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1 **Effects of tritiated water on locomotion of zebrafish larvae: a new insight in tritium toxic**  
2 **effects on a vertebrate model species**

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14

15 **Abstract**

16 Tritium (<sup>3</sup>H), a radioactive isotope of hydrogen, is ubiquitously present in the  
17 environment. In a previous study, we highlighted a mis-regulation of genes involved in  
18 muscle contraction, eye transparency and response to DNA damages after exposure of  
19 zebrafish embryo-larvae from 3 hpf to 96 hpf at 0.4 and 4 mGy/h of tritiated water (HTO).  
20 The present study aimed to link this gene mis-regulation to responses observed at higher  
21 biological levels. Zebrafish embryo-larvae were exposed to 0.4 and 4 mGy/h. Analyses on  
22 spontaneous tail movement, locomotor activity and heart rate were performed. Histological  
23 sections of eyes were made to evaluate the impact of HTO on eye transparency and whole  
24 embryo immunostainings were realized to assess DNA double strand breaks repair using  
25 gamma-H2AX foci. We found a decrease of basal velocity as well as a decrease of response in  
26 96 hpf larvae exposed at 0.4 mGy/h after a tactile stimulus as compared to controls.  
27 Histological sections of larvae eyes performed after the exposure to 4 mGy/h did not show  
28 obvious differences in lens transparency or retinal development between contaminated and

29 control organisms. Gamma-H2AX foci detection revealed no differences in the number of  
30 gamma-H2AX foci between contaminated organisms and controls, for both dose rates.  
31 Overall, results highlighted more detrimental effects of HTO exposure on locomotor  
32 behavior in 96 hpf larvae exposed at the lowest dose rate. Those results could be linked to  
33 mis-regulation of gene involved in muscle contraction found in a previous study at the same  
34 dose rate. It appears that not all effects found at the molecular scale were confirmed using  
35 higher biological scales. These results could be due to a delay between gene expression  
36 modulation and the onset of physiological disruption or homeostatic mechanisms to deal  
37 with tritium effects. However, crossing data from different scales highlighted new pathways  
38 to explore, i.e. neurotoxic pathways, for better understanding HTO effects on organisms.

39

#### 40 **Keywords**

41 Tritiated water; Zebrafish embryo-larvae; locomotor-related behavior ; heart-beats ; eye  
42 histology; DNA double strand breaks

43

#### 44 **1. Introduction**

45 Organisms are largely exposed to radionuclides originating from natural or anthropic  
46 sources. Among them, tritium ( $^3\text{H}$ ) can be found in the environment coming from both  
47 sources. As a radioactive isotope of hydrogen, tritium enters in the water cycle and  
48 therefore is present in all environmental compartments (Adam-Guillermin et al., 2010). The  
49 decay of tritium, following a half-time of 12.32 years, emits a beta particle of low energy  
50 (mean = 5.7 keV) and low penetration range (mean = 0.56  $\mu\text{m}$  in water) (HPA, 2007). This last  
51 property implies that the energy deposition in tissues is very localized and gives to tritium a  
52 high ionization capacity that can be considered negligible for external exposure (Mathur-De  
53 Vre and Binet, 1984). However, if tritium is integrated in organism tissues, the effects could  
54 be more detrimental than external exposure.

55 The tritium activity background, i.e. without nuclear facilities influences, in southern  
56 France rivers ranges from 0.12 to 0.86 Bq/L (Ducros et al., 2018). In marine environment, the  
57 background of tritium activities ranges from 0.02 to 0.15 Bq/L (Eyrolle-Boyer, 2017). In

58 France, tritium is mainly discharged in the environment by nuclear power plants and nuclear  
59 reprocessing plants in normal operations in the form of tritiated water (HTO) or tritiated  
60 hydrogen (HT). The average activity measured in nuclear power plants liquid effluents in  
61 normal operations is of  $2 \times 10^6$  Bq/L making tritium one of the most discharged radionuclides  
62 in terms of activity compared to other radionuclides (Adam-Guillermin et al., 2010).  
63 However due to the tritium properties of rapid dilution in the water cycle, activities  
64 measured in rivers downstream of the nuclear facilities range from 1 to 65 Bq/L (Eyrolle-  
65 Boyer, 2017). In the English Channel, downstream of the nuclear facilities tritium activity is  
66 about 10 Bq/L and gradually decrease along the coast (Eyrolle-Boyer, 2017). In the future,  
67 the development of new nuclear facilities, such as European Pressurized Reactor (EPR) or  
68 International Thermonuclear Experimental Reactor (ITER), is expected to increase tritium  
69 discharge in environment. That is why, understanding the risks of tritium on aquatic  
70 organisms is of growing concern in the scientific community.

71 The effects of tritiated water exposure were studied over a large range of species, from  
72 mammals through the effects on reproduction in mice or rats (Cahill and Yuile, 1970; Laskey  
73 et al., 1973; Lowry Dobson and Cooper, 1974) and human cells to assess DNA damages  
74 (Gonen et al., 2014) to aquatic invertebrates or vertebrates (Adam-Guillermin et al., 2012).  
75 For those organisms, studies ranged from *in vitro* (Stuart et al., 2016) to *in situ* (Gagnaire et  
76 al., 2017) through laboratory studies mainly focusing on developmental and reproductive  
77 effects (Adam-Guillermin et al., 2012). Despite the wide range of species in which effects of  
78 HTO exposure were studied, there is still a lack of knowledge of tritium modes of action at  
79 the molecular scale, the transgenerational effects, the adaptation or the behavior of  
80 organisms (Adam-Guillermin et al., 2012, 2010).

