The stimulated level found in larvae exposed to 170  $\mu\text{Gy/h}$  was also greater to the one  
370 measured in larvae exposed to 22  $\mu\text{Gy/h}$  ( $p < 0.01$ ).

371

#### 372 **4. Discussion**

373 To assess tritiated thymidine effects on zebrafish development, hatching success,  
374 embryo and larva morphometry, as well as their spontaneous tail movements and heartbeat  
375 were studied. ROS production and expression of different genes of interest were also  
376 monitored.

377 This work showed that tritiated thymidine, while not causing significant increase of  
378 the embryo-larval mortality, does affect the development of zebrafish. Indeed, the  
379 embryonic development seemed to be slowed down, even if this effect was only transient,  
380 with larvae being smaller after 3 days of exposure at the two lowest dose rates. These  
381 observations were expected, as radiations are known to have early effects on development,  
382 with zebrafish larvae being small after being irradiated for 24 h at a dose rate of 41.6  $\mu\text{Gy/h}$   
383 of Cs-137<sup>23</sup>. The only difference is that in the case of that study<sup>21</sup>, the effect increased with  
384 the dose rate, while in the present study, effects only increased up to the intermediate dose  
385 rate and no differences were found after exposure to the highest dose rate. More  
386 specifically, these observations have already been reported in the case of tritium, where  
387 zebrafish larvae exposed for 3 days to HTO presented smaller yolk sacs and shorter lengths  
388 after exposure to a dose rate of around 150  $\mu\text{Gy/h}$ . These results show that tritiated  
389 thymidine might have a higher toxicity than HTO, considering this effect appeared at lower  
390 dose rate for organic form of tritium<sup>10</sup>. However, the fact that the yolk sac was smaller in  
391 contaminated larvae compared to control ones does not match with a slower development.  
392 Indeed, if the embryo development was reduced, the yolk sac in irradiated embryos would

393 be bigger than in the control, which is not the case here. Our results indicated that more  
394 yolk has been consumed by embryos after exposure while the development was not  
395 accelerated, leading to the hypothesis that the energy allowance is different after exposure,  
396 with more energy being allowed to possible metabolic processes induced by the exposure,  
397 as the repair or renewing of damaged cells, as those processes have already been observed  
398 in individuals exposed to radiations<sup>6,11,24,29</sup>. Though, this change in the energy allowance  
399 seems to be reverted when the dose rate used in the exposure reached a high value: the  
400 individuals exposed to the highest dose rates seemed to develop at the same rate than the  
401 control ones, while still showing a delay in hatching. Considering that there is a strong  
402 relationship between ATP production and energy use, quantifying this production could  
403 help identify if this hypothesis is correct<sup>31</sup>. This could, for example, be done by measuring  
404 the NADH<sub>2</sub> in zebrafish embryos and larvae, considering that this compound is strongly  
405 linked to the ATP production<sup>32</sup>. This could mean that, the contamination becoming too  
406 strong, the individuals stopped trying to fight its possible deleterious effects, their energy  
407 allowance thus returning to a control level.

408 The delay of the hatching is also in accordance with the development being slowed,  
409 and this effect has also already been observed after exposure to gamma radiations and  
410 tritium<sup>3,12,23</sup>. In teleost fish, hatching is the result of the combination of two processes: the  
411 chorion digestion by two enzymes, the high- and low choriolytic enzymes (HCE and LCE),  
412 and the tearing of the egg membrane by the twisting of the embryo once the membrane has  
413 been digested<sup>16</sup>. The analysis of the spontaneous tail movements showed an increase of the  
414 burst activity, the burst duration and the burst count in the embryos exposed to the highest  
415 dose rate, while the exposure to the two lowest dose rates did not induce any changes. This  
416 result means that the hatching delay could result from a defect of the synthesis or efficiency  
417 of chorionases as the embryos either twisted in a similar way than the control ones, or even

418 more than those. An analysis of the choriolytic enzymes synthesis or activity would thus  
419 help understand more about the mechanisms leading to the effect that was observed on the  
420 hatching kinetic. Regarding the increase in the burst activity, such effect has already been  
421 observed after exposure to gamma radiation at a dose rate of 5000  $\mu\text{Gy/h}$  but not at lower  
422 or higher dose rates, while this effect was not observed after exposure to HTO, which  
423 means that in the case of this effect, tritiated thymidine seems to behave like gamma  
424 radiation, but at a lower dose rate<sup>24,29</sup>.

