Tritiated thymidine induces developmental delay, oxidative stress and gene overexpression in developing zebrafish (Danio rerio)

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Abstract

Tritium is a betta emitter radionuclide. Being an isotope of hydrogen, it is easily transferred to different environmental compartments, and to human and non-human biota. Considering that tritium levels are expected to rise in the upcoming decades with the development of nuclear facilities producing tritium using fission processes, investigating the potential toxicity of tritium to human and non-human biota is necessary. Tritiated thymidine, an organic form of tritium, has been used in this study to assess its toxicity on fish embryo development. Zebrafish embryos (3.5 hpf; hours post fertilization) have been exposed to tritiated thymidine at three different activity concentrations (7.5; 40; 110 kBq/mL) for four days. These experiments highlighted that zebrafish development was affected by the exposure to organic tritium, with smaller larvae at 3 dpf after exposure to the two lowest dose rates (22 and 170 µGy/h), a delayed hatching after exposure to the two highest dose rates (170 and 470 µGy/h), an increase in the spontaneous tail movement (1 dpf) and a decrease in the heartbeat (3 dpf) after exposure to the highest dose rate. The results also highlighted an increase in ROS production in larvae exposed to the intermediate dose rate. A dysregulation of many genes, involved in apoptosis, DNA repair or oxidative stress, was also found after 1 day of exposure to the lowest tritium dose rate. Our results thus suggest that exposure to tritiated thymidine from a dose rate as low as 22 µGy/h can lead to sublethal effects, with an effect on the development, dysregulation of many genes and increase of the ROS production. This paper provides valuable information on toxic effects arising from the exposure of fish to an organic form of tritium, which was the main objective of this study.

1. Introduction

Tritium is a radioactive isotope of hydrogen[1]. In the environment, it comes from different sources: the interaction between the cosmic rays and atmospheric oxygen and nitrogen atoms is the only natural source of tritium, while there are more types of anthropogenic releases, including nuclear power plants, nuclear reactors, but also nuclear incidents or weapon testing[1,2]. Despite those numerous anthropogenic sources of tritium, those releases are still being closely monitored, those monitoring mostly focusing on the activity concentrations in the different environmental compartments. In France, for example, in non-nuclearized aquatic ecosystems, tritium levels range from 3 to 4 Bq/L, while in nuclearized areas, those levels rise and reach 2.5 to 12.85 Bq/L, with a mean value of 6.31 Bq/L[3]. In the environment, tritium can be found in three different forms: free in the water (HTO), atmospheric tritium (HT) or organically bound tritium (OBT)[1]. This last form can be separated into two different ones: the exchangeable OBT (eOBT), where tritium is weakly bound to oxygen, sulphur or nitrogen atoms, and the non-exchangeable OBT (neOBT), where tritium is strongly bound to a carbon atom[3]. Numerous studies have shown that tritium can be transferred to biota, accumulate in living tissues and, depending on its chemical form (free or organic), can be incorporated in the DNA during its synthesis[4–10]. This ability made the study of its possible toxic effects even more relevant. Considering that radiations emitted by tritium have a low penetrating power, the only exposure source considered in this study is the internal exposure (*i.e.*, the exposure to the tritium that has been translocated in the embryos and larvae).

As for many other contaminants, tritium effects on early development stages have been studied on numerous species. For example, it was shown that tritium induced a delay in hatching of zebrafish embryos (*Danio rerio*), no matter what chemical form was used[11,12]. Literature also reports that tritium can induce a delay in development in species like

zebrafish, blue mussel (*Mytilus edulis)* and grass puffer (*Fugu niphobles)*, while it seems to have the opposite effect on the prussian carp (*Carrasius gibelis)*[3,10,13]. While affecting the macroscopic development, it seems that it can also affect microscopic and molecular processes. For example, a study showed that the spontaneous tail movement in 24 hpf zebrafish embryos decreased after an exposure to HTO at 3.7 kBq/mL, while the same exposure increased the heartbeat of the individuals at 60 and 120 hpf[12].

Other studies also highlighted that exposure to tritium leads to an increase in the reactive oxygen species (ROS) in bacterial cells, fathead minnow (*Pimephales promelas*) and zebrafish, while the GPx activity also increased with the tritium dose rate in fathead minnows[7,10,14]. Finally, it was shown that genes involved in detoxication and neuro-transmission in zebrafish were affected after exposure to tritium, but only in a transient way, while a RNA sequencing study carried out on zebrafish embryos and larvae showed that many genes were either over- or under-expressed after exposure[11,15]. Genes involved in somite differentiation, oxidative reduction processes and peripheral nervous system axonogenesis were down-regulated. In contrast, genes involved in the response to oxidative stress, circadian rhythm, response to light stimulus, and regulation of DNA methylation were up-regulated[15]. Genes involved in processes linked to the muscle contraction or the sarcomere assembly were up- and down-regulated depending on both the developmental stage and the dose rate used for the exposure. Thus, when studying the toxic effects of various compounds on living organisms, multiple biological scales should be used, as it could give more information on the possible modes of action of the contaminant to induce toxicity[3,16,17].

Even though all those studies give multiple information on HTO toxic effects on various aquatic species, very few studies focused on the toxic effects that could arise from an exposure to organic tritium, which is the main objective of our study[8,18]. Another

objective was to determine and confirm toxic mechanisms specific to tritium and, more generally, radiations, by comparing the toxicity induced by tritiated thymidine to the toxicity induced by HTO or gamma irradiation. To do so, three tritiated thymidine dose rates were used (22, 170 and 470 µGy/h). Zebrafish is used as a biological model because it is a convenient species, with adults being able to lay hundreds of eggs, but also because these eggs are transparent, easy to handle, and because its genome is fully sequenced[19,20]. Early development stages were also chosen as it is well known that those are usually considered as being more sensitive to most contaminants, but also because is it a commonly used model in toxicological studies[3,10,11,15,21–26]. Zebrafish embryos were exposed three activity concentrations (7.5; 40; 110 kBq/mL) to study toxicological effects than can be classified in three different categories depending on the biological scale concerned. First, the macroscopic part of the study will focus on the development of the larvae, while a second part of the study will focus on toxicity markers as the spontaneous tail movement and the heartbeat. Finally, molecular studies will focus on the ROS production index and the expression of 30 different genes. The selected genes are involved in multiple biological processes such as DNA repair, apoptosis, oxidative stress regulation and eye development. As multiple effects have already been observed after the exposure of fish to different radionuclides, with for example the deregulation of various genes involved in the response to oxidative stress and DNA damage, but also with disruption of the development, the main hypothesis behind this study is the induction of disruptions by tritiated thymidine on the same biological targets, but with different severities.