81 The present work is part of a project which aims to identify the modes of action of  
82 tritiated water. Explaining that activities chosen for exposures are widely above those found  
83 in natural environment, under nuclear facilities influence or not. To reach this objective, an  
84 aquatic vertebrate model was used, the zebrafish, *Danio rerio*. The embryo larval stages  
85 were chosen, as early developmental stages are supposed to be more sensitive to pollutant  
86 and as the effects of pollutants on this early stage could induce detrimental effects during  
87 the rest of the fish development (Frayssé et al., 2006). Furthermore, these early stages are  
88 recommended in OECD guidelines for the testing of chemicals (OECD, 2013). Zebrafish is a

89 very convenient laboratory model especially when the use of the embryo-larvae stages is  
90 required, as in this project. Indeed, adults can spawn hundreds of transparent eggs in a  
91 single spawning (Spence et al., 2008). The genome of this species is fully sequenced, and a  
92 wide variety of mutants are now available (Scholz et al., 2008), which is useful when focusing  
93 on modes of action. Furthermore, zebrafish is an emerging model for studying behavior and  
94 many tools are now available to assess plenty of behavioral parameters in adult and embryo-  
95 larvae of zebrafish (Ahmad et al., 2012). In this project, a previous study was conducted and  
96 focused on the effects of HTO exposure on zebrafish early life stages at the molecular scale  
97 using a transcriptomic analysis by mRNAseq (Arcanjo et al., 2018). This study revealed an  
98 effect of HTO exposure on the expression of genes involved in muscle contraction, eye  
99 transparency, circadian clock, response to reactive oxygen species and repair of DNA  
100 damages. The aim of the present study was to complete the results of this previous work by  
101 investigating if the effects observed at the molecular scale were confirmed at higher  
102 biological scales including tissue and individual, more relevant from an ecological point of  
103 view. To reach this objective, 3 hpf (hours post-fertilization) zebrafish eggs were exposed to  
104 the same dose rates that were previously used (i.e. 0.4 and 4 mGy/h). Both of these dose  
105 rates corresponded to a band of dose rates within which there is some chance of observing  
106 some deleterious effects on young fish (e.g. reduction in resistance to infection and  
107 reproductive success) (ICRP, 2012). In term of activity concentrations, these two doses rates  
108 corresponded to tritiated water activities of  $1.22 \times 10^5$  and  $1.22 \times 10^6$  Bq/mL, respectively. As  
109 the first part of the study highlighted differential expression of genes involved in muscle  
110 contraction (skeletal and cardiac) (Arcanjo et al., 2018), 24 hpf embryos to 96 hpf larvae  
111 were challenged with behavioral analyses including spontaneous tail movements, heart rate  
112 and free-swimming behavior. Histological observations of eyes from 48 hpf to 96 hpf were  
113 also performed to make the link with modulation of genes involved in eye transparency.  
114 Finally, a whole mount immunostaining was performed to detect gamma-H2AX foci, an early  
115 marker of double strand break repair process (Rogakou et al., 1998), as some genes involved  
116 in DNA repair pathways were found mis-regulated in a previous study (Arcanjo et al., 2018).

117

## 118 **2. Materials and methods**

### 119 ***2.1. Fish maintenance and eggs production***

120 Adult wild type zebrafish (AB genetic background) were maintained in a Tecniplast©  
121 ZebTEC system in 8L tanks (20 adults per tank) with a temperature of 28°C, males and  
122 females being kept separated. A photoperiod of 12/12h dark-light cycles was used. Eggs  
123 were produced by mating couples in 1.7L Breeding Tank Beach Style Design (Tecniplast©) in  
124 a thermoregulated room at 28°C. Males and females were kept apart in the same breeding  
125 tank during the night before the mating. The morning of mating, couples were allowed to  
126 mate, and eggs were sampled 30 min after the beginning of the mating in order to obtain  
127 synchronised eggs. All eggs were pooled together, sorted, counted and kept in petri dish  
128 with embryo medium (60mg/L of Instant Ocean© sea salt diluted in ultrapure water  
129 (Westerfield, 1995)), at  $28 \pm 0.5^\circ\text{C}$  until the beginning of the experiments.

130

## 131 ***2.2. Eggs contamination and sampling***

132 Tritiated water source (97% purity, 185 MBq, PerkinElmer, Courtaboeuf, France) was diluted  
133 in embryo medium to obtain final activity concentrations of  $1.22 \times 10^5$  and  $1.22 \times 10^6$  Bq/mL.  
134 At 3 hpf, eggs were contaminated respecting a density of 5 eggs per 2 mL of HTO solution in  
135 three experimental units (three replicates). To perform the behavioural and physiological  
136 experiments, the histological sections and the whole mount immunostaining, 1620 eggs  
137 were used for both conditions, control and HTO-contaminated (135 eggs \* 3 replicates \* 2  
138 treatments \* 2 dose rates). The eggs were kept in the dark at  $27.7 \pm 0.3^\circ\text{C}$  in a thermo-  
139 regulated incubator confined inside a glove box. Control eggs were placed in embryo  
140 medium and kept in the same manner as contaminated organisms ( $27.8 \pm 0.07^\circ\text{C}$ ) in the  
141 same room outside the glove box. Water in contaminated and control experimental units  
142 was renewed once (at 72h) during the experiment to ensure water quality and constant  
143 tritiated water activity. Samples of contamination medium were made to monitor tritium  
144 activity along the experiments in the three experimental units. These samples were analysed  
145 by liquid scintillation counting. Briefly, samples were diluted with tritium-free water (Les  
146 Abatilles, Arcachon, France) to obtain a volume of 1 mL. A liquid scintillation cocktail (Ultima  
147 Gold LLT, PerkinElmer) was then added to a final volume of 20 mL. Samples were analysed  
148 using a Quantulus 1220 (DL = 5 Bq/L, PerkinElmer, WinQ software) and spectrum were  
149 analysed using the EASY View Spectrum analysis Program (PerkinElmer). To avoid cross-  
150 contamination between the two HTO activity concentrations, experiments were conducted

