



## **Effects of in vivo exposure to tritium: a multi-biomarker approach using the fathead minnow, *Pimephales promelas***

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**Effects of *in vivo* exposure to tritium: a multi-biomarker approach using the fathead minnow,**  
***Pimephales promelas***

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## Highlights

- Following a previously published field investigation, this study aims to characterize tritium effects on fish health under controlled conditions in a laboratory setting.
- Fathead minnows were exposed to tritium activity concentrations up to 180,000 Bq/L.
- At the highest levels of exposure, tritium increased DNA damage and modulated the immune responses.
- Other markers were affected, including the neural system, oxidative stress and fatty acid composition.
- No effects are reported on the measured health indices and anti-oxidant activities.

## Abstract

Tritium ( $^3\text{H}$ ) is a radioactive isotope of hydrogen. In the environment, the most common form of tritium is tritiated water (HTO). However, tritium can also be incorporated into organic molecules, forming organically bound tritium (OBT). The present study characterized the effects of tritium on the health of the fathead minnow, *Pimephales promelas*. Fish were exposed to a gradient of HTO (activity concentrations of 12,000, 25,000 and 180,000 Bq/L) and OBT using food spiked with tritiated amino acids (OBT only, with an activity concentration of 27,000 Bq/L). A combined exposure condition where fish were placed in 25,000 Bq/L water and received OBT through feed was also studied. Fish were exposed for 60 days, followed by a 60 day depuration period. A battery of health biomarkers were measured in fish tissues at seven time points throughout the 120 days required to complete the exposure and depuration phases. HTO and OBT were also measured in fish tissues at the same time points. Results showed effects of increasing tritium activity concentrations in water after 60 days of exposure. The internal dose rates of tritium, estimated from the tissue free-water tritium (TFWT) and OBT activity concentrations, reached a maximum of 0.65  $\mu\text{Gy/h}$ , which is relatively low considering background levels. No effects were observed on survival, fish condition, and metabolic indices (gonado-, hepato- and spleno- somatic indexes (GSI, HSI, SSI), RNA/DNA and proteins/DNA ratios). Multivariate analyses showed that several biomarkers (DNA damages, micronucleus (MN) frequency, brain acetylcholinesterase (AChE), lysosomal membrane integrity (LMI), phagocytosis activity and

reactive oxygen species (ROS) production) were exclusively correlated with fish tritium internal dose rate, showing that tritium induced genotoxicity, as well as neural and immune responses. The results were compared with another study on the same fish species where fish were exposed to tritium and other contaminants in natural environments. Together with the field study, the present work provides useful data to identify biomarkers for tritium exposure and better understand modes of action of tritium on the fathead minnow.

## **Keywords**

Fathead minnow, *Pimephales promelas*; tritium internal dose rate; *in vivo* exposure; genotoxicity; immune system response; oxidative stress response; neural response; fatty acid composition

## Introduction

Tritium ( $^3\text{H}$ ) is a radioactive isotope of hydrogen. Naturally, the main source of tritium is from cosmic rays interacting with nitrogen in the atmosphere. Anthropogenically, it can be produced by the in-reactor neutron activations of lithium, hydrogen and deuterium. Releases of tritium to the environment occur during normal functioning of nuclear power plants ( $6.0 \times 10^2$  to  $4.0 \times 10^3$  kBq/L in the effluents in France and  $<1.5 \times 10^3$  kBq/L in Canada) and nuclear fuel reprocessing plants ( $10^5$  kBq/L in the effluents in France) (HPA 2007, ASN 2010, OPG 2017). In both countries, the average tritium activity concentrations in receiving waters is very low at 1-4 Bq/L (ASN 2010, OPG 2017). The European and Canadian limits of tritium in water for human consumption are 0.1 and 7 kBq/L, respectively (CNSC 2008, European\_Union 2013). The International Thermonuclear Experimental Reactor (ITER) being built next to the Cadarache facility in France is an international research and engineering reactor due to come into service in 2030. It is expected that tritium releases to water and air will increase after this reactor is in operation (ASN 2010). In the environment, tritium is often found in the form of tritiated water (HTO), which can be incorporated into organic molecules, forming organically bound tritium (OBT) (Bogen et al. 1979). With the anticipated increase in tritium releases, there is a need to better understand tritium effects and particularly its toxic modes of action (ASN 2010).

Tritium is a low energy  $\beta$  emitter with a half-life of 12.3 years, which causes high ionization density within a relatively small volume of matter (ASN 2010). Therefore, if enough tritium is ingested, the energy deposited could lead to health consequences. The toxicity of HTO has been studied on aquatic invertebrates and vertebrates (Adam-Guillermin et al. 2012). However, most of the studies reported acute toxicity effects using high dose rates (Real et al. 2004). Studies of the effects of tritium on fish have focused mostly on life-history endpoints linked to reproduction and development (Ichikawa & Suyama 1974, Hyodo-Taguchi & Etoh 1993, Sazykina & Kryshev 2003). Therefore, there is a lack of data on biological effects induced on other biological scales and from chronic low levels of exposure, especially for aquatic vertebrates such as fish (Adam-Guillermin et al. 2012).

Chronic low levels of ionizing radiation exposure are not necessarily detrimental. In fact, they were previously shown to induce adaptive responses in fish and amphibians (Smith & Moccia 2001, O'Neill-

Mehlenbacher et al. 2007, Audette-Stuart et al. 2011, Audette-Stuart & Yankovich 2011, Mothersill et al. 2014).

Tritium exposure has been shown to cause DNA breaks in aquatic invertebrates such as the marine mussel, *Mytilus edulis* (Hagger et al. 2005, Jha et al. 2005, 2006, Jaeschke et al. 2011), and in vertebrates including medaka (Suyama et al. 1981), fathead minnow (Gagnaire et al. 2017) and fish cells (Stuart et al. 2016). Molecular changes including DNA lesions, measured through the comet assay or micronucleus frequency (MN), a biomarker of chromosome damages, are referred to as genotoxicity markers because they indicate that ionizing radiation can affect cells and, if the DNA is left unrepaired, potentially have an effect on individual health or reproductive fitness. However, other kinds of effects were reported, such as the suppression of immune response in rainbow trout (Strand et al. 1982) as well as hormonal effects (Erickson 1971). Immune parameters such as phagocytosis (Fournier et al. 2000), lysosomal membrane integrity (Bado-Nilles et al. 2013), necrosis and reactive oxygen species production (ROS) (Gagnaire et al. 2014) have also been described as biomarkers that can be modified by several kinds of contaminants and can, therefore, be useful to assess the effects of tritium on aquatic vertebrates. If tritium can induce genotoxicity and immunotoxicity, it can be hypothesized that defense from oxidative stress, detoxification and general metabolism would respond to tritium exposure. Therefore, biomarkers of the antioxidant system (catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX)) and also of the neural system (acetylcholinesterase (AChE)), which are commonly used in ecotoxicological studies (Sanchez & Porcher 2009), may be modified by tritium. Moreover, the combined use of these biochemical markers, which are sensitive molecular and cellular indicators, and physiological markers (metabolic and somatic indices and fatty acid composition), which can show effects occurring at the individual level, is a promising way to relate responses observed at different levels of biological organization (Kerambrun et al. 2012).