2. Material and methods

2.1. Fish

All experimentations presented in this paper were previously approved by IRSN Animal Care Committee and followed French regulations for animal experimentation (protocol P2019-19; registration number of IRSN laboratory: C13-013-07). Three to four months old wild-type zebrafish (AB genetic background) were supplied by Paris Sorbonne University (France) and were kept in a zebrafish housing system (Zebtec Techniplast® Stand Alone). The light:dark photoperiod chosen was 12h:12h and fishes were fed three times a day with Gemma Wean 0.3 (Sketting). All water parameters were monitored daily (pH 7.5 ± 0.2; conductivity 450 ± 50 µS.cm-1, temperature 27.5 ± 0.5 °C). As males and females were kept in separated tanks to increase the effectiveness of the mating process, the mating was also done once every three weeks during the periods during which no experimental procedures were planned. All matings were done in the husbandry using the water from the housing system and using fishes that were between 4 and 13 months old.

2.2. Embryo exposure and tritium contamination

Embryo medium (pH 7.5, conductivity 500 µS/cm in Milli-Q water) was prepared using InstantOcean® sea salt at a concentration of 60 mg/L and was used to dilute the tritiated thymidine solution and as a negative control[27]. Tritiated thymidine solutions (97 % purity, 185 MBq, PerkinElmer, Courtaboeuf, France) were prepared in three different activity concentrations (7.5; 40 and 110 kBq/mL), chosen according to a previous study in order to obtain 3 different internal dose rates: 22; 170; 470 µGy/h[5]. As multiple experiments were necessary to study the effects of tritiated thymidine at one dose rate, a 10 % variability was accepted in the initial measured activity concentration between each experiment.

All animals were exposed to tritiated thymidine as presented elsewhere[5]. Briefly, at 3.5 hpf, zebrafish embryos were placed in two to three glass crystallizers covered with

Parafilm at a density of two eggs per mL of working solution for four days. Each crystallizer was considered as an exposure replicate. The embryos were kept in the dark in thermo-regulated incubators (Heraterm, ThermoScientific) at 27 ± 1 °C and 80 % of the working solution was replaced after 48 hours of exposure. Temperature (°C) and relative humidity (%) were monitored every 30 minutes during the exposure (HOBO data logger U12, Onset), and beakers were filled with tritium-free water (Abatilles water, France, 40 mL) to monitor tritium exchanges in the incubator.

For both development and hatching studies, two 25-well plastic plates were added in the incubators. In the development plate, 4 wells were filled with 5 embryos each in 2.5 mL of working solution, while the wells that did not contain any embryos were kept empty. In the hatching plate, 8 wells were also filled with the same amount of embryos and working solution. Both plates were covered with Parafilm® to avoid any significant evaporation of the medium.

2.3. Tritium activity concentration measurements

In order to monitor the evaporation of the medium from both the crystallizers and the plates, tritium activity concentration was measured in the beakers (1 replicate/crystallizer, once a day) and in the crystallizers (3 replicates/crystallizer, after 0; 24; 48 and 96 hours of exposure). The sampling and the measurement was adapted from Schiano Di Lombo et al.[5]. The water sampling, differing depending on the activity concentration used in the experiment, is presented in Table 1. All 1 mL samples were diluted in 19 mL of the liquid scintillation cocktail (Ultima Gold LLT, PerkinElmer). The liquid scintillation measurements were done using a Quantulus (Quantulus 1220, Perkin Elmer) monitored by WinQ (1220-307 WinQ, Perkin Elmer). Each measurement was done five times and lasted 75 minutes.

2.4. Monitoring of the embryo-larval development

2.4.1. Hatching and morphometric measurements

After 24, 48, 50, 52.5, 69, 71, 73, 75, 77, 93 and 96 hours of exposure, embryos and larvae were counted in each well of the plate. Hatching time 50 % (HT50, time at which 50 % of the individuals have hatched for a same replicate) has been calculated using REGTOX® (n = 500 simulations, http://www.normalesup.org/~vindimian/fr.index.html) and was used in the statistical analyses.

Morphometric measurements were made by following the procedure already presented[28]. Briefly, 1 to 4 dpf individuals were pictured every day at the same hour using a camera (Nikon D5000) connected to a binocular microscope (ZEISS SteREO Discovery V20, magnification 93x). Pictures were first analysed to qualify if the individual was normal or malformed, and the egg and yolk sac diameters (1 to 2 dpf) were measured using ImageJ, as well as the total length of the larvae, the yolk sac and the eye diameters (3-4 dpf).

2.4.2. Spontaneous tail movements of 1 dpf embryos

The spontaneous tail movement was measured in 1 dpf embryos using a protocol adapted from the one presented by Murat et al. in 2019[29]. Six pools of 10 embryos per condition were used and, in a room set at 28 °C, 10 embryos were immobilized in 2 % w/v methylcellulose in PBS and set under the light on a stereomicroscope (Nikon SMZ800) connected to a high-resolution camera (acA1300-60gm, Basler, Germany). After two min of acclimatation, a 5 min-video was recorded using EthoVision XT16 (Noldus, Netherlands). The burst activity, the burst duration, and the burst count (%) was determined using DanioScope (Noldus, Germany).

2.4.3. Heart rate of 3 dpf larva

Measurement of the heart rate was measured in 3 dpf larvae using a protocol adapted from Murat et al.[29]. Six pools of 5 larvae per condition were used. In a room set at 28 °C, 5 larvae were immobilized in 2 % w/v methylcellulose with 0.02 % MS-222 (Sigma-Aldrich, France) in a 55 mm petri dish. After an acclimatation period of 2 min under the light on the stereomicroscope (Nikon SMZ800) connected to the high-resolution camera (acA1300-60gm, Basler, Germany), a 25 seconds video was recorded using EthoVision XT16 (Noldus, Germany). Heart rate (BPM) was measured using DanioScope (Noldus, Germany).

