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**Effects of gamma ionizing radiation exposure on *Danio rerio* embryo-larval stages -  
comparison with tritium exposure**

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## Abstract

The objective was to investigate the effects of ionizing radiation induced in zebrafish early life stages by coupling responses obtained at the molecular (genotoxicity, ROS production, gene expression) and phenotypic (tissue alteration, embryo-larval development) levels. Here we present results obtained after exposure of 3 hpf larvae to 10 days of gamma irradiation at  $3.3 \times 10^1$ ,  $1.3 \times 10^2$  and  $1.2 \times 10^3$   $\mu\text{Gy/h}$ , close to and higher than the benchmark for protection of ecosystems towards ionizing radiations of  $10^1$   $\mu\text{Gy/h}$ . Dose rates used in these studies were chosen to be in the 'derived consideration reference level' (DCRL) for gamma irradiation where deleterious effects can appear in freshwater fish. Also, these dose rates were similar to the ones already tested on tritium (beta ionizing radiation) in our previous work, in order to compare both types of ionizing radiation. Results showed that gamma irradiation did not induce any effect on survival and hatching. No effect was observed on DNA damages, but ROS production was increased. Muscle damages were observed for all tested dose rates, similarly to previous results obtained with tritium (beta ionizing radiation) at similar dose rates. Some molecular responses therefore appeared to be relevant for the study of gamma ionizing radiation effects in zebrafish.

## Keywords

Gamma irradiation; tritium; zebrafish, *Danio rerio*; embryo-larvae stages; molecular responses.

## 1. Introduction

Recommendations from international organizations underline the need for a radiological protection of the environment towards deleterious effects of ionizing radiation (IAEA 2014, ICRP 2007). They aim to guarantee for the present and future human generations (i) a durable use of natural ecosystems for agricultural needs, forest exploitation, fishing, tourism; (ii) to prevent the effects of ionizing radiations on flora and fauna. In line with that, considering that the status of ecosystem can impact human health at long-term, European Union supported a politic protecting the environment from harmful effects of ionizing radiation (directive 2013/59/Euratom). Several approaches are proposed to evaluate the potential effects of ionizing radiation on ecosystems. The system recommended by the ICRP is based on a range of dose rates called the 'Derived Consideration Reference Levels' (DCRL) for 'Reference Animals and Plants' (RAP), in which deleterious effects to specific wildlife groups (eg. Pelagic fish, Crustacean...) are considered (ICRP 2008). The ERICA and PROTECT projects enabled to define a benchmark value or reference no effect dose rate (10  $\mu\text{Gy/h}$ ) for ecosystems (considered as generic) exposed chronically to ionizing radiation (Garnier-Laplace et al. 2010).

In the environment, natural populations are exposed to different radionuclides, emitting different kinds of radiations ( $\alpha$ ,  $\beta$  or  $\gamma$ ). The effects of ionizing radiation depend on the type of radiation, the absorbed dose, the duration of exposure but also on the sensitivity of the exposed organism or organ. Therefore, it is necessary to take into account these variations in biological efficiency in the environmental risk assessment of ionizing radiations. To this end, the concept of Relative Biological Effectiveness (RBE) was developed. RBE corresponds to the ratio between the dose of reference radiation ( $\gamma$  or X) needed to produce a level of effect and the dose of the studied radiation ( $\alpha$  or  $\beta$ ) needed to produce the same kind of effect (similar mode of action) and the same level of effect on a common endpoint (Dobson and Kwan 1978, Hall et al. 1967). RBE depends on several factors, including the type of irradiated cell, the effect considered and the distribution (homogenous or not) of radionuclides. In the area of radioprotection of non-human species, there is no consensus on the choice of RBE values, due to a lack of data, notably in the domain of low dose rates. Therefore, on the basis of literature data, a RBE of 5 was proposed for alpha emitters, but with values ranging between 1 and 377, showing the complexity to precisely determine the biological efficiency of a radiation (Chambers et al. 2006). For tritium, the literature reports a controversy concerning the RBE value (Higley et al. 2012). Therefore, work are needed to better

characterize tritium effects and mode of action on non-human species and to compare to a reference radiation such as gamma ionizing radiation.

Fish represent a relevant vertebrate model to study effects of ionizing radiation, as they are considered to be among the most radiosensitive animals in aquatic species (ICRP 2008). Several kinds of effects have been observed in fish exposed to gamma ionizing radiation such as disruption of immune response (Strand et al. 1982), apoptosis (Zhao et al. 2019), genomic instability (Hurem et al. 2017a) and effects on hormonal levels (Erickson 1971). These alterations could have consequences on survival (especially for early life stages), fecundity and development. In a previous work, we also exposed zebrafish larvae, *Danio rerio*, a well-known model organism in toxicology and ecotoxicology (OECD 2004), during 4 days at low and high dose rates ( $3,3 \cdot 10^1$  and  $2,4 \cdot 10^4$   $\mu\text{Gy/h}$ ) of external chronic gamma irradiation and showed effect on hatching, gene expression and muscular tissue integrity (Gagnaire et al. 2015b).