The fathead minnow, *Pimephales promelas*, a species of freshwater fish native to Canada, has shown to be tolerant to a wide range of water types. Moreover, this species has been used for toxicity testing since the 1960s (Environment Canada 2011). In chronic low dose field tests (Gagnaire et al. 2017), fathead minnows were exposed to increasing HTO activity concentrations with cross-contamination with metals. It was found that several biomarkers related to immunity, DNA integrity, neural system

and fatty acids presented changes that were correlated to fish internal tritium dose rate (Gagnaire et al. 2017). Therefore, this study was followed up with a controlled laboratory study in which fathead minnows chronically exposed to low dose tritium spanning a similar range as that found at the field sites were tested to assess genotoxic, immune and neurological responses. Two forms of tritium (HTO and OBT) were assessed because tritium toxicity may also vary as a function of its chemical form (Kim et al. 2015). Minnows were housed in aquaria and exposed over 60 days to water with a range of HTO activity concentrations. Fish were given either non-tritiated feed or feed containing tritiated amino acids. After this exposure period, all fish were maintained in non-tritiated water and given non-tritiated food for another 60 day depuration period. Throughout the study, tritium internalization and biomarkers for genotoxicity, oxidative stress, immunity and fitness were evaluated.

## Material and Methods

### Fish exposure

The laboratory study took place between September 2014 and January 2015. Approximately five-month old male fathead minnows ( $3.93 \pm 0.13$  g,  $6.00 \pm 0.08$  cm) were received from Aquatics Research Organisms Inc. (Hampton, NH) and progressively allowed to adapt to the reconstituted water. During the study, the fish were kept in reconstituted water prepared by adding 11.0 g of calcium chloride ( $\text{CaCl}_2$ ), 8.4 g of sodium bicarbonate ( $\text{NaHCO}_3$ ), 3.0 g of magnesium sulfate ( $\text{MgSO}_4$ ), 0.4 g of potassium chloride (KCl) and 0.1 g of sodium bromide (NaBr) to 100 L of deionized (reverse osmosis) water. The fish were housed in adjacent rooms that were maintained at  $21 \pm 1^\circ\text{C}$ . The temperature in the tanks was  $19.8 \pm 0.7^\circ\text{C}$  due to frequent water changes; however, all treatment groups including controls were on the same water change schedule, so the potential effects of temperature changes were identical for all groups. Each 40 L or 20 L aquaria was equipped with circulation and filtration systems. Prior to being exposed to tritium, the fish were acclimated to the tank environment for 21 days, after which HTO was added to cover an activity concentration range from  $<100$  Bq/L up to a maximum of 180,000 Bq/L. The aquaria were referred to as C (control), 12K, 25K and 180K to reflect the nominal HTO tritium activity concentrations of 12, 25 and 180 kBq/L. The control aquaria were separated from the test aquaria in a different ventilated room to minimize cross-contamination. To assess the effects of HTO through water and OBT through food on their own and combined, there were three different test conditions (HTO, OBT and a combination of the two), as previously used (Gagnaire et al. 2017). To measure the effects of only OBT, fish in dedicated tanks were fed commercial fish pellets spiked with tritiated amino acids (activity concentration of  $27.8 \pm 5.7$  kBq/L) and the aquaria were filled with non-tritiated water (OBT). The HTO test group received non-tritiated commercial fish pellets, but were housed in water at activity concentrations of either 12, 25 or 180 kBq/L. Finally, the third test group combined OBT and HTO, the fish were fed tritiated feed to an activity concentration of  $27.8 \pm 5.7$  kBq/L and the tanks contained water at 25 kBq/L of HTO (25KOBT). The tritium exposure period was for 60 days, after which time the tritium activity concentrations returned to background values ( $<100$  Bq/L) for an additional 60-day depuration period in all tanks.



Fish then received non-tritiated feed. The tank water was changed weekly and the fish were fed daily by adding an amount of commercial feed to achieve up to 1% of the total fish biomass in each tank. Water quality parameters for pH, nitrogen compounds, temperature and hardness were regularly measured throughout the study. Fish mortality was recorded. Ten individual fish were randomly sampled from each exposure group for analysis of tritium, physical and biological markers at 60 days and at 120 days. In addition, 10 fish were measured (fork and standard lengths), the fish and their organs were weighed and a blood smear was prepared (to evaluate MN frequency) at day 0, 15, 30, 60, 75, 90 and 120, and tritium was measured in their tissues.

## Physico-chemical parameters

### *HTO measurements*

HTO activity concentrations within the aquaria were measured weekly throughout the experiment. Water samples were collected from each aquarium and measured using Ultima Gold scintillation cocktail as previously described (Gagnaire et al. 2017). A standard of 145 Bq/mg tritium was used to determine counter efficiency. Blank samples were also added. The detection limit was 50 Bq/L.

### *Preparation of tritiated feed*

Fish were fed with Silver Cup 1.0 mm pellet fish feed from Martin Mills Inc. (Elmira, ON). The OBT-spiked feed was prepared as previously described (Gagnaire et al. 2017). Non-tritiated control food was also prepared in the same way so that fish received the same food quality. The feed was analyzed for tritium and an average value of 27.8 +/- 5.7 kBq.L OBT was obtained.

### *TFWT and OBT measurements and estimated dose rate in fish samples*

Ten minnows per treatment group were randomly collected for the analysis of tissue free-water tritium (TFWT) and OBT activity concentrations on days 0, 15, 30, 60, 75, 90 and 120. Carcasses were also pooled (composite samples of 2-4 fish), and analyzed at days 0, 60 and 120.

For TFWT measurements, frozen fresh samples were used as previously described (Gagnaire et al. 2017). The samples were counted for 100 minutes on a Tri-Carb 3110 TR LSC. The dilution factor

was taken into account when the activity concentrations were calculated. The protocol for OBT measurement in animals is described in Kim et al. (2015) and Gagnaire et al. (2017). Samples of non-tritiated Silver Cup feed were also prepared as described in Gagnaire et al. (2017).

Estimated internal dose rates were calculated from fish internal activity concentrations of TFWT and OBT using an internal dose coefficient (DC) of  $5.38 \times 10^{-3}$   $\mu\text{Gy/h}$  per Bq/g (EDEN software v2) (Beaugelin-Seiller 2016, Paquet & Harrison 2018) under the hypothesis of a uniform repartition of tritium in tissues and using a radiation weighting factor of 3 in order to consider the relative biological effectiveness of tritium beta emissions compared to gamma radiation (Hunt et al. 2009), as previously described (Gagnaire et al. 2017). The weighting factor of 3 has been debated in the literature, but we chose to follow the same methodology as in the field experiment (Gagnaire et al. 2017) to allow for a direct comparison of results.

## Biological parameters

### *Fish dissection and sample collection*

At several time points (D0, D60, and D120), ten fathead minnows per treatment group were randomly collected for analysis. For D0, 10 fish were analyzed before the beginning of the experiment.