2.5. Gene expression analysis

2.5.1. RNA extraction and quantification

RNAs from the samples were extracted following the protocol published previously[29]. Briefly, 3 pools of 25 to 30 embryos (1 dpf) and 3 pools of 15 larvae (4 dpf) were grinded using 300 µL of Trizol in ceramic beads tubes (CK14 tubes, Bertin Instruments, France) with the Precellys 24 grinding machine (Bertin Instruments, France). After homogenisation, all samples were stored at – 80 °C. For RNA extraction, samples were thawed, and all sample handling and centrifugations were done at 4 °C. Chloroform (60 µL) was added to the homogenized samples in Trizol, followed by vortexing and centrifugation at 13000g for 15 min at 4 °C. The upper aqueous phase was collected in a new tube and 60 µL of chloroform was added before centrifuging in the same conditions as before. The upper aqueous phase was collected in a new tube and 150 µL of isopropyl alcohol was added before the samples were vortexed and stored at – 80 °C for 30 min. The samples were then centrifuged during 30 min at 13000 g. The supernatant was removed,

and the RNA sample was washed using 150 µL of ethanol 80 % (v/v) by centrifuging the sample for 5 min at 13000 g. The supernatant was then removed and once the RNA sample had been air-dried, it was diluted in 15 µL of RNAse and DNAse free water. All RNA concentrations (ng/µL) and quality (RNA Integrity Number, RIN) were assessed using RNA Nano Chips (BioAnalyzer 2011, Agilent Technologies). All samples were used as all RINs were above 9.

2.5.2. cDNA synthesis and preamplification and TaqMan assay

Reverse transcription was done on 1 µg of extracted RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), following the manufacturer's instructions. After the reverse transcription was completed, 100 of cDNA were used for the rest of the experiment. The cDNA samples were preamplified using Custom TaqMan PreAmp Pool and TaqMan PreAmp Master Mix (2X; Applied Biosystems) while following the manufacturer's instructions. Gene expression was quantified using the preamplified cDNA samples in a 384-well microfluidic TaqMan Array Card in which the expression of 31 genes was measured for 4 samples with 3 analytical replicates (32 format). The array cards were processed in the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems). All Ct results were combined on the ExpressionSuite Software for QuantStudio 6 & 7 Flex Real Time PCR System and exported to DataAssist v3.01 (Applied Biosystems) for analysis while using the 18S RNA gene expression as an endogenous control. Ct values were used to obtain ΔCt values, each value corresponding to the gene expression of each biological replicate, normalized using the reference gene. ΔCt were then used to calculate $2^{(-\Delta Ct)}$, which was in turn used to determine the fold change. All genes studied in this experiment are presented in Supplementary Information (Table S1).

2.6. Oxidative stress measurements

Oxidative stress was studied by measuring ROS production. The protocol used was the one presented elsewhere[30]. Briefly, for each condition, 20 larvae were sampled after 96 hours of exposure. All larvae were individually placed in 96-well plates. All of them received 100 µL of HBSS buffer (ThermoFisher) and 50 µL H_2DCFDA (2 mg/L in DMSO 0.4 %). The first ten larvae received 50 µL DMSO at 0.4 % while the other ten larvae received 50 µL Phorbol myristate acetate (PMA, 0.8 mg/L in DMSO 0.4 %). Fluorescence emitted by the reaction between H_2DCFDA and ROS was measured over 300 min (TECAN Infinite M1000). Basal and stimulated levels were calculated on the linear part of the curves for larvae incubated with DMSO and PMA, respectively. ROS production index was calculated as the ratio PMA/DMSO.

2.7.Statistical analyses

All statistical analyses and figures were made in R environment using RStudio using the *PMCMR, PMCMRplus, car, lmtest, scales, SciViews, ggplot2, ggpubr, ggpmisc, plotly, plot3D, FSA, rcompanion, multcomp, drc and lme4* packages. The threshold chosen for significative results was 0.05.

For all development and oxidative stress analyses, linear mixed models (GLMM/LMM) were used. The treatment was set as the fixed variable, while variables as the crystallizer was used as a random variable. In more specific experiments as the measurement of the spontaneous tail movement and the heartbeat, the trial was also added as a random variable, which was also the case for the larval position when performing statistical analyses on the larval yolk measurement. For other developmental and hatching analyses, basic GLM were used as there was no variables identified as random. When the normality of the residuals and the homogeneity of the variance were not met ($p < 0.05$),

data was transformed using either Log10, square root or boxcox transformations. The treatment effect was then checked and, when necessary, the Tukey-HSD post-hoc test was used. For the gene expression data, all analyses were made using Data Assist 3.01, where Ct were used to obtain the fold change by calculating ΔCt and $2^{(-\Delta Ct)}$. All ΔCt were compared to the control values with the use of a t-test, and all p-values were adjusted using the Benjamini-Hochberg False Discovery Rate.

Except for the gene expression, all results have been represented as boxplots. Regarding the horizontal lines, the lowest one represents the first quartile, while the middle one represents the median value, and the highest one represents the third quartile. When data points are outside of the boxplot lines, they are considered as being outliers, but were not removed from the statistical analysis when there was no experimental reason to do so. In the boxplots, the dots correspond the data points obtained during the experiments. When different graphs are presented for the same analysis, it means the results come from different experiments, and different batches of embryos.

3. Results

All exposure experimentations led to cumulative mortality rates below 20 % in the control condition. Exposure to tritiated thymidine at all dose rates tested did not induce any significant induction of mortality (data not shown).

3.1. Hatching

The hatching results are presented in Figure 1. Even if a complete hatching was observed after 96 hours of exposure, the hatching was delayed when the larvae were exposed to the intermediate and the greater tritiated thymidine activity concentrations ($4x10^4$ and $1x10^5$ Bq/mL; 170 and 470 µGy/h) when compared to the control (p = 0.02 and

294 $p < 0.01$, respectively). Even if the larvae exposed to the lowest activity concentration
295 ($7.5x10^3$ Bq/mL; 22 µGy/h) did not show a significantly longer hatching time compared to
296 controls, it also did not show any significant differences with the hatching time of the larvae
297 exposed to the intermediate activity concentration.
298
299 **3.2. Developmental defects**
300 **3.2.1. Development measurements**
301 The exposure of the embryos and larvae to tritiated thymidine at all dose rates tested
302 did not induce any skeletal malformations, nor peri-cardiac or peri-vitelline oedema (data
303 not shown).
304 The egg and yolk sac diameters measured on controls and embryos exposed to 22
305 µGy/h and 170 µGy/h for 1 and 2 days are presented in Table 2, alongside the yolk sac and
306 eye diameters and total length of the larvae exposed to those same dose rates for 3 and 4
307 days. First, it is seen that the embryos exposed to the lowest activity concentration for 1
308 day had a larger yolk sac than the control embryos ($p = 0.02$). This effect was only transient
309 as it is not observed after 4 days of exposure. Most of the morphometric differences were
310 observed for the 3 dpf larvae. Indeed, larvae exposed to both the lowest and the
311 intermediate activity concentrations were shorter than the control larvae ($p = 0.03$ and $p <$
312 0.001, respectively). The yolk sac and eye diameters were also smaller for the larvae
313 exposed to the intermediate activity concentration when compared to the control ($p = 0.006$
314 and 0.004, respectively). All those differences were only temporary as no significant
315 differences were observed for the 4 days old larvae. Through the whole experiment, no
316 significant differences were observed between both thymidine activity dose rates (22 and
317 170 µGy/h).

