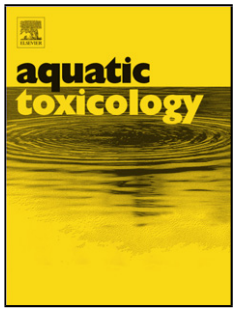

Title: RIBE at an inter-organismic level: a study on genotoxic effects in *Daphnia magna* exposed to waterborne uranium and a uranium mine effluent

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RIBE at an inter-organismic level: a study on genotoxic effects in *Daphnia magna* exposed to waterborne uranium and a uranium mine effluent

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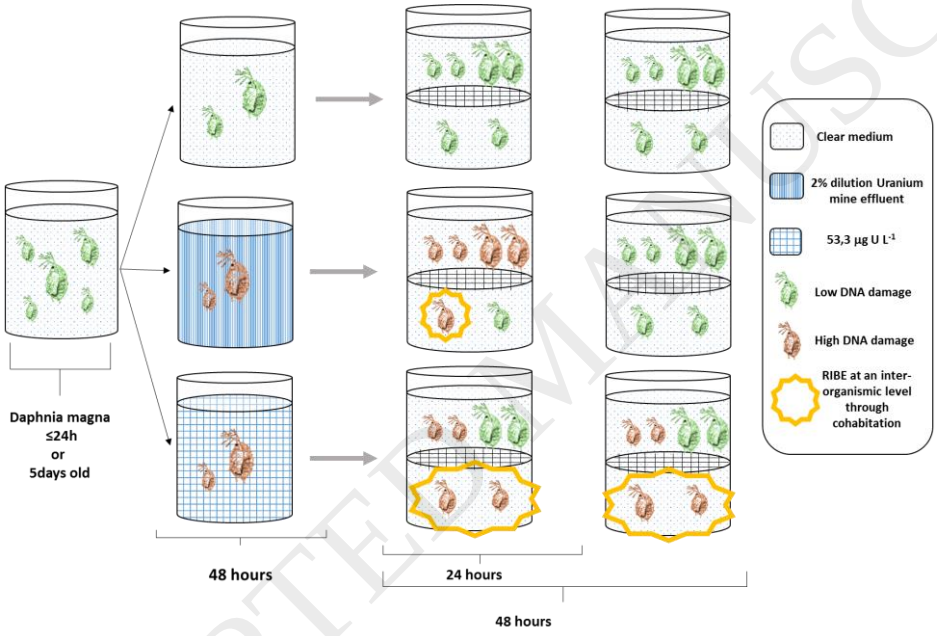
Graphical Abstract:



Highlights:

- D. magna was exposed to waterborne Uranium and a mine effluent to assess RIBE
- Assess if inter-organismic RIBE plays a role in environmental risk assessment
- The uranium mine effluent caused a less pronounced RIBE effect
- RIBE depends on the age of exposed organisms and on cohabitation duration
- Recovery of DNA damages was observed in exposed and non-exposed organism

Abstract

The induction of RIBE (Radiation Induced Bystander Effect) is a non-target effect of low radiation doses that has already been verified at an inter-organismic level in fish and small mammals. Although the theoretical impact in the field of environmental risk assessment (ERA) is possible, there is a gap of knowledge regarding this phenomenon in invertebrate groups and following environmentally relevant exposures. To understand if RIBE should be considered for ERA of radionuclide-rich wastewaters, we exposed *Daphnia magna* (<24h and 5d old) to a 2% diluted uranium mine effluent for 48h, and to a matching dose of waterborne uranium (55.3 μg L^{-1}). Then the exposed organisms were placed (24 and 48h) in a clean medium together with non-exposed neonates. The DNA damage observed for the non-exposed organisms was statistically significant after the 24h cohabitation for both uranium (neonates p=0.002; 5d-old daphnids p=<0.001) and uranium mine effluent exposure (only for neonates p=0.042). After 48h cohabitation significant results were obtained only for uranium exposure (neonates p=0.017; 5d-old daphnids p=0.013). Although there may be some variability associated to age and exposure duration, the significant DNA damage detected in non-exposed organisms clearly reveals the occurrence of RIBE in Daphnia. The data obtained and here presented are a valuable contribution for the discussion about the relevance of RIBE for environmental risk assessment.

Keywords: Bystander effect, Daphnia magna; Uranium mine effluent; Environmental risk assessment; Radiobiology;

1. Introduction

Ionizing radiation (IR) is the energy released in the form of particles or rays from radioactive materials, which has ionizing capacity (Agency for Toxic Substances and Disease Registry, 1999). Therefore, by interacting with molecules it has the ability of expelling electrons from their atoms (Agency for Toxic Substances and Disease Registry, 1999). Concerns with exposures to IR are mainly linked with nuclear tests, nuclear power plant accidents (Chernobyl and Fukushima) or with diagnostic or radiation medical treatments. However, most radiation exposures are low dose/low dose rate exposures that can come from many sources as cosmic radiation, soil background radiation, normal activity of nuclear power plants and hazardous wastes/wastewaters that are mainly discarded in dumping sites and/or waterbodies (Agency for Toxic Substances and Disease Registry, 1999).

For a better understanding of the problem of environmental discharges of uranium mining activities, we may look, for example, to the deactivated uranium mines located in the Central region of Portugal. Near these sites, significant concentrations of uranium as well as other contaminants are still being detected in wastewaters, which might be due to the uprising of aquifers or by the precipitation on solid wastes, even after chemical treatment (e.g. 1380 μg U L-1 (Bessa et al., 2016); 3143 μg U L-1 (Lourenço et al., 2017)). These effluents are intermittently discharged on the nearby watercourses where, despite underlying dilution, they may still affect the aquatic ecosystems (Antunes et al., 2007; Marques et al., 2011, 2009).