151 separately. The statistical analyses performed to compare mean activity concentration  
152 between experimental units showed no differences for both activities tested in this study.  
153 Measured activity concentrations along experiments and for all experimental units were of  
154  $1.10 \times 10^5 (\pm 5.72 \times 10^3)$  Bq/mL and  $1.35 \times 10^6 (\pm 8.89 \times 10^4)$  Bq/mL respectively for the lowest and  
155 the highest activity concentration tested.

156 Concerning the sampling of organisms needed for the behavioral and histological  
157 analyses as well as for the gamma-H2AX immunostaining, contaminated organisms were  
158 washed three times in non-tritiated embryo medium to eliminate tritium contamination  
159 around the eggs that could lead to over-estimate radiological dose. Control organisms were  
160 handled in the same way. This protocol was optimised for zebrafish (Arcanjo et al., 2019).

161

### 162 **2.3. Behavioral analyses**

163 All behavioral analyses were preceded by an acclimation period of 30 min for all  
164 organisms (control and contaminated) in the behavioral room. Acclimation was performed in  
165 a dark thermoregulated incubator (AquaLytic, Germany) at  $28 \pm 1^\circ\text{C}$ . Videos for the  
166 assessment of spontaneous tail movements of chorionated embryos at 24 hpf and heart rate  
167 of 48 hpf and 72 hpf larvae were performed under a stereomicroscope (Nikon SMZ800)  
168 connected to a high-resolution camera (acA1300-60gm, Basler, Germany) under the  
169 behavioural room light and temperature ( $28^\circ\text{C}$ ). Recording were performed using Media  
170 Recorder Software (v.4.0.542.1, Noldus Information Technology, Netherlands) and the  
171 analysis of videos was performed using the DanioScope Software (v.1.1.110, Noldus  
172 Information Technology, Netherlands). Concerning the swimming behavior of 96 hpf larvae,  
173 the DanioVision Observation Chamber (Noldus Information Technology, Netherlands)  
174 coupled with the DanioVision Temperature Control Unit (Noldus Information Technology,  
175 Netherlands) at  $28^\circ\text{C} (\pm 0.1)$  were used and videos were analysed using the EthoVision XT  
176 software (v.13.0.1216, Noldus Information Technology, Netherlands). All videos used for the  
177 swimming behavior analyses were performed in the dark under infra-red light. Results for all  
178 behavioral tests were compared to their respective control.

179

180 *2.4.1. Spontaneous tail movements of 24 hpf embryos*

181 For the assessment of the spontaneous tail movement in 24 hpf embryos, three  
182 replicates of 30 organisms were used. As the 30 organisms cannot be recorded together  
183 under the stereomicroscope, replicates were divided in three sub-replicates of 10 organisms.  
184 After the acclimation period, sub-replicates were placed on a petri-dish containing 1%  
185 agarose gel (Sigma-Aldrich), then 20  $\mu$ L of methyl cellulose was added to avoid movement of  
186 eggs during the recording time. Organisms were then placed under the stereomicroscope  
187 and after an acclimation period of 2 min before the videos were recorded (5 min of video,  
188 frame rate = 25 Hz). Control and contaminated replicates were alternated. All the recordings  
189 of the spontaneous movement in 24 hpf embryos took place between 10:20 am and 01:00  
190 pm. For the statistical analysis, the burst activity was used. It corresponds to the percentage  
191 of time the embryo was moving along the duration time of the video. Based on the data  
192 distribution, values below 1 and above 40 were considered as outliers and were excluded  
193 from the data set.

194

195 *2.4.2. Heart rate of 48 hpf and 72 hpf larvae*

196 For the heart rate analysis, protocol was almost the same as described for the activity  
197 assessment, except that three replicates of 10 organisms were used for each stage. These  
198 replicates were divided in three sub-replicates of 5 organisms. After the acclimation period,  
199 sub-replicates were placed on a petri-dish containing 1% agarose gel (Sigma-Aldrich), then  
200 20  $\mu$ L of methyl cellulose and 1  $\mu$ L of MS-222 final concentration 0.02 % (w.v.) (Tricaine  
201 methanesulfonate, Molekula) were added to avoid movement of larvae during the recording  
202 time. After the end of video, larvae were placed in clean embryo medium and started to  
203 swim normally. When anesthetised, organisms were then placed under the  
204 stereomicroscope and, after an acclimation period of 2 min, the videos were recorded (30  
205 sec of video, frame rate = 25 Hz). Control and contaminated replicates were alternated. All  
206 the recordings of the heart rate in 48 hpf and 72 hpf larvae took place between 10:15 am  
207 and 11:30 am. For the statistical analysis, based on the data distribution, a number of beats  
208 per second below 1.5 and above 3.5 were considered as outliers and were excluded from the  
209 data set.