425 Exposure to the highest dose rate of tritiated thymidine led to a significant decrease of  
426 the heart rate. Even though the larvae were not smaller at this time point, this effect could  
427 indicate a delay in the development as it is known that in the case of zebrafish, the heart  
428 rate gradually increases with time<sup>24,33</sup>. The decrease of the heartbeat after exposure to a  
429 contaminant has already been observed after exposure to gamma radiations at a dose rate  
430 equivalent to the one used in this study (*i.e.* 500  $\mu\text{Gy/h}$ ), but also after exposure to some  
431 organic contaminants as butyl benzyl phthalate, propranolol, thifluzamide,  
432 penconazole<sup>16,26,33,34</sup>. No other markers studied in this work can help understand the  
433 mechanisms lying under the appearance of such an effect, but as the cardiac development  
434 in zebrafish is quite a complex system, we could for example hypothesize that there was  
435 an issue during the development, or that the thyroid gland was affected by tritium, as it has  
436 an important role on the regulation of the heartbeat and is also one of the targets already  
437 identified in the case of radiations<sup>3,12,35,36</sup>.

438  
439 While this work highlighted different macroscopic effects that tritiated thymidine had  
440 on zebrafish in its early life stages, it also showed the presence of an increase in the ROS  
441 production and of a dysregulation of many genes. Such an increase of the ROS production  
442 has already been observed after exposure to radiation dose rates as low as 134  $\mu\text{Gy/h}$  (Cs-

443 137), but also after exposure of fathead minnows to HTO and organic tritium (25 and 180  
444 kBq/L)<sup>7,11</sup>. In a more general way, several studies reported that low-dose radiations had  
445 effects that have been associated with ROS production<sup>37,38</sup>. ROS are considered as being  
446 unstable and highly reactive compounds, including O<sub>2</sub><sup>-</sup> or H<sub>2</sub>O<sub>2</sub>, and these compounds are  
447 also considered as being able to induce oxidative stress and oxidative damage, which is  
448 thus in accordance with the hypothesis that organic tritium could lead to the induction of  
449 oxidative stress in zebrafish<sup>39</sup>. Though, considering that using H<sub>2</sub>DCFDA as a probe only  
450 gives an indirect information on the H<sub>2</sub>O<sub>2</sub> production, that means that our results only  
451 showed a part of the response to oxidative stress.

452       Regarding the gene expression measured after 24 hours of exposure, the results  
453 indicated that there is a significant over-expression of the genes only after exposure to the  
454 lowest dose rate, while the exposure to the intermediate dose rate only led to a tendency  
455 towards the over-expression of a few genes, and the exposure to the highest dose rate led  
456 to no gene dysregulation. This result, alongside the absence of a significant increase in the  
457 ROS production index could, for example, highlight a decrease in the metabolic activity of  
458 the individuals. This discussion will only focus on the results observed after exposure to  
459 the lowest dose rate. The analysis of the genes being over-expressed ( $p < 0.05$ ) or nearly  
460 overexpressed ( $p < 0.1$ ) showed that many of those genes are involved in the same  
461 processes. For example, the apoptotic process (*baxa*, *bbc3*, *casp9*, *bcl2*), DNA binding  
462 (*cry5*, *crx*, *efl1*, *h2ax*, *tbpl1*) and repair (*cry5*, *ddb2*, *h2ax*, *xpc*, *xrcc1*) seem to be affected,  
463 as most of the genes involved in these processes and studied here are overexpressed (either  
464 significantly or only as a tendency). These observations are in accordance with results  
465 obtained in various studies that showed that processes linked to DNA synthesis or repair  
466 are easily affected by the exposure to tritium or other radionuclides, even if in most studies,  
467 the gene expression is also dysregulated in later development stages (*e.g.* 96 hpf)<sup>15,17</sup>. This