When IR interacts with cells it can damage DNA, but also proteins and lipids. It can affect gene expression, mitochondrial processes, DNA repair mechanisms, induce apoptosis and form free radicals (Criswell et al., 2003; Jeggo and Löbrich, 2006; Nikitaki et al., 2016; Rodemann and Blaese, 2007). Regarding genotoxic effects, these are mainly due to the ability of IR to promote single and/or double strand breaks, and loss of bases on the DNA molecule (Little, 2000).

For many years, it has been though that all the above-mentioned damages were induced only in irradiated cells. However, this erstwhile dogma, has been challenged since Nagasawa and Little (Nagasawa and Little, 1992), studying sister chromatid exchanges in irradiated Chinese hamster ovary cells, reported that low doses of α-radiation may induce genetic damages in the cell nuclei of non-irradiated cells. Since then, hundreds of studies demonstrated that there are similar injury responses in neighbouring cells, not targeted by IR, as in those directly exposed (Hei et al., 2008; Little, 2006; Unscear, 2006). These out-of-field effects, were named non-targeted-effects (NTE) and encompass the

well-established radiation-induced genomic instability (RIGI) and radiation-induced bystander effect (RIBE) (Choi and Yu, 2015). In this work we focused on RIBE.

RIBE represents a one-way stress communication, where non-irradiated cells/organisms exhibit responses that are caused by signals sent by nearby irradiated cells (Mothersill et al., 2006). *In vitro* studies indicated bystander effects as an ubiquitous consequence of radiation exposure, which may or may not be harmful to the non-irradiated cells (Mothersill and Seymour, 2004; Unscear, 2006). The plethora of *in vitro* studies concluded that the mechanisms underlying RIBE encompass both the transmission of signals through gap-junction intercellular communication (Autsavapromporn et al., 2013; Harada et al., 2009), and the release of soluble molecules/factors into the medium by the irradiated cells (Mothersill et al., 2001).

There are several factors that may influence the occurrence of RIBE, such as the irradiation itself (dose, dose rate, dose fractionation), the type of cells and tissues irradiated and also the biological endpoints evaluated (Bahreyni Toossi et al., 2017). However, until now we were not able to understand the influence of these factors in the induction of RIBE (Bahreyni Toossi et al., 2017). The responses in bystander cells vary and encompass injuries such as cell death, DNA damage and neoplastic transformations, but can also benefit the bystander population by inducing radio-adaptive responses (RAR) and hormesis (Yum et al., 2010).

However, such phenomenon does not necessarily occur only at the cellular level, and *in vivo* assays have reported that bystander effects also occur at tissue and organism levels (Belyakov et al., 2005; Koturbash et al., 2006). In the last decade it has been reported that RIBE can also occur at an inter-organismic level; i.e., damage responses were detected in non-irradiated organisms that were housed together or shared the same medium as organisms previously exposed to low radiation doses (Mothersill et al., 2006; Surinov et al., 2005; Yum et al., 2009). However, the species used in those studies were exclusively vertebrates. All these studies supported the idea that bystander signals are waterborne soluble stable molecules (Liu et al., 2006; Mothersill et al., 2007). Results became even more ecologically relevant when it was reported that RIBE can be communicated not only through a waterborne route between organisms of different species, but also through diet, namely by ingestion of irradiated *Lumbriculus variegatus* by rainbow trout (Smith et al., 2013).

In addition to the above-mentioned studies, there is an extreme paucity of data regarding this phenomenon in invertebrates and at low doses of high LET radiation. Only few studies reported bystander effects induced by alpha particles. Those who did, were

performed on zebrafish embryos (Choi et al., 2012; Yum et al., 2009). Radio-adaptive responses were reported in bullfrog tadpoles (*Rana catesbeiana*) housed for one week with tadpoles which were previously exposed to tritiated water (Audette-Stuart and Yankovich, 2011). Despite all the studies carried out far, the Committee of US government on the Biological Effects of Ionizing Radiation in its last report on low levels of IR (Council, 2006) concluded that it was too early to assess whether non targeted effects of IR, including bystander effects, had any relevance for risk assessment. The reason for this may be the scarcity of *in vivo* studies, and also of studies addressing the relevance of these effects in more realistic environmental scenarios.

All these studies have made us realize the true knowledge gap regarding the induction of this phenomenon by low doses of natural α-emitting radionuclides and complex naturally ooccurring mixtures containing radionuclides and other metals or stressors (with the exceptions of co-exposures to IR and copper and aluminum (Mothersill et al., 2014; Olsvik et al., 2010; Salbu et al., 2008; Smith et al., 2015)). Therefore, we consider that it is of high relevance to study RIBE not only under exposure to high LET-emitting radioisotopes such as uranium, but also to complex uranium mine effluents. This will give a great contribution to the environmental risk assessment of radioactive wastes and wastewaters, which often reach freshwater resources, through intermittent point discharges.

Considering all that has been referred to so far, this study intends to answer the following questions: 1) Can RIBE at inter-organismic level be detected in *Daphnia magna*? 2) Does time of cohabitation and age of irradiated organisms modulate RIBE in *D. magna*? 3) Does bystander effects between organisms change the current paradigm used in predicting the risks of radionuclide-rich wastewaters? Furthermore, the following questions will also be addressed: a) Is the DNA damage induced by exposure to both uranium/uranium mine effluent repaired during the period of cohabitation in clean medium? b) Does the age at which the organisms are exposed influence the repair of DNA damage?

These questions were answered in an experiment with *D. magna*. Daphnids of two different ages (less than 24h and 5d old) were exposed to low doses of waterborne uranium and to uranium mine effluent. Then the organisms were transferred to clean medium and were allowed to cohabit with non-exposed organisms (age of less than 24h). The genotoxic effects of IR were assessed through alkaline comet assay. This technique proved to be consistent with other methods available to evaluate DNA damage in several

freshwater organisms exposed to genotoxicants (Lee and Steinert, 2003) and it was successfully used in bystander assays (Przybyszewski et al., 2004).