318 The egg and yolk sac diameters measured on embryos exposed to 0 and 470 µGy/h for
319 1 and 2 days are presented in Table 3, alongside the yolk sac and eye diameter and lengths
320 of the larvae exposed to those same dose rates for 3 and 4 days. When larvae were exposed
321 to tritiated thymidine at this high dose rate, no significant morphometric changes were
322 observed ($p > 0.05$).
323
324 **3.2.2. Spontaneous tail movement**
325 After exposure to the two lowest activity concentrations (22 µGy/h and 170 µGy/h),
326 no significant changes in the spontaneous tail movement were observed compared to the
327 control individuals (data not shown). After exposure to the highest activity concentration
328 (470 µGy/h, Figure 2), a significant increase was observed in the burst activity, the total
329 burst duration, the burst count, and the burst count per minute. The mean burst duration
330 was the only spontaneous tail movement measurement that was not affected by the
331 exposure to tritiated thymidine (data not shown).
332
333 **3.2.3. Heart rate**
334 Heart rate was measured in 3 dpf larvae exposed to 0, 170 µGy/h and 470 µGy/h of
335 tritiated thymidine (Figure 3). After exposure to tritiated thymidine at the lowest dose rate
336 (22 µGy/h, data not shown), no significant change was observed in the heart rate compared
337 to the control. After exposure to the intermediate dose rate (170 µGy/h), there were no
338 significant changes in the heart rate, but a marginal increase was still observed ($p = 0.07$).
339 Finally, after exposure to the highest dose rate (470 µGy/h), a significant decrease in the
340 heart rate was observed ($p < 0.01$).
341
342 **3.3. Gene expression analysis**

The gene expression analysis results are presented in Figure 4. The number of dysregulated genes decreased when the tritiated thymidine dose rate increased. When embryos were exposed to the highest dose rate of tritiated thymidine (470 µGy/h), the changes in gene expression were not significant. At 170 µGy/h, a marginal of over-expression was observed for *casp9, cyp1a* and *sod2* ($p < 0.1$, 1.5 to 2.5-fold increase). Finally, embryos exposed to the lowest dose rate (22 µGy/h) showed genes that were dysregulated, with all genes being overexpressed. Genes as *aanat2, baxa, bbc3, bcl2, cryba, ddb2, h2ax, mmp13, mpx* and *xpc* showed a marginal overexpression ($p < 0.1$, fold changes varying between 1.4 and 4.4), while genes as *ache, casp9, crx, cry5, cyp1a, efl1a, gstp1, hspb9, lyz, saga, sod2, tbpl1* and *xrcc1* were all significantly overexpressed ($p < 0.05$, fold changes between 1.4 and 4.4). Other genes as *atp1a1l, eef1a2, gadd45bb, oxt, per2* and *tnni2b.2* were also studied, but no significant variations were observed ($p > 0.1$, data not shown).

The same genes were also studied after 4 days of exposure, but no significant dysregulation was observed in the gene expression, for all three dose rates ($p > 0.1$, data not shown).

3.4. Oxidative stress measurements

The ROS production index measured in larvae after exposure to tritiated thymidine is presented in Figure 5, as well as the fluorescence levels measured after exposure of the larvae to PMA. The ROS production index only significantly rose after the exposure to the intermediate dose rate (170 µGy/h, $p = 0.001$). The basal fluorescence level (larvae only exposed to DMSO) and the stimulated fluorescence level (larvae exposed to PMA) were also analysed to obtain more information. The basal fluorescence level did not change with any of the tritiated thymidine dose rates ($p > 0.05$, data not shown), while the stimulated

fluorescence level increased significantly after exposure to all three dose rates ($p < 0.01$). The stimulated level found in larvae exposed to 170 µGy/h was also greater to the one measured in larvae exposed to 22 µGy/h ($p < 0.01$).

4. Discussion

To assess tritiated thymidine effects on zebrafish development, hatching success, embryo and larva morphometry, as well as their spontaneous tail movements and heartbeat were studied. ROS production and expression of different genes of interest were also monitored.

This work showed that tritiated thymidine, while not causing significant increase of the embryo-larval mortality, does affect the development of zebrafish. Indeed, the embryonic development seemed to be slowed down, even if this effect was only transient, with larvae being smaller after 3 days of exposure at the two lowest dose rates. These observations were expected, as radiations are known to have early effects on development, with zebrafish larvae being small after being irradiated for 24 h at a dose rate of 41.6 µGy/h of Cs-137[23]. The only difference is that in the case of that study[21], the effect increased with the dose rate, while in the present study, effects only increased up to the intermediate dose rate and no differences were found after exposure to the highest dose rate. More specifically, these observations have already been reported in the case of tritium, where zebrafish larvae exposed for 3 days to HTO presented smaller yolk sacs and shorter lengths after exposure to a dose rate of around 150 µGy/h. These results show that tritiated thymidine might have a higher toxicity than HTO, considering this effect appeared at lower dose rate for organic form of tritium[10]. However, the fact that the yolk sac was smaller in contaminated larvae compared to control ones does not match with a slower development. Indeed, if the embryo development was reduced, the yolk sac in irradiated embryos would

be bigger than in the control, which is not the case here. Our results indicated that more yolk has been consumed by embryos after exposure while the development was not accelerated, leading to the hypothesis that the energy allowance is different after exposure, with more energy being allowed to possible metabolic processes induced by the exposure, as the repair or renewing of damaged cells, as those processes have already been observed in individuals exposed to radiations[6,11,24,29]. Though, this change in the energy allowance seems to be reverted when the dose rate used in the exposure reached a high value: the individuals exposed to the highest dose rates seemed to develop at the same rate than the control ones, while still showing a delay in hatching. Considering that there is a strong relationship between ATP production and energy use, quantifying this production could help identify if this hypothesis is correct[31]. This could, for example, be done my measuring the $NADH_2$ in zebrafish embryos and larvae, considering that this compound is strongly linked to the ATP production[32]. This could mean that, the contamination becoming too strong, the individuals stopped trying to fight its possible deleterious effects, their energy allowance thus returning to a control level.