The effects of tritium were also investigated at the molecular and cellular levels in zebrafish larvae early life stages using of a battery of molecular responses of immunotoxicity, neurotoxicity and genotoxicity. Tritiated water (HTO) effects were studied at dose rates of  $3,4 \cdot 10^1$ ,  $1,36 \cdot 10^2$  and  $1,26 \cdot 10^3$   $\mu\text{Gy/h}$  until larvae reached 10 dpf (days post fertilization), in order to observe a key phase of larval development until the total resorption of yolk bag, and therefore to investigate potential effects on metabolism (Gagnaire et al. 2020a). The links between responses observed at the molecular, tissular and individual levels were also investigated using specific tools such as RNAseq (Arcanjo et al. 2018) and behavioral analyses (Arcanjo et al. 2020). Tritiated thymidine was also studied at dose rates of  $8,7 \cdot 10^1$  to  $2,4 \cdot 10^3$   $\mu\text{Gy/h}$  (Adam-Guillermine et al. 2013). This previous work allowed to better understand the mode of action of tritium on zebrafish early life stages. However, results were difficult to compare to those obtained for gamma ionizing radiation, as the exposure conditions were not the same in duration and dose rates.

Therefore, the aim of the study presented in this paper was to complete the previous study realised on gamma irradiation (Gagnaire et al. 2015b) by studying the same dose rates as we used for HTO exposure (Gagnaire et al. 2020a), and to continue the exposure until 10 dpf, in order to study the potential effects of gamma irradiation on metabolism and to compare the mode of action of HTO and gamma irradiation. Dose rates tested were in the range of the DCRLs for fish ( $4 \cdot 10^1$ - $4 \cdot 10^2$   $\mu\text{Gy/h}$ ) for which deleterious effects are supposed to appear (ICRP 2008). Biological effects were evaluated in terms of immunotoxicity and oxidative stress (production of reactive oxygen species (ROS)) and genotoxicity (DNA strand breaks

113 measured by the comet assay). These markers were previously measured on HTO and gamma  
114 irradiation, which justifies their choice. Moreover, they are classical molecular responses used  
115 in a wide range of fish species in ecotoxicology (van der Oost et al. 2003). Moreover, the  
116 expression of genes linked to the studied mechanisms was followed. Molecular responses  
117 were related to individual responses by the analysis of muscle histopathology and embryo-  
118 larval development. The whole responses were compared to those obtained on zebrafish  
119 larvae contaminated to HTO at 1, 4, 7 and 10 dpf and also on zebrafish contaminated with  
120 tritiated thymidine at 4 dpf in order to compare and better understand the mode of actions of  
121 different types of ionizing radiations.

## **2. Material & Methods**

### **2.1 Chemicals**

The chemicals used in this study were purchased from Sigma-Aldrich (St Quentin-Fallavier, France) and Molecular Probes (Invitrogen, Cergy-Pontoise, France), as mentioned before (Gagnaire et al. 2015b).

### **2.2 Egg production**

All experimental procedures of this study were approved by IRSN Animal Care Committee and followed French regulations for animal experimentation (protocol P2017-05; registration number of IRSN laboratory: C13-013-07).

The eggs were obtained using zebrafish aged of 6-12 months (AB strain, GIS Amagen, Gif sur Yvette, France) as described before in Gagnaire et al. (2020a). Genitors were maintained in a Tecniplast ® rearing system with a temperature of  $28 \pm 1^\circ\text{C}$  and a photoperiod of 12h/12h. They were fed three times per day with GemmaWean fish feed (Planktovie, France). Embryos were obtained from at least 40 animals by putting together 2 females and 2 males. All of the eggs spawned by all couples of genitors were pooled; egg viability was confirmed when the blastula stage was reached without visible abnormalities at 3 hpf (hours post fertilization). Experiments were started when the viability rate at 3 hpf was at least 90%.

