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

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Abstract

Tritium, a radioactive isotope of hydrogen of natural and anthropogenic origin, is ubiquitously present in the environment. Effluents of nuclear center of production are significant anthropogenic sources. With the upcoming project of thermonuclear fusion, tritium releases in the environment may increase. It is therefore important to characterize the ecological risk linked to tritium. The effects of tritiated water (HTO) were therefore studied in zebrafish larvae exposed during 10 days to different dose rates: $1.1x10^2$, $4.1x10^2$ and $3.8x10^3$ µGy/h for larvae, corresponding respectively to a water contamination of 10^4, 10^5 and 10^6 Bq/mL of HTO. Those dose rates were higher than 10 µGy/h which is the threshold recommended to start monitoring ecosystems where radiological contaminants are present. Mortality, embryo-larval development, immune-, geno and neuro-toxicity, and alterations of tissues were investigated. Results showed that tritiated water exposure induced DNA damage, ROS production and modulated the expression of genes involved in detoxification processes. Moreover, modifications of the muscular tissues (degradation of myofibrils at 4 dpf and disorganization of mitochondria at later stages) were observed. Results differed with HTO dose rates and with developmental stages. These results will drive future research for the development of new HTO-sensitive biomarkers and will allow us to progress in the characterization of the modes of action of tritium in fish.

Keywords

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

Highlights

- This study evaluated the effects of tritiated water on zebrafish embryo-larvae
- Larvae were exposed to different dose rates: $1.1x10^2$, $4.1x10^2$ and $3.8x10^3$ µGy/h
- Several endpoints were evaluated: embryo-larval mortality and development, muscular tissue alteration, geno-, immuno-, neuro-toxicity, reactive oxygen species production, and gene expression
- The results showed that tritiated water, depending on the dose rate and the stage of development considered, induced molecular changes, cellular modifications, as well as tissue alterations

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 µGy/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,

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

In this study, we conducted experiments on zebrafish embryo-larvae in order to assess tritiated water effects at dose rates close to and higher than the environmental protection criteria of 10 µGy/h (Garnier-Laplace et al. 2010). 3 hpf eggs were placed in tritiated water at 10^4, 10^5 and 10^6 Bq/mL for 10 days. These activity concentrations corresponded to theoretical dose rates of $4x10^1$, $4x10^2$ and $4x10^3$ µGy/h (Adam-Guillermin et al. 2013), in order to assess tritium effects A battery of biomarkers were followed, including neurotoxicity (acetylcholinesterase (AChE) activity), defence system (phenoloxidase-like (PO) activity, an enzyme involved in pathogen lysis, as described before (Gagnaire et al. 2013) and reactive oxygen species (ROS) production), and DNA damage (using comet assay). The expression of genes related to these diverse mechanisms (immunotoxicity, neurotoxicity, genotoxicity) was also quantified. These responses measured at low levels of biological organization were related to endpoints measured at the individual level (mortality, hatching success of embryos and developmental parameters). Histopathology of tail muscle was also studied. The total dose rates were calculated for eggs and larvae by considering an external dose rate calculated using HTO water concentration and an internal dose rate calculated using tritium activities measured in individuals, using a protocol developed in a related study (Arcanjo et al. 2019). Results were then compared to observations obtained after exposure of zebrafish larvae to gamma irradiation in previous studies.

2. Material & Methods

2.1. Chemicals

The chemicals used in this study were purchased from Sigma-Aldrich (St Quentin-Fallavier, France), as mentioned before (Gagnaire et al. 2015): BSA, Bradford reagent, H_2DCFDA, L-Dopa, DMSO, HBSS, DTNB, ATCi, trypan blue, ultrapure water sodium cacodylate, glutaraldhehyde, osmium tetroxide, toluidine blue. PMA was purchased from Molecular Probes (Invitrogen, Cergy-Pontoise, France).

2.2 Production of eggs

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

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

2.3 Exposure of eggs to tritiated water

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

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

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% (HT_{50}), 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 (Fraysse 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, Brillant 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

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

2.10 Microscopical observations

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

2.11 Statistical analyses

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

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

3. Results

3.1 Tritium dose rate in eggs and larvae

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

Despite these slight differences, the mean pondered dose rate of exposure all stages included was calculated as $1.1x10^2$, $4.1x10^2$ and $3.8x10^3$ µGy/h for nominal exposure HTO concentration of 10^4, 10^5 and 10^6 Bq/mL, respectively (Table 2).