D. magna is a model species in aquatic ecotoxicology and risk assessment. Moreover, cladocerans in general and this species in particular are very important in freshwater food webs and there are not many studies addressing bystander effects in aquatic invertebrates. Furthermore, since chemical signaling in this species is documented (Stabell et al., 2003), it was expected that bystander signals could also be exchanged and perceived between exposed and non-exposed daphnids.

2. Material and methods

2.1. Water sampling and preparation of uranium dilutions for the test

Mine water samples were directly collected from the pond in the open mine pit of the Quinta do Bispo uranium mine (Portugal) into polyethylene bottles, and immediately frozen and kept at −20 °C. Waterborne uranium solutions were prepared using distilled water and uranium, as uranyl nitrate hexahydrate (UO2(NO3)2.6H2O) was obtained from Panreac.

2.2.Culture conditions

D. magna is maintained in our laboratory in 800mL flasks at a density of 30 individuals per bottle in ASTM artificial freshwater medium (ASTM Standard, 2007), at 20ºC (±1ºC) with a natural photoperiod (≈16L:8D). Every two days the medium is renewed and the animals fed with green algae *Raphidocelis subcapitata* ($3x10^5$ cells/mL) and supplemented with an organic seaweed extract of *Ascophyllum nodosum* (Algea Fert Solid).

The health status of the cultures was previously confirmed, as the OECD criteria on survival (less than 20% mortality at the end of 21days) and reproduction rates (> 60 living offspring produced per parent animal surviving at the end of 21days (OECD, 2004)) were fullfilled. All the experimental assays started with newly released neonates (less than 24h) from the third brood.

2.3. Experimental design

In a previous study of our research group (Reis et al., submitted) it was recorded that a concentration of 55.3 μg U L^{-1}, a 2% dilution of an uranium mine effluent with ASTM hardwater medium and an exposure time of 48h caused significant DNA damages in *D. magna*. Thus, these were the concentrations and the exposure time considered appropriate for this study.

A schematic representation of the experimental design (Parts A and B) is illustrated in Fig.1.

Part A) – Sixty neonates, with age less than 24h, were exposed for 48h to the previously defined concentrations of uranium (u), uranium mine effluent (e) and also to clean ASTM medium (c-negative control). At the end of the exposure DNA integrity was analyzed in 20 randomly selected exposed organisms. The remaining organisms were washed in clean medium for 5 minutes, transferred to flasks containing clean ASTM medium and left to cohabit with neonates (less than 24h old) for 24 and 48h at a density of 2:1. The flasks were specifically prepared for this experiment (Fig.1 in Appendix). Each flask consisted of two continuous compartments separated horizontally by a nylon net (170 μm mesh openings). Three replicates for each cohabitation condition were performed, each one containing 40 previously exposed daphnids on the top and 20 unexposed daphnids at the bottom part of the flask. At all steps, daphnids were kept in starvation and at a density of 2.5 mL of medium *per* daphnia *per* day, fulfilling the criteria of the OCDE protocol (above mentioned) for acute chemical tests in *D. magna* (OECD, 2004). After 24 and 48h of cohabitation, DNA damage was evaluated in exposed and bystander organisms.

Part B) The same approach of the experimental design described in part A was followed, but instead five days old daphnids were used.

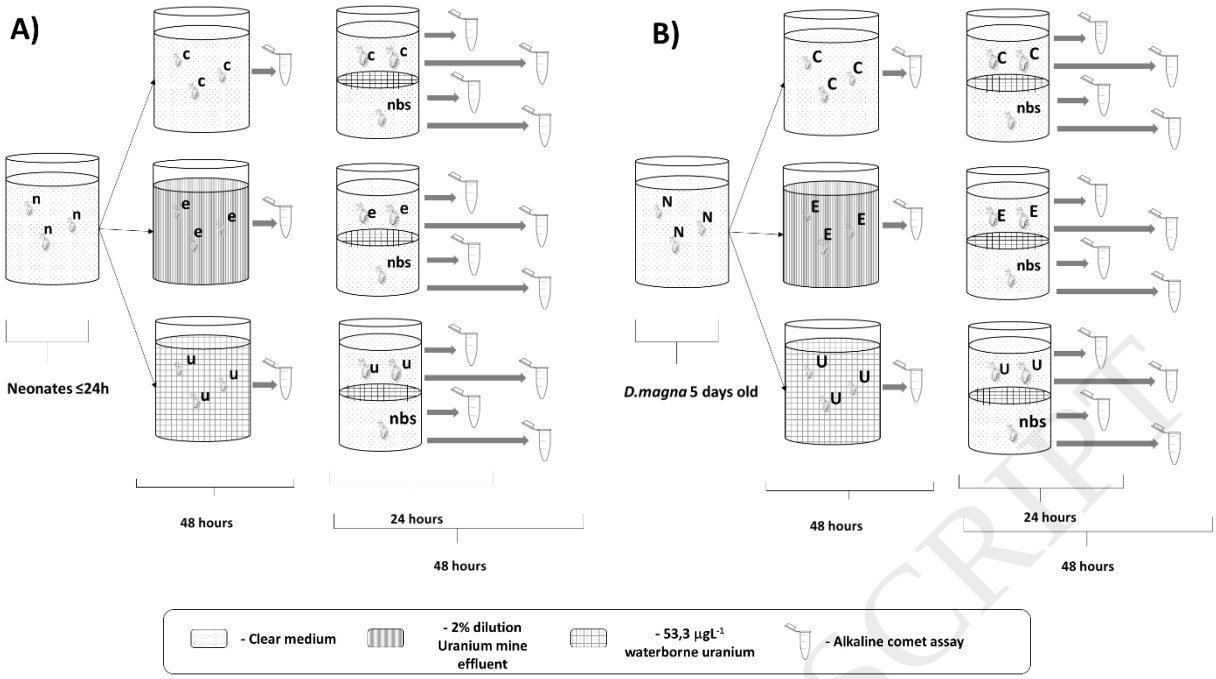


Fig.1- Schematic representation of the experimental design (part A and B). **n** - newly released neonates (less than 24h old); **c** – Control - daphnids exposed to clean ASTM medium for 48h; **e** – daphnids exposed to a 2% dilution of a uranium mine effluent for 48h; **u** – daphnids exposed to waterborne uranium at a concentration of 55.3 μg U L^{-1} for 48hours;); **nbs** - bystander neonates (less than 24h old) **N** – *D. magna* five days old; **C** – Control - 5 days old daphnids exposed to clean ASTM medium for 48h; **E** – 5 days old daphnids exposed to a 2% dilution of a uranium mine effluent for 48hours; **U** – 5 days old daphnids exposed to waterborne uranium at a concentration of 55.3 μg U L^{-1} for 48h.