### **2.3 Egg exposure to gamma irradiation**

Eggs used in these experiments were aged of 3 hpf. They were maintained in embryo medium (Westerfield 2007). Irradiations took place in incubators at a temperature of  $28 \pm 1^\circ\text{C}$  and a photoperiod of 12h dark/12h light. Eggs were randomly distributed on a standard 25 well-plate (5 eggs in 2 mL of embryo medium; VWR). Three dose rates were studied (one source by incubator). Plates were placed circularly around the central  $^{137}\text{Cs}$  gamma source (either liquid in a polystyrene tube (20 MBq in HCl 0.1 M) or solid (1.85 GBq)). Eight plates were used for each source. Only 3 or 5 wells on each plate were used in order that each well received a similar dose rate of gamma irradiation, depending on their position towards the source. The same experimental design was used for controls (25 well-plate, 5 eggs in 2 mL). Therefore, 160 eggs were used for each condition. Eggs/larvae were randomly sampled in different wells for all analyses. Dose rates of the three sources were characterized using RPL glass dosimeter measurements (ChiyoadaTechnologies, Japan) at means of  $3.3 \times 10^1$ ,  $1.34 \times 10^2$  and  $1.24 \times 10^3$   $\mu\text{Gy/h}$  (Dubourg, pers. comm.). Experiments lasted 10 days. Water was

renewed after 3 and 7 days of experiments. From days 6 to 10, a special feed for larvae was given to each well (GemmaMicro 75, Planktovie, France).

#### **2.4 Monitoring of embryonic developmental parameters**

Mortality was monitored daily. At 2 dpf, larvae started to hatch and were monitored each day until 10 dpf (Frayssé et al. 2006). Hatching time 50% (HT<sub>50</sub>), which is the time needed to reach 50% of hatched eggs, was calculated using REGTOX<sup>®</sup> ([http://www.normalesup.org/~vindimian/fr\\_index.html](http://www.normalesup.org/~vindimian/fr_index.html)).

At 1, 2, 3, 4, 7 and 10 dpf, pictures were acquired in order to measure total length, and egg and yolk bag diameters and analyzed on individual eggs or larvae as described before (Gagnaire et al. 2015b).

#### **2.5 Comet assay**

The comet assay in alkaline conditions was used to detect DNA strand breaks (single- and double-strand breaks, and alkali-labile sites) as described before (Gagnaire et al. 2015b) on eggs and larvae controls and irradiated according to the procedure of Singh et al. (1988) modified by Devaux et al. (1998). Three pools of ten 1 dpf-eggs and ten individual 4, 7 and 10 dpf larvae were used for each condition (control and 3 dose rates of gamma irradiation). The tail moment (measurement of fluorescence intensity in comet tail) was determined for all samples.

#### **2.6 ROS production**

Production of Reactive Oxygen Species (ROS), used here as a biomarker of immunotoxicity, was *in vivo* measured on 4, 7 and 10 dpf larvae, as described before (Gagnaire et al. 2013). Briefly, 20 larvae per condition were individually placed in HBSS buffer in a black 96-well plate. All larvae received H<sub>2</sub>DCFDA. In order to determine basal and stimulated activities, half of the larvae received DMSO and the other half received PMA. Fluorescence was monitored during 300 min (TECAN Infinite M1000). The ROS production index was calculated (PMA/DMSO).

#### **2.7 Gene expression**

RNA was extracted from three pools of fifteen 4, 7 and 10 dpf larvae per condition and the methodology described in Gagnaire et al. (2015b) was used to obtain the gene expression data. Briefly, Brilliant III Ultra-Fast SYBR<sup>®</sup> Green QPCR Master Mix was used in addition of



cDNA sample, primers (reverse and forward) and a referent dye on a Mx3000P real-time PCR machine (Stratagene, Agilent). 35 cycles of amplification were used (30 sec at 95°C, 1 min at 60°C and 1 min at 72°C). The expression of several genes involved in immune response (lysozyme C), apoptosis (bcl2-associated X protein), oxidative stress (myeloid specific peroxidase), detoxication (cytochrome P450 CYP1A, glutathion-S-transferase, metallothionein) and nervous system (acetylcholinesterase, choline acetyltransferase) were followed. The gene of elongation factor 1 was followed as a reference gene. The list of primers used has been previously published in Gagnaire et al. (2015b). The relative expression ratio of mRNA of every gene normalized by the reference gene was calculated using the threshold cycle Ct on REST-384© software (Pfaffl et al. 2002). The expression of the housekeeping gene efl (elongation factor 1) was validated between all conditions: Ct means  $\pm$  SE for controls and 4, 7 and 10 dpf larvae irradiated at the three dose rates were not different ( $18.9 \pm 0.6$  for controls and  $19.2 \pm 0.8$  for  $3.3 \times 10^1$   $\mu$ Gy/h ( $p=0.07$ , t test);  $18.8 \pm 0.6$  for controls,  $18.8 \pm 0.6$  for  $1.34 \times 10^2$   $\mu$ Gy/h and  $19.1 \pm 0.4$  for  $1.24 \times 10^3$   $\mu$ Gy/h ( $p=0.21$ , ANOVA).

## 2.8 Histological observations

Three larvae aged of 4, 7 and 10 dpf of each condition (controls and irradiated groups) were sampled for microscopical observations and individually analyzed as described before (Gagnaire et al. 2020a, Gagnaire et al. 2015b). However, for technical reasons, not all larvae of all ages and all groups could be analyzed.

