Gamma radiation induces dose-dependent oxidative stress and transcriptional alterations in the freshwater crustacean *Daphnia magna*

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Abstract

Among aquatic organisms, invertebrate species such as the freshwater crustacean *Daphnia magna* are believed to be sensitive to gamma radiation, although information on responses at the individual, biochemical and molecular level is scarce. Following gamma radiation exposure, biological effects are attributed to the formation of free radicals, formation of reactive oxygen species (ROS) and subsequently oxidative damage to lipids, proteins and DNA in exposed organisms. Thus, in the present study, effects and modes of action (MoA) have been investigated in *D. magna* exposed to gamma radiation (dose rates: 0.41, 1.1, 4.3, 10.7, 42.9 and 106 mGy/h) after short-term exposure (24 and 48 hrs). Several individual, cellular and molecular endpoints were addressed, such as ROS formation, lipid peroxidation, DNA damage and global transcriptional changes. The results showed that oxidative stress is one of the main toxic effects in gamma radiation exposed *D. magna*, mediated by the dose-dependent increase in ROS formation and consequently oxidative damage to lipids and DNA over time. Global transcriptional analysis verified oxidative stress as one of the main MoA of gamma radiation at high dose rates, and identified a number of additional MoAs that may be of toxicological relevance. The present study confirmed that acute exposure to gamma radiation caused a range of cellular and molecular effects in *D. magna* exposed to intermediate dose rates, and highlights the need for assessing effects at longer and more environmentally relevant exposure durations in future studies.

Keywords: Gamma radiation, *Daphnia magna*, oxidative stress, mode of action, gene expression.

1. Introduction

The increased use of nuclear technologies in the past decades has increased the concern on the impacts of man-made radionuclides in the environment, especially after the nuclear accident in Chernobyl in 1986 and more recently at Fukushima. In addition, other anthropogenic activities as routine discharges from nuclear power plants, nuclear weapons testing, mining, and nuclear waste from research facilities enhance the discharge of radionuclides into the aquatic environment thereby causing significant exposure of aquatic organisms (Unscear 2008).

Most radionuclides are gamma emitting, and gamma radiation can result in direct damage to biomolecules, such as double-strand breaks in genomic DNA (Ward, 1995), genotoxic DNA alterations (Parisot et al., 2015), chromosomal aberrations and mutations (Dallas et al., 2012), or indirectly damage macromolecules through the production of free radicals and reactive oxygen species (ROS) (Reisz et al., 2014). As a consequence, effects on a genetic and cellular level can result in significant impacts at the individual and population level, such as increased mortality and morbidity, reproduction impairment, shortening of life span and growth inhibition (Dallas et al., 2012; Fuller et al., 2015; Won et al., 2014). Although gamma radiation is known to induce toxicity in several aquatic invertebrates, knowledge of low dose effects on this diverse group of organisms is still limited compared to more extensively studied organisms such as fish and mammals. An overview of the effects of ionising radiation on aquatic invertebrates has already been carried out (Dallas et al., 2012; Fuller et al., 2015), highlighting the need for information regarding mechanisms of toxicity, early and sub-lethal effects in several groups of invertebrates, in for example the subphylum Crustacea. Crustaceans, such as the water flea *Daphnia magna*, have been identified as key models for the development of environmental radiation protection frameworks (ICRP, 2008).

Daphnia magna are small freshwater filter-feeding crustaceans that occupy a key position in the aquatic food web, not only as important phytoplankton grazers, but also as major food

sources for fish and invertebrate predators (Shaw et al., 2008). Daphnids are one of the most used invertebrate species in freshwater ecotoxicology and ecology mainly due to their comparatively short generation time, ease of culturing under laboratory conditions, capacity to reproduce through parthenogenesis and sensitivity to various environmental stressors (Watanabe et al., 2008). Accordingly, daphnids have been routinely used as standard model organisms in regulatory toxicity testing and detailed test guidelines have been developed (OECD, 2004, 2008; US EPA, 1996). Knowledge of the ecology, phylogeny, toxicology, and physiology of daphnia species in combination with a fully sequenced genome (wfleabase.org) has enabled a high number of exposure studies with different stressors in this species. Recent development of genomic tools, such as genetic linkage maps, cDNA libraries, expressed sequence tags databases and microarrays, have further enhanced the understanding of environmental-induced modulation of gene functions that may give rise to effects of ecological relevance (Kim et al., 2015; Shaw et al., 2008; Watanabe et al., 2008).

Previous studies have shown that exposure to acute doses of gamma radiation can cause significant mortality (Fuma et al., 2003), cause reduction in mobility and growth in daphnids, as well as a decrease in carbon incorporation in connection to reduced activity, filtering and ingestion rates (Nascimento et al., 2015, 2016; Nascimento and Bradshaw, 2016). Chronic exposure to gamma radiation can negatively impact survival, growth (decrease in body mass and length), metabolic dynamics (reduced resistance to starvation, decrease in mean-life span, alterations in respiration rate and mitochondrial activity) and reproduction (reduction in fecundity, delay in brood release and reduction in brood size) in daphnids, effects that were aggravated in subsequent generations (Gilbin, 2008; Marshall, 1962, 1966; Parisot et al., 2015; Sarapultseva and Gorski, 2013; Sarapultseva et al., 2017). Radiation-induced genotoxicity after chronic exposure was also reported in *D. magna* in the form of significant DNA alterations and transmission to progeny across generations (Parisot et al., 2015).

One of the most well-known toxic mechanisms of gamma radiation is the generation of ROS (e.g. superoxide radicals, hydroxyl radicals and hydrogen peroxide), either through direct interaction with the water in cells (formation of free radicals, recombination of radicals) or indirectly by the generation of secondary ROS by subsequent chemical cascades. The production of these radicals in excess can overwhelm the antioxidant capacity of cells and lead to oxidative stress due to oxidization of cellular components, instigating cell damage and other deleterious effects (Reisz et al., 2014). Some of the most common examples of biochemical and physiological damages associated with oxidative stress are lipid peroxidation (LPO) (formation of malonaldehyde-like species and 4-hydroxyalkenals), protein oxidation (e.g. carbonylation and cysteine oxidation) and DNA damage (e.g. single and double-strand breaks, 8-hydroxydeoxyguanosine and other oxidized bases), that have been described as some of the mechanisms involved in the damage caused by gamma radiation (Dallas et al., 2012; Fuller et al., 2015; Reisz et al., 2014). Even though it is well documented that gamma radiation can cause oxidative stress responses in several aquatic organisms (Dallas et al., 2012; Fuller et al., 2015; Won et al., 2014), detailed knowledge about the mode of action (MoA) of gamma radiation and linkage to phenotypical effects in crustaceans are still limited. Thus, acute toxicity of gamma radiation-induced oxidative stress was examined in *D. magna* by focusing on ROS formation, lipid peroxidation and DNA damage. In addition, alterations in the global gene expression were investigated to identify potential MoAs of gamma radiation in *D. magna*.

2. Material and Methods

2.1. Test Organism

Daphnia magna used in this study have been maintained in the NIVA laboratory for more than 20 years (DHI strain NIVA, Oslo, Norway). *Daphnia magna* was cultured in EPA moderately hard media (MHRW, 96.0 mg/L $NaHCO_3$, 60.0 mg/L $CaSO_4.2H_2O$, 60.0 mg/L $MgSO_4$, 4.0

mg/L KCl, pH 7.2), which was renewed twice a week. Daphnids were fed daily with a suspension of the unicellular algae *Pseudokirchneriella subcapitata* and supplemented by an amount of dried baker's yeast (20 mg/mL). Cultures were kept in a climate room with light conditions set to 16:8 hr light: dark photoperiod and temperature 20 ± 1ºC, according to the OECD 202 guidelines (OECD, 2004). Under these conditions, female daphnids reproduce by parthenogenesis every three days. All cultures and exposures were initiated using third to fifth brood neonates aged <24 h old.

2.2. Gamma radiation exposure

Gamma radiation exposures were conducted at the FIGARO ^{60}Co facility at the Norwegian University of Life Sciences (NMBU, Ås, Norway). *D. magna* neonates (<24h old) were exposed for 24 and 48 hrs to external gamma radiation under controlled climate conditions in accordance with the OECD 202 guidelines (OECD, 2004), with slight modifications to accommodate the experimental conditions used in this study. Neonates were exposed in 24-well plates (FalconTM, Oslo, Norway) to 7 different gamma dose rates varying from 0.41 to 106 mGy/h (see Supplementary Table A1 for more information on dose rates and total doses), along with a control placed behind lead shielding in the same room (background radiation). Experiments were conducted at the same temperature as that used for maintenance of *D. magna* cultures and in the dark, and exposure conditions as temperature, pH and dissolved oxygen were monitored for each dose rate throughout exposure. Immobilization and moulting frequency were recorded at 24 and 48 hrs. Due to relatively large sample size required for some of the parameters analysed, exposed daphnids were obtained across different experiments spaced in time, but subjected to the same experimental conditions. Three to six replicate plates were used for each endpoint, each plate with 10-12 daphnids depending on endpoint (see Supplementary Table A1 for more information on replication used). Field dosimetry (air kerma rates measured

with an ionization chamber) was traceable to the Norwegian Secondary Standard Dosimetry Laboratory (Norwegian Radiation Protection Authority, NRPA, Oslo, Norway) (Bjerke and Hetland, 2014). Dose rates to water in the centre of microplate wells (front row) were estimated according to Bjerke and Hetland (2014) and used as a proxy for the dose rates to exposed *D. magna*. Actual air kerma rates were measured using an Optically Stimulated Luminescence (OSL) based nanoDots dosimetry (Landauer) by positioning the nanoDots at the front of the microplates without use of build-up caps. Air kerma dose rates were calculated applying a conversion factor suggested by Hansen and Hetland (2015). Total doses were calculated from measured dose rates (mGy/h), multiplied by total exposure time (Supplementary Table A2).