2.4. DNA damage evaluation

DNA damage was evaluated through alkaline comet assay. Three replicates were used for each treatment (e, c and u after exposure, before and after co-habitation for both exposed and bystander organisms as well as bystander neonates (nbs). Capital letters were used to distinguish organisms of part B of the experiment, each one containing a pool of twenty neonates/three 5d old daphnids. The organisms were placed in a 1.5 mL microtube containing 800 μL of a solution consisting of phosphate-buffered saline (PBS), 10% (v/v) dimethyl sulfoxide (DMSO) and 20 μM ethylene diamine tetra-acetic acid disodium salt (Na_2EDTA). Organisms were gently macerated with a pestle to release the cells. Microtubes were centrifuged at 200g for 10min at 4ºC and the supernatant almost completely removed leaving about 50 μL to resuspend the pellet. Then, 15 μL of the cell suspension was mixed with low melting point agarose (0.5% (w/v)) at 37ºC and placed on top of pre-coated slides (1% normal melting point agarose). The slides were placed in lysing solution (2.5 M NaCl + 100 mM EDTA + 10 mM Tris–HCl + 1% DMSO + 10% TritonX-100) for at least one hour at 4ºC, protected from the light. After lysis, slides were subjected to denaturation in alkaline buffer (0.3M NaOH and 1mM EDTA, pH 13) for 15 min and to electrophoresis for 10 min at 0.7 V/cm, 300 mA. Slides were neutralized in

Tris-HCL (0.4 M), and then submerged for a few seconds in absolute ethanol, left to dry for at least 24h and stored in the dark until observation. The assay was conducted under yellow light to prevent UV-induced DNA damage.

Before observation, slides were stained with 100 μL of ethidium bromide (20 μg L^{-1}) and scored using a fluorescent microscope (amplification 400X). To avoid bias, the observations were blind, i.e., scored without any previous knowledge of the origin of the slide, and always by the same person, and proceeded as follows: Visual scoring of cellular DNA on each slide was based on the categorization of 100 cells randomly selected. The comet like formations were visually graded into 5 classes (Fig.2), depending on DNA damage, and scored as described by García (García et al., 2004), where class 0: no visible DNA damage, i.e., compact DNA in the cell nucleus, and class 4: significant loss of DNA integrity, i.e., indistinguishable cell nucleus.

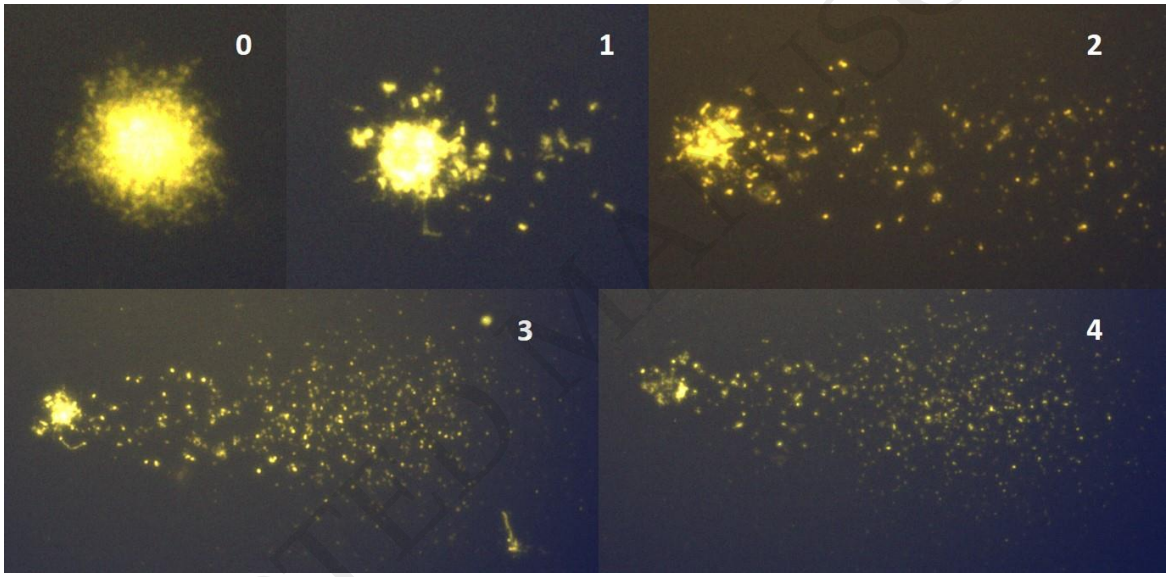


Fig.2- Alkaline comet assay: visual scoring of DNA damage in *Daphnia magna*, from 0 to 4 according to comet appearance. (Amplification: 400X)

2.5. Chemical analysis of the effluent

2.5.1. Determination of radionuclides and trace metals

The method of analysis used in the measurement of radionuclides and trace metals in the selected effluent is described by Lourenço et al. (Lourenço et al., 2017).

2.5.2. Estimation of radiation dose exposure

Estimates of radiation doses were calculated using the RESRAD-BIOTA software, version 1.5. Dose estimates were based on the activity of each radionuclide individually present in the 2% effluent dilution and the sum of all radionuclides.

