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Tritiated Water Exposure in Zebrafish (*Danio rerio*): Effects on the Early-Life Stages

Beatrice Gagnaire, Caroline Arcanjo, Isabelle Cavalie, Virginie Camilleri,
Olivier Simon, Magali Floriani, Daniel Orjollet, Christelle Adam-Guillermin

► **To cite this version:**

Beatrice Gagnaire, Caroline Arcanjo, Isabelle Cavalie, Virginie Camilleri, Olivier Simon, et al.. Tritiated Water Exposure in Zebrafish (*Danio rerio*): Effects on the Early-Life Stages. *Environmental Toxicology and Chemistry*, 2019, 39 (3), pp.648-658. 10.1002/etc.4650 . hal-02507245

HAL Id: hal-02507245

<https://hal.science/hal-02507245>

Submitted on 7 Jul 2020

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1 **Tritiated water exposure in zebrafish, *Danio rerio*: effects on the early-life stages**

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3 Authors:

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5 Gagnaire B.*¹, Arcanjo C.¹, Cavalé I.¹, Camilleri V.¹, Simon O.¹, Floriani M.¹, Orjollet D.²,
6 Adam-Guillermin C.³

7
8
9 Affiliation:

10
11 ¹ Institut de Radioprotection et de Sureté Nucléaire (IRSN), PSE-ENV/SRTE/LECO,
12 Cadarache, Saint-Paul-lez-Durance 13115, France

13 ² Institut de Radioprotection et de Sureté Nucléaire (IRSN), PSE-ENV/SRTE/LR2T,
14 Cadarache, Saint-Paul-lez-Durance 13115, France

15 ³ Institut de Radioprotection et de Sureté Nucléaire (IRSN), PSE-SANTE/SDOS/LMDN,
16 Cadarache, Saint-Paul-lez-Durance 13115, France

20 **Abstract**

21 Tritium, a radioactive isotope of hydrogen of natural and anthropogenic origin, is ubiquitously
22 present in the environment. Effluents of nuclear center of production are significant
23 anthropogenic sources. With the upcoming project of thermonuclear fusion, tritium releases in
24 the environment may increase. It is therefore important to characterize the ecological risk
25 linked to tritium. The effects of tritiated water (HTO) were therefore studied in zebrafish
26 larvae exposed during 10 days to different dose rates: 1.1×10^2 , 4.1×10^2 and 3.8×10^3 $\mu\text{Gy/h}$ for
27 larvae, corresponding respectively to a water contamination of 10^4 , 10^5 and 10^6 Bq/mL of
28 HTO. Those dose rates were higher than $10 \mu\text{Gy/h}$ which is the threshold recommended to
29 start monitoring ecosystems where radiological contaminants are present. Mortality, embryo-
30 larval development, immune-, geno and neuro-toxicity, and alterations of tissues were
31 investigated. Results showed that tritiated water exposure induced DNA damage, ROS
32 production and modulated the expression of genes involved in detoxification processes.
33 Moreover, modifications of the muscular tissues (degradation of myofibrils at 4 dpf and
34 disorganization of mitochondria at later stages) were observed. Results differed with HTO
35 dose rates and with developmental stages. These results will drive future research for the
36 development of new HTO-sensitive biomarkers and will allow us to progress in the
37 characterization of the modes of action of tritium in fish.

38

39 **Keywords**

40 Zebrafish embryo-larvae; tritiated water; biomarkers; DNA damage; ROS production.

41

42 **Highlights**

- 43 - This study evaluated the effects of tritiated water on zebrafish embryo-larvae
- 44 - Larvae were exposed to different dose rates: 1.1×10^2 , 4.1×10^2 and 3.8×10^3 $\mu\text{Gy/h}$
- 45 - Several endpoints were evaluated: embryo-larval mortality and development,
46 muscular tissue alteration, geno-, immuno-, neuro-toxicity, reactive oxygen species
47 production, and gene expression
- 48 - The results showed that tritiated water, depending on the dose rate and the stage of
49 development considered, induced molecular changes, cellular modifications, as well as
50 tissue alterations

51

52 1. Introduction

53 The main source of naturally produced tritium is the reaction between cosmic rays and
54 atmospheric gases (Boyer et al. 2009). Anthropogenic sources of tritium, including nuclear
55 reactors, add to this. The nuclear industry releases tritium mostly in the form of tritiated water
56 (HTO) (Boyer et al. 2009, CNSC 2008). The future use of nuclear fusion reactor (ITER
57 project) is expected to meaningfully increase the anthropogenic contribution and aquatic
58 ecosystems are expected to be the main receptors (ASN 2010). Aquatic systems are inhabited
59 by a large variety of species. For these reasons, it seems important to assess the ecological
60 risk of this radionuclide, especially on aquatic ecosystems. All sources combined, worldwide,
61 the annual tritium production reaches about 200 g, corresponding to 7.2×10^{16} Bq/year (ASN
62 2010).

63 The approaches of ecological risk assessment used for chemicals are usually also used for
64 ionizing radiation (Garnier-Laplace et al. 2006), using the benchmark of 10 μ Gy/h for
65 ecosystem protection (Garnier-Laplace et al. 2010). However, ecotoxicity, toxicity
66 mechanism and sub-lethal effects of tritiated waterborne exposure has been poorly studied,
67 particularly for aquatic vertebrates (Adam-Guillermin et al. 2012). It is therefore important to
68 acquire a better knowledge of effects of tritium on fish.

69 Tritium toxicity to fish has been mostly reported on macroscopic endpoints for early life
70 stages (Adam-Guillermin et al. 2012, Sazykina and Kryshev 2003). DNA lesions constitute
71 one of the primary damages from which tritiated water effects can propagate from cell to
72 individual (Mathur-De Vré and Binet 1984). Tritium is known to increase micronucleus
73 frequency (Jaeschke et al. 2011). However, effects other than genotoxicity including effects
74 on hormonal levels and on anti-inflammatory mechanisms were reported (Erickson 1971,
75 Strand et al. 1982). Studies also showed that tritiated thymidine can induce neurotoxic effects
76 (Adam-Guillermin et al. 2013). Neurologic response and immune system response, through
77 the defense from oxidative stress, can therefore be altered by tritium exposure. Effects on
78 these mechanisms can have consequences on survival and/or development of organisms.