210

### 211 *2.4.3. Swimming behavior of 96 hpf larvae*

212           The swimming behavior of the 96 hpf larvae was assessed using 48 organisms per  
213 treatment distributed in four 24-wells plates. Organisms were placed individually in each  
214 well with 2 mL of embryo medium; controls and contaminated animals were placed in the  
215 same plate. All four plates were prepared few minutes before the beginning of their  
216 respective recording time. Once placed in the DanioVision Observation Chamber, an  
217 acclimation period of 10 min was respected before the beginning of the recording. Videos of  
218 10 min were recorded with a tapping stimulus (level=8, on the side of the well plate) at t+5  
219 min after the beginning of the recording. All the recordings of the swimming behavior in 96  
220 hpf larvae took place between 11:00 am and 12:40 pm. Analysis were made between control  
221 and contaminated during the first five minutes of the video to assess the swimming activity  
222 basal level. The response to the tactile stimulus was assessed by comparing responses 1  
223 second before and 1 second after the stimulus for all treatment. Then the delta of the  
224 response (after-before) was used to compare the response to the tactile stimulus between  
225 control and contaminated larvae.

226

### 227 ***2.5. Histological analysis of eyes***

228           The histological sections of zebrafish eyes from 48 hpf to 96 hpf were performed  
229 after the exposure to 4 mGy/h of HTO only. Sections of 400 nm were realized using an  
230 ultramicrotome UCT (Leica Microsystems GmbH, Wetzlar, Germany) and stained with  
231 toluidine blue. Sections were then observed with an optic microscope (DM750, Leica) with  
232 ICC50 camera (Leica) coupled with the LAS EZ software. Images focusing on retina and lens  
233 were made using the 100x objective. Both eyes for two larvae were used for the three stages  
234 studied representing 12 organisms.

235

### 236 ***2.6. Whole mount gamma-H2AX immunostaining***

#### 237 *2.6.1. Immunostaining*

238 The whole mount gamma-H2AX immunostaining were performed using at least 20  
239 embryos at 24 hpf for each treatment (control and contaminated). After the sampling, eggs  
240 were manually dechorionated and fixed in 2 mL of PFA 4% in PBS 1X (Sigma-Aldrich) during 3  
241 h at 4°C. Once fixed, embryos were kept in 100% methanol (VWR International) at -20°C until  
242 the beginning of the staining.

243 The staining begins by the rehydration of embryos with successive washes with  
244 decreasing concentrations of methanol in PBS 1X (70%; 50%; 25% methanol). Embryos were  
245 then permeabilized with Proteinase K in PTW buffer (10 µg/mL final concentration) for 3 min  
246 at room temperature. After a brief wash in PTW buffer, they were fixed in PFA 4% in PBS 1X  
247 (Sigma-Aldrich) for 20 min at 4°C. The samples were blocked in a 0.2% BSA and 1% DMSO  
248 PBST solution for 3 h at room temperature, and then incubated with the primary antibody  
249 (1:200 gamma-H2AX in blocking solution, GeneTex GTX127342) at 4°C overnight. The  
250 samples were washed five times in PTW buffer, incubated with the secondary antibody  
251 (1:500 Sigma-Aldrich anti rabbit-FITC F0382) 2 h at room temperature and then accurately  
252 washed with PTW buffer. The samples were kept protected from light at 4°C until analysis by  
253 confocal microscopy.

254

### 255 *2.6.2. Confocal observations*

256 Confocal microscopy analyses were performed using a laser scanning confocal  
257 microscope (Carl Zeiss, France) using a 20x dry objective (N.A. 0.8), with a pinhole setting of  
258 1 A.U. For FITC fluorescence, excitation was performed at 488 nm and emission acquired  
259 between 510/550 nm. For DAPI detection, samples were stained in a 0.1% Tween and  
260 300nM DAPI PBS solution for 1 h and 30 min, excitation was performed at 405 nm and  
261 detection between 420 and 500 nm. The samples were mounted on 35 mm imaging dish  
262 with a polymer coverslip bottom (Ibidi) and covered by a 2% methyl-cellulose solution.

263

### 264 *2.6.3. Image analysis*

265 The images acquired at the confocal microscope were analysed using the open-  
266 source software Fiji ImageJ. For each image, the smooth filter was applied (this filter  
267 replaces each pixel with the average of its 3x3 neighbourhood) to increase the ratio

268 signal/noise, and then the level of background was chosen (values between 5 and 10).  
269 Several regions of interest (ROI) were designed on the nuclei detected in the DAPI channel,  
270 using the ImageJ tool ROI manager. The ROI were transferred and combined with the FITC  
271 channel. The function Find Maxima was used to identify and count the foci; this algorithm  
272 determines local maxima that pass the threshold (noise tolerance) in the selected area. The  
273 values derived from the initial background analysis were used as a threshold. The ratio  
274 between the number of maxima identified by the function and the number of selected nuclei  
275 was reported as foci per nucleus.

276

## 277 **2.7. Statistical analyses**

278 All statistical analyses were performed using the R software (v 3.4.3) (R Core Team,  
279 2017). Behavioral parameters, i.e. tail spontaneous movements, heart rate and locomotor  
280 activity, were analysed using linear mixed effect models fitted with the *nlme* package  
281 (Pinheiro et al., 2017). Treatment and time (at which the video was recorded reported as  
282 “time effects” in the text) were included as fixed effects while replicates were included as  
283 random effects. Residuals normality and homogeneity assumptions were assessed  
284 employing QQplot and fitted values vs. standardized residuals plot. Since they were not  
285 satisfied random permutation tests were finally applied using the *pgirmess* package  
286 (Giraudoux et al., 2018).

287 HTO activity in water and gamma-H2AX foci were analysed using Student t tests, or  
288 Wilcoxon tests if validity condition were not satisfied, and using two ways ANOVA. Normality  
289 and homogeneity assumptions were assessed using Shapiro-Wilk tests and Bartlett tests  
290 respectively. Hypotheses were bilateral, and significance level was set to 0.05.