3.2 Effects on mortality, hatching and embryo-larvae development

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

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

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

3.3 Effects on DNA damage measured by the comet assay

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

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

4. Discussion

4.1 Dose rates calculated for tritiated water

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

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

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

No differences of mortality and hatching were observed between conditions. In zebrafish, tritiated thymidine induced a delay in hatching at $1.91x10^3$ and $1.05x10^4$ µGy/h (Adam-Guillermin et al. 2013). Moreover, we showed no abnormalities in larvae exposed to HTO at the same dose rates, while abnormalities were observed in zebrafish larvae after exposure to tritiated thymidine (Adam-Guillermin et al. 2013). Exposure of medaka embryos for 30 days to $1.2x10^3$ µGy/h of HTO led to vertebral malformations and effects on fecundity of adults (Hyodo-Taguchi and Etoh 1993). Moreover, the hatchability of common flounder, *Paralichtys olivaceus*, was modified after 4 days of exposure to $1.21x10^5$ µGy/h of tritium (Ichikawa and Suyama 1974). Thus, tritium effects on hatching process seem to be related to the form used, the total dose received and the biological stage.

Tritiated water induced some effects on macroscopical endpoints depending on the dose rate studied. At 10^4 Bq/mL (i.e. $1.1x10^2$ µGy/h), exposed animals presented differences with the controls for early stages (<4 dpf). At 10^5 Bq/mL (i.e. $4.1x10^2$ µGy/h), differences between controls and exposed animals were seen only at older stages (3-10 dpf). At 10^6 Bq/mL (i.e. $3.8x10^3$ µGy/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 $1.21x10^5$ and $1.21x10^6$ µGy/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. $1.1x10^2$ µGy/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 $2.08x10^1$ µGy/h (Suyama et al. 1981) and of DNA damage and MN frequency at
344 $1.25x10^1$ µGy/h in mussels (Jha et al. 2005). Tritiated thymidine also induced an increase of
345 DNA damage in 4 dpf larvae exposed at $1.05x10^4$ µGy/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. $4.1x10^2$ µGy/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. $3.8x10^3$ µGy/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. $4.8x10^3$ µGy/h). No effect was shown on ROS stimulation index at 10^4
376 Bq/mL (i.e. $1.1x10^2$ µGy/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*, *cyp1a* and
380 *gstp1*) were modified after 4 and 7 days of exposure to 10^4 Bq/mL (i.e. $1.1x10^2$ µGy/h) and
381 10^6 Bq/mL (i.e. $4.8x10^3$ µGy/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. $4.1x10^2$ µGy/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 $4.8x10^3$ µGy/h). Therefore, the nervous system does not seem to be affected by tritium.
394

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

4.4 Comparison to gamma irradiation

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

Gamma irradiation at $3.33x10^{1}$ µGy/h decreased ROS stimulation index due to an increase of ROS basal levels in irradiated larvae (Gagnaire et al. 2015). It should be noted that this dose rate is close to the benchmark value of 10^{1} µGy/h recommended for ecosystem protection towards ionizing radiations (Garnier-Laplace et al. 2010). Moreover, 24 hpf eggs exposed to $2.37x10^{4}$ µGy/h showed an increase in DNA damage (Gagnaire et al. 2015). Taken together, the present results confirmed the existence of an oxidative stress conducting to DNA damage after fish exposure to radionuclides like tritium. The induction of an oxidative stress can therefore be considered as a common mode of action to both kind of exposure: external irradiation and HTO contamination.

Neither HTO nor gamma irradiation induced any effect on phenoloxidase activity. HTO modulated the expression of genes involved in detoxication, while gamma irradiation at $3.33x10^{1}$ µGy/h decreased EROD activity in 4 dpf larvae (Gagnaire et al. 2015). Detoxication could therefore be a mechanism common to several types of radionuclides and relevant in the study of effects of such contaminants in aquatic organisms.

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

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

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 $1.1x10^2$ to $3.8x10^3$ µGy/h, which is in the range or even higher than the derived consideration
474 reference levels (DCRLs) for fish of $4.1x10^1$-$4.1x10^2$ µGy/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 µGy/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 Effectivness (RBE) of tritium
488 (Adam-Guillermin et al. 2012).
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491 **Figure captions**

492

493 **Figure 1:** Hatching Time (HT_{50}) in control and contaminated zebrafish larvae for 10^4 (A), 10^5
494 (B) and 10^6 (C) Bq/mL of HTO. Error bars correspond to 95% confidence intervals of the
495 HT_{50} values.

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497 **Figure 2:** muscular tissue of 4 dpf zebrafish larvae observed by TEM in control (A) and
498 contaminated at 10^4 (B), 10^5 (C) and 10^6 (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.

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501 **Figure 3:** muscular tissue of 4 dpf zebrafish larvae observed by TEM in control (A) and
502 contaminated at 10^4 (B), 10^5 (C) and 10^6 (D) Bq/mL of HTO. My: myofibrils, S: sarcomere,
503 Z: Z stria, Mi: mitochondria.