## 2.9 Statistical analyses

Results (ROS and developmental parameters data) were expressed as means  $\pm$  standard error (se=standard deviation/square root(n)). Analyses were realized using STATISTICA Software v12 (StatSoft, Inc., Tulsa, OK, USA), with a significance level of  $p \leq 0.05$ . Normality was checked using Shapiro–Wilk tests. In the case of non-normal data, data were Boxcox-transformed. For normal data, differences between conditions (controls and irradiated larvae) were tested with t-tests or ANOVAs followed by an *a posteriori* post-hoc LSD (least significant difference) test in the case of rejection of  $H_0$ . For non-normal data, Kruskal-Wallis or Mann-Whitney U tests were used.

For statistical analysis of comet data, we used previously described method using R software (R\_Core\_Team 2017) on raw data of the individual cell tail moment (Gagnaire et al. 2020a). Briefly, we constructed a linear mixed-effects model using the *nlme* package (<https://CRAN.R-project.org/package=nlme>) with condition (control or irradiated larvae) as

224 fixed factor and replicates (slides) as random factor. Then, random permutation tests were  
225 applied using the *pgirmess* packages (<https://CRAN.R-project.org/package=pgirmess>).  
226

### 3. Results

#### 3.1 Mortality, hatching and embryonic development

Mortality and hatching of embryos were monitored every day. No significant difference was shown neither for mortality, nor for hatching in controls and irradiated groups (data not shown).

Larvae irradiated to  $3.3 \times 10^1$   $\mu\text{Gy/h}$  presented an increase of 11% of yolk-bag diameter only at 10 dpf compared to controls (t test,  $p=0.0066$ ) (Table 1). Larvae irradiated to  $1.34 \times 10^2$  and  $1.24 \times 10^3$   $\mu\text{Gy/h}$  presented variations for earlier stages: 1 dpf egg diameter was decreased of 11% (Kruskal Wallis test,  $p=0$ ) and 4 dpf length was increased of 5% for larvae irradiated to  $1.24 \times 10^3$   $\mu\text{Gy/h}$  compared to other conditions (ANOVA,  $p=0.0056$ ) (Table 1). At 7 dpf, larvae irradiated to  $1.34 \times 10^2$  and  $1.24 \times 10^3$   $\mu\text{Gy/h}$  had a yolk-bag diameter decreased of 18% and 11% compared to controls, respectively (ANOVA,  $p=0.034$ ), but no more effect was shown on larval length (Table 1). No significant difference was shown at 10 dpf.

#### 3.2 DNA damages

DNA damages were measured by comet assay at 1, 4, 7 and 10 dpf. Larvae irradiated to the three dose rates showed no significant differences compared to controls for all sampling points (Table 2).

#### 3.3 ROS production

ROS production was also evaluated at 1, 4, 7 and 10 dpf. At 7 dpf, larvae irradiated to  $3.3 \times 10^1$   $\mu\text{Gy/h}$  presented a significantly higher ROS basal activity compared to controls (U Mann Whitney test,  $p=0.027$ ); at 10 dpf, these larvae presented a ROS production index significantly higher than controls (t test,  $p=0.0148$ ), related to an increase of stimulated activity (U Mann Whitney test,  $p=0.0014$ ) (Table 3). Larvae irradiated to  $1.34 \times 10^2$  and  $1.24 \times 10^3$   $\mu\text{Gy/h}$  presented a ROS production index significantly higher than controls only at 4 dpf (ANOVA,  $p=0.0035$ ), also related to an increase of stimulated activity (Kruskal Wallis test,  $p=0.002$ ) (Table 3Table 3).

#### 3.4 Muscle histopathology

For technical reasons, all samples could not be analyzed. Larvae irradiated to  $1.34 \times 10^2$   $\mu\text{Gy/h}$  showed visible effects on mitochondria and myofibrils at 4 and 7 dpf (Figure 1B and C). Larvae irradiated to  $1.24 \times 10^3$   $\mu\text{Gy/h}$  showed more important effects compared to  $1.34 \times 10^2$

μGy/h at 7 and 10 dpf, with missing mitochondria and lesions on the myofibrils attachment point (Figure 1D and E).

### 3.5 Gene expression

The expression of the eight genes (and elongation factor) was followed in 1, 4, 7 and 10 dpf larvae. At  $3.3 \times 10^1$  μGy/h, only *bax* gene was significantly repressed in 7 dpf larvae compared to controls; in 10 dpf larvae, the same non-significant trend was observed (Table 4). At  $1.34 \times 10^2$  μGy/h, *ache* and *mt2* genes were repressed at 7 dpf and *cyp1a* gene was repressed at 10 dpf in irradiated larvae compared to controls; for *cyp1a* gene, the same non-significant trend was observed at 7 dpf (Table 4). At  $1.24 \times 10^3$  μGy/h, *mpx* gene was repressed at 7 dpf and overexpressed at 10 dpf, while *gstp1* gene was repressed at 10 dpf in irradiated larvae compared to controls (Table 4).