468 seems to indicate that DNA might be damaged as a result from the exposure, which was  
469 expected considering the fact that it has been shown that a significant portion of the tritium  
470 ends up bound to the DNA<sup>5</sup>. However, no conclusions can be drawn concerning DNA  
471 integrity since DNA damage was not experimentally measured in the present study.  
472 Besides, two genes *mpx* and *sod2* involved in the regulation of oxidative stress were also  
473 upregulated, confirming that there might be ROS overproduction in zebrafish embryos  
474 following tritiated thymidine exposure. This would also be in accordance with different  
475 studies where genes involved in the oxidative stress regulation were overexpressed after  
476 exposure to HTO<sup>10,15</sup>. In contrast, it differs with the downregulation of the *mpx* gene that  
477 was observed in zebrafish embryos and larvae after being irradiated at a dose rate of 1240  
478  $\mu\text{Gy/h}$  (Cs-137), thus showing that tritiated thymidine might behave more like HTO, even  
479 though all genes showed dysregulation at low dose rates only, which was not the case after  
480 exposure to HTO.<sup>11,15</sup>.

481

## 482 **5. Conclusion**

483 The toxicity of tritiated thymidine on early life stages at different biological levels was  
484 investigated herein. While this study reported that tritiated thymidine did not induce any  
485 significant mortality, it was also shown that it can affect zebrafish embryo-larval  
486 development in different ways. Indeed, organic tritium affected hatching kinetic, with  
487 hatching being delayed and a transient decrease in the development kinetics. Those  
488 developmental effects were observed at all dose rates tested, even though the nature of the  
489 effect depended on the tritiated thymidine activity concentration. This work also showed  
490 an increase in the ROS production levels after four days of exposure. This study also  
491 highlighted a dysregulation of many genes after one day of exposure, even if the latter was  
492 only transient, as no effect were observed after four days of exposure and after exposure to

493 greater dose rates. The dysregulated genes are involved in different cellular processes, *i.e.*,  
494 development, oxidative stress, DNA repair and apoptosis. It confirms that tritium does have  
495 an effect on zebrafish individuals at different molecular scales and, more specifically, on  
496 various molecular targets and processes. Linking all the results presented in this paper is  
497 not possible, as the investigation of other markers as, for example, the protein levels, would  
498 be needed to do so. The study of other biological markers, as the protein expression, is also  
499 considered necessary to confirm the gene dysregulation results presented in this paper. This  
500 study does not highlight any specific toxic effects arising from the exposure to tritium in  
501 its organic phase. As the experimental design of this study limited the exposure length to  
502 four days, later development stages could not be studied. Those stages should be the focus  
503 of a later study to confirm the results presented in this paper. The study of the same toxicity  
504 markers and other ones, as for example carcinogenic effects or reproductive behavior,  
505 could also give more information on tritiated thymidine toxicity and possible specific  
506 effects.

507

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513

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622

623 **Tables**

624

625 **Table 1.** Amount of solution sampled in the beakers (1 replicate/beaker) and the crystallizers (3  
626 replicate/crystallizer) depending on the activity concentration (Bq/mL,  $\pm 10\%$ ) used during the  
627 experiment

<b>Nominal activity concentration (<math>\times 10^3</math> Bq/mL)</b>	<b>Beaker sampling</b>	<b>Crystallizer sampling</b>
<b><math>7.5 \pm 0.8</math></b>	1 mL	10 $\mu$ L diluted in 990 $\mu$ L of tritium-free water
<b><math>40 \pm 4.0</math></b>	1 mL	10 $\mu$ L diluted in 990 $\mu$ L of tritium-free water
<b><math>110 \pm 11.0</math></b>	100 $\mu$ L diluted in 900 $\mu$ L of tritium-free water	10 $\mu$ L of a 1:10 solution in 990 $\mu$ L of tritium-free water