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

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DC (µGy/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}

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524 **Table 2**: tritium dose rates (µGy/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 ± 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	$9.1x10^1 \pm 1.2x10^1$ ab	$1.7x10^2 \pm 1.3x10^1$ b	**$9.5x10^1 \pm 1.3x10^1$ a**	**$8.5x10^1 \pm 1.1x10^1$ a**	$1.1x10^2 \pm 5.2x10^1$
10^5	$5.2x10^2 \pm 1.3x10^1$ c	$4.6x10^2 \pm 1.3x10^1$ bc	**$2.8x10^2 \pm 9.8$ a**	**$3.7x10^2 \pm 1.6x10^1$ ab**	$4.1x10^2 \pm 1.1x10^1$
10^6	$3.8x10^3 \pm 1.5x10^2$ ab	**$3.3x10^3 \pm 1.5x10^2$ a**	$4.7x10^3 \pm 2.4x10^2$ b	**$3.4x10^3 \pm 1.5x10^2$ a**	$3.8x10^3 \pm 8.5x10^2$

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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 10^4, 10^5
531 and 10^6 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, $p \leq 0.05$; a, b: significantly different, a<b. n.d.: not determined.
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	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 10^4 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 10^5 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 10^6 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.
535

	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 10^4 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 10^5 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 10^6 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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Table 4: mean tail moments (comet assay) obtained for zebrafish 24 hpf embryos (n=3 pools of 10) and 4, 7 and 10 dpf larvae (n=10) control and exposed (at 10^4, 10^5 and 10^6 Bq/mL of HTO) groups. Mix model (R), $p \leq 0.001$; Bold values indicate a significant difference between conditions. a, b: significantly different, a<b. n.d.: not determined.

	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 10^4 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 10^5 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 10^6 Bq/mL	1.78 ± 0.13	2.09 ± 0.13 b	1.47 ± 0.09	0.83 ± 0.07

Table 5: PO-like (U/larva) and AChE activities (nmol ATCi/min/mg proteins) in zebrafish larvae contaminated during 4 days at 10^4, 10^5 and 10^6 Bq/mL of HTO. Values are means of 10 replicates for each condition; standard error is presented. T-test or U Mann Witney test, $p<0.05$.

	PO-like activity (U/larva)	AChE activity (nmol ATCi/mg/mg proteins)
Control	1.11 ± 0.09	167.9 ± 21.3
HTO 10^4 Bq/mL	1.03 ± 0.07	195.7 ± 38.0
Control	1.29 ± 0.12	68.1 ± 10.8
HTO 10^5 Bq/mL	1.08 ± 0.04	63.9 ± 10.4
Control	1.63 ± 0.35	173.7 ± 16.0
HTO 10^6 Bq/mL	1.94 ± 0.28	146.7 ± 10.4

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.
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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**	**a**	1.21 ± 0.06		1.15 ± 0.07	
HTO 10^5 Bq/mL	1.22 ± 0.02	b	1.28 ± 0.05		1.19 ± 0.09	
Control	**1.14 ± 0.10**	**a**	1.26 ± 0.17		0.87 ± 0.08	
HTO 10^6 Bq/mL	1.58 ± 0.12	b	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 10^4, 10^5 and 10^6 Bq/mL of HTO (n=9). Analysis
582 with REST-384©, $p \leq 0.05$; 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**
ache	1.38	1.74	0.99	1.39	1.19	1.57	1.28	1.31	**1.63**
bax	1.61	1.28	0.74	1.29	0.77	1.27	1.12	0.75	1.48
chat	1.29	1.40	0.79	-1.40	1.29	1.29	2.13	1.07	1.43
cyp1a	1.63	1.55	**42.74**	1.52	0.83	0.86	-1.17	0.78	1.30
gstp1	0.93	1.34	**0.02**	1.36	1.27	0.95	1.02	0.74	0.69
lyz	1.13	0.76	1.34	1.79	0.84	**1.45**	-1.76	0.92	1.29
mpx	1.20	**0.37**	1.02	1.34	0.59	**2.45**	1.50	0.83	1.52
mt2	**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.

	10^{4} Bq/mL				10^{5} Bq/mL				10^{6} Bq/mL			
Stage	**24 hpf**	**4 dpf**	**7 dpf**	**10 dpf**	**24 hpf**	**4 dpf**	**7 dpf**	**10 dpf**	**24 hpf**	**4 dpf**	**7 dpf**	**10 dpf**
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)	$2.2x10^{3}$	$1.6x10^{4}$	$1.6x10^{4}$	$2.0x10^{4}$	$1.2x10^{4}$	$4.5x10^{4}$	$4.7x10^{4}$	$8.8x10^{4}$	$9.1x10^{4}$	$3.2x10^{5}$	$7.8x10^{5}$	$8.2x10^{5}$
Develop-ment	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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