The delay of the hatching is also in accordance with the development being slowed, and this effect has also already been observed after exposure to gamma radiations and tritium[3,12,23]. In teleost fish, hatching is the result of the combination of two processes: the chorion digestion by two enzymes, the high- and low choriolytic enzymes (HCE and LCE), and the tearing of the egg membrane by the twisting of the embryo once the membrane has been digested[16]. The analysis of the spontaneous tail movements showed an increase of the burst activity, the burst duration and the burst count in the embryos exposed to the highest dose rate, while the exposure to the two lowest dose rates did not induce any changes. This result means that the hatching delay could result from a defect of the synthesis or efficiency of chorionases as the embryos either twisted in a similar way than the control ones, or even

418 more than those. An analysis of the choriolytic enzymes synthesis or activity would thus
419 help understand more about the mechanisms leading to the effect that was observed on the
420 hatching kinetic. Regarding the increase in the burst activity, such effect has already been
421 observed after exposure to gamma radiation at a dose rate of 5000 µGy/h but not at lower
422 or higher dose rates, while this effect was not observed after exposure to HTO, which
423 means that in the case of this effect, tritiated thymidine seems to behave like gamma
424 radiation, but at a lower dose rate[24,29].

425 Exposure to the highest dose rate of tritiated thymidine led to a significant decrease of
426 the heart rate. Even though the larvae were not smaller at this time point, this effect could
427 indicate a delay in the development as it is known that in the case of zebrafish, the heart
428 rate gradually increases with time[24,33]. The decrease of the heartbeat after exposure to a
429 contaminant has already been observed after exposure to gamma radiations at a dose rate
430 equivalent to the one used in this study (*i.e.* 500 µGy/h), but also after exposure to some
431 organic contaminants as butyl benzyl phthalate, propranolol, thifluzamide,
432 penconazole[16,26,33,34]. No other markers studied in this work can help understand the
433 mechanisms lying under the appearance of such an effect, but as the cardiac development
434 in zebrafish is quite a complex system, we could for example hypothesize that there was
435 an issue during the development, or that the thyroid gland was affected by tritium, as it has
436 an important role on the regulation of the heartbeat and is also one of the targets already
437 identified in the case of radiations[3,12,35,36].

438

439 While this work highlighted different macroscopic effects that tritiated thymidine had
440 on zebrafish in its early life stages, it also showed the presence of an increase in the ROS
441 production and of a dysregulation of many genes. Such an increase of the ROS production
442 has already been observed after exposure to radiation dose rates as low as 134 µGy/h (Cs-

137), but also after exposure of fathead minnows to HTO and organic tritium (25 and 180 kBq/L)[7,11]. In a more general way, several studies reported that low-dose radiations had effects that have been associated with ROS production[37,38]. ROS are considered as being unstable and highly reactive compounds, including $O_2^{-\cdot}$ or H_2O_2, and these compounds are also considered as being able to induce oxidative stress and oxidative damage, which is thus in accordance with the hypothesis that organic tritium could lead to the induction of oxidative stress in zebrafish[39]. Though, considering that using H_2DCFDA as a probe only gives an indirect information on the H_2O_2 production, that means that our results only showed a part of the response to oxidative stress.

Regarding the gene expression measured after 24 hours of exposure, the results indicated that there is a significant over-expression of the genes only after exposure to the lowest dose rate, while the exposure to the intermediate dose rate only led to a tendency towards the over-expression of a few genes, and the exposure to the highest dose rate led to no gene dysregulation. This result, alongside the absence of a significant increase in the ROS production index could, for example, highlight a decrease in the metabolic activity of the individuals. This discussion will only focus on the results observed after exposure to the lowest dose rate. The analysis of the genes being over-expressed ($p < 0.05$) or nearly overexpressed ($p < 0.1$) showed that many of those genes are involved in the same processes. For example, the apoptotic process (*baxa, bbc3, casp9, bcl2*), DNA binding (*cry5, crx, efl1, h2ax, tbpl1*) and repair (*cry5, ddb2, h2ax, xpc, xrcc1*) seem to be affected, as most of the genes involved in these processes and studied here are overexpressed (either significantly or only as a tendency). These observations are in accordance with results obtained in various studies that showed that processes linked to DNA synthesis or repair are easily affected by the exposure to tritium or other radionuclides, even if in most studies, the gene expression is also dysregulated in later development stages (*e.g.* 96 hpf)[15,17]. This

seems to indicate that DNA might be damaged as a result from the exposure, which was expected considering the fact that it has been shown that a significant portion of the tritium ends up bound to the DNA[5]. However, no conclusions can be drawn concerning DNA integrity since DNA damage was not experimentally measured in the present study. Besides, two genes *mpx* and *sod2* involved in the regulation of oxidative stress were also upregulated, confirming that there might be ROS overproduction in zebrafish embryos following tritiated thymidine exposure. This would also be in accordance with different studies where genes involved in the oxidative stress regulation were overexpressed after exposure to HTO[10,15]. In contrast, it differs with the downregulation of the *mpx* gene that was observed in zebrafish embryos and larvae after being irradiated at a dose rate of 1240 µGy/h (Cs-137), thus showing that tritiated thymidine might behave more like HTO, even though all genes showed dysregulation at low dose rates only, which was not the case after exposure to HTO.[11,15].

5. Conclusion

The toxicity of tritiated thymidine on early life stages at different biological levels was investigated herein. While this study reported that tritiated thymidine did not induce any significant mortality, it was also shown that it can affect zebrafish embryo-larval development in different ways. Indeed, organic tritium affected hatching kinetic, with hatching being delayed and a transient decrease in the development kinetics. Those developmental effects were observed at all dose rates tested, even though the nature of the effect depended on the tritiated thymidine activity concentration. This work also showed an increase in the ROS production levels after four days of exposure. This study also highlighted a dysregulation of many genes after one day of exposure, even if the latter was only transient, as no effect were observed after four days of exposure and after exposure to

greater dose rates. The dysregulated genes are involved in different cellular processes, *i.e.*, development, oxidative stress, DNA repair and apoptosis. It confirms that tritium does have an effect on zebrafish individuals at different molecular scales and, more specifically, on various molecular targets and processes. Linking all the results presented in this paper is not possible, as the investigation of other markers as, for example, the protein levels, would be needed to do so. The study of other biological markers, as the protein expression, is also considered necessary to confirm the gene dysregulation results presented in this paper. This study does not highlight any specific toxic effects arising from the exposure to tritium in its organic phase. As the experimental design of this study limited the exposure length to four days, later development stages could not be studied. Those stages should be the focus of a later study to confirm the results presented in this paper. The study of the same toxicity markers and other ones, as for example carcinogenic effects or reproductive behavior, could also give more information on tritiated thymidine toxicity and possible specific effects.