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629

630

631 **Table 2.** Developmental characteristics (mm) measured on control and contaminated  
632 zebrafish early life stages (0, 22 and 170  $\mu\text{Gy/h}$ ). Measurements were made on embryos (1  
633 and 2 dpf) and larvae (3 and 4 dpf), and the number in parenthesis corresponds to the  
634 number of individuals used for each measurement. Bold numbers and \* indicate a  
635 significant difference with the control (GLM and GLMM,  $p < 0.05$ )

Measurement (mm)	Control	22 $\mu\text{Gy/h}$	170 $\mu\text{Gy/h}$
Egg diameter (1 dpf)	1.25 $\pm$ 0.06 (19)	1.26 $\pm$ 0.06 (20)	1.25 $\pm$ 0.05 (19)
Yolk sac diameter (1 dpf)	0.52 $\pm$ 0.03 (19)	<b>0.55 <math>\pm</math> 0.04*</b> (20)	0.52 $\pm$ 0.04 (19)
Egg diameter (2 dpf)	1.26 $\pm$ 0.07 (11)	1.24 $\pm$ 0.03 (12)	1.22 $\pm$ 0.05 (11)
Yolk sac diameter (2 dpf)	0.50 $\pm$ 0.03 (11)	0.49 $\pm$ 0.04 (12)	0.50 $\pm$ 0.04 (11)
Larva length (3 dpf)	3.22 $\pm$ 0.16 (17)	<b>3.03 <math>\pm</math> 0.21*</b> (14)	<b>2.97 <math>\pm</math> 0.13*</b> (16)
Yolk sac diameter (3 dpf)	0.42 $\pm$ 0.06 (17)	0.39 $\pm$ 0.06 (14)	<b>0.37 <math>\pm</math> 0.07*</b> (16)
Eye diameter (3 dpf)	0.26 $\pm$ 0.02 (17)	0.25 $\pm$ 0.04 (14)	<b>0.22 <math>\pm</math> 0.04*</b> (16)
Larva length (4 dpf)	3.22 $\pm$ 0.16 (15)	3.30 $\pm$ 0.18 (16)	3.31 $\pm$ 0.20 (17)
Yolk sac diameter (4 dpf)	0.35 $\pm$ 0.08 (15)	0.37 $\pm$ 0.04 (16)	0.38 $\pm$ 0.05 (17)
Eye diameter (4 dpf)	0.26 $\pm$ 0.04 (15)	0.27 $\pm$ 0.02 (16)	0.27 $\pm$ 0.03 (17)

636

637

638

639 **Table 3.** Developmental characteristics (mm) measured on control and contaminated  
640 zebrafish early life stage (0 and 470  $\mu\text{Gy/h}$ ). Measurements were made on embryos (1 and  
641 2 dpf) and larvae (3 and 4 dpf), and the number in parenthesis corresponds to the number  
642 of individuals used for each measurement. No significant differences were observed  
643 between both conditions (GLM and GLMM,  $p < 0.05$ )

Measurement (mm)	Control	470 $\mu\text{Gy/h}$
Egg diameter (1 dpf)	1.21 $\pm$ 0.07 (18)	1.19 $\pm$ 0.06 (16)
Yolk sac diameter (1 dpf)	0.51 $\pm$ 0.04 (18)	0.50 $\pm$ 0.03 (16)
Egg diameter (2 dpf)	1.20 $\pm$ 0.06 (15)	1.20 $\pm$ 0.07 (13)
Yolk sac diameter (2 dpf)	0.48 $\pm$ 0.05 (15)	0.45 $\pm$ 0.05 (13)
Larva length (3 dpf)	3.46 $\pm$ 0.23 (16)	3.32 $\pm$ 0.29 (17)
Yolk sac diameter (3 dpf)	0.47 $\pm$ 0.09 (16)	0.44 $\pm$ 0.09 (17)
Eye diameter (3 dpf)	0.30 $\pm$ 0.04 (16)	0.28 $\pm$ 0.04 (17)
Larva length (4 dpf)	3.61 $\pm$ 0.26 (15)	3.56 $\pm$ 0.17 (17)
Yolk sac diameter (4 dpf)	0.37 $\pm$ 0.09 (15)	0.35 $\pm$ 0.04 (17)
Eye diameter (4 dpf)	0.30 $\pm$ 0.04 (15)	0.31 $\pm$ 0.04 (17)