2.3. ROS formation

Intracellular ROS production in *D. magna* exposed to gamma radiation was determined *in vivo* as described by Ma et al. (2012) and Xie et al. (2007) using the probes 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Invitrogen, Molecular Probes Inc., Eugene, OR, USA) and dihydrorhodamine 123 (DHR 123, Invitrogen, Molecular Probes Inc., Eugene, OR, USA), and adapted to the experimental conditions used in this study. Stock solutions of 20 mM H_2DCFDA and 5 mM DHR 123 were prepared in DMSO and kept at -20°C prior to use. On the day of the analysis, H_2DCFDA and DHR123 stock solutions were diluted in MHRW to a final working solution of 2 mM. After 24h and 48 hrs exposure to gamma radiation, daphnids were collected and transferred in 200 µL MHRW to a 96-well black microplate (Corning Costar, Cambridge, MA, USA), with 10-12 replicates per dose rate. Only surviving daphnids were used for the determination of ROS. For each dose rate, 5 µL of either H_2DCFDA or DHR 123 working solutions were immediately added to each well (50 µM final concentration) and the microplate covered with aluminium foil and incubated for 6 hrs under laboratory conditions. Fluorescence was recorded hourly on a microplate fluorescent reader Fluoroskan Ascent 2.5,

ThermoFisher Scientific, USA) with excitation/emission of 485/538 nm. Natural fluorescence of irradiated MHRW in combination with the probes (without presence of daphnids) for each dose rate was also analysed and the resulting fluorescence subtracted. The relative fluorescence obtained for both probes at each dose rate was expressed as fold induction comparative to the control. Two independent experiments were run to determine the formation of ROS in daphnia exposed to gamma radiation. Hydrogen peroxide (H_2O_2, CAS number: 7722-84-1, purity ≥30%) was used as positive control for both probes following the same procedure, in concentrations ranging from 1–50 μM.

2.4. Lipid peroxidation

Lipid peroxidation (LPO) was assessed by determining malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) concentrations upon decomposition by polyunsaturated fatty acid peroxides, following the method described by Erdelmeier et al. (1998). Briefly, after 24 and 48 hrs exposure to gamma radiation, 5 to 6 groups of 36 daphnids were pooled, frozen in liquid nitrogen and stored at -80ºC until further analysis. Pooled daphnids were homogenized using a Precellys tissue Homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) in 0.02 M Tris-HCl containing 0.5 M BHT (pH 7.4) at 4ºC. The resulting homogenate was centrifuged at 3000 *g* for 10 minutes at 4ºC and the supernatant used for protein determination and LPO analysis. LPO analysis was based on the reaction of two moles of N-methyl-2-phenylindole (3:1 mixture of acetonitrile/methanol), a chromogenic reagent, with one mole of either MDA or 4-HNE under acidic conditions (methanesulfonic acid) at 45°C for 60 min to yield a stable chromophore with maximum absorbance at 586 nm. Malondialdehyde bis-(1,1,3,3-tetrametoxypropane) was used as a standard. Protein content was determined using the Bradford method (Bradford, 1976) with Immunoglobulin G (IgG) as a standard. Lipid peroxidation was expressed as fold induction comparative to the control.

2.5. Comet Assay

The alkaline Comet Assay was performed on haemolymph cells from exposed daphnids, according to the method by Pellegri et al. (2014) and adapted to the high throughput single cell gel electrophoresis described in Gutzkow et al. (2013). After 24 and 48 hrs exposure, pools of 24 daphnids (3 biological replicates) were placed in PBS buffer without Ca^{2+}/Mg^{2+} (pH 7.4) and haemolymph cells extracted by mechanical dissociation using a metal grinder. After haemolymph extraction, the buffer containing the cells was filtered using a 55 µM nylon mesh and the resulting cell suspension centrifuged at 300 *g* for 5 minutes (4ºC). The pellet was gently resuspended in PBS buffer without Ca^{2+}/Mg^{2+} (pH 7.4) and the final cell suspension adjusted to $1x10^6$ cells/mL. Cell viability was checked using the trypan blue exclusion assay. Cells were resuspended in 1:10 0.75 % low melting point agarose at 37 ºC and triplicates (3×4 µL) from each biological replicate were immediately applied on a cold GelBond®film. Lysis was performed overnight in lysis buffer (2.5 M NaCl, 0.1 M Na_2EDTA, 0.01 M Tris, 0.2 M NaOH, 0.034 M N-laurylsarcosine, 10 % DMSO, 1 % Triton X-100, pH 10) at 4ºC. For unwinding, films were immersed in cold electrophoresis solution (0.3 M NaOH, 0.001 M Na_2EDTA, pH > 13) for 40 min. Electrophoresis was carried out in cold, fresh electrophoresis solution for 20 min at 8 ºC, 25 V giving 0.8 V/cm across the platform, with circulation of electrophoresis solution. After electrophoresis, films were neutralized with neutralisation buffer (0.4 M Tris–HCl, pH 7.5) for 2×5 min, fixed in ethanol (>90 min in 96 % ethanol) and dried overnight. Films were stained with SYBR®Gold Nucleic Acid Gel Stain (Life Technologies, Paisley, UK) in TE-buffer (1 mM Na_2EDTA, 10 mM Tris–HCl, pH 8) before examination at a 20× magnification under an Olympus BX51microscope (light source: Olympus BH2-RFL-T3, Olympus Optical Co., Ltd.; camera: A312f-VIS, BASLER, Ahrensburg, Germany). Fifty randomly chosen cells per replicate (150 cells per biological replicate, total 450 cells per dose

rate) were scored using the Comet IV analysis software (Perceptive Instruments Ltd., Bury St. Edmunds, UK). Tail intensity (% Tail DNA), defined as the percentage of DNA migrated from the head of the comet into the tail, was used as a measure of DNA damage induced by gamma radiation because it has been shown to be the most meaningful endpoint to assess genotoxicity (Kumaravel and Jha, 2006). The mean percentage (%) of DNA in the tail per biological replicate was calculated using the median values of % tail DNA from the 50 comets from each technical replicate. Treatment with hydrogen peroxide (H_2O_2, CAS number: 7722-84-1) was used as the positive control following the same procedure, in concentrations ranging from 1 to 10 μM.

2.6. Microarray gene expression analysis

After 24 hrs exposure to gamma radiation, six daphnids were pooled for each replicate (n=5), sampled in RNALater (Sigma-Aldrich) and stored at -80°C until use. Total RNA was isolated using the ZR Tissue & Insect RNA MicroPrep kit in combination with on-column DNase I treatment (Zymo Research Corp., Irvine, CA) as previously described (Song et al., 2016). The purity (260/280>1.8, yield > 100 ng) and integrity (clear RNA peaks, flat baselines) of RNA were assessed using Nanodrop ND-1000 (Nanodrop Technologies, Wilminton, DE) and Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively.

Transcriptomic analysis was performed using Agilent custom 60,000-feature *D. magna* oligonucleotide microarrays and 50 ng input RNA according to Agilent's standard protocol "One-Color Microarray-Based Gene Expression Analysis, version 6.5", with modifications (Song et al., 2016). Raw microarray data (signal intensity) was extracted from scanned images using the Feature Extraction software v10.7 (Agilent), and data corrected for baseline variance (normexp method), inter-array variance (quantile method), filtered for low expression probes and technical replicate probes merged using the Bioconductor package LIMMA (Smyth, 2005) in the R statistical environment v3.1.2, as previously described (Jensen et al., 2016).

Differentially expressed genes (DEGs) were determined using LIMMA by contrasting gamma-exposed groups to the control (p<0.05). Gene ontology (GO) enrichment analysis was performed towards crustacean GO databases using a hypergeometric test (p<0.05) implemented in Cytoscape v3.1.1 (Smoot et al., 2011) via the Bingo plugin v2.4 (Maere et al., 2005). The *D. magna* DEGs were further mapped to *Drosophila melanogaster* orthologs in order to perform Reactome pathway enrichment analysis (p<0.05) using the Cytoscape plugin ClueGO v2.1.4 (Bindea et al., 2009). Venn diagram analyses were performed using Venny (http://bioinfogp.cnb.csic.es/tools/venny/) and Sumo software package (http://angiogenesis.dkfz.de/oncoexpress/software/sumo/). No multiple testing corrections were performed to avoid loss of DEGs and GO/pathways that may potential have high relevance for gamma-induced stress response profiles (Song et al., 2014; 2016).