Fathead minnows were measured for length, weighed and dissected for endpoint measurements. Fish were euthanized via rapid cooling followed by decapitation according to an animal care protocol approved by the Chalk River Laboratories (CRL) Animal Care Committee. Gonado-Somatic (GSI), Hepato-Somatic (HSI), Spleno-Somatic (SSI) indexes and Fulton's condition factor (K) were calculated as previously described (Amara et al. 2009, Gagnaire et al. 2017). Gender was confirmed during dissection.

Blood was used for micronuclei (MN) measurements. The brain, the muscle and the liver were used for enzymatic measurements. Other parts of the muscle and the liver were used for fatty acid composition analysis. Cell viability, phagocytosis, ROS levels and lysosomal membrane stability were assessed in spleen by flow cytometry within 24 hours of collection. Gonad samples were prepared, kept at 4°C overnight, and then used to conduct the viability and comet assay; another part of gonad

samples was used to perform the RNA/DNA and protein/DNA determinations. Details are provided in Gagnaire et al. (2017).

#### *Biological analyses*

Cellular mortality, lysosomal membrane integrity (LMI), ROS basal and phorbol myristate acetate (PMA)-activated levels, and phagocytosis activity were determined using flow cytometry according to protocols described in Gagnaire et al. (2015a, 2017). The comet assay and the micronucleus frequency assay were performed as previously described (Festarini et al. 2016, Gagnaire et al. 2017). For enzymatic assays, brain, liver and muscle samples were homogenized immediately after sampling and the S9 fraction was frozen until analyses. All of the protocols for measuring hepatic GPX, CAT and SOD and brain and muscle AChE are presented in details in Gagnaire et al. (2017). Proteins, RNA and DNA quantification were performed on gonads as previously described (Gagnaire et al. 2017). Samples for fatty acids analysis were sent to Lipid Analytical Laboratories (Guelph, Ontario, Canada).

#### Statistical analyses

Results are presented as mean  $\pm$  standard error (se). For the first raw analyses, normality of data was checked using the Shapiro–Wilk test and when not normal, data were Box-Cox transformed. Differences between conditions were tested with t-tests or ANOVA when the data were normal and with Kruskal-Wallis or Mann-Whitney Rank Sum tests when data were not normal. Analyses were performed using the STATISTICA Software version 12 (StatSoft, Inc., Tulsa, OK, USA), with significance at  $p < 0.05$ .

All multivariate analyses were computed using the R software (version 3.4.3) (R\_Core\_Team 2017) and RStudio environment (version 1.1.442) (RStudio\_Team 2015). Simple linear regressions were used at D60 and D120 in order to assess the individual evolution of each biomarker as a function of increasing tritium exposure. Tritium exposure was characterized by a unique value for each condition (Control, 12K, 25K, OBT, 25KOBT, 180K). These values were the median of internal dose rates measured on individual fish (n comprised between 7 and 16). As the medians were highly dispersed (0.000258 to 0.65  $\mu\text{Gy/h}$ ), they were log10 transformed in order to be used in regression models.

240 Before performing the regression models, the linearity hypothesis was visually evaluated for each  
241 biomarker using scatter plots of type “biomarker vs log10 of internal dose rate median” using the  
242 *ggplot2* package. This was satisfied for all comparisons. Finally, the significance of the linear  
243 relationship was evaluated using a permutation test using the *lmp* function of the *lmp* package.  
244

## Results

### Physico-chemical parameters

#### *HTO in water and TFWT in fish*

At D15 and D30, we saw that TFWT values were consistently the same as the tank water HTO activity concentrations. At D60, however, the fish were transported, from the aquaria to the dissection room, in background water instead of tank water. For those fish, the measured TFWT were much lower because of the fast depuration rate. For dose calculation of the D60 fish, the TFWT was assumed to be the same as HTO of the tank water at the time of sampling.

Measured HTO activity concentrations in water are presented in Table 1. Average measured values were 63, 127, 10058, 21629, 20645 and 154314 Bq/L for control, OBT, 12K, 25K, 25KOBT and 180K tanks, respectively. The OBT activity concentration in tritiated feed was  $27.8 \pm 5.7$  Bq/L for both the OBT and 25KOBT conditions.

#### *TFWT, OBT and estimated dose rate in fish*

TFWT and OBT internal concentrations and estimated internal dose rates for all conditions and all time points are presented in Table 1. TFWT internal values followed the HTO activity concentrations in water (Control<OBT<12K<25KOBT=25K<180K). OBT internal concentrations were 24 to 50 times lower than TFWT internal concentrations at D60 (except for the OBT condition) and followed a different gradient (Control<12K<25K<OBT<25KOBT<180K). At D120, TFWT internal concentrations had long returned to background level. Note that for the OBT condition, TFWT stayed at background level throughout the study. At D120, OBT internal concentrations were 2 times lower than at D60 and were 1.9 to 17 times higher than TFWT internal concentrations.

OBT internal concentrations in 25KOBT compared to 25K were 2.4 times higher at D60 and 1.5 times higher at D120 (Table 1).

The highest value of internal dose rate was 0.65  $\mu$ Gy/h, obtained for 180K fish at D60 (Table 1).

The OBT formation and uptake was studied in fish exposed in the conditions 25K, OBT and 25KOBT (Figure 1). The contribution of water and food to the OBT buildup was investigated over a period of

60 days. It was noted that in the early stage of the exposure period (15 days), OBT formation (from water) was the major contributor to the buildup of OBT (Figure 1). Towards the end of the exposure period, however, OBT uptake (from food) was predominating (Figure 1). In contrast, the rate of loss of OBT in fish tissues over a 60 day depuration period showed a similar pattern regardless of the source of the buildup (Figure 1).

## Biological parameters

### *Survival*

The mortality rates were at a maximum of 4% between days 0 and 60, and 2% between days 60 and 120, without any differences between groups (data not shown).

### Physiological responses

Results were analyzed in the context of researching effects of different conditions and type of exposure on biomarkers.

### *Biological indices*

None of the evaluated biological indices (K, HSI, SSI, GSI) showed any significant differences among groups for all time points studied.

Values were significantly higher at D60 compared to D120 for K (t test,  $t_1=11.7$ ,  $p=5 \times 10^{-21}$ ). The fish weight to length ratio change for the last time point was not associated with a tritium exposure.

### *RNA/DNA and proteins/DNA*

Analyses were performed only at D60. No significant differences among groups were shown for the different parameters.

### *Fatty acids using C18:1 trans and SFA/MUFA as markers*

At D60, no differences were shown for parameters measured in the liver and for C18:1 trans in the muscle. The ratio SFA/MUFA in the muscle showed significant differences, with values for controls

being lower than values for the 25KOBT group (Kruskal Wallis test,  $H_5=15.4$ ,  $p=0.008$ ) (Figure 2). Neither of the measured parameters presented differences among groups at D120. Values were significantly higher at D60 compared to D120 for SFA/MUFA in liver (t test,  $t_1=2.05$ ,  $p=0.042$ ).