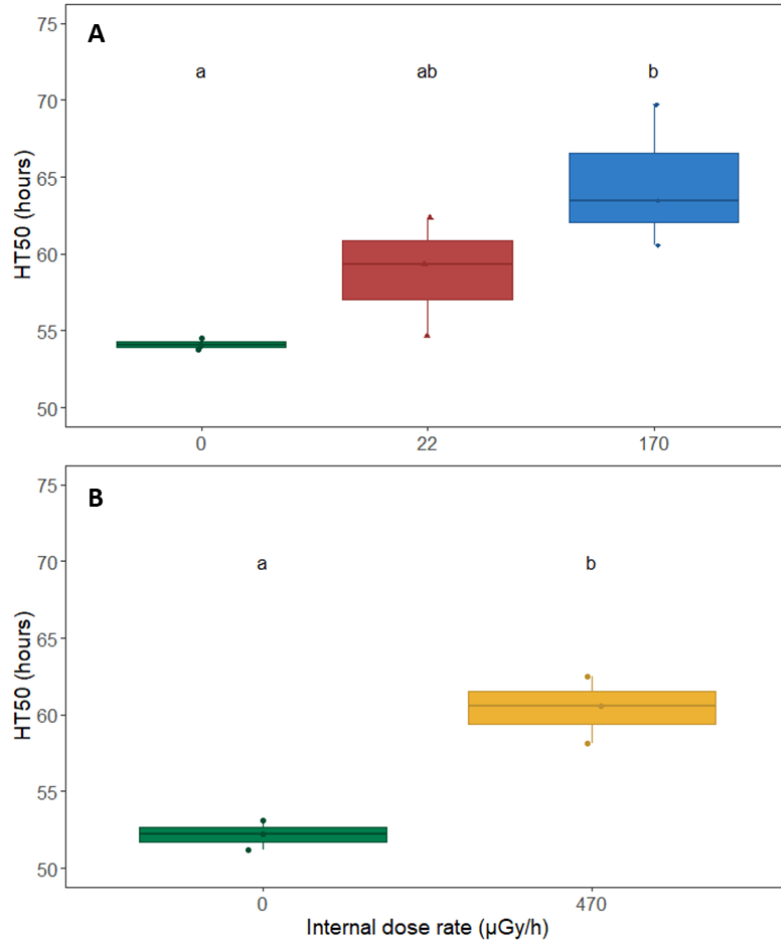
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647 **Figures**