2.7. Quantitative real-time PCR analysis

A selection of 13 target genes considered relevant to potential MoAs of gamma radiation was further verified using quantitative real-time reverse transcription polymerase chain reaction (qRTPCR) essentially as described by Song et al. (2016). The qRTPCR analysis was conducted on a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Oslo, Norway) using the same RNA as used in the microarray analysis (n=5). Primers used for cDNA amplification were designed using the online software Primer3 v4.0.0 (http://primer3.ut.ee/) and purchased from Invitrogen™ (Carlsbad, California, USA) (Supplementary Table A3). Briefly, cDNA was made from total RNA (82.5 ng) using qScript™ cDNA SuperMix (Quanta BioSciencesTM, Gaithersburg, MD, USA), and amplified in a 20 µl reaction (1 ng cDNA, 400 nM forward/reverse primer and 15 µl PerfeCTa® SYBR® Green FastMix® (Quanta BioSciencesTM)) using the Bio-Rad CFX384 platform (Bio-Rad Laboratories, Hercules, CA). Four biological replicates (each containing two technical replicates), no-reverse-transcriptase

(NRT) and no-template controls (NTC) were included in the amplification. Pooled cDNA (0.25–4 ng) was used to generate a standard curve for determination of amplification efficiency. The relative expression was calculated using the Pfaffl method (Pfaffl, 2001). Gene expression data for target genes was normalized to the geometric mean expression of three reference genes, beta actin (β-actin), cyclophilin (Cyp) and glyceraldehyde 3-phosphate dehydrogenase (Gadph), to compensate for any difference in initial RNA quantity and in reverse transcriptase efficiency. The normalized expression of each target gene was further normalized to the mean expression of the control.

2.8. Statistical Analysis

Statistical analyses were performed using XLStat2016® (Addinsoft, Paris, France) and GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Data was tested for normality and homogeneity of variances using Shapiro-Wilk and Levene´s tests, respectively, to check if all parameters satisfied the assumptions associated with parametric tests. Differences between dose rates and time of exposure were compared for ROS production, LPO and DNA damage data using a 2-way ANOVA followed by the post-hoc Tukey test. Gene expression results were analysed for significant differences between dose rates either with one-way analysis of variance (ANOVA) or Kruskal–Wallis One Way Analysis of Variance on Ranks. If significant, pairwise multiple comparison procedures were conducted, using the Tukey test or the Dunn's method. For qPCR data, outliers were removed using the ROUT test implemented in GraphPad. A Pearson correlation analysis was also performed between the mean relative gene expression values obtained by qPCR compared to mean relative gene expression values for the same genes from the microarray analysis for all exposure groups. Statistical significance was set at $p<0.05$ for all statistical analyses.

3. Results

3.1. Effects on mortality and exposure parameters

Following gamma radiation exposure (dose rates: 0.41, 1.1, 4.3, 10.7, 42.9 and 106 mGy/h), no significance difference in mortality, visual morphological or behavioural changes were observed between control and irradiated daphnia for all doses rates tested at 24 and 48 hrs. The temperature, pH and dissolved oxygen of the MHRW exposure media was 20.0 ± 0.05ºC, 8.1 ± 0.05 and 8.6 ± 0.02 mg/L during the exposure period, respectively.

3.2. ROS formation

The formation of ROS was analysed regarding differences between dose rate and time of exposure using a two-way ANOVA (Supplementary Table A4). Results show that for the H_2DCFDA fluorescence probe only the effect of dose rate was significant for the results obtained (p<0.0001), while for the DHR 123 probe, both time and dose rate where significant for the differences seen in exposed daphnids (p=0.0384 and p<0.0001, respectively). Exposure to gamma radiation for 24 hrs caused a significant increase in ROS formation in *D. magna* at 4.3 (1.4-fold), 10.7 (1.4-fold) and 42.9 mGy/h (1.5-fold), when measured by the H_2DCFDA fluorescence probe (Figure 1A). Similar results were obtained with the DHR 123 probe (Figure 1C), with significant ROS levels at dose rates higher than 1.1 mGy/h after 24 hrs exposure (up to a 1.7-fold increase at 42.9 mGy/h). The results obtained for both probes showed no significant ROS formation at the highest dose rate tested (106 mGy/h). After 48 hrs exposure, a significant increase in ROS formation was observed at 10.7 mGy/h and higher dose rates (up to 1.3-fold) in daphnids incubated with H_2DCFDA (Figure 1B), even though no clear dose-response relationship was observed. In daphnids incubated with the DHR 123 probe, a significant increase in ROS formation was only detected at 1.1, 10.7 and 106 mGy/h (p<0.05), with a maximum 1.6-fold induction at 1.1 mGy/h (Figure 1D). Temporally, a decrease in ROS

formation from 24 to 48 hrs exposure was detected with DHR 124 only at 4.3 and 42.9 mGy/h (Figure 1C-D).

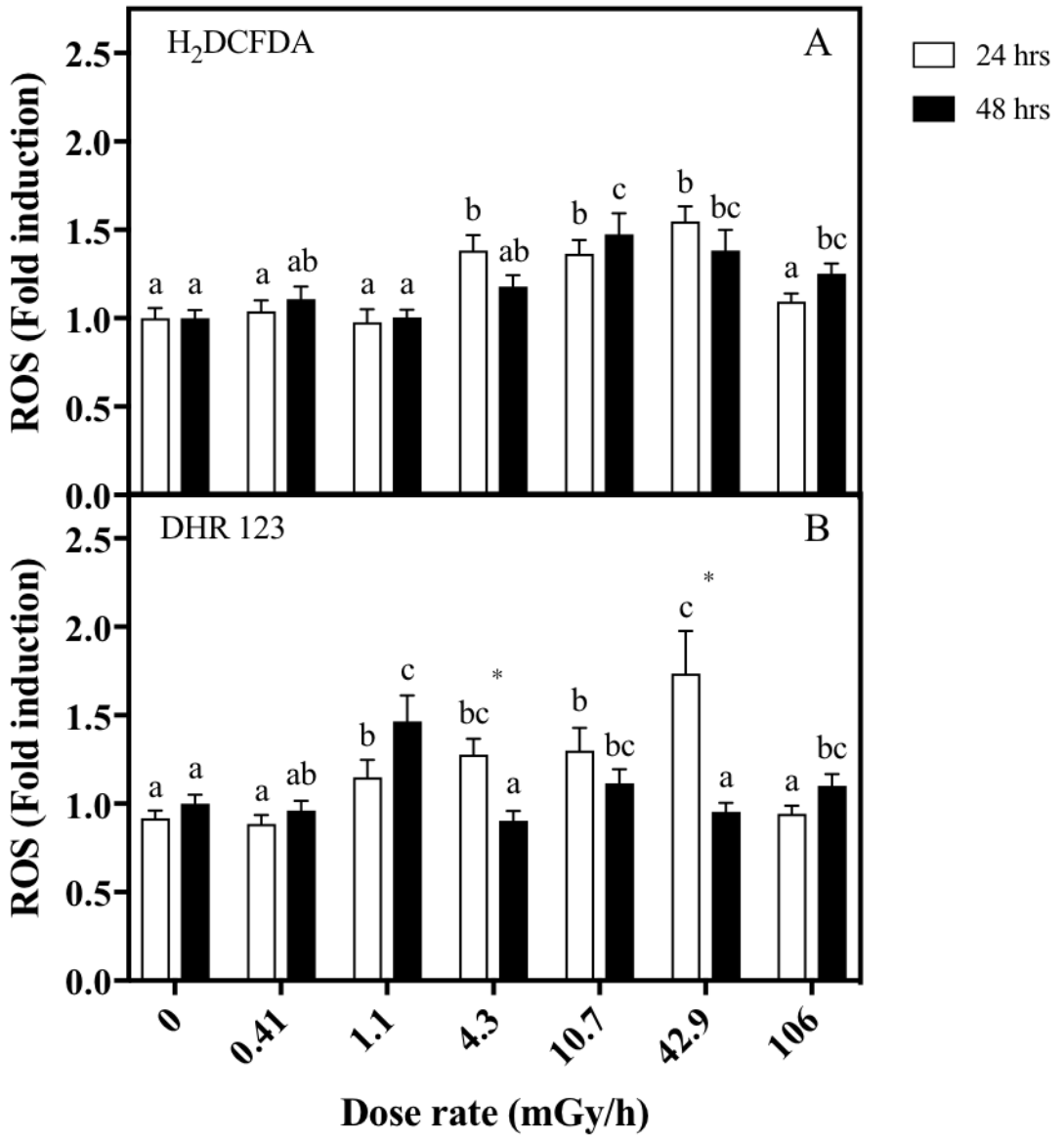
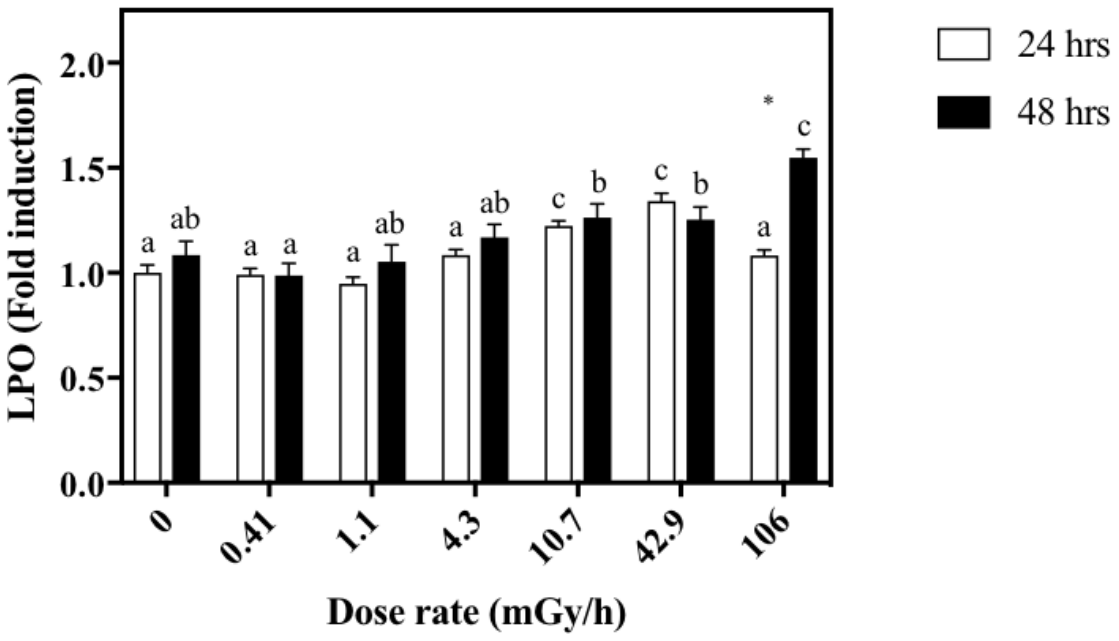