#### Genotoxic responses

##### *Comet assay*

At D60, both 25KOBT and 180K groups presented significantly higher DNA damages compared to the control and OBT groups (Figure 3). At D120, a tendency for increased DNA damages was noted, with values higher for the OBT, the 25KOBT and the 180K groups as compared to the 12K and the 25K groups, and values for both these groups were higher than for the control group (Figure 3). Values were significantly higher at D60 compared to D120 (Mann-Whitney test U,  $U_1=826$ ,  $p=0.011$ ).

##### *Micronucleus frequency*

At D60, the 25KOBT and the 180K groups presented significantly higher MN frequencies than all other groups (Figure 4). At D120, no differences between groups were found (Figure 4). Values were higher at D60 compared to D120 (t test,  $t_1=2.17$ ,  $p=0.03$ ).

#### Immune, oxidative stress and neurotoxic responses

Cell mortality presented no significant variations at either D60 or D120. LMI presented significant variations at D120: values were lower in the 12K group compared to the 180K group (Figure 5). At D60, the differences were not significant ( $p=0.09$ ), but a trend of higher values for the OBT, 25KOBT and 180K groups as compared to the controls appeared. Values were higher at D60 compared to D120 (t test,  $t_1=4.7$ ,  $p=8 \times 10^{-6}$ ).

The ROS production index presented no significant differences at D60. However, at D60, both basal and activated ROS levels were significantly lower in controls than in the 25KOBT and the 180K groups (Kruskal Wallis test,  $H_5=22.1$  and  $19.9$ , respectively;  $p=0.001$ ). At D120, the ROS production index values were significantly lower in the 180K group compared to the control and the 12K groups

(one way ANOVA,  $F_1=3.28$ ,  $p=0.012$ ) (Figure 6). At D120, results for basal and activated ROS levels were globally the same as D60 (Kruskal Wallis test,  $H_5=22.6$  and  $20.8$ , respectively;  $p=0.001$ ). Phagocytosis activity presented no significant differences at D60. At D120, values were significantly higher in the 180K group compared to controls (Figure 7). None of the evaluated enzymatic activities (AChE in brain and muscle, SOD and GPX in brain and liver, CAT in liver) showed any significant differences among groups for all time points studied. Values were significantly higher at D60 compared to D120 for muscle AChE (t test,  $t_1=6.9$ ,  $p=3.3 \times 10^{-10}$ ), and lower at D60 compared to D120 for brain AChE, GPX and SOD (t test,  $t_1=-4.32$ ,  $-12.2$  and  $-5.9$ , respectively;  $p=4.7 \times 10^{-5}$ ,  $2.8 \times 10^{-12}$  and  $8.7 \times 10^{-8}$ , respectively).

### *Multivariate analyses*

The General Linear Models (GLM) allowed for the evaluation of the potential effects of tritium radiological dose rate in fish tissues.

The model showed that several parameters were positively or negatively correlated with fish tritium dose rate (Table 2). At D60, DNA damages ( $F_1=0.073$ ,  $p=2 \times 10^{-16}$ ) and MN frequency ( $F_1=1.54$ ,  $p=10^{-20}$ ) were positively correlated to dose rate. At D120, several parameters were positively correlated to tritium dose rate in fish tissues: DNA damages ( $F_1=0.11$ ,  $p=10^{-20}$ ), brain AChE activity ( $F_1=0.42$ ,  $p=0.0224$ ), spleen LMI ( $F_1=84.5$ ,  $p=0.0144$ ), spleen phagocytosis activity ( $F_1=7.41$ ,  $p=0.0014$ ), spleen ROS activated and basal levels ( $F_1=377.8$  and  $387.1$ ,  $p=0.0026$  and  $0.0002$ , respectively), whereas ROS index was negatively correlated ( $F_1=-0.32$ ,  $p=0.0158$ ) (Table 2).



## Discussion

### *TFWT and OBT in fish tissues*

As for the field study (Gagnaire et al. 2017), the increasing gradient of tritium activity concentration in the water was also found in fish tissues for TFWT and OBT at D60. After the beginning of depuration, when tritium was absent from the water, fish tissue TFWT was also rapidly depurated; however, OBT was still present in fish tissues even after 60 days of depuration (D120).

As previously discussed (Gagnaire et al. 2017), TFWT levels in fish tissues are assumed to match water levels, with theoretical concentration factors close to 1 (Melintescu et al. 2011). In our study, we saw equilibrium between TFWT and tank water HTO at D15 and D30. At those time points, concentration factors were between 0.76 and 0.81, which is higher than the concentration factors obtained for the field study (Gagnaire et al. 2017). Moreover, exchangeable OBT is supposed to equilibrate with internalized TFWT, leading to an OBT/TFWT ratio close to 1 in aquatic organisms at equilibrium (Melintescu et al. 2011) and in fish (Smith et al. 2006). This was not the case in the present study. As for the field study (Gagnaire et al. 2017), this was expected as it takes time for OBT to accumulate in fish tissues and it also takes a long time for it to depurate (Kim et al. 2015). However, we used our data to evaluate HTO transfer and transformation of internalized TFWT in OBT because this is currently poorly documented (Melintescu & Galeriu 2011).

### *Origin of OBT accumulated in fish tissues*

OBT in fish can be formed from two different sources: water and food. To differentiate the sources we have used the terms formation and uptake. Firstly, formation is defined as the buildup of OBT through surrounding water by metabolism. Secondly, uptake is defined as the buildup of OBT through ingestion of OBT contained in the food.

Our results indicated that the food chain will be the main contributor to OBT buildup in fish under long-term exposure, such as natural environmental conditions around nuclear facilities. Once fish are no longer exposed to tritium, the depuration time for OBT would be expected to be similar regardless of the form of tritium exposure.

A series of trout experiments conducted over 140 days, when fish were contaminated with HTO and/or OBT, reported that OBT was building up considerably faster in tissues when fish were receiving OBT spiked feed (assuming 25% assimilation) compared to being exposed to the same HTO level in their tank water (Kim et al. 2013, Kim et al. 2015). This means that OBT uptake was a greater contributor compared to OBT formation. The rate of OBT build up was also found to change with the fish growth rate, with faster growth rates resulting in lower OBT activity concentration in tissues.

In the current study, the OBT activity in the feed represented a smaller fraction than the HTO in the water compared to the trout study. If the exposure conditions were mirrored, for a water HTO activity concentration of about 25 kBq/L, fish would receive OBT in the feed of about 100 kBq/L (again assuming 25% of assimilation). In this study, however, for a HTO activity concentration of about 25 kBq/L, the fish were receiving feed at about 27 kBq/L, which corresponds to about 7 kBq/L if 25% of assimilation is assumed. Despite this difference in study design, the results obtained in the current study agree well with the trout study results in the sense that, for chronic exposure, OBT contaminated feed also plays a predominant role in OBT buildup in the tissue.

