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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 μ Gy/h), a delayed hatching after exposure to the two
34 highest dose rates (170 and 470 μ 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 μ 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 hydrogen¹. 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^{1,2}. 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/L³. 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)¹. 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 atom³. 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 synthesis⁴⁻¹⁰.
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^{11,12}.
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*)^{3,10,13}. 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 hpf¹².

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^{7,10,14}. 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^{11,15}. 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-regulated¹⁵. 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^{3,16,17}.

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^{8,18}. 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^{19,20}.
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^{3,10,11,15,21-26}. 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 ± 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 control²⁷. 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 elsewhere⁵. 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 ± 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.⁵. 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 presented²⁸. 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 2019²⁹. 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.²⁹. 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 previously²⁹.
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 μ 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 μ L of RNase and DNase free water. All RNA
222 concentrations (ng/ μ 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 μ 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 Δ Ct values, each value corresponding to the gene
240 expression of each biological replicate, normalized using the reference gene. Δ 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 elsewhere³⁰. 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 μ L of HBSS buffer (ThermoFisher) and 50 μ L H₂DCFDA (2 mg/L in DMSO
249 0.4 %). The first ten larvae received 50 μ L DMSO at 0.4 % while the other ten larvae
250 received 50 μ L Phorbol myristate acetate (PMA, 0.8 mg/L in DMSO 0.4 %). Fluorescence
251 emitted by the reaction between H₂DCFDA 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 ΔCt and $2^{(-\Delta\text{Ct})}$. All ΔCt 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 (4×10^4 and 1×10^5 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 (7.5×10^3 Bq/mL; 22 μ 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 μ Gy/h and 170 μ 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 μ 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 μ Gy/h), the
346 changes in gene expression were not significant. At 170 μ 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 μ 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 μ 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-137²³. The only difference is that in the case of that study²¹, 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 tritium¹⁰. 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^{6,11,24,29}. 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 correct³¹. This could, for example, be done by measuring
404 the NADH₂ in zebrafish embryos and larvae, considering that this compound is strongly
405 linked to the ATP production³². 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^{3,12,23}. 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 digested¹⁶. 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^{24,29}.

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^{24,33}. 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^{16,26,33,34}. 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^{3,12,35,36}.

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)^{7,11}. In a more general way, several studies reported that low-dose radiations had
445 effects that have been associated with ROS production^{37,38}. ROS are considered as being
446 unstable and highly reactive compounds, including O₂⁻ or H₂O₂, 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 zebrafish³⁹. Though, considering that using H₂DCFDA as a probe only
450 gives an indirect information on the H₂O₂ 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)^{15,17}. 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 DNA⁵. 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^{10,15}. 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.^{11,15}.

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

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

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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)	0.55 \pm 0.04* (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)	3.03 \pm 0.21* (14)	2.97 \pm 0.13* (16)
Yolk sac diameter (3 dpf)	0.42 \pm 0.06 (17)	0.39 \pm 0.06 (14)	0.37 \pm 0.07* (16)
Eye diameter (3 dpf)	0.26 \pm 0.02 (17)	0.25 \pm 0.04 (14)	0.22 \pm 0.04* (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)

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638

639 **Table 3.** Developmental characteristics (mm) measured on control and contaminated
640 zebrafish early life stage (0 and 470 μ 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 μ 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)