291

## 292 **3. Results**

### 293 **3.1. Behavioral and physiological analysis**

294 In 24 hpf embryo, the spontaneous movements of the tail were not significantly  
295 different in controls and in contaminated organisms for both dose rates (Figure 1). A

296 significant effect of the time was found for both dose rates and treatment as a diminution of  
297 tail movement (data not shown).

298 The heart rate of 48 hpf and 72 hpf larvae were also analysed. Beats per second (BPS)  
299 in control and contaminated organisms were not significantly different for both stages and  
300 dose rates (Figure 2). A significant effect of the time was highlighted for both dose rates in  
301 48 hpf larvae and only for 4 mGy/h in 72 hpf larvae as an increase of BPS (data not shown).

302 Concerning the swimming behavior of 96 hpf larvae after a 0.4 mGy/h exposure,  
303 contaminated organisms showed a significant lower velocity (mm/s) during the first five  
304 minutes of the tracking videos than control organisms (Figure 3A). This difference was not  
305 found in 96 hpf larvae exposed to 4 mGy/h (Figure 3B). A second experiment was conducted  
306 with 96 hpf larvae exposed at 0.4 mGy/h and the same trend was highlighted (Figure S1).  
307 Following the tapping stimulus (i.e. one second before and one second after), control and  
308 contaminated 96 hpf larvae showed significant differences in velocity (mm/s). Indeed, the  
309 velocity of 96 hpf larvae was significantly lower one second before the tapping stimulus than  
310 one second after in control and contaminated organisms for both dose rates (Figure 4). The  
311 delta of the response to the stimulus (i.e. after-before) between control and contaminated  
312 organisms was compared for both dose rates. Results showed that after an exposure to 0.4  
313 mGy/h, the delta of the response in contaminated 96 hpf larvae was significantly lower than  
314 the delta observed in control organisms (Figure 5). After an exposure to 4 mGy/h, no such  
315 difference was found (Figure 5).

316

### 317 ***3.2. Histological observations of eyes***

318 The histological observations were performed on larvae eyes from 48 hpf to 96 hpf  
319 exposed to a dose rate of 4 mGy/h. The comparison between pictures did not show notable  
320 alterations in the retinal structure when comparing control and contaminated organisms for  
321 all stages (Figure S2). Indeed, as expected when normal development occurs, the five layers  
322 composing the retina were not distinguishable from each other in 48 hpf larvae but well  
323 defined in 72 hpf and 96 hpf larvae (Figure S2). When focusing on the lens, no remarkable  
324 difference appeared in lens development between 4 mGy/h exposed larvae and controls for  
325 all the three stages. In 48 hpf larvae, lens was fully detached from the ectoderm and showed

326 a mono layer of epithelial cells on the anterior part of the lens while in the central part  
327 elongated primary lens fibre were visible as a concentric layer (Figure 6). On later stages (i.e.  
328 72 hpf and 96 hpf larvae), the lens structure became more homogenous, with cells forming a  
329 concentric structure still visible in 72 hpf larvae but not in 96 hpf larvae (Figure 6).

330

### 331 **3.3. Whole mount gamma-H2AX immunostaining**

332 For both dose rates, the comparison between control and contaminated organisms  
333 was performed using the foci per nucleus ratio. For control and samples exposed to 0.4  
334 mGy/h, a total of 850 and 779 cells were counted, respectively. A similar number of cells  
335 were counted in control and 24 hpf embryos exposed to 4 mGy/h (824 for control and 936  
336 for contaminated samples). For both dose rates, no significant difference was highlighted  
337 between control and contaminated 24 hpf embryos (Figure 7).

338

## 339 **4. Discussion**

### 340 **4.1. Behavior and heart rate**

#### 341 *4.1.1. HTO exposure did not change spontaneous tail movement*

342 At 24 hpf, the spontaneous movement of the zebrafish embryo tail was investigated.  
343 Results did not show significant differences between control and contaminated organisms  
344 for both dose rates. These results suggested that genes coding for proteins involved in  
345 muscle contraction found, via a transcriptomic analysis, to be affected in 24 hpf eggs for  
346 both dose rates (Arcanjo et al., 2018) did not induce any effect on spontaneous tail  
347 movement at the same developmental stage. The spontaneous movements of the tail  
348 originate from the spinal cord and are dependant of functional motoneuron innervation  
349 (Brustein et al., 2003) as well as muscle contraction. Furthermore, a well-known  
350 neurotoxicant such as chlorpyrifos was shown to increase tail movements in zebrafish  
351 embryo from 24 to 26 hpf from 0.625 to 10 mg/L (Selderslaghs et al., 2010). Our result  
352 suggests that this pathway did not seem to be affected by HTO exposure in 24 hpf eggs for  
353 both dose rates tested, questioning the neurotoxic effect of HTO. However, genes coding for  
354 proteins involved in neurotransmitter and Ca<sup>2+</sup> transport were modulated in 24 hpf eggs

355 after exposure to 0.4 and 4 mGy/h of HTO (Arcanjo et al., 2018). Furthermore, in 24 hpf eggs  
356 exposed to 4 mGy/h of HTO an over-expression of the *ache* gene, coding for the  
357 acetylcholinesterase protein that play a role in muscle relaxation or muscular and neuronal  
358 development (Behra et al., 2002), was found compared to control (Gagnaire et al, in prep).  
359 Together, these results suggest that the neuro-muscular functions could be impaired after  
360 HTO exposure and it would be interesting to perform further investigations with older  
361 developmental stages. Indeed, as genes mis-regulation were found at 24 hpf, it was probably  
362 too soon to see any phenotypical impairments at the same time. That is why we also  
363 performed locomotor analysis on 96 hpf larvae to assess the latency between the gene  
364 expression impairments and the phenotypical observations.