2.6. Statistical analyses

The statistical analysis was performed using one-way ANOVA with Holm-Sidak and Tukey's all-pair comparison tests as post hoc comparison tests. Two levels of significance were assessed: $p \leq 0.05$ and $p \leq 0.01$. All the statistical analyses were performed with IBM SPSS Statistics v17.0 software. Table S1 of annex displays all the statistical results of this study.

3. Results

3.1. Effluent characterization

The chemical analyzes of the effluent revealed a complex mixture with a plethora of metals and radionuclides (Tab.1). Some of the metals (e.g. Al and Mn) were detected in concentrations above the limit values in wastewater discharges, according to the Portuguese Law by Decree 236/98 (Ministério do Ambiente, 1998). Some of the radionuclides values were so high that they exceeded the national legislation on water for human consumption (Law by Decree 23/2016) (Ministério do Ambiente, 2016) by more than ten and three times (^{238}U, ^{234}U and ^{210}Po, ^{226}Ra, respectively). Although we consider that this standard is overprotective, in Portugal there are no legal limits specified for radionuclides, except for water intended for human consumption.

Table 1- Chemical characterization of uranium mine effluent from Quinta do Bispo (Mangualde, Portugal). Error bars within the table stand for measurement error.

Metals	In solution (μg/L)	Particulate (μg/g)	**Radionuclides**	In solution (mBq/L)	Particulate (Bq/Kg)
Be	110 ± 10	50.1 ± 5	**^{238}U**	37000 ± 2000	1852 ± 71
Cr	< L.D.	---	**^{235}U**	1700 ± 100	71 ± 5
Mn	7230 ± 720	---	**^{234}U**	31000 ± 2000	1769 ± 68
Co	330 ± 30	<L.D.	**^{230}Th**	102 ± 7	380 ± 31
Ni	540 ± 50	50.1 ± 5	**^{226}Ra**	1580 ±90	2185 ± 107
Cu	100 ± 10	68.2	**^{210}Po**	290 ± 20	3955 ± 218
Zn	1040 ± 100	3.63 ± 0.36	**^{232}Th**	2.3 ± 0.4	22 ± 4
Se	---	9.65 ± 0.97			
Sr	220 ± 20	2.13 ± 0.21			
Cd	< L.D.	9.02 ± 0.9			
Ba	20	---			
Pb	10	4 ± 0.4			
Fe	1790 ± 180	<L.D.			
Al	15800 ± 1600	12 ± 1.2			
U	2180 ± 220	3.31 ± 0.33			

3.2. Estimated radiation doses

The estimated radiation doses received by the daphnids exposed to a 2% dilution of the uranium mine effluent (Tab.2), was below the dose limit of ICRP's DCRL value for crustaceans (10 - 100 mGy/d) (ICRP, 2008).

Table 2- Dose estimates (Gy·d^{-1}) received by neonates of *D. magna* exposed to 2% dilution of the uranium mine effluent. Radiation doses provided by each radionuclide and by the mixture (sum of all individual doses) are shown.

Radionuclides	**^{238}U**	**^{235}U**	**^{234}U**	**^{230}Th**	**^{226}Ra**	**^{210}Po**	**^{232}Th**
Gy·d^{-1} discriminated	8.78E-04	4.15E-05	8.34E-04	2.10E-07	9.49E-04	1.04E-05	6.28E-06
Gy·d^{-1} total				2.72E-03			

3.3. Radiation Induced Bystander Effect (RIBE) – part A

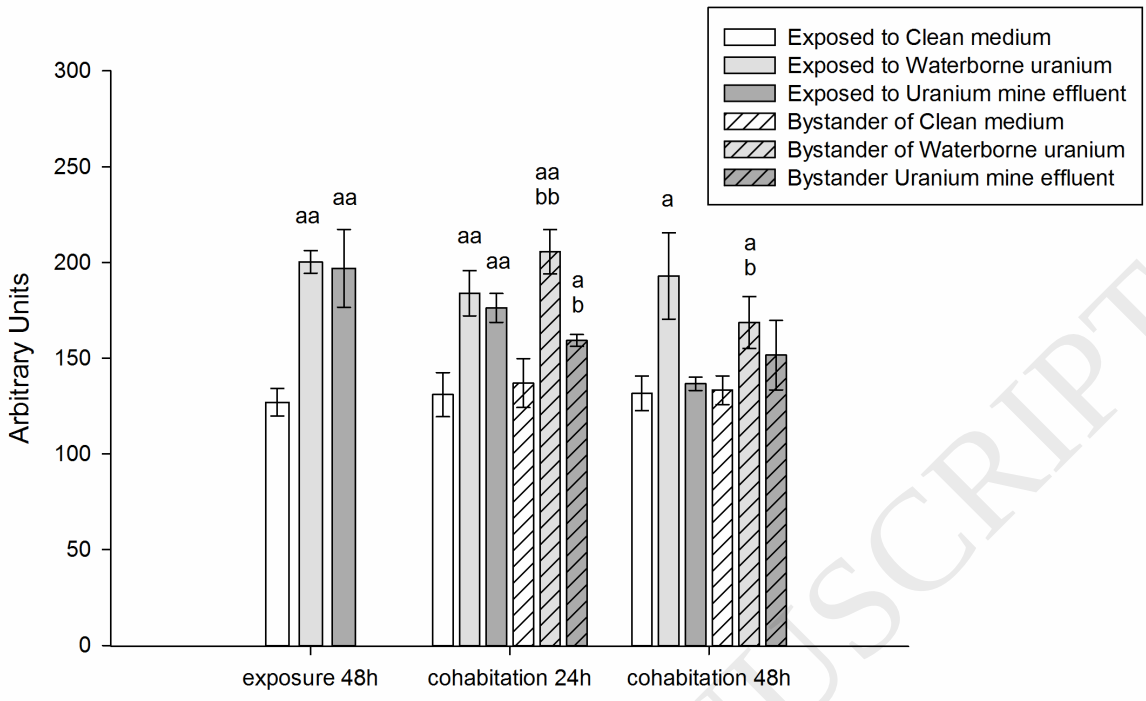


Fig.3 - Weighted average of the DNA damage (arbitrary units) in part A experiment. Letters indicate significant differences among treatments: One lowercase- p≤0.05; two lowercases- p≤0.01. Error bars represent standard deviation.