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Tables

Table 1. Amount of solution sampled in the beakers (1 replicate/beaker) and the crystallizers (3 replicate/crystallizer) depending on the activity concentration (Bq/mL, ± 10 %) used during the experiment

Nominal activity concentration (x10^3 Bq/mL)	**Beaker sampling**	**Crystallizer sampling**
7.5 ± 0.8	1 mL	10 µL diluted in 990 µL of tritium-free water
40 ± 4.0	1 mL	10 µL diluted in 990 µL of tritium-free water
110 ± 11.0	100 µL diluted in 900 µL of tritium-free water	10 µL of a 1:10 solution in 990 µL of tritium-free water

Table 2. Developmental characteristics (mm) measured on control and contaminated zebrafish early life stages (0, 22 and 170 µGy/h). Measurements were made on embryos (1 and 2 dpf) and larvae (3 and 4 dpf), and the number in parenthesis corresponds to the number of individuals used for each measurement. Bold numbers and * indicate a significative difference with the control (GLM and GLMM, $p<0.05$)

Measurement (mm)	Control	22 µGy/h	170 µGy/h
Egg diameter (1 dpf)	1.25 ± 0.06 (19)	1.26 ± 0.06 (20)	1.25 ± 0.05 (19)
Yolk sac diameter (1 dpf)	0.52 ± 0.03 (19)	**0.55 ± 0.04*** (20)	0.52 ± 0.04 (19)
Egg diameter (2 dpf)	1.26 ± 0.07 (11)	1.24 ± 0.03 (12)	1.22 ± 0.05 (11)
Yolk sac diameter (2 dpf)	0.50 ± 0.03 (11)	0.49 ± 0.04 (12)	0.50 ± 0.04 (11)
Larva length (3 dpf)	3.22 ± 0.16 (17)	**3.03 ± 0.21*** (14)	**2.97 ± 0.13*** (16)
Yolk sac diameter (3 dpf)	0.42 ± 0.06 (17)	0.39 ± 0.06 (14)	**0.37 ± 0.07*** (16)
Eye diameter (3 dpf)	0.26 ± 0.02 (17)	0.25 ± 0.04 (14)	**0.22 ± 0.04*** (16)
Larva length (4 dpf)	3.22 ± 0.16 (15)	3.30 ± 0.18 (16)	3.31 ± 0.20 (17)
Yolk sac diameter (4 dpf)	0.35 ± 0.08 (15)	0.37 ± 0.04 (16)	0.38 ± 0.05 (17)
Eye diameter (4 dpf)	0.26 ± 0.04 (15)	0.27 ± 0.02 (16)	0.27 ± 0.03 (17)

638

639 **Table 3.** Developmental characteristics (mm) measured on control and contaminated
640 zebrafish early life stage (0 and 470 µGy/h). Measurements were made on embryos (1 and
641 2 dpf) and larvae (3 and 4 dpf), and the number in parenthesis corresponds to the number
642 of individuals used for each measurement. No significant differences were observed
643 between both conditions (GLM and GLMM, $p<0.05$)

Measurement (mm)	Control	470 µGy/h
Egg diameter (1 dpf)	1.21 ± 0.07 (18)	1.19 ± 0.06 (16)
Yolk sac diameter (1 dpf)	0.51 ± 0.04 (18)	0.50 ± 0.03 (16)
Egg diameter (2 dpf)	1.20 ± 0.06 (15)	1.20 ± 0.07 (13)
Yolk sac diameter (2 dpf)	0.48 ± 0.05 (15)	0.45 ± 0.05 (13)
Larva length (3 dpf)	3.46 ± 0.23 (16)	3.32 ± 0.29 (17)
Yolk sac diameter (3 dpf)	0.47 ± 0.09 (16)	0.44 ± 0.09 (17)
Eye diameter (3 dpf)	0.30 ± 0.04 (16)	0.28 ± 0.04 (17)
Larva length (4 dpf)	3.61 ± 0.26 (15)	3.56 ± 0.17 (17)
Yolk sac diameter (4 dpf)	0.37 ± 0.09 (15)	0.35 ± 0.04 (17)
Eye diameter (4 dpf)	0.30 ± 0.04 (15)	0.31 ± 0.04 (17)

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Figures

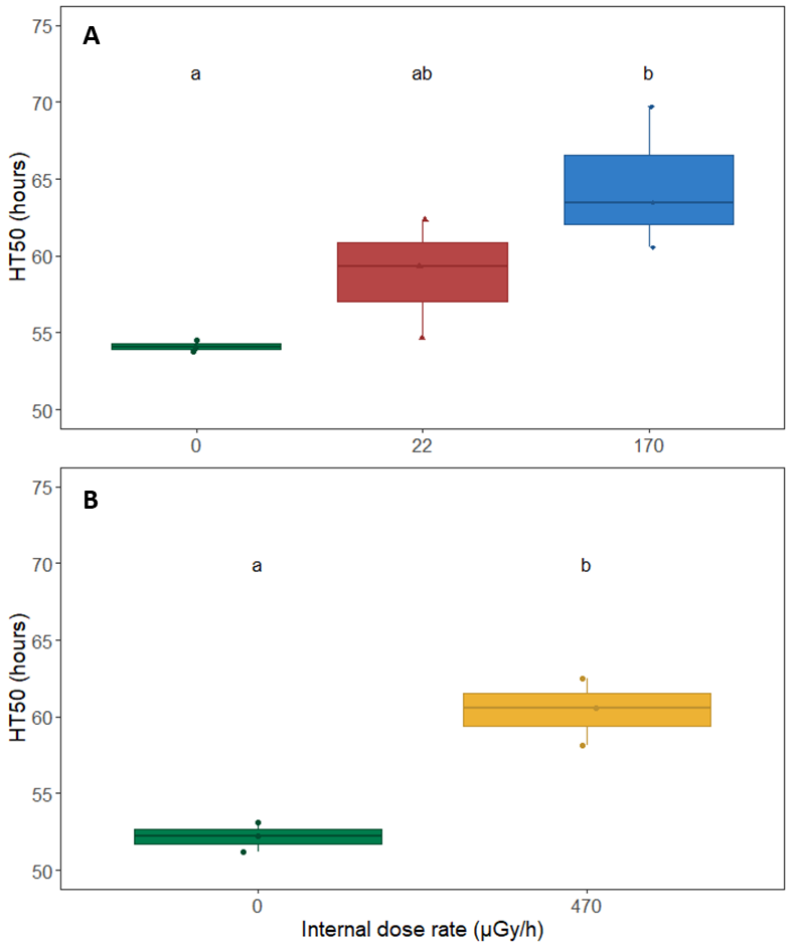

Figure 1. Average hatching time (HT50, hours, N = 8) measured in control and exposed zebrafish embryos to tritiated thymidine (panel A: 22 and 170 µGy/h; panel B: 470 µGy/h). ANOVA, a<b, $p<0.05$.