365 Interestingly, the statistical analysis performed on the spontaneous tail movement  
366 highlighted a significant effect of time for both HTO exposures; meaning that the time during  
367 which experiments were made has a significant effect on the measured parameter (burst  
368 activity). Indeed, the first spontaneous movement appeared around 17 hpf. In  
369 dechorionated embryos, the spontaneous movements reach a peak around 19 hpf and then  
370 slowly decrease by 26 hpf (Saint-Amant, 2006). Such a decrease of the spontaneous tail  
371 movement was also reported in literature in control organisms (Huang et al., 2010; Wang et  
372 al., 2013) confirming that time influence this parameter. In the present study, as  
373 experiments were performed on stages between 24 hpf and 27 hpf, it is not surprising to  
374 find a significant effect of time in the obtained data.

375

#### 376 4.1.2. HTO exposure did not change the number of beats per seconds

377 After an exposure to 0.4 mGy/h of HTO, no significant differences in BPS were  
378 highlighted between control and exposed organisms for both stages. The same result was  
379 found for the highest dose rate of 4 mGy/h. Furthermore, the statistical analyses showed a  
380 significant effect of time for both dose rate when 48 hpf larvae were investigated and only  
381 for the highest dose rate for the 72 hpf larvae suggesting that the time at which the  
382 experiments are recorded could influence the heart rate. Together, the results found for the  
383 cardiac activity after HTO exposure suggested no effect of HTO on this parameter for the  
384 developmental stages investigated. As previously said, it appears that the down-regulation

385 of genes involved in both muscle contraction and event leading to the muscle contraction  
386 found in a previous study in 24 hpf eggs (Arcanjo et al., 2018) had no effects on cardiac  
387 activity on 48 hpf and 72 hpf larvae. These results could indicate that the organisms can cope  
388 with tritium toxicity with early gene expression changes, indicating the low toxicity of  
389 tritium, at least for the considered life stages and exposure period. Using gamma radiation, it  
390 is possible to observe effects on heart rate with dose as low as 0.5 mGy/h in 48 hpf larvae  
391 (Murat El Houdigui et al, under review). To go further, it would be interesting to perform  
392 dedicated experiments to find the moments of the day were larvae show constant BPS to  
393 avoid time effect.

394

#### 395 *4.1.3. HTO exposure induces effects on locomotor behavior*

396 To get insight into the swimming abilities of 96 hpf larvae after HTO exposure, a free-  
397 swimming behavioral test was performed in 96 hpf larvae, followed by a tactile stimulus on  
398 the side of the plate containing organisms. Results highlighted that during the first five  
399 minutes of the video tracking, the 0.4 mGy/h exposed larvae showed a mean velocity  
400 significantly lower than the velocity of control larvae. The response to a tactile stimulus was  
401 then investigated and showed that controls and HTO-contaminated organisms (for both  
402 dose rates) responded to the tactile stimulus by a significant enhancement of their velocity  
403 one second after the stimulus. The delta of the response to the tactile stimulus was also  
404 studied. It appeared that the 0.4 mGy/h exposed 96 hpf larvae showed a delta of the  
405 response significantly lower than control organisms.

406 The effects found on 96 hpf larvae locomotor activity could be explained by the  
407 potential impact of HTO exposure on muscle structure and events leading to muscle  
408 contraction. Indeed, muscles impairments as disorganization of sarcomeres myofilaments  
409 (96 hpf) and disruption of genes involved in muscle contraction (24 hpf for both dose rate)  
410 were reported in a previous study using the same HTO dose rates (Arcanjo et al., 2018). Also  
411 in zebrafish, gamma exposure to 0.03 mGy/h and 24 mGy/h induced degradation of  
412 myofibrils which were more deleterious for the higher dose rate tested (Gagnaire et al.,  
413 2015). In 96 hpf larvae TEM muscle observation reveals myofibers and neuromuscular  
414 junction alterations after gamma irradiation at 5 and 50 mGy/h (Murat El Houdigui et al,

415 under review). Furthermore, after HTO or gamma exposure, vertebral malformations have  
416 been documented in medaka, *Oryzias latipes*, exposed to 35 mGy/h (HTO) and 18 mGy/h ( $\gamma$ )  
417 during embryonic stages (Hyodo-Taguchi and Etoh, 1993) which could lead to locomotor  
418 impairments. To explain the locomotor effects of HTO on zebrafish larvae, it would be also  
419 interesting to focus on the lateral line, a mechanosensory system in fish and on the  
420 Mauthner cells which mediated the trunk and tail contraction during the response to a  
421 tactile stimulus (Eaton et al., 1977). Indeed, after uranium exposure, severe tissue  
422 alterations were reported in the lateral line system in zebrafish (Faucher et al., 2012).

423           However, the impairments of the muscular system, both at the molecular and tissue  
424 scales, cannot explain the observed effects alone, as the locomotor activity in 96 hpf larvae  
425 exposed to 4 mGy/h was not affected by HTO exposure. As a proper locomotor activity  
426 depends on both the muscular and the nervous systems (Drapeau et al., 2002), the potential  
427 neurotoxic effect of HTO exposure has also to be considered. After gamma irradiation,  
428 zebrafish larvae show neuronal as well as muscles impairments (Murat El Houdigui et al,  
429 under review). After HTO exposure, a transcriptomic analysis highlight down-regulation of  
430 genes coding for proteins involved in peripheral nervous system axonogenesis (*her4.3* and  
431 *her4.4*) in 24 hpf embryos after exposure at 4 mGy/h (Arcanjo et al., 2018). In studies  
432 focusing on chemicals such as nicotine (Parker and Connaughton, 2007; Svoboda et al.,  
433 2002), caffeine (Chen et al., 2008) or ethanol (Carvan et al., 2004), effects on the locomotor  
434 behavior of zebrafish larvae after a tactile stimulus, dose dependant decrease of response  
435 was shown in contaminated organisms comparing to controls. The mode of action evoked to  
436 explain these results are linked to an effect on the development and on the alteration of the  
437 axonal pathfinding of the secondary spinal motoneurons as defects on the neuromuscular  
438 junction (Chen et al., 2008; Svoboda et al., 2002).