648

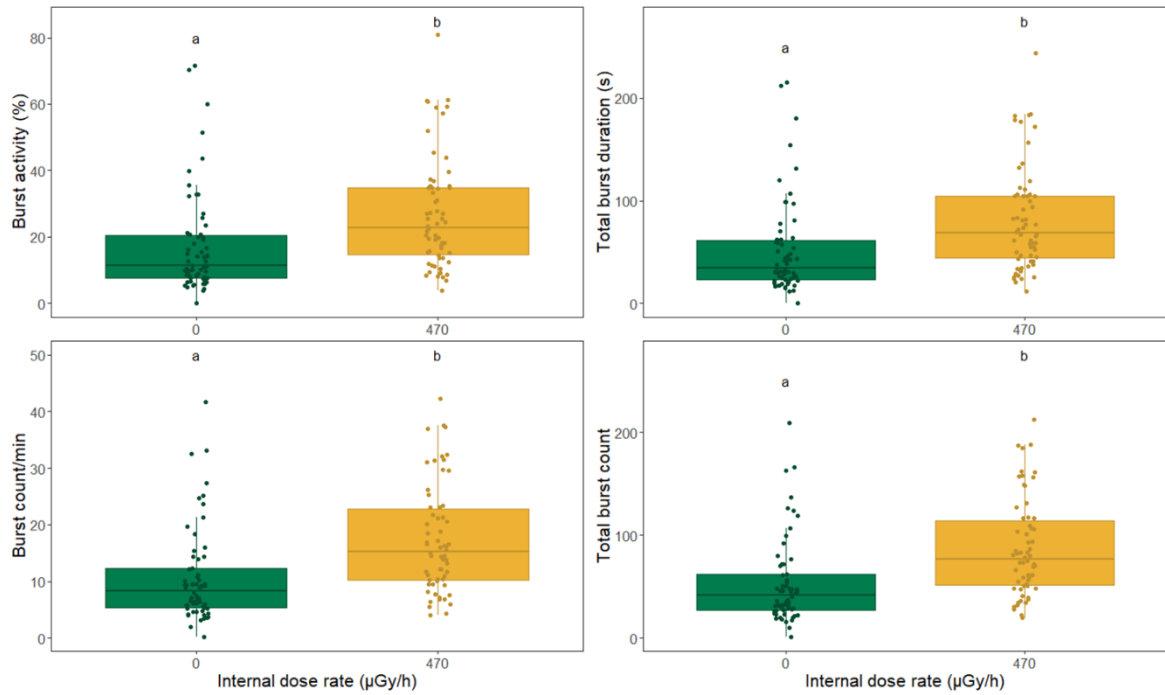


649

650 **Figure 1.** Average hatching time (HT50, hours, N = 8) measured in control and exposed  
651 zebrafish embryos to tritiated thymidine (panel A: 22 and 170 μGy/h; panel B: 470 μGy/h).  
652 ANOVA, a<b, p<0.05.

653

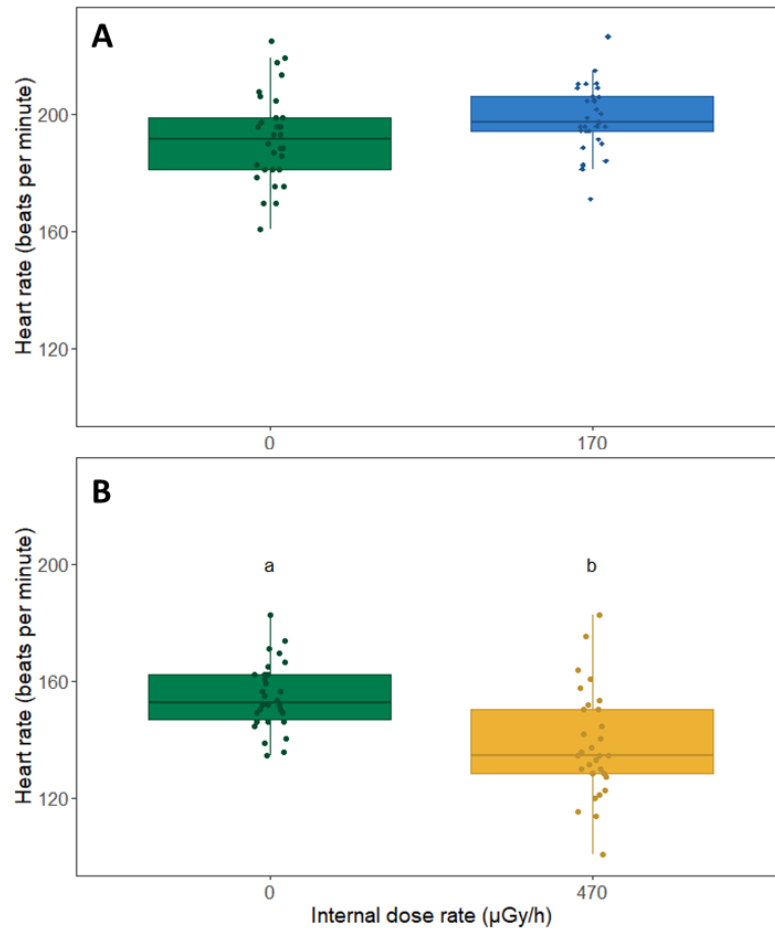
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656 **Figure 2.** Burst activity (% , panel A), total burst duration (s, panel B), burst count per  
 657 minute (panel C) and total burst count (panel D) measured in control and contaminated  
 658 zebrafish embryos (0 and 470 μGy/h) after 24 hours of exposure to tritiated thymidine.  
 659 GLMM/LMM, N = 60, a<b, p < 0.05.