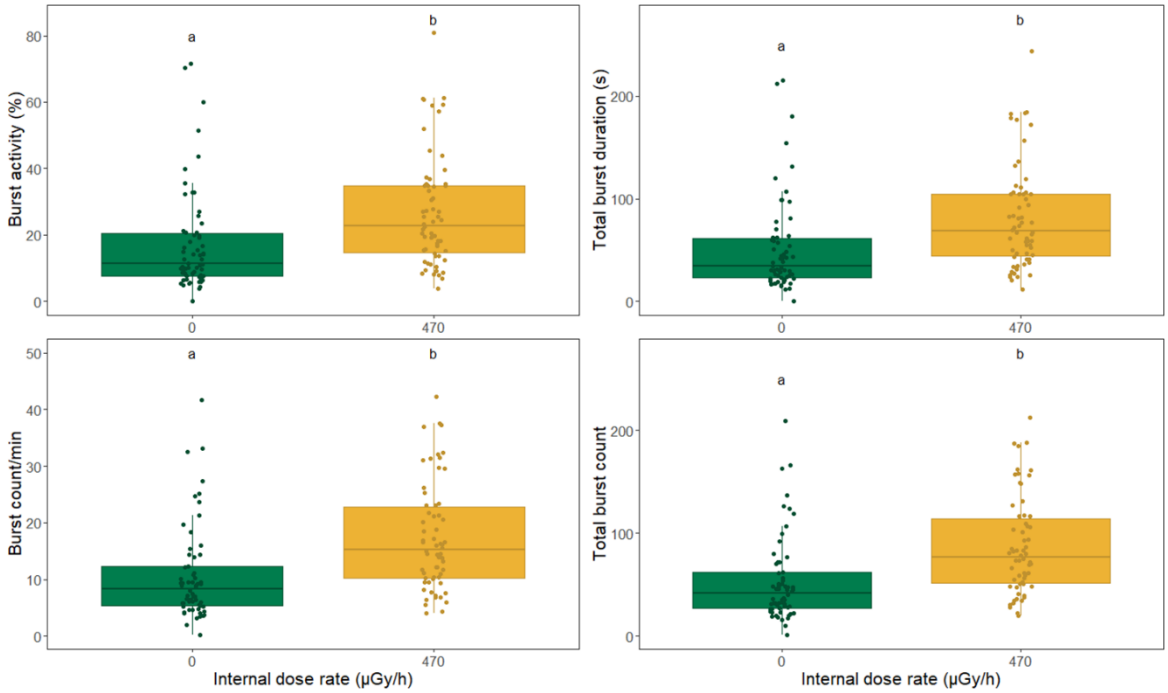

Figure 2. Burst activity (%, panel A), total burst duration (s, panel B), burst count per minute (panel C) and total burst count (panel D) measured in control and contaminated zebrafish embryos (0 and 470 µGy/h) after 24 hours of exposure to tritiated thymidine. GLMM/LMM, N = 60, a<b, $p < 0.05$.

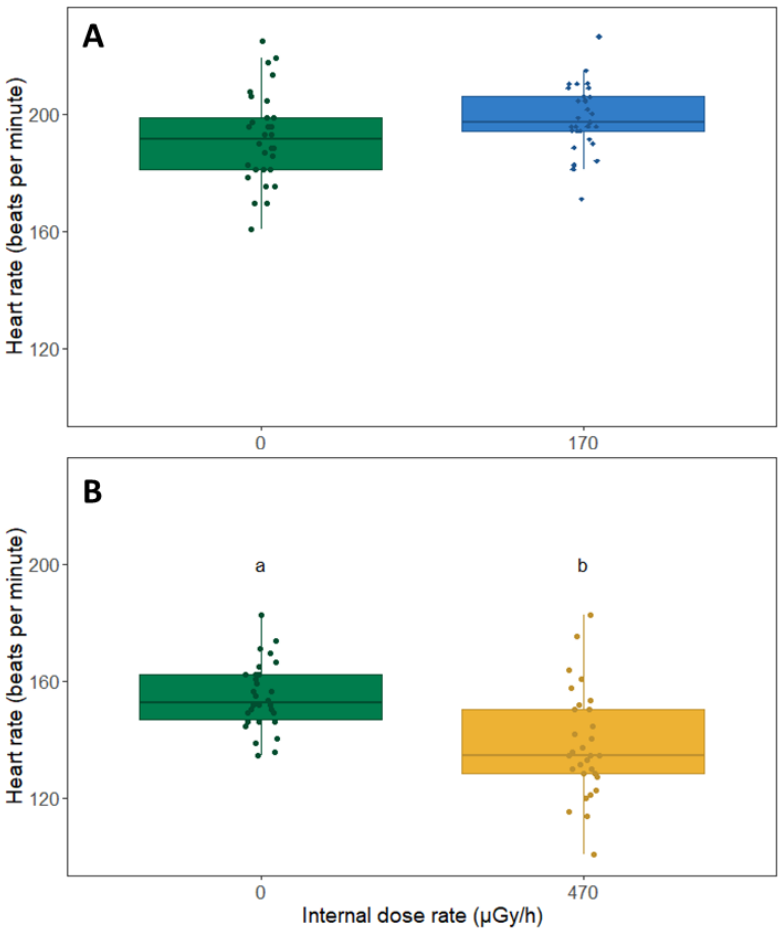

Figure 3. Heart rate (BPM, N = 30) measured in control and contaminated 3 dpf zebrafish larvae (0, 170 µGy/h, panel A and 470 µGy/h, panel B) after three days of exposure to tritiated thymidine. GLMM/LMM, a<b, $p < 0.05$.