Figure 1 – Intracellular reactive oxygen species (ROS) formation measured by A) 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and B) dihydrorhodamine 123 (DHR 123) in *Daphnia magna* after 24 hrs and 48 hrs exposure to gamma radiation (average ± SEM). Letters represent statistical differences between dose rates for each exposure period (p<0.05). Asterisk represent statistical differences between exposure period for each dose rate (p<0.01).

335 H_2O_2 was used as a positive control to evaluate the performance of the ROS formation bioassay
336 in *D. magna* using two fluorescent probes H_2DFFDA and DHR 123. The results obtained
337 showed a significant concentration dependent increase in ROS formation after 24 hrs exposure
338 to H_2O_2 (Supplementary Figure A1).
339
340 **3.3. Lipid peroxidation**
341 The two-way ANOVA showed that both time and dose rate had a significant effect on LPO
342 data in exposed daphnids (Supplementary Table A4) and that their interaction was also
343 significant (p<0.0001). Exposure to gamma radiation caused LPO in exposed daphnids after 24
344 hrs exposure only at 10.7 and 42.9 mGy/h (1.2- and 1.3–fold, respectively, Figure 2A). After
345 48 hrs exposure, a dose-dependent increase in LPO was detected (Figure 2B), reaching a 1.5–
346 fold increase at the highest dose rate (106 mGy/h, p<0.05). A significant temporal increase in
347 LPO was only detected at 106 mGy/h, with a 1.4-fold increase from 24 hrs to 48 hrs exposure.



348
349 **Figure 2 –** Lipid peroxidation in *Daphnia magna* (5 to 6 groups of 36 pooled daphnids) was
350 measured as malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) after exposure to
351 gamma radiation for 24 hrs and 48 hrs (average ± SEM). Letters represent statistical differences

between dose rates for each exposure period (p<0.05). Asterisk represent statistical differences between exposure period for each dose rate (p<0.0001).

3.4. Comet assay

Similarly to LPO, time and dose rate also had a significant effect on DNA damage (Supplementary Table A4) and that their interaction was also significant (p<0.0001), as shown by the two-way ANOVA. Gamma radiation caused a small, but statistically significant increase in DNA-damage measured as single strand breaks (SSB) and alkali labile site formation in the haemolymph after 24 hrs of exposure at the highest doses (10.7, 42.9 and 106 mGy/h) compared to untreated controls. All dose rates except for 4.3 mGy/h caused DNA-damage after 48 hrs exposure. As for temporal variation, an increase in DNA-damage was observed at 0.41, 1.1 and 10.7 mGy/h at 48 hrs exposure compared to 24 hrs. H_2O_2 was used as a positive control and the results obtained showed a significant concentration-dependent increase in DNA damage in haemolymph from daphnids after 24 hrs, thus assuring a good quality control of the assay (Supplementary Figure A2). Cell viability was assessed using the trypan blue staining with cell viability >90 % at all dose rates used. Images of comets from haemolymph cells isolated from control and gamma radiation exposed daphnids are shown in Supplementary Figure A3.

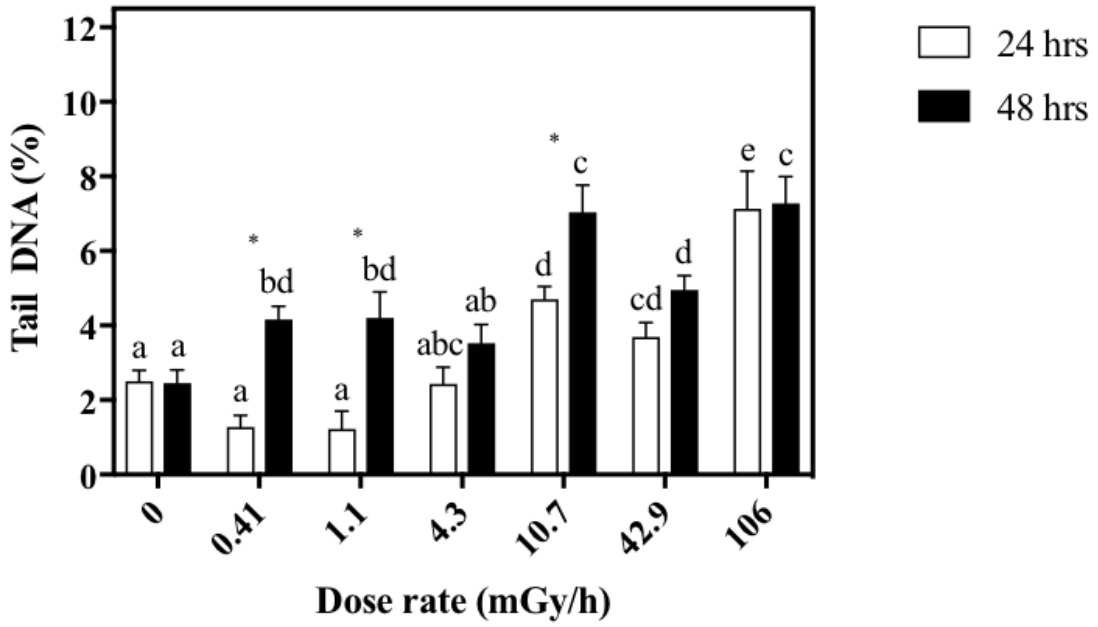
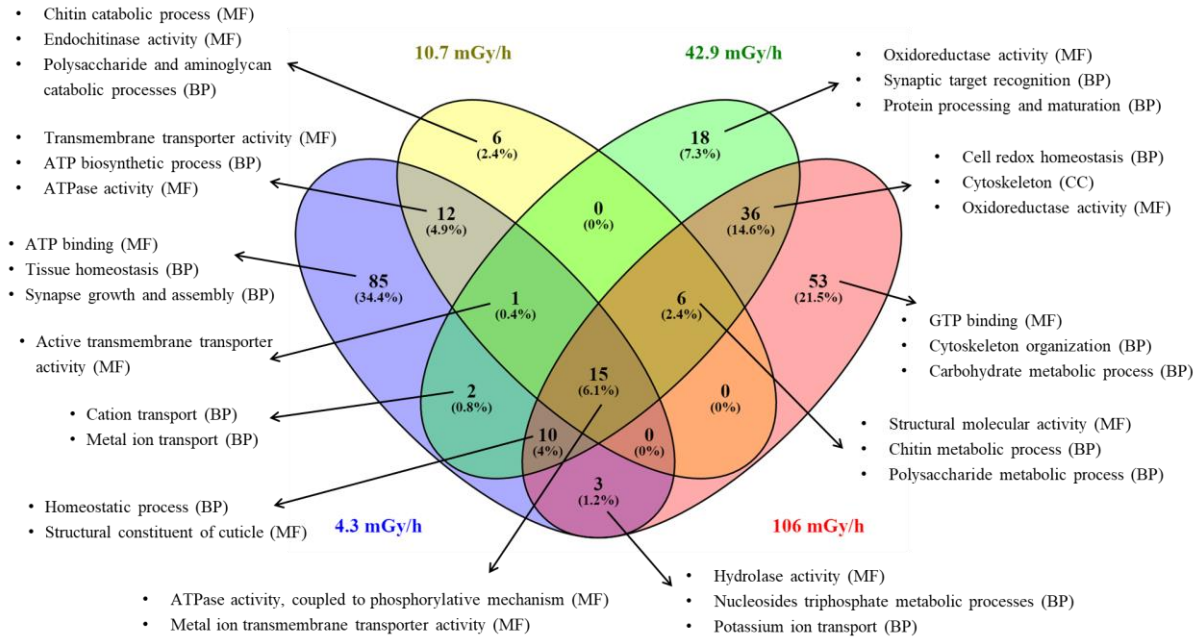