#### *Effects on biological parameters*

No significant differences among groups for any of the assessed biological indices were found. Similarly, no effect of tritium was shown for either the RNA/DNA or proteins/DNA ratios in gonads and kidneys. Similar results were obtained after exposure of younger fathead minnows to tritium and other pollutants in a field experiment (Gagnaire et al. 2017). As several of these parameters are a direct or indirect indication of the metabolic growth rate of the organism (Amara et al. 2009), we could hypothesize that the dose rates were too low or that the duration of the experiment was not long enough to see an impact on these integrative indices.

In the present study, SFA/MUFA ratio in the muscle was not correlated to fish tritium dose rate. In the field study, the same ratio in liver and also C18:1 trans in muscle were positively correlated to fish tritium dose rate (Gagnaire et al. 2017). Ionizing radiation exposure was shown to modulate fatty acid composition, and the changes are known to be tissue-specific and influenced by diet (Nawar 1973, Audette-Stuart et al. 2012). In the field study, the dietary fatty acids intake differed from the laboratory

study because the fish were feeding on organic matter suspended in the water column in addition to the provided feed. The differences noted between the field and the laboratory study may, therefore, reflect differences in diet. Our laboratory findings did not confirm laboratory results obtained in trout exposed to HTO where a decrease of SFA/MUFA ratio was observed in liver and muscle (Festarini et al. 2016). Muscle SFA/MUFA ratio cannot be considered as a direct indicator of tritium effects in fish. The genotoxic biomarkers evaluated in this study presented significant differences in tritium exposed fish: DNA damages measured by the comet assay were positively correlated to tritium internal dose rates for both sampling times. The same result was obtained for MN frequency but only at D60. This result showed that the effect on MN frequency was reversible as they returned to background values at the end of the depuration period. These results suggest that OBT remaining in the tissues at D120 can induce DNA strand breaks in the gonad, but not MN frequency in the blood. However, it should be noted that while sperm cells turnover is really quick (a few days), the turnover of fish erythrocytes is longer (100-400 days) (Soldatov 2005). Damages on blood cells could therefore have been repaired at D120. The same results were obtained in the field experiment, with a reversible effect on MN frequency and non-reversible effects, within 60 days of depuration, on DNA damages (Gagnaire et al. 2017). Moreover, in the field study, both of these parameters were positively correlated with fish tritium dose rate (Gagnaire et al. 2017); in the present lab study, the same result was obtained for MN frequency at D60. The effects observed on these genotoxic biomarkers can therefore be related to tritium.

Tritium was previously shown to increase MN frequency and DNA damages in adult mussels exposed at 12.5  $\mu\text{Gy/h}$  of HTO (Jha et al. 2005, 2006) and 79  $\mu\text{Gy/h}$  of tritiated glycine (Jaeschke et al. 2011), doses 19 and 121 times higher, respectively, than the highest one estimated in our study, showing that fathead minnow is more sensitive to tritium than invertebrates considering DNA damages. Cytogenetic effects were also shown in medaka eggs and embryo-larvae of marine mussel, *Mytilus edulis*, exposed to high dose rates of tritium (Suyama et al. 1981, Hagger et al. 2005). DNA damages also increased after exposure of sticklebacks around former uranium mining sites (Le Guernic et al. 2016). Increased DNA damages were also shown after exposure of daphnids, *Daphnia magna*, to dose rates of gamma-rays as low as 7  $\mu\text{Gy/h}$  (Parisot et al. 2015). Increased DNA damages were also shown

after exposure of zebrafish larvae to 33  $\mu\text{Gy/h}$  of gamma-rays (Gagnaire et al. 2015b) and 400  $\mu\text{Gy/h}$  of tritium (Gagnaire et al, unpublished data). At 400  $\mu\text{Gy/h}$ , still on zebrafish larvae, the expression of genotoxic markers *h2afx* and *ddb2* involved in DNA repair were both induced in response to HTO exposure (Arcanjo et al., unpublished data). In the same way, DNA damages and MN frequency increased in fish cell lines exposed to tritium (Stuart et al. 2016). Gamma-H2AX was also correlated to tritium dose rate in the field experiment (Gagnaire et al. 2017). These results suggest the establishment of defense against DNA damage by the over-expression of DNA repair genes. Taken all together, these results confirm that radionuclides can induce genotoxicity and that the cells respond by upregulating DNA repair genes.

The immune system also responded to tritium exposure. The response was, however, often detected later in the study, mostly after the depuration period. At D120, LMI and phagocytosis activity were higher and ROS production index was lower in the 180K fish. LMI and phagocytosis activity were positively correlated with tritium dose rate in fish tissues at D120, while ROS index was negatively correlated with tritium dose rate in fish tissues. The same increase in LMI was found in the field study at both sampling times (Gagnaire et al. 2017). Even if LMI was shown to be affected by metals (Bado-Nilles et al. 2013), we could hypothesize that the effects observed on LMI were, in part, due to tritium. Contrary to the lab study findings, no statistically significant effects on phagocytosis were shown in the field study; however, this biomarker did correlate with the HTO conditions in the depuration period (Gagnaire et al. 2017). An increase of phagocytosis indicates an enhanced capacity of the organism to protect against infections, but could also lead to an increase of oxidative stress (Whyte 2007). Although ROS production was not investigated in the field study, the laboratory study showed that increased phagocytosis did not correlate with an increase in oxidative stress. It has been shown that tritium induced no effect on ROS production index in zebrafish larvae exposed to HTO; however, ROS activated level increased in HTO larvae after 4, 7 and 10 days of exposure to 280-380  $\mu\text{Gy/h}$  (Gagnaire et al, unpublished data). The ROS index was also shown to be affected by gamma radiation exposure (Gagnaire et al. 2015b). Tritium has been shown to negatively affect the primary immune response in juveniles of rainbow trout after 20 days of exposure to 83  $\mu\text{Gy/h}$  (Strand et al. 1982), suggesting that the fish immune system seems to be an important target of tritium.

In this study, none of the antioxidant parameters (SOD, CAT, GPX) showed any variations with tritium concentrations, but brain AChE was correlated to fish tritium dose rates, as in the field study (Gagnaire et al. 2017). For the field study, SOD and CAT activities were also correlated to fish tritium dose rate, but at different time points depending on the organ (Gagnaire et al. 2017). However, in the field study, metals were also present in the water of the tested sites in addition to tritium. Many studies have shown that alterations of these parameters are mostly caused by metals or pesticides (Sanchez et al. 2005, Richetti et al. 2011). The fact that these activities presented variations in the presence of metals associated with tritium and not with tritium alone leads us to think that these markers are not suitable for the assessment of chronic low level tritium exposure in fish.

#### *Relationships between observed effects and tritium internal dose rate*

Research on the effects of tritium on fish has mostly addressed reproduction and fecundity. Strand et al. (1982) showed mortality and malformations in trout eggs after 21 days of HTO exposure at 12  $\mu\text{Gy/h}$  (total dose of 6 mGy). In medaka, the number of germ cells changed in embryos exposed to 7080  $\mu\text{Gy/h}$  for 10 days (total dose of 1.7 Gy) (Etoh & Hyodo-Taguchi 1983), and the number of primary spermatogonia (Hyodo Taguchi & Egami 1977), vertebral malformations and fecundity (Hyodo-Taguchi & Etoh 1986, 1993) were affected at 1200  $\mu\text{Gy/h}$  in adult fish exposed for 30 days (total dose of 864 mGy). In the puffer fish, *Fugu niphobles*, HTO induced a decrease of egg hatching at 12100  $\mu\text{Gy/h}$  following a 130 h exposure period (total dose of 1.54 Gy) (Ichikawa & Suyama 1974). All of these observations were made at much higher levels than the doses studied in the present study. In fact, at D60, the highest dose rate calculated in our study was 0.65  $\mu\text{Gy/h}$ , which corresponds to a total dose of 936  $\mu\text{Gy}$ .