All of the results concerning tritium and gamma irradiation are presented in Tables 5 and 6, respectively. HTO data were previously published (Gagnaire et al. 2020a) but the dose rate was recalculated here without the weighting factors (see explanations in (Gagnaire et al. 2020a)) and results were summed up here to allow a direct comparison with gamma irradiation results. For HTO, dose rates of  $3.43 \times 10^1$ ,  $1.36 \times 10^2$  and  $1.26 \times 10^3$  μGy/h for nominal concentrations of HTO of  $1.22 \times 10^4$ ,  $1.22 \times 10^5$  and  $1.22 \times 10^6$  Bq/mL, respectively (Table 5), so very close to dose rates studied in gamma irradiation ( $3.3 \times 10^1$ ,  $1.34 \times 10^2$  and  $1.24 \times 10^3$  μGy/h, Table 6).

## 4. Discussion

### 4.1 Effects of gamma irradiation on embryonic development, DNA damage, ROS production, gene expression and muscle integrity

In the present work, no effect of gamma irradiation was seen on mortality or hatching. Zebrafish exposed to 38 mGy/h presented an increase in mortality (Hurem et al. 2017b), showing that this endpoint is sensitive as higher dose rates than the one tested in our study. However, an increase of 4 dpf larval length was shown at  $1.34 \times 10^2$  and  $1.24 \times 10^3$   $\mu\text{Gy/h}$  and a decrease of yolk bag diameter was shown in 7 dpf larvae at  $1.34 \times 10^2$   $\mu\text{Gy/h}$ . This result was previously observed in zebrafish larvae irradiated in the same conditions at  $2.4 \times 10^4$   $\mu\text{Gy/h}$  (Gagnaire et al. 2015b). Differences on larval length in our study was transient and may be due to a difference in kinetic of vitellin reserve consumption in the different conditions. Malformations were also observed in 4 dpf zebrafish exposed at 0.4 mGy/h (Hurem et al. 2017b). It would be interesting to continue the observation of animals to see if this increase of 11% of yolk bag diameter has an impact on age at metamorphosis or on adult stage. Previous experiments showed an acceleration of hatching in zebrafish larvae irradiated to dose rates comprised between  $3.3 \times 10^1$  and  $4.1 \times 10^4$   $\mu\text{Gy/h}$  (Gagnaire et al. 2015b, Pereira et al. 2011, Simon et al. 2011), while a decrease was observed at  $1.5 \times 10^4$   $\mu\text{Gy/h}$  when eggs were irradiated earlier (2 hpf) (Hurem et al. 2017b) and an absence of effects was shown after exposure to 0.5-50 mGy/h on zebrafish larvae irradiated starting at 1 hpf (Murat El Houdigui et al. 2019). These whole results seem to indicate that gamma irradiation induces an increase in consumption of energetic reserves and therefore an increase of development and of global metabolism of larvae. It would be interesting to deep further in these results at the physiological level using DEBtox approaches to characterize which mechanism leading to this altered phenotype is impaired (Kooijman et al. 2009).

Gamma irradiation has often been shown to induce DNA damages in zebrafish larvae at different dose rates, such as  $3.3 \times 10^1$   $\mu\text{Gy/h}$  (Gagnaire et al. 2015b) and from  $4.16 \times 10^2$  to  $4.16 \times 10^5$   $\mu\text{Gy/h}$  (Simon et al. 2011). DNA damages also appeared in progeny when parents were exposed to gamma irradiation (Guirandy et al. 2019) or when exposure occur during gametogenesis (Hurem et al. 2018). However, at  $2.4 \times 10^4$   $\mu\text{Gy/h}$ , DNA damages increased at 1 dpf and decreased at 4 dpf, suggesting that repair mechanisms occurred (Gagnaire et al. 2015b). The absence of effects of gamma irradiation on DNA damage in the present work is unexpected, as DNA damages are one of the first effect observed in animals exposed to radiations. DNA strand breaks are known to repair very quickly, therefore we can hypothesize

that for older larvae (7 and 10 dpf), the repair mechanisms suspected in the previous study on 4 dpf larvae could have been induced to a large extent, as physiological functions are more developed in older animals. Moreover, the background DNA damage levels was high in controls for 1 and 4 dpf, explaining the difficulty to show some effects for  $1.34 \times 10^2$  and  $1.24 \times 10^3$   $\mu\text{Gy/h}$ . An interesting complement would be to study the repair mechanisms initiated for example by gamma H2AX protein, which has been shown to be a sensitive endpoint in fish early life stages exposed to gamma irradiation (Honjo and Ichinohe 2019, Sayed et al. 2017).