79 In this context, this study aims to augment our knowledge on absorbed dose rates, effects and
80 action mechanisms of tritiated water on fish physiology in the case of chronic contaminations
81 at low and high dose rates of tritium. Fish are known to be relevant sentinels for monitoring
82 the effects of environmental pollution; fish have been used for studies on toxicology and
83 environmental risk assessment for several years (van der Oost et al. 2003). Among them,
84 zebrafish, *Danio rerio*, is commonly used as an ecotoxicological model (Hill et al. 2005).
85 Moreover, the use of early-life stages, considered as the most sensitive part of fish life-cycle,

86 has been proposed as a relevant bioassay to assess toxic effects of contaminants (Oberemm
87 2000, OECD 2004a, b, Scholz et al. 2008).

88 In this study, we conducted experiments on zebrafish embryo-larvae in order to assess
89 tritiated water effects at dose rates close to and higher than the environmental protection
90 criteria of 10 $\mu\text{Gy/h}$ (Garnier-Laplace et al. 2010). 3 hpf eggs were placed in tritiated water at
91 10^4 , 10^5 and 10^6 Bq/mL for 10 days. These activity concentrations corresponded to theoretical
92 dose rates of 4×10^1 , 4×10^2 and 4×10^3 $\mu\text{Gy/h}$ (Adam-Guillermin et al. 2013), in order to assess
93 tritium effects. A battery of biomarkers were followed, including neurotoxicity
94 (acetylcholinesterase (AChE) activity), defence system (phenoloxidase-like (PO) activity, an
95 enzyme involved in pathogen lysis, as described before (Gagnaire et al. 2013) and reactive
96 oxygen species (ROS) production), and DNA damage (using comet assay). The expression of
97 genes related to these diverse mechanisms (immunotoxicity, neurotoxicity, genotoxicity) was
98 also quantified. These responses measured at low levels of biological organization were
99 related to endpoints measured at the individual level (mortality, hatching success of embryos
100 and developmental parameters). Histopathology of tail muscle was also studied. The total
101 dose rates were calculated for eggs and larvae by considering an external dose rate calculated
102 using HTO water concentration and an internal dose rate calculated using tritium activities
103 measured in individuals, using a protocol developed in a related study (Arcanjo et al. 2019).
104 Results were then compared to observations obtained after exposure of zebrafish larvae to
105 gamma irradiation in previous studies.

106

107

108 2. Material & Methods

109 2.1. Chemicals

110 The chemicals used in this study were purchased from Sigma-Aldrich (St Quentin-Fallavier,
111 France), as mentioned before (Gagnaire et al. 2015): BSA, Bradford reagent, H₂DCFDA, L-
112 Dopa, DMSO, HBSS, DTNB, ATCi, trypan blue, ultrapure water sodium cacodylate,
113 glutaraldehyde, osmium tetroxide, toluidine blue. PMA was purchased from Molecular
114 Probes (Invitrogen, Cergy-Pontoise, France).

115

116 2.2 Production of eggs

117 All experimental procedures performed in this study were previously approved by IRSN
118 Animal Care Committee and followed French regulations for animal experimentation
119 (protocols: P2015-13 and P2017-05; registration number of IRSN laboratory: A13-013-07).

120 The eggs were obtained using 20 couples of 6-months old zebrafish (GIS Amagen, Gif sur
121 Yvette, France) as described before in Gagnaire et al. (2015). Genitors were maintained in a
122 Tecniplast ® rearing system with a photoperiod of 12h/12h and a temperature of $28 \pm 0.5^\circ\text{C}$.
123 They were fed three times per day with Tetramin ® fish food (Tetra, Melle, Germany) and
124 Artemias. Embryos were obtained by putting together 2 females and 2 males. All of the eggs
125 spawned by all the genitors were pooled; egg viability was confirmed when the blastula stage
126 was reached at 3 hpf (hours post fertilization) without visible abnormalities. The eggs were
127 considered to be of sufficient quality for the experiment when viability was at least 80% at 24
128 hpf.

129

130 2.3 Exposure of eggs to tritiated water

131 The tritium HTO source is an aqueous solution (97 % purity, 185 MBq, PerkinElmer,
132 Courtaboeuf, France). It was used after being diluted in embryo medium (Westerfield 2007)
133 to the targeted concentrations of 10^4 , 10^5 or 10^6 Bq/mL.

134 Egg contamination was done as described before (Arcanjo et al. 2018). Two hundred 3 hpf
135 eggs were randomly distributed in a 25 well-plate or in crystallizing dishes (5 eggs for 2 mL
136 of embryo medium). One 25 well-plate and three crystallizing dishes were used for each dose
137 rate and for the control group. Tritium contamination of embryo-larvae was done in glove
138 box. Exposed eggs (as well as controls) were incubated in the dark at a temperature of $28 \pm$
139 1°C . Eggs were exposed during 10 days to 10^4 , 10^5 or 10^6 Bq/mL of tritiated water with water
140 renewal every three days.

141

142 2.4 Calculation of dose rate

143 In order to calculate dose rates, internalization of tritium was assessed using a protocol which
144 aimed to minimize the exchanges between organisms and ambient medium developed in a
145 related study (Arcanjo et al. 2019). Dose rates were calculated using tritium activity
146 concentrations measured in the exposure medium (external dose rate) and in egg or larvae
147 (internal dose rate) applying dose coefficients (DC) calculated with EDEN v2 software
148 (IRSN), according to the hypothesis of an homogeneous tritium distribution in tissues (Table
149 1). A ponderation coefficient of 3 was applied in order to take into account beta rays as
150 recommended in ERICA tool (assessment of impacts of radiation on non-human organisms)
151 (Brown et al. 2008).

152

153 2.6 Following of embryo-larvae development

154 Mortality was monitored daily. Starting at 48 hpf, when larvae started to hatch, larvae were
155 examined each day until 10 dpf. Hatching observations were used to calculate hatching time
156 50% (HT₅₀), which represents the time necessary for half of eggs to hatch. This was
157 calculated using REGTOX ® (http://www.normalesup.org/~vindimian/fr_index.html).

158 At 1, 3, 4, 7 and 10 dpf, pictures were acquired in order to measure total length and egg and
159 yolk bag diameters (Frayse et al. 2006). Pictures were realized and analysed as described
160 before (Gagnaire et al. 2015).

161

162 2.7 DNA damage

163 The alkaline comet assay was used in order to detect DNA strand breaks (single- and double-
164 strand breaks, and alkali-labile sites) as described before (Gagnaire et al. 2015) according to
165 the procedure of Devaux et al. (1998).