439           Together, these results suggest that HTO exposure had more detrimental effect after  
440 an exposure to 0.4 mGy/h than to 4 mGy/h on the locomotor activity in 96 hpf larvae. This  
441 lack of concentration-dependant response could be due to the onset of more efficient  
442 defence mechanisms following a threshold that is not reach for the lowest dose rate. Indeed,  
443 in a previous study, we found more differentially expressed genes involved in DNA repair  
444 pathways at 4 mGy/h than at 0.4 mGy/h (Arcanjo et al., 2018). However, at 0.4 mGy/h, the  
445 events triggering the impairment of the locomotor activity in larvae remained to be



446 confirmed. Indeed, the velocity of the control larvae in the 0.4 mGy/h experiment was  
447 higher than the velocity in control larvae for the experimentation with the highest dose rate.  
448 A second experiment were performed at 0.4 mGy/h, and the same trend was highlight with  
449 a mean velocity in control of the same range as for the first experiment. The difference in  
450 control larvae mean velocity between dose rates could be explained by biological variation  
451 between the pool of eggs used. Indeed, to avoid HTO cross-contamination, the experiments  
452 for both dose rate were conducted separately in time, meaning that pools for both  
453 experiments were not from the same spawn. This could lead to find a significant difference  
454 in velocity where there is not. Nevertheless, the impact of HTO on the velocity of larvae  
455 could cause delayed response to threats by impairing the escaping/avoidance behavior or  
456 decrease success of prey capture and could therefore have a detrimental impact on fish  
457 fitness.

458 In the literature, it is known that many variables could influence zebrafish larvae  
459 locomotor behavior, i.e. larvae age, size of well, time of the day or experiment performed in  
460 light or dark (MacPhail et al., 2009; Padilla et al., 2011). In the present study, we chose to  
461 assess locomotor activity on 96 hpf larvae to be consistent with experiments on gene  
462 expressions where genes involved in muscle contraction were mis-regulated in 24 hpf  
463 embryos. Furthermore, in locomotor activity experiments performed in the dark, as in the  
464 present study, response in 4 dpf, 5 dpf and 6 dpf larvae are similar to each other, which is  
465 not the case in light experiments (Padilla et al., 2011), reinforcing the use of 96 hpf larvae in  
466 our study. Time of the day when the experiments are performed could also influence larvae  
467 locomotor response. Indeed, in 6 dpf larvae, the locomotor activity assessed in the dark  
468 reached stability in early afternoon around 01:00 pm (MacPhail et al., 2009). Even though  
469 our experiments were performed in late morning (from 11:00 am to 12:40 pm) when it  
470 seems that locomotor activity is still stabilizing, we were able to find effects of HTO exposure  
471 on larvae velocity.

472

#### 473 **4.2. Histology of the eyes**

474 As cataract is one of the complications after beta radiation therapy in Human and  
475 that cataract incidence was shown to increase in Chernobyl birds (Barron et al., 1970;

476 Mousseau and Møller, 2013), the potential development of cataract in HTO exposed  
477 zebrafish larvae was investigated. Furthermore, as irregularities in the development of  
478 zebrafish larvae eye were reported after gamma radiation in zebrafish larvae exposed only  
479 via their parents, exposed only as larvae or both (Hurem et al., 2017), eye layer development  
480 was assessed. Histological sections of eyes lens and retina were performed. As genes  
481 involved in eye transparency were found down-regulated in 24 hpf eggs after an exposure to  
482 4 mGy/h in a previous study (Arcanjo et al., 2018), all sections were made at this dose rate.  
483 Results highlighted no distinguishable defects in larvae (from 48 hpf to 96 hpf) when  
484 comparing control and contaminated organisms. Indeed, as expected in normal zebrafish  
485 eyes development, in 48 hpf larvae the lens is fully detached from the cornea and show  
486 different kind of cells, an anterior monolayer of epithelial cells and in more centrally the  
487 primary lens fibre starting to elongated (Dahm et al., 2007). At 72 hpf, histological sections  
488 of lens showed a more homogeneous appearance with lens fibre cell even more elongated  
489 with degraded nucleus (Dahm et al., 2007). Finally, at 96 hpf, sections of the lens also  
490 showed a homogenous pattern with no appearance of nuclei. At this stage the  
491 differentiation of lens fibre cells is terminated, and lens is totally transparent (Goishi et al.,  
492 2006). Concerning the histological sections of retina, no obvious differences could be seen  
493 between control and contaminated organisms regardless of stages. Indeed, the five retinal  
494 layers were well distinguishable in 72 hpf and 96 hpf larvae. These five layers are composed  
495 of three nuclear layers (ONL, INL and GCL) and two plexiform layers (IPL and OPL) (Chhetri et  
496 al., 2014). No remaining nuclei was found in lens, as already shown by histological section of  
497 zebrafish eyes with cataract (Dhakal et al., 2015; Goishi et al., 2006). Therefore, these results  
498 did not support the molecular alterations previously shown (Arcanjo et al., 2018).