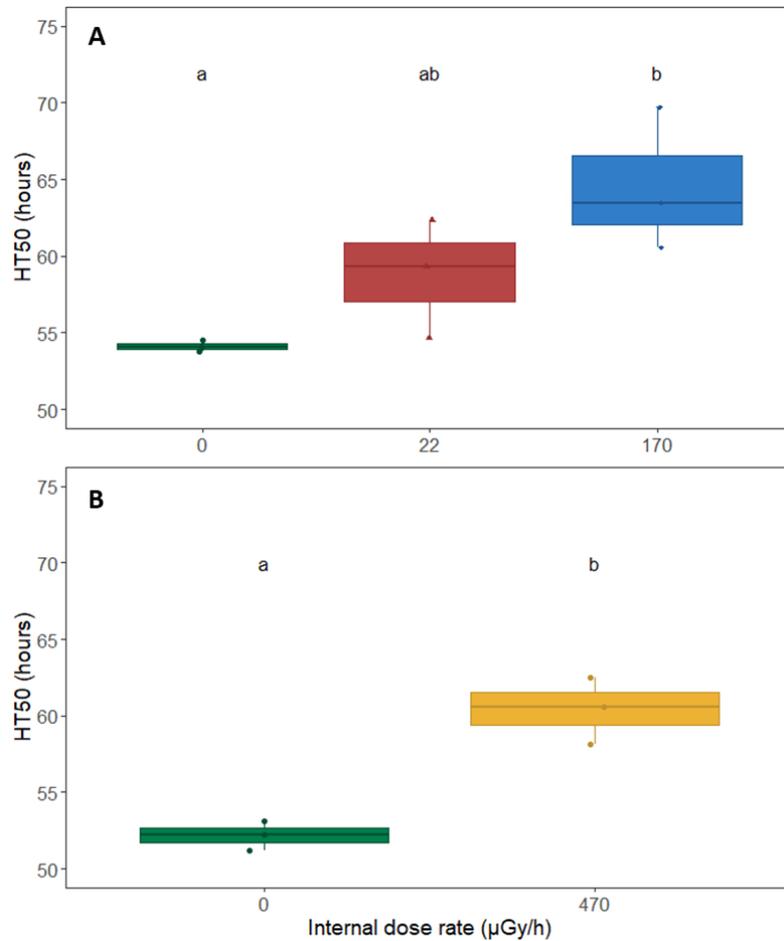
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647 **Figures**

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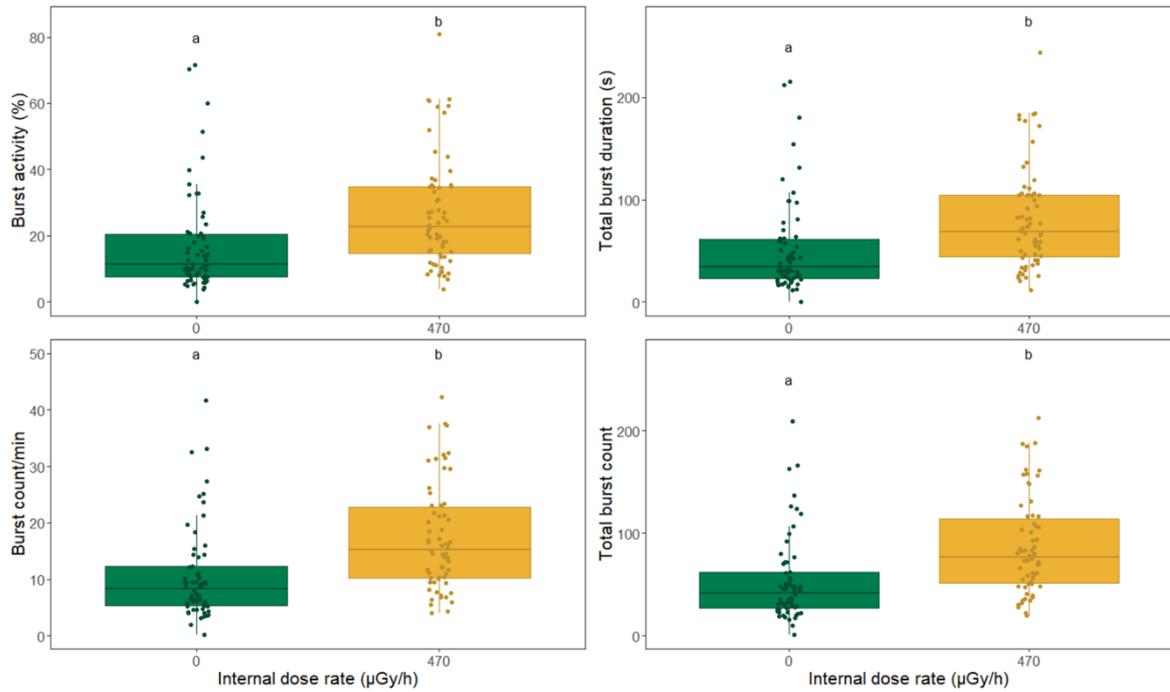


649

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.