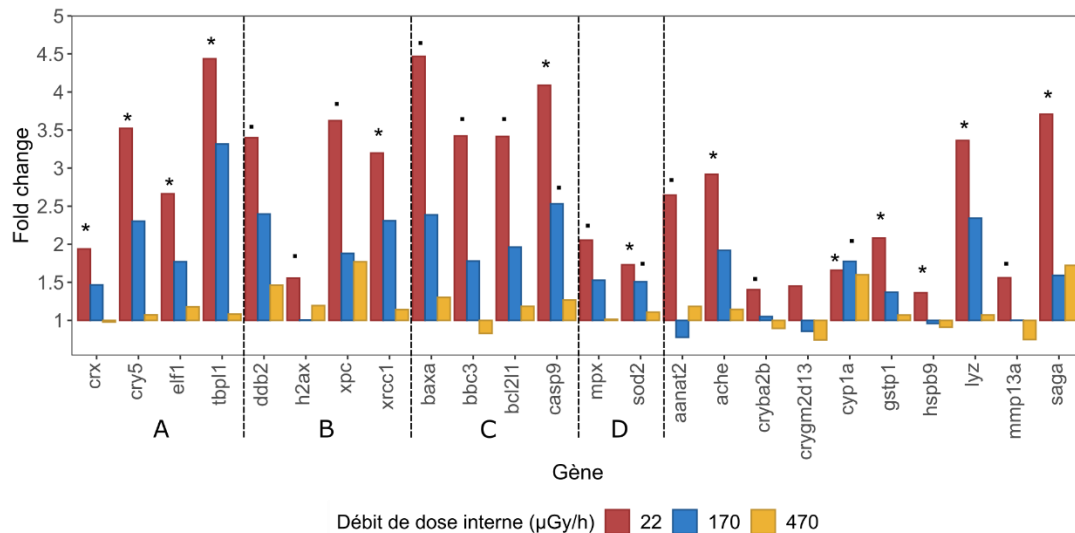
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661

662 **Figure 3.** Heart rate (BPM, N = 30) measured in control and contaminated 3 dpf zebrafish  
 663 larvae (0, 170 μGy/h, panel A and 470 μGy/h, panel B) after three days of exposure to  
 664 tritiated thymidine. GLMM/LMM, a<b, p < 0.05.