In 10 dpf larvae exposed to  $3.3 \times 10^1$   $\mu\text{Gy/h}$ , an increase in ROS production due to a decrease of ROS basal level was observed, while no effect was seen on stimulated level. The exact same kind of result was already shown for other pollutants, such as endocrine disruptors (Bado-Nilles et al. 2014b) and uranium (Gagnaire et al. 2015a, Gagnaire et al. 2014, Le Guernic et al. 2016). However, an increase of ROS basal level was shown in zebrafish progeny when parents were exposed to gamma irradiation (Guirandy et al. 2019, Hurem et al. 2017a). On the contrary, 4 dpf larvae exposed to  $1.34 \times 10^2$  and  $1.24 \times 10^3$   $\mu\text{Gy/h}$  showed also an increase of ROS production index, but this was due to an increase of the stimulated level, as shown before for agricultural products (Bado-Nilles et al. 2014a). However, for both dose rates, no effect was shown at 7 and 10 dpf. Larvae irradiated to high dose rates of gamma irradiation seem therefore able to regulate ROS production, hypothetically by the development of compensatory mechanisms. In previous experiments, irradiation to  $2.4 \times 10^4$   $\mu\text{Gy/h}$  did not induce ROS production, which confirms this hypothesis (Gagnaire et al. 2015b). However, at low dose rate ( $3.3 \times 10^1$   $\mu\text{Gy/h}$ ), effects persist at 10 dpf and ROS production index was decreased at 4 dpf (Gagnaire et al. 2015b). ROS production is a global indicator of stress, used to assess the exposure to stressful conditions (including pollutants), but the response can vary during time and can be transient (Hakim 1993). Our results show the non-linearity of responses of ROS production after a gamma irradiation.

Several histological alterations were shown for larvae exposed to the highest dose rates. Degradation of myofibrils with alteration of actin and myosin filaments were already shown in previous studies after irradiation at  $3.3 \times 10^1$  et  $2.4 \times 10^4$   $\mu\text{Gy/h}$  during 4 dpf, but without any effects on the mitochondria (Gagnaire et al. 2015b). These results showed tissue alterations that can affect muscle contractility and cellular respiration and have consequences on larvae motility. At other dose rates, zebrafish larvae locomotion was impacted (Murat El Houdigui et al. 2019). It would be interesting to complete the results with behavioural tests of motility and response to a stimulus to confirm this hypothesis.

Genes involved in detoxication were also repressed in the late stages of zebrafish larvae (*Cyp1a*, *gstp1* and *mt2*) at different dose rates, which was already seen before (Hurem et al. 2017b). Moreover, similarly to results previously obtained, *mpx* and *ache* seem to be the most modulated by gamma irradiation (Gagnaire et al. 2015b). It is interesting to note that at  $1.24 \times 10^3$   $\mu\text{Gy/h}$ , *mpx* gene modulation happened at 7 and 10 dpf, where no effect was shown on ROS production, and did not happen at 4 dpf, where the ROS production index was increased. This result could be explained by a delay of response between gene expression and ROS production. Taken together, these results confirm the induction of an oxidative stress by gamma irradiation.

It is important to underline that in their majority, obtained responses followed a non-monotonous dose-response. To explain this result, the hypothesis of plurality of molecular targets could be made: responses at low dose rates could be different from responses at high dose rates because molecular targets could be differentially activated depending on the dose, therefore producing different kinds of responses, as it was shown for endocrine disruptors (Lagarde et al. 2015). It seems important to take into account these non-linear responses in risk assessment, as NOEC or LOEC values are mostly defined for monotonous dose-response relationships.

#### **4.2 Comparison of results for HTO/tritiated thymidine/gamma radiation exposure**

In our previous work on HTO, dose rates were calculated by applying dose coefficients to external and internal dose rates, according to the hypothesis of an homogeneous tritium distribution in tissues (Gagnaire et al. 2020a). A ponderation coefficient of 3 was applied in order to take into account beta radiation efficiency as recommended (Brown et al. 2008). However, in the present paper, we removed the ponderation coefficient of 3 to calculation of doses rates, in order to allow direct comparison between HTO and gamma irradiation results.

Our results showed that gamma irradiation at high dose rate accelerated hatching, while HTO had no effect on this parameter; on the contrary, tritiated thymidine induced a delay of hatching for equivalent dose rates (dose rates of  $1.8 \times 10^2$ - $4.4 \times 10^2$ - $2.4 \times 10^3$   $\mu\text{Gy/h}$ ) and also some malformations. Developmental parameters, even if they were modified in a transitory way, confirmed the hypothesis of an acceleration of development for gamma irradiation and a slowing of development for tritium. However, for tritium, effects differed according to the form used. Developmental parameters do not seem to be relevant indicators of HTO effects on zebrafish larvae, contrarily to gamma irradiation and tritiated thymidine.