The maximal dose rate studied in the laboratory setting was higher than the highest dose rate calculated in the field study. In the field study, over a 60 day exposure period, the total dose was 216  $\mu\text{Gy}$  (Gagnaire et al. 2017). Both of these values are considerably lower than the studies reporting effects of tritium on fish. The values of dose rates observed in these field and laboratory studies were also lower than the benchmark used for ecosystem protection of 10  $\mu\text{Gy/h}$  (Garnier-Laplace et al.

2010). It follows that the low dose rates and cumulative doses studied were not expected to induce deterministic effects on fathead minnows, and our observations are in agreement with this.

The minimum Low Observed Effect Dose Rate (LOEDR) for tritium has been shown to reach 0.62  $\mu\text{Gy/h}$  for invertebrate larval development and 12.1  $\mu\text{Gy/h}$  on fish development (Adam-Guillermin et al. 2012). The highest dose rate obtained in this study for 180K fish at D60 was 0.65  $\mu\text{Gy/h}$ , 18 times lower than the LOEDR for fish development. As for the field study (Gagnaire et al. 2017), at this low dose rate, several biomarkers were found to be exclusively modulated by tritium internal dose rate, including blood MN frequency (at D60), gonad comet tail moment, spleen LMI, phagocytosis activity and ROS production, and muscle SFA/MUFA ratio (at D120).

## Conclusion

The present study aimed to evaluate the effects of HTO and OBT on *P. promelas* in laboratory conditions. Fish health was not affected by tritium exposure as no effects were shown on survival, condition and physiological indices. Considering the low dose rates (maximum of 0.65  $\mu\text{Gy/h}$ ), this result is not surprising, as the DCRL (Derived Consideration Reference Levels) in which deleterious effects are expected on young fish are supposed to be 4 mGy/h (ICRP 2008). However, at these low dose rates, several biomarkers were correlated with the internal tritium dose rate. Therefore, this study showed that tritium induced genotoxicity, neural and immune responses as we saw modulations of DNA damages, micronucleus frequency, brain AChE, lysosomal membrane integrity and phagocytosis activities. No effects were observed on SOD, CAT, GPX activities and RNA/DNA/protein ratios.

In this study, as no effects appeared on health indices, we can conclude that no adverse effect on fitness was shown at tritium concentrations 25 times and 1800 times higher than Canadian and European drinking water limits, respectively. This result indicates nonlinear dose-response relationships for these indices. Effects on these indices may be observed at higher tritium concentrations when the exposure threshold is reached.

When comparing our results with those obtained in another study using the same fish species exposed to tritium in natural environments also containing other contaminants, results were similar for several biomarkers: immune (phagocytosis activity, ROS production, lysosomal membrane integrity) and genotoxic parameters (MN frequency and DNA damages). Therefore, the multi-biomarker approach used in both of these studies allowed us to identify the most relevant biological pathways involved in response to tritium exposure in fathead minnows in laboratory and field settings, and consists in an interesting approach for the assessment of radionuclide effects in aquatic ecosystems.

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523



524 **Figure Legend**

525

526 **Figure 1:** Relative OBT build up and loss patterns during exposure and depuration periods.

527 **Figure 2:** SFA/MUFA ratio in the muscle of fathead minnows at D60 and D120. Kruskal-Wallis test,  
528  $p < 0.01$ ;  $a < b$ .

529 **Figure 3:** Results of the comet assay, expressed as mean tail moment, obtained in the gonad of fathead  
530 minnows at D60 and D120. Kruskal-Wallis test,  $p < 0.0001$ ;  $a < b < c < d$ .

531 **Figure 4:** Micronucleus frequency obtained from the blood of fathead minnows sampled at D60 and  
532 D120. Two-way ANOVA,  $p < 0.0001$ ;  $a < b$ .

533 **Figure 5:** Lysosomal membrane integrity (LMI) expressed as mean fluorescence intensity (MFI),  
534 measured in the spleen of fathead minnows at D60 and D120. Two-way ANOVA,  $p < 0.05$ ;  $a < b$ .

535 **Figure 6:** ROS index (ratio activated/basal ROS levels expressed as MFI) measured in the spleen of  
536 fathead minnows at D60 and D120. Two-way ANOVA,  $p < 0.05$ ;  $a < b$ .

537 **Figure 7:** Phagocytosis activity expressed as percentage of cells containing three beads or more,  
538 measured in the spleen of fathead minnows at D60 and D120. Two-way ANOVA,  $p < 0.001$ ;  $a < b$ .