166

167 2.8 Measurement of biomarkers of neurotoxicity and immunotoxicity

168 AChE and PO-like activities and ROS production were measured as described before
169 (Gagnaire et al. 2015).

170

171 2.9 Following of gene expression

172 RNA was extracted from three pools of fifteen 4, 7 and 10 dpf larvae per condition and the
173 methodology described in Gagnaire et al. (2015) was used to obtain the gene expression data.
174 Briefly, Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix was used in addition of
175 cDNA sample, primers (reverse and forward) and a referent dye on a Mx3000P real-time PCR

176 machine (Stratagene, Agilent). 40 cycles of amplification were used (30 sec at 95°C, 1 min at
177 60°C and 1 min at 72°C) followed by a final step for melting curve analyses. The relative
178 expression ratio of mRNA of every gene normalized by the reference gene was calculated
179 using the threshold cycle Ct on REST-384© version 2 software (Relative Expression
180 Software Tool, <http://www.gene-quantification.de/rest-384.html>) (Pfaffl et al. 2002). The
181 expression of the housekeeping gene *ef1* (elongation factor 1) was validated between all
182 conditions: Ct means \pm SE for controls and 4, 7 and 10 dpf larvae contaminated at 10^4 , 10^5 or
183 10^6 Bq/mL were not different (data not shown).

184

185 2.10 Microscopical observations

186 Three 4, 7 and 10 dpf larvae were used for microscopical observations as described before
187 (Gagnaire et al. 2015).

188

189 2.11 Statistical analyses

190 Results were expressed as means \pm standard error (se). Normality assumption was verified
191 using Shapiro–Wilk tests. When data were not normal, they were transformed using Boxcox
192 formula. Differences between controls and contaminated larvae were tested with t-tests or
193 ANOVAs followed by an *a posteriori* least significant difference (LSD) post-hoc test in the
194 case of rejection of H_0 when data were normal. Kruskal-Wallis or Mann-Whitney U tests
195 were used when data were not normal. Analyses were performed on STATISTICA Software
196 v12 (StatSoft, Inc., Tulsa, OK, USA). Significance was set at $p \leq 0.05$.

197 For comet assay statistical analysis, raw data of the individual cell tail moment were used.
198 The analyses were performed using R software (R_Development_Core_Team_ 2004). Tail
199 moments equal to zero were considered as artefact of the image analysis. However, they
200 represent “true” zeros, i.e. cells with no or few DNA damage, so, if any, they were replaced
201 by the smallest value of the slide considered. To deal with the nested design of the comet
202 assay, a linear mixed-effects model was constructed using the *nlme* package
203 (<https://CRAN.R-project.org/package=nlme>) with treatment (control or HTO) as fixed factor
204 and replicates (slides) as random factor. Then random permutation tests were applied using
205 the *pgirmess* packages (<https://CRAN.R-project.org/package=pgirmess>) and the number of
206 permutations was fixed to 2000.

207

208

209 **3. Results**

210 3.1 Tritium dose rate in eggs and larvae

211 After exposure to 10^4 Bq/mL of HTO, tritium dose rate was lower in 7 and 10 dpf larvae
212 when compared to 4 dpf larvae. After exposure to 10^5 Bq/mL, tritium dose rate was lower in 7
213 and 10 dpf larvae when compared to 24 hpf eggs. After exposure to 10^6 Bq/mL, tritium dose
214 rate was lower in 4 and 10 dpf larvae compared to 7 dpf larvae. For the three concentrations,
215 the mean tritium activity was significantly higher in eggs compared to larvae (1.12 and 1.15
216 times higher for 10^5 and 10^6 Bq/mL, respectively) (Table 2).

217 Despite these slight differences, the mean pondered dose rate of exposure all stages included
218 was calculated as 1.1×10^2 , 4.1×10^2 and 3.8×10^3 $\mu\text{Gy/h}$ for nominal exposure HTO
219 concentration of 10^4 , 10^5 and 10^6 Bq/mL, respectively (Table 2).

220

221 3.2 Effects on mortality, hatching and embryo-larvae development

222 No significant differences in mortality were observed between control and exposed groups for
223 all HTO concentrations on all experiments (data not shown; mean cumulated mortality for all
224 experiments at 10 dpf of 14.7% for controls and 16.4% for HTO).

225 No differences in HT_{50} between controls and contaminated groups were shown for all of the
226 HTO concentrations tested (Figure 1).

227 Morphological analyses performed on eggs and larvae showed differences in several stages
228 depending on the tritium level. At 10^4 Bq/mL, 24 hpf egg diameter and 3 and 7 dpf yolk bag
229 diameter were significantly higher and 4 dpf larvae length was significantly lower in exposed
230 animals when compared to controls. At 10^5 Bq/mL, 3 and 7 dpf yolk bag diameter and 3 dpf
231 larvae length were significantly lower and 10 dpf yolk bag diameter was significantly higher
232 in exposed animals compared to controls. At 10^6 Bq/mL, 24 and 48 hpf egg diameters and 48
233 hpf and 7 dpf yolk bag diameters were significantly higher in exposed animals when
234 compared to controls (Table 3).

235

236 3.3 Effects on DNA damage measured by the comet assay

237 After an exposure to 10^4 Bq/mL, only 4 dpf exposed larvae presented significantly less DNA
238 damage than controls. After an exposure to 10^5 or 10^6 Bq/mL, only 4 dpf exposed larvae
239 presented significantly higher DNA damage than controls (Table 4).

240

241

242

243 3.4 Effects on biomarkers of immunotoxicity and neurotoxicity

244 No effect of HTO was observed on PO-like and AChE activities after 4 days of exposure,
245 whatever the dose rate used (Table 5).

246 At 10^4 Bq/mL of HTO, ROS stimulation index showed no significant differences whatever
247 the stage studied (Table 6). ROS basal and stimulated levels were higher in exposed animals
248 compared to controls only for 7 dpf larvae (data not shown). At 10^5 Bq/mL of HTO, ROS
249 stimulation index was higher in exposed 4 dpf larvae compared to controls. ROS stimulated
250 levels were higher in contaminated animals compared to controls for 4, 7 and 10 dpf larvae,
251 while ROS basal level was higher in contaminated animals compared to controls only for 7
252 dpf larvae (data not shown). At 10^6 Bq/mL of HTO, ROS stimulation index was higher in
253 exposed 4 dpf larvae compared to controls. ROS basal level was lower in 7 dpf contaminated
254 larvae compared to controls (data not shown).