DNA damages were observed, mostly at 4 dpf, for both types of ionizing radiation and chemical forms studied. Increase of ROS production was also shown for the different stressors, but not for all stages or all dose rates studied. These results confirm the known data on the effects of ionizing radiation in terms of oxidizing stress and genotoxicity (Adam-Guillermine et al. 2012, Gagnaire et al. 2017, Gagnaire et al. 2020b, Hu et al. 2016).

For both gamma irradiation and tritium, genes involved in detoxication and neurotransmission were punctually altered, but only gamma irradiation induced effects on EROD and AChE activities (Gagnaire et al. 2015b). Gamma irradiation at  $5 \times 10^3$  and  $5 \times 10^4$   $\mu\text{Gy/h}$  also induced modification of expression of genes involved in nervous central system and decrease of locomotion when exposure happened in the gastrulation stage of zebrafish (Murat El Houdigui et al. 2019). The nervous system did not seem to be a target of HTO in zebrafish larvae, contrary to gamma irradiation. However, AChE activity was modified in adult fathead minnows exposed to HTO after depuration (Gagnaire et al. 2020b). Moreover, genes involved in muscle contraction were modified by HTO (Arcanjo et al. 2018), and swimming behavior of larvae was altered but only for one dose rate (Arcanjo et al. 2020). Gamma irradiation seemed to induce more deleterious effects on nervous system compared to HTO, but the pathway of alteration was similar for both stressors.

In the present study, only HTO modified the expression of *lyz* gene, involved in immune response. Immune parameters were also modified in adult minnows exposed to HTO (Gagnaire et al. 2020b). These results showed on the basis of these parameters some differences of responses to different kind of radioactive stressors.

Regarding the tissue level, gamma irradiation (Gagnaire et al. 2015b), as well as HTO (Gagnaire et al. 2020a), were previously shown to induce myofibril alterations on 4 dpf larvae. In the present study, important alterations were observed on mitochondria. This result could be related to the increase of ROS production also seen in the present study. The exact same results were observed after exposure to HTO at 7 and 10 dpf (Gagnaire et al. 2020a). Therefore, the mechanisms of action of these different kinds of radioactive stressors on zebrafish tail muscle were similar in early life stages.

Therefore, ROS production, DNA damages and detoxication process, as well as histology of muscle, responding similarly to both to tritium and gamma irradiation, seemed to be relevant and could be used in further studies in the context of environmental risk assessment of ionizing radiation. Our work also showed some specific response to tritium (immune system) and gamma irradiation (nervous system).



It is important to take into account the fact that tools available to calculate dose rates are based on the hypothesis of a homogeneous distribution of dose deposition, which may be the case for HTO and gamma irradiation but not for tritiated thymidine that is incorporated in DNA; however, as we have no other tool, we assume that tritium results obtained can be compared to gamma irradiation. An interesting perspective would be to better characterize tritium internal distribution at the cellular level (DNA molecule) and, with the help of modelisation (microdosimetry and toxicodynamics), to better understand the link between dose rates and effects (Sakata et al. 2019, Tang et al. 2019).

### **4.3 Comparison between the effects of gamma and beta ionizing radiation**

In the present work, we obtained data that were missing for the same dose rates as the ones already tested for HTO. The study of molecular responses showed that oxidative stress and DNA damages are the major toxic mode of action of gamma irradiation as well as tritium. For both stressors, the tail muscle integrity was also altered in the same way. However, these effects are generally not dose-dependent, which could indicate a compensation of several mechanisms of defense and toxicity, or the overwhelming of cell repair capacity. The effects seemed more pronounced for gamma irradiation than for tritium for equivalent dose rates (e.g. muscular alterations). Some molecular responses seemed more specific of gamma irradiation (e.g. AChE pattern modification at several biological levels) compared to tritium (modification of immune response). The whole results suggest some common pattern of responses, but also some differences of mode of action between HTO, tritiated thymidine (internal contamination) and gamma irradiation (external irradiation) on zebrafish larvae.

For beta emitters and in particular for tritium, a RBE of 3 was recommended (Adam-Guillermin et al. 2012, ASN 2010, Brown et al. 2008). However, there is no consensus on this value in the whole scientific community, due to the uncertainty of models (Harrison et al. 2002, Melintescu et al. 2007). The recent work of ICRP on biological effects of tritium on non-human species showed that RBE values obtained are of 1.5-2 towards X rays and 2-2.5 towards gamma rays (ICRP 2019). For environmental radioprotection, ICRP proposed to weight absorbed dose rates resulting of exposure of biota to radiation of low linear energy transfer (including beta radiation from tritium) by a RBE of 1. However, they recommend, when the level of exposure to tritium is susceptible to induce potentially deleterious effect on a population of plants or animals, that the use of a higher RBE can be considered (ICRP 2019).