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540

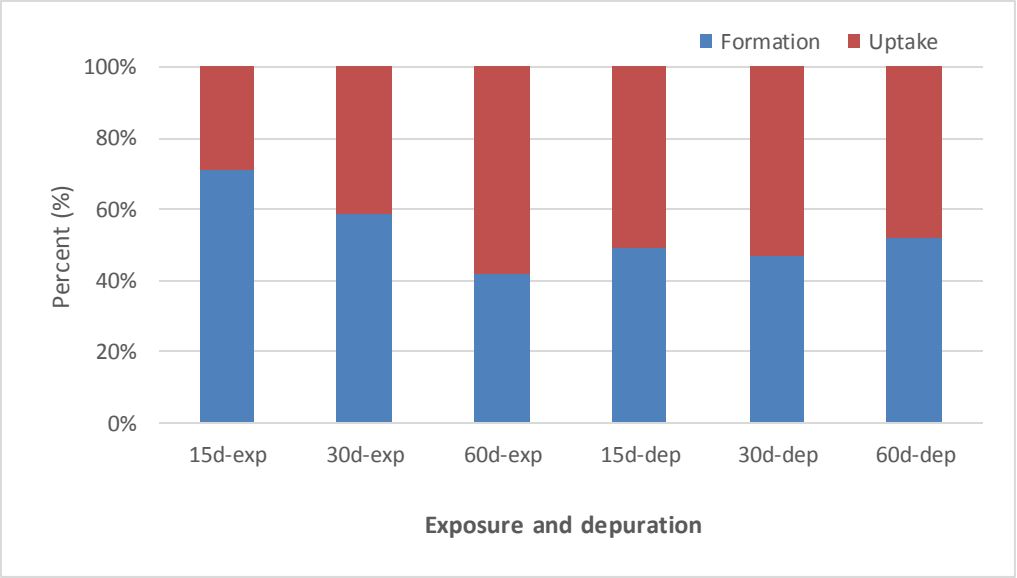


Figure 1

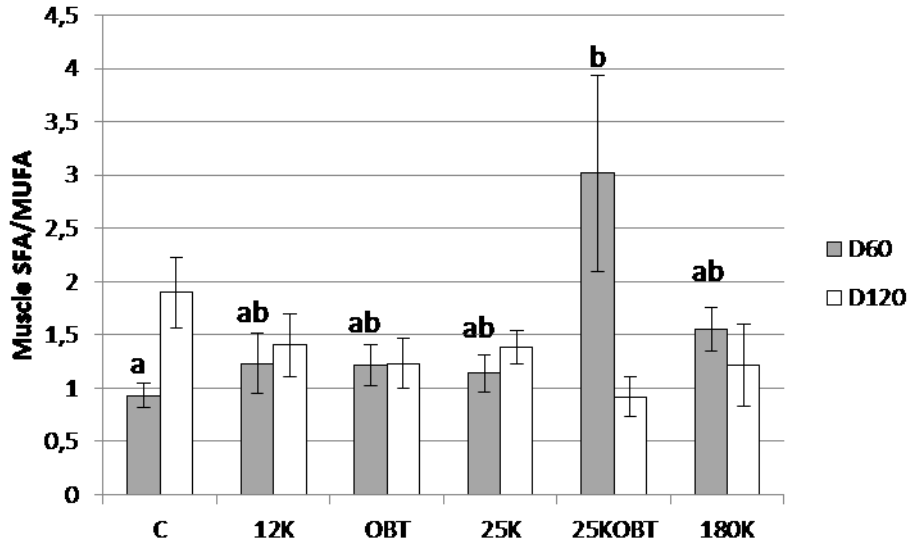


Figure 2

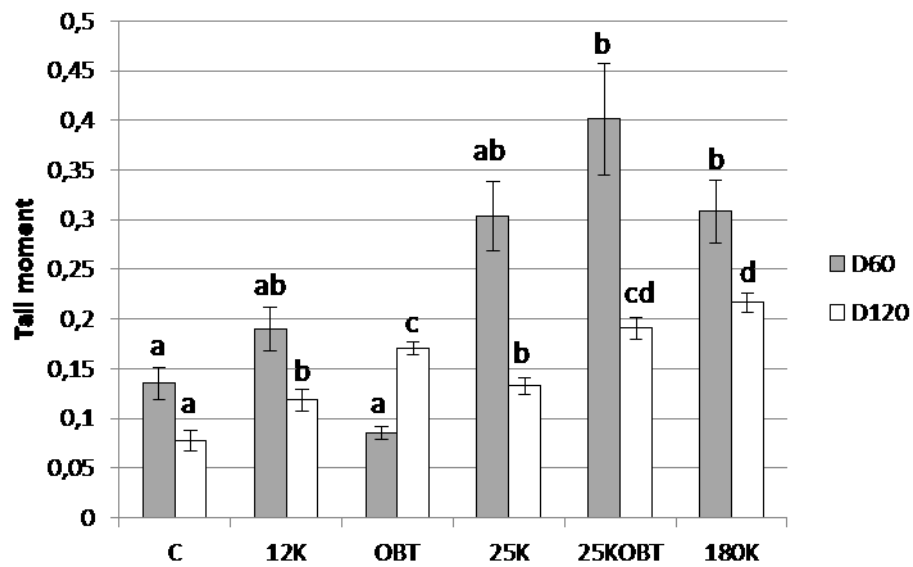


Figure 3

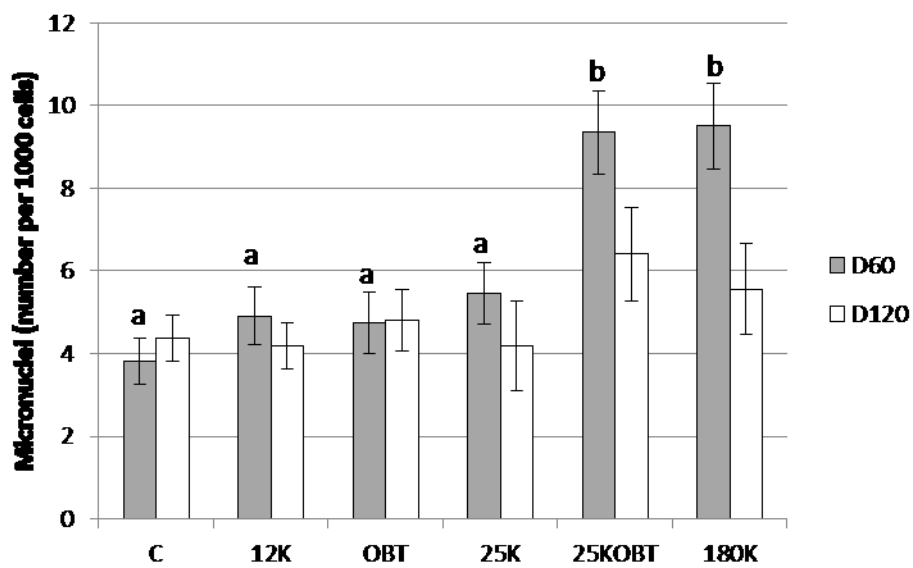


Figure 4

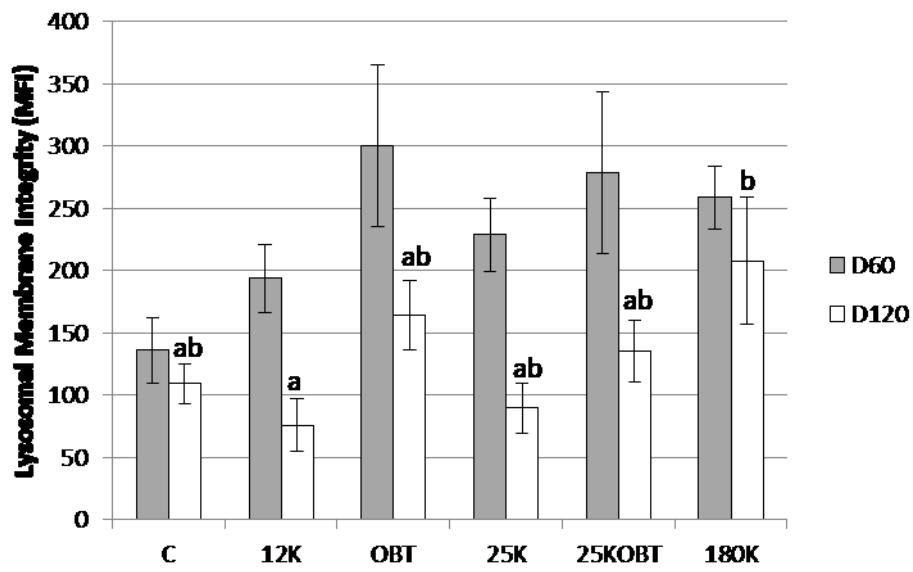


Figure 5

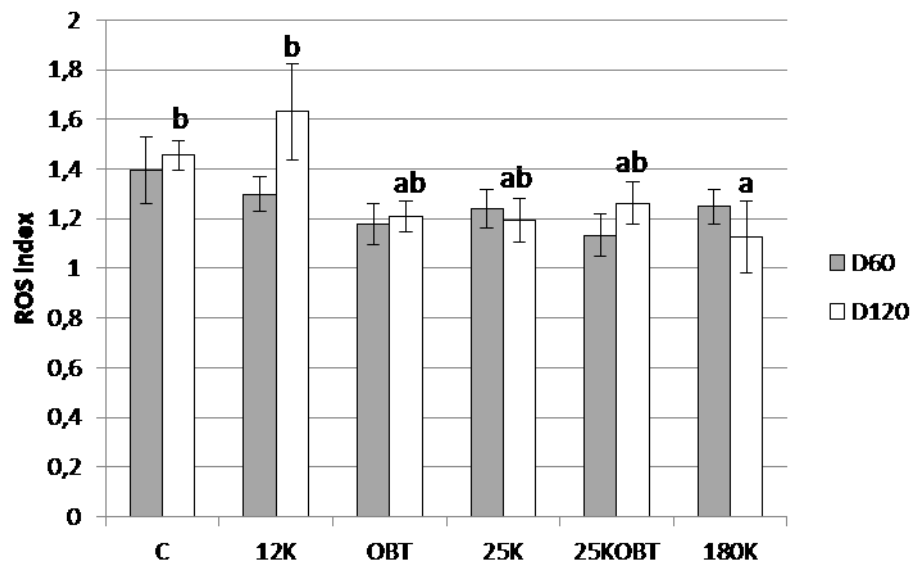


Figure 6

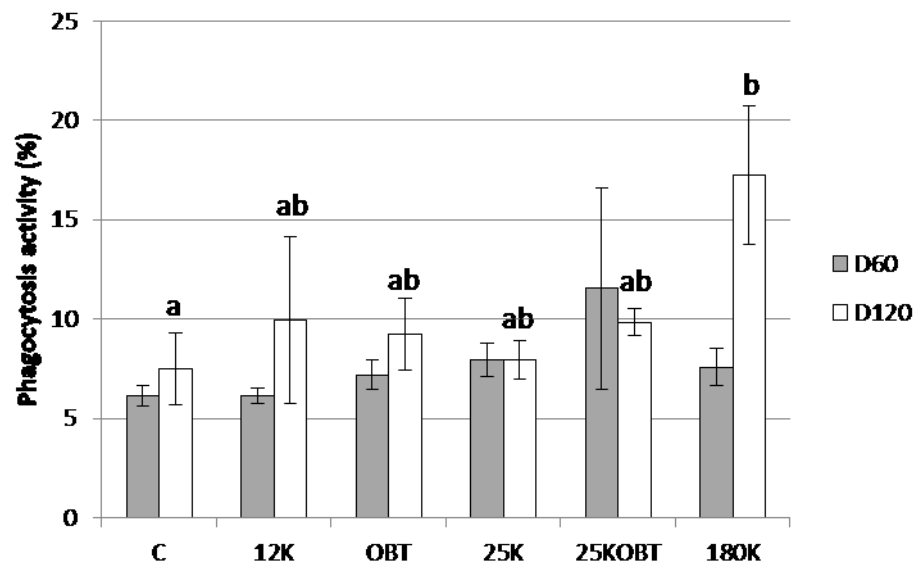


Figure 7

**Table 1:** HTO water concentration, HTO and OBT activity concentrations (Bq/kg fresh) and associated estimated dose rate ( $\mu\text{Gy/h}$ ) in fish during the experiment. <dl: less than detection limit. Values presented contain at least ten replicates  $\pm$  standard error. The water equivalent factor (WEF) was calculated using our fish water content (Water content=75%, WEF=0.6). At D60, concentration factor was not calculated because as fish were transported in background water instead of tank water, their measured TFWT were much lower because of the fast depuration rate. At D120, HTO water activity concentrations were either below or above DL; therefore, only one value corresponding to the mean of all values obtained is presented.

Time point	Condition	HTO water activity concentration Bq/L	Internal activity concentration			Concentration factor (TFWT fish/HTO water)	Estimated dose rate
			TFWT Bq/kg (fresh)	OBT Bq/kg (fresh)	Total tritium (TFWT+OBT) Bq/kg (fresh)		Total tritium (3H DR) $\mu\text{Gy/h}$
D60	C	63	15 $\pm$ 2	22 $\pm$ 5	42 $\pm$ 6	---	2.3E-04 $\pm$ 3.2E-05