Figure 3 – DNA damage in *Daphnia magna* after exposure to gamma radiation for 24 hrs and 48 hrs (total 450 cells per dose rate, average ± SEM). Letters represent statistical differences between dose rates for each exposure period (p<0.05). Asterisk represent statistical differences between exposure period for each dose rate (p<0.001).

3.5. Global transcriptional alterations

A massive number of transcriptional alterations were found in *D. magna* exposed 24 hrs to 42.9 and 106 mGy/h (3308 and 3352 DEGs, respectively), the highest dose rates tested, compared to the intermediate dose rates of 1.1, 4.3 and 10.7 mGy/h (458, 534 and 1220 DEGs) (Supplementary Table A5). Interestingly, exposure to the lowest gamma radiation dose rate of 0.41 mGy/h, resulted in a higher number of DEGs than the intermediate dose rates (2679 DEGs), suggesting a transcriptional response of *D. magna* also at low-dose rates (Supplementary Table A5). The Venn diagram analysis (Supplementary Figure A4) revealed that only 35 DEGs were identified to be common between all dose rates, whereas the majority of transcriptional changes were due to up-regulation of the DEGs. The complete list of DEGs

385 that were regulated in *D. magna* after exposure to gamma radiation can be found in the
386 Supplementary Table A6.
387
388 **3.6. Functional enrichment analysis**
389 Functional enrichment analysis showed that a total of 128, 40, 88 and 123 GO functions were
390 over-represented after exposure to 4.3, 10.7, 42.9 and 106 mGy/h, with the majority being dose
391 rate specific (Figure 4). No significant GO enrichment was identified at the two lowest dose
392 rates tested (i.e., 0.41 and 1.1 mGy/h). Briefly, exposure to 4.3 mGy/h seems to modulate DEGs
393 involved in ATP binding, tissue homeostasis, and synapse growth and assembly. Exposure to
394 10.7 mGy/h resulted in the differential regulation of genes related to chitin catabolic process,
395 endochitinase activity and polysaccharide and aminoglycan catabolic processes, while 42.9
396 mGy/h regulated genes involved in oxidoreductase activity, synaptic target recognition and
397 protein processing and maturing. The highest dose (106 mGy/h) regulated DEGs associated
398 with GTP binding, cytoskeleton organization and carbohydrate metabolic process. Functions
399 such as ATPase activity coupled to phosphorylative mechanism and metal ion transmembrane
400 transporter activity were commonly regulated by all dose rates. The complete list of GO
401 functions affected by the different dose rates used in this study can be found in the
402 Supplementary Table A7.
403
404



405

406 **Figure 4 –** Venn diagram analysis of overrepresented gene ontology (GO) functions that were
407 regulated in *Daphnia magna* after 24 hrs exposure to gamma radiation (p<0.01). A selection of
408 toxicologically relevant GO functions was identified and displayed. BP – Biological process,
409 MF – Molecular function, CC – Cellular component.

410

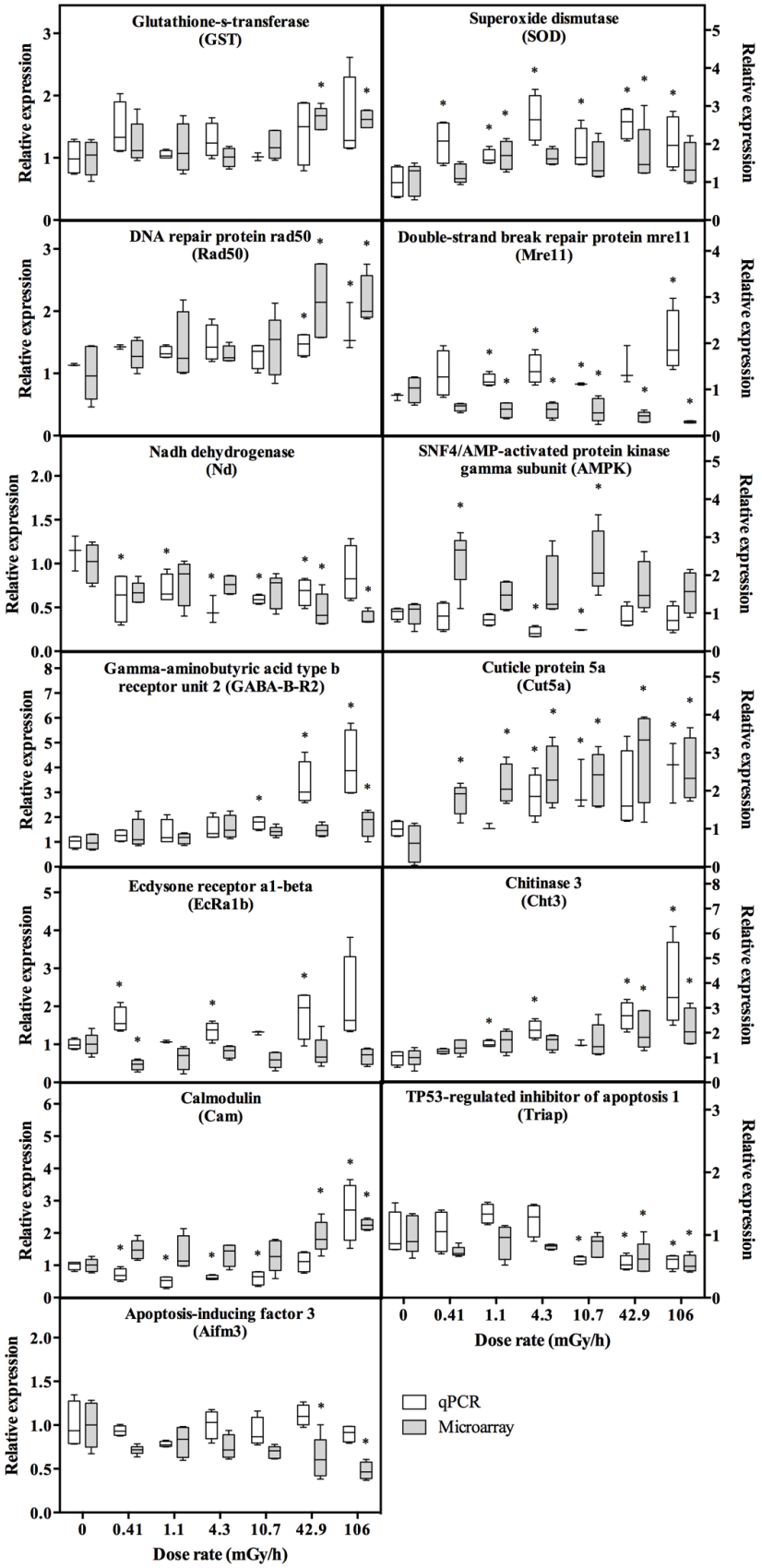
411 Pathway enrichment analysis further revealed a total of 73 (0.41 mGy/h), 6 (1.1 mGy/h), 11
412 (4.3 mGy/h), 37 (10.7 mGy/h), 119 (42.9 mGy/h) and 132 (106 mGy/h) pathways affected by
413 gamma radiation. Signal transduction, immune system and gene expression were identified as
414 the top functional categories with the most supporting pathways, while categories such as
415 transmembrane transport of small molecules (106 mGy/h) and DNA replication (42.9 mGy/h)
416 were only affected at specific dose rates (Supplementary Figure A5). Venn diagram analysis
417 allowed the identification of specific and common pathways affected by the different dose rates
418 (Supplementary Figure A6). In general, the higher number of pathways identified was at 106
419 mGy/h (e.g. G1/S DNA damage Checkpoints, p53-Independent DNA damage response, p53-
420 Independent G1/S DNA damage checkpoint, Ubiquitin mediated degradation of
421 phosphorylated Cdc25). The two highest dose rates tested displayed a higher number of

common pathways (total 58 pathways) than the remaining dose rates combined (e.g. calmodulin induced events, DNA damage/telomere stress induced senescence and GABA synthesis, release, reuptake and degradation). No pathway was commonly regulated across all dose rates. Pathways such as cell death signaling via NRAGE, NRIF and NADE, NRAGE signals death through JNK and P75 NTR receptor-mediated signaling were mainly affected by the lowest and highest dose rates used in this study (0.41 and 106 mGy/h), while pathways related to DNA double strand break response, recruitment and ATM-mediated phosphorylation of repair and signaling proteins at DNA double strand breaks were regulated by all dose rates except 1.1 mGy/h. Several toxicologically relevant pathways and supporting DEGs representative of potential MoAs of gamma radiation were identified (Supplementary Table A8), such as DNA repair and cell cycle regulation, neurotransmitter signaling, mTOR signaling, oxidative stress and antioxidant defense, molting and developmental signaling, cell death, oxidative phosphorylation and calcium signaling. The complete list of pathways affected by the different dose rates used in this study can be found in the Supplementary Table A9.