We designed the work presented in this paper in order to calculate a RBE for tritium. Unfortunately, the RBE could not be calculated because i) the responses for ROS and DNA damages did not increase in a monotonous manner with the dose rate, both for tritium as for gamma irradiation, and ii) some responses were specific. Moreover, dose rates inducing no effects on followed parameters (NOEDR) are missing. However, on the basis of obtained results in the present study and in previous studies, the value of 1 proposed by ICRP seems to be relevant, as effects seemed to be more important after gamma irradiation than after tritium exposure for equivalent dose rates.

## 5. Conclusions

The effects of a gamma irradiation were studied on zebrafish larvae during the critical period of the larval development until 10 dpf for three dose rates, the lowest one ( $3.3 \times 10^1$   $\mu\text{Gy/h}$ ) being close to the benchmark value for ecosystem protection towards radiations (10  $\mu\text{Gy/h}$ ). Effects on mortality, hatching, development, DNA damages, immune responses, gene expression and tissue integrity were searched.

The work presented here, added with previous work on gamma irradiation and tritium, allowed to show that ionizing radiation induced effects on zebrafish early life stages. Some pathways were seen to be shared with the different kinds of ionizing radiations tested, as effects on muscular tissue. However, the effects can differ considering the form studied for a same dose rate. Indeed, effects of tritium were not the same for HTO (transitory morphometric modifications) and tritiated thymidine (slowing of hatching, malformations of larvae). The form of tritium used plays a preponderant role in its toxicity.

These results allow to show the interest of using molecular responses in complement to macroscopical observations better understand the mechanisms activated during zebrafish larvae exposure to different kinds of ionizing radiations. Some results still need to be acquired in order to calculate a RBE for tritium, with the help of microdosimetry to better characterize the internal dose.

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## Figure captions

**Figure 1:** muscular tissue of zebrafish larvae observed by TEM in control (A) and irradiated at  $1.34 \times 10^2$   $\mu\text{Gy/h}$  at 4 (B) and 7 (C) dpf. E: endomysium, M: mitochondria, My: myofibrils, S: sarcomere, Z: Z stria.

**Table 1:** measurements in mm of egg diameter, total length and yolk bag (YB) diameter in zebrafish eggs and larvae control and irradiated to  $3.3 \times 10^1$ ,  $1.34 \times 10^2$  and  $1.24 \times 10^3$   $\mu\text{Gy/h}$ . Values are expressed as means of at least 10 replicates. Bold values indicate a significant difference between conditions.  $p \leq 0.05$ ; a, b: significantly different,  $a < b$ . n.d.: not determined.

**Table 2:** mean tail moments (comet assay) obtained for 1 dpf zebrafish embryos ( $n=3$  pools of 10) and 4, 7 and 10 dpf larvae ( $n=10$ ) control and irradiated to  $3.3 \times 10^1$ ,  $1.34 \times 10^2$  and  $1.24 \times 10^3$   $\mu\text{Gy/h}$ . Mix model (R),  $p \leq 0.001$ . n.d.: not determined in the present study.

**Table 3:** ROS basal and stimulated levels and stimulation index in zebrafish larvae irradiated at  $3.3 \times 10^1$ ,  $1.34 \times 10^2$  and  $1.24 \times 10^3$   $\mu\text{Gy/h}$  during 10 days. Values are expressed as means of 20 replicates (10 for stimulated levels and 10 for basal levels); standard error is presented.  $p < 0.05$ ; Bold values indicate a significant difference between conditions. a, b, c: significantly different,  $a < b < c$ .

**Table 4:** relative gene expression (normalized to reference gene *ef1*) compared to control in zebrafish larvae irradiated at  $3.3 \times 10^1$ ,  $1.34 \times 10^2$  and  $1.24 \times 10^3$   $\mu\text{Gy/h}$  during 10 days ( $n=9$ ). Analysis with REST-384©,  $p \leq 0.05$ ; bold values indicate a significant difference compared to

control; underlined values indicate a non-significant difference compared between exposed and control of a factor 2 or superior.

**Table 5:** synthesis of results obtained for exposition of zebrafish larvae to tritiated thymidine and HTO (adapted from Adam-Guillermin et al. (2013) and Gagnaire et al. (2020a)). For explanations concerning weighting factors, please refer to Gagnaire et al. (2020a). C: control; Ø: no significant effect; -: not relevant; n.d.: not determined. Diam: Diameter; YB: Yolk Bag.

**Table 6:** synthesis of results obtained for exposition of zebrafish larvae to five dose rates of gamma irradiation. C: control; Ø: no significant effect; -: not relevant; n.d.: not determined. Diam: Diameter; YB: Yolk Bag; HT<sub>50</sub>: hatching time 50%.

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