499 Together, the results showed no effect of HTO on lens transparency or retinal  
500 development at the analysed developmental stages. This results suggest that the transient  
501 down-regulation of genes involved in eye transparency at 24 hpf (Arcanjo et al., 2018) did  
502 not have any effect on eye development. Another hypothesis would be that toxico-dynamics  
503 are different between molecular and tissular effects. To check this last hypothesis, it would  
504 be interesting to follow eye development over the whole larvae development until the adult  
505 stage. In addition, considering the observed behavioural changes, it would be an asset to  
506 investigate behavioral tests dedicated to visual parameters as the optokinetic response

507 (OKR) or the optomotor response (OMR) in 96 hpf larvae. Indeed, at this stage the retinal  
508 structure and the ocular muscles are functional, which leads to visible eye movements which  
509 could indicate a behavioral changes, liable to occur even in the absence of any structural  
510 defects (Chhetri et al., 2014). Indeed, the OKR was used to isolated zebrafish mutant with  
511 visual defect and highlighted two mutants lacking obvious morphological difference from  
512 controls (Brockerhoff et al., 1995).

513

#### 514 **4.3. gamma-H2AX foci detection**

515           The effects of HTO exposure on DNA damages were already investigated using  
516 a wide range of species as mollusc (Hagger et al., 2005; Jha et al., 2005), aquatic vertebrates  
517 (Gagnaire et al., 2017; Suyama et al., 1981) or fish cell culture (Stuart et al., 2016). In the  
518 present study, we focused on DNA double strand breaks in 24 hpf embryos using an  
519 immunostaining method to assess the number of gamma-H2AX foci, an early marker of DNA  
520 double strand break repair (Rogakou et al., 1998). Results showed that, comparing to their  
521 respective controls, no significant differences were found for both dose rates. After an  
522 exposure to tritium at much lower activities than in the present study, no difference  
523 between control and contaminated organisms were neither found in fish cells and *in situ*  
524 exposed fathead minnows (Gagnaire et al., 2017; Stuart et al., 2016) using gamma-H2AX foci  
525 detection. At the same stage, i.e. 24 hpf, no DNA damages were found using the comet assay  
526 for both dose rates (Gagnaire et al, in prep). Using fish cells (3B11 and FHMT-W1), no effects  
527 of HTO exposure was found when investigating the gamma-H2AX foci intensity after  
528 exposure from 0.01 to 100 Bq/mL (Stuart et al., 2016). After gamma irradiation, no  
529 significant difference in the gamma-H2AX detection was found after exposure at dose rate  
530 close to those in the present study (5 mGy/h) for the same stage while an increase of foci  
531 was detected at 50 mGy/h (Murat El Houdigui et al, under review). By exposing directly ZF4  
532 zebrafish cells to chronic gamma radiation, an increase of gamma-H2AX foci per nucleus was  
533 found after 24h at 4 mGy/h while no effects was found at 0.4 mGy/h (Pereira et al., 2011).

534           Overall, the results found in the present study combined with those in studies  
535 investigating gamma-H2AX foci detection after exposure to HTO or gamma radiation suggest  
536 that organisms could cope with DNA damages potentially occurring after an exposure to

537 ionizing radiations. These results could be due to an enhancing of DNA repair pathways at  
538 those dose rates to balance the effects of ionizing radiations. This hypothesis is consistent  
539 with previous findings at the molecular scale highlighting an up-regulation of genes involved  
540 in DNA repair pathways, in 24 hpf eggs after an exposure to HTO at 4 mGy/h (Arcanjo et al.,  
541 2018). However, this hypothesis remains to be confirmed by further investigation on the  
542 DNA repair pathways after ionizing radiation exposure.

543

## 544 **5. Conclusion**

545 In this study, observed effects at the molecular scale, i.e. genes involved in  
546 muscle contraction, eye transparency and response to DNA damage, have not all been  
547 confirmed at higher biological scales using stages from 24 hpf to 96 hpf. However it was  
548 possible to confirm these early effects for locomotion. Results highlighted that the 0.4  
549 mGy/h exposure could have more detrimental effects than the 4 mGy/h exposure on the  
550 locomotor activity. These results could be partly explained by the impact on muscle  
551 (structure and genes) previously highlighted for both dose rate, but not entirely. Therefore,  
552 an impact on the nervous system coupled with muscular impairments could be more  
553 relevant for the explanation of effects on the locomotor behavior. Furthermore, the ability  
554 to cope with damages after HTO exposure could also be advanced to explain the observed  
555 effect on locomotion. Concerning the other parameters followed in this study, it could be  
556 interesting to explore the responses by using older larvae as there may be a delay between  
557 gene expression changes and the onset of physiological disruption or homeostatic  
558 mechanisms onset at those early stages which could be exceeded along time. Despite that,  
559 the present study and the whole project related to, allowed to link responses observed at  
560 different organization levels, from molecule to individual, contributed to improve the  
561 knowledge of tritiated water exposure effects. From an ecological risk assessment point of  
562 view, the present study did not highlight detrimental effects at the individual scale with  
563 tritium activity as high as  $1.22 \times 10^9$  Bq/L. Activities used in the present study are widely  
564 higher than those found in aquatic environment suggesting that environmental exposure to  
565 tritium could lead to poor detrimental effects in organisms. However, it is not excluded that  
566 chronic exposure to environmental activities of tritium could lead, over time, to detrimental

567 effects at the individual level. Indeed, at the same activities as is the present work, molecular  
568 effects were highlight and could, with time, generate detrimental effects.

569

570

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579

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737 **List of figures**

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