3.7. Quantitative real-time RT-PCR verification

The expression of thirteen target genes involved in relevant toxicity pathways were verified by qPCR, namely glutathione s-transferase (*GST*), superoxide dismutase (*SOD*), DNA repair protein rad50 (*Rad50*), double-strand break repair protein mre11 (*Mre11*), Nadh dehydrogenase (*Nd*), SNF4/AMP-activated protein kinase gamma subunit (*AMPK*), gamma-aminobutyric acid type b receptor subunit 2 (*GABA-B-R2*), cuticle protein5a (*Cut5a*), ecdysone receptor a1-beta (*EcRa1b*), chitinase 3 (*Cht3*), calmodulin (*Cam*), TP53-regulated inhibitor of apoptosis 1 (*Triap*) and apoptosis-inducing factor 3 (*Aifm3*). The transcriptional patterns obtained by qPCR for the 13 target genes were in close agreement with those of the microarray (Fig. 5), with a general tendency of increased expression with increasing dose rate. The only exceptions were

447 the genes *Mre11* and *AMPK*, in which the patters obtained by the microarray were the opposite
448 of those reflected by the qPCR. The similarity of transcriptional patterns obtained for the
449 microarray and qPCR analyses was also evidenced by the significant correlation obtained for
450 all genes ($r=0.446$, $p<0.0001$).


Glutathione-s-transferase (GST)
Superoxide dismutase (SOD)
DNA repair protein rad50 (Rad50)
Double-strand break repair protein mre11 (Mre11)
Nadh dehydrogenase (Nd)
SNF4/AMP-activated protein kinase gamma subunit (AMPK)
Gamma-aminobutyric acid type b receptor unit 2 (GABA-B-R2)
Cuticle protein 5a (Cut5a)
Ecdysone receptor a1-beta (EcRa1b)
Chitinase 3 (Cht3)
Calmodulin (Cam)
TP53-regulated inhibitor of apoptosis 1 (Triap)
Apoptosis-inducing factor 3 (Aifm3)
Relative expression
Dose rate (mGy/h)
0 0.41 1.1 4.3 10.7 42.9 106
qPCR
Microarray

451

Figure 5 – Gene response in *Daphnia magna* after 24h exposure to gamma radiation determined by quantitative real-time reverse transcription polymerase chain reaction (qPCR, white box, N=4-5) in comparison with microarray (grey box, N= 4-5). *Represents significant statistical differences compared to the respective control ($p<0.05$).

4. Discussion

Even though aquatic ecosystems are continuously exposed to low levels of naturally occurring radionuclides, the anthropogenic inputs of man-made radionuclides in these ecosystems has increased the need to study their impact on aquatic organisms. These concerns have intensified especially after the Fukushima nuclear power plant accident in 2011, where large amounts of radioactive iodine and caesium were released into the surrounding aquatic environment, resulting in increasing concentrations in many aquatic species at dose rates above suggested benchmark levels (Buesseler et al., 2012; Johansen et al., 2015; Nair et al., 2014). Nonetheless, there is still a lack of information about the toxic effects of ionizing radiation on invertebrate species, despite their essential role in aquatic ecosystems. In this context, this study aimed to understand the mechanism of toxicity of gamma radiation in the freshwater crustacean *D. magna* by identifying alterations in oxidative stress markers and their relation to alterations seen at the transcriptional level.

D. magna at the organismal level could tolerate gamma exposure up to 106 mGy/h for 48 hrs (total dose 5 Gy) without any sign of acute mortality, morbidity, or apparent developmental effects. No mortality, visual morphological or behavioural changes were detected in daphnids at any of the dose rates tested after the 48 hrs exposure to gamma radiation. This is in agreement with other studies, which have reported no effects in survival in *D. magna* as a result of acute exposure to gamma radiation generated by ^{137}Cs, at doses higher than those used in this study (total doses from 2 to 28 Gy and 5 to 200 Gy) (Nascimento et al., 2015, 2016). In fact, the

estimated 50 % effect dose for mortality reported for gamma radiation (^{60}Co source) in *D. magna* after exposure is 1600 Gy and 1500 Gy for 24 and 48 hrs, respectively (Fuma et al., 2003). On the other hand, Sarapultseva and Dubrova (2016) observed a significant shortening in the life span of *D. magna* after acute exposure to ^{60}Co (total doses of 100, 1000 and 10000 mGy), nonetheless, these effects were observed 4 to 7 days following radiation exposure. Even though there were no significant effects in mortality in irradiated daphnia, the gamma radiation dose rates used in this study can be considered high, especially when compared to the suggested ecosystem screening benchmark of 0.24 mGy/h for the protection of freshwater ecosystems from radioactive substances (Garnier-Laplace et al., 2010). The total doses used are, however, within the range of those found in highly contaminated sites, such as reservoir at Mayak PA in Russia, used as waste ponds for decades, where the absorbed dose rates for zooplankton and phytoplankton were estimated as 3.8 and 40 Gy/day, respectively (Triapitsyna et al., 2012). Another example is the Techa River also at Mayak, where doses to biota have been estimated as high as 200-800 Gy after the accident in 1957 (Kryshev et al., 1998).

4.1. ROS formation

Relative simple and rapid fluorescence assays for detecting ROS production have proven useful for the prediction of whole-organism toxicity, as previously seen in *D. magna* exposed to nano-TiO_2 under solar ultraviolet radiation (Ma et al., 2012). As anticipated, gamma radiation generated an apparent dose rate-dependent increase in ROS in daphnids after 24 h exposure (No Observed Effect Dose Rate, NOEDR of 1.1 mGy/h), particularly at dose rates higher than 1.1 mGy/h. Interestingly, no significant ROS production was detected at the highest dose of 106 mGy/h, as shown by both of the fluorescent probes. This lack of ROS formation can be potentially related to the combined protective action of radical scavenging antioxidants such as glutathione (GST), metallothionen and thioredoxin and/or induction of antioxidant enzymes

such as catalase (CAT), superoxide dismutase (SOD and glutathione-S-transferase (GST), among others (Reisz et al., 2014). This hypothesis is supported by the results obtained by transcriptional analysis which showed up-regulation of several antioxidant genes after 24 hrs exposure to gamma radiation at the highest dose rate. In fact, the *SOD* gene was up-regulated at all dose rates (qPCR) and at 1.1 and 42.9 mGy/h (microarray), suggesting that antioxidant enzymes were induced both at low and high dose rates. *GstS1* and *GstD5* were both up-regulated at the two highest dose rates (microarray), whereas no alterations were detected in *Gst* transcripts by qPCR. Thioredoxin peroxidase was also up-regulated at 0.41 mGy/h in addition to thioredoxin domain-containing protein at both 0.41 and 106 mGy/h. The induction of these antioxidant genes in *D. magna* after gamma radiation exposure confirms their central role in reducing oxidative stress caused by gamma radiation exposure at both low and high dose rates. Nonetheless, one cannot exclude the hypothesis that at intermediate dose rates, the antioxidant defence mechanisms triggered were insufficient to counterbalance the production of ROS, as seen at 42.9 mGy/h, or that other ROS-metabolizing molecules and detoxification enzymes not detected by the microarray analyses were affected. The induction of enzymatic and non-enzymatic antioxidants (SOD, CAT, GR (glutathione reductase), GPx (glutathione peroxidase), GST and GSH) has also been shown in other crustacean species (*Paracyclopina nana*, *Tigriopus japonicas, Brachionus koreanus* and *Mesocyclops hyalinus*) in response to increased ROS production by gamma (^{137}Cs and ^{60}Co) radiation (Han et al., 2014a, b; Won and Lee, 2014). After 48 hrs of exposure to gamma radiation, a dose-dependent ROS formation was observed in irradiated daphnids with a NOEDR of 1.1 mGy/h, similarly to what was seen at 24 hrs. In contrast to the response at 24 hrs, a significant ROS production was detected at 106 mGy/h after 48 hrs exposure, which may reflect temporal activation of direct ROS formation and activation of intracellular ROS-producing systems (e.g. mitochondria) at high doses (Reisz et al., 2014). Although the present study is the first to document gamma radiation-induced ROS

in *D. magna*, it has been documented for other aquatic invertebrates elsewhere (see review by Won et al., 2014).