255

256 3.5 Effects on gene expression

257 The genes for which the expression was modified in presence of HTO depended on the
258 concentration studied.

259 At 10^4 Bq/mL of HTO, *mt2* (metallothionin) was 2.72 times more expressed in 4 dpf
260 contaminated larvae compared to controls. At 10^5 Bq/mL, only *mpx* (myeloid specific
261 peroxidase) was 2.7 times less expressed in 4 dpf contaminated larvae compared to controls.
262 After exposure at 10^6 Bq/mL of HTO, *cyp1a* (cytochrome P450 CYP1A) was 42.74 times
263 more expressed and *gstp1* (glutathione-S-transferase) was 50 times less expressed in 4 dpf
264 contaminated animals compared to controls; at the same activity concentration, *lyz* (lysozyme
265 C), *mpx* and *mt2* were 1.45, 2.45 and 1.57 times more expressed in 7 dpf contaminated
266 animals compared to controls, respectively, and *ache* (acetylcholinesterase) was 1.63 times
267 more expressed in 10 dpf contaminated larvae compared to controls (Table 7).

268

269 3.6 Histological observations

270 TEM observations on the tail muscular part of 4 dpf larvae showed a similar pattern of
271 responses for all concentration studied. A global degradation of myofibrils with an alteration
272 of actin and myosin filaments on A and I disks was observed (Figure 2). Z stria was still
273 observable, but the connections seemed to be broken (Figure 2). These alterations were rarely
274 observed at 10^4 (Figure 2B) and 10^5 Bq/mL of HTO (Figure 2C) compared to controls (Figure
275 2A). However, at 10^6 Bq/mL of HTO, the alterations were observed with a higher frequency
276 compared to lower concentrations (Figure 2D).

277 For 4 and 7 dpf larvae exposed to all dose rates, mitochondria seemed to be more numerous
278 compared to controls, indicating a high energetic activity in cells. However, no structural
279 differences appeared in mitochondria between contaminated 4 dpf larvae and controls (data
280 not shown). On the contrary, for 7 dpf larvae, important alterations were observed on muscle
281 mitochondria (Figure 3). For the two highest dose rates, mitochondrium cristae were
282 disorganized or missing; only external membranes of mitochondria were present. Some
283 mitochondria seemed to have disappeared from endomysium, leaving numerous free spaces in
284 muscular tissue (Figure 3C, D). For the lowest dose rate, mitochondria seemed normal but
285 numerous free spaces in the muscular tissue were present compared to controls (Figure 3B).
286 Only 10 dpf larvae exposed to 10^4 Bq/mL of HTO showed the same alterations in a more
287 important way compared to 7 dpf larvae, while for 10 dpf larvae exposed to 10^5 Bq/mL of
288 HTO, the degradations seemed less present compared to 7 dpf larvae (data not shown). Not
289 enough 10 dpf larvae were available at 10^6 Bq/mL of HTO to perform TEM analyses.

290

291 All of the results were summed up in Table 8.

292

293

294 4. Discussion

295 4.1 Dose rates calculated for tritiated water

296 Our results showed that tritium accumulates in eggs and larvae, as it was already shown for
297 tritiated thymidine (Adam-Guillermin et al. 2013). Our results also showed differences in
298 dose rates calculated in all stages, values being globally higher in eggs or young larvae (4
299 dpf). These differences could indicate that the HTO transfer behavior mechanisms can vary
300 depending on the life stage considered and the activity concentration of tritium in the
301 medium.

302 Exchanges of HTO between water and organisms are rapid, as maximal dose rates were
303 obtained for 24h eggs. Several studies showed that tritium accumulated in adult organisms
304 equilibrates with HTO concentration of the water and that OBT fraction (tritium bounded to
305 organic matter) equilibrates with the concentration of HTO internalized (Gagnaire et al. 2017,
306 Gagnaire et al. in press, Galeriu and Melintescu 2011, Kim et al. 2019, Kim and Korolevych
307 2013, Kim et al. 2015, Melintescu et al. 2015). A companion paper study presented in details
308 the discussion about internalization results obtained after tritium contamination for two stages
309 (24h eggs and 4 dpf larvae) and two HTO concentrations (10^5 and 10^6 Bq/mL) (Arcanjo et al.
310 2019).

311

312 4.2 Macroscopic effects of tritiated water (hatching, morphological measurements)

313 No differences of mortality and hatching were observed between conditions. In zebrafish,
314 tritiated thymidine induced a delay in hatching at 1.91×10^3 and 1.05×10^4 $\mu\text{Gy/h}$ (Adam-
315 Guillermin et al. 2013). Moreover, we showed no abnormalities in larvae exposed to HTO at
316 the same dose rates, while abnormalities were observed in zebrafish larvae after exposure to
317 tritiated thymidine (Adam-Guillermin et al. 2013). Exposure of medaka embryos for 30 days
318 to 1.2×10^3 $\mu\text{Gy/h}$ of HTO led to vertebral malformations and effects on fecundity of adults
319 (Hyodo-Taguchi and Etoh 1993). Moreover, the hatchability of common flounder,
320 *Paralichthys olivaceus*, was modified after 4 days of exposure to 1.21×10^5 $\mu\text{Gy/h}$ of tritium
321 (Ichikawa and Suyama 1974). Thus, tritium effects on hatching process seem to be related to
322 the form used, the total dose received and the biological stage.

323 Tritiated water induced some effects on macroscopical endpoints depending on the dose rate
324 studied. At 10^4 Bq/mL (i.e. 1.1×10^2 $\mu\text{Gy/h}$), exposed animals presented differences with the
325 controls for early stages (<4 dpf). At 10^5 Bq/mL (i.e. 4.1×10^2 $\mu\text{Gy/h}$), differences between
326 controls and exposed animals were seen only at older stages (3-10 dpf). At 10^6 Bq/mL (i.e.
327 3.8×10^3 $\mu\text{Gy/h}$), exposed animals presented lower egg and/or yolk-bag diameters at early and

328 old stages. Effects on developmental parameters including egg diameter has also been shown
329 on three-spined stickleback, *Gasterosteus aculatus* (Walden 1971), and in puffer, *Fugu*
330 *niphobles* (Ichikawa and Suyama 1974), eggs exposed to 1.21×10^5 and 1.21×10^6 $\mu\text{Gy/h}$ of
331 HTO, respectively. Indeed, HTO induced some effects on zebrafish larvae development,
332 indicating an effect on metabolism. It would be interesting to study the responses of older
333 larvae to HTO in order to see if the effects persist after 10 dpf.