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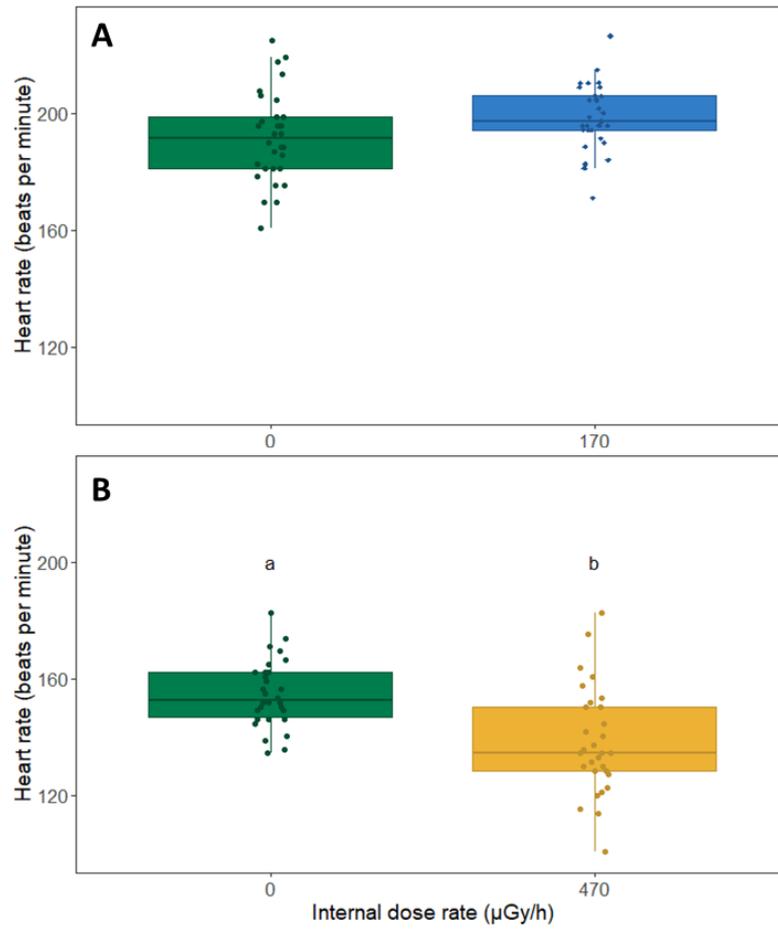
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656 **Figure 2.** Burst activity (% , panel A), total burst duration (s, panel B), burst count per
 657 minute (panel C) and total burst count (panel D) measured in control and contaminated
 658 zebrafish embryos (0 and 470 μGy/h) after 24 hours of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