Results of the comet assay of part A experiment are shown in Fig.3. The absence of mortality in all conditions, the basal damage in all controls and the significant level of DNA damage recorded in both 48h exposures to waterborne uranium and uranium mine effluent, was in agreement with previous data obtained by our research group (Reis et al., submitted). Thus, this allowed us to validate the test.

Regarding the organisms exposed to waterborne uranium (**u** in Fig.1) no DNA damage recovery was observed after 24 or 48h in clean medium during cohabitation. In addition, the DNA damage detected by the comet assay remained significantly increased when compared to the control c (Fig. 1) (p=0.005 and p=0.012, for 24h and 48h, respectively) (Fig.3). Yet, in the organisms exposed to uranium mine effluent (**e** in Fig.1), the scenario was different. After 48h in clean medium, there was a recovery of DNA damage, as revealed by the increase in DNA integrity to levels similar (p=0.421) to those of the control and it differed significantly from that of the same organisms immediately after the 48h exposure (p=0.005) (Fig. 3).

As for the bystander organisms (**nbs** in fig.1): there was a clear induction of DNA damage in the organisms that cohabitated with organisms exposed to waterborne uranium (**u** in Fig.1), i.e., the so-called RIBE (**b** in Fig.3). This DNA damage response in

bystander organisms reached its maximum after 24h of cohabitation (p=0.002), and it was slightly attenuated after 48h (p=0.017). In the uranium mine effluent bystander organisms, the RIBE was not so evident as in organisms exposed to waterborne uranium. However, there was a significant induction of DNA damage after 24h of cohabitation (p=0.042), but no significant damages were recorded after 48h.

3.4. Radiation Induced Bystander Effect (RIBE) – part B

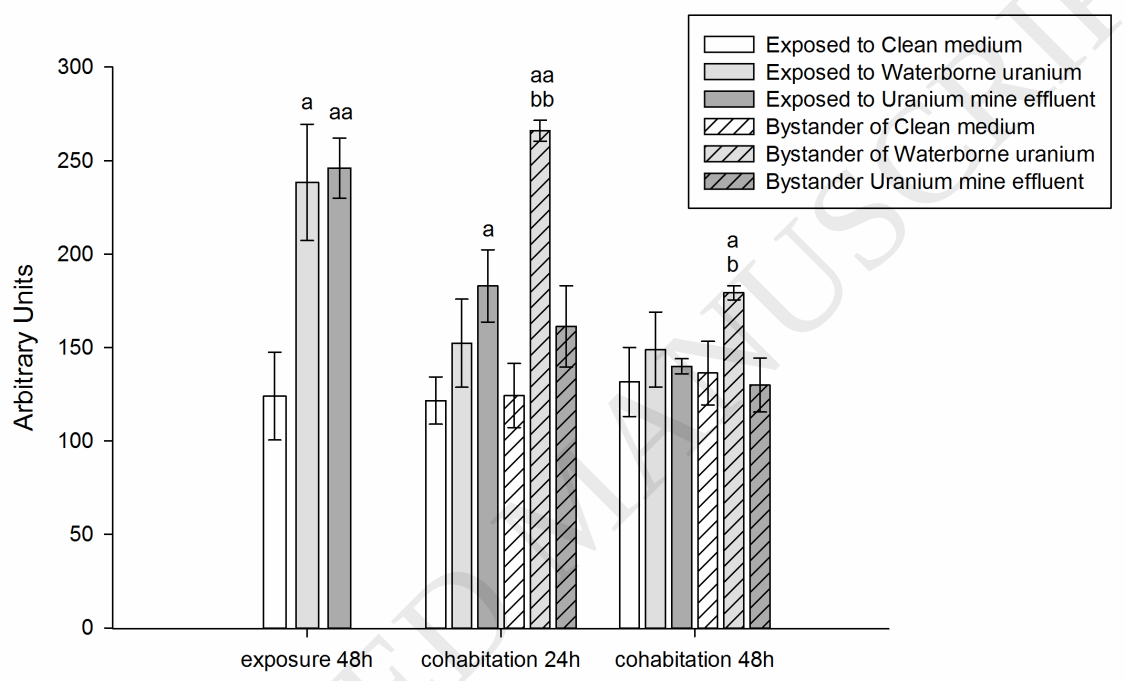


Fig.4 - Weighted average of the DNA damage (arbitrary units) in part B of experimental design. Letters indicate significant differences among treatments: One lowercase- p≤0.05; two lowercases- p≤0.01 Error bars represent standard deviation.

The assay performed with 5d old daphnids (Part B) (Fig.4) produced similar results as the one with neonates, yet some differences were detected. For instance, in the organisms exposed to waterborne uranium a more pronounced bystander response was observed, mainly in the first 24h of cohabitation (p=<0.001). In addition, and unlike the exposed neonates (with less than 24h) from part A, there was a clear recovery of DNA damage. After 24h in clean medium, the DNA damage of exposed organisms returned to levels similar to those of the control (p=0.118) (Fig.4).

With regard to the induction of genotoxic effects in bystander organisms exposed to uranium mine effluent, there was a clear difference from Part A, since despite the

increase in DNA damage, the high standard deviation does not allow to statistically confirm the occurrence of a bystander effect (p=0.082 for 24h of cohabitation) (Fig.4).

It is also interesting to note that, especially after 48h of exposure to the effluent, the level of genotoxic damage observed was higher in 5d old daphnids than in newly released neonates (p=0.031).