4.2. Lipid peroxidation

Excessive ROS formation can induce oxidative stress and cause damage to lipids, proteins and DNA thus disturbing normal cellular functions (Reisz et al., 2014). Lipid peroxidation in particular, is characterized by the oxidative deterioration of polyunsaturated fatty acids present in cellular membranes, which can result in membrane destabilization and further oxidative damage (Halliwell and Gutteridge, 2007). Results from the present study verify that exposure to gamma radiation increased LPO at 10.7 and 42.9 mGy/h (24 and 48 hrs) when measured as MDA and 4-HNE, which were consistent with the observations on ROS formation at the same dose rates and exposure period. At 106 mGy/h, an increase in LPO was only observed after 48 hrs exposure, thus suggesting that the antioxidant protective system was capable of limiting oxidative damage only at lower dose rates and shorter exposure times. At the remaining dose rates, the production of ROS apparently exceeded the antioxidant capacity of cells. Nonetheless, the hypothesis that the rate of ROS produced at 10.7 and 42.9 mGy/h were not high enough to trigger the antioxidant defence mechanisms and counteract their oxidative damage cannot be excluded as a possible explanation for the LPO levels seen in irradiated daphnids. Although this is the first study to report gamma radiation-induced LPO formation in invertebrates, disruption of the integrity of membranous lipid bilayers in mammalian cells (Azzam et al., 2012) and plants (Jan et al., 2012) suggest that LPO may be a conserved MoA of gamma radiation across species.

4.3. DNA damage

The genotoxicity measured as increase in DNA of haemocytes from *D. magna* by the Comet assay suggest that gamma radiation caused significant decrease in DNA integrity, especially at the highest dose rates. Although this increase was small and variable along the dose rates tested, an overall dose rate-response relationship resembling that of ROS and LPO was observed. It's well established that radiation induced-ROS attack DNA, generating a variety of DNA lesions, such as oxidized bases and strand breaks (single and double DNA strand breaks). If not properly removed, DNA damage by direct interaction and enhanced ROS formation by radiation can accumulate to the point where it leads to mutagenesis (Maynard et al. 2009). Ionizing radiation can lead to a broad spectrum of DNA lesions (Goodhead, 1989), including increased incision in the backbone of DNA while repairing. Since the damage persisted in exposed daphnids for 48 hrs, it may suggest that induction of DNA repair capacity was not sufficiently effective to counteract the damage caused by ionizing radiation in haemolymph cells. It has been suggested that low doses of radiation may not activate DNA repair, thus leading to recovery processes being triggered only above acritical level of damage. This may result in the elimination of the damaged cells by apoptosis or mitotic death (Hayes 2008; Zaichkina et al., 2004) and possibly a selection of less damaged cells is analysed at low dose rates. Radiation-induced DNA damage has been previously reported in *D. magna* exposed to ^{137}Cs source (Parisot et al., 2015). In this case, an overall accumulation and transmission of DNA alterations was registered across three successive *D. magna* generations in a time and dose-dependent manner, at dose rates from 0.0007 to 35.4 mGy/h. These authors hypothesized that DNA repair mechanisms become efficient only after organisms receive a sufficient cumulative dose of radiation, especially under chronic exposure (Parisot et al., 2015). Dose-dependent modulation of genes such as DNA-PK, PCNA, Ku70 and Ku80, involved in DNA repair in the rotifer *B. koreanus* and the copepods *T. japonicus* and *P. nana*, suggest that exposure to ^{137}Cs (total doses from 10 to 200 Gy) also cause DNA damage in other invertebrates (Han et al., 2014a, b; Won and Lee, 2014). In the present

study, several DEGs and pathways related to DNA repair and cell cycle regulation were affected by gamma radiation, probably as a consequence of handling destabilized and damaged DNA. From the several DEGs identified herein, the up-regulation of DNA repair proteins rad 50 (*Rad50*) (42.9 and 106 mGy/h) and MRE11-like (*mre11*) (1.1, 4.3, 10.7 and 106 mGy/h), constituents of a repair complex implicated in multiple DNA repair mechanisms (Brodsky et al., 2004), confirm that daphnids repairing systems responded effectively to exposure to gamma radiation, initiating a recovery of cellular damages especially at higher dose rates. *mre11* seemed to be more responsive than *Rad50* at low dose rates, albeit inconsistencies between he microarray and qPCR data for *mre11* suggest that additional effort is required to characterize the transcription regulation of this gene in *D. magna* in response to gamma radiation.

4.4. Energy production and homeostasis

Another important cellular target of ionizing radiation and consequent ROS formation is the mitochondria. Gamma radiation has been associated with mitochondrial dysfunction in the form of mitochondria-dependent ROS formation, increased mitochondrial membrane potential and promoted respiration and ATP production (Kam and Banati, 2013; Reisz et al., 2014), processes that can lead to further propagation of ROS and oxidative stress. In the present study, several genes related to the mitochondria were differentially regulated in daphnids exposed to gamma radiation. Several DEGs involved in mitochondrial electron transport chain (ETC) were suppressed by gamma radiation, namely genes encoding NADH dehydrogenase (*Nd*) in complex I, succinate dehydrogenase subunit A (*SdhA*) in complex II, cytochrome c oxidase subunit 1 (*COX1*), cytochrome c oxidase subunit 2 (*COX2*), cytochrome c oxidase subunit 3 (*COX3*) and cytochrome c oxidase copper chaperone (*COX17*) in complex IV, and ATP synthase subunit mitochondrial (*sun*) in complex V. Only the gene encoding succinate dehydrogenase B (*SdhB*) in complex II was induced by gamma radiation (0.41, 42.9 and 106

mGy/h). No DEGs involved in ETC complex III were differentially regulated in the irradiated daphnia. The *Nd* gene was also found to be significantly down-regulated by qPCR at 4.1, 1.1, 4.3 10.7 and 42.9 mGy/h, even though the microarray analysis only showed significant suppression at the two highest dose rates used. These results suggest that gamma radiation may interfere with mitochondrial membrane function in daphnids, modulate oxidative phosphorylation (OXPHOS) and ultimately cause loss of aerobic energy supply or even cell death (Joshi and Bakowska, 2011). The reduction of mitochondrial membrane potential and associated ATP synthesis in response to gamma radiation has been documented in several mammalian and fish species (Kam and Banati, 2013, O'Dowd et al., 2006, Song et al., 2014), although the knowledge of the MoA in crustaceans is still limited.

A potential imbalance of energy homeostasis in daphnids exposed to gamma radiation was also evidenced by the enrichment of a pathway involved in the mechanistic target of rapamycin (mTOR) signaling. In vertebrate species, alterations in cellular energy balance impact mTOR signaling via AMPK, a Serine Threonine kinase consisting of a catalytic α-subunit and two regulatory subunits, β and γ (Huang and Fingar, 2014; Roux and Topisirovic, 2012). In the present study, the SNF4/AMP-activated protein kinase gamma subunit (*SNF4Agamma*) gene was induced (microarray analysis) in irradiated daphnia probably due to an alteration in the intracellular AMP/ATP ratio associated with mitochondrial dysfunction (Lippai et al., 2008). This result was the opposite of that found by qPCR, in which the *SNF4Agamma* gene was down-regulated at 4.3 and 10.7 mGy/h. The inhibition of the mTOR signaling pathway can also stimulate autophagy due to a rise in free cytosolic calcium, as well as the stimulation of the lipid mechanism (Huang and Fingar, 2014). A dysregulation of mTOR as a possible mechanism of radiotoxicity has already been reported in zebrafish embryos exposed to the same gamma source as that used in this study (Hurem et al., 2017), however its function in irradiated *D. magna* needs to be further explored.

4.5. Cell death

Apoptosis has been extensively documented in cells upon exposure to gamma radiation, normally as a consequence of oxidative stress and associated cell cycle arrest, DNA damage, impairment of DNA repair and mitochondrial dysfunction (Reisz et al., 2014). Several genes involved in the modulation of several apoptotic pathways were significantly regulated by gamma radiation. For example, the down-regulation of apoptosis-inducing factor 3 (*Aifm*, microarray: 42.9 and 106 mGy/h) and p53-regulated inhibitor of apoptosis 1 (*Triap*, qPCR: 10.7, 42.9 and 106 mGy/h), two genes involved in the modulation of the mitochondrial apoptotic pathway, is suggestive of a potential induction of apoptosis, however, not through major signaling pathways. In addition, the enrichment of pathways related to neuronal cell death was also identified in *D. magna* after exposure to 0.41 and 106 mGy/h, highlighting the onset of cognitive dysfunction in daphnids following radiation exposure. Taken together, results suggest that different apoptotic signaling pathways were regulated in daphnids in response to gamma radiation, which seems to be consistent with the identified DNA damage and repair, cell cycle disruption, mitochondrial dysfunction and neurotransmission impairment. The induction of apoptosis after exposure to the same gamma source as that used in this study has already been documented in fish, namely Atlantic salmon and zebrafish, in which the regulation of different apoptotic signaling was also highlighted in response to upstream mechanisms as for example oxidative stress and DNA damage and repair (Song et al., 2014, Hurem et al., 2017).