334

335 4.3 Sub-individual responses to HTO

336 Tritiated water induced effects on DNA damage depending on the studied dose rate. At 10^4
337 Bq/mL (i.e. 1.1×10^2 $\mu\text{Gy/h}$), a decrease of DNA damage was observed in 4 dpf contaminated
338 larvae. An explanation of this result at the low dose rate could be an hormesis effect of
339 ionizing radiation, as it was already shown in fish (Mothersill and Seymour 2009). No effect
340 was shown for all dose rates in 7 and 10 dpf larvae. This result could indicate that repair
341 mechanisms are induced after 4 dpf in response to HTO. Other studies have shown that
342 tritium can induce genotoxicity, with an increase of chromosome aberrations in medaka eggs
343 exposed to 2.08×10^1 $\mu\text{Gy/h}$ (Suyama et al. 1981) and of DNA damage and MN frequency at
344 1.25×10^1 $\mu\text{Gy/h}$ in mussels (Jha et al. 2005). Tritiated thymidine also induced an increase of
345 DNA damage in 4 dpf larvae exposed at 1.05×10^4 $\mu\text{Gy/h}$ (Adam-Guillermin et al. 2012).
346 DNA damage were also induced *in vitro* in fish lymphocytes exposed to 10^{-1} - 10^2 Bq/mL of
347 HTO (Stuart et al. 2016) and in fathead minnow, *Pimephales promelas*, after and *in vivo* and
348 *in situ* exposure to tritium (Gagnaire et al. 2017, Gagnaire et al. in press). However, no DNA
349 damage was induced in rainbow trout, *Onchorynchus mykiss*, after exposure to 7.10^3 Bq/L
350 (Festarini et al. 2019). These results make us hypothesize that zebrafish could be more
351 resistant to HTO effects on DNA damage compared to other species.

352 These results corroborate those described by other authors, showing a non-linear genotoxic
353 response to HTO (Bannister et al. 2016, Stuart et al. 2016). This kind of responses could be
354 due to reparation and/or decrease of damages due to efficient cell elimination by apoptosis.
355 This hypothesis is realistic as a transcriptomic analysis performed in a companion study of 24
356 hpf and 4 dpf larvae exposed to HTO showed that some genes involved in DNA damage
357 repair and anti-apoptotic response (e.g. *bcl2l1*, *xpc*, *gadd45bb* et *xrcc1*) were over-expressed
358 in exposed animals (Arcanjo et al. 2018).

359 As a whole, these results indicated an increase of DNA damage in presence of tritium, which
360 is coherent with the known mechanisms of action of ionizing radiations (Adam-Guillermin et
361 al. 2012).

362

363 In our study, ROS production was modified by HTO with again a response depending on the
364 dose rate tested. Four dpf larvae exposed to 10^5 Bq/mL (i.e. 4.1×10^2 μ Gy/h) presented an
365 increase in ROS stimulation index as a result of an increase of both basal and stimulated ROS
366 levels. At this dose rate, the expression of *mpx* (myeloid specific peroxidase) gene, which is
367 involved in reduction of H_2O_2 during the oxidative burst, was lower in contaminated larvae,
368 indicating that HTO induced effects on elements playing a role in oxidative stress. Indirect
369 effects of HTO, by a modulation of ROS levels, could therefore be responsible of the DNA
370 damage observed. Moreover, ROS stimulated levels were decreased in 7 dpf animals exposed
371 to 10^6 Bq/mL (i.e. 3.8×10^3 μ Gy/h). The decrease of ROS PMA-stimulated levels by HTO
372 could indicate that fish may not be able to stimulate defence capacities to an infectious
373 disease.

374 For other HTO dose rates tested, *mpx* expression was increased only in 7 dpf larvae exposed
375 to 10^6 Bq/mL (i.e. 4.8×10^3 μ Gy/h). No effect was shown on ROS stimulation index at 10^4
376 Bq/mL (i.e. 1.1×10^2 μ Gy/h). Even though there is no direct link between ROS production
377 levels and DNA damage, our results showed that the study of ROS production allows to better
378 understand the mechanisms deployed in response to HTO exposure in zebrafish larvae.

379 Interestingly, the expression of several genes related to detoxication process (*mt2*, *cypla* and
380 *gstp1*) were modified after 4 and 7 days of exposure to 10^4 Bq/mL (i.e. 1.1×10^2 μ Gy/h) and
381 10^6 Bq/mL (i.e. 4.8×10^3 μ Gy/h). MT are known to play a role in cell protection towards
382 oxidative stress (Sato and Kondoh 2002). Detoxication could therefore be a mechanism
383 relevant in the study of effects of HTO in aquatic organisms.

384 The expression of *lyz* gene was slightly increased only in 7 dpf larvae contaminated to 10^5
385 Bq/mL (i.e. 4.1×10^2 μ Gy/h), possibly indicating a stimulation of the innate immune system.
386 However, this response was transient as it was observed neither in older larvae nor at other
387 dose rates.

388

389 No effect of HTO was observed on PO-like activity after 4 days of exposure for all dose rates.
390 This biomarker does not seem relevant for the study of HTO effects on fish.

391 No effect was observed on AChE activity after 4 days of exposure to all dose rates of HTO. A
392 slight increase in *ache* expression was shown only in 10 dpf larvae exposed to 10^6 Bq/mL (i.e.
393 4.8×10^3 μ Gy/h). Therefore, the nervous system does not seem to be affected by tritium.

394

395 Histological observations of 4 dpf larvae showed global degradation of myofibrils of tail
396 muscle for all dose rates, with an increase of observations of these alterations with the dose
397 rate. The affected part being the contractile part of the muscle, an increase in alterations could
398 eventually have consequences on larvae motility. In 7 and 10 dpf larvae, the alterations to
399 myofibrils observed at 4 dpf seemed to be repaired, probably due to the important cell
400 replacement in this organism in embryo-larval development. However, the intensity of the
401 alterations observed on mitochondria for 7 and 10 dpf larvae suggests an important functional
402 alteration or accelerated aging, possibly leading to the end of the energetic process of
403 muscular tissue and alteration in cell respiration (Delbart, 2000). This phenomenon could be
404 due to a progressive muscular dystrophia. The increase of mitochondria alterations could also
405 be linked to the increase of ROS basal level in exposed larvae, which could be toxic for the
406 organelle when excessively present. However, for 10^5 Bq/mL of HTO, the alterations seemed
407 to be less important on 10 dpf larvae compared to 7 dpf larvae, while for 10^4 Bq/mL of HTO
408 alterations seemed more important at 10 dpf compared to 7 dpf. As for DNA damage, we can
409 hypothesize that this result could be due to an increase of mitochondria destruction by cell
410 autophagy and apoptosis in 10 dpf larvae as a compensatory response to HTO induced-stress.
411 It would be interesting to study older larvae to demonstrate if the alterations are still present
412 or repaired by the organism.