4. Discussion

One of the main questions that we wanted to answer with this study was: *"Can RIBE at inter-organism-level be detected in Daphnia magna?"* The data gathered and herein presented showed clearly the induction of RIBE in neonates of *D. magna* housed together with previously exposed daphnids. This was true for the exposure to both waterborne uranium and to uranium mine effluent. Despite the differences observed in the bystander effect in the two exposure scenarios, it is clear that this work contributes to a better understanding of RIBE at the inter-organism level. Furthermore, this study becomes even more relevant because it assesses effects in key species that plays an important role in freshwater food chains as primary consumers and in a environmentally relevant scenario. Focusing on RIBE at an inter-organismic level, our results were in accordance with other studies published and available in the literature on chemo-signaling in *D. magna* (Alekseev and Kazantseva, 2016; Stabell et al., 2003) and also previous evidence of this phenomenon in other species. For example, Surinov et al., (Surinov et al., 2005) reported that after the exposure to volatile compounds of the urine of rats irradiated with low doses of X-rays, the non-exposed animals exhibited the same injury responses (decreased thymus-dependent humoral immune response) as those that were exposed. RIBE between species of fish, e.g. rainbow trout (*Oncorhynchus mykiss*) (Liu et al., 2006; Mothersill et al., 2010), zebrafish (*Danio rerio*) (Mothersill et al., 2007) and medaka (*Orzias latipes*) (Mothersill et al., 2009) were also detected when non-exposed fish were housed together or left to swim in medium previously used by organisms irradiated with low doses of X-rays (0.5 Gy). Although this study did not intend to investigate the signals/mechanisms underlying RIBE between organisms, our results provide some more evidence that water is likely the conducting element of the chemical signal(s) responsible for the induction of the bystander effect, meaning that the signal may be water-soluble.

Genotoxicity is known to be the most sensitive endpoint in crustaceans exposed to radioactive compounds (Fuller et al., 2015) and it was already observed in *D. magna* exposed to concentrations as low as 22.2 $\mu g\ L^{-1}$ U (Plaire et al., 2013). Therefore, in this study, it was expected that a significant level of DNA damage would be recorded after 48h of exposure of daphnids of both ages. It should be noted that significant levels of DNA damage were observed after exposure to a very high dilution of the effluent. Also, the daily value of radiation to which daphnids were exposed (2.72E-03 $Gy \cdot d^{-1}$) was below the limit ICRP DCRL value for crustaceans (10 - 100 mGy/d) (ICRP, 2008). Nonetheless, Lourenço et al. (Lourenço et al., 2017), showed that in a scenario of multiple stressors, genotoxic effects may occur even if the organisms are exposed to radiation doses below the predicted biological risk limit. The results herein presented also suggest that, at least when exposed to the effluent, neonates evidenced less DNA damage. This may be due to the fact that 5d old daphnids take up more radioactive metal from the medium (Vanni and Lampert, 1992) than 24h neonates being, therefore, more exposed to stressors. When exposed to the effluent, age-related damage to organisms may also be related to metal stressors. That was also observed by Hoang and Klaine (Hoang and Klaine, 2007) in a study addressing the age-sensitivity of *D.magna* (age range: 3h to 10d old) to some metals (Cu, Zn, Se and As). In this study, the authors reported a peak of sensitivity in 3-4 days old organisms. Despite age-related differences, it should be noticed that for the same life stage, after 48h of exposure, both uranium and the effluent triggered a similar response in daphnids. In a previous study with Atlantic salmon (*Salmo salar*) similar levels of genotoxic damage were observed when the organisms were exposed to low levels of ionizing radiation (IR) and were co-exposed to IR and aluminum (which is the most abundant metal in our effluent) (Salbu et al., 2008).

Regarding the ability to repair DNA damage, after being exposed to waterborne uranium and placed in clean medium, neonates were unable to repair damage, which contrasted with the significant recovery observed in 5d old organisms. We hypothesize that the complexity of the damages resulting from the chemical and radiological action of uranium, coupled with different rates of cell proliferation are responsible for these discrepancies. Neonates have a faster growth rate and a higher metabolic activity, which gives less time for cells to repair damage. These differences related to the age of exposed organisms are in agreement with those reported by David et al. (David et al., 2011). These authors observed the response of two life-stages of D. *magna* (adults and neonates) to genotoxicants and recorded a higher number of transcripts encoding genes involved in the response to DNA damage, in adults. That could be reflected in a higher

level of genotoxic damage, but also in adult's greater capacity to respond and repair damage, as more mRNA for DNA repair genes was detected at this life stage.

Unlike the observed age-related differences in DNA damage recovery, already discussed for waterborne uranium exposure, in the organisms exposed to the uranium mine effluent, both states of maturity, presented high efficiency of DNA repair, which may be partly explained by the presence of some metal ions in the effluent, which are important modulators of biological responses and act as important cofactors in DNA repair mechanisms (Anastassopoulou and Theophanides, 1995). Another explanation may be the lower bioavailability of metals and radionuclides, or the lower sensitivity of daphnids to toxicants due to the physico-chemical properties of the effluent. At high hardness levels the responses of organisms to uranium may be reduced, because the uranyl ions compete with calcium and magnesium for binding sites at the cell surface (Markich, 2002). However, given the complexity of the effluent, it is difficult to claim that the higher ability to repair DNA, observed in organisms exposed to the effluent was only due to a single chemical propriety, such as hardness (the ASTM medium used to dilute uranium is also a hard-medium and, on the other hand, the effluent was highly diluted). As so, we can only say that due to its complexity it is not possible to predict the synergistic and, above all, the antagonistic effects that may occur between contaminants.

As regards the recovery from genotoxic damage, one aspect should be referred taking into account the method used in this study. Comet assay allows the measurement of breaks in the DNA molecule and not mutations that could have occurred during the repair process. As so, future works on this subject, should be complemented with different methods of genotoxicity assessment such as HRM (High Resolution Melt) or RFLP (Restriction Fragment Length Polymorphism) analyses, once the DNA integrity evaluation does not guarantee that there are no mutations. Only mutations can be transmitted to offspring and have long-term effects on populations.