	12K	10058 $\pm$ 1298	8193 $\pm$ 219	192 $\pm$ 13	8396 $\pm$ 227	---	4.5E-02 $\pm$ 1.2E-03
	25K	21629 $\pm$ 612	16477 $\pm$ 135	292 $\pm$ 10	16780 $\pm$ 142	---	9.0E-02 $\pm$ 7.6E-04
	OBT	127 $\pm$ 71	29 $\pm$ 3	419 $\pm$ 20	452 $\pm$ 19	---	2.4E-03 $\pm$ 1.0E-04
	25KOBT	20645 $\pm$ 3033	16748 $\pm$ 669	691 $\pm$ 23	17449 $\pm$ 683	---	9.4E-02 $\pm$ 3.7E-03
	180K	154314 $\pm$ 6239	118713 $\pm$ 1141	2377 $\pm$ 99	121153 $\pm$ 1203	---	6.5E-01 $\pm$ 6.5E-03
D120	C	<100	38 $\pm$ 13	31 $\pm$ 1	69 $\pm$ 13	0.49	3.7E-04 $\pm$ 6.9E-05
	12K	<100	49 $\pm$ 11	94 $\pm$ 5	147 $\pm$ 13	0.63	7.9E-04 $\pm$ 6.9E-05
	25K	<100	35 $\pm$ 8	180 $\pm$ 12	227 $\pm$ 17	0.45	1.2E-03 $\pm$ 9.1E-05
	OBT	<100	19 $\pm$ 2	176 $\pm$ 4	197 $\pm$ 6	0.24	1.1E-03 $\pm$ 3.0E-05
	25KOBT	<100	18 $\pm$ 1	275 $\pm$ 13	301 $\pm$ 11	0.23	1.6E-03 $\pm$ 6.0E-05
	180K	<100	58 $\pm$ 3	990 $\pm$ 52	1046 $\pm$ 52	0.75	5.6E-03 $\pm$ 2.8E-04

**Table 2:** Results of GLM analyses used to explain the evolution of biomarkers as a function of tritium fish dose rate (3H DR). The table only presents the significant results obtained at D60 and D120. ‘positive’, ‘negative’ indicates the trend of the interaction between the biomarker and the parameter explaining its evolution.  $p < 0.05$  for all trends indicated.

<b>Time</b>	<b>Biomarker</b>	<b>3H DR</b>
<b>D60</b>	MN frequency	positive
	DNA damages	positive
<b>D120</b>	DNA damages	positive
	LMI	positive
	Phagocytosis activity	positive
	ROS activated level	positive
	ROS basal level	positive
	ROS index	negative
	Brain AChE	positive

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