4.6. Ca^{2+} homeostasis and other potential mechanisms

The gene pathway analysis highlighted other potential MoA of gamma radiation in daphnids. A general activation of genes associated with Calcium signaling pathways such as Ca-dependent events, Calmodulin induced events and CaM pathway were observed in daphnids

651 exposed to 42.9 and 106 mGy/h. Calmodulin (*CaM*), the ubiquitously expressed and highly
652 conserved protein that is essential for numerous cellular processes and is the key mediator of
653 Ca^{2+} signals (Altshuler et al., 2015; Song et al., 2016), was significantly down-regulated by
654 0.41, 1.1, 4.3 and 10.7 mGy/h and up-regulated at 106 mGy/h (qPCR). Cells tightly regulate
655 their cytoplasmic calcium concentrations, as Ca^{2+} ions are used in a several concentration-
656 dependent processes, which in crustaceans can be directly related to molting, mTOR signaling
657 and intracellular calcium influx (Altshuler et al., 2015). Accordingly, these results seem to point
658 to a dose rate-dependent disruption in Ca^{2+} homeostasis by gamma radiation, which may play
659 an important role in the activation/suppression of several processes in *D. magna*, as for example
660 mitochondrial dysfunction, mTOR signaling, neurochemical signaling and endocrine
661 regulation.
662 Exposure to gamma radiation also affected the neurochemical signaling system in exposed
663 daphnids, as neuronal system-related pathways were significantly enriched at the two highest
664 dose rates used (42.9 and 106 mGy/h). Among these, pathways related to glutamate and GABA
665 signaling were identified as the most significant, as highlighted by the up-regulation of the
666 gamma-aminobutyric acid type b receptor subunit 2-like (*GABA-B-R2*) gene by both the
667 microarray (106 mGy/h) and qPCR (10.7, 42.9 and 106 mGy/h) analysis at the highest dose
668 rates used. GABA-mediated signaling has been extensively studied in crustacean species due
669 to its role in synaptic transmission and neural inhibition (Northcutt et al., 2016), as well as its
670 involvement in the regulation of cell development (Salat and Kulig, 2011). Even though no
671 studies have focused on the neurotransmitter related-effects of gamma radiation in crustaceans,
672 there is evidence that the modulation of these pathways is related to cognitive dysfunction
673 following radiation exposure in mammals (see Wu et al., 2012 and references herein).
674 Nonetheless, the molecular mechanisms underlying the up- and downstream signaling of these
675 pathways in response to gamma radiation still remain to be elucidated in *D. magna*.

Another novel finding in the present study was that multiple genes associated with the endocrine regulation of molting in *D. magna* were differentially expressed after exposure to gamma radiation. These transcriptional alterations suggest that as low as 0.41 mGy/h gamma may disrupt molting signaling by inhibiting the synthesis of ecdysteroids, thus potentially leading to suppressed transcriptional regulation of molting through the EcR. Inhibition of ecdysteroid synthesis may be attributed by increased intracellular calcium influx, which has been shown to suppress ecdysteroid synthesis in crustaceans (Chang and Mykles, 2011). On the contrary, high dose-rate of gamma potentially induced the expression of cuticle proteins, which are necessary for the generation of new exoskeletons in *D. magna* (Song et al., 2017). Two examples of the effects of gamma radiation in daphnids exoskeleton is the significant induction of genes encoding for the cuticle protein 5a (*Cut5a*) and chitinase 3 (*Cht3*) at both low and high dose rates, as shown by both the microarray and qPCR analysis. However, whether these molecular responses can lead to impaired molting at the organismal still needs to be verified.

5. Conclusions

The present study showed that acute exposure to gamma radiation resulted in significant alterations at the cellular and molecular level in the crustacean *D. magna*. Results showed a significant dose and time-dependent increase in ROS formation in daphnids, which is consistent with the MoA of gamma radiation in cells. Moreover, the LPO and DNA damage observed in gamma-irradiated daphnids showed dose rate and cumulative dose and time dependent effects, which seems to be connected not only to oxidative stress, but also to radiolysis mechanisms. Transcriptional analysis further highlighted oxidative stress as one of the main MoA of gamma radiation, especially at high dose rates, suggesting a strong causal relationship between cellular and molecular disturbances upon gamma radiation exposure. This include the induction of oxidative damages to DNA and lipids through excessive ROS formation, as well as causing

mitochondrial ETC dysfunctions and cellular energy imbalance, possibly through direct damage to the mitochondrial membranes by ROS and/or as a result of potentially increased calcium influx to the mitochondria. Additional toxicological relevant MoAs were evidenced by microarray analysis, further suggesting that downstream responses such as antioxidant defense, cell cycle regulation and DNA repair, apoptotic cell death, abnormal neurotransmission and disruption of molting signaling may also be affected. However, since no adverse effects were observed due to the short exposure duration, whether these were adaptive (compensatory) responses or toxicity pathways leading to adversity still need to be investigated. Further assessment using relevant functional endpoints are also necessary to help understand the mechanistic link between these molecular alterations and organism level responses. In addition, it still remains to be verified if the alterations observed are also relevant at lower dose rates, including a purported low dose-rate effect at 0.41 mGy/h, and if the dose rates used in this study are sufficient to induce cumulative effects in daphnids at longer and more environmentally relevant exposure durations, as well as over a range of successive generations. Overall, the results obtained allowed the identification of a suite of biomarker genes associated with several biological mechanisms that could be used in future evaluation of toxicity and MoA of ionizing radiation in *D. magna*. Accordingly, based on both functional and transcriptional responses observed in irradiated *D. magna*, several putative MoAs for gamma radiation are thus proposed (Figure 6).

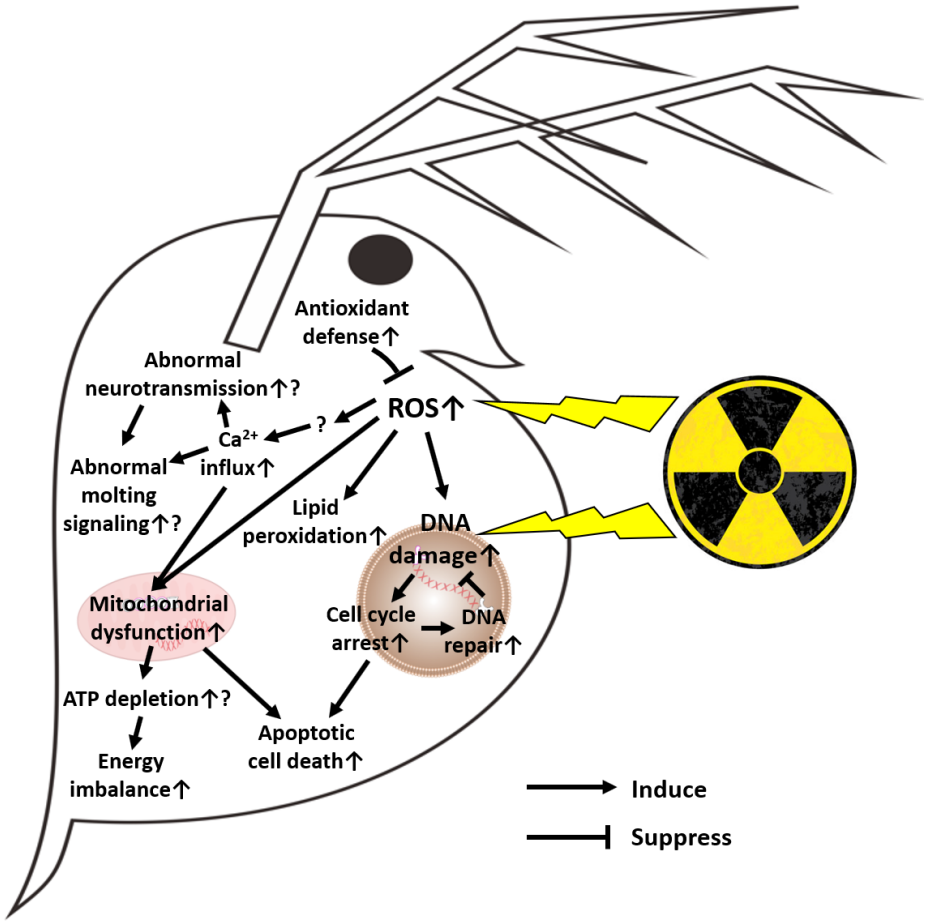


Figure 6 – Putative toxicity mechanisms of gamma radiation in *Daphnia magna*.

Conflict of interest

The authors declare the inexistence of any conflict of interest.

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