665

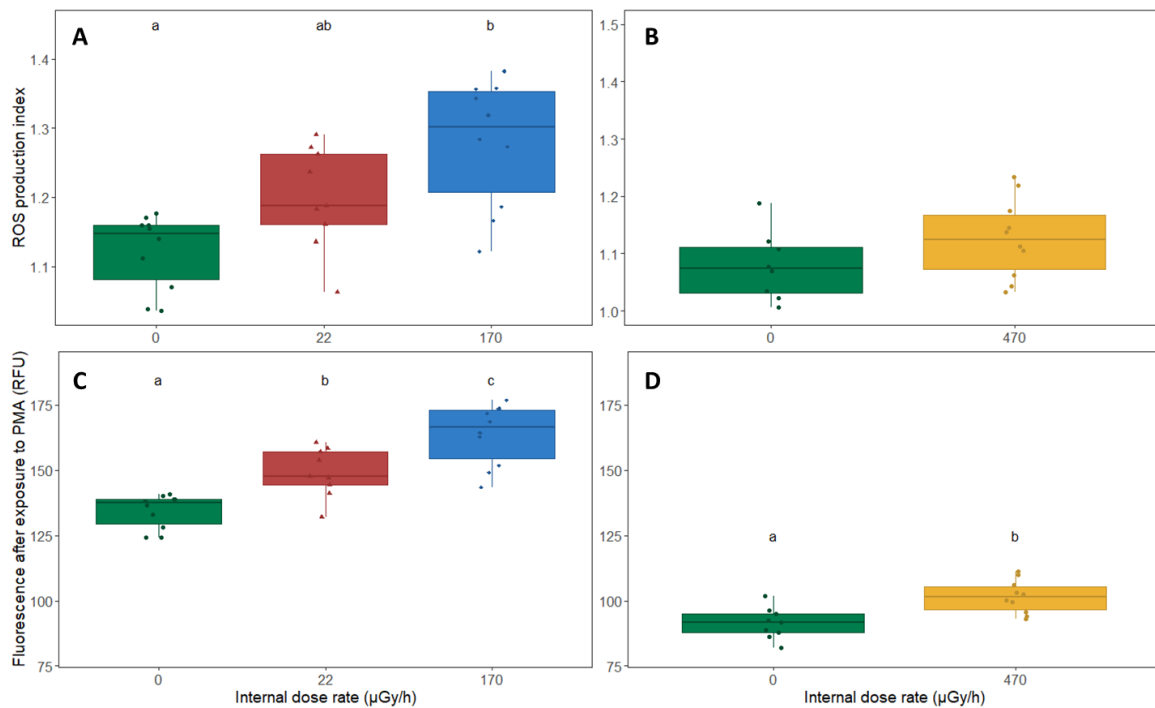


666

667 **Figure 4.** Fold change in gene expression in comparison to controls of 24 different genes  
 668 in 1 dpf fish embryos exposed for 24 h to tritiated thymidine (22, 170 and 470 µGy/h).  
 669 Genes studied are involved in different biological processes such as DNA binding (A),  
 670 DNA repair (B), apoptosis (C) and response to the oxidative stress (D). Statistics: t-test and  
 671 adjustment of the p-value using the Benjamini-Hochberg False Discovery Rate. (-p < 0.1;  
 672 \* p < 0.05 when comparing to the control).

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676 **Figure 5.** ROS production index (panels A and B) and fluorescence measured after  
 677 exposure to PMA (panels C and D) in control and contaminated zebrafish larvae (left



678 panels: 22 and 170  $\mu\text{Gy/h}$ , right panels: 470  $\mu\text{Gy/h}$ ) after 4 days of exposure to tritiated  
679 thymidine. ANOVA,  $a < b < c$ ,  $p < 0.05$ . Each point represents a data point acquired during  
680 the experiment.

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**Supplementary Information**

684

**Table S1.** Genes chosen for the Real Time PCR Analysis and their corresponding Assay IDs. All genes were chosen by using the gene library provided by ThermoFisher.

685

<b>Gene Name</b>	<b>Assay_ID</b>
<i>aanat2</i>	Dr03432880_m1
<i>ache</i>	Dr03093481_m1
<i>atp2a1l</i>	Dr03085336_m1
<i>baxa</i>	Dr03432977_m1
<i>bbc3</i>	Dr03168831_s1
<i>bcl2l1</i>	Dr03080274_m1
<i>casp9</i>	Dr03121787_m1
<i>crx</i>	Dr03131743_m1
<i>cry5</i>	Dr03138452_m1
<i>cryba2b</i>	Dr03160512-m2
<i>crygm2d13</i>	Dr03426056_gH
<i>cyp1a</i>	Dr03112444_m1
<i>ddb2</i>	Dr03429621_m1
<i>eef1a2</i>	Dr03076192_m1
<i>elf1</i>	Dr03118707_m1
<i>gadd45bb</i>	Dr03083476_m1
<i>gstp1</i>	Dr03118988_m1
<i>h2ax</i>	Dr03145737_s1
<i>hspb9</i>	Dr03436228_s1
<i>lyz</i>	Dr03099437_m1
<i>mmp13a</i>	Dr03438515_g1

<i>mpx</i>	Dr03075659_m1
<i>oxt</i>	Dr03112593_m1
<i>per2</i>	Dr03093697_m1
<i>plekhs1</i>	Dr03093697_m1
<i>saga</i>	Dr03435203_m1
<i>sod2</i>	Dr03100019_m1
<i>tbpl1</i>	Dr03088430_m1
<i>tnni2b.2</i>	Dr03111539_g1
<i>xpc</i>	Dr03148843_m1
<i>xrcc1</i>	Dr03115324_m1

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