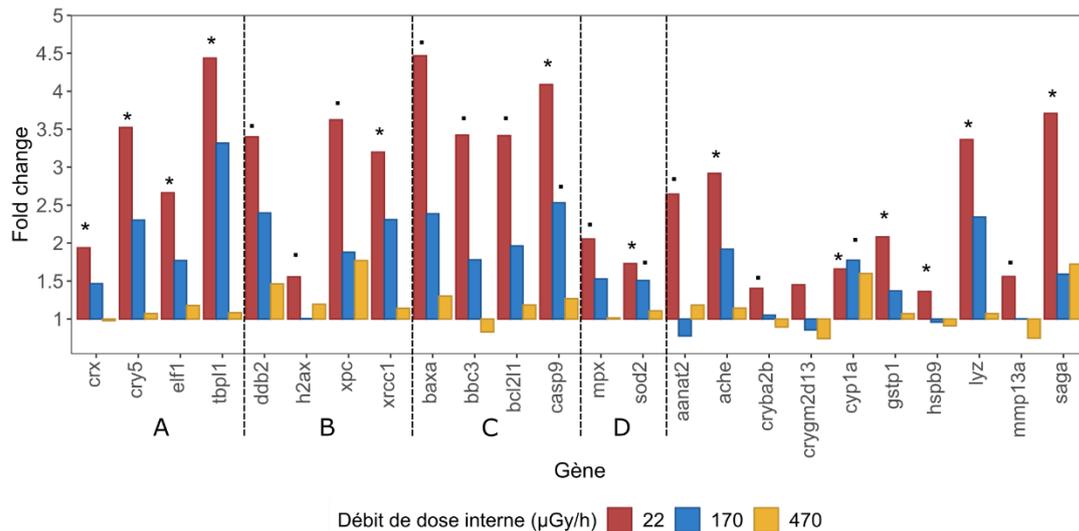
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661

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.

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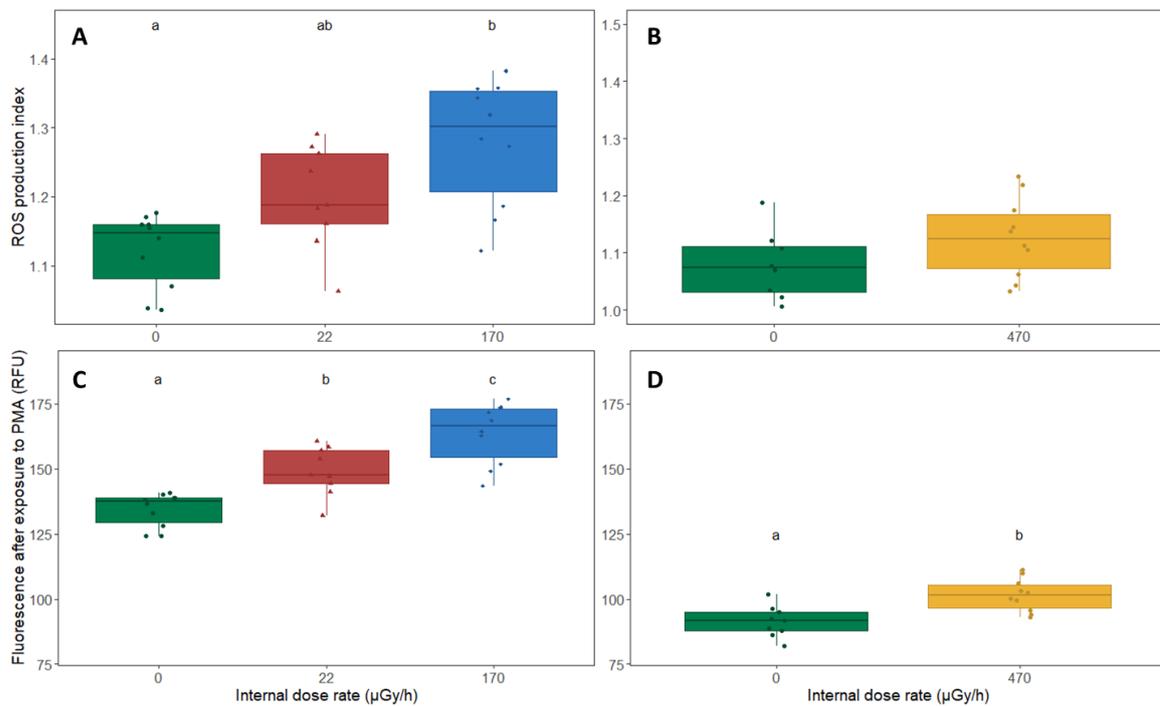


666

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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675

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

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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.

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Gene Name	Assay_ID
<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