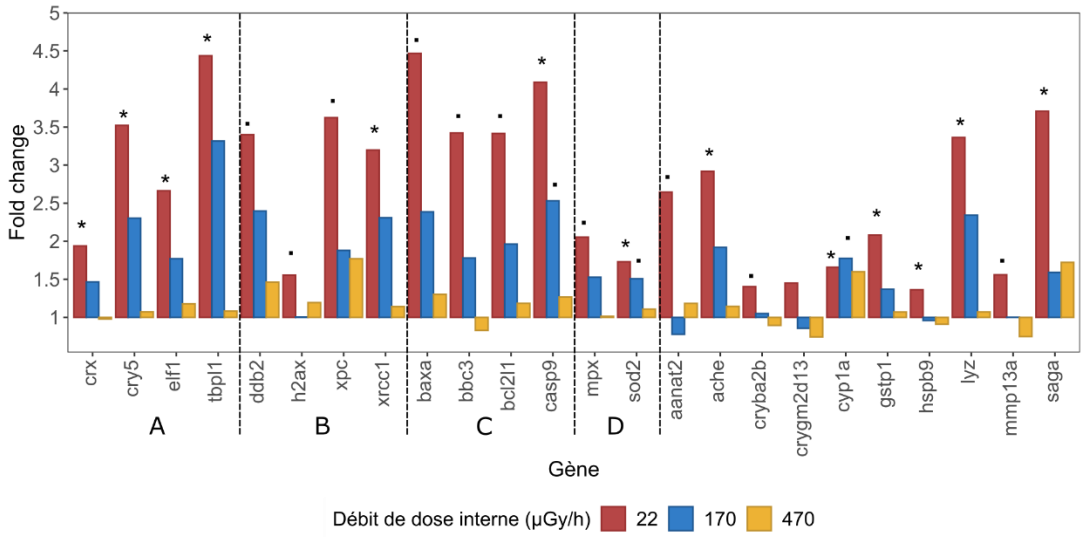

Figure 4. Fold change in gene expression in comparison to controls of 24 different genes in 1 dpf fish embryos exposed for 24 h to tritiated thymidine (22, 170 and 470 µGy/h). Genes studied are involved in different biological processes such as DNA binding (A), DNA repair (B), apoptosis (C) and response to the oxidative stress (D). Statistics: t-test and adjustment of the p-value using the Benjamini-Hochberg False Discovery Rate. (. p < 0.1; * p < 0.05 when comparing to the control).

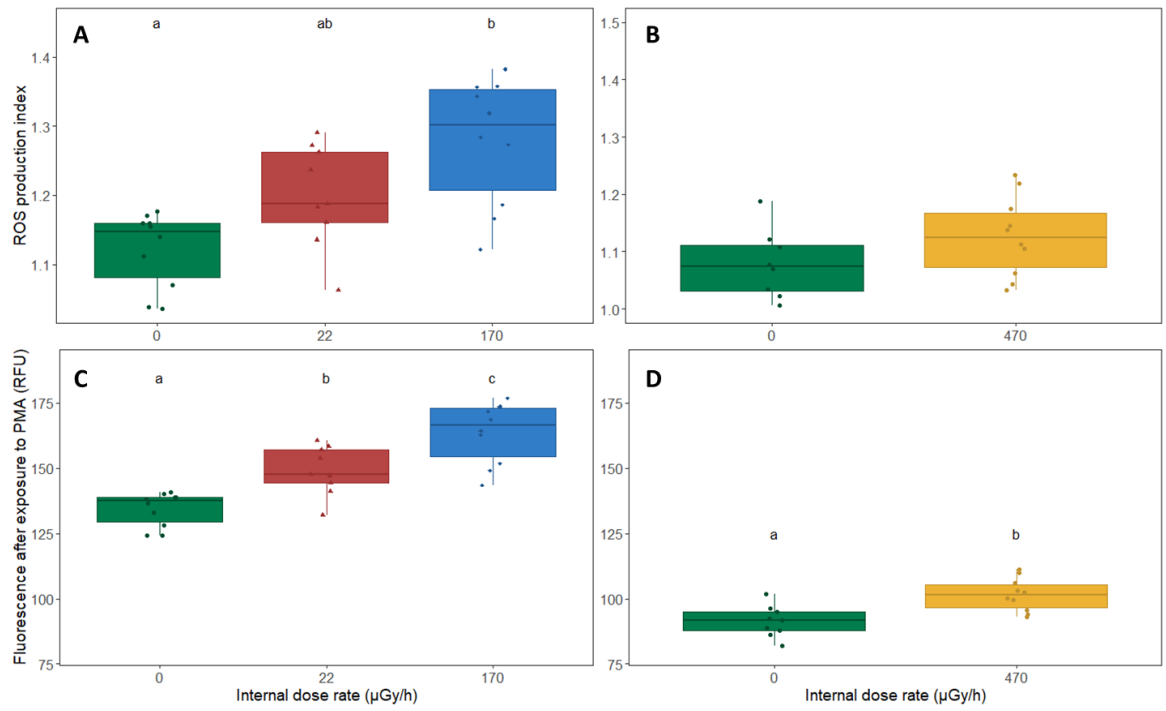

Figure 5. ROS production index (panels A and B) and fluorescence measured after exposure to PMA (panels C and D) in control and contaminated zebrafish larvae (left

678 panels: 22 and 170 µGy/h, right panels: 470 µGy/h) after 4 days of exposure to tritiated
679 thymidine. ANOVA, a<b<c, $p < 0.05$. Each point represents a data point acquired during
680 the experiment.

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Supplementary Information

Table S1. Genes chosen for the Real Time PCR Analysis and their corresponding Assay IDs. All genes were chosen by using the gene library provided by ThermoFisher.

Gene Name	Assay_ID
aanat2	Dr03432880_m1
ache	Dr03093481_m1
atp2a1l	Dr03085336_m1
baxa	Dr03432977_m1
bbc3	Dr03168831_s1
bcl2l1	Dr03080274_m1
casp9	Dr03121787_m1
crx	Dr03131743_m1
cry5	Dr03138452_m1
cryba2b	Dr03160512-m2
crygm2d13	Dr03426056_gH
cyp1a	Dr03112444_m1
ddb2	Dr03429621_m1
eef1a2	Dr03076192_m1
elf1	Dr03118707_m1
gadd45bb	Dr03083476_m1
gstp1	Dr03118988_m1
h2ax	Dr03145737_s1
hspb9	Dr03436228_s1
lyz	Dr03099437_m1
mmp13a	Dr03438515_g1

mpx	Dr03075659_m1
oxt	Dr03112593_m1
per2	Dr03093697_m1
plekhs1	Dr03093697_m1
saga	Dr03435203_m1
sod2	Dr03100019_m1
tbpl1	Dr03088430_m1
tnni2b.2	Dr03111539_g1
xpc	Dr03148843_m1
xrcc1	Dr03115324_m1

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