413

414 4.4 Comparison to gamma irradiation

415 Results of previous studies on zebrafish larvae exposed to gamma irradiation showed that
416 gamma irradiation induced either a delay in hatching after acute exposure (0.3-2 Gy during 1
417 min) (Pereira et al. 2011), or an acceleration of hatching and abnormalities after 96h exposure
418 at 3.33×10^1 and 2.37×10^4 μ Gy/h (Gagnaire et al. 2015, Simon et al. 2011). Gamma irradiation
419 at 2.37×10^4 μ Gy/h also induced effects on 4 dpf zebrafish larvae, with a decrease of yolk bag
420 diameter in exposed animals, indicating an increase in energetic reserve consumption
421 (Gagnaire et al. 2015). We saw here that HTO induced no effects on hatching and slight
422 effects on developmental parameters (increase of yolk-bag diameter, decrease of length)
423 rather indicating a disruption of energy consumption in these larvae. On the contrary,
424 tritiated thymidine induced drastic effects (malformations, hatching delay) at dose rates to
425 DNA of 1-7 mGy/h, indicating an important toxicity of this form for these high dose rates
426 (Adam-Guillermin et al. 2013). Therefore, different types of exposure to radionuclides have
427 different consequences on hatching process and development.

428

429 Gamma irradiation at $3.33 \times 10^1 \mu\text{Gy/h}$ decreased ROS stimulation index due to an increase of
430 ROS basal levels in irradiated larvae (Gagnaire et al. 2015). It should be noted that this dose
431 rate is close to the benchmark value of $10^1 \mu\text{Gy/h}$ recommended for ecosystem protection
432 towards ionizing radiations (Garnier-Laplace et al. 2010). Moreover, 24 hpf eggs exposed to
433 $2.37 \times 10^4 \mu\text{Gy/h}$ showed an increase in DNA damage (Gagnaire et al. 2015). Taken together,
434 the present results confirmed the existence of an oxidative stress conducting to DNA damage
435 after fish exposure to radionuclides like tritium. The induction of an oxidative stress can
436 therefore be considered as a common mode of action to both kind of exposure: external
437 irradiation and HTO contamination.

438
439 Neither HTO nor gamma irradiation induced any effect on phenoloxidase activity. HTO
440 modulated the expression of genes involved in detoxication, while gamma irradiation at
441 $3.33 \times 10^1 \mu\text{Gy/h}$ decreased EROD activity in 4 dpf larvae (Gagnaire et al. 2015). Detoxication
442 could therefore be a mechanism common to several types of radionuclides and relevant in the
443 study of effects of such contaminants in aquatic organisms.

444
445 At the tissular level, HTO induced alterations in myofibrils after 4 days of exposure. It is
446 interesting to point out that the same alteration was already observed in a more pronounced
447 way with gamma irradiation, conducting to a dilatation of endomysium (Gagnaire et al. 2015),
448 not observed with HTO. The alterations to muscle seem to be a common mode of action of
449 HTO and gamma irradiation. However, HTO induced no effect on neurotransmission. On the
450 contrary, gamma irradiation induced disruptions of this mechanism at the biochemical and
451 molecular levels (Gagnaire et al. 2015). Moreover, HTO modulated the expression of *lyz*
452 gene, while gamma irradiation did not (Gagnaire et al. 2015). These results showed again that
453 tritium and gamma irradiation have different mechanisms of action in zebrafish.

454
455 It is important to consider that energy deposition following an exposure to tritiated thymidine
456 directly impacts the DNA, contrary to HTO and gamma irradiation for whom the dose is
457 distributed in a more homogenous way in the cell, therefore more diluted (Adam-Guillermin
458 et al. 2012). The doses calculated at the scale of an egg or a larvae for HTO, tritiated
459 thymidine and gamma irradiation are therefore not directly comparable.

460

461 **5. Conclusion**

462 The present study investigated the effects of a contamination to HTO at different dose rates
463 on biomarkers and macroscopical parameters of zebrafish larvae. The results showed that
464 tritium was internalised in eggs and in larvae at all dose rates tested. They also showed that
465 differences in the sensitivity of biomarkers measured can exist. Some effects were observed
466 on the parameters of development. We showed that HTO could modify zebrafish parameters
467 including ROS production and induced muscle alterations and DNA damage. These
468 parameters could therefore be relevant parameters for assessing tritium-related stress.
469 However, the responses to HTO were not linear with the increasing dose rate, probably due to
470 apoptosis. On the contrary, PO-like and AChE activities did not seem relevant to study of
471 tritium effects in larvae. However, effects differed considering the dose rate of HTO used.
472 HTO effects on zebrafish larvae were observed at several levels at dose rates ranging from
473 1.1×10^2 to 3.8×10^3 $\mu\text{Gy/h}$, which is in the range or even higher than the derived consideration
474 reference levels (DCRLs) for fish of 4.1×10^1 - 4.1×10^2 $\mu\text{Gy/h}$ (ICRP 2008). These DCRLs
475 correspond to a “band of dose rate within which there is likely to be some chance of
476 deleterious effects of ionizing radiation occurring to individuals” (ICRP 2008). Our results
477 seem to validate the accuracy of this DCRL. However, the dose rates we tested were greatly
478 higher than the benchmark value of 10 $\mu\text{Gy/h}$ for the protection of all ecosystems towards
479 ionizing radiations (Garnier-Laplace et al. 2010). As at the lowest dose rate of HTO tested, we
480 saw few effects, hence our results do not seem to question the relevance of this protection
481 threshold.
482 When comparing results of HTO to gamma irradiation, some mechanisms seem common for
483 both types of exposure (muscle alterations, DNA damage, oxidative stress), but gamma
484 irradiation seem more deleterious to zebrafish larvae at lower or higher dose rates than the
485 ones tested on HTO on the present study. Some more experiments are planned to acquire the
486 whole results presented here on similar dose rate of irradiation gamma and of tritiated
487 thymidine in order to refine a value for the Relative Biological Effectiveness (RBE) of tritium
488 (Adam-Guillermin et al. 2012).

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491 **Figure captions**

492

493 **Figure 1:** Hatching Time (HT₅₀) in control and contaminated zebrafish larvae for 10⁴ (A), 10⁵
494 (B) and 10⁶ (C) Bq/mL of HTO. Error bars correspond to 95% confidence intervals of the
495 HT₅₀ values.