In view of the above we are in a position to respond to questions a and b, raised in the introduction section. Accordingly, a) Yes; for both waterborne uranium and uranium mine effluent, the exposed organisms repaired damages during cohabitation in clean medium, however recovery was more pronounced in the effluent. b) Yes; at least in waterborne uranium exposure, the recovery was age-dependent (neonates were not able to repair DNA damage so effectively).

The present study demonstrated that low doses of radiation from natural α-emitting radionuclides such as uranium are able to induce genotoxic effects in non-exposed organisms as a bystander effect. In all waterborne uranium treatments a genotoxic

bystander response in neonates was observed. However, the magnitude of the response differed, especially between times of cohabitation. In both experiments (A and B) the level of DNA damage in bystander organisms was significant after 24h of cohabitation, but decreased after 48h of cohabitation. There are several aspects that could interfere with these observations: (a) the chemical signal is not stable and starts to degrade with time; (b) the emission of the bystander signal ends as exposed organisms are able to repair damage; (c) bystander organisms quickly repair the DNA damage that result from cohabitation with exposed organisms. It is worth referring that Mothersill et al (Mothersill et al., 2007), in a study with *Oncorhynchus mykiss* irradiated with low doses of X-ray, observed that 6h after irradiation, the bystander signals emitted by irradiated fish lost the strength/ability to induce a response in partner rainbow trout. Regarding the different life stages of daphnids, when organisms were exposed to waterborne uranium, our data reveals higher levels of DNA damage in bystander organisms that cohabitated with 5d old organisms. This was probably due to the size of the daphnids, which are able to release to the medium higher amounts of signals than neonates. As already mentioned, this is the first time that a study of inter-organismic RIBE is performed with cladocerans, an thus, it is impossible to make comparisons with other studies. Still, our data are not in agreement with the study of Mothersill et al (Mothersill et al., 2010), which showed that for rainbow trout the magnitude of the bystander effect did not depend on the life stage at which irradiation occurred.

As for the highly diluted uranium mine effluent the results suggest that, at least in the early life-stages, and in real environmental scenarios, the RIBE phenomenon can also occur. Tests in which organisms are exposed to such complex mixtures, such as uranium mine effluents, frequently give rise to data difficult to analyse (Lourenço et al., 2017; Pyle et al., 2002). For example, in the present study, despite the fact that metals are recognized for their ability to affect cell signaling pathways and to promote the formation of ROS (Jomova and Valko, 2011), DNA damages were lower than in waterborne uranium treatments. Complexation, lower bioavailability or inhibition of the bystander signal by the chemical components of the effluent are likely explanations for the results obtained. Even so, it may be difficult to discuss these issues, since bystander signaling mechanisms are not fully understood. However, considering that NO (nitric oxide) is pointed out as a key element of RIBE (Hei et al., 2008; Prise and O'Sullivan, 2009; Shao et al., 2003), we hypothesize that some of the metals present in the effluent (e.g. Zn, Co and Ni) may be the responsible for lowering or impairing the production of bystander signals, once some divalent transition metals, (e.g. Cu, Zn, Co, Ni) inhibit NO synthase catalysis (Perry and Marletta, 1998).

Considering the data and the discussion made above and answering to the second main question of this study, “Do factors such as time of cohabitation or the age of irradiated organisms modulate RIBE in D. magna?”, we can say yes, inter-organismic RIBE in *D. magna* was influenced by cohabitation time. Bystander DNA damage reached a peak after 24h of cohabitation, and damage decreased with time. Given the age of organisms from both exposures, the 5d old daphnids were able to induce more bystander damage than <24h old neonates.

Having answered all the other questions raised, we are now in position to answer to a major question behind the present work: to what extent bystander effects between organisms change the current paradigm (only based on direct effects of low doses and low dose rates, at the individual level) used to predict the risks of radionuclide-rich wastewaters? The relevance of non-targeted effects (including the bystander effect) of IR is a difficult and non-unanimous issue that has already been addressed for human health risk assessment (e.g.(Morgan and Sowa, 2009)) and, more recently, for environmental radiation protection (Mothersill et al., 2017). However, it was suggested that it was worth considering, if the effects on individuals could be amplified to the population level. Although it is not our intention with this study to make definitive statements on this issue, some points deserve attention.

First, we want to emphasize the contribution of the present work for the discussion of RIBE, using natural α-emitting radionuclides such as those present in a uranium mine effluent. This approach allowed to complement studies that only used IR or radiation exposures combined with few metals, which shows a more realistic view of the possible relevance and non-linearity/complexity of bystander effects.

Based on all the results herein presented, we can say that the induction of bystander effects between organisms through cohabitation seems to be more pronounced when organisms are exposed only to uranium rather than to a complex uranium mine effluent. As so, and despite the differences observed in bystander induction between effluent and waterborne uranium, it seems that for the first tier of the risk assessment framework, uranium assays can be used, because they are overprotective. Nevertheless, the importance of studies using natural environmental radioactive samples should never be neglected since, as we have seen, a scenario of multiple stressors can induce damage in biota even at levels below the predicted biological risk limits for radiation.

Despite the maintenance of this physiological trait i.e. the ability of irradiated organisms to induce bystander effects, the rapid disappearance of such effects, especially in the uranium mine effluent exposures (but also in waterborne uranium) legitimizes doubts

about the ecological relevance of this trait in a multigenerational scenario for organisms that are submitted to bystander signals.

5. Conclusions

D. magna single-event exposure to both uranium mine effluent and waterborne uranium is able to induce bystander effects in non-exposed daphnids through cohabitation. This is the first time that this phenomenon has been observed and described in invertebrates and it complements similar data for vertebrates. Despite a mild bystander effect, this study brings new insights to the discussion of the relevance of RIBE to environmental risk assessment in an ecologically relevant exposure scenario. There is some variation in RIBE depending on exposure (waterborne uranium versus uranium mine effluent), cohabitation time and age of exposed daphnids,. Although it is thought to be desirable and important for evolution (Mothersill and Seymour, 2009), the true evolutionary background and purpose of this trait and the way in which it contributes to the fitness of populations are not yet understood.

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