496

497 **Figure 2:** muscular tissue of 4 dpf zebrafish larvae observed by TEM in control (A) and
498 contaminated at 10⁴ (B), 10⁵ (C) and 10⁶ (D) Bq/mL of HTO. A: A disk, E: endomysium, I: I
499 disk, M: M stria, My: myofibrils, S: sarcomere, Z: Z stria, Mi: mitochondria.

500

501 **Figure 3:** muscular tissue of 4 dpf zebrafish larvae observed by TEM in control (A) and
502 contaminated at 10⁴ (B), 10⁵ (C) and 10⁶ (D) Bq/mL of HTO. My: myofibrils, S: sarcomere,
503 Z: Z stria, Mi: mitochondria.

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Table 1: external and internal dose coefficients (DC) calculated with EDEN v2 software (IRSN) for zebrafish egg and larvae.

DC ($\mu\text{Gy/h per Bq/g}$)	24 hpf	4 dpf	7 dpf	10 dpf
Internal DC	6.71×10^{-3}	6.71×10^{-3}	6.71×10^{-3}	6.71×10^{-3}
External DC	3.77×10^{-5}	5.21×10^{-5}	1.03×10^{-4}	1.15×10^{-4}

524 **Table 2:** tritium dose rates ($\mu\text{Gy/h}$) based on internal concentrations in 24 hpf eggs and 4, 7 and 10 dpf larvae exposed to HTO. Values are expressed
 525 as means \pm se, n=7-15 per condition. Bold values indicate a significant difference between conditions. Letters indicate significant difference between
 526 stages for a given HTO water concentration (Kruskal-Wallis test, $p \leq 0.05$): a<b<c.

527

Nominal HTO concentration (Bq/mL)	24 hpf	4 dpf	7 dpf	10 dpf	Mean
10^4	$9.1 \times 10^1 \pm 1.2 \times 10^1$ ab	$1.7 \times 10^2 \pm 1.3 \times 10^1$ b	$9.5 \times 10^1 \pm 1.3 \times 10^1$ a	$8.5 \times 10^1 \pm 1.1 \times 10^1$ a	$1.1 \times 10^2 \pm 5.2 \times 10^1$
10^5	$5.2 \times 10^2 \pm 1.3 \times 10^1$ c	$4.6 \times 10^2 \pm 1.3 \times 10^1$ bc	$2.8 \times 10^2 \pm 9.8$ a	$3.7 \times 10^2 \pm 1.6 \times 10^1$ ab	$4.1 \times 10^2 \pm 1.1 \times 10^1$
10^6	$3.8 \times 10^3 \pm 1.5 \times 10^2$ ab	$3.3 \times 10^3 \pm 1.5 \times 10^2$ a	$4.7 \times 10^3 \pm 2.4 \times 10^2$ b	$3.4 \times 10^3 \pm 1.5 \times 10^2$ a	$3.8 \times 10^3 \pm 8.5 \times 10^2$

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530 **Table 3:** measurements in mm of egg diameter, total length and yolk bag (YB) diameter in zebrafish egg and larvae control and exposed (at 10⁴, 10⁵
531 and 10⁶ Bq/mL of HTO) groups. Values are expressed as means of at least 10 replicates. Bold values indicate a significant difference between
532 conditions. T test, p≤0.05; a, b: significantly different, a<b. n.d.: not determined.

	24 hpf		48 hpf		3 dpf		534
	Egg diameter (μm)	YB diameter (μm)	Egg diameter (μm)	YB diameter (μm)	Length (μm)	YB diameter (μm)	
Control	1177.5 ± 11.4 a	489.6 ± 12.4	465.8 ± 6.2	1173.0 ± 11.0	2943.5 ± 126.7	347.6 ± 21.1 a	
HTO 10⁴ Bq/mL	1213.4 ± 10.5 b	478.3 ± 5.3	474.2 ± 6.0	1201.8 ± 13.5	3127.5 ± 46.0	399.1 ± 12.5 b	
Control	1175.3 ± 12.4	502.8 ± 5.8	1164.1 ± 8.9	494.0 ± 8.7	3208.9 ± 39.3 b	410.7 ± 9.9 b	
HTO 10⁵ Bq/mL	1127.5 ± 21.2	508.6 ± 12.3	1152.3 ± 21.2	498.1 ± 13.6	3017.5 ± 57.1 a	358.7 ± 10.9 a	
Control	1156.4 ± 11.3 a	509.4 ± 5.2	1155.1 ± 16.1 a	456.7 ± 6.6 a	n.d.	n.d.	
HTO 10⁶ Bq/mL	1226.1 ± 10.3 b	506.0 ± 8.2	1213.2 ± 14.9 b	503.9 ± 11.2 b	n.d.	n.d.	

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	4 dpf		7 dpf		10 dpf	
	Length (μm)	YB diameter (μm)	Length (μm)	YB diameter (μm)	Length (μm)	YB diameter (μm)
Control	3480.0 ± 30.8 b	351.1 ± 8.8	3950.5 ± 52.7	325.2 ± 6.4 a	3688.6 ± 78.9	312.5 ± 11.6
HTO 10⁴ Bq/mL	3335.1 ± 63.4 a	329.5 ± 15.0	4027.5 ± 35.8	361.9 ± 12.5 b	3585.2 ± 112.2	294.7 ± 11.7
Control	3406.6 ± 35.1	325.6 ± 10.2	3995.6 ± 37.1	387.2 ± 9.7 b	4029.2 ± 70.4	291.6 ± 7.8 a
HTO 10⁵ Bq/mL	3351.9 ± 39.2	339.1 ± 8.5	3915.2 ± 57.7	322.0 ± 8.3 a	3876.6 ± 76.3	317.1 ± 8.5 b
Control	3300.8 ± 47.4	325.7 ± 10.4	3779.4 ± 63.9	282.4 ± 8.8 a	n.d.	n.d.
HTO 10⁶ Bq/mL	3379.1 ± 57.6	322.1 ± 6.3	3625.1 ± 60.6	339.6 ± 8.5 b	n.d.	n.d.

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537 **Table 4:** mean tail moments (comet assay) obtained for zebrafish 24 hpf embryos (n=3 pools
 538 of 10) and 4, 7 and 10 dpf larvae (n=10) control and exposed (at 10⁴, 10⁵ and 10⁶ Bq/mL of
 539 HTO) groups. Mix model (R), p≤0.001; Bold values indicate a significant difference between
 540 conditions. a, b: significantly different, a<b. n.d.: not determined.

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	24 hpf	4 dpf	7 dpf	10 dpf
Control	4.80 ± 0.31	3.68 ± 0.14 b	0.87 ± 0.06	0.42 ± 0.03
HTO 10⁴ Bq/mL	4.93 ± 0.21	1.39 ± 0.06 a	1.47 ± 0.10	0.39 ± 0.03
Control	1.82 ± 0.09	0.84 ± 0.04 a	2.20 ± 0.11	n.d.
HTO 10⁵ Bq/mL	1.87 ± 0.09	1.30 ± 0.07 b	2.47 ± 0.13	n.d.
Control	1.46 ± 0.06	0.86 ± 0.04 a	1.15 ± 0.08	0.78 ± 0.06
HTO 10⁶ Bq/mL	1.78 ± 0.13	2.09 ± 0.13 b	1.47 ± 0.09	0.83 ± 0.07

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545 **Table 5:** PO-like (U/larva) and AChE activities (nmol ATCi/min/mg proteins) in zebrafish
 546 larvae contaminated during 4 days at 10⁴, 10⁵ and 10⁶ Bq/mL of HTO. Values are means of
 547 10 replicates for each condition; standard error is presented. T-test or U Mann Witney test,
 548 p<0.05.

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	PO-like activity (U/larva)	AChE activity (nmol ATCi/mg/mg proteins)
Control	1.11 ± 0.09	167.9 ± 21.3
HTO 10⁴ Bq/mL	1.03 ± 0.07	195.7 ± 38.0
Control	1.29 ± 0.12	68.1 ± 10.8
HTO 10⁵ Bq/mL	1.08 ± 0.04	63.9 ± 10.4
Control	1.63 ± 0.35	173.7 ± 16.0
HTO 10⁶ Bq/mL	1.94 ± 0.28	146.7 ± 10.4

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555 **Table 6:** ROS stimulation index in *D. rerio* larvae contaminated during 10 days at 10^4 , 10^5
556 and 10^6 Bq/mL of HTO. Values are expressed as means of 20 replicates (10 for stimulated
557 levels and 10 for basal levels); standard error is presented. T test, p0.05; Bold values indicate
558 a significant difference between conditions. a, b: significantly different, a<b.

559

ROS stimulation index	4 dpf			7 dpf			10 dpf		
Control	1.17	±	0.06	1.18	±	0.03	1.22	±	0.02
HTO 10^4 Bq/mL	1.24	±	0.10	1.20	±	0.03	1.22	±	0.03
Control	1.08	±	0.08	1.21	±	0.06	1.15	±	0.07
HTO 10^5 Bq/mL	1.22	±	0.02	1.28	±	0.05	1.19	±	0.09
Control	1.14	±	0.10	1.26	±	0.17	0.87	±	0.08
HTO 10^6 Bq/mL	1.58	±	0.12	1.12	±	0.16	0.78	±	0.09

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580 **Table 7:** relative expression of genes (normalized to reference gene *ef1*) compared to control
 581 in larvae contaminated during 10 days at 10⁴, 10⁵ and 10⁶ Bq/mL of HTO (n=9). Analysis
 582 with REST-384©, p≤0.05; bold values indicate a significant difference compared to control.

	4 dpf			7 dpf			10 dpf		
	10 ⁴ Bq/mL	10 ⁵ Bq/mL	10 ⁶ Bq/mL	10 ⁴ Bq/mL	10 ⁵ Bq/mL	10 ⁶ Bq/mL	10 ⁴ Bq/mL	10 ⁵ Bq/mL	10 ⁶ Bq/mL
<i>ache</i>	1.38	1.74	0.99	1.39	1.19	1.57	1.28	1.31	1.63
<i>bax</i>	1.61	1.28	0.74	1.29	0.77	1.27	1.12	0.75	1.48
<i>chat</i>	1.29	1.40	0.79	-1.40	1.29	1.29	2.13	1.07	1.43
<i>cypla</i>	1.63	1.55	42.74	1.52	0.83	0.86	-1.17	0.78	1.30
<i>gstpl</i>	0.93	1.34	0.02	1.36	1.27	0.95	1.02	0.74	0.69
<i>lyz</i>	1.13	0.76	1.34	1.79	0.84	1.45	-1.76	0.92	1.29
<i>mpx</i>	1.20	0.37	1.02	1.34	0.59	2.45	1.50	0.83	1.52
<i>mt2</i>	2.72	0.98	0.87	1.04	1.39	1.57	1.50	1.37	1.48

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585 **Table 8:** synthesis of results obtained for exposition of zebrafish to three dose rates of HTO. C: control; ∅: no significant effect; -: not tested;
 586 ND: not determined. Diam: Diameter.

Stage	10 ⁴ Bq/mL				10 ⁵ Bq/mL				10 ⁶ Bq/mL			
	24 hpt	4 dpt	7 dpt	10 dpt	24 hpt	4 dpt	7 dpt	10 dpt	24 hpt	4 dpt	7 dpt	10 dpt
Dose rate (μGy/h)	9.1x10 ¹	1.7x10 ²	9.5x10 ¹	8.5x10 ¹	5.2x10 ²	4.6x10 ²	2.8x10 ²	3.7x10 ²	3.8x10 ³	3.3x10 ³	4.7x10 ³	3.4x10 ³
Total dose (μGy)	2.2x10 ³	1.6x10 ⁴	1.6x10 ⁴	2.0x10 ⁴	1.2x10 ⁴	4.5x10 ⁴	4.7x10 ⁴	8.8x10 ⁴	9.1x10 ⁴	3.2x10 ⁵	7.8x10 ⁵	8.2x10 ⁵
Development	Egg diam > C	Length < C	RV diam > C	∅	∅	∅	RV diam < C	RV diam > C	Egg diam > C	∅	RV diam > C	ND
DNA damage	∅	< C	∅	∅	∅	> C	∅	ND	∅	>T	∅	∅
Gene expression	-	MT2 > C	∅	∅	-	Mpx < C	∅	∅	-	Cyp1a>T GSTp1<T	Mpx > C Lyz > C MT2 > C	Ache > C
ROS production	-	∅	Basal and stimulated activities > C	∅	-	Stimulated activity > C Index > C	Stimulated and basal activities > C	Stimulated activity > C	-	Index > C	Stimulated activity < C	∅
Histology of muscle	-	Rare alterations of myofibrils	Mitochondria alterations	Mitochondria alterations	-	Rare alterations of myofibrils	Mitochondria alterations	Mitochondria alterations	-	Alterations of myofibrils	Mitochondria